In vitro studies to assess the potential bioactivity of quercetin as an anti-bacterial and anti-cancer agent

Thesis submitted for the award of the degree of

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In

Cell Biology and Molecular Genetics

under the Faculty of Allied Health and Basic Sciences

By

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2020

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Mary Shobha Rani I.

ABBREVIATIONS

AO	Acridine Orange
ANOVA	Analysis of Variance
ARSE	Amoxicillin Resistant *Staphylococcus epidermidis*
ATCC	American Type Culture Collection
BCL-XL	B- cell lymphoma- extra large
CSC	Cancer Stem Cells
COLO 320	Human Caucasian Colon Adenocarcinoma
DAD	Diode Array Detector
DMSO	Dimethyl Sulphoxide
DOX	Doxorubicin
DNA	Deoxyribo Nucleic Acid
ESI-MS	Electrospray Ionization Mass Spectrometry
EDTA	Ethylenediaminetetraacetic acid
FITC	Fluorescein isothiocyanate
^{1}H NMR	Proton Nuclear Magnetic Resonance
HPLC	High Performance Liquid Chromatography
Hep G2	Liver Hepatocellular cells

HCT-16	Hematocrit
IC	Inhibitory Concentration
JC	Tetraethylbenzimidazolylcarbocyanine iodide
JNK	Jun N- terminal Kinases
LCMS	Liquid Chromatography Mass Spectroscopy
3T3-L1	Mouse Fibroblast cells
MRM	Modified Radical Mastectomy
MCF-7	Michigan Cancer Foundation-7
MIC	Minimum Inhibitory Concentration
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MFI	Mean Fluorescence Intensity
NFkB	Nuclear Factor Kappa B
NMBP	National Medicinal Plants Board
PBS	Phosphate Buffered Saline
PC-3	Prostate Cancer cell line
PI	Propidium Iodide
R_f	Retention Factor
ROS	Reactive Oxygen Species

SDS	Sodium Dodecyl Sulphate
UV-Vis	Ultra-Violet Visible Spectroscopy
Yau	Arbitrary units of Fluorescence
ZOI	Zone of Inhibition

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Abstract

Bacterial infections and cancers are the two foremost health apprehensions in the modern society. The control of these two modalities is a challenge to the scientific community as they gain resistance to the existing therapies. Medicinal plants continue to be the major contributing factors in drug discovery till today. Hence, the aim of the study was to explore the medicinal plants and their bioactive compounds towards these two concerns.

Secondary metabolites are the major carriers of biological activities; therefore, the present study was focussed on the predominant group of metabolites, the flavonoids. 'Quercetin', a bio flavonol of diverse fruits and vegetables is extracted from the leaf material of *Anethum graveolens* L. and *Raphanus sativus* L. The process of quercetin extraction involved three methods (Maceration, Digestion and Column chromatography), out of which column chromatography was found effective. High Performance Liquid Chromatography reports proved that the fraction yielded by column chromatography was 98% pure along with the standard. The compound was further identified by spectral analysis *viz*., UV-Visible, Infra-red, ^{1}H Nuclear Magnetic Resonance and Liquid Chromatography Mass Spectrometry.

The confirmed fractions of quercetin from both the plant species are studied for antibacterial and anticancer potentials.

Generally, broad spectrum antibiotics act diversely on different pathogens. Similarly, quercetin when tested against *Pseudomonas aeruginosa, Staphylococcus aureus* and *Escherichia coli* displayed varied mechanism of action. Preliminary screening of antibacterial activity proved that quercetin fraction has more or less similar activity with broad spectrum antibiotics tested and in case of *E.coli,* quercetin exhibited better activity than the tested antibiotics. Antibiotics compared in the study act on cell wall and membrane, so, quercetin was also presumed to be acting on the same components of the bacteria tested. It was found

that quercetin showed twitching motility restriction which is a major phenomenon involved in biofilm formation. Surprisingly, quercetin also presented 99% biofilm inhibition in *S. aureus*. The release of absorbing materials and proteins indicated quercetin damaged the cell membrane at 10 and 100 µg/ml concentration. This made us to focus on the activity of quercetin on DNA and was ruled out by DNA fragmentation assay. Quercetin showed marked damage to DNA indicating its abrupt action. All the evidences draw a conclusion that quercetin has broad spectral activity on the pathogenic organisms which are the major challenging targets in the world.

Cancers treatment involves surgery, radiation and chemotherapies with adverse side effects. Amongst, chemotherapy is widely applied as a sole therapy, before and after surgery and involves combination of treatments for enhancing its effectiveness. This throws out a challenge to researchers in finding out novel combinations with lesser side effects. Literature proves that diet plays an important role in providing active ingredients that suppresses cancer. We focussed our study on the combination of quercetin from edible greens with the standard chemo drugs used for top listed cancers (Breast, Colon and Prostate). The results of the study proved that quercetin alone could effectively present cytotoxic activity against breast and colon cancers as that of standard drugs. The same quercetin when combined with standard drugs at lesser concentrations further enhanced its activity than the treatment with standard drugs alone. The observations were further carried out for screening its potency on cell cycle, mitochondrial membrane potential and caspase activation. Quercetin effectively arrested various phases of cell cycle and stood as growth arrest initiator; also displayed its activity on mitochondrial membrane potential and activated caspase-3 which is a hallmark of apoptosis. Therefore, these novel combinations and observations seem to be the best choices in reducing the burden of toxic drugs in both bacterial infections and caner. Further, *in vivo* studies have

to be performed to substantiate the present result. Thesis presents the process of quercetin purification and its action on selected bacteria and cancer cell lines.

CHAPTER 1

INTRODUCTION

Our planet earth is occupied with thousands of healing plants. Since biblical times, human civilisation is in close interaction with the environment by using the elements of the plants. It can be quoted that 'early humans recognised and exploited the plants around them for fuel, cloth, shelter, food, and medicine and also well versed with their benefits' [Halberstein, 2005]. Through the course of period, medicinal plants are transformed into one of the oldest sciences in countries like India, China, Greece and Egypt [Hamilton, 2004]. Till now, 2, 50, 000 to 3, 00,000 plant species were recognised on the earth and out of which only 7% of vascular flora have been exploited for their medicinal potential. About 100 plant species are involved in 25% of drug manufacturing process in and around the world. India has 25 biotic provinces, 15 agro-climatic zones and 10 biogeographic regions and two biodiversity hot spots of world's biodiversity. In India, 50,000 plant species were recognised and out of which 10% are known for their medicinal value [Myers, 2000].

The medicinal values of plants are mainly attributed by their active components named 'phytochemicals'. Every part of the plant has enormous phytochemicals that contribute various medicinal properties as therapeutics. From a single plant species, a minimum of two novel metabolites can be found [Kroymann, 2011]. This implies that plants are the library of natural compounds with potentially useful chemicals [Edreva *et al*., 2008]. Since ages, the living beings have exploited plants for bioactive compounds either as direct use or in making drugs to cure diseases [De Luca *et al*., 2012]. These components are called as 'secondary metabolites' and Albert Kossel, Noble Laureate, was the first person to define the term "secondary metabolite" in 1910 [Jones and Kossel, 1953]. Thirty years later, Czapek coined them as end-products, described them as nitrogen metabolism derivatives and called them as 'secondary modifications'. In the middle of twentieth century, advances in chromatographic techniques allowed to recover more of these compounds and was established as a basis for natural product chemistry [Bourgaud *et al*., 2001].

1.1. Natural products

An impressive number of modern drugs are derived from natural sources based on their traditional value. The four major sources to get natural products are: (1) whole organism (plant/animal/microorganism), (2) part of an organism (leaves/ flowers/any part of plant), (3) An extract of an organism and (4) pure compounds isolated from plant (flavonoids, alkaloids, terpenoids, glycosides and sugars etc.). In most of the instances, natural products refer to as secondary metabolites, derived from plants and are generated under stress conditions [Sarkar, 2006]. Drug discovery process from natural products includes finding a healthy plant or microbial source in an adequate manner followed by bioassay guided fractionation; isolation and purification of active constituents. The active constituents, either in their original or modified forms, showing significant *in vivo* activity (animal models) are considered as lead molecules for preclinical development with prevailing activity and decreased toxicity [Cragg and Newman, 2005].

1.2. Extraction of plant-derived natural products

The story of extraction of natural products dates back to the Mesopotamian and Egyptian times, where production of perfumes or pharmaceutically active oils and waxes were the major businesses. From papyrus writings, in 1600 BC, beer and wine were considered as solvents for achieving high solubility of a solute in perfume production. A maximum of forty repetitive extraction procedures were necessary to get quality product. Till middle ages there was no development in availability of solvent and ethanol became a solvent in 900 AD [Bart, 2011]. Therefore extraction of an active constituent from plant source is always a challenging task for the researchers.

Extraction is defined as the process of removal of desirable soluble constituent from a mixture leaving out unwanted materials with the help of solvent. The standard procedures and

the solvents are the key players in extraction of active fragment from crude material (medicinal plant parts). Several methods of extractions are maceration, percolation, reflux, soxhlet, ultrasonic and microwave assisted extraction. Variation in extraction process depends on particle size (wet or dry material), solvent, temperature, length of extraction, plant tissue and solvent to sample ratio.

Isolation is another step involved in getting functional compound from plants is based on its utility to cure diseases. The main chemical groups of bioactive compounds are glycosides, tannins, resins, flavonoids, lignins and alkaloids. Pure bioactive compound can be achieved by more than one isolation procedures based on homogeneity, the following are the list of various methods involved in extraction, isolation and identification of bioactive compounds [Table 1.1-1.3]. [Mendonca- Filho, 2006; Zhang, 2018].

Table 1.1: Different extraction methods and their advantages

Type of extraction	**Application**
Maceration	Simple, long extraction time, low efficiency
Percolation	Advantageous over maceration with continuous replacement of fresh solvent
Reflux	More efficient than percolation or maceration, less extraction time
Soxhlet extraction	Advantageous over reflux and percolation with high extraction efficiency, uses less solvent and time
Ultrasonic assisted extraction	Advantageous over the above methods, uses ultrasonic wave energy, improves extraction efficiency with less solvent and energy consumption
Microwave assisted extraction	Uses microwaves, efficient in improving yield

Table 1.2: Types of Chromatography and their applications

Technique	**Application**
Thin Layer Chromatography (TLC)	Identifies compounds from a mixture and determines the purity of a substance
Column Chromatography (CC)	Separates fractions based on differential adsorption of compounds in the column at different rates
Flash Chromatography (FC)	Separates mixtures of molecules into their individual constituents
High Performance Thin Layer Chromatography (HPTLC)	Separates compounds by more flexible, reliable and cost-efficient manner
High Performance Liquid Chromatography (HPLC)	Uses high pressures of up to 400 atmospheres to separate compounds from a mixture

Table 1.3: Structure elucidation by biophysical/analytical methods

Spectroscopy technique	**Application**
Ultra-Violet-Visible Spectroscopy (UV-Vis)	Applies to quantitative determination of different analytes such as transition metal ions, highly conjugated organic compounds and biological macromolecules
Infrared Spectroscopy (IR)	Simple and reliable technique widely used in chemical, biological and industrial research for molecular characterization
Nuclear Magnetic Resonance Spectroscopy (NMR)	Widely used for elucidating the structure of a compound by either matching against spectral libraries

	or infer the basic structure directly for unknown compounds
Liquid Chromatography-Mass Spectroscopy (LC/MS)	Widely used in the field of bioanalysis in identifying more than 85% of natural chemical compounds for polar and thermally liable compounds.

The study of natural products is called 'Phytochemistry' which is defined as the study of chemical constituents of plants called primary and secondary metabolites. Primary metabolites are necessary for growth and development (carbohydrates, proteins, lipids, nucleic acids and hormones) and secondary metabolites (terpenes, phenolics, glycosides and alkaloids) are derived from primary metabolites and are essential to protect plants from biotic and abiotic conditions. Among the secondary metabolites, 'phenolics' are the most abundant phytochemicals of plants ranging from simple molecules to highly polymerized substances and are categorised based on the carbon skeleton as below [Table 1.4] [Taiz, 2006] .

Table 1.4: Classification of Phenolic compounds based on carbon skeleton

Carbon atoms and basic skeleton	**Phenolic classes**
C6	Simple phenols
C6-C1	Phenolic acids
C6-C2	Acetophenone, Phenyle acetic acid
C6-C3	Phenylepropanoids, hydroxycinnamic acid, coumarins
C6-C4	Naphthoquinone
C6-C1-C6	Xanthone
C6-C2-C6	Stibene, anthraquinone
C6-C3-C6	Flavonoids, isolflavonoids
(C6-C3)2	Lignans, neolignans
(C6-C3-C6)2	Biflavonoids

Among the phenolic classes, 'flavonoids' are the large group with varied pharmacological activities. It was first believed as vitamin and named as 'vitamin P' in oranges, later

identified as flavonoid 'rutin' and subsequent findings reported nearly 4000 varieties of flavonoids [Stalikas, 2007; Koes, Quattrocchio, 1994]. The basic flavonoid structure includes 15 carbons, consisting of two benzene rings linked via a heterocyclic pyrene ring C. The classification of flavonoids is based on ring structure, includes Flavone (apigenin and leteolin), Flavonol (quercetin, kaempeferol, myricitin and finestin), Flavonone (hesperidin and naringenin), Catechins (Proanthocyanides: gallocatechin, epicatechin, epigallocatechin, epicatechin 3-gallate and epigallocatechin 3-gallate), Anthocyanidin (cyanidin and delphidin), Flavandio, Aurone (4'-chloro-2-hydroxyaurone) and Isoflavone (biochanin A and formononetin). These classes differ based on the C ring substitution and its position on A and B rings (Figure 1.1) [De Rijke *et al*., 2006; Middleton, 1998].

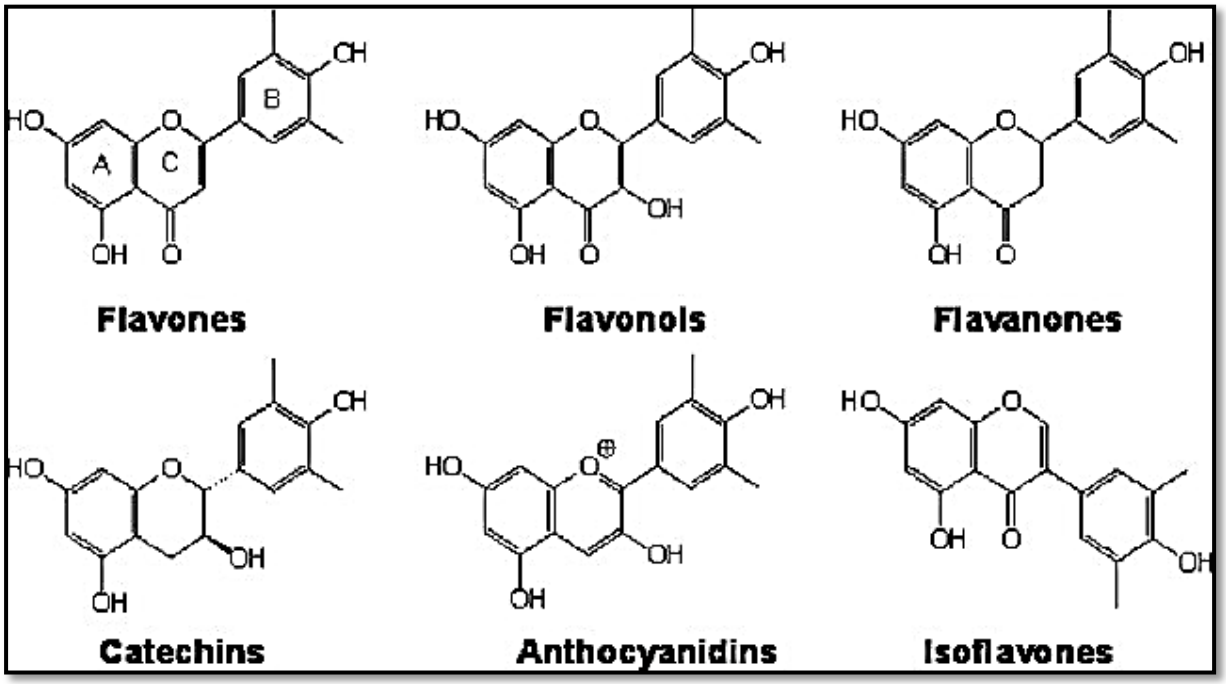


Figure 1.1: Classification of flavonoids based on ring structure (Source: Peter CH Hollman)

Quercetin, a distinctive bioactive flavonoid of 'flavonol' subgroup, is a dietary component (distinct fruits and vegetables) that has attracted the attention of dietitians and medicinal

chemists due to its numerous health benefits. Hence, in the current study, quercetin is considered as molecule of choice to explore its potential.

1.3. Physicochemical properties of Quercetin

Quercetin, is a derivative of 'quercetum' and is an unique important subclass of flavonoid since 1857 (Figure 1.2). International Union of Pure and Applied Chemistry's (IUPAC) nomenclature to quercetin is 3, 3′, 4′, 5, 7-pentahydroxy-2-phenylchromen-4-one; other names were hesperidin and vitamin P. Quercetin is aglycone form of variety of other glycosides found in various fruits and vegetables. Quercetin glycoside is formed by the attachment of a glycosyl group (rhamnose or glucose) at position 3 by replacing one of the OH groups. Quercetin glycoside is greatly soluble in alcohols and lipids, insoluble in cold water and poorly soluble in hot water [Häkkinen, 1999; Williamson and Manach, 2005; Wiczkowski *et al*., 2008].

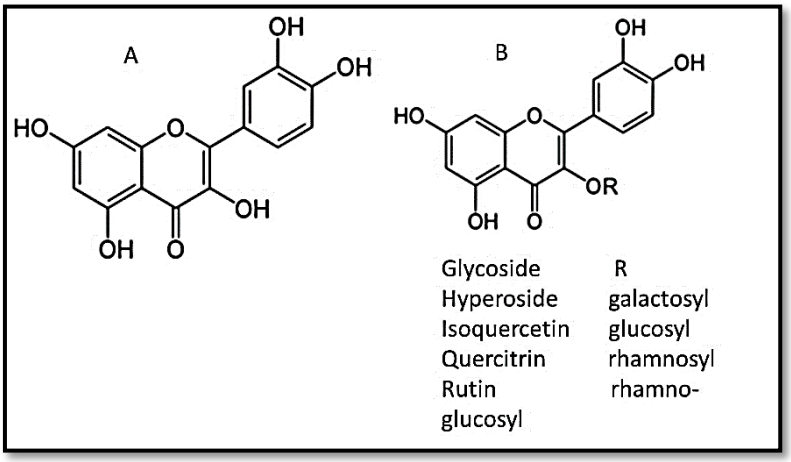


Figure 1.2: Structure of quercetin (A) and Glycoside quercetin (B)

(Source: Wikipedia)

1.4. Quercetin role in antibacterial potency and causes of bacterial resistance

Quercetin is extensively studied by researchers over the past 30 years and is known to have antibacterial activity against pathogenic bacteria encountered in hospital environment [Ganesh Ghosh and Danish Khan, 2016; Renu Narendra Jaisinghani, 2017; Sergio Dias da Costa Junior, 2018; Chunlian Tan *et al*., 2019]. In particular, quercetin shows effective bactericidal potency in infections related to dermal, respiratory, gastrointestinal and urinary system. And also, shows antiviral properties on adenovirus, herpes simplex virus and respiratory syncytial virus [Cushnie and Lamb AJ, 2005; Ramos *et al*., 2006; Coles, 2016]. Even though the molecule is well studied and known for its antimicrobial potencies, limited literature is available on its mechanism of action. Therefore, the study is designed to discover the possible mechanistic action of quercetin on life threatening bacteria encountered in Intensive Care Units (ICU), to name *Pseudomonas aeruginosa* (Figure 1.3), *Staphylococcus aureus* (Figure 1.4) and *Escherichia coli* (Figure 1.5). The possible antibacterial potency of quercetin is predicted to be on motility patterns, biofilm formation, release of absorbed materials and proteins; and DNA fragmentation. The said mechanisms are well versed with a few antibiotics used to treat them and hence are used to screen quercetin's best potency in treating bacterial infections.

1.4.1. Discovery of *P. aeruginosa*

Figure 1.3: ***P. aeruginosa*** **(**Source: 2019 AR Threats report)

Carle Gessard, a chemist and French bacteriologist discovered *P. aeruginosa* in 1882. It is a gram-negative proteobacteria belongs to family of Pseudomonadaceae, having a rod shaped structure with 0.5-0.8 μm width and 1.5-3 μm height with one flagellum for motility. The habitat of *P. aeruginosa* is in soil, water, on plants, on epidermis of animals, as a plankton, or as a biofilm [Centers for Disease Control and Prevention, 2018]. The bacterium can also thrive well in nutrient deficient, harsh temperatures and gets adapted to changing environmental conditions. It is an opportunistic pathogenic bacteria responsible for both acute and chronic infections. The clinically immunocompromised patients are at the highest risk because of more colonization and experience significant amounts of trauma. Antimicrobial resistance is a serious restriction in treatment options of the patients around the world, which has become a critical and deadly issue causing increased mortality rates (50%) [Favero *et al*., 1971; Pollack, 2000].

Since ages, *P. aeruginosa* infections are treated by combination of drugs *viz.*, carboxypeptidase and trans peptidases of β-lactam antibiotics majorly focusing on the cell wall constructing enzymes. Penicillin-binding proteins, another group of antibiotics mainly retard the synthesis of peptidoglycan by inhibiting the cell's ability to synthesize cell wall [Beta Lactam Antibiotics, 2011]. The β-lactams pass through porins of the outer membrane and play an essential role. Membrane impermeability is another proven mechanism in gaining resistance towards aminoglycosides, β-lactams and quinolones [Lambert, 2002]. Beyond its natural resistance to many drugs, it has ability to form biofilm, a complex biological system. Many antibiotics have been proven to be less effective on biofilm-forming bacteria. This remarkable ability of *P. aeruginosa* to form biofilms in discrete environments renders antibiotic treatments inefficient and therefore promotes chronic infectious diseases [Bjarnsholt, 2013]. Numerous phytochemicals from plants such as alkaloids, flavonoids, terpenoids, quinones and phenolics play a major role against pathogenicity and also showed

to be involved in inhibition of biofilm [Amaya, 2012]. Solvents used for extraction also have a principle role in biofilm inhibition. It was noted from the literature that alcohol extracts showed potent results than water extracts [Daneshfar, 2008]. Hence, the present study is designed to open the mechanistic potential of flavonol component quercetin from *Anethum graveolens* L. and *Raphanus sativus* L. with modern methods of extraction using ethanol as primary solvent.

1.4.2. Discovery of *S. aureus*

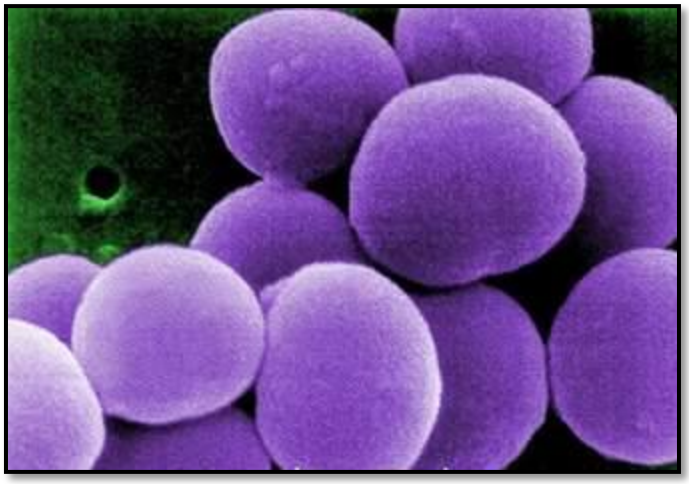


Figure 1.4: *Staphylococcus aureus* (Source: Rosenbach 1884)

Alexander Ogston, a Scottish surgeon in 1880 discovered major cause of pus and hypothesized that acute abscesses were caused by "micrococci". In 1882, he named the clustered micrococci as "staphylococci", derived from Greek word '*staphle*' (bunch of grapes).

In 1884, a German surgeon, Anton J. Rosenbach isolated two strains of staphylococci, named as *Staphylococcus aureus* and *Staphylococcus albus* with a size of about 1 µm [Ogston, 1881; Ogston, 1883; Rosenbach, 1884]. First antibiotic, Penicillin was discovered by Alexander Fleming (1928) from *Penicillium notatum* contaminating the pure culture of *S. aureus* in the plate. Resistance to Penicillin has emerged (1959), followed by the discovery of Methicillin,

which was believed to control the resistant organism. Later methicillin resistant *staphylococcus* strains were emerged (1961), became a challenging threat and noted as multiple drug resistance organism (MDR) (late 1970s) [Mohammad Fareed Khan, 2017].

S. aureus is a gram positive bacterium with peptidoglycan cell wall. Approximately 30% of normal healthy population is affected by *S. aureus* and can easily colonize the skin, nose and pharynx. It is capable to escape the innate immune responses (antimicrobial peptide-mediated killing and phagocytic) that can assist its survival in blood and other tissues during persistent infections. It occupied the first place in community acquired infections, hospitalized and immune-compromised patients [Thammavongsa *et al*., 2009; Lowy, 2003]. The increased mortality rates across the globe ranges from 20-40% despite the antibiotics' availability [Mylotte *et al*., 1987]. Several study reports say that *S. aureus* is the primary cause of nosocomial infections and have become an immediate concern to be addressed [CDC NNIS System, 2001; Diekema *et al*., 2002]. In the current study, a new flavonoid isolated from plant sources is studied to monitor the resistance mechanism of this highly contagious organism.

1.4.3. Discovery of *E.coli*

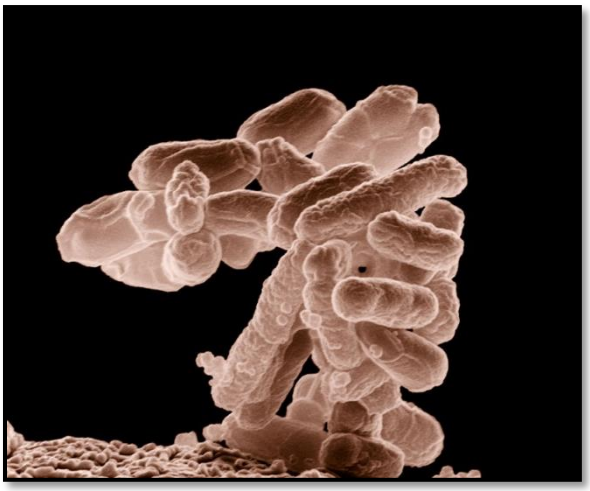


Figure 1.5: ***Escherichia coli*** (Source: Castellani and Chalmers 1919)

Theodor Escherich (1885) discovered the organism and named it as *Escherichia coli*. *E. coli* is a facultative rod shaped gram negative bacteria found in lower intestine of mammals and animals. The size ranges from 1-2 μm long with 0.5 μm radius. The hundreds of strains possess fimbriae; lipopolysaccharides and peptidoglycan cell wall. The pathogen is known to cause life-threatening infections worldwide and can be transmitted from person to person through fecal shedding and accounts for an estimation of 11% of infections [Erickson *et al.*, 2019; Atnafie *et al.*, 2017; Thomas and Elliott 2013]. Food poisoning also causes the infection, pneumonia; and 75 to 95% of Urinary Tract Infections (UTI) are initiated by *E.coli.* Some of the strains of *E.coli* release 'Shiga' a toxin, damages the lining of intestine and are called as 'Shiga toxin producing *E.coli'*. Another strain of *E.coli* (O157:H7) causes diarrhea, vomiting and abdominal cramps and causes acute kidney failure in children and also life-threatening conditions like adult kidney failure, fever, bleeding, confusion and seizures.

A wide variety of antimicrobial agents, β-lactams, fluoroquinolones, aminoglycosides and trimethoprim-sulfamethoxazole, effectively inhibit *E.coli*. β- lactams disrupt cell wall synthesis by binding to penicillin binding proteins essential for transpeptidation and carboxypeptidation reactions. Fluoroquinolones interfere during DNA supercoiling and promote DNA gyrase-mediated double-stranded DNA breaks. Most common risk factors associated with this MDR *E.coli* were noticed in diabetes patients followed by renal pathologies such as chronic renal disease, hydro ureter nephrosis and posterior urethral valve. Biofilm formation is another risk factor that rises the chance of *E.coli* to cause UTI [Stamm, 2008; Elder, 2007].

Developing novel antimicrobial agents will be cornerstones of clinical medicine and will save several lives from life threatening infections. This will stop the spread of antibiotic resistant pathogenic bacteria around the world and consequent failure of antibiotic therapy, especially in ICU, is posing an increased mortality rates [Palmer and Kishony, 2013].

1.4.4. Causes of bacterial resistance

1.4.4.1. Bacterial motility

Bacteria generally exhibit swarming, swimming and twitching motilities. Jorgen Henrichsen was the first person to report the swarming motility pattern in *Pseudomonas*, *Proteus*, *Vibrio*, *Serratia* and *Salmonella* which bacteria [Henrichsen, 1972]. Swarming involves coordinated (as a group) and rapid movement of bacterial population by flagella and causes bacterial multicellular behavior [Harshey and Rasika, 2003] that depends on viscosity of culture medium and nutrient composition. Swarming motility is well studied in *P. aeruginosa* [Tremblay *et al*., 2007], *E.coli* [Burkart *et al*., 1998], *Salmonella* [Harshey and Rasika, 1994], *Bacillus* [Kearns Daniel and Losick, 2004] and *Vibrio* [Mc Carter and Linda, 2004]. Swimming motility helps bacteria move individually by rotating flagella in liquid environments (*E.coli, Salmonella* and *Bacillus subtilis*) [Riley *et al*., 2018]. Twitching motility is a kind of surface motility powered by extension and retraction of type IV pili that confers slow movement (*P. aeruginosa, Neisseria gonorrhoeae* and *Myxococcus xanthus*). Twitching motility helps the organism in biofilm formation [Burrows and Lori, 2012].

The various motility pattern displayed by bacteria help in microbial colonization followed by spreading across the medium. It also aids in formation of biofilms or structured surface associated communities that increase various environmental and clinical problems due to enhanced resistance to antimicrobial agents [Costerton *et al*., 1999; Mah and OToole, 2001]. Thereby novel agent for reducing resistance and control of biofilm is required to be studied.

1.4.4.2. Biofilm formation and its discovery

Anton von Leeuwenhoek (17^{th} century) first observed the presence of microbial aggregates in plaque scrapings from his teeth. Bill Costerton (1978) coined the term ‘biofilm’; Wilderer and Charaklis (1989) described that biofilm is ‘indefinable community of microbes connected

with surface of tooth or material and distributed indiscriminately in a matrix'. Later, Donlan and Costerton (2002) together described the salient features of biofilm. They derived a statement that biofilm is a "sessile community of cells that are irreversibly attached to surface embedded in extracellular matrix and acquire altered phenotype with respect to growth rate and gene transcription" [Socransky and Haffajee, 2000].

Biofilms are classified based on its location (*Supragingival* – coronal to gingival margin; *Subgingival* – apical to gingival margin) and pathogenicity (*Cariogenic* – occurs in gram-positive and are acidogenic; *Periopathogenic* – occurs in gram – negative and are basophilic). The five stages in biofilm formation are mentioned in figure 1.6 [Ximénez-Fyvie *et al*., 2000].

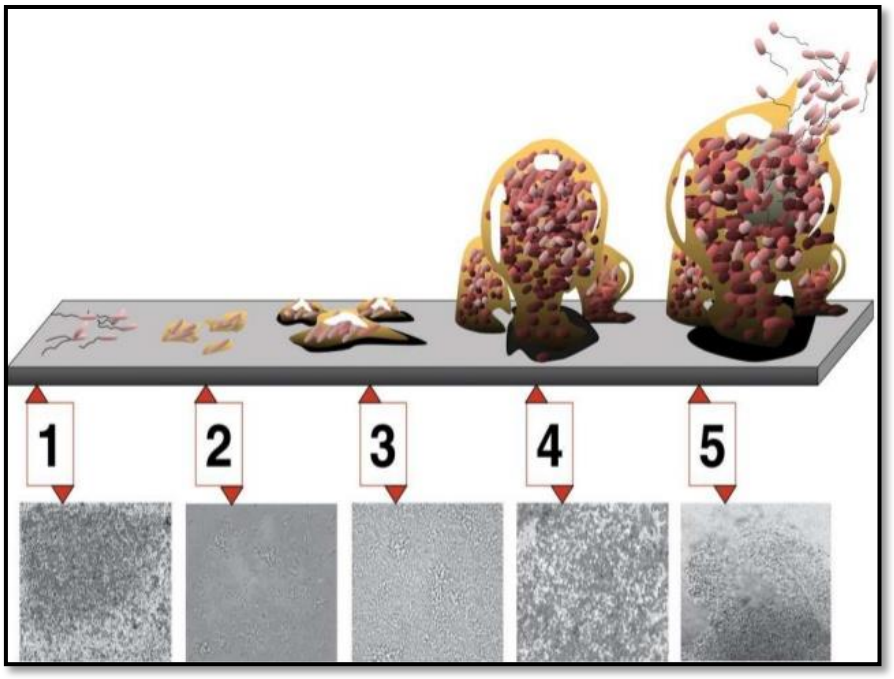


Figure 1.6: Stages of biofilm formation: (1) Initial attachment, (2) Irreversible attachment, (3) Maturation I, (4) Maturation II, and (5) Dispersion.

(Source: O'Toole *et al*., 2000)

Either as alone or as a group, bacterium forms biofilms, attaches to solid surfaces by encasing in exopolysaccharide matrix [Monroe and Don, 2007; Whittaker and Klier, 1996]. Reports say that formation of biofilms (on for example: catheters, contact lenses and artificial hips) have great impact on drug discovery due to their increased resistance to antimicrobial agents (10-100 times) [Prosser *et al*., 1987]. An estimate of 65% nosocomial infections are related to biofilms [Evans and Holmes, 1987; Costerton *et al*., 1995; Archibald LK and Gaynes, 1997]. As there is an unwinding bond between biofilms and nosocomial infections, much research has to be directed towards new antimicrobial agents on these surface-attached communities.

1.4.4.3. Other components of bacteria

Anton van Leeuwenhoek (1676) first discovered the structure of bacteria. Integrity of bacterial cell shows a critical role in the viability of cell. Cell wall is the principle stress bearing and shape-maintaining element in gram-positive and gram-negative bacteria. Cell wall of gram-negative bacteria is thin (10 nm thick) and based on growth stages, composed of two to five layers of peptidoglycan. Cell wall of gram-positive bacteria is made up of 20-40 nm thick layer of peptidoglycan and derives the structural framework of the cell and teichoic acid makes up 50% of cell wall material and controls the overall surface charge of the wall. Any incident that interferes with assembly of peptidoglycan precursor, transport of object across the membrane will integrate into the cell wall and compromise the reliability of the wall. This kind of damage to cell wall disturbs the state of cell electrolytes that triggers cell death. Regulated cell death has a vital role in various developmental processes like competence and biofilm development. Several antibiotics are involved in cell wall disruption for instance, penicillin which acts by binding to transpeptidases and inhibits cross-linking of peptidoglycan subunits [Potera, 1999; Höltje, 1998; Lewis, 2013]. Hence, bacterial cell with

damaged cell wall fails to undergo division and dies. In our study, an attempt was made to find out the antibacterial potency of quercetin on cell damage.

Williamson (1970) demonstrated DNA fragmentation for the first time in primary neonatal liver cultures. The hallmark of cell death is double stranded DNA breaks/ DNA fragmentation. Unlike DNA fragmentation in higher eukaryotic cells, DNA fragmentation in microorganisms is rarely studied. It is stated that bactericidal antibiotics can trigger the production of hydroxyl radicals which contribute to cell death. Factors like differentiation processes, starvation and ecological pressure cause DNA fragmentation [Schneider and Sahl, 2010]. In the present study, we made an attempt to knock out the action of quercetin on bacterial DNA.

1.5. Quercetin role in cancer reduction

Cancer is a multifactorial, multifaceted and multi-mechanistic disease that starts anywhere in human body where cells misbehave and continuously divide in the absence of growth factors. World Health Organisation (WHO) reports state that out of 7.5 million population, 0.8 million population in India are affected by cancer. Multiple genetic and environmental risk factors increase the incidence of cancer and WHO estimates 11.5 million deaths by 2030 [Fernández *et al*., 2008]. The commonest four most frequently causing cancers are lung, breast, colorectal and stomach. Among and according to global scenario, the most prominent cancers are breast and colorectal cancers and till now 2,069 anticancer clinical trials were already done by United States National Cancer Institute and 150 drug combinations have been successfully applied against cancer [Parkin *et al*., 2001; Lee and Xiao, 2005].

The World Cancer Research Fund Network (American Institute for Cancer Research and World Cancer Research Fund, United Kingdom; and World Cancer Research Fund, Hong Kong) is dedicatedly trying to reduce the chance of developing cancer through diet, nutrition,

physical activity and body composition. Quercetin, the most predominant component present in various fruits and vegetables, has antioxidant effect that contributes for its anticancer activity. The anticancer activity of quercetin is based on its binding to cellular receptors and proteins. Furthermore, recent investigations have showed synergistic effects when quercetin combined with chemotherapeutic agents such as cisplatin, which may further improve the outcomes of the traditional chemotherapy [Sharma Poonam and Majee Chandana, 2015]. In this context, the present research is designed to study the mechanism of combined action of quercetin and drugs (Anastrozole, Capecitabine and Bicalutamide) on most common cancers of women (breast and colorectal) and men (colorectal and prostate).

1.5.1. Breast Cancer

Breast cancer is the second common cancer after skin cancer that develops in cells either in lobules or ducts of the breast fatty or fibrous connective tissue within the breast. Uncontrolled cancer cells invade healthy breast tissue and can travel to lymph nodes (under arm) and account for 25% of mortality rates. These lymph nodes are the primary pathways that take part in metastasis. The risk factors connected with breast cancer incidence are age, gender, gene mutations, alcohol consumption and early menstruation [Rauf *et al*., 2018].

1.5.2. Colorectal Cancer

Colorectal cancer is reflected as one of the predominant cancers that accounts for 10% of cancer related mortality in both men and women. It starts in the colon or rectum of lower digestive system. The risk factors connected with this are a prior history of colon polyps, bowel diseases, family history of colorectal cancer and certain genetic disorders like familial adenomatous polyposis, obesity, diabetes and smoking [Sun Yi-Sheng *et al*., 2017].

1.5.3. Prostate cancer

Prostate cancer of prostate gland is the second most frequent malignant cancer after lung cancer in men around the world with 3.8% mortality rate. Initially it grows slowly and confined to the gland and doesn't cause any serious harm. In slow growing cancer type and in certain conditions, it may need minimal treatment than aggressive types which spread quickly. Lack of proper diet and physical activity lead to the development of prostate cancer. The risk factors connected with it are age, ethnicity, family history, diet, obesity and smoke habit [Ernst J Kuipers, 2015].

1.5.4. Mechanism of cancers

1.5.4.1. Apoptosis

Cancer cell death exhibits several mechanisms of which the wide spread are apoptosis and necrosis. Apoptosis is a highly regulated process that remove unwanted cells, activated by intracellular and extracellular signals which regulate internal (mitochondrial) and external (death receptor) pathways. Initiator caspases (caspase 2, 8, 9 and10) and executioner caspases (caspase 3, 6 and 7) initiate apoptotic cells to shrink and undergo plasma membrane changes that signal the macrophage response. Prevention of cancer is one of the important features of apoptosis. Loss of apoptotic control permits cancer cells to survive longer, triggers tumour progression, deregulates cell proliferation, stimulates angiogenesis and interferes with differentiation. Irrespective of cause or type of cancer, aiming at apoptosis is an important target for all cancer types. The intrinsic mechanism of apoptosis uses mitochondria and its proteins and extrinsic pathway uses extracellular signals to induce apoptosis. A new non-toxic anticancer treatment is essential due to increasing resistance of cancer cells to chemo and radiotherapy. Additionally, some of the chemotherapeutic agents are toxic to both normal and tumour cells. Interestingly, plant based compounds are minimal to non-toxic to normal

cells and a quarter of all modern medicines are derived from plants either directly or indirectly [Rawla and Prashanth, 2019]. Hence in the present study plant derived quercetin and combination with drugs were used to see the impact on cancer and normal cells.

1.5.4.2. Cell cycle

Cell division, growth, development and differentiation are tightly controlled by a conserved biological mechanisms. Deregulation of the cell cycle is a hallmark of the transformation of normal cells into tumour cells. Cell cycle has two distinct phases like mitosis (M) and interphase that include G1 (pre-DNA synthesis), S (DNA synthesis) and G2 (pre-division) phases followed by interphase G0 (quiescence). Increasing evidence reveals that cell cycle arrest is often tracked by or associated with apoptotic death of cancer cells. Arresting cell cycle is the key entrant for any anticancer drug derived from natural compounds. Knowing the underlying mechanism of action of natural compounds is a prerequisite for application of these compounds in basic and translational research before going for clinical trials [Rao and Kurkjian 2012; Dall'Acqua, 2014].

1.5.4.3. Mitochondrial membrane potential

In eukaryotes, mitochondrion is well studied due to its vital role in regulation of cellular metabolism and multifaceted functions associated with diabetes, cancer and neurodegenerative diseases. Several significant roles of mitochondria include amino acid metabolism, energy production and involvement in respiratory cycle. Lethal functions are mainly regulated by a process called mitochondrial outer membrane permeation. In healthy cells, mitochondria regulate cell cycle whereas in cancer cells, to meet the higher metabolic demand of rapidly proliferating cells, dysregulation occurs. By using therapeutics that target mitochondrial metabolism of altered cancer cells is highly advantageous [Pfeffer, 2018; Jeena

et al., 2018]. Studies suggest that targeting mitochondria is an attractive strategy in cancer therapy which is considered in the present study.

1.5.4.4. Caspase activation

Caspases involved in apoptosis are cysteine dependent aspartate directed proteases. Out of 11 caspases, 7 are involved in apoptosis. Expression levels of caspases in tumour cells may have an impact on their activity since a lower protein concentration may have a lower ability of apoptosis. The apoptotic caspase cascades are divided into two groups: one is initiator caspases (2, 8, 9 and 10) that enter early into the cascade and are responsible for activating effector caspases (3, 6 and 7). Caspase-3 shares many of the typical characteristics of all known caspases. Caspase-3 is activated in the apoptotic cell both by extrinsic and intrinsic pathways. Hence, activation of Caspase-3 is considered to be one of the targets in the study [Weber, 2008].

Keeping all the above gaps in mind, the widely grown and consumed plants of Karnataka were chosen as material for quercetin extraction to knock out its best biological activity against two major concerns encountered in the present trend.

1.6. Plants selected for the study

Figure 1.7: *Anethum graveolens* L.

Table 1.5: Scientific Classification of *A. graveolens*

Kingdom	Plantae
Order	Apiales
Family	Apiaceae
Genus	*Anethum*
Species	*Graveolens*
Botanical name	*Anethum graveolens* L.

Anethum graveolens L., an annual herb, belongs to Apiaceae (Umbellilferae) family, native to southern Europe and is growing in Mediterranean region, central and southern Asia, Its traditional use dates back to 5,000 years as aromatic herb and spice and is cultivated widely throughout the world for its foliage as a cold weather crop. It is commonly called as "Indian dill" or "Sowa" and is widely grown in Karnataka and spread widely in Bijapur with an average yield of 646 tonnes per year. The ethanomedicinal properties of this plant are antihelmintic, anti-convulsive, wound healing, appetizer, decrease indigestion, flatulence and strengthen the stomach [Zargari, 1996; Ishikawa *et al*., 2002; Kaur and Arora, 2000].

Figure 1.8: *Raphanus sativus* L.

Table 1.6. Scientific Classification of *R. sativus*

Kingdom	Plantae
Order	Brassicales
Family	Brassicaceae
Genus	*Raphanus*
Species	*Sativum*
Botanical name	*Raphanus sativus* L.

Raphanus sativus L., belongs to Brassicaceae (Cruciferae) family commonly called as Radish. It is an annual, temperate and fast growing herb originated from Europe and Asia. It is grown as vegetable in Kolar, Karnataka with an annual yield of 42,276 tonnes. The plant, as a whole has traditional importance in many countries: and are demanded to have medicinal properties. Root and leaves, have pungent taste and are used as one of the traditional Japanese foods. Plant is used to treat liver and respiratory illnesses [Paredes, 1984; Caceres, 1987].

CHAPTER 2

REVIEW OF LITERATURE

2.1. Methods involved quercetin extraction from various plant sources

Quercetin is considered as the main bioactive molecule in the present study and hence, the various methods used for its extraction from various plant sources is briefly described hereunder.

Table 2.1: Isolation and extraction methods of quercetin from various plant sources

S. No.	Plant source	Methods employed	Solvents used	Reference
1.	*Allium cepa* L. (dry scales)	Liquid extraction	Ethyl acetate and water	Marcin Horbowicz, 2002.
2.	*Gingko birch* L. (leaves)	Soxhlet extraction	Ethanol	Chiu *et al.* 2002.
3.	*Bergenia crassifolia* L. (leaves)	Liquid extraction	Ethanol and water	Fedoseeva *et al*., 2006.
4.	*Hypericum perforatum*	Water bath	Methanol	Biesaga *et al.*, 2007.
5.	*Euonymus alatus* L. (spindle wood tops)	Solvent extraction	Ethanol	Yang Yi and Zhang, 2008.
6.	*Chrysanthemum indicum* L. (flowers)	Triple extraction method	Methanol	Zhang *et al.*, 2008.
7.	Tomato and grapes (whole fruit)	Reflux method	Methanol and hydrochloric acid	Kumar *et al*., 2009.
8.	*Rosa multiflora* L. (flowers)	Liquid extraction	Ethanol	Pang *et al*., 2009.
9.	*Michelia champaca* L. (leaves and stem bark)	Column chromatography	Methanol	Thida Cho, 2013.
10.	*Polygonum hydropiper* L. (leaves and stems)	Flow extraction method and High Performance Liquid Chromatography (HPLC)	Water	Lekar *et al*., 2014.
11.	*Trigonella foenum graecum* L. (leaves)	Column chromatography	Ethanol, hexane and ethyl acetate	Bharathi Sambandam *et al*., 2016.
12.	*Ginkgo biloba* (synthetic powder)	Solid phase extraction	Methanol	Wang *et al*., 2017.
13.	*Lagerstroemia speciosa* L. (leaves)	High Performance Liquid Chromatography-Diode Array Detector-Mass Spectroscopy	Methanol	Sai Saraswathi *et al*., 2017.
14.	*Aesculus indica* L. (fruit)	Column chromatography	Chloroform	Muhammed Zahoor *et*

				al., 2018.
15.	Onion and Broccoli (bulb)	Green extraction method (HPLC) by natural deep eutectic solvents	Methanol	Yunliang Dai and Kyung Ho Row, 2019.
16.	*Allium ascalonium* L. (flesh and skin)	Ultra sound assisted extraction	Acetonitrile and water	Thuy *et al*., 2020.

2.2. Literature on infectious diseases and emergence of antibacterial agents

Infectious diseases are the greatest blights in past of mankind till the discovery of first antibacterial agent (1930). Gerhard Domagk (1935) first discovered the antibacterial agent and named as 'Prontosil' [Brisker, 2005; Bosch and Rosic, 2008; Stork, 2005]. This antibacterial agent was later initiated as a prodrug, metabolized *in vivo* in to its active metabolite, sulfanilamide [Fuller, 1937]. This initiated the discovery of 'sulfa drugs' and its discovery unshered in the era of antibiotics.

Antibacterial agents/ antibiotics belong to broad class of antimicrobial agents. These antibiotics are naturally occurring molecules produced by a variety of microorganisms [Williams, 2002]. Based on the mechanism of action, antibiotics are largely divided as inhibitors of a) cell wall biosynthesis b) protein synthesis c) deoxyribonucleic acid (DNA) or ribonucleic acid (RNA) synthesis and d) folate synthesis [Walsh, 2003].

2.2.1. Antibiotics

The Greek word 'antibiotic' refers to anti (against) and bios (life); a substance which kills an organism. These antibiotics act as bacteriostatic (prevent growth of bacteria) and bactericidal (kill the bacteria). Antibiotics control bacterial infections, whereas, antimicrobial agent works as antiviral, antifungal and antiparasitic [Sommer and Dantas, 2011]. There is a proportionate equilibrium between the growing number of infections to mankind and the development of antibacterial agents in twentieth century. Alexander Fleming discovered broad spectrum

antibiotic, penicillin for the first time, and over decades, streptomycin, chloramphenicol and tetracycline a new class of antibiotics were discovered [Sharma *et al.*, 2013]. World's population was using antibiotic drugs for centuries and bacteria started getting resistance in various forms. The use of antibacterial agents and complexity of resistance go hand in hand. Scientists are focussing to develop new antibiotics [Nikaido, 2009] as the emergence of antibiotic resistance is an essential scenario due to overwhelming use of antibiotics. Pathogenic organism exhibits resistance to antibiotics and emerge as multidrug resistant (MDR). This is becoming the world's biggest concern for patients with various infections [Alekshun and Levy, 2009].

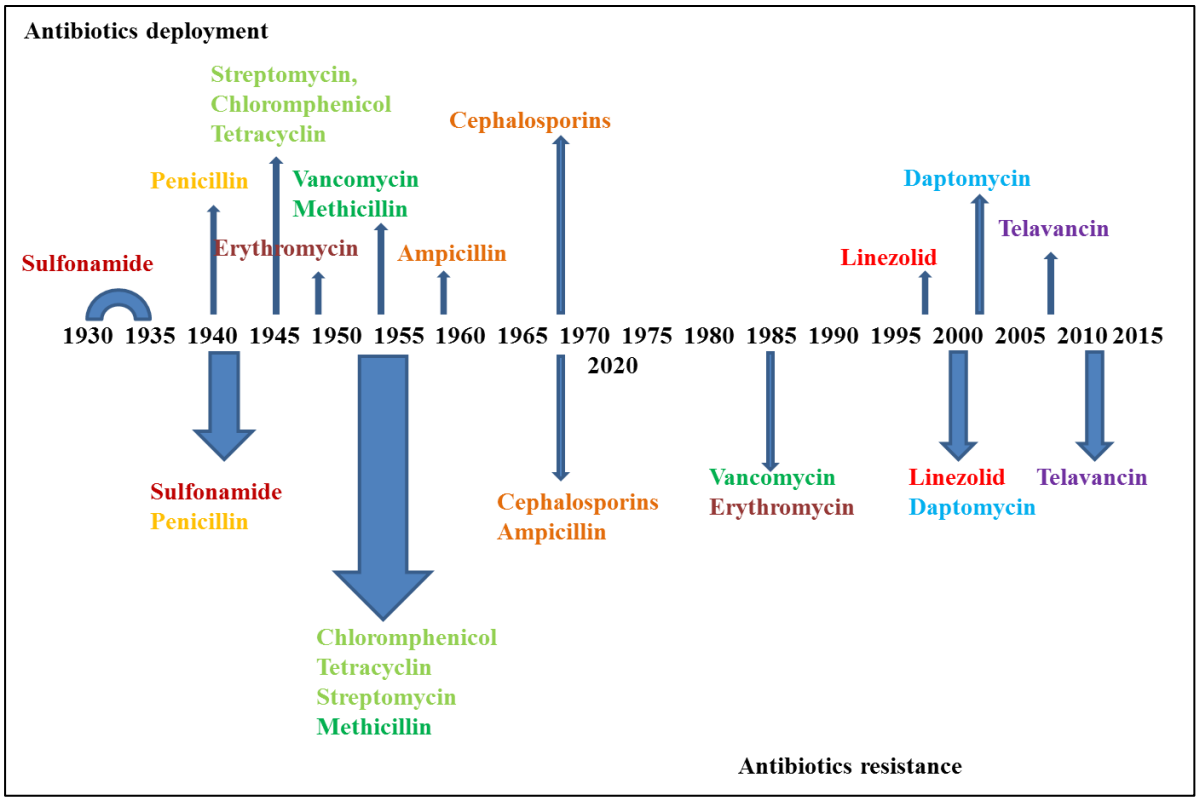


Figure 2.1: Timeline of antibiotic deployment (top) and development of antibiotic resistance (bottom)

Development of synthetic antibiotics has dropped from pharma industry, as a result new antibiotics with novel mechanism of action has been compromised in the last decade.

Therefore, there is a need to focus on plants for novel antimicrobial agents to address the mounting resistance. The other ways to control resistance is the judicious use of antibiotics and use of narrow spectrum antibiotics at appropriate times. In search of better drug/ drugs, medical researchers are finding ways for potential antibiotics from plants [Brew *et al*., 2008].

2.2.2. Antibacterial activity of quercetin extracted from various plant sources

Ganesh Ghosh and Danish Khan in 2016, from Arunachal Pradesh worked on impact of gallic acid, rutin, quercetin and mannitol on pathogenic microbes. Results proved that antimicrobial activity of flavonoids (quercetin) was enhanced against *S. aureus, Klebsiella, E.coli* and *B. cereus* when tested in combination like quercetin with rutin, mannitol and gallic acid. The outcome of antioxidant activity was effective when tested with these combinations. Natural antioxidants upon combination yielded better antimicrobial activity was the conclusion and lack of mechanism of action was the limitation.

Renu Narendra Jaisinghani in 2017 from Mumbai worked on antibacterial potentials of quercetin. Quercetin presented potent antibacterial activity against *S. aureus* and *P. aeruginosa* with a concentration of 20 µg/ml. Quercetin showed moderate activity against *E. coli* and *Proteus vulgaris* and did not show any activity against *Shigella flexneri* and *Lactobacillus casei* var Shirota. Conclusions drawn were quercetin could be used as a food preservative with its limitations of mechanistic-based laboratory and clinical studies.

Sergio Dias da Costa Junior *et al*., in 2018, from Brazil worked on antibacterial and antibiofilm activities of quercetin against clinical isolates of *S. aureus* and *S. saprophyticus*. Results of antimicrobial activity by quercetin proved that, quercetin inhibits methicillin susceptible *S. aureus* at 250 µg /ml and vancomycin resistant *S. aureus* at 500-1000 µg /ml. Based on the study results, Quercetin also inhibited biofilm production even in sub-inhibitory concentrations. The conclusions and limitations drawn from the study stated the need for

identification of resistance profile followed by biofilm inhibition as these aspects are responsible for bacterial survival; *in vivo* studies need to be addressed for quercetin usage in treating infections caused by *Staphylococcus sp.*

Chunlian Tian *et al*., in 2019 in China worked on antioxidant, antibacterial and analgesic activities of flavonoid fractions of *Tribulus terrestris* L. leaves. The resultant 14 fractions from HPLC-DAD-ESI-MS showed good antioxidant activity. The conclusions were flavonoid fractions can be challenging candidates for pharmaceutical companies in drug discovery.

Artur Adamczak *et al*., in 2019, worked on antibacterial activity of flavonoids by microdilution method. The aim of their work was to evaluate the antibacterial activity of 13 different flavonoid components and 6 organic acids. The results proved that all the tested flavonoids and organic acids exhibited antimicrobial activity with low to moderate activity. It was also found that antibacterial activity was high on gram-negative bacteria like *E. coli* and *P. aeruginosa* than gram positive ones like *E. faecalis* and *S. aureus*. They also concluded that the level of sensitivity of bacterial species to plant substances is very diverse and strongly depends not only on the type of active compounds but also on the strains tested.

2.3. History of cancer and emergence of anticancer drugs

In 460- 370 BC, Greek Physician Hippocrates, Father of Medicine coined the word cancer. Galen (130-200 AD), another Greek physician used the word “oncos” to describe tumors. During 1940s, anticancer drugs entered the world with the history of use of nitrogen mustard, a poison gas to slaughter soldiers in the trenches of the First World War. The exposed soldiers were suffered with lymphocyte destruction and required blood transfusions. This selective action against a particular type of cell suggested that nitrogen mustard might be used to treat lymphoma, a tumour of the lymph system. Nitrogen mustard worked and

rechristened as ‘mustine’, the first licensed chemotherapy agent. Synthetic drugs arrived successively in later years and combination of drugs became important in modern chemotherapy for most cancers. The toxicity of these drugs sometimes creates a significant problem in the treatment of cancer.

Plants, reservoirs of natural chemicals have enormous potential to provide newer drugs to fight cancer. The anticancer properties of plants have been recognised for centuries. Various therapies have been proposed to treat cancer; many of them used plant derived products, *viz.*, Vinblastine, Vincristine and Vindesine, the Epipodophyllotoxins (etoposide and teniposide), the Taxens (paclitaxel and docetaxel) and Camptothecin derivatives (campotecin and irinotecan). Isolation of active compounds from plants has led to the development of anticancer drugs. The National Cancer Institute (NCI – United States) has screened approximately 35,000 plant species for potential anticancer activities. Among them, 3,000 plant species have been demonstrated reproducible anticancer property [Desai *et al.*, 2008]. Between 1981 and 2007, a lot of investigations were renowned for their contribution to find out new drugs and almost half of the approved drugs were originated from natural sources. Near about 13 approved natural drugs were documented from 2005 to 2007. In 2014, 44 small molecules of natural origin were approved as drugs. Out of which, 10 were modified as synthetic drugs, though natural, that account for 25%. Combinational approach can further enhance the natural products’ efficiency [Thomson, 1978; Cragg *et al.*, 2005; Jachak and Saklani 2007; Katiyar *et al*., 2012; Newman, 2016].

Researchers worldwide are now focussing to discover plant-based anticancer drugs. In one such report, Shalabi *et al*., in 2015, from Egypt proved anticancer and apoptogenic potential of *Aloe vera* and *Calligonum comosum* extract against Hepatocellular carcinoma cell line. Fruits and vegetables are tested to have chemo-preventive and cancer curative potential, one among it is, pomegranate extract, effective against oestrogen positive breast cancer cell line

MCF-7 [Jeune *et al.,* 2005]. *Mentha* species (mint) is known to exhibit significant anticancer activity against cell lines, COLO-205, MCF-7, NCI-H322 and THP-1 [Sharma *et al*., 2014]. In India, researchers are focusing on conventional and locally available foods to cure cancer and suggesting to consume accordingly.

2.3.1. Anticancer activity of quercetin extracted from different plant sources

Shikha Srivastava *et al*., in 2016, in Bangalore, studied quercetin standard and its interactions with DNA, cell cycle and activating mitochondrial pathway of apoptosis. The findings presented that quercetin induced cytotoxicity in leukaemia and breast cancer cells in a dose-dependent manner and arrested S phase of cell cycle. *In vivo* studies on mice proved that quercetin induced tumour regression in concentration dependent manner and administration of quercetin to mice increased the life span to five-fold compared to untreated mice. The conclusions drawn were quercetin might interact with DNA directly and involve apoptosis induction process. Combination of quercetin and their potential in cancer therapeutics was the limitation of the study.

Pei-Hsun Tsai *et al*., 2016, in Taiwan worked on effect of quercetin (Qu) and Luteolin (Lu) in cancer stem cells and metastatic potential of isolated prostate cancer cells. The results of the study proved that Lu and Qu are promising and prospective candidates that function as anti-CSC and anti-metastasis agents. Furthermore along with JNK inhibitor SP600125, they provide a new avenue for therapeutic treatment in future.

Akbar Minaei *et al*., in 2016, from Iran studied the co-delivery of nano-quercetin enhances doxorubicin-mediated cytotoxicity against MCF-7 cells. The study results proved that co-treatment of nano-quercetin and doxorubicin on MCF-7 breast cells by phytosome technology increased the percentage of apoptosis. Phytosome technology might increase the permeability of tumour cells to chemical agents in treating breast cancer.

Ahmed S Sultan *et al*., in 2017 from Saudi Arabia worked on quercetin and its impact on triple-negative breast cancer cells via inhibiting fatty acid synthase and beta catenin. Results revealed that quercetin treated cells induced anticancer/ apoptotic effects by enhancing morphological alterations, fragmentation of DNA and Caspase-3 activation. *In vitro* treatment with quercetin induced suppression of tumour when treated for 25 days. The conclusions of the study were quercetin induced apoptosis via targeting fatty acid synthesis through Caspase-3 dependent mechanism.

In 2019, Akhilendra Kumar Maurya and Manjula Vinayak in Varanasi studied the improved synergistic anticancer efficacy of quercetin in combination with PI-103, rottlerin and G0 6983 against MCF-7 and RAW 264.7 cells. The results of the study found that quercetin had improved the cytotoxicity against MCF-7 cells in combinations with (rottlerin) ROT+G0 6883 30-55% and PI-103 at 69-75% and PI-103 at 45-88% with time dependent manner in RAW 264.7 cells. This observation was in correlated with morphological changes noticed of MCF-7 cells. The conclusions drawn from the study were, quercetin enhances the synergistic anticancer activity in combination with PI-103, rottlerin and G0 6983 in both MCF-7 and RAW 264.7 cells. Quercetin can be used along with other chemotherapeutic drugs in order to improve the efficacy in cancer prevention.

Ayaan Mukherjee *et al*., in 2019 from India, worked on derivatives of quercetin on colon carcinoma. The results of the study indicated that the semisynthetic derivative enabled the installation of different substitutions at C-3 and C-5 positions of quercetin. These derivatives had a better cell-penetrating ability when compared to quercetin and represented 96 fold increased cytotoxicity in HCT-16 colon cancer cells. The *in vivo* studies proved that colon cancer model mice exhibited striking survival rates and reduction in tumour weight. This study has drawn a conclusion that semisynthetic derivatives of quercetin can be developed as

good antitumor drugs. The study finds its limitations in developing other structural moieties and encouraging the researchers to explore different applications of modified quercetin.

Eun Ji Ha *et al.*, in 2019 from Korea worked on quercetin- induced apoptosis by co-treatment with autophagy inhibitor (Chloroquine). The results indicated that cytosolic reactive oxygen species (ROS) followed by mitochondrial ROS levels increased in quercetin-treated J/Neo cells; whereas ROS increase was abrogated in J/BCL-XL cells suggesting fact that ROS elevations downstream BCL-XL sensitive mitochondrial damage and dysfunction. Furthermore, Jurkat clones presented equal susceptibility to cytotoxicity of quercetin, excluding an involvement of extrinsic pathway of apoptosis. Quercetin along with chloroquine resulted in significant increase in BAK activation and mitochondrial mediated apoptosis. The study makes a conclusion that quercetin induces intrinsic apoptosis and cyto-protective autophagy and autophagy inhibition in Jurkat T cells.

Vinnarasi *et al.*, in 2020 from Tamilnadu, India, worked on structural insights into the anticancer activity of quercetin on G-tetrad mixed G-tetrad and G-quadruplex DNA by quantum chemical and molecular dynamics. The resulted findings proved that quercetin makes structural changes in DNA tetrads. Among the DNA tetrads, quercetin stacked with GCGC tetrad implicated more interaction energy. The results of the study proved the requisite nature of quercetin with DNA tetrads and inhibiting telomerase activity. The conclusions of the study were quercetin stabilizes G-quadruplex DNA and regulates telomerase activity and potentially termed to be good anticancer agent.

CHAPTER 3

AIMS AND OBJECTIVES

The present study is undertaken to isolate and identify quercetin from *A. graveolens* L. and *R. sativus* L. plants and to screen its antibacterial and anticancer potential. The following objectives are formulated in order to accomplish the aim:

1. Extract and purify compound quercetin from leaves of *Anethum graveolens* L. and *Raphanus sativus* L. by means of column chromatography, thin layer chromatography and HPLC.

2. Identify and characterize the isolated compound using spectroscopic technique like UV-VIS, IR, NMR and LC-MS.

3. Test the isolated compound for antibacterial property on most common pathogenic pure bacterial cultures such as *Pseudomonas aeruginosa, Staphylococcus aureus* and *E. coli* (cultures obtained from Hi-media Laboratories).

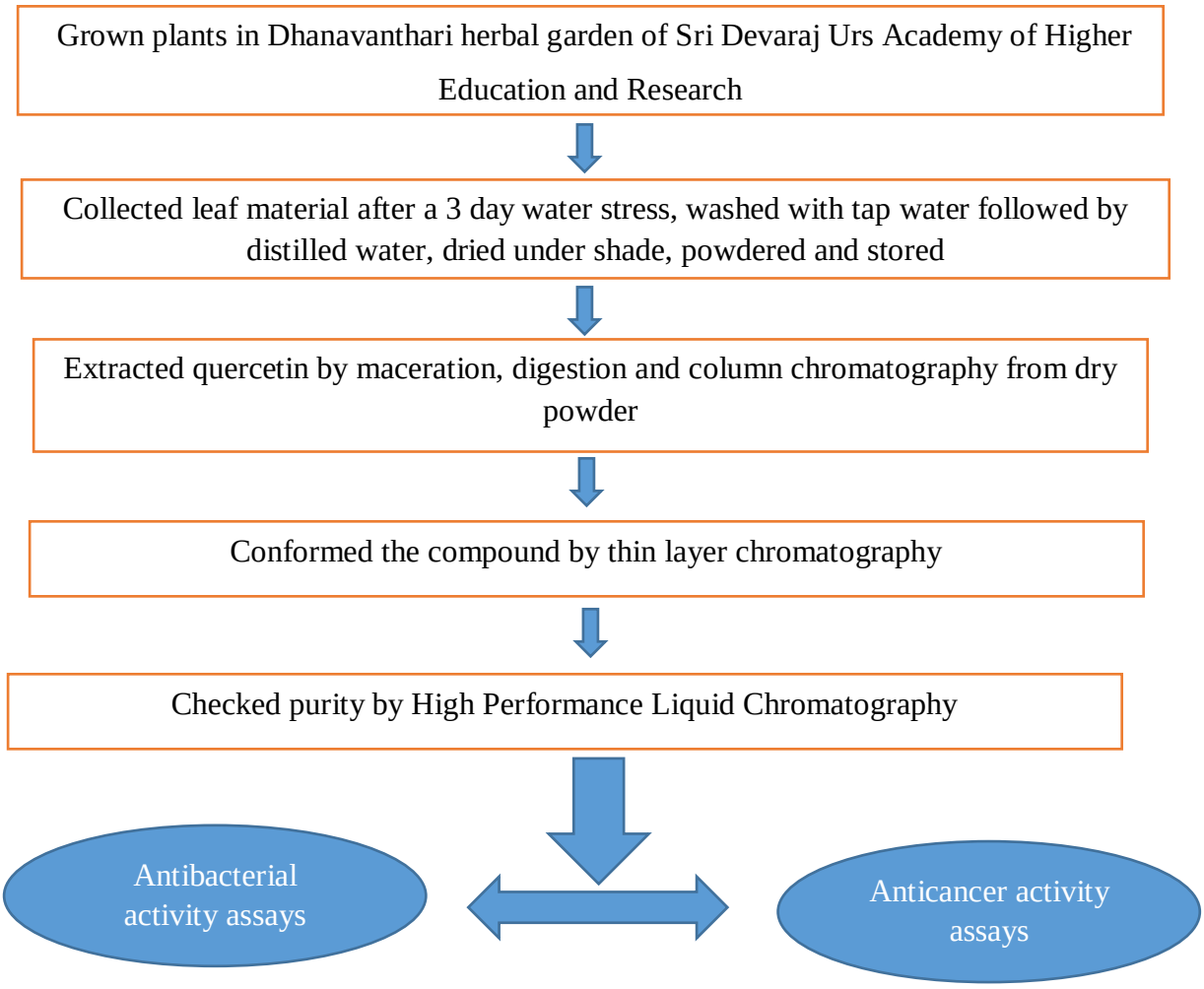
4. Test the isolated compound for anticancer property on selected cell-lines of Breast, Colon and Prostate using the acridine orange-ethidium bromide dual staining method (cell lines available from National Center for Cell Science, PUNE).

CHAPTER 4

MATERIALS AND METHODS

4.1. Study design

The process of quercetin extraction from *A. graveolens* and *R. sativus* is undertaken by applying the following procedures.



1. Antibacterial activity
2. Determination of bacterial motility
3. Determination of bacterial biofilm formation
4. Measurement of absorbing materials and proteins
5. Determination of DNA fragmentation

1. Determination of cytotoxicity
2. Apoptosis by dual stain method
3. Analysis of cell cycle arrest
4. Determination of Mitochondrial membrane potential
5. Determination of Caspase-3

Seeds of *A. graveolens* and *R. sativus* were collected from local market and were grown in pesticide free environment. The leaf material was authenticated by Dr. Madhava Shetty, Assistant Professor, Department of Botany, S.V. University, Tirupathi with voucher specimen numbers of 1236 *(A. graveolens*) and 2232 (*R. sativus*).

Before collecting leaves, a 3 day water stress was given to the plants and healthy leaves were collected in the morning hours, washed with tap water, followed by distilled water, shade dried, powdered and stored in air tight container.

All the chemicals and synthetic drugs used were procured from Sigma Aldrich.

Microbial strains were obtained from Hi-media laboratories and cell-lines were obtained from National Centre for Cell Science (NCCS), Pune.

4.2.2. Maintenance of cultures

Three reconstituted bacterial cultures (*P. aeruginosa, S. aureus* and *E.coli*) were sub-cultured on to nutrient agar plates, glycerol stocks were made and kept at -80^0C.

Lyophilized cancer cell lines of Breast (MCF-7), Colon (COLO 320DM); Prostate (PC-3) and normal mouse fibro blast cells (3T3-L1) were reconstituted and maintained in DMEM (Dulbecco's Modified Eagle's medium) and FBS (Fetal bovine serum) medium, sub cultured and stored at -80^0 C.

4.2.3. Preparation of plant material

About 25 g of leaf powder was taken in 100 ml of ethanol, kept on constant shaking at 120-130 rpm for 48 h. The residue was filtered using Whatmann No. 1 filter paper followed by evaporating the solvent in an air-circulating oven at 54°C until total dryness. About 12 g of obtained dried extract was collected and stored at 4°C for further analysis.

4.2.4. Qualitative phytochemical analysis of leaf material

The phytochemical components of ethanol extracts were qualitatively detected by following standard procedures [Roghini and Vijayalakshmi, 2018].

4.2.4.1. Test for alkaloids

Extract was treated with 2 ml of concentrated hydrochloric acid followed by addition of a few drops of Mayer's reagent. The appearance of green or white precipitate confirms alkaloids.

4.2.4.2. Test for flavonoids

Extract was added with 1 ml of 2N NaOH (sodium hydroxide), yellow colour appearance confirms flavonoids

4.2.4.3. Test for glycosides

Extract was taken in 3 ml of chloroform followed by addition of 10% ammonia solution. Pink colour indicates the presence of glycosides.

4.2.4.4. Test for phenolics

Extract was mixed with 2 ml of distilled water followed by a few drops of 10% ferric chloride. Formation of blue green colour indicates the presence of phenolics.

4.2.4.5. Test for terpenoids

Extract was mixed with 2 ml of chloroform and concentrated sulphuric acid, reddish brown colour at the interface indicates the presence of terpenoids.

4.2.4.6. Test for cardiac glycosides

Extract was supplemented with 2 ml of glacial acetic acid and a few drops of ferric chloride. This was under layered with 1 ml of concentrated sulphuric acid. Formation of brown ring at the interface indicates the presence of cardiac glycosides.

4.2.4.7. Test for steroids

To 1 ml of extract equal proportion of chloroform and a few drops of concentrated sulphuric acid was added. Brown ring appearance indicates steroids and bluish brown indicates phytosteroids.

4.2.4.8. Test of saponins

Extract was mixed with 6 ml of water and rinsed vigorously, the appearance of froth indicates the presence of saponins.

4.2.4.9. Test for proteins

Extract was added with 5 ml of biuret reagent, purple colour formation indicates the presence of proteins.

4.2.4.10. Test for tannins

Extract was added with 2 ml of 5% ferric chloride, formation of dark blue or greenish blank indicates the presence of tannins.

4.2.5. Quantification of total flavonoid content

Total flavonoid content was assessed according to aluminium chloride colorimetric method. The leaf extracts were diluted with ethanol and mixed with 0.1ml of 1M potassium acetate and 2.8 ml of distilled water. Blank was also made in a similar way by replacing aluminium chloride with distilled water. After 40 min, absorbance was measured at 415 nm. Quercetin standard was used to compare the percentage of flavonoid content present in the test material [Chang *et al.*, 2002].

4.2.6. Methods of quercetin extraction and identification

4.2.6.1. Maceration

Leaf material (2 g) was taken and mixed with 20 ml of ethanol and kept on orbital shaker for 48 h. Later, the mixture was strained by filtration, the filtrate was air dried and stored in air tight container [Azwanida, 2015].

4.2.6.2. Digestion

Digestion is a form of maceration with slight warming during extraction process, provided active ingredients of plants do not alter with temperature. Leaf material (2 g) was mixed with 20 ml of ethanol and kept at 50^0 C. Later the extract was sieved and stored in air tight container [Azwanida, 2015].

4.2.6.3. Column Chromatography

These ethanol extracts were used for column chromatography according to the procedure described by Manguro *et al.,* Briefly, 100 mg of extract was dissolved in ethanol, loaded on the silica column, ran with combinations of hexane, ethanol and ethyl acetate (1:1, 1:2 and 1:5) and the fractions were collected with 2 min time interval, pooled the fractions and concentrated by evaporation [Lawrence *et al*., 2013].

4.2.6.4. Thin Layer Chromatography

Ethanol dissolved sample and standards were tested for flavonoid with various solvents and their combinations. The resultant combination found was n-butanol: acetic acid: water (2:2:6). All plates were visualized for dark bands that were produced after keeping the plates in iodine chamber. R_f value of the different spots that were observed was calculated [Harborne, 1998].

4.2.6.5. High Performance Liquid Chromatography (HPLC)

Reversed phase gradient elution method was performed. The fractions of leaf material obtained from various extraction methods were subjected to HPLC. Quercetin was purchased from Sigma Aldrich and methanol, Orthophosphoric acid, water and other chemicals were of analytical grade [Chandrappa *et al*., 2014].

Mobile phase A: 1ml of Orthophosphoric acid was mixed with 2 L of water.

Mobile phase B: Methanol

Table 4.1: Chromatographic conditions

Column	:	250x 4.6, 5 µm, C 18
Column Temperature	:	25^0C
Injection volume	:	10 µl
Diluent	:	Methanol
Flow	:	2.0 ml/min
Run time	:	25 min
UV Detection	:	370 nm

4.2.6.6. UV-Vis spectrophotometry and FTIR measurements

Spectrophotometric analysis of the isolated quercetin was performed by UV-Vis Lambda 20 Spectrophotometer between 200 and 700 nm wavelength with a band width of 2 mm. Infrared (IR) spectrum of quercetin was recorded with Fourier Transform Infrared (FTIR) absorption spectrophotometer. The spectrum was recorded in the region between 4000 cm^{-1} and 400 cm^{-1} [Lu Ross *et al.*, 2011].

4.2.6.7. ^{1}H Nuclear Magnetic Resonance (NMR) Spectroscopy

NMR is primarily related to the magnetic properties of certain atomic nuclei; notably the nucleus of the hydrogen atom and will demonstrate the number of atoms in neighbouring groups. Hence, it was used as one of the tools for quercetin identification in the present study [Selvaraj *et al*., 2013].

4.2.6.8. Mass spectrometry-based Multiple Reaction Monitoring (MS-MRM)

The qualitative analysis of the quercetin was performed using Multiple Reaction Monitoring (MRM) by QTRAP 6500, AB-SCIEX mass spectrophotometer. The samples were diluted to 1:5 ratio in 0.1% formic acid in water and analysed in MS1, MS2 and MRM modes for the presence of quercetin using the selected ion pair transitions [Ming-Hua Hao *et al*., 2018].

4.2.7. Antibacterial assays

4.2.7.1. Antibacterial activity by agar well diffusion method

Antibacterial activity of quercetin extracted from both the plant species was detected by agar well diffusion method for ATCC strains of *P. aeruginosa* (27853), *S. aureus* (25923) and *E. coli* (25922). Quercetin was prepared in DMSO (Dimethyl Sulphoxide) with concentrations ranging from 1 µg/ml to 10 µg/ml concentrations with 1 µg/ml variation followed by 20, 30, 40 50, 100, 150, 200 and 250 µg/ml concentrations and screened for antibacterial activity against the high-end antibiotic discs at different concentrations as listed in below table. All the experiments were done in triplicates and results were noted [Nakamura *et al*., 2015].

Table 4.2: Antibiotic disks and their concentrations used in the study

P. aeruginosa		***S. aureus***		***E. coli***	
Antibiotic	**Conc. of disk in µg**	**Antibiotic**	**Conc. of disk in µg**	**Antibiotic**	**Conc. of disk in µg**
Piperacillin	100	Azithromycin	15	Ampicillin	10
Ceftazidime	30	Erythromycin	15	Cephalexin	30
Gentamycin	10	Ciprofloxacin	5	Amikacin	30
Tazobactum	30	Penicillin	30	Amoxicillin	20
Amikacin	30	Trimethoprim	1.25	Cefepime	30
Aztreonam	30	Cefoxitin	30	Chloramphenicol	30
Ciprofloxacin	5	Tetracycline	30	Sulfamethoxazole	23
Imipenem	10	Vancomycin	30	Cefuroxime	23
Levofloxacin	5	Gentamycin	10	Trimethoprim	30

4.2.7.2. Determination of motility assays

After obtaining minimum inhibitory concentration (MIC) from antibacterial activity results, motility of *P. aeruginosa*, *S. aureus* and *E. coli* were tested for quercetin fraction of both the plants. MIC of 10 µg/ml was taken as initial concentration and another concentration of 100 µg/ml was taken as reference in order to know the efficiency of the compound. Extracts of the said concentrations were dissolved in DMSO and used to screen for motility patterns (Swarming, Swimming and Twitching). Briefly, nutrient agar plates were prepared with 10 µg/ml and 100 µg/ml of quercetin and point inoculated with 50 cells. After incubation, the appearance of spreading zones from the point of inoculation from the bottom of petri dish is noted for all the assays and results were noted [Sameer Mohideen Gani *et al*., 2016].

4.2.7.3. Effect of quercetin on biofilm

Anti-biofilm activity of extracted quercetin was performed with 10 and 100 µg/ml concentrations as arrived by previous methods. Biofilm formation was facilitated by aliquoting 200 µl of cultures individually and incubated for 8 h at 37^0C for attachment of cells in a 96 well plate. The test compound was added and incubated for 2 h along with positive controls. The wells were then washed with phosphate buffered saline (PBS) solution

and stained with 5% crystal violet. The absorbance was recorded in ELISA reader at 570 nm and reduction of biofilm formation was calculated by using the following formula. Experiments were repeated thrice and results were noted [Sarangapani Sreelatha and Ayyavoo Jayachitra, 2018].

$$\text{Percentage Inhibition} = \frac{\text{OD of the control- OD of the test}}{\text{OD of the control}} \times 100$$

4.2.7.4. Measurement of absorbing materials

Fresh bacterial cultures (*P. aeruginosa, S. aureus* and *E.coli)* were inoculated in nutrient broth and exponential phase cells were collected by centrifugation at 5,000 rpm for 15 min. The collected cells were washed (three times) and suspended in 0.1M PBS (pH 7.4). The three culture suspensions (10^4 cells) were incubated with 10 and 100 µg/ml concentrations of quercetin for 4 and 24 h at 37^0C at 120 rpm. After incubation, centrifuged at 6,000 rpm for 5 min and the supernatants read at 260 nm and 280 nm [Xu *et al.*, 2010].

4.2.7.5. DNA fragmentation assay

DNA fragmentation assay was performed according to standard procedure [145]. About 10^4 cells of all the bacterial cultures (*P. aeruginosa, S. aureus* and *E.coli*) were treated with 10 and 100 µg/ml concentrations of quercetin for 4h, centrifuged at 5,000 rpm for 5 min and discarded the supernatant. To lyse the pelleted cells, 20 µl of TES (1 M Tris, 20 mM EDTA and 1%SDS) and 10 µl of RNase were added followed by incubation for 1 h at 37^0C. Added 10 µl of proteinase K, incubated at 50^0C for 1 h and 30 min. The sample was analysed on 1% agarose gel and documented by using UV-trans illuminator (Bio-Rad) [Pattern, 2017].

4.2.8. Anticancer assays

4.2.8.1. Determination of cytotoxicity

Briefly, 200 µl of Breast (MCF-7), Colon (COLO 320) and Prostate (PC-3) cell suspension (2x 10^4 cells/mL) was added into a 96 well plate and grown for 24 h [Juillerat- Jeanneret *et al*., 2008]. The test compound (quercetin) extracted from both the plants and standard drugs (Anastrozole, Capecitabine and Bicalutamide) at 50 µg/ml, 100, 150, 200, 250 µg/ml concentrations were made individually, added into well of 100 µl each and incubated in a 5% CO_2 incubator for 24 h, 48 and 72 h. Media was removed and MTT- (4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) was added to a final concentration of 0.5 mg/ml of total volume and incubated for 2 h. Later MTT was removed followed by addition of 100 µl DMSO for solubilisation, absorbance was measured at 570 nm and IC 50 was determined. The same experiment was repeated for combination of quercetin from plant source and standard drugs, absorbance was recorded and IC 50 values were determined. The rest of the experiments were followed from combinations and concentrations mentioned in the below table at 72 h time interval.

Table 4.3: Concentrations and combinations used in the study

Treatment groups	1. Cell control: only cells
	2. Standard control (std): (Anastrozole /Capecitabine)
	3. S1 (Quercetin from *A. graveolens*)
	4. S2 (Quercetin from *R. sativus*)
	5. S1 + Standard control (Anastrozole /Capecitabine)
	6. S2 + Standard control (Anastrozole /Capecitabine)
Concentrations	1. Standard control (Anastrozole) at 38 µg/ml on breast cancer cell lines
	2. Standard control (Capecitabine) at 45 µg/ml on colon cancer cell lines
	3. S1+ Anastrozole at 16 µg/ml on breast cancer (MCF-7) cell lines
	4. S2+ Anastrozole at 31 µg/ml on breast cancer (MCF-7) cell lines
	5. S1+ Capecitabine at 43 µg/ml on colorectal cancer (COLO 320) cell lines
	6. S2+ Capecitabine at 46 µg/ml on colorectal cancer (COLO 320) cell lines

4.2.8.2. Dual stain method

Cell lines were cultured to a concentration of 2x 10^4/ml and100 µl was seeded in a 96 well plate, treated with the combination of quercetin as mentioned in table 4.3 and incubated for 72 h followed by addition of 0.25% trypsin in PBS. When cells had sloughed off, 25 µl was taken onto a glass slide, added 1µl dual fluorescent staining solution (100 µg/ml of Acridine Orange and 100 µg/ml of Ethidium Bromide) and covered with coverslip. The morphology of apoptotic cells was examined and cells were counted within 20 min under fluorescent microscope. The observations were noted for three times [Lecoeur, 2002].

4.2.8.3. Cell Cycle assay

Cancer cell lines were retained in a 6 well plate with cell density of 2x10^5/ 2ml and were incubated for 24 h at 37^0C in a CO_2 incubator followed by treatment as mentioned in table 4.3 and incubated for 72 h [Cardenas *et al*., 2008]. Later, clarified cells were washed with PBS and 2 ml of culture medium was added to all the wells, harvested directly into 12x7 mm polystyrene tubes and centrifuged at 300 xg at 25^0C for 5 min, decanted the supernatant. Cells were fixed in 1ml of cold 70% ethanol dropwise and mixed thoroughly to avoid clumping. Further, cells were fixed for at least 30 min on ice and pelleted at high speed for 5 min. Aspirated the supernatant carefully and washed twice with PBS. To ensure the DNA staining, pelleted cells were treated with 50 µl Ribonuclease A to get rid of RNA. To each tube, propidium iodide (PI) of 400 µl per million cells was added, mixed, incubated for 5 to 10 min at room temperature and were analysed by flow cytometry.

4.2.8.4. Mitochondrial membrane potential analysis

Cancer cell lines were maintained in a 6 well plate with a cell density of 3x10^5/ml, incubated for 24 h at 37^0C in a CO_2 incubator followed by combination treatment (table 4.3) for 72 h [Cossarizza *et al*., 1993]. At the end of treatment, cells were transferred into 12x75 mm

polystyrene tubes and centrifuged at 300 xg, at 25^0C for 5 min. The cell pellet was washed with PBS (pH 7.4), supplemented with 0.5 ml JC-1 dye (5, 5′, 6, 6′-tetrachloro-1, 1′, 3, 3′-tetraethylbenzimi- diazole carbocyanine iodide) and incubated for 10-15 min at 37^0C in a CO_2 incubator. Cells were washed twice with 2 ml of 1x assay buffer (Tris-20mM, Nacl-150mM), the pellet was re-suspended in 0.5 ml of 1x assay buffer and analysed by flow cytometry.

4.2.8.5. Caspase Assay

Cancer cell lines were taken in a 6 well plate with a cell density of 0.5 x 10^6/2ml and incubated for a period of 24 h in a CO_2 incubator at 37^0C. Aspirated the media and cells were fed with the combinations provided in table 4.3 and incubated for 72 h [Dai and Krantz, 1999]. Cells were splashed with PBS, 300 µl of trypsin- EDTA was added and incubated for 3-4 min at 37^0C. Then 2 ml of growth medium was supplied, cells were picked directly into 12x75 mm polystyrene tubes and centrifuged at 300 xg, 25^0C for 5 min. Washed the cell pellet twice with PBS. The PBS free cells were fixed in 1 ml of pre chilled cold 70% ethanol and left on ice for 30 min. Pelleted the cells at high speed, discarded the supernatant and washed the cells once with PBS. Then 5 µl of FITC (Fluorescein isothiocyanate) Caspase-3 antibody was added, mixed and left at room temperature for 30 min in dark. Cells were then washed with 1X PBS contained 0.1% sodium azide, mixed the cells thoroughly with PBS and analysed by flow cytometry.

4.2.8.6. Determination of cytotoxicity on normal cell-line

Mouse fibroblast cell lines, 3T3-L1, were grown in DMEM and FBS medium and 200 µl cell suspension was seeded in a 96 well plate at required cell density ($2x10^4$ cells). To these cells, 100 µl of different concentrations (50 µg/ml, 100, 150, 200, 250 µg/ml) of standard drugs (Anastrozole and Capecitabine) and combination with quercetin (1:1) were added. Plates

were incubated for 72 h at 37°C in a 5% CO_2 atmosphere followed by addition of MTT (0.5 mg/ml) and further incubated for 3 h. Later, 100 µl of solubilisation solution (DMSO) was added to all the wells and gently stirred on a gyrator shaker and absorbance was read at 570 nm. The IC 50 value was calculated [Juillerat-Jeanneret *et al.*, 2008].

4.3. Statistical analysis

The data obtained was calculated for mean ±standard deviation. The statistical differences of all the treated groups at different time intervals were determined by ANOVA for comparison with various combinations of test compound and drug, $p < 0.05$ is considered as significant.

CHAPTER 5

RESULTS

Despite the advances in medical care, bacterial infections and cancer remain the most serious health care concerns presenting highest mortality rates. This grabs the attention of scientific community to look for novel treatment options. In continuation of such efforts, our study meets the need of existing medical care demands in contributing new knowledge to scientific literature. As per the study objectives, extraction and isolation of 'quercetin' from leaves of *A. graveolens* and *R. sativus* was successful. Attempts of screening for its antibacterial and anticancer potencies were found effective with their approach in treating infections. The results of the said objectives were described in below sections.

Preliminary screening of plant extracts for their phytochemical constituents and the results of the same is depicted in below table.

Table 5.1: Distribution and qualitative estimation of phytochemicals in leaves of *A. graveolens* and *R. sativus*

Name of the Phytochemical	***A. graveolens***	***R. sativus***
Alkaloids	Present	Present
Cardiac glycosides	Absent	Present
Glycosides	Present	Present
Flavonoids	Present	Present
Phenolics	Present	Present
Proteins	Present	Absent
Terpenoids	Absent	Present
Tannins	Present	Absent
Saponins	Absent	Absent
Steroids	Absent	Absent

Quantitative estimation of total flavonoids by Aluminium chloride method resulted from the leaves of *A. graveolens* and *R. sativus* as 128 mg and 190 mg respectively.

5.1. Process of quercetin extraction

The process of quercetin extraction was done by three different methods (Maceration, Digestion and Column chromatography). The fractions from the three methods were subjected to TLC and compared with standard quercetin's R_f values. The R_f value of standard is 0.6 and extracted quercetin by maceration, digestion and CC methods were as follows: 0.45, 0.4 and 0.57 for *A. graveolens* and 0.46, 0.4 and 0.5 for *R. sativus* respectively. All the fractions were further subjected to HPLC for checking the purity of the compound. Among the methods followed, CC method was found effective with purity of 98% as that of standard and were depicted in the below figures (5.1-5.3).

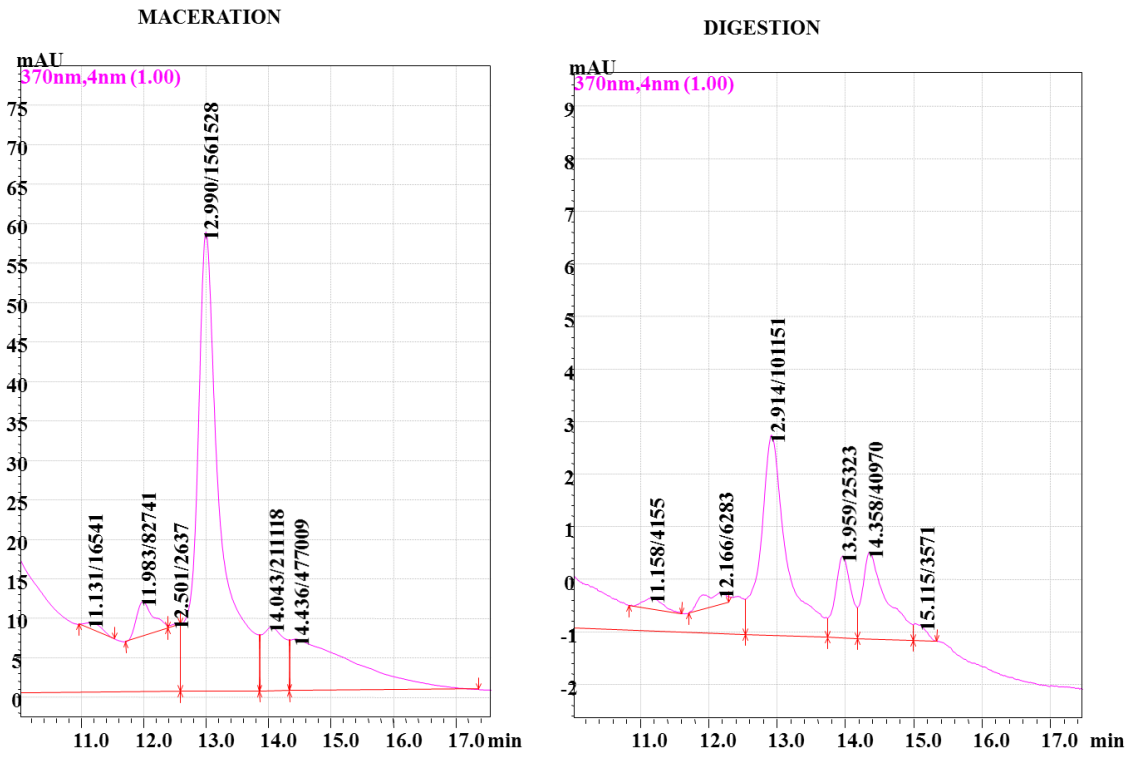


Figure 5.1: HPLC chromatogram of quercetin fraction obtained from leaves of *A. graveolens* by maceration and digestion methods

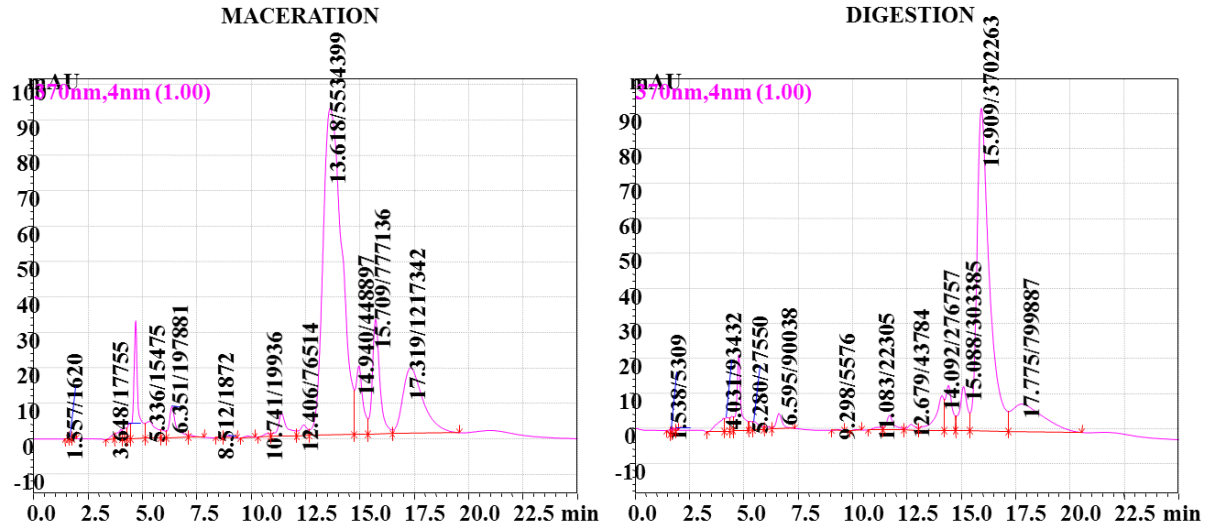


Figure 5.2: HPLC chromatogram of quercetin fraction obtained from leaves of *R. sativus* by maceration and digestion methods

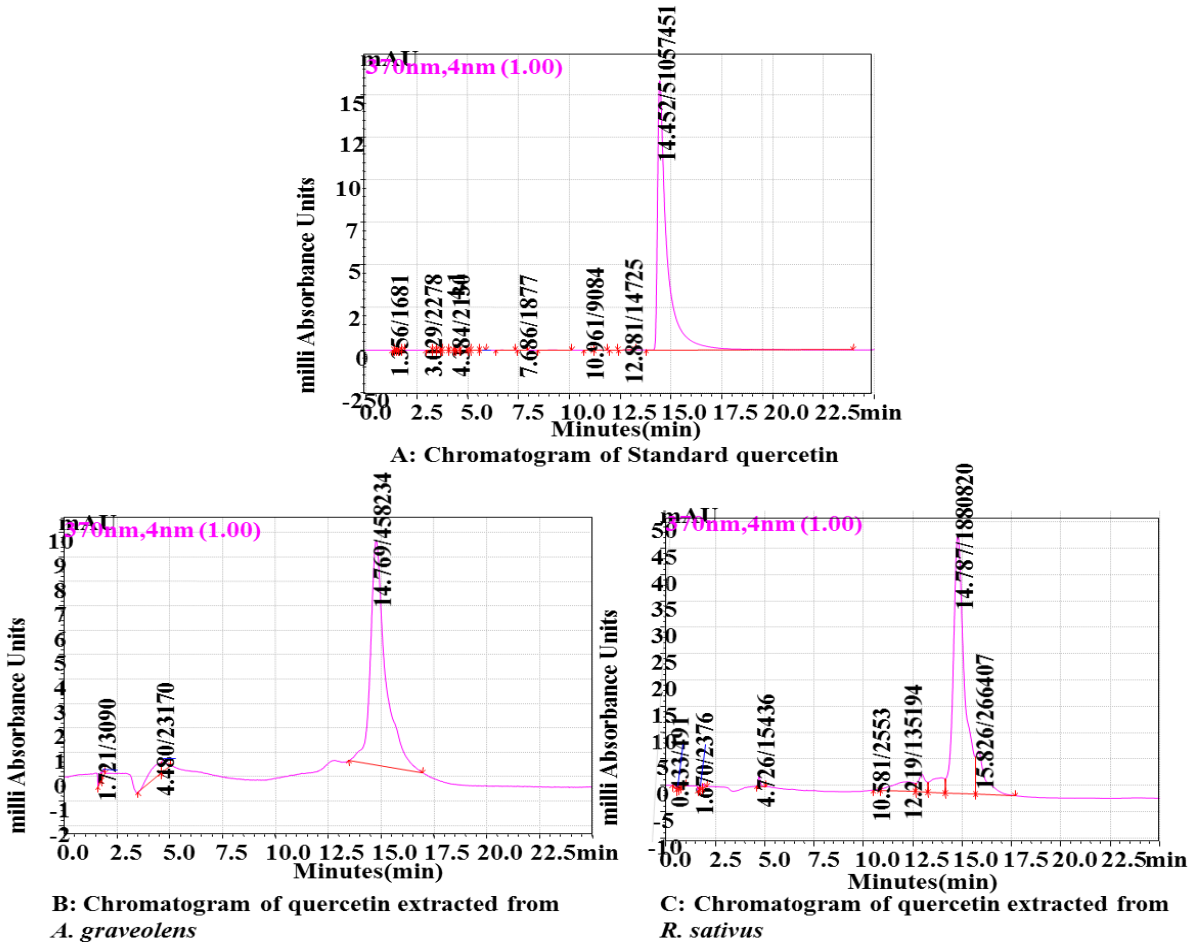


Figure 5.3: HPLC chromatograms of plant quercetin and standard quercetin by column chromatography

5.2. Spectrophotometric methods for identification of quercetin

- UV-visible spectra of fractions from leaves of *A. graveolens* (A) and *R. sativus* (B) showed maximum absorption peaks at 270 nm and 375 nm which is similar to standard quercetin and are depicted hereunder (Figure 5.4 and 5.5) .

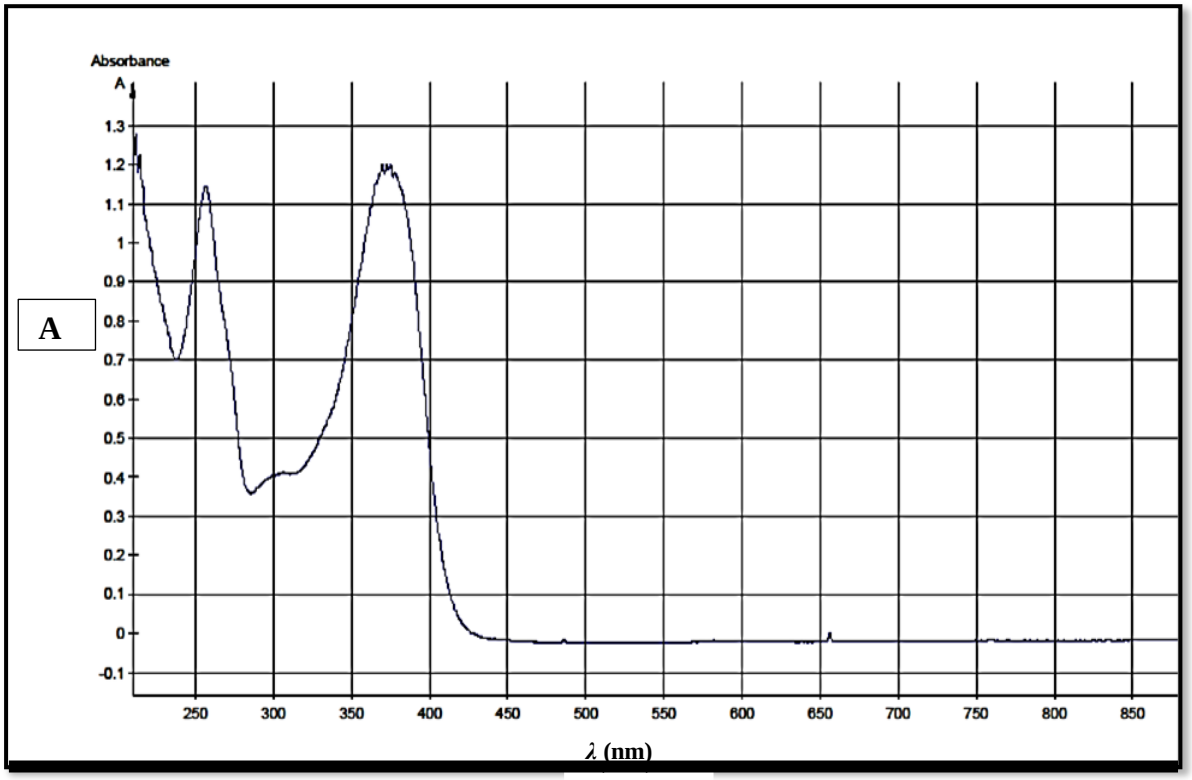
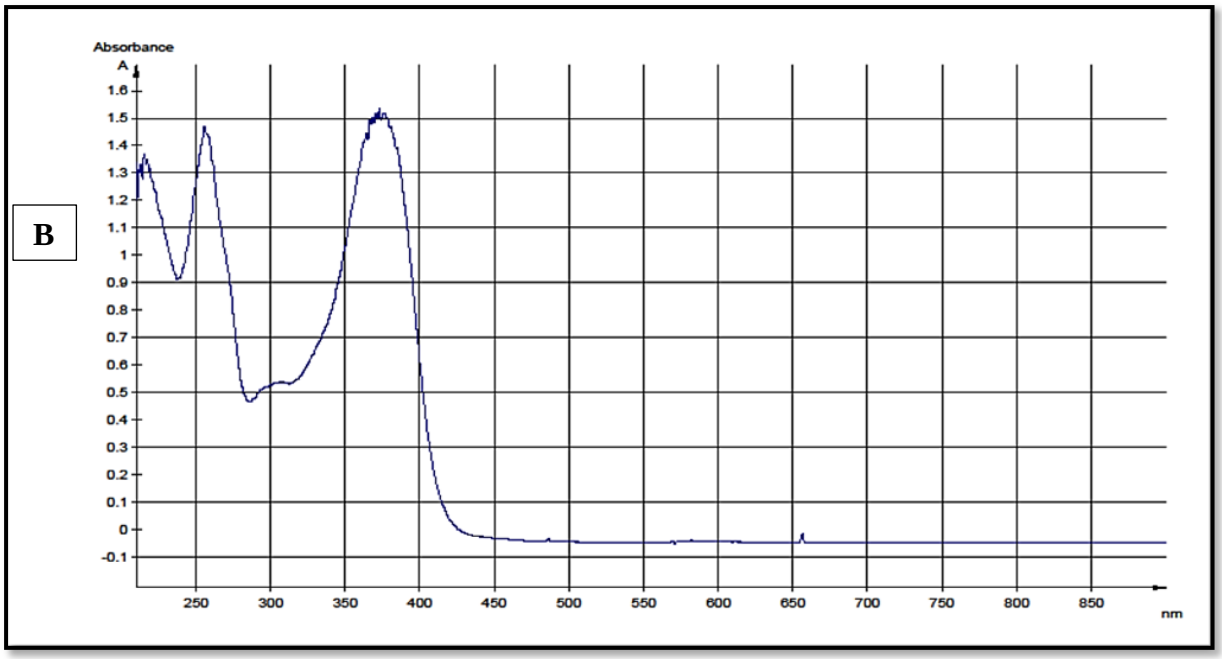


Figure 5.4: UV-visible spectrum of quercetin from *A. graveolens*



λ (nm)

Figure 5.5: UV-visble spectrum of quercetin from *R. sativus*

- The FTIR spectra of quercetin fractions from *A. graveolens* (A) and *R. sativus* (B) were shown below with its characteristic bands (Figure 5.6 and 5.7).

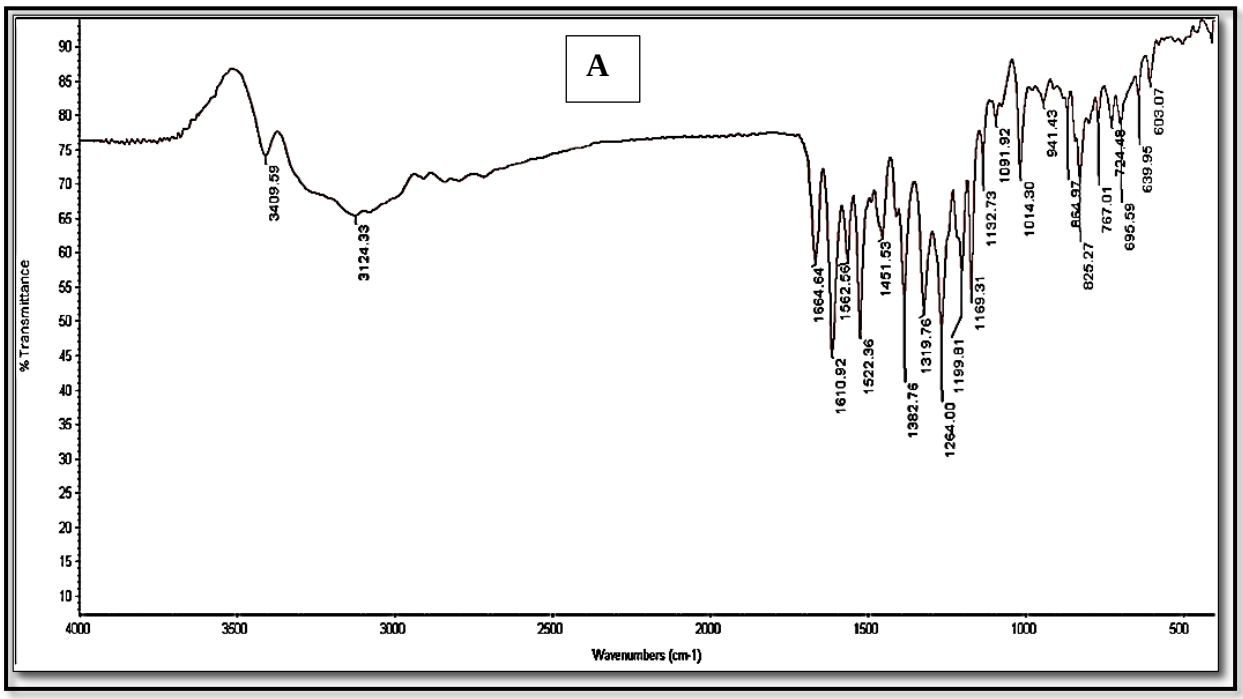


Figure 5.6: FTIR spectrum of quercetin from *A. graveolens*

The results of the FTIR were as follows; with respect to *A. graveolens* OH group stretches were found at 3409 and 3124 cm^{-1}, OH bending of the phenol function was detected at 1382 cm^{-1}. The C=O aryl ketonic stretch absorption was evident at 1664 cm^{-1}. C=C aromatic ring stretches were found at 1610, 1562, 1522 cm^{-1}.

The in plane bending of C-H in aromatic hydrocarbon was detected at 1319 cm^{-1} and out plane bending bands were found at 941, 864, 852, 767, 724, 695, 639, 603 which were attributed to C-O stretch in the aryl ether ring, the C-O stretching in phenol and C-CO-C stretch and bending in ketone respectively.

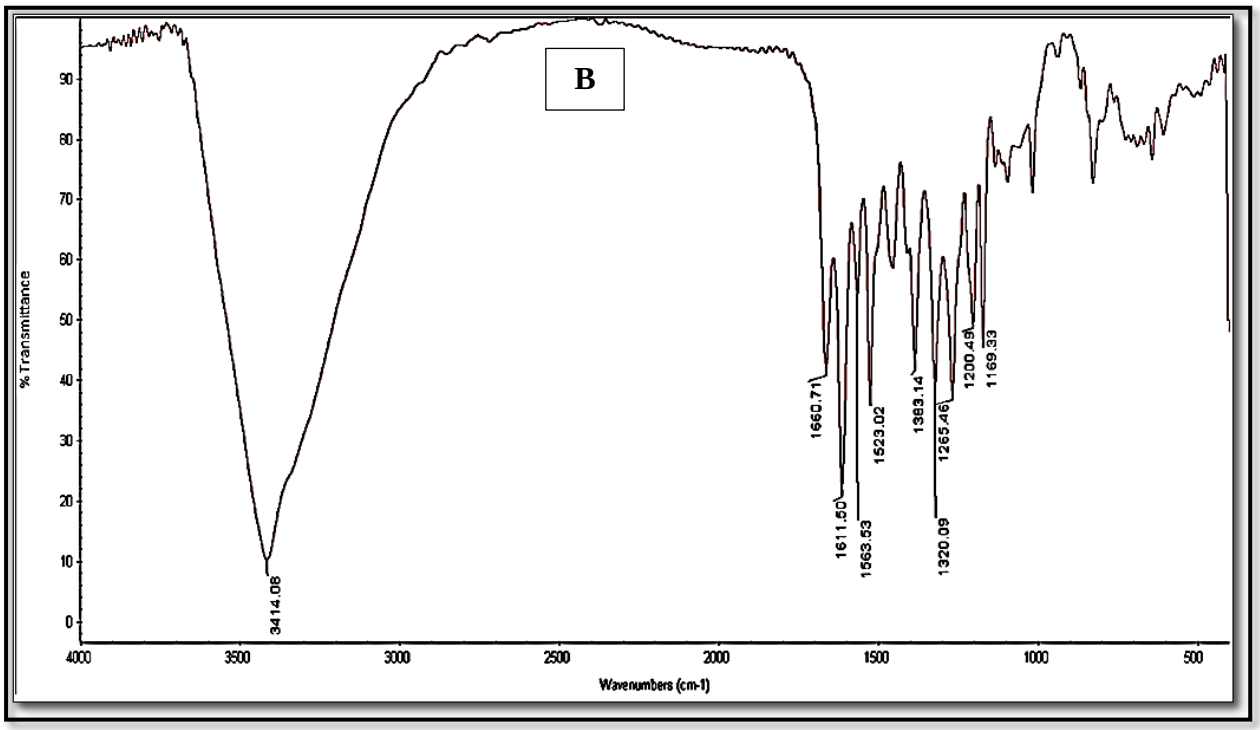


Figure 5.7: FTIR spectrum of quercetin from *R. sativus*

Where as in *R. sativus*, OH group stretches were found at 3414 cm^{-1}, OH bending of the phenol function was detected at 1383 cm^{-1}. The C=O aryl ketonic stretch absorption was evident at 1660 cm^{-1}. C=C aromatic ring stretches were found at 1611, 1563, 1523 cm^{-1}.

The in plane bending of C-H in aromatic hydrocarbon was detected at 1320 cm^{-1} and out plane bending bands were found at 942, 865, 853, 768, 725 respectively which were attributed to C-O stretch in the aryl ether ring, the C-O stretching in phenol and C-CO-C stretch and bending in ketone group. These results confirmed that the compound extracted was quercetin.

- The peaks of proton NMR of quercetin fractions are depicted hereunder (Figure 5.8).

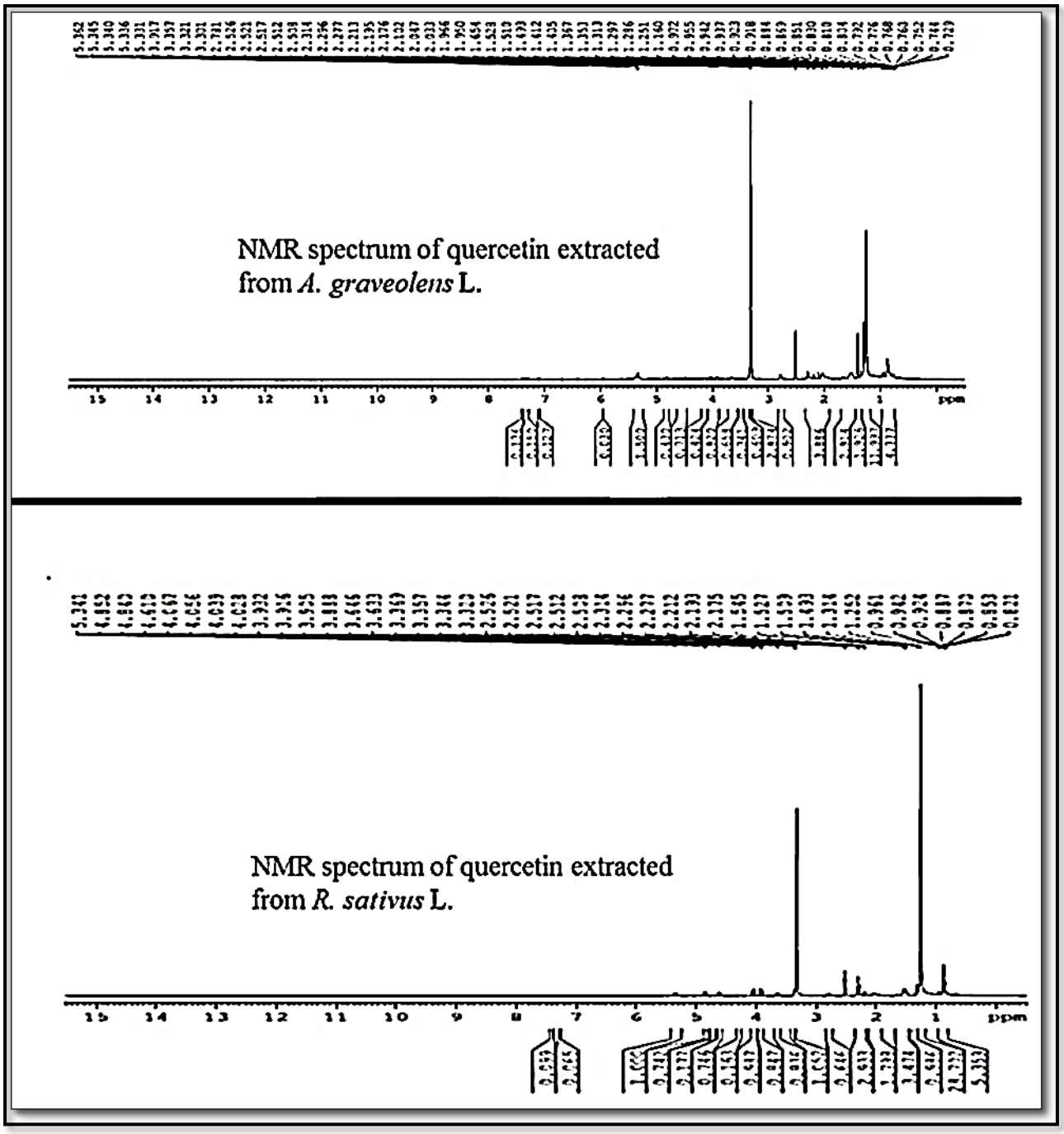


Figure 5.8: NMR spectra of quercetin extracted from both the plants

- LC-MS results presented the fact that mass/charge (*m/z*) ratio of isolated samples (A : quercetin from *A. graveolens* (Figure 5.9) and quercetin from *R. sativus* (Figure 5.10) were in agreement with the standard m/z ration i.e 303.3 (Figure 5.11)

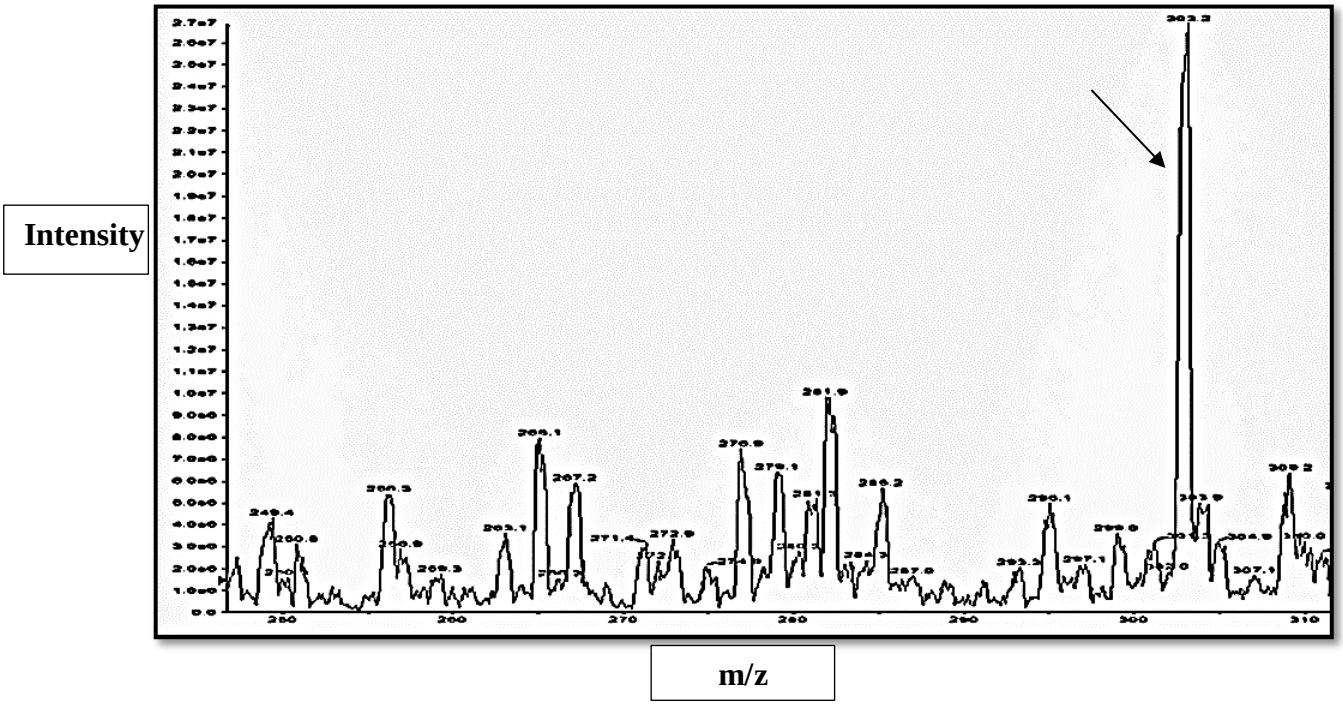


Figure 5.9: LCMS spectrum of quercetin from *A. graveolens*

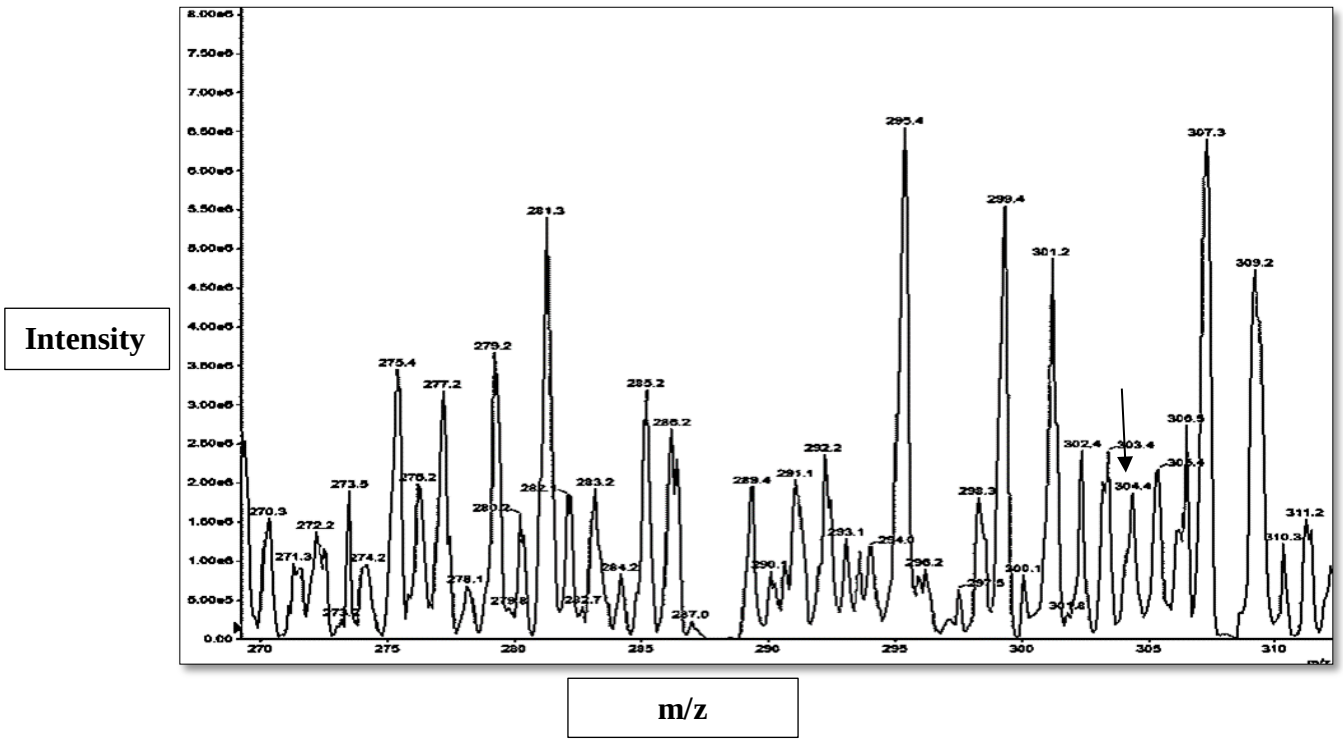


Figure 5.10: LCMS spectrum of quercetin from *R. sativus*

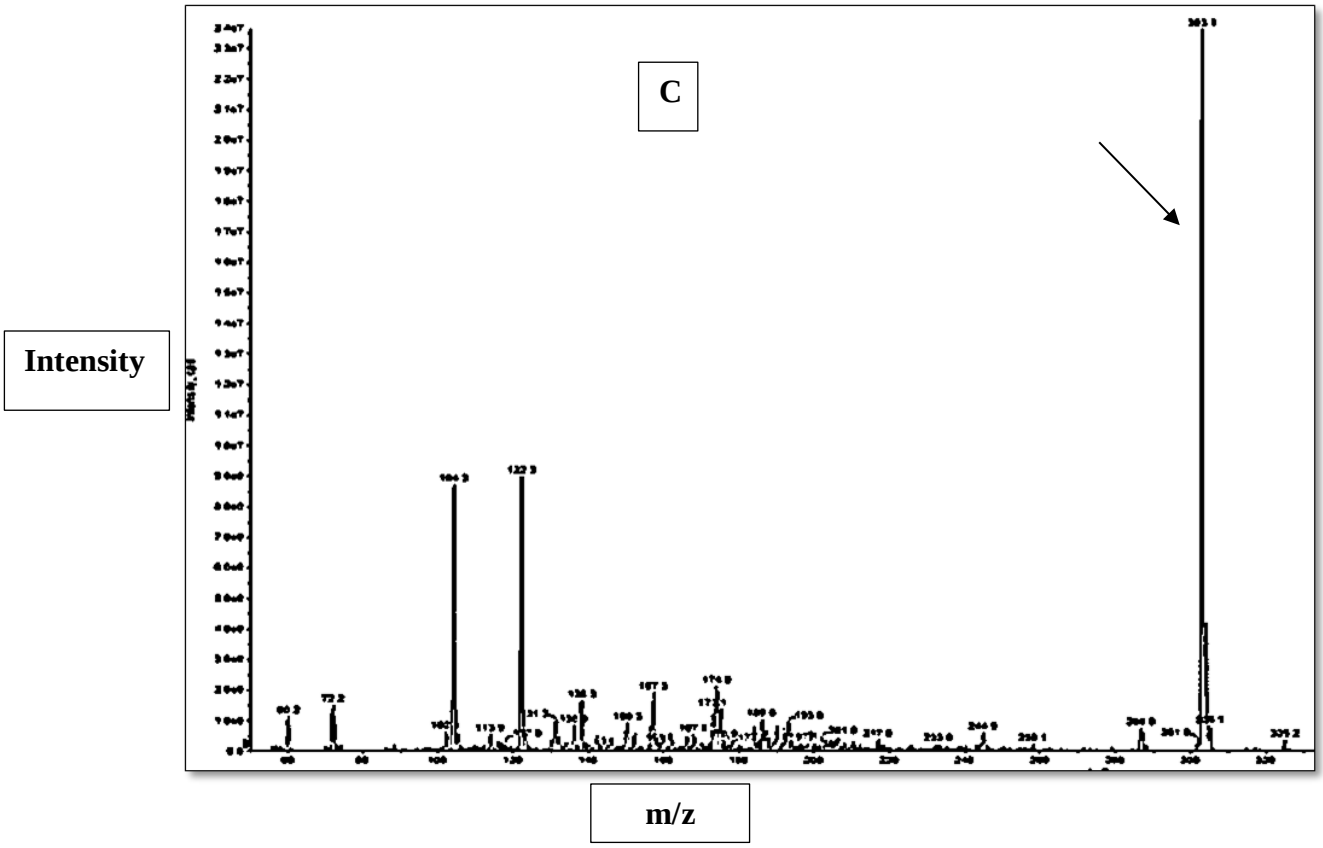


Figure 5.11: LCMS spectrum of quercetin from standard

5.3. Bactericidal activity of Quercetin

Quercetin extracted from leaf material displayed potent antibacterial activity on the tested bacteria. The initial screening by agar well diffusion method proved that quercetin from both the plants has broad spectral activity with Zone of Inhibition (ZOI) from 10 µg/ml to 250 µg/ml concentrations.

The maximum ZOI was noted at 250 µg/ml on all the organisms. ZOI of quercetin from *A. graveolens* is as follows: on *S. aureus*, the noted 22 mm is in agreement with Cefoxitin, Vancomycin and Ciprofloxacin; on *P. aeruginosa*, 20 mm is in coordination with Piperacillin, Ceftazidime and Imipenem and on *E. coli*, 18 mm is in coordination with Ampicillin, Sulphomethexazole and Chloramphenicol.

Whereas, ZOI of quercetin from *R. sativus* is as follows: on *S. aureus* the noted 24 mm is in consistent with Penicillin, on *P. aeruginosa*, 20 mm is in consistent with Piperacillin and

Imipenem; on *E.coli*, 27 mm noted ZOI was the maximum among the tested 9 antibiotics (Tables 5.2 and 5.3). To summarize, quercetin shared similar antibacterial activity compared to the tested antibiotics and in case of *E.coli*, quercetin extracted from *R. sativus* presented higher ZOI than all the high-end antibiotics tested. The above results draw a conclusion that quercetin is on par with the standard antibiotics and hence further investigations were carried out to know the exact mechanism of action.

Table 5.2: Antibacterial activity of quercetin extracted from *A. graveolens* and *R. sativus*

Concentration in µg/ml	***P. aeruginosa* (ZOI in mm)**		***S. aureus* (ZOI in mm)**		***E.coli* (ZOI in mm)**	
	A.graveolens	***R.sativus***	***A.graveolens***	***R.sativus***	***A.graveolens***	***R.sativus***
10	06	06	06	06	06	06
20	06	06	06	6.5	07	07
30	07	6.5	07	6.5	08	7.2
40	07	07	08	7.5	09	7.5
50	7.5	07	10	8.5	12	10
100	08	08	14	12	14	13
150	9.5	09	16	15	16	18
200	13	12	18	18	17	23
250	20	20	22	24	18	27

Table 5.3: Antibacterial activity exhibited by antibiotics on selected pathogens

P. aeruginosa		***S. aureus***		***E. coli***	
Antibiotic	**ZOI (mm)**	**Antibiotic**	**ZOI (mm)**	**Antibiotic**	**ZOI (mm)**
Piperacillin	20	Azithromycin	19	Ampicillin	18
Ceftizidime	19	Erythromycin	19	Cephelexin	23
Gentamycin	18	Ciprofloxacin	22	Amikacin	20
Tazobactum	21	Penicillin	25	Amoxicillin	22
Amikacin	18	Trimethoprim	16	Sepifime	19
Aztronam	24	Cefoxitin	22	Chloramphenicol	18
Ciprofloxacin	23	Tetracycline	18	Sulphomethoxazole	18
Imipenem	20	Vancomycin	22	Cefuroxime	23
Levofloxacin	18	Gentamycin	16	Trimethoprim	21

5.3.1. Limiting bacterial motility by quercetin

Bacteria exhibit gliding, spirochaetal, swarming, swimming and twitching motility patterns by flagella, cilia and pili. These motilities help the bacteria to change the direction, get the

nutrients, avoid and escape from the unfavourable conditions. Hence, restricting its motility plays a vital role in controlling its spread and colonization. The study results showed that plant quercetin restricted swarming, swimming and twitching motility patterns as low as 10 µg/ml and as high as 100 µg/ml. Comparison of all the results draws a conclusion that twitching motility (by pili) is found to be restricted more than the other two motilities (Figure 5.12). The impact of quercetin in restricting motilities of each bacterium is discussed hereunder based on spread area from point of inoculation as compared with control without quercetin.

- In *P. aeruginosa*, quercetin from both the plant leaf material equally restricted swarming motility. In swimming and twitching, quercetin extracted from *A. graveolens* has shared better restriction ability than from *R. sativus*. The overall restriction ability was high in twitching motility by quercetin from *A. graveolens*.
- In *S. aureus*, quercetin from *A. graveolens* and *R. sativus* similarly restricted all the three motilities. Whereas, the restriction is higher in swimming and twitching motilities.
- In *E.coli*, quercetin from both the plant leaf material exhibited similar restriction motilities, but highest restriction was found in both swimming and twitching motilities.

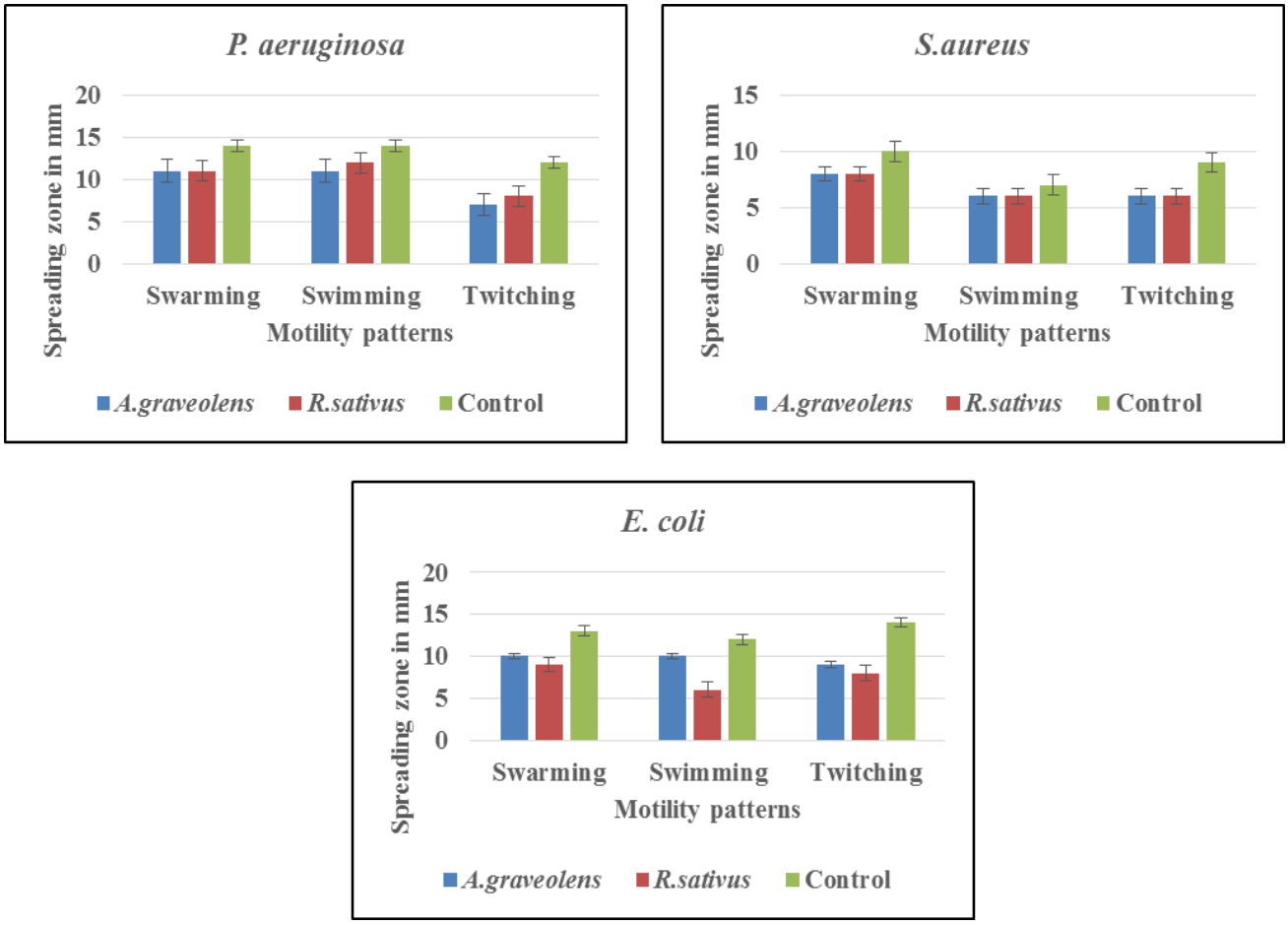


Figure 5.12: Impact of quercetin extracted from plant sources on bacterial motility

5.3.2. Regulation of biofilm formation by quercetin

The formation of biofilm is an important tool for developing resistance in bacteria. Hence, control of biofilm formation is an interesting strategy. Quercetin extracted from both the plant species in the current study had restricted biofilm formation in a dose dependent manner (Figure 5.13). It was noted that the highest biofilm activity was achieved by quercetin extracted from *R. sativus* on *S. aureus* with 99% inhibition, followed by quercetin from *A. graveolens* with 98% inhibition. The lowest biofilm inhibition was noted in *E. coli* with 44% from both the quercetin fractions. The overall conclusion was, quercetin could successfully inhibit biofilm formation with minimal concentration which is a novel observation of the study.

Table 5.4: Inhibition of biofilm formation by quercetin fractions extracted from plant source

Conc. (µg)	Biofilm inhibition by quercetin from *A. graveolens* (%)			Biofilm inhibition by quercetin from *R. sativus* (%)		
	P. aeruginosa	***S. aureus***	***E. coli***	***P. aeruginosa***	***S. aureus***	***E. coli***
10	30	90	28	30	92	27
20	32	92	29	31	91	28
30	34	93	30	32	92	29
40	42	94	32	39	92	29
50	45	95	34	43	96	34
60	50	95	35	49	96	34
70	52	96	40	50	97	40
80	59	98	42	52	97	41
90	60	98	43	59	98	42
100	64	98	44	63	99	44

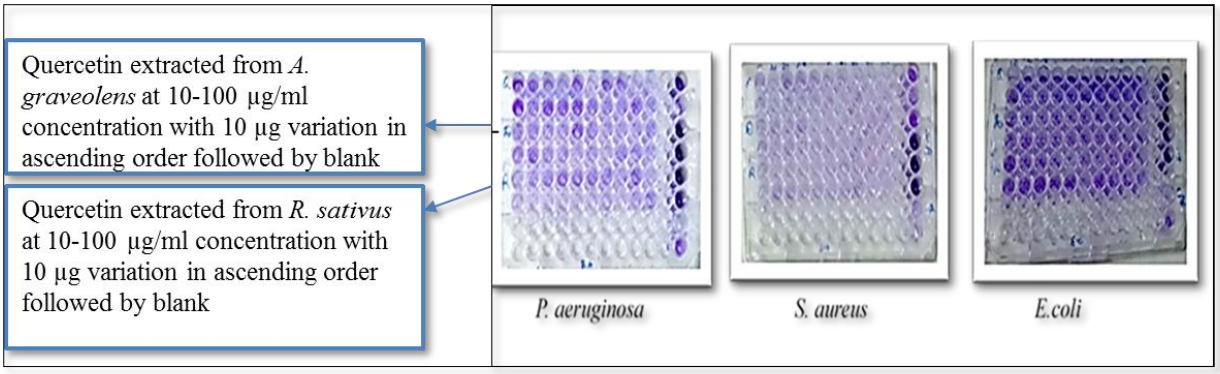


Figure 5.13: Anti-biofilm activity of quercetin extracted from plant sources

5.3.3. Induced effect of quercetin on release of absorbing materials and proteins

Release of absorbing materials and proteins take place in bacteria through the cytoplasmic membrane that control the permeability of different compounds. Cell membrane acts as a barrier for maintenance of the energy status of the cell, transport of solute, control and regulation of metabolism. Hence, measure of marked leakage of cytoplasmic material is an indicative of gross and irreversible damage to the cytoplasmic membrane. The tested bacteria lost the cell constituents at lowest (10 µg/ml) and highest concentration (100 µg/ml) concentrations of quercetin, indicating damage to cytoplasmic membrane (Figure 5.14). Any compound to be considered as an antibiotic shall be its maximum efficacy at minimum concentration which is proven in the study and the results are as follows.

At 260 nm, quercetin from *A. graveolens* exhibited marked release of cellular contents at 10 µg/ml and at 100 µg/ml with promising results in *E.coli* and *S. aureus*. At 280 nm also quercetin from *A. graveolens* exhibited membrane permeability by all the tested bacteria at 10 µg/ml and at 100 µg/ml and *S. aureus* showed highest permeability.

At 260 nm; quercetin from *R. sativus* exhibited marked release of cellular components at 10 and 100 µg/ml concentration with highest in *E.coli* than the other two. At 280 nm; quercetin from *R. sativus* exhibited membrane permeability by all the bacteria at 10 µg/ml and 100 µg/ml concentration with highest in *S. aureus*.

The overall activity of quercetin proved that it can induce loss of materials at 260 and 280 nm and interestingly was higher in *S. aureus* and *E.coli* than from *P. aeruginosa* by both the plant extracts.

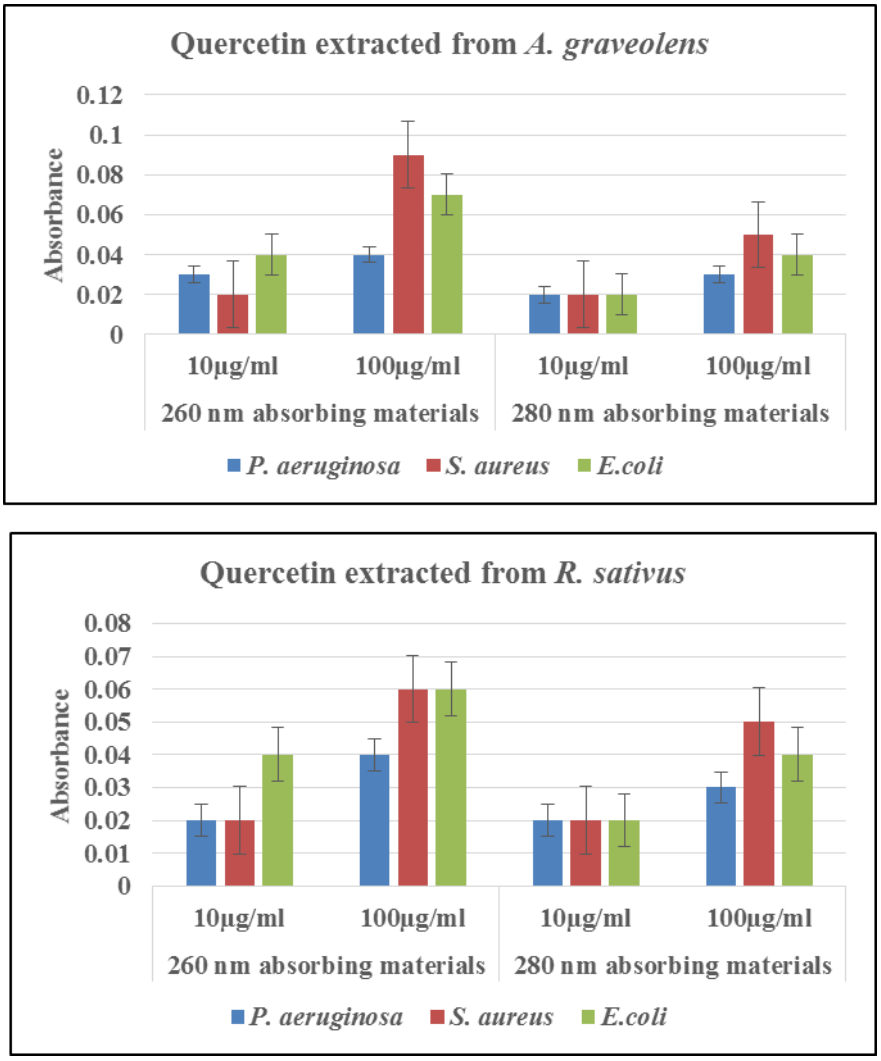


Figure 5.14: Impact of quercetin on release of proteins and nucleic acids

5.3.4. Quercetin induced DNA fragmentation

Naturally occurring compounds are important agents to test for any modality as they exhibit limited toxicity. The shredding of bacterial genomic DNA is one of the early events of destruction. The bacteria (*P. aeruginosa*, *S. aureus* and *E.coli)* screened for the DNA fragmentation capacity when treated with quercetin showed cleavage of DNA at lowest as well as highest concentrations (10 and 100 µg/ml) after 24 h incubation. Among all tested bacteria, *P. aeruginosa* and *E.coli* showed more cleaved DNA fragments than *S. aureus* which is depicted in the figure (Figure 5.15).

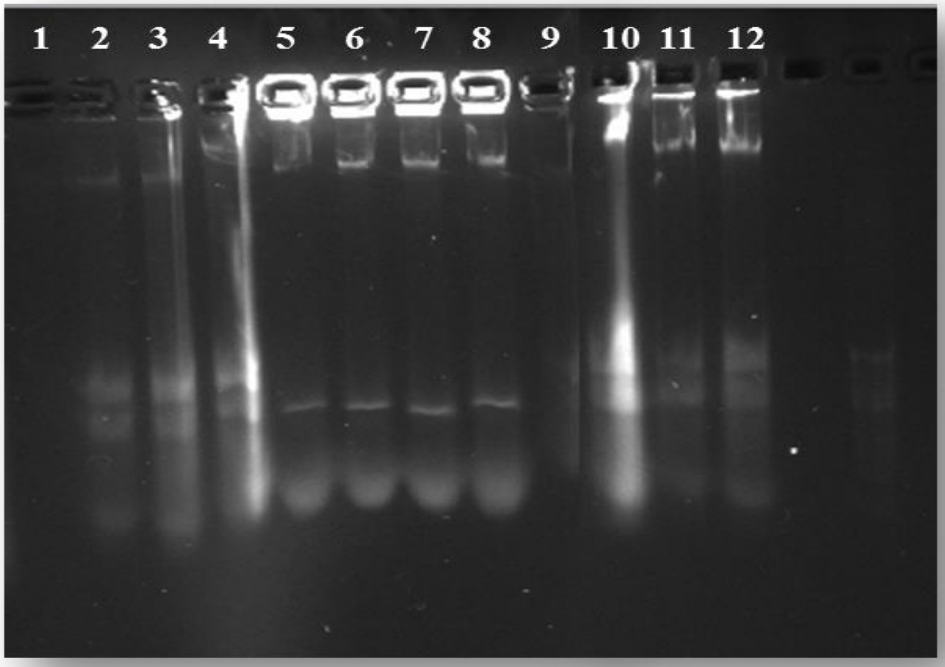


Figure 5.15: Quercetin induced DNA fragmentation on tested bacteria. (1, 2: quercetin extracted from *A. graveolens* on *P. aeruginosa*; 3, 4: quercetin extracted from *R. sativus* on *P. aeruginosa*; 5, 6: quercetin extracted from *A. graveolens* on *S. aureus;* 7, 8: quercetin extracted from *R. sativus* on *S. aureus*; 9, 10: quercetin extracted from *A. graveolens* on *E.coli* and 11, 12: quercetin extracted from *R. sativus* on *E.coli*).

5.4. Quercetin as an anticancer agent

Determining the anticancer potential of quercetin draws our attention in screening its cytotoxic ability by MTT assay. The assay was applied for all the cancer cell lines and the detailed description of the results was given hereunder for each cell line.

In Breast cancer cell line, quercetin fractions from *A. graveolens* (S1), *R. sativus* (S2) and Anastrozole exhibited cytotoxicity at three time points (24h, 48 and 72h) and showed maximum activity at 72 h time interval. IC 50 values were calculated from the obtained observations and depicted in figure 5.16, 5.17 and 5.18 and table 5.5.

The combinations of quercetin (S1 and S2) with the Anastrozole and Capecitabine showed significant cytotoxicity at lowest concentrations than standard drug and these observations were reported first in the study (Figures 5.19 and 5.20).

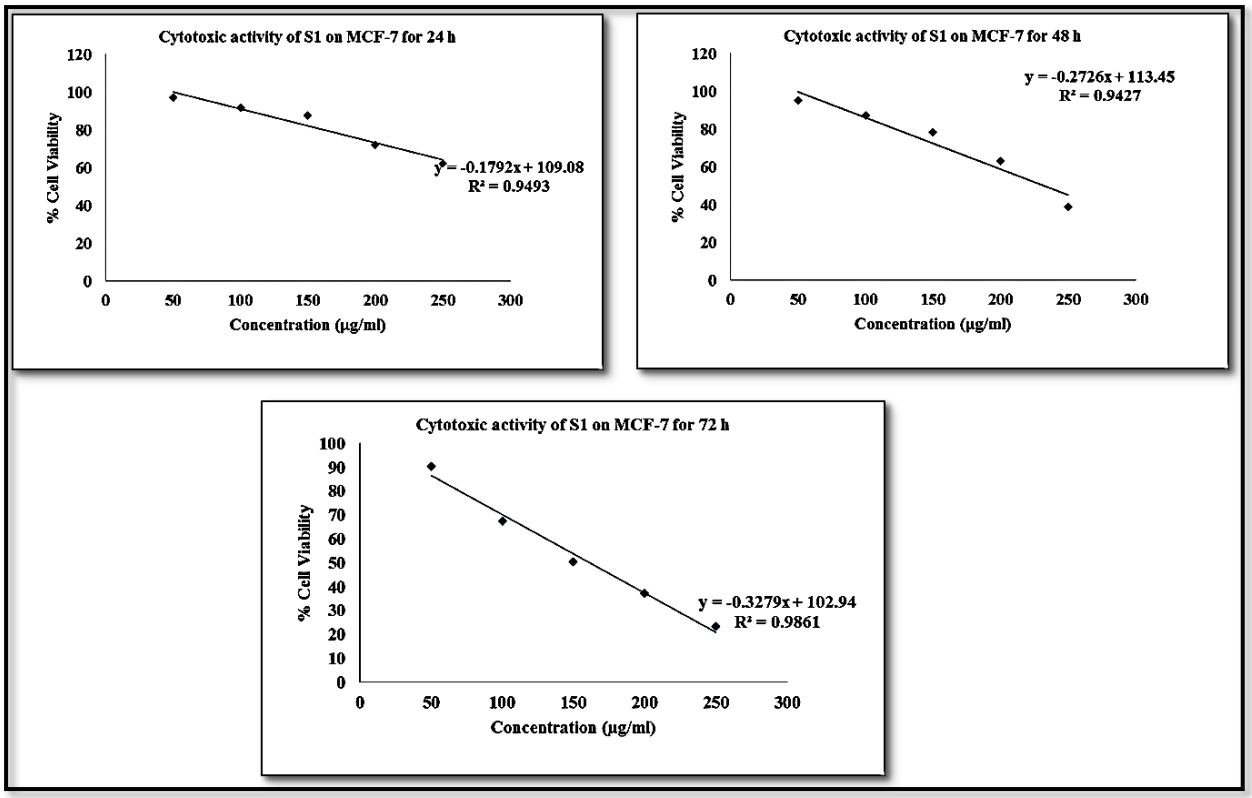


Figure 5.16: Cytotoxicity of S1 on MCF-7 at three time points

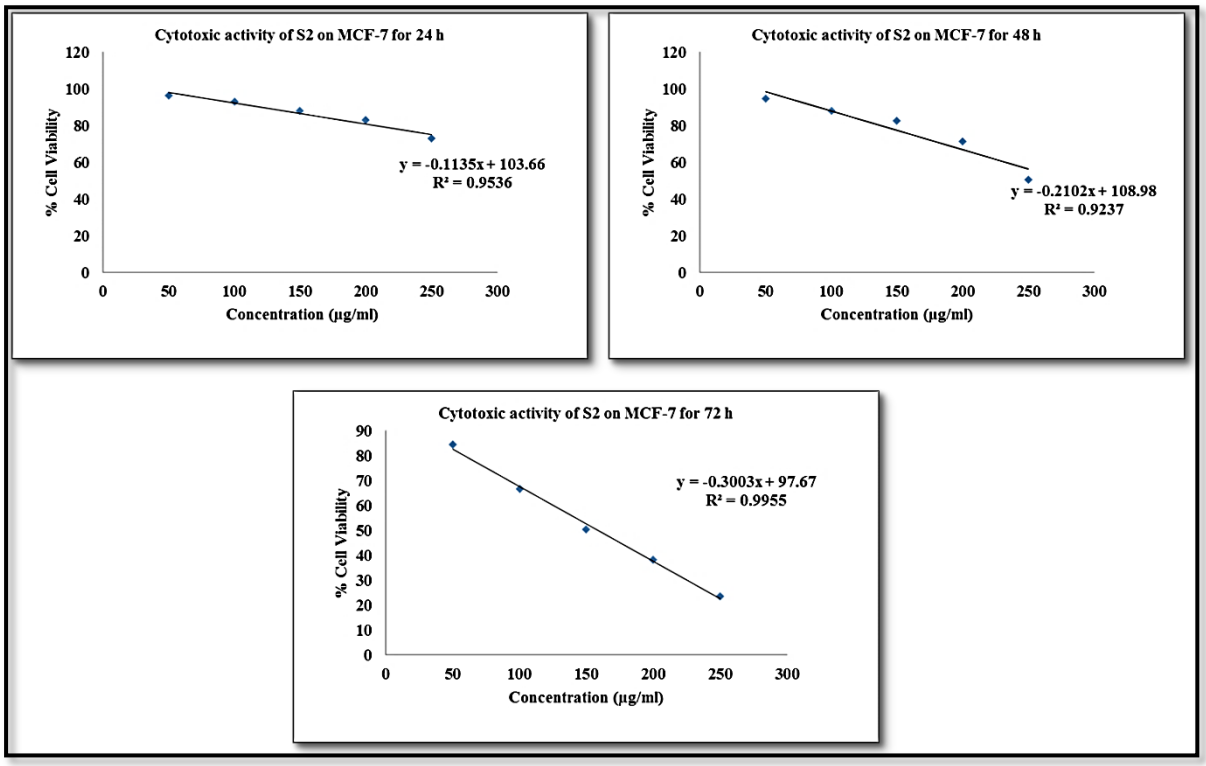


Figure 5.17: Cytotoxicity of S2 on MCF-7 at three time points

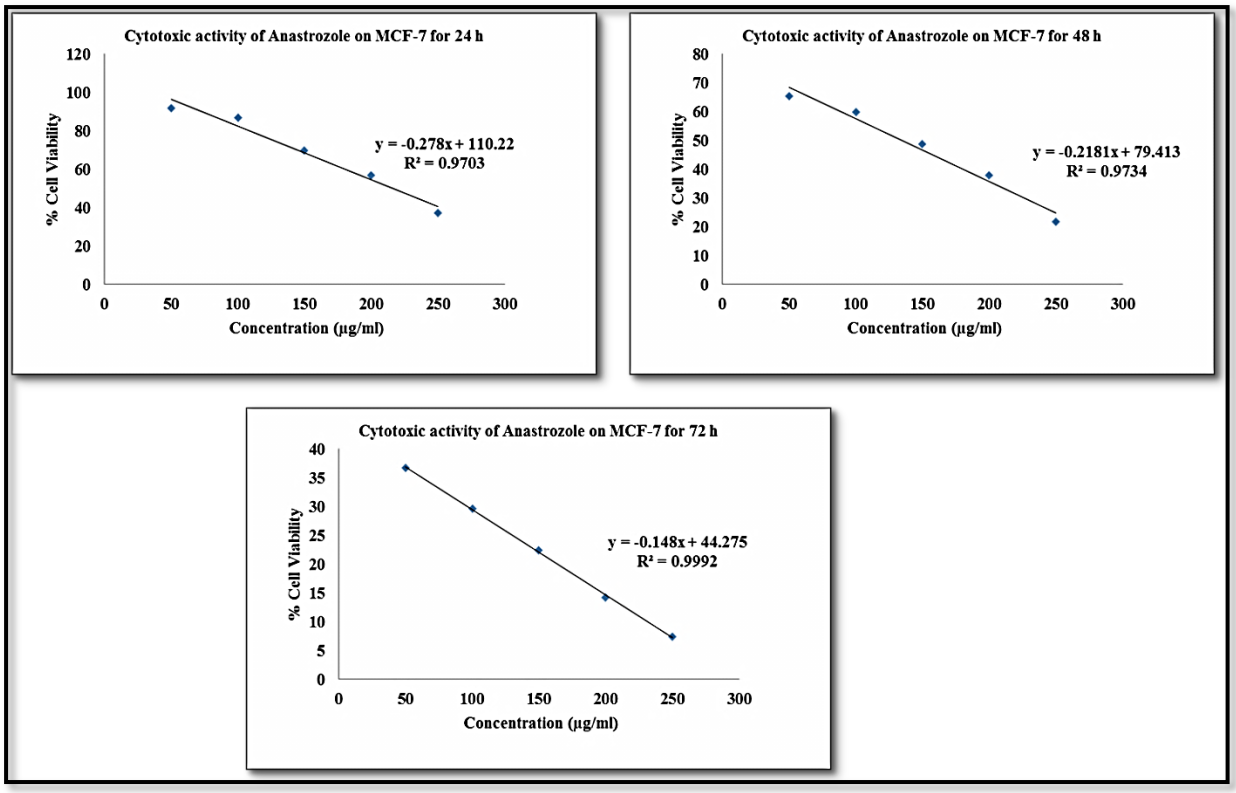


Figure 5.18: Cytotoxicity of Anastrozole on MCF-7 at three time points

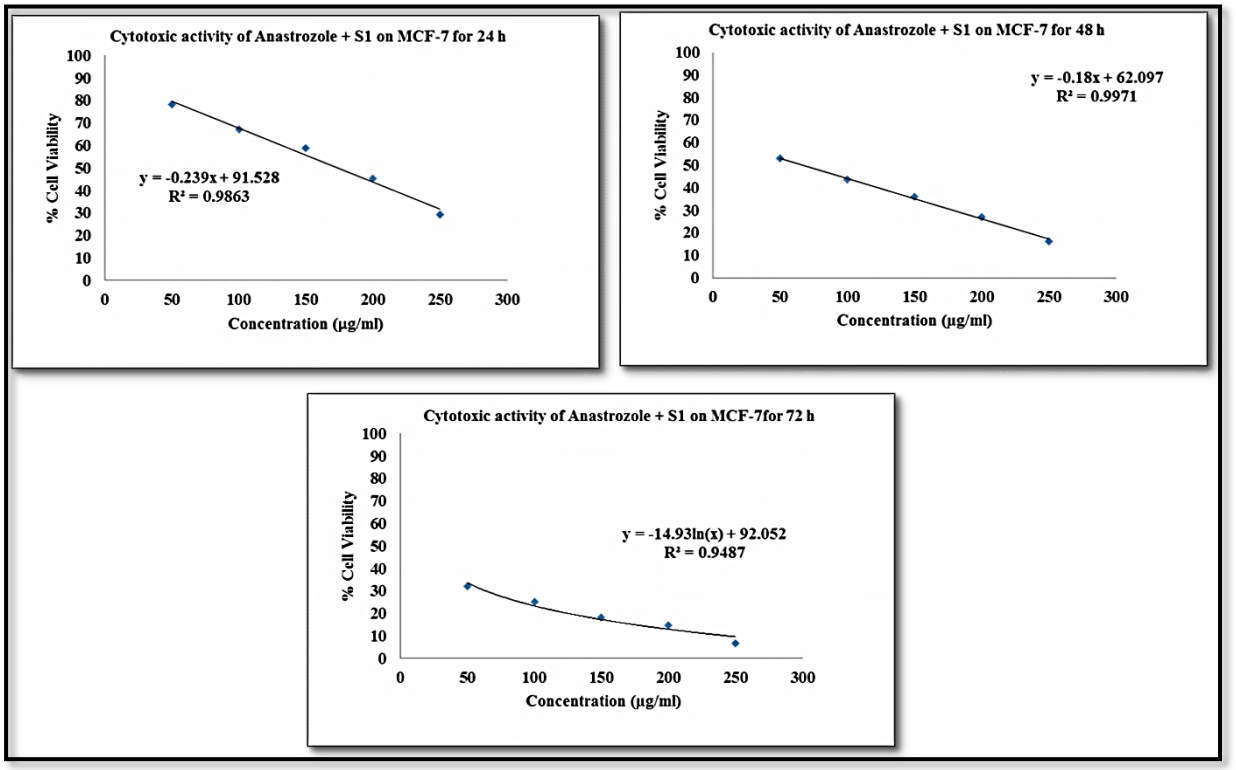


Figure 5.19: Cytotoxicity of combination of Anastrozole and S1 on MCF-7 at three time points

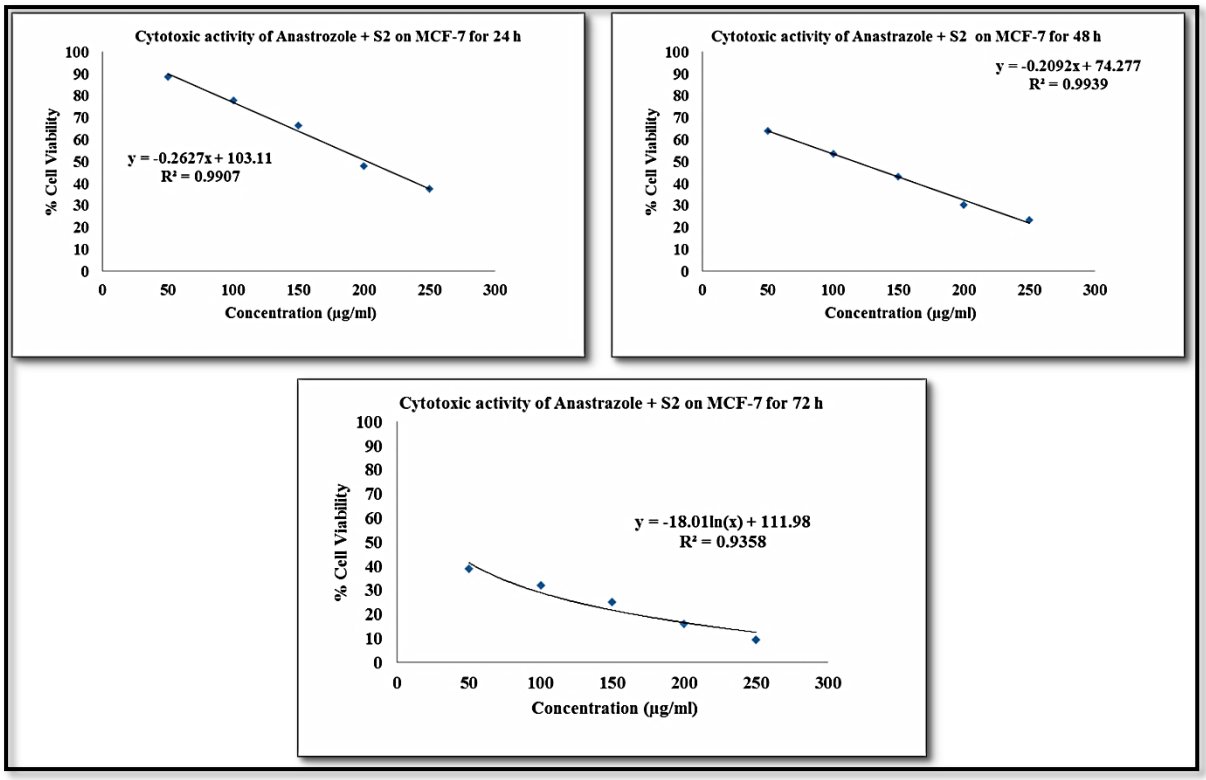


Figure 5.20: Cytotoxicity of combination of Anastrozole and S2 on MCF-7 at three time points

Table 5.5: IC 50 values of test compounds on MCF-7 cell line at different time intervals

S. No.	Test compounds and combinations	IC 50 in µg/ml for 24 h	IC 50 in µg/ml for 48h	IC 50 in µg/ml for 72 h
01	Quercetin from *A. graveolens* (S1)	329	233	161
02	Quercetin from *R. sativus* (S2)	474	280	158
03	Anastrozole(Std)	216	134	38
04	S1+ Std	173	67	16*
05	S2+ Std	202	116	31*

Note: ANOVA is performed for comparison of groups and found significant with P value < 0.05

Similar to the breast cancer results, in colon cancer cell line, quercetin fractions of *A. graveolens* (S1) and *R. sativus* (S2) and Capecitabine proved to have cytotoxic potential at three time points (24 h, 48 and 72 h) and showed maximum activity at 72 h time interval

(Figures 5.21, 5.22 and 5.23). However, the same quercetin when combined with Capecitabine, showed cytotoxicity same as that of standard drug with less Capecitabine concentration (Figures 5.24 and 5.25; table 5.6).

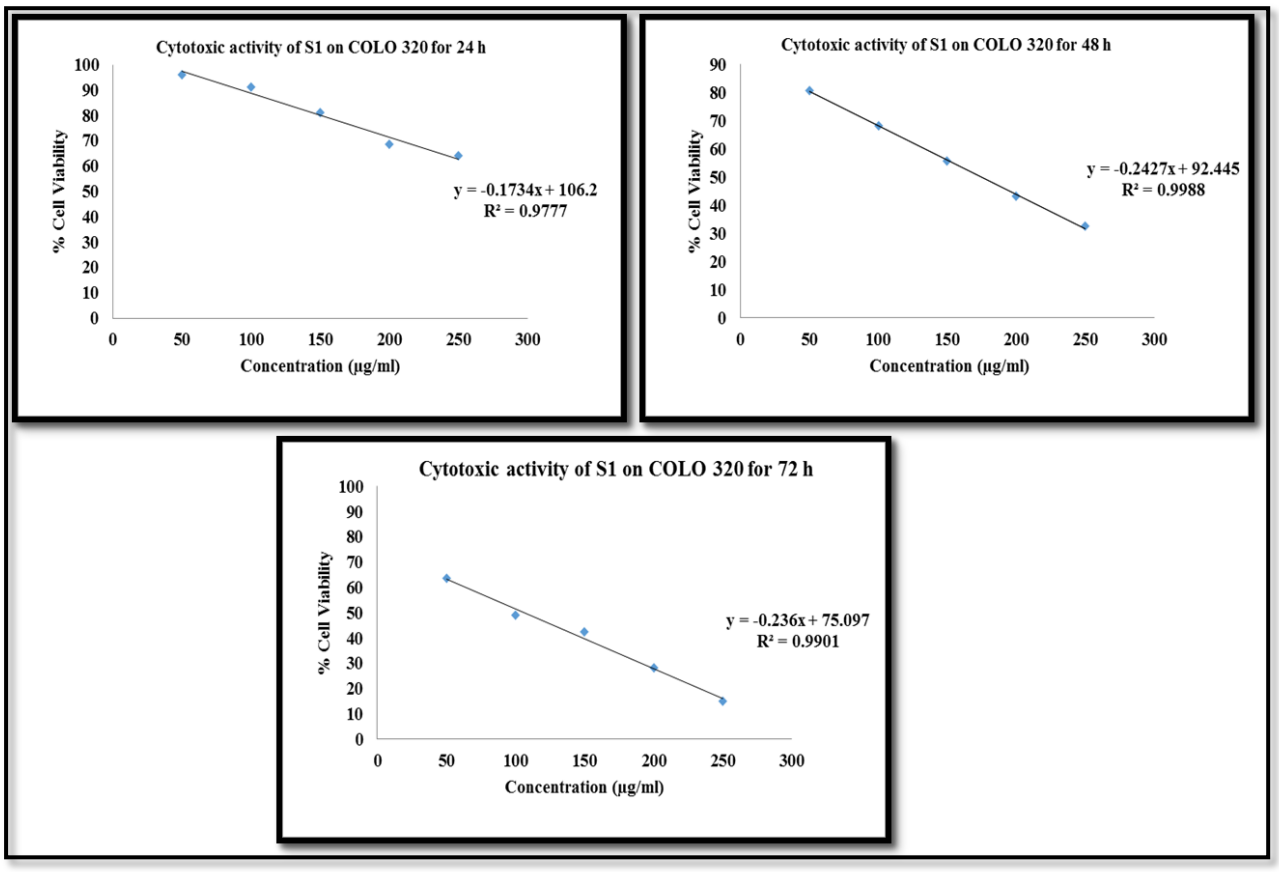


Figure 5.21: Cytotoxic activity of S1 on COLO 320 at three time points

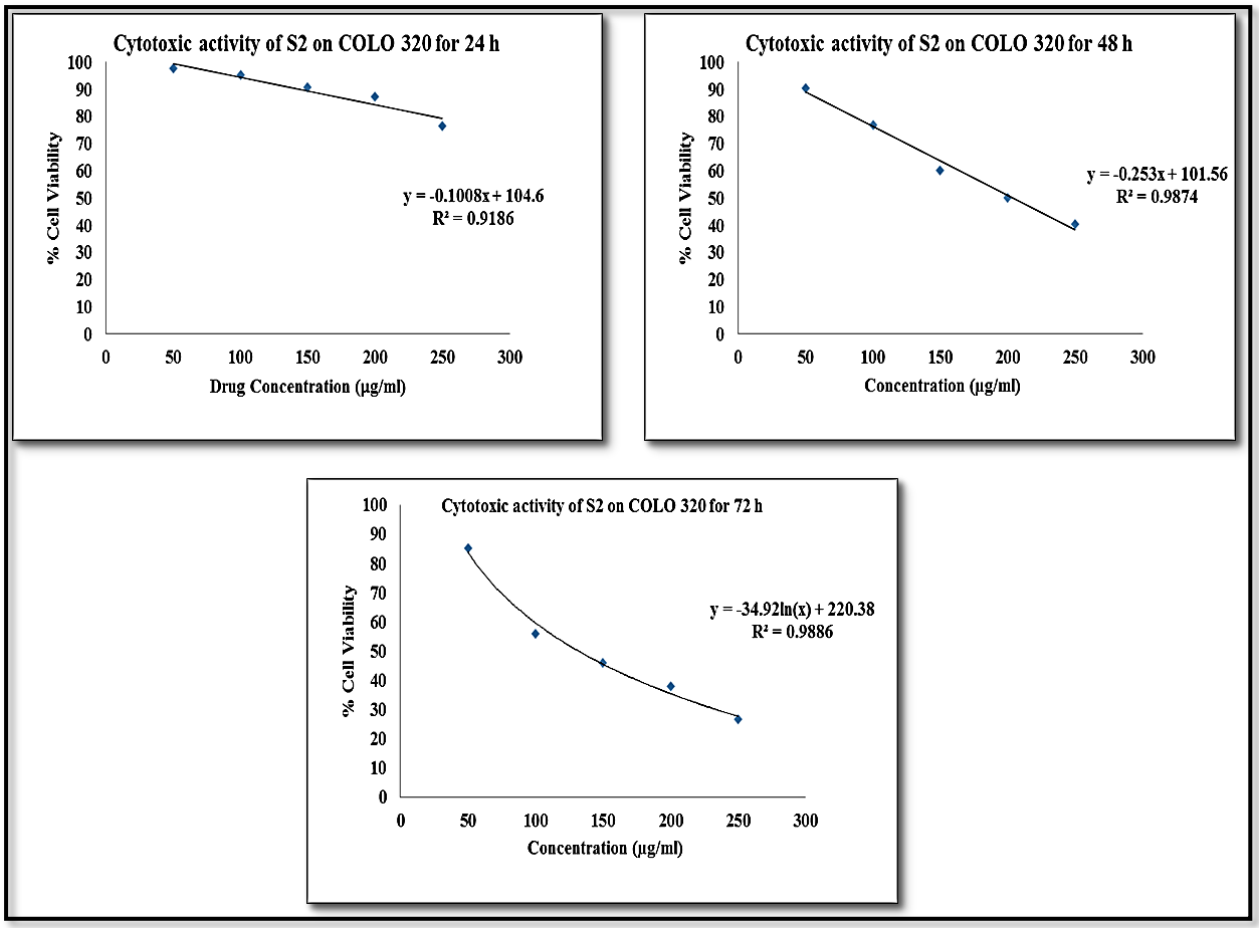


Figure 5.22: Cytotoxicity of S2 on COLO 320 at three time points

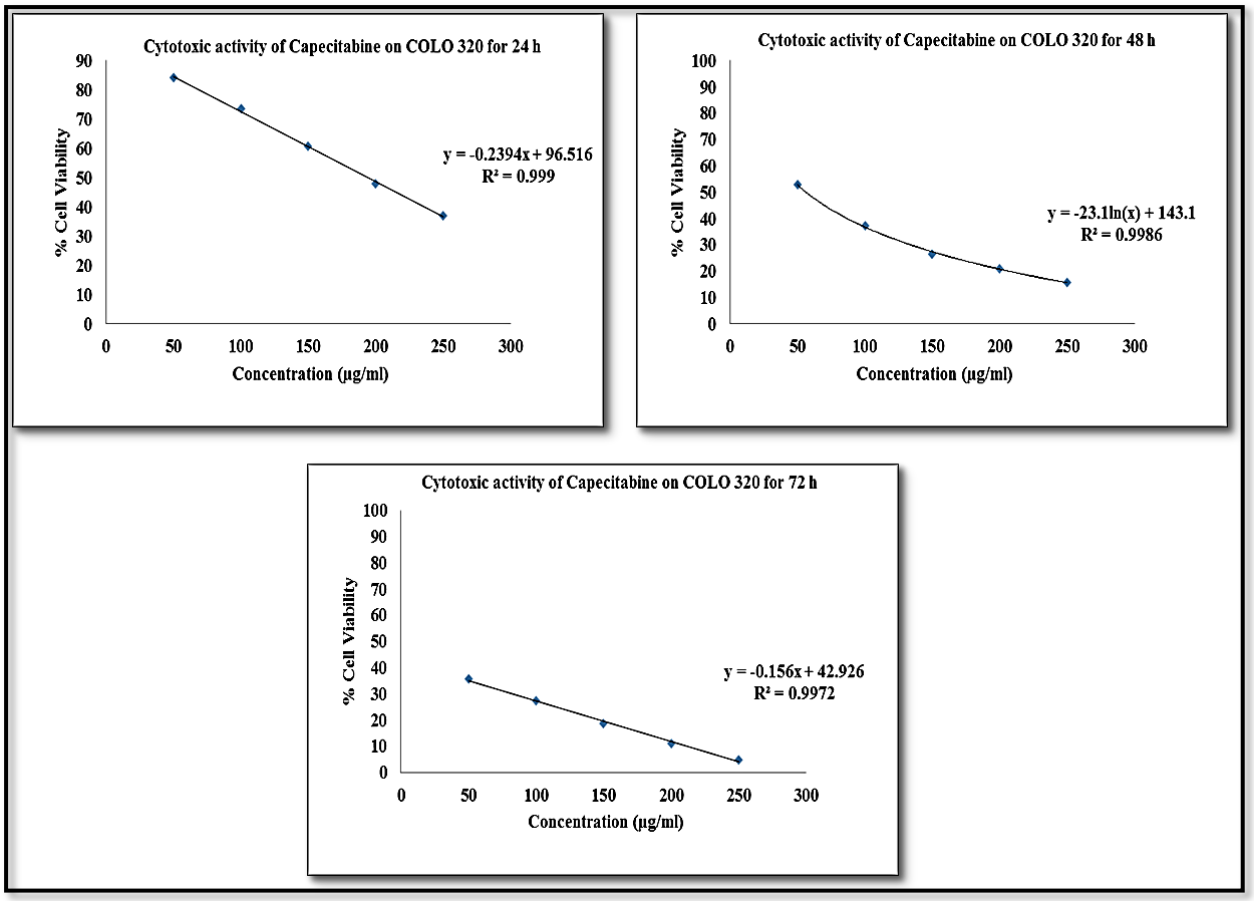


Figure 5.23: Cytotoxicity of Capecitabine on COLO 320 at three time points

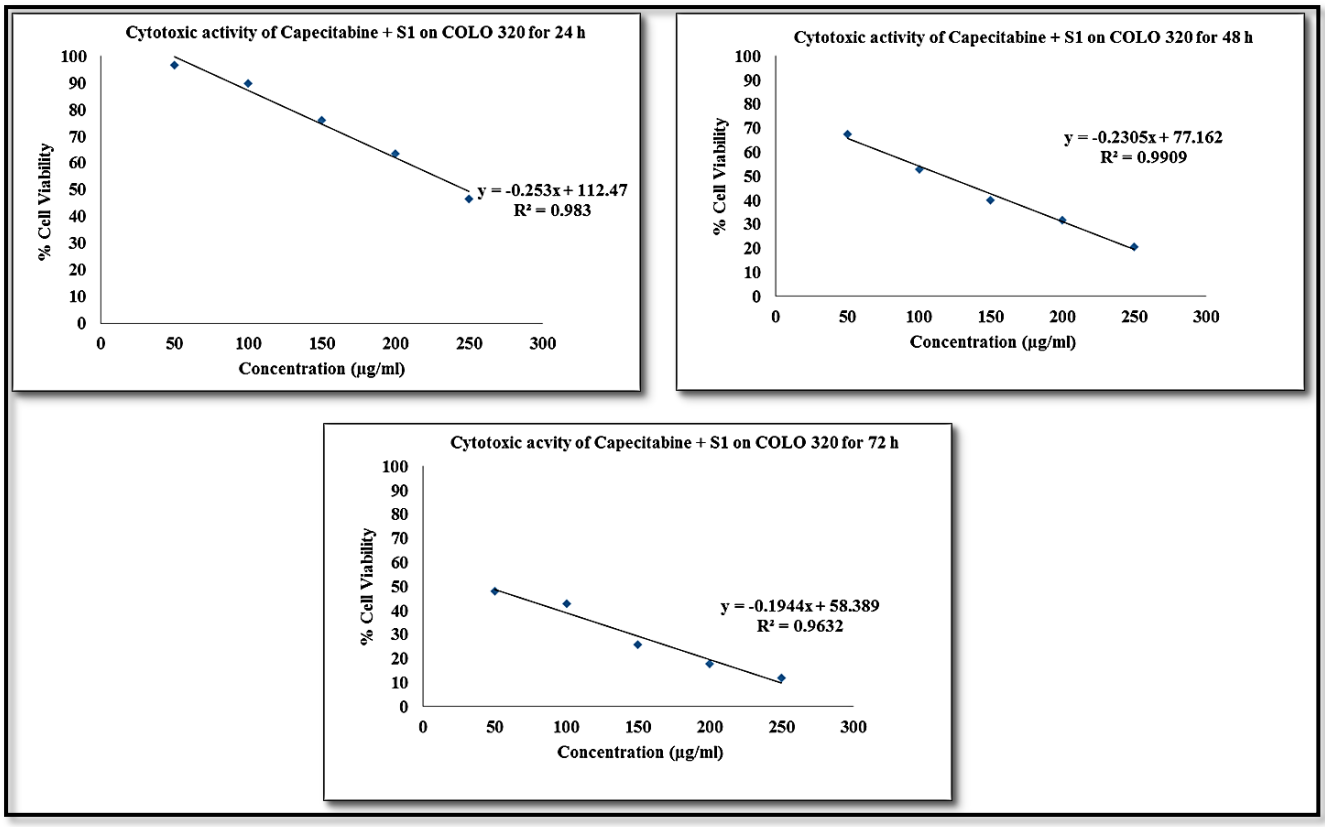


Figure 5.24: Cytotoxicity of combination of Capecitabine and S1 on COLO 320 at

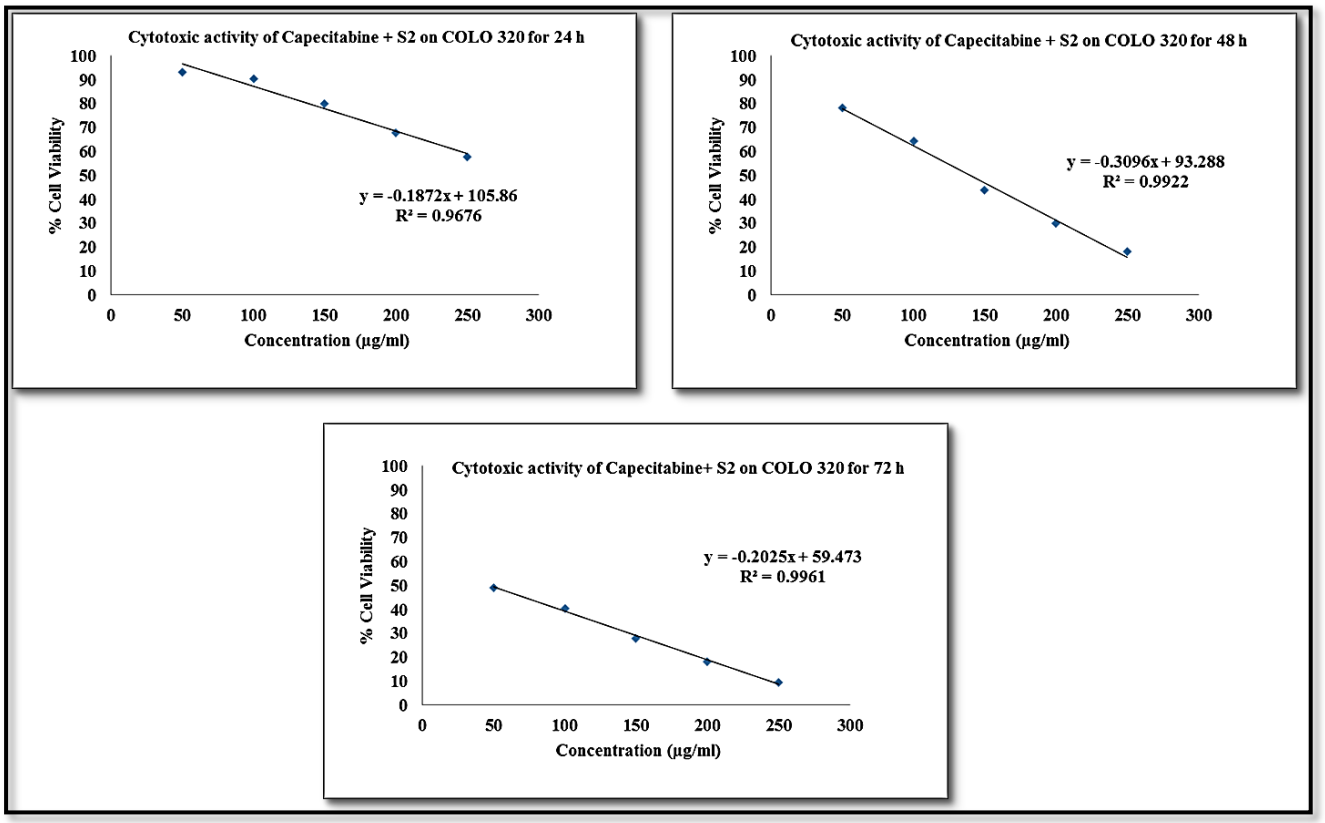


Figure 5.25: Cytotoxicity of combination of Capecitabine and S2 on COLO 320 at three time points

Table 5.6: Cytotoxic potential of test compounds on COLO 320 cell line at different time intervals

S. No.	Test	IC 50 in µg/ml for 24 h	IC 50 in µg/ml for 48 h	IC 50 in µg/ml for 72 h
01	Quercetin from *A.graveolens* (S1)	324	175	106
02	Quercetin from *R. sativus* (S2)	546	203	131
03	Capecitabine (Std)	194	56	45
04	S1+ Std	246	118	43*
05	S2+ Std	298	140	46*

Note: ANOVA is performed for comparison of groups and found significant with P value < 0.05

In Prostate cancer cell line, quercetin fractions from *A. graveolens* (S1) and *R. sativus* (S2) and Bicalutamide displayed cytotoxic activity at three different time points (24 h, 48 and 72 h) with maximum efficiency at 72 h time interval (Figures 5.26, 5.27 and 5.28). The same quercetin when combined with Bicalutamide exhibited higher cytotoxicity than the treatment with quercetin (Figures 5.29 and 5.30; table 5.7).

Table 5.7: Cytotoxic potential of test compounds on PC-3 cell line at different time intervals

S. No.	Test	IC 50 in µg/ml for 24 h	IC 50 in µg/ml for 48 h	IC 50 in µg/ml for 72 h
01	Quercetin from *A.graveolens* (S1)	479	261	183
02	Quercetin from *R. sativus* (S2)	681	271	180
03	Bicalutamide (Std)	172	86	20
04	S1+Std	230	157	110
05	S2+ Std	315	181	140

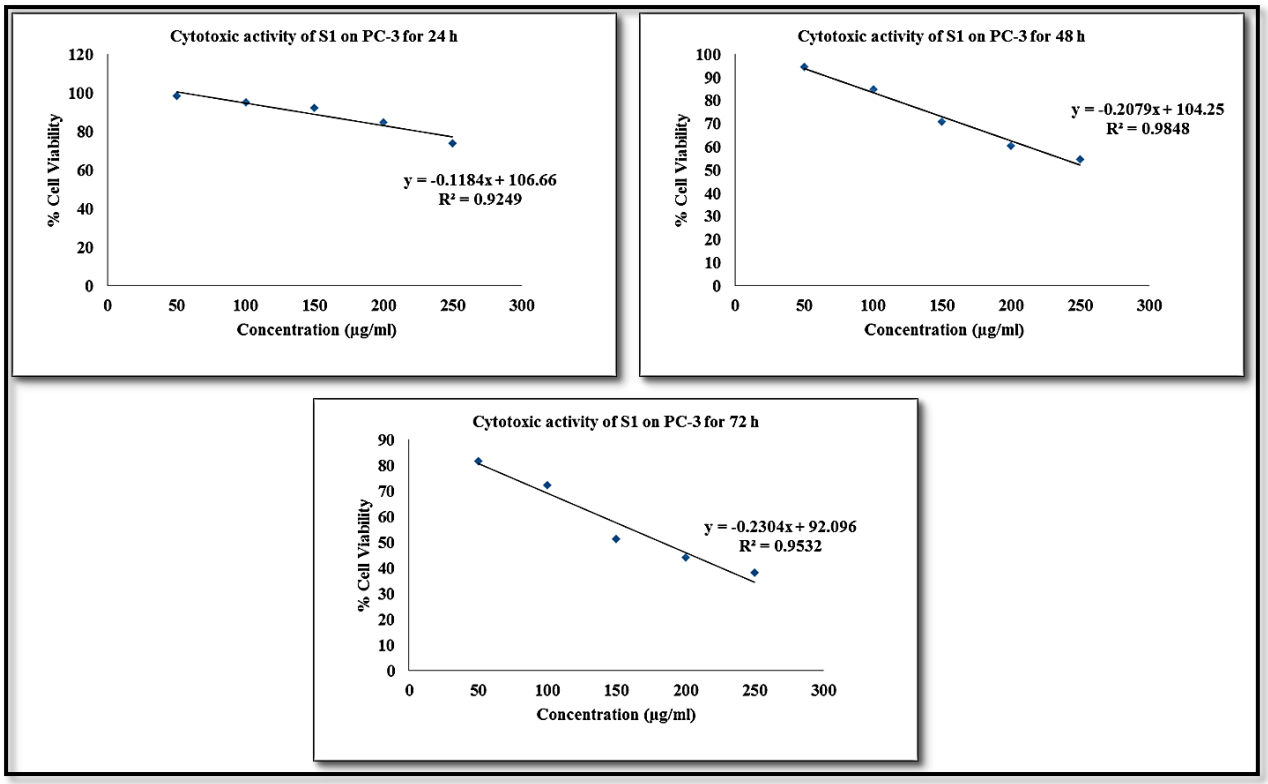


Figure 5.26: Cytotoxicity of S1 on PC-3 at three time points

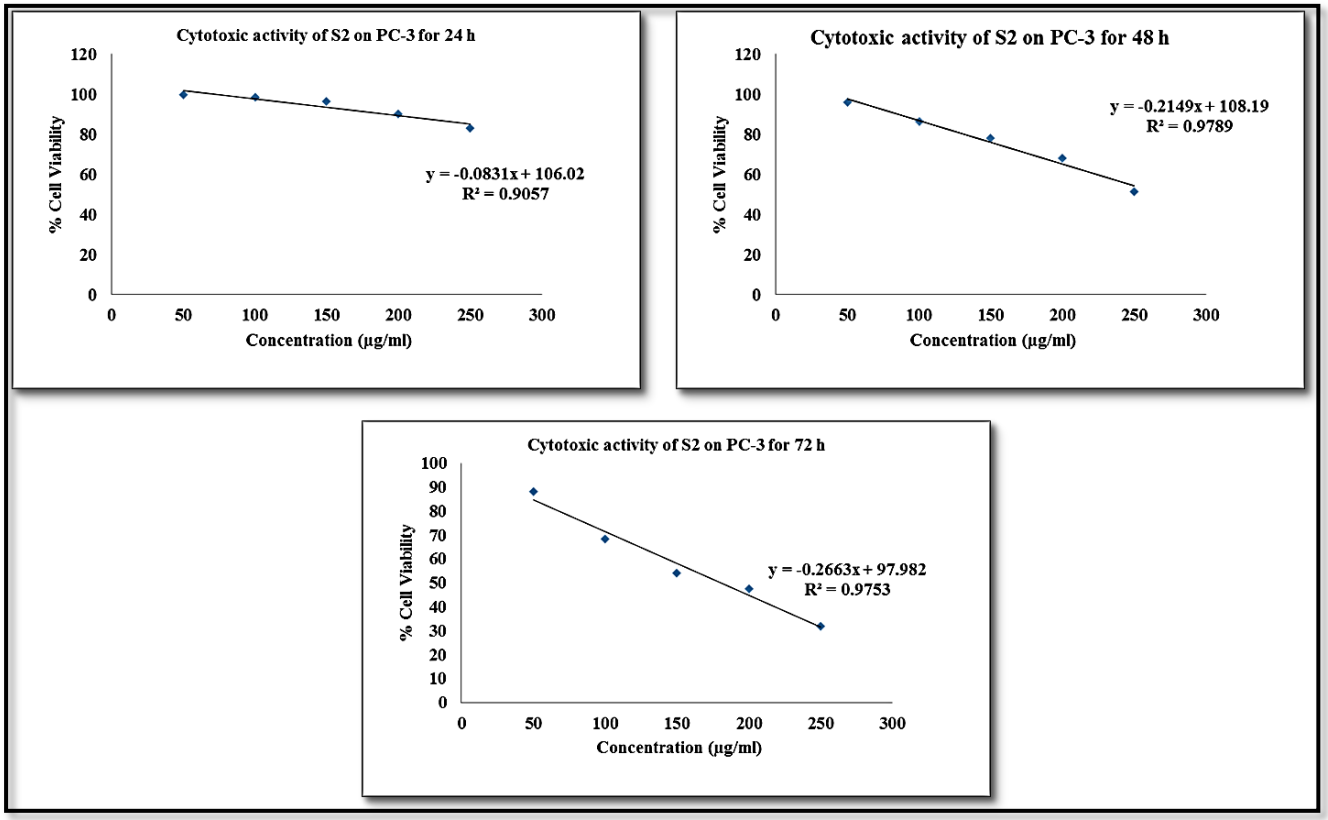


Figure 5.27: Cytotoxicity of S2 on PC-3 at three time points

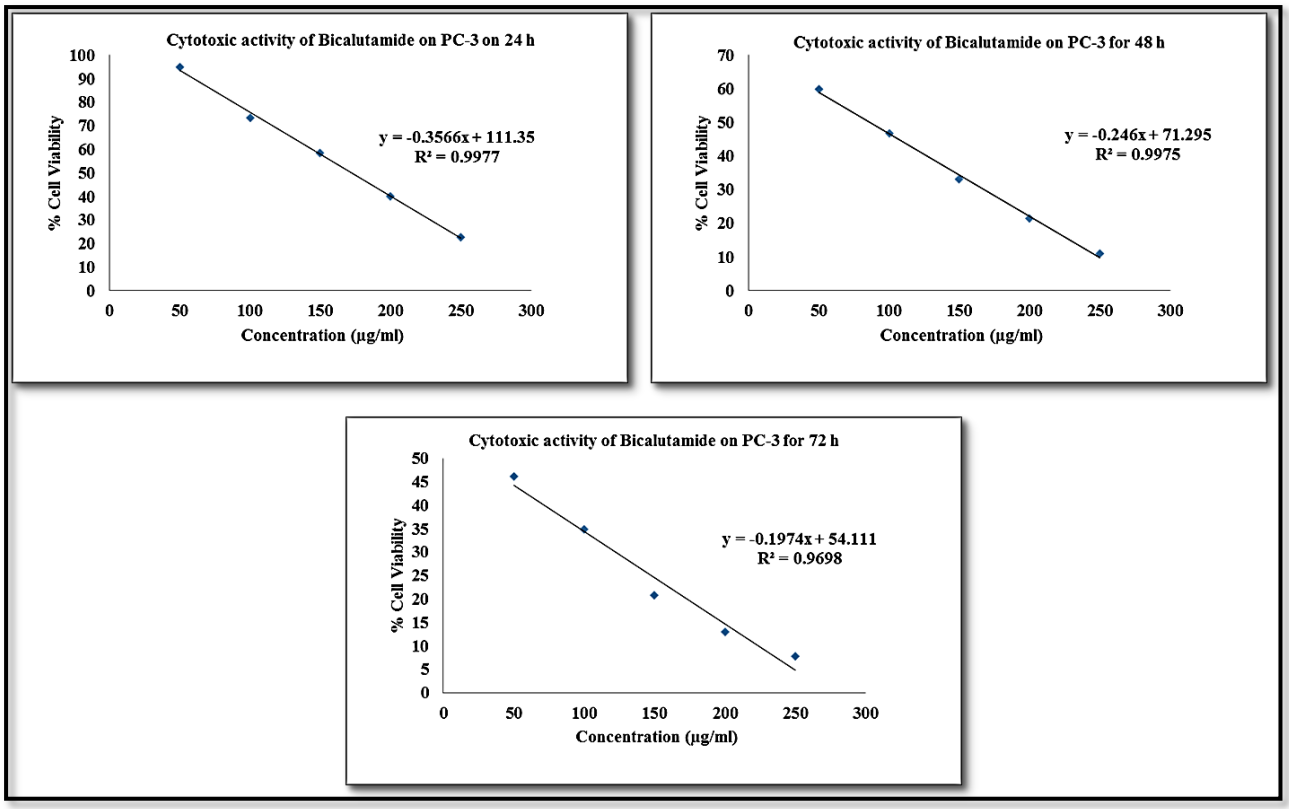


Figure 5.28: Cytotoxicity of Bicaluatmide and S1 on PC-3 at three time points

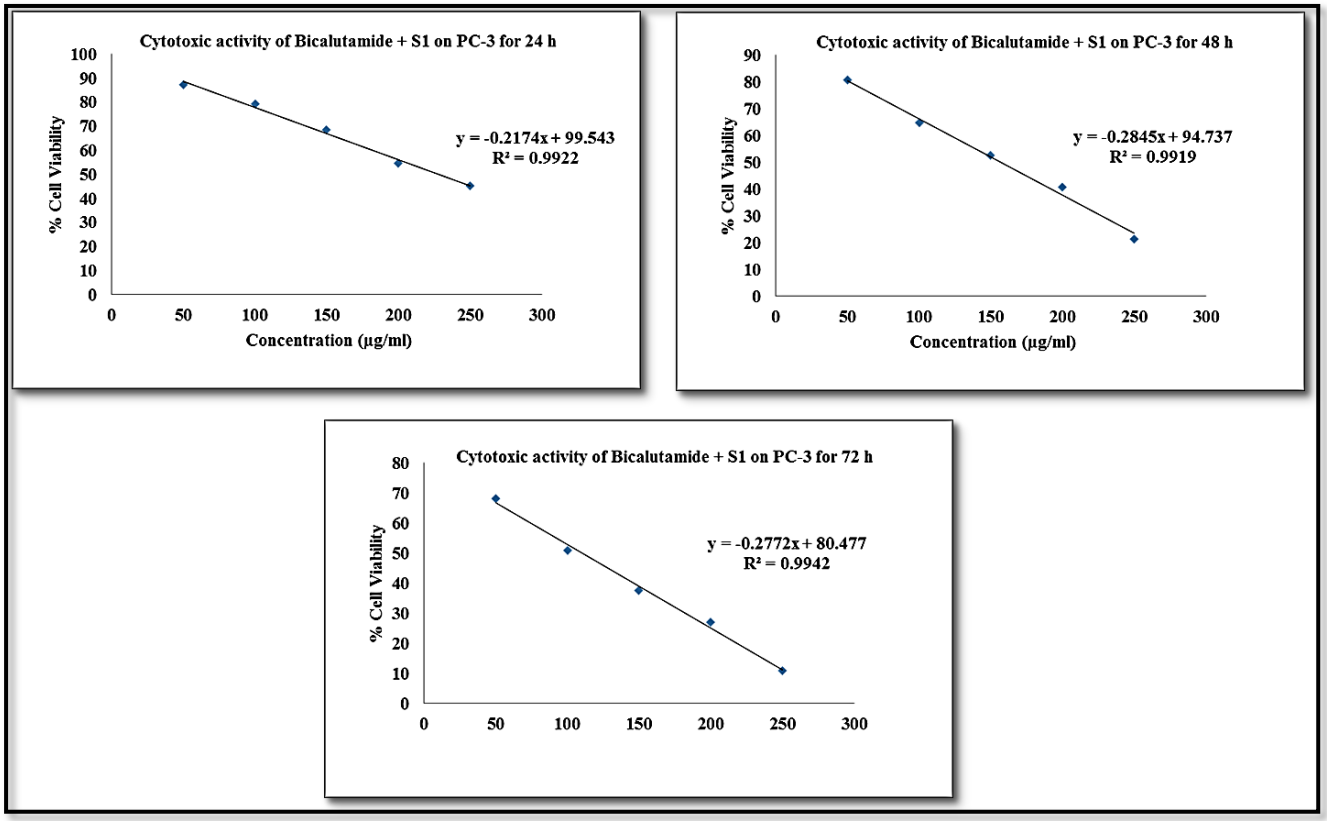


Figure 5.29: Cytotoxicity of combination of Bicaluatmide and S1 on PC-3 at three time points

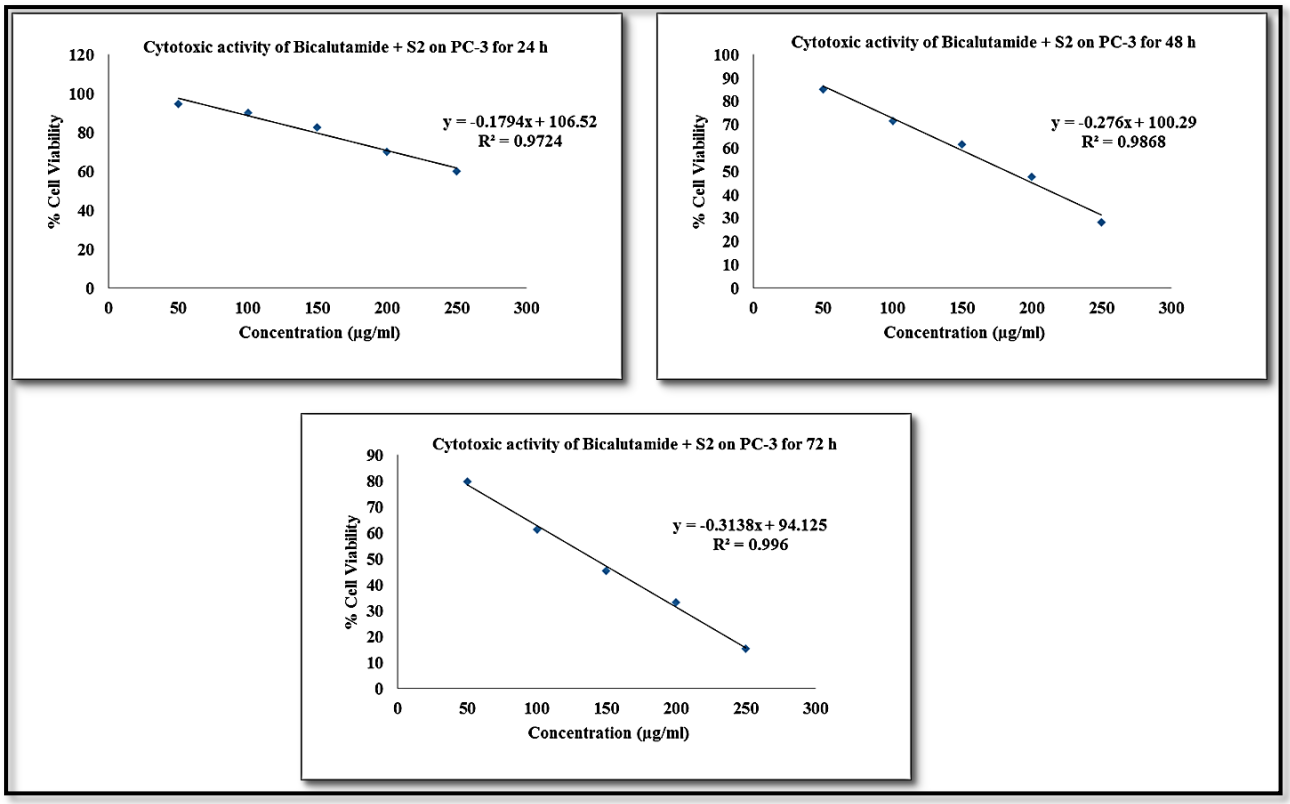


Figure 5.30: Cytotoxicity of combination of Bicalutamide and S2 on PC-3 at three time points

The comparative evaluation of MTT assay on all tested cancer cell lines proves that quercetin fractions when treated alone and in combination with standard drugs have significant anti proliferative potentials against breast and colon cancer cell lines. Even though the fractions had shown cytotoxic potential on prostate cancer cell lines, the effect was higher in standard drug alone than in combinations. Hence, the combinations of quercetin fractions from two plant species were made with Anastrozole and Capecitabine and further tested on breast and colon cancer cells.

5.4.1. Quercetin prompts apoptosis with drug combination against MCF-7 and COLO 320

MCF-7 (breast) and COLO 320 (colon) cells were treated with various concentrations of quercetin (Table 4.8) for 72 h, stained with AO/ Etbr and cells were analysed by fluorescent microscope. Quercetin from *A. graveolens* and *R. sativus* in combination with Anastrozole and Capecitabine on breast and colon cancer presented cell shrinkage and condensation of chromatin (Figures 5. 31 and 5.32).

Table 5.8: Percentage of apoptosis in breast and colon cancers

Breast Cancer cell line	**Percentage of Apoptosis in test combinations (table 3.3)**	
	S1+Anastrozole	S2+ Anastrozole
MCF-7	48 %	58%
Colon cancer cell line	S1+ Capecitabine	S2+ Capecitabine
COLO 320	48%	54%
Control	0.5%	0.5%

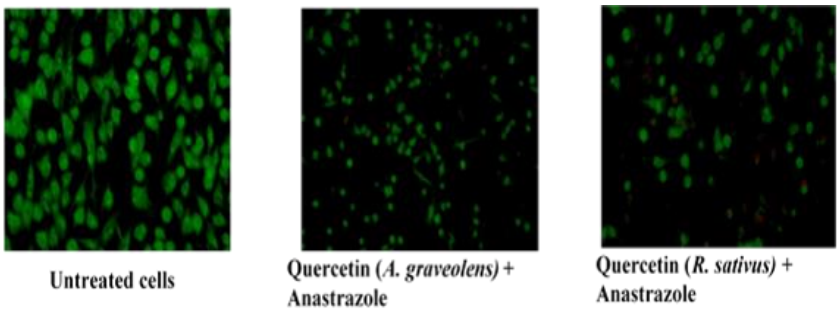


Figure 5.31: Acridine orange/ Ethidum Bromide staining of *in vitro* grown cultures of MCF-7 cells in treatment with test compound and standard drug.

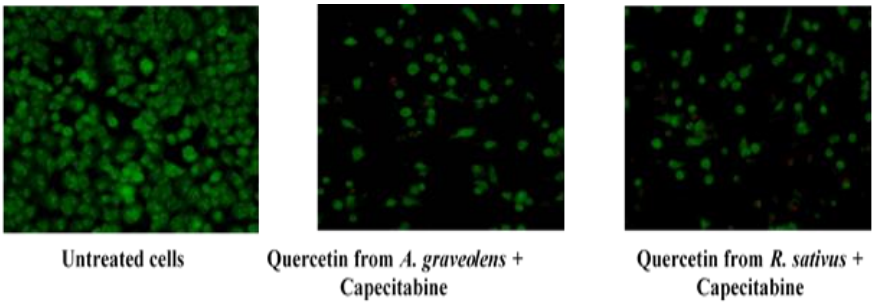
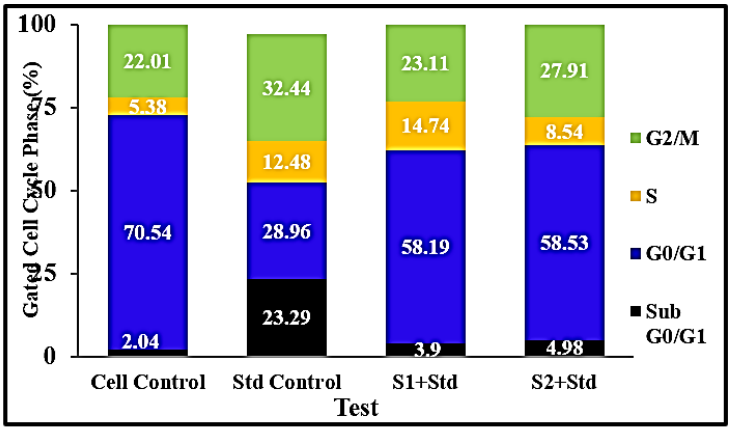


Figure 5.32: Acridine orange/ Ethidum Bromide staining of *in vitro* grown cultures of COLO 320 cells in treatment with test compound and standard drug.

5.4.2. Quercetin and standard drugs boost cell cycle arrest at various stages

Further, in MTT assay (cell viability), growth arrest was explained by cell cycle analysis. Changes in cell cycle after 72 h of stimulation with combination of quercetin and drugs were assessed by flow cytometry. Treatment of MCF-7 cells with quercetin (*A. graveolens*) and Anastrozole (1:1 ratio) at 16 µg/ml concentration showed 58% G1 phase arrest i.e., two times the amount of activity exhibited by standard drug Anastrozole alone with same concentration. Likewise, more number of cells were arrested in G2/ M phase at 16 and 31 µg/ml concentrations of quercetin from *A. graveolens* and *R. sativus* which is effective than the G2/M arrest exhibited by standard drug, a novel identification. No significant differences were noted in subG0/G1 (apoptotic phase) of cell cycle with quercetin. Standard drug showed significant increase in sub G0/G1 as compared to untreated (Figure 5.33).



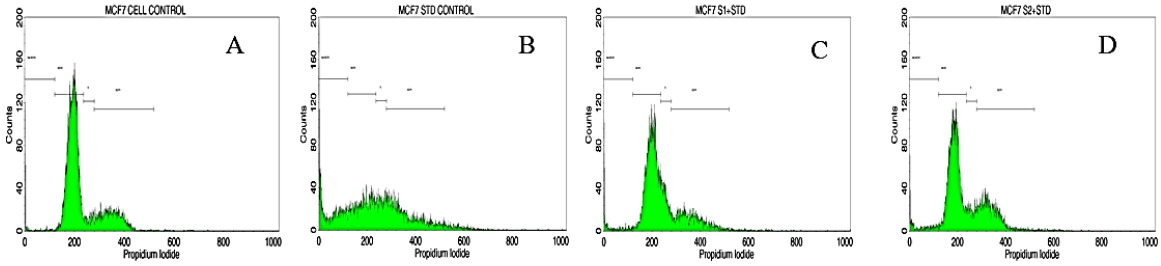


Figure 5.33: PI (Propidium iodide) histogram of the gated cell singlets distinguishes cells at the sub G0/G1, G0/G1, S and G2/M cell cycle phases and images of flow cytometric analysis showing: A (cell control), B (Std control- Anastrozole), C (S1+Std) and D (S2+Std) against MCF-7 cells.

The results of cell cycle by flow cytometry on COLO 320 cells suggested that compared to untreated, combination treatment clearly established cell cycle arrest in sub G0/G1, G0/G1 phase and also G2/M phase of cell cycle. There were no significant differences noticed in synthetic phase (S) of cell cycle with S1+Std, S2+Std compared to untreated. This is found to be the first report to explain the effectiveness of the selected drugs combined with natural quercetin at different stages of cell cycle at lowest concentrations (Figure 5.34).

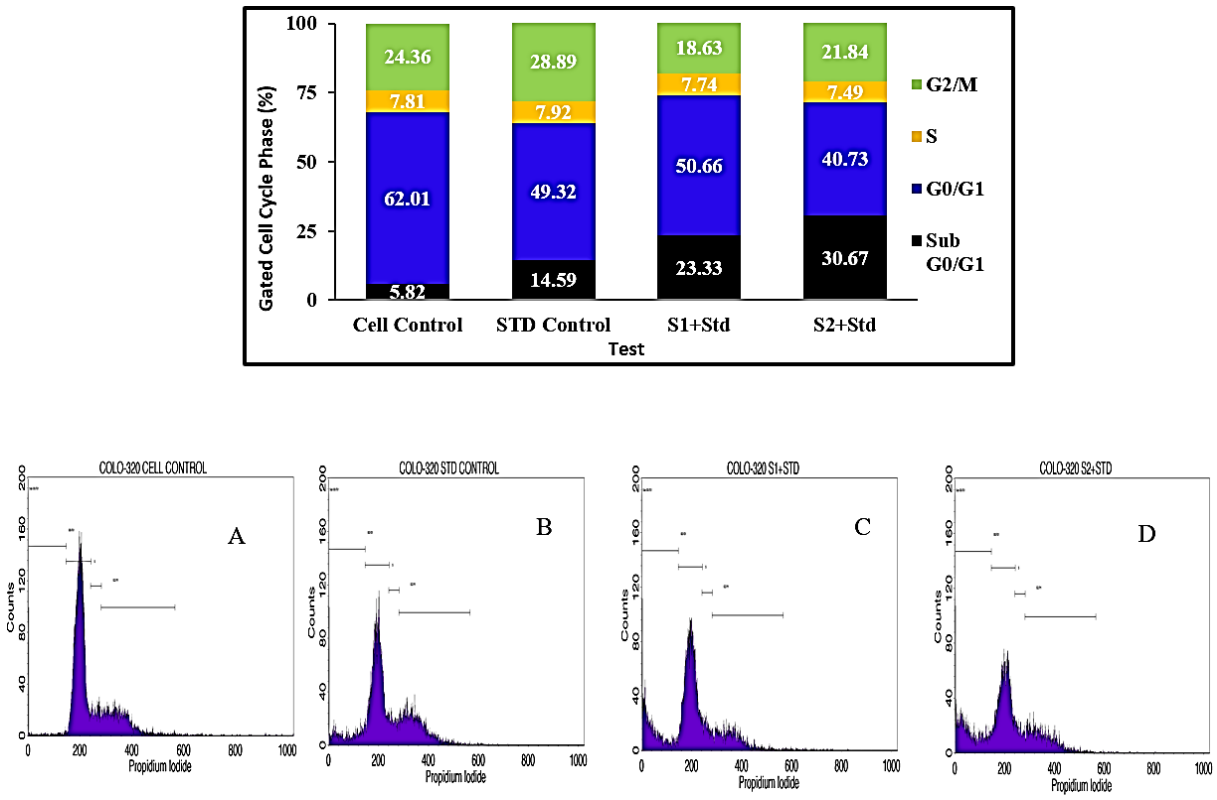


Figure 5.34: PI (Propidium iodide) histogram of the gated cell singlets distinguishes cells at the sub G0/G1, G0/G1, S and G2/M cycle phases and images of flow cytometric analysis showing: A(cell control), B(Std control-Capecitabine), C (S1+ Std) and D (S2+Std) respectively against COLO 320cells.

5.4.3. Quercetin induces depolarization to mitochondrial membrane potential

A sensitive cationic and lipophilic JC-10 fluorescent probe was used to monitor the mitochondrial membrane potential alteration in the cells. Quercetin in combination with drugs showed significant reduction of mitochondrial membrane integrity against MCF-7 and COLO 320 cells by showing the shift of cells from upper right quadrant - red (FL2) to lower right quadrant –green (FL1). This suggested the participation of mitochondrial apoptotic mechanism in cell death upon treatment with test compounds against the MCF-7 and COLO 320 cells. It is observed that S1 has significant mitochondrial membrane depolarization/integrity potential compared to S2 by conjugating with standard drugs at1:1 ration against MCF-7 and COLO 320 cell lines after 72 h incubation (Figures 5.35-5.38).

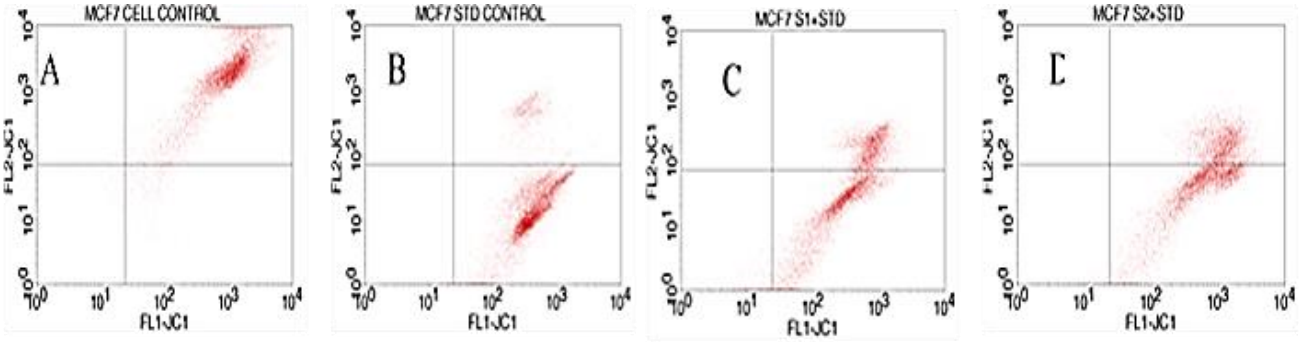


Figure 5.35: JC1 expression study of MCF-7 cell line by flow cytometry. The quadrants depicting mitochondrial membrane depolarization in different groups *viz*., A (cell control), B (Std control- Anastrozole), C (S1+Std) and D (S2+ Std).

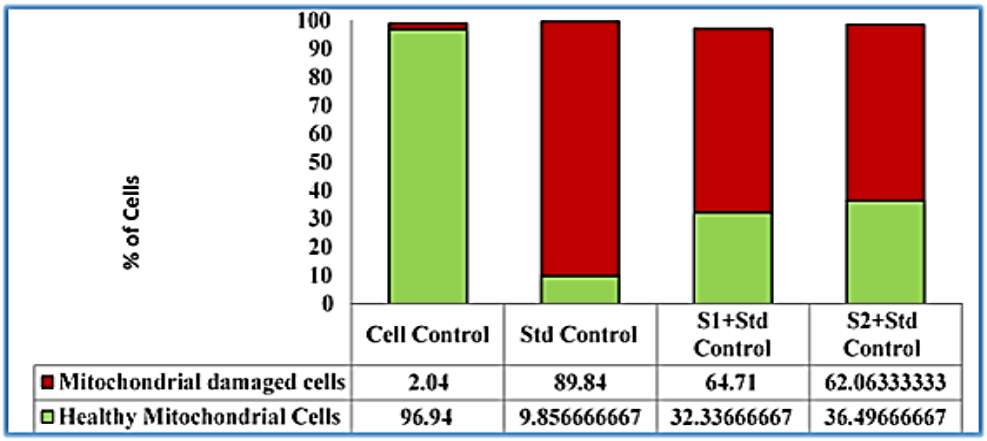


Figure 5.36: Histogram showing the healthy and damaged mitochondrial cells upon treatment with combination of Anastrozole (Std control) and quercetin from *A.graveolens* (S1) and *R.sativus* (S2) standard against MCF-7 cells.

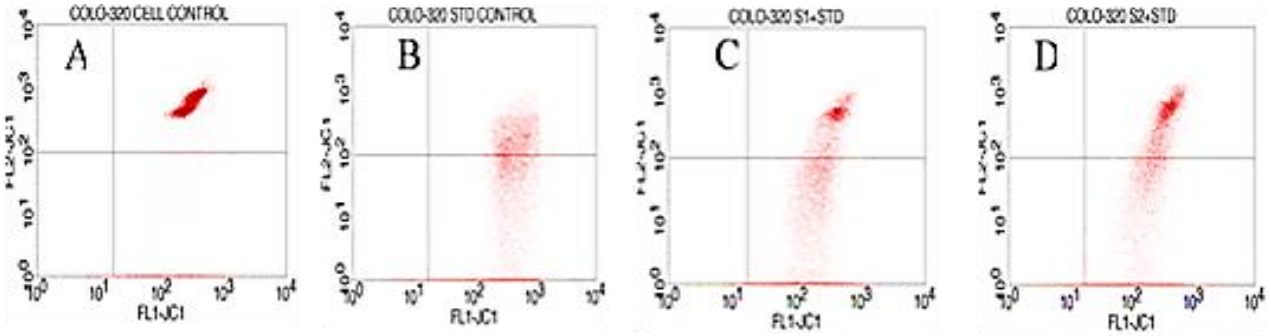


Figure 5.37: JC1 expression study of COLO 320 cell line by flow cytometry. The quadrants depicting mitochondrial membrane depolarization in different groups *viz*., A (cell control), B (Std control-Anastrozole), C (S1+Std) and D (S2+ Std).

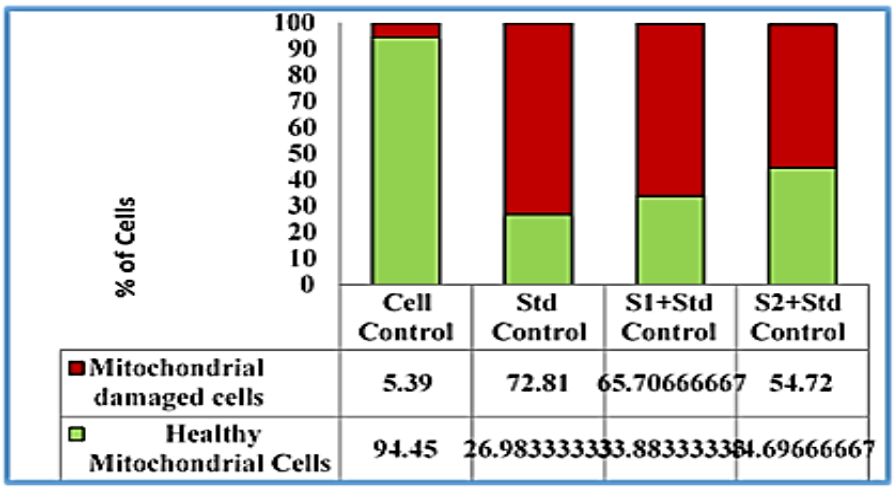


Figure 5.38: Histogram showing the healthy and damaged mitochondrial cells upon treatment with combination of Capecitabine (Std control) and quercetin from *A. graveolens* (S1) and *R. sativus* (S2) against COLO 320 cells.

5.4.4. Quercetin with Anastrozole and Capecitabine combination induces activation of expression of Caspase-3

Flow cytometry data of the test compounds (S1+Anastrozole and S2+Anastrozole) showed Caspase- 3 expression at 37.82 and 32.10 in terms of Mean Fluorescence Intensity (MFI) after 72 h of incubation. Statistical data of Caspase-3 expression suggested that its level is decreased in untreated MCF-7 cells (8.82MFI) compared to Anastrozole (41.33MFI) (Table 5.9 and figure 5.39).

Table 5.9: Table with mean fluorescence intensity of Caspase-3 activity against on MCF-7 cells.

Caspase-3	Relative Mean Fluorescence Intensity y.a.u (arbitrary units of fluorescence)
Cell control	8.82 y.a.u
Anastrozole	41.33 y.a.u
S1+Anastrozole	37.82 y.a.u
S2+Anastrozole	32.1 y.a.u

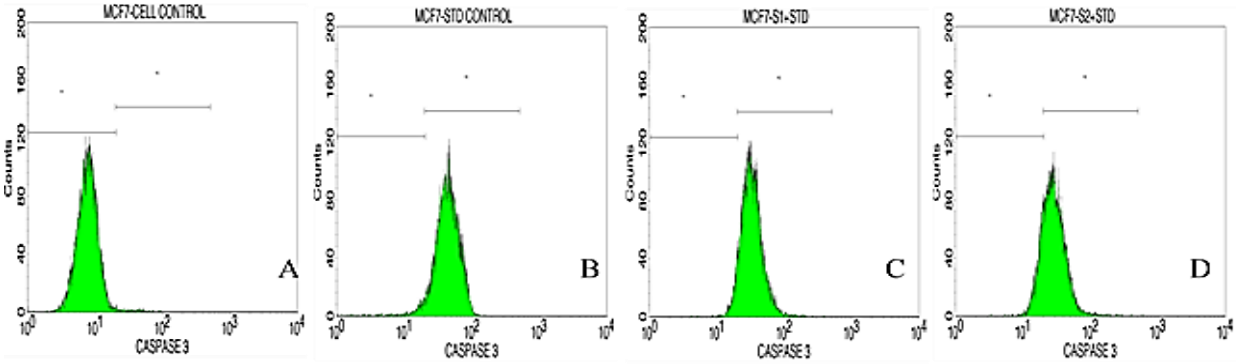


Figure 5.39: Caspase-3 expression study on MCF-7 cells: A (cell control), B (Std control-Anastrozole), C (S1+Std) and D (S2+ Std).

The data on Caspase-3 expression indicated that expression levels are low in untreated COLO 320 cells (8.69MFI) compared to Capecitabine (61.88MFI). The test compounds (S1+Capecitabine and S2+Capecitabine) showed Caspase-3 expression at 47.79 and 42.45 MFI after 72 h of incubation (Table 5.10 and figure 5.40).

Table 5.10: Table with mean fluorescence intensity of Caspase-3 activity on COLO 320 cells

Caspase-3	Relative Mean Fluorescence Intensity
Cell control	8.69
Capecitabine	61.88
S1+Capecitabine	47.79
S2+Capecitabine	42.45

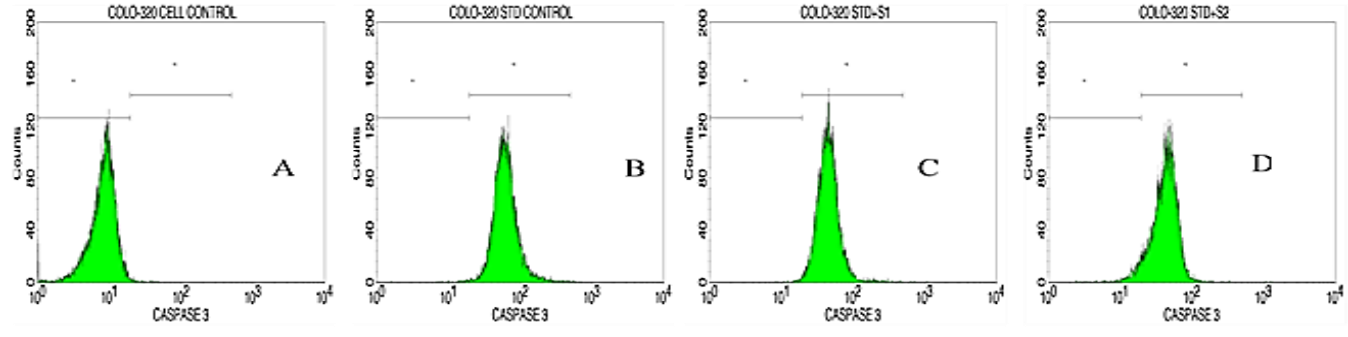


Figure 5.40: Caspase-3 expression study on COLO 320: A (cell control), B (standard Anastrozole), C (S1+Std) and D (S2+ Std).

5.4.5. The non-toxic nature of combinational treatment on normal cell line, 3T3-L1

The cytotoxic effect of quercetin in combination with Anastrozole and Capectiabine was evaluated on 3T3-L1. The results showed that the combination of quercetin and standard drugs were not cytotoxic to normal cells even after 72 h of incubation at IC 50 above 100 µg/ml and up to 250 µg/ml (Figure 5.41).The final conclusion drawn from the study suggest that,S1(quercetin from *A. graveolens*) and S2 (quercetin from *R. sativus*) in combination with standard drugs on 3T3-L1 cell lines exhibited IC 50 values greater than the IC 50 values obtained on MCF-7 and COLO 320 cancer cell lines after the incubation period of 72 h. Hence, it is proved that quercetin does not have any cytotoxicity against mouse normal fibroblast cells (3T3-L1).

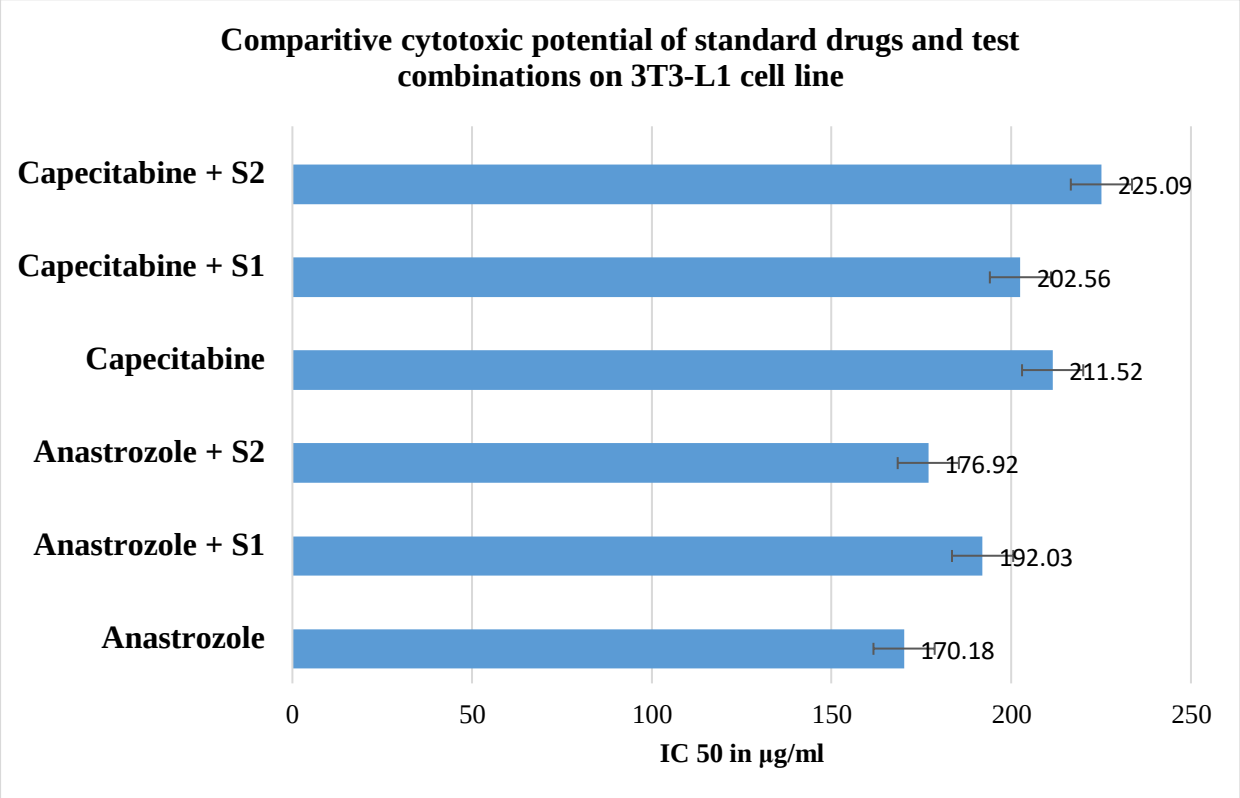


Figure 5.41: Cytotoxic activity of Anastrozole and Capecitabine in combination with quercetin on 3T3-L1 cells

CHAPTER 6

DISCUSSION

The study is designed for achieving four objectives; first is to isolate quercetin from plant sources, second is to identify the compound, third is to determine its antibacterial activity and fourth is to determine its anticancer activity. The active compound was fractioned by column chromatography and characterized by spectrophotometric techniques. The *in vitro* mechanism of action of extracted quercetin fractions were tested on bacteria and cell lines.

6.1. Selection of plant material

Infectious diseases are significant cause of mortality and morbidity worldwide. Over 100 years, notably, plants, the reservoirs of phytochemicals, are used as first aid source. Rigorous resistance in bacteria and cancer; and reduced number of new drugs paves a way for identifying new sources, in particular, edible plants that have added advantage of incorporating them in diet. Prescribed diet with rich antioxidants plays a major role in reducing the cancer cell proliferation and side effects of chemotherapy. Hence, in the present study, the selection of plants were appropriate for using them as drug or as a vegetable.

A. graveolens and *R. sativus*, the widely used greens either as salads or as an ingredient in food preparation, are a good material of choice for investigation. The crude extract of the selected plants are known to have traditional importance over 5,000 years in many countries (China, Japan, Korea, Southeast Asia and Egypt) [Jana and Shekhawat, 2010; Tung-Ting Sham *et al.,* 2013]. Therefore, diversity of secondary metabolites of plants, particularly quercetin, should be investigated more extensively for its therapeutic activities as it has unique biological properties [Anand David *et al.,* 2016].

6.2. Extraction and identification of quercetin

Qualitative and quantitative methods of extraction of bioactive molecules from plants are important approaches for any drugs' exploration. Pharmaceuticals and agro industries apply these methods to get a lead molecule or combination of drug.

The tedious extraction process involves many stages that include; content of bioactive compound, environmental conditions, time of harvest, soil conditions, solvent ratio, type and procedure of extraction. Apart from several methods of extractions, a comparison of conventional and traditional methods are employed in the study that draws a conclusion that maceration and digestion methods yielded quercetin with more extraction time and less purity. On contrary, CC extraction yielded better purity of quercetin in less time. In support to that, a study by Durga and his co-investigators proved that CC is a simple extraction method for quercetin from *Trigonella foenum-graecum* leaves [Durga *et al.,* 2017]. Other study reports by Thida Choo and Mohammed Zahoor also detected quercetin in *Michelia chapaca* L. leaves; and fruits of *Aesculus indica* L. by using CC. In contrast, a study by Conglei Pan *et al.,* showed that High-Speed Counter Current Chromatography (HSCCC) is a convenient method of quercetin extraction from *Gynostemma pentaphyllum* [Conglei Pan and Haitao Lü, 2019]. Quercetin, a flavonoid, from the leaves of selected plants of present study is as well in agreement with the results of Holderbaum *et al.* (2015) who stated that leaves possess higher flavonoid content. Furthermore, Horbowicz and Jang *et al.* stated that high yield of quercetin depends on the solvent used for extraction. Cemili Ozcan stated that solvents like with methanol: acetonitrile: formic acid combination brings in good amount of quercetin from Rose Hip (*Rosa canina*), Bettle (*Urtica dioica*), *Terebinth chica* and Purslane (*Portulace oleracea*) [Cemile Ozcan *et al.,* 2012]. All these references boldly explain that quercetin extraction in pure form depends on the solvents used. In the present study, the preferred combination of solvents i.e., ethanol and hexane yielded pure compound and marks as a suitable solvent combination for quercetin extraction [Chourasiya, 2012].

Identification of any molecule as a drug lies in its biophysical and biochemical characterization. The extracted fractions from leaves of *A. graveolens* and *R. sativus* had been subjected to spectral analysis (UV-Vis, FTIR, ^{1}H NMR, and LC-MS spectroscopy) for their

identification. The FTIR spectra of quercetin were in co-ordination with the peak values of standard confirmed in a study performed by Chorasiya [Chourasiya *et al.,* 2012]. The characterization studies of quercetin by proton NMR and UV-vis spectroscopy from Selvaraj and Benzie findings are in consistent with our study results [Selvaraj *et al.,* 2013; Benzie and Strain, 1996]. With respect to UV- vis spectra, both the samples showed maximum absorption from 230 nm to 450 nm which are similar to standard quercetin absorption range. Based on the spectral analysis, the identified and confirmed quercetin from both the plant extracts was tested for its biological activity. LC-MS analysis reported the fragment pattern of quercetin with m/z of 303.03 from both the leaf materials are corresponding to the fragment patterns of reference quercetin and also with other study reports of of Xu *et al.,* 2012.

6.3. Role of quercetin in control of bacterial infections

Screening of natural compounds allows them for assessment of bioactive compounds for new therapeutic routes. The observed resistance in bacteria to antibiotics makes them multidrug resistance (MDR) which is a serious threat to living beings. This observed resistance is a growing challenge in medicine field; and several *in vitro* studies are to be carried out to discover an elucidation [Chandra *et al.,* 2017]. It is reflected that metabolites of plants might be major alternatives for modifying resistance [Gupta and Birdi, 2017]. Several groups of metabolites like alkaloids, phenolics, carotenoids, tannins and flavonoids exhibit antimicrobial properties [Barbieri *et al.*, 2017]. Amongst, flavonoids are favourable bioactive group with less toxicity. Various other compounds of this group (flavones, flavanols and flavanones etc.) are dominant in plant sources exhibiting strong anti-oxidant and anti-inflammatory properties [Jungbauer and Medjakovic, 2012; Goncalves *et al.*, 2017]. Hence, dietary flavonoids benefit in avoidance of chronic and degenerative diseases in human. Even though their absorption is low (5-10%) in intestine, a novel formulation of quercetin based on

a phytosome delivery system was developed and Industrialized by Indena Pvt Ltd. These phytosomes are utilised to promote the solubilities of poorly bioavailable compounds that improve their bioavailability [Riva *et al.,* 2019]. Flavonoid selected in the present study is 'quercetin' and is ubiquitous in fruits, vegetables, seeds, skin of certain fruits [Kaşıkcı and Bağdatlıoğlu, 2016]. The availability of quercetin in leafy greens of *A. graveolens* and *R. sativus* is an addition to the current sources of quercetin and its antibacterial activity is a potent contribution in scientific literature.

The capacity of antibacterial drug depends on selective killing or inhibiting the bacterial growth with minimal or no side effects to the host. In a broader sense, on any prokaryotic and eukaryotic cell, antibacterial agents exhibit the following mechanisms: adsorption, changes in mobility, inhibition of growth and cell wall biosynthesis, modification of cell permeability, lysis and leakage of cell constituents and general effects on metabolism. Surprisingly, a few antibacterial agents are noted to have anticancer properties as well (Actinomycin D and Bleomycin). Biofilm formation and motilities such as swarming, swimming and twitching are the major advantages of any bacterium to gain drug resistance. These activities are shown by a few pathogenic bacteria *viz.*, *P. aeruginosa*, *S. aureus* and *E. coli*. Hence, the purified quercetin from the selected plants was tested for its antibacterial properties against these bacteria causing 25,000 deaths due to multi-drug resistance as novel therapy options are limited [Ebejer *et al.,* 2016].

Preliminary screening of antibacterial activity of 'quercetin' fraction proved that highest zone of inhibition was seen on *E.coli* (27 mm) and *S. aureus* (24 mm) by S2 and on *P. aeruginosa* (20 mm) by both S1and S2. To our surprise, quercetin activity on *E. coli* seems to be higher than the antibiotics tested (Ampicillin, Cephalexin, Amikacin, Amoxicillin, Sepifime, Chloramphenicol, Sulfamethoxazole and Trimethoprim). Plant based quercetin showed broad spectral activity as that of Cefoxitin, Vancomycin, Piperacillin, Ceftazidime, Ampicillin and

Imipenem broad spectrum antibiotics whose major action is on disruption of cell wall and cell membrane [O'Toole and Kolter, 1998]. As reported in introduction about the crucial role of these antibiotics, the study findings knocked the potential of quercetin to be considered in treatment choices when further clinical trials are performed.

Scientists working on microbes focus on how the drug acts on motile or non-motile or biofilm forming bacteria. Motility plays an important role in the virulence of bacteria at differing environmental conditions. Hence, restricting its motility is important in controlling virulence. The main forms of bacterial motility are swarming (flagella), swimming (flagella) and twitching (type IV pilus). These help a bacterium in the initial attachment and development of a biofilm [Pollitt *et al.,* 2015]. Among the motility patterns tested, quercetin exhibited maximum restriction of swimming and twitching motilities in *S. aureus* and *E.coli*. Twitching is considered as an important motility factor involved in formation of biofilms on abiotic surfaces. Generally, in natural habitats, bacteria raise biofilms with extra cellular matrix as organised communities of cells and attach to a surface. Majorly focused research is to restrict the biofilm formation developed by twitching [Watnick and Kolter, 2000]. The current findings demonstrates the activity of quercetin on motility patterns which stood as a novel findings in the reported literature.

Formation of biofilm in many bacteria displays protection by imbibing resistance to antibiotics [Sharma *et al.,* 2019]. Quercetin tested on the bacterial pathogens exhibited marked biofilm inhibition of *S. aureus* than *P. aeruginosa* and *E.coli*. The result was in agreement with the study quoted that flavonoids have strong antibiofilm activity on *S. aureus* [Manner *et al.*, 2013]. It was reported from another study that the red wine flavonoids, quercetin, apigenin and chrysin, had also shown antibiofilm activity on *S. aureus* [Singh *et al.,* 2017]. Though there are studies on flavonoids and its antibiofilm activity on *P. aeruginosa*, the exact bioactive flavonol group is not studied [Paczkowski *et al.*, 2017]. In our

study, a minimal quercetin concentration of 10-100 µg/ml inhibited *P. aeruginosa* biofilm, and increasing concentration might further enhance the activity of quercetin on *P. aeruginosa*. Reports also suggest that treatment to the infections caused by *E.coli, S. aureus, P. aeruginosa, Bacillus* and *Salmonella* make difficult due to the formation of biofilms [Petruzzi *et al.,* 2018]. Most of the common form of gram positive and gram negative bacteria *viz P. aeruginosa, E.coli* and *S. aureus* can from biofilms on medical devices. Among them, *S. aureus* cause 40-50% of prosthetic heart valve infections, 70% catheter biofilm infections and 87% blood stream infections [Chen *et al.,* 2013]. Hence control of biofilms is an important approach that is addressed in the present study and quercetin showed 99% biofilm inhibition on *S. aureus* which is useful and novel information to pharmaceutics.

To explain the mechanism of action of any anitimicrobial agent is to see its toxic effects on the membrane structure and function. As quercetin displayed antibiofilm activity, there is a need to know whether it has an impact on the disruption of cell boundaries. Any antibacterial agent that is acting on bacterial membrane result in loss of absorbing material, nucleic acid, at 260 nm and proteins at 280 nm [Carson *et al.,* 2002]. The results of the study are supportive to the data that measurement of absorbing materials at 260 and 280 nm by UV-visible spectroscopy in quercetin treated bacteria. Our findings presented increased leakage of contents in a dose dependent manner on all the tested strains and in fact were more in *S. aureus*. This report was in agreement with the study results of Vani *et al.,* (2014) who stated that spices (cinnamon, ginger, cloves and cumin) have cell membrane disruption ability. A study by Tang *et al.,* (2017) reported that black pepper essential oil caused leaching of materials at 260 nm and 280 nm against *S. aureus* indicating the membrane damage. These observations prove the fact that quercetin aids in the disruption of cell membrane.

Cell membrane leakage and DNA damage are the two independent activities that result in cell death. Quercetin fraction when tested on the selected organisms resulted in DNA

fragmentation. DNA fragmentation due to double strand or single strand break is the hallmark of cell death. Though there are limited studies available on the detection of DNA fragmentation capacity of phytochemicals on bacteria, flavonoids have DNA damaging ability but could not identify the active bioactive compound [Babii *et al.,* 2015; Anandhi *et al.,* 2014; Kwasi *et al.,* 1997]. Together, the studies evidenced that plant compounds have active moieties that act on nucleic acid and lead to cell death which is in agreement with the current study results. The study stands first in identifying bioactive compound inducing DNA fragmentation in bacterial cells.

6.4. Role of quercetin on cancer progression

The anticancer properties of plants are documented since ancient times. Isolation of bioactive compounds from plants helps in identifying novel drug formulations. The goal of the present study was extended afar in controlling the cancer by the combinational approach. Chemotherapy, Surgery and radiation therapy are the most important approaches of cancer treatment with fundamental drawbacks. Chemotherapy displays side effects and damage healthy cells along with cancer cells, surgery is effective when tumour location is not surrounded by sensitive tissues and radiation is potent when tumour location is specified exactly. However, new techniques like combinational approach employed in chemotherapy could reduce the obstacles in the treatment and will be of more interest in the drug development [Shabani, 2016]. The non- toxic flavonoids are safe and easily available from food source making them identified as ideal candidates for cancer treatment. The cytotoxic activity of flavonoids has proved less or no effect on normal cells and inspired researchers in developing potential flavonoid based chemotherapies for cancer treatment [Plochmann *et al.,* 2007; Ben Sghaier *et al.,* 2011]. The accurate mechanism of action of flavonoids in contributing anticancer activity is yet to speak systematically. The unique properties of

flavonoids like binding to cell membrane, penetrate easily into cultured cells and control metabolic activities make them the right choice for cancer treatment [Chan *et al.,* 2000].

The thrust in research is looking for the best natural source that reduces the chemotherapy burden on the patient as chemotherapy is the most chosen option. Hence, the combination of natural quercetin (*A. graveolens* and *R. sativus*) and synthetic drugs were used in the present study. Cytotoxic reports of the present study has proven that quercetin showed measurable control on colon and breast cancer cell lines with an inhibitory concentration of 106 µg/ml (S1), 131 µg/ml (S2) on colon cancer, 161 µg/ml (S1) and 158µg/ml (S2) on breast cancer over synthetic drugs. Upon combination with standard drug, quercetin significantly enhanced its efficacy with an IC 50 value of less than 50 µg/ml, which seem to be a good concentration and can be considered for formulation of any drug. A good number of supportive studies proved that quercetin improved its potency in combination with drugs like Cisplatin, Doxorubicin, Camptothecin, 5-Fluorouracil, Sorafenib, Oxaliplatin and Gemcitabine against various kinds of cancers which are in agreement with the current results [Androutso poulos *et al.,* 2009; Chan *et al.,* 2013; Li *et al.,* 2013; Jakubowicz *et al.,* 2014; . Kavithaa *et al.,* 2014; Zhao *et al.,* 2014]. It is interpreted that the combination of phytochemicals with anticancer drugs might be a strategy for cancer treatment. We are first to demonstrate the cytotoxic potential of quercetin in combination with Anastrozole, a breast cancer drug and Capecitabine, a colon cancer drug. The combinations can be further carried out *in vivo* for clinical trials to ensure its efficacy and accuracy.

A vast number of investigations have to fill the existing gap between the edible greens and their action upon consumption. Cancer cells undergo complex molecular pathways (pro-inflammatory and inflammatory) and application of monotherapy triggers cancer cells to look for alternate pathways for recurrence and metastasis [Meng *et al.,* 2016]. This has drawn our attention to focus on combination therapy to overcome these issues and to reduce the dose of

each agent. Quercetin is assumed as strong antioxidant with its free radicle scavenging activity by binding to metal ions leading to reduced risk of cancer development and progression [Sakanashi *et al.,* 2008]. As quoted earlier in the literature, several *in vitro* reports proved that quercetin effectively minimized the number of cells in massive tumours and induced apoptosis. Our study reports also proved that, quercetin in combination with Anastrozole (16 and 31µg/ml) and Capecitabine (43 and 46 µg/ml) on breast and colon cancer cell lines exhibited apoptosis with a percentage rate of 48 and 58 in breast cancers and 48 and 54 in colon cancers respectively. The reports of the study are better than the other study reports who presented higher apoptotic results with higher concentrations [Gibellini *et al.,* 2011; Abotaleb *et al.,* 2018]. In contrast, a study by Monaserio *et al.,* (2004) presented no apoptotic cell death by 22 varieties of flavonoids on leukemia U937 cell line. Hence, the observed apoptotic effect by the combination of quercetin and synthetic drug is a notable component which should be further studied to elaborate its precise mechanism of action.

Cell division is a highly regulated process for a cell to divide into daughter cells and endures duplication of genetic material. The process involves four phases: G0 (quiescence phase); G1, S, G2 and M (proliferation phase). These phases are necessary for normal and cancer cell growth, development and proliferation [Monasterio *et al.,* 2004]. Hence, targeting cell cycle stands at most priority in control of cancer by natural products in basic and translational research before going for clinical trials [Bai *et al.,* 2017; Rao *et al.,* 2012]. Among the phases, arresting mitotic phase (M) is critical, in this study we addressed the effects of drug combinations on M phase and observed a clear control in both breast and colon cancers. These results were in agreement with other study groups who used alkaloids and colchicine that block mitotic phase of cell cycle in leukemia [Dall'Acqua, 2014; Palmer *et al.,* 1960; Wani *et al.,* 1971]. In continuation to that, the combination of quercetin with Anastrozole and Capecitabine induced S phase arrest in breast and cancer. The findings are in agreement

with similar study reports where quercetin blocked S phase in breast and colon cancers. However, reports derived from other studies convey that, cancer types and application of compound might account for disparity of cell cycle arrest [Mukhtar *et al.,* 2014; Zhang *et al.,* 2012; Yuan *et al.,* 2012; Zhang *et al.,* 2008; Mertens-Talcott *et al.,* 2005]. Hence, understanding the cell cycle mechanism is of significant importance that helps to overcome resistance of cancer cells for their uncontrolled proliferation which is addressed in this study.

The combination of quercetin with ellagic acid lessened proliferation and cell cycle arrest in MOLT-4 cells of leukaemia [Mertens-Talcott *et al.,* 2003]. Quercetin with doxorubicin combination showed a significant decrease in cell proliferation in MCF-7 and MDA-MB-231. However, in the present study combination of quercetin with Anastrozole and Capecitabine at low concentration revealed significant anti-proliferative activity, a unique observation than other reports. Another study also proved that quercetin narrowed T47D breast cancer cell lines sensitivity with an IC 50 value at 160 µM [Staedler *et al.,* 2011]. The combination of quercetin and Anastrozole against breast cancer displayed G2/M and S phase arrest; Capecitabine in combination with quercetin exhibited G0/G1 and G2/M phase arrest on colon cancer at 16 µg/ml concentration and 43 µg/ml concentrations that is testified for the first time in the current study.

Mitochondrial membrane potential is a dominant driving force for mitochondrial ATP synthesis. Depolarization below a certain level may impair mitochondrial function, a major criterion for mitophagy. Mitochondria in majority of cancer cells become dysfunctional via changes in energy metabolism, elevated ROS generation and increased trans-membrane potential. Therefore, targeting mitochondria using pharmacological agents is an attractive therapeutic strategy to destroy cancer cells [Srivastava *et al.,* 2016]. Quercetin co-treatment with autophagy inhibitor enhanced mitochondrial damage and autophagy inhibition in Jurkat T-cells [Ha *et al.,* 2019]. Isorhamnetin glycoside (IGP) from *Opuntia ficus-indica* L. Mill on

colon cancer implied membrane permeabilization of 30% and G2/M phase arrest which is similar to our study results [Antunes-Ricardo *et al.,* 2019]. Quercetin protected mitochondrial impairment in an *in vitro* model of hepatocyte steatosis [Rafiei *et al.,* 2019]. A study reports, quercetin acts via mitochondrial pathway in cervical cancer cells through p53 induction and NF-kB inhibition at 80 µm [Vidya Priyadarsini *et al.,* 2010]. However, we demonstrated enhanced effect of quercetin with Anastrozole and Capecitabine on lower doses on breast (16 and 31 µg/ml) and colon cancer (43 and 46 µg/ml). Study investigations made on quercetin with curcumin combination presented apoptotic induction in chronic leukaemia cells that lowered toxic effects on normal cells [Mutlu Altundağ *et al.,* 2018]. The same combination had also impact on apoptosis through mitochondrial pathway on gastric cancer MGC-803 cells indicating the combination has better potential [Zhang *et al.,* 2015]. These results are at par with our study results where combinations (quercetin and Anastrozole combination, quercetin and Capecitabine) showed decreased cell proliferation, apoptosis through acting on mitochondrial mechanism on breast and colon cancer cells, a novel combination.

Caspases are crucial mediators of programmed cell death and among them the activated Caspase-3 functions as death protease in different cell types. The frequent activation of Caspase-3 raises a question of whether this protease is required for cell death or for the morphological changes associated with apoptosis. Since cancer is a multi-step process, anti-tumour activities modulate molecular targets like growth factors, cytokines and apoptosis [Aggarwal *et al.,* 2007]. The study findings illustrate that quercetin with drugs initiate many triggering pathways. Caspases, an important scenario in extrinsic and intrinsic pathways of apoptosis, when treated with quercetin in association with Anastrozole and Capecitabine on MCF-7 and COLO 320 cancer cells are activated. Tangeretin flavone when treated breast carcinoma cells, MDA-MB-231, made cell cycle arrest and caspase activation [Fan *et al.,* 2019]. Quercetin induced apoptosis via activation of caspases in human hepatoma cell line

HepG2 [Ana Belen Granado- Serraw *et al.,* 2006]. They also mentioned in their study that a continuous daily intake of polyphenols like quercetin might provoke apoptotic cell death. As Caspase 3 plays a central role in execution-phase of cell apoptosis, continuous attempts are happening from the decades to develop molecules capable of directly activating Caspase 3 for cancer treatment [Gregoraszczuk *et al.,* 2015]. This was an impressive tactic in the present study, where Caspases 3 activation by novel drug combinations (quercetin and Anastrozole on MCF-7 cell line; quercetin and Capecitabine on COLO 320 cell line) is proved at lower concentrations and can be effective activators of caspases leading to apoptosis.

The combination of natural components and synthetic drugs to treat cancers is a corner stone for cancer therapy. Even though, monotherapy is a common treatment modality, it becomes less effective than the combination therapy approach [Bayat Mokhtari *et al.,* 2017]. The rationale of combination therapy is to use drugs that work by different mechanisms for optimal use without side effects. A successful treatment with anticancer drug remains a challenge due to non-selective cytotoxicity of the drug. Over all, chemotherapy with cytotoxic and cytostatic drugs remains a primary treatment option. In addition, chemotherapeutic agents available today are highly cytotoxic to normal cells, resulting in a series of adverse effects [Hojjat- Farsangi *et al.,* 2015]. A promising new compound in anticancer therapy is based not only on its ability to induce tumour cell death but also its selectivity, i.e., on its capacity to minimally affect normal cells. The results of the study suggest that plant quercetin when combined with synthetic drugs is highly selective in cancer treatment by showing no toxicity towards normal cell lines when compared to cancer cell lines. Hence, this combination, when tested on other models, can be a new cancer treatment option for future clinical trials in future.

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In vitro studies to assess the potential bioactivity of quercetin as an anti-bacterial and anti-cancer agent

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CHAPTER 7

SUMMARY AND CONCLUSIONS

- Leaves of *A. graveolens* L. and *R. sativus* L. plants were used for quercetin extraction.
- Among the methods, column chromatography was found effective in isolating the pure compound.
- Isolated fractions of quercetin were checked for purity in High Performance Liquid Chromatography (HPLC) and found 98% accuracy as compared with standard from both the plants.
- The employed spectrophotometric techniques (UV-Vis, IR, ^{1}H NMR and LC-MS) were effective in identifying the quercetin fraction.
- Quercetin shared antibacterial activity similar to broad spectrum antibiotics and quercetin from *R. sativus* exhibited highest inhibitory zone (27 mm) than all the high-end antibiotics tested against *E.coli*.
- Quercetin showed restriction on all motility patterns with maximum efficiency on twitching motility in *S. aureus*.
- Quercetin exhibited 98% (*A. graveolens*) and 99% (*R. sativus*) biofilm inhibition on *S. aureus* which is a novel observation.
- Quercetin tested at 10 µg/ml and 100 µg/ml concentrations displayed loss of absorbing materials and proteins at 260 and 280 nm.
- Quercetin resulted in fragmenting the DNA of tested bacteria (*P. aeruginosa* and *E.coli*). This is the first study to report the activity of quercetin on bacterial DNA.
- Quercetin in combination with Anastrozole on breast cancer (MCF-7); Capecitabine on colon cancer (COLO 320) showed potent cytotoxic effect and hence can be considered as an adjuvent for synthetic drugs in chemotherapy.
- Quercetin in combination with drugs displayed morphological changes in nuclei of both breast and colon cancer cells indicating damage to the cells.
- Quercetin, as an adjuvant with standard drugs exhibited cell cycle arrest.

a. The combination of quercetin (S1) and Anastrozole on breast cancer cell lines (1:1 ratio) at 16 µg/ml (MIC) concentration showed G1 phase arrest twice (58%) than the Anastrozole alone (28%) at the similar concentration. This gives an idea that combination of drug with quercetin has better action on cancer cells at cell cycle level.

b. The observations of cell cycle by flow cytometry on COLO 320 cells suggested that compared to standard drug, the combination (S1 at 43 µg/ml + Capecitabine and S2 at 46 µg/ml+ Capecitabine) arrested cell cycle at sub G0/G1 phase as well as G2/M phase of cell cycle. This is the first knock out report to mention combination study on colon cancer that effectively arrested different phases of cell cycle.

- Quercetin in combination with Anastrozole and Capecitabine showed significant reduction of mitochondrial membrane integrity on MCF-7 and COLO 320 cell-lines. This suggested the quercetin participated in mitochondrial apoptotic mechanism of cell death. It is observed that S1 has significant mitochondrial membrane depolarization/integrity compared to S2 by conjugating with standard drugs at 1:1 ratio against both the cell lines.
- Decreased Caspase-3 expression was noted in untreated MCF-7 Cells (8.82MFI) compared to Anastrozole (41.33MFI). The test compounds (S1+Anastrozole and S2+ Anastrozole) showed Caspase-3 expression at 37.82 and 32.10 in terms of Mean Fluorescence Intensity (MFI). Similarly, the expression of Caspase-3 is less in untreated COLO 320 cells (8.69MFI) compared to Capecitabine (61.88MFI). The test compounds (S1+ Capecitabine and S2+ Capecitabine) showed Caspase-3 expression at 47.79 and 42.45 in terms of Mean Fluorescence Intensity (MFI).
- Quercetin in combination with Anastrozole and Capecitabine showed less toxicity to normal cell line (3T3-L1) and this combination seems to have a synergic effect with chemotherapy drugs.

- Food is a major source of proteins, carbohydrates, fats and minerals and is incorporated with many bioactive components that benefit us. One of such bioactive compounds is quercetin, that showed potent antibacterial properties against most common pathogenic bacteria. Also, quercetin if given as an adjuvant with chemo drugs, might reduce the toxic burden on the patient and can be a combatant against diseases.

New Knowledge generated

- Identified new sources of quercetin
- As an antibacterial agent, quercetin showed broad spectrum antibacterial activity, restriction of motility, inhibition of biofilm formation and fragmented DNA on pathogens causing nosocomial infections.
- As an anticancer agent, quercetin induced cytotoxicity in cell lines of most common cancers (breast and colon). The combination of quercetin with chemotherapeutic drugs, Anastrazole and Capecitabine is reported for the first time.

Limitations of the study

- Study did not involve more varieties of cell lines of a particular disease (breast and colon)
- Combinations would be further validated if *in vivo* studies are performed