DETECTION OF NOVEL METABOLITES IN GARLIC (*Allium sativum* L.) THROUGH *IN SILICO* ANALYSIS AND ITS VALIDATION

By

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(2013-11-106)

THESIS

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DECLARATION

I hereby declare that the thesis entitled **"Detection of novel metabolites in garlic (*Allium sativum* L.) through *in silico* analysis and its validation"** is a bonafide record of research work done by me during the course of research and that it has not been previously formed the basis for the award to me of any degree, diploma, fellowship or other similar title, of any other university or society.

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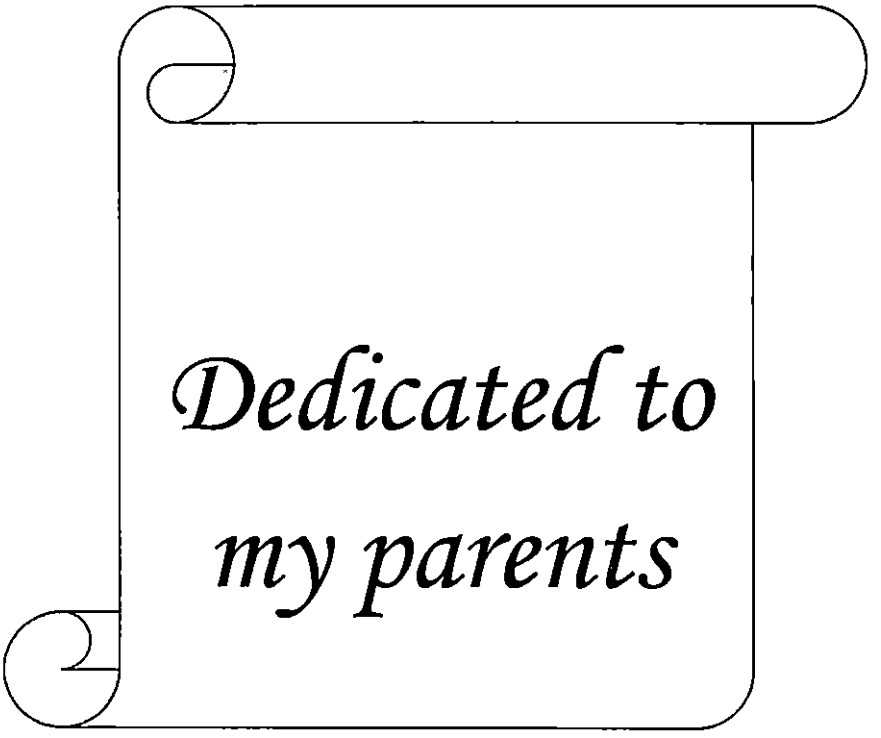
Dedicated to
my parents

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ABBREVIATIONS

%	Percentage
>	Greater than
β	Beta
°C	Degree Celsius
µg	Microgram
µl	Microlitre
ADMET	Absorption Distribution Metaboilsm Elimination Toxicity
AGE	Aged Garlic Extract
ALDR	Aldose Reductase
CDK	Cyclin Dependent Kinase
CHARMM	Chemistry at Harvard Macromolecular Mechanics
CPBMB	Centre for Plant Biotechnology and Molecular Biology
CT	Cycle Threshold
CYP2D6	Cytochrome P450 2D6 enzyme
DIC	Distributed Information Centre
DPP4	Dipeptidyl Peptidase-4
EDTA	Ethylene Diamine Tetra Acetic acid
EGFR	Epidermal Growth Factor Receptor
ER	Estrogen Receptor
FBS	Fetal Bovine Serum
g	Gram
HDL	High Density Lipoprotein
IR	Insulin receptor
KAU	Kerala Agricultural University
l	Litre
LDL	Low Density Lipoprotein
SACSHD	Alliin/ S allyl cysteine sulfoxide High Dose
SACSLD	Alliin/ S allyl cysteine sulfoxide Low Dose
SACHD	S allyl cysteine High Dose

SACLD	S allyl cysteine Low Dose
FAHD	Ferulic acid High Dose
FALD	Ferulic acid Low Dose
pCAHD	p-Coumaric acid High Dose
pCALD	p-Coumaric acid Low Dose
M	Molar
OSCs	Organosulfur compounds
mg	Milligram
ml	Millilitre
MTT	3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide
TS	Thymidylate Synthase
OD	Optical Density
PBS	Phosphate-buffered saline
PCR	Polymerase Chain Reaction
PDB	Protein Data Bank
PR	Progesterone receptor
RT-qPCR	Real Time quantitative Polymerase Chain Reaction
RPMI	Rosewell Park Memorial Institute
UV	Ultra violet
VEGFR1	Vascular Endothelial Growth Factor Receptor 1

Introduction

1. Introduction

Garlic (*Allium sativum* L.) is one of the earliest plants documented in literature for its beneficial therapeutic effects. Garlic was used extensively for its healing properties in ancient India as mentioned in the Vedas. Conventionally, it has been used to treat infections, wounds, diarrhea, rheumatism, heart disease, diabetes and many other disorders. Clinically, garlic has been investigated for a variety of conditions, namely hypertension, hypercholesterolemia, diabetes and also for the prevention of arteriosclerosis and cancer. The garlic plant (*Allium sativum*) is a bulbous one that belongs to family Alliaceae and genus *Allium*. The biological activities of garlic arise from its active chemical components. Garlic contains more than 2000 biologically active substances such as volatile, water-soluble and oil-soluble organosulfur compounds along with essential oils, dietary fiber, sugars, flavonoids and pectin. The most important ones are alliin, s-allyl cysteine, s-allyl mercapto cysteine etc.

Of the many favorable actions of garlic, inhibition of the growth of cancer is perhaps the most prominent. Setiawan *et al.* (2005) observed a negative dose response relationship between the monthly intake of garlic and the risk of stomach cancer in Shanghai and Qingdao in China. Based on the US Food and Drug Administration's evidence based review system for scientifically evaluating the risk of diverse types of cancer, human studies have revealed garlic's antitumorigenic potential in stomach, colon, rectal, breast, lung, and endometrial cancers (Kim and Kwon, 2009). Garlic has also been regarded as a potent anti-atherogenic food. Its capacity to lower blood cholesterol is believed largely to be due to reduction in LDL-cholesterol through inhibition of hepatic hydroxymethylglutaryl-CoA reductase activity by alliin and allicin (Sangeetha and Darlin, 2006). According to reports by Ryan *et al.* (2001) garlic appeared as the most used vegetable to cure diabetes. Research postulated that garlic may act as an antidiabetic agent by increasing either the pancreatic secretion of insulin from the β-cells or release of bound insulin (Thomson *et al.* 2007).

Though the medicinal properties of garlic are well known and are being exploited for quite a long time, the exact phytocompounds of garlic behind the

action of curing the disease by interacting with the protein targets is not much studied or reported. Use of bioinformatics (*in silico*) tools such as molecular docking will help to screen out potential phytocompounds from garlic that could be later developed as useful drugs to cure several diseases. This tool can also be used to find out target proteins which are implicated in various lifestyle diseases. Singh and Singh (2010) performed *in silico* analysis of organosulfur compounds from garlic to check the bioavailibility and the toxicity of the compounds. It was suggested that although computational toxicology is not intended to replace the development and use of experimental, laboratory-based approaches, these *in silico* approaches are intended to complement each other, save time and make research cost effective. Deshmukh *et al.* (2013) performed *in silico* reverse docking to find out novel therapeutic targets for ajoene, a compound of garlic.

Thus the present study entitled "Detection of novel metabolites in garlic (*Allium* sativum L.) through *in silico* analysis and its validation" was taken up to identify the actual compounds in garlic responsible for its medicinal properties. Some of the results of *in silico* work were further validated through wet lab studies such as cytotoxicity assays and real time PCR analysis.

Review of Literature

2. Review of literature

Garlic (*Allium sativum*) belonging to the family alliaceae is an important spice crop most widely used in India. Garlic bulb consists of 10-12 bulblets or segments called cloves. The bulb can be consumed as a spice or condiment in the form of garlic paste, pickle, chutney, curried vegetables, curry powder, meat preparation, etc. Value added products of garlic are represented by garlic powder, flavors, flakes and volatiles. Originated from Central Asia about 6000 years ago, garlic is one of the earliest of cultivated plants (Zohary and Hopf, 2000).

The name "*Allium sativum*" is derived from the Celtic word "all", meaning burning or stinging, and the Latin "sativum" meaning planted or cultivated. The English word, garlic, is derived from the Anglo-Saxon "gar-leac" or spear plant, referring to its flowering stalk. (Pickering, 1979).

China, India, Korea, Spain, Egypt and USA are the major garlic producing countries. China ranks first with an area of 8.50 lakh hectares and a production of 200.00 lakh tonnes. India ranks second with an area of 2.02 lakh hectares and the production of 11.50 lakh tonnes (Manoharan and Ramalakshmi, 2015). In India, Madhya Pradesh is the leading state in area (60,000 ha) and Gujarat is the leading state in production (2,77,000 MT), whereas maximum productivity is in Jammu and Kashmir (13.91 MT ha-1).

2.1 History of medicinal properties of garlic

Garlic has many medicinal properties and has been used as a remedy for various diseases and ailments throughout history. Some of the earliest references to this medicinal plant were found in the Avesta, a collection of Zoroastrian holy writings that was probably compiled during the sixth century BC (Dannesteter, 2003).

Sanskrit records document the use of garlic remedies approximately 5000 years ago, whereas the Chinese have been using it for at least 3000 years. Medical applications of garlic have been documented in ancient medical texts from Egypt,

Greece, Rome, China and India. Hippocrates, the Father of Medicine, prescribed garlic for various pathological conditions (Rivlin, 2001).

Garlic was administered to provide strength and increase work capacity for labourers in many cultures (Moyers, 1996). There is evidence that during the earliest Olympics, which originated in Greece, garlic was fed to the athletes before they competed (Lawson and Bauer, 1998). It was a "performance enhancing" agent used in competitive athletics. The Romans perceived garlic as an aid to improve strength and endurance; it was fed to both soldiers and sailors and was part of a ship's manifest when it set out to sea (Green and Polydoris, 1993).

Pedanius Dioscorides, the chief physicist of Greece and the author of a five-volume treatise 'De Materia Medica' (written in between 50 and 70 AD) recommended garlic because it cleans the arteries. Garlic was also recommended for disorders of the gastrointestinal tract, for the alleviation of joint disease and seizures (Riddle, 1996). Another Greek physicist, Pliny the Elder, mentioned in his five-volume treatise 'Historica Naturalis' about 23 uses of garlic for a variety of disorders among which the most important was to confer significant protection against toxins and infections (Bergner, 1996).

In ancient Chinese medicine, garlic was prescribed to aid respiration and digestion. In India, the leading medical text of history, Charaka-Samhita, recommends garlic for the treatment of heart disease and arthritis 2000 years ago (Woodward, 1996). All the 3 ancient medical traditions Unani, Tibbi and Ayurveda made extensive use of garlic for its healing power.

Thus, a bulb of garlic represented a whole pharmacy due to the broad spectrum of effects at the time when antibiotics and other pharmacy products did not exist. Garlic was referred with different names that are still in use such as, "Russian penicillin", "natural antibiotic", "vegetable viagra", "plant talisman", "rustic's theriac", "snake grass", etc. (Petrovska and Cekovska, 2010).

During the last century, scientific research and clinical trials to determine the effects of garlic consumption were intensified and now the medicinal use of garlic is common. Medicinal properties of garlic are known widely now and over a thousand scientific reports enumerate its functional activities which include free radical scavenging, immune stimulation, curing cardiovascular diseases, anti-cancer, and anti-infectious properties (Harris *et al.*, 2001; Khanum *et al.*, 2004; Borek, 2006; Herman-Antosiewicz *et al.* 2007; Singh *et al.* 2007).

Evidences ensure possible applications of garlic in cancer therapy, reactive oxygen species (ROS) associated diseases and certainly in immune-nutrition (Butt *et al.*, 2007). Its potential in combating lifestyle related disorders like hypercholesterolemia, dyslipidemia, and high blood pressure that lead to several cardiovascular pathologies has been the focus of major research interventions (Mahmoodi *et al.*, 2006; Kojuri *et al.*, 2007; Wojcikowski *et al.* 2007). However, the scientific explanation of the mode of action of active phytocompounds in garlic is still not well elucidated.

2.2 Chemistry of garlic

Garlic has a higher nutritive value than other *Alliums*. It has a high concentration of sulfur containing compounds. The major sulfur-containing compounds in intact garlic are δ-glutamyl-S-allyl-L-cysteines and S-allyl-L-cysteine sulfoxides (alliin). These compounds act as precursors of several other compounds (Matsuura, 1997). Garlic contains 65% water, 28% carbohydrates (fructans), 2.3% organo-sulfur compounds, 2% proteins (allinase, peroxidase and miracynase), 1.2% amino acids (cysteine, glutamine, isoleucine and methionine) and 1.5% fiber. It also contains volatile oils, minerals (selenium, germanium, calcium, copper, iron, potassium, magnesium, chromium, manganese, boron, barium, aluminium, sodium, phosphorus, zinc), bioflavonoids (quercetin, cyanidin, kaempferol, allistatin I, allistatin II) and vitamins like C, E , A, B1, B2, and niacin which help to protect cells from the harms of free radicals and

oxidation agents (Augusti, 1996). The list of major compounds from garlic is shown in Table 2.1.

The active substance allicin (diallyl thiosulfate) is responsible for the typical pungent smell (Macpherson *et al.*, 2005). Allicin is formed when alliin, a sulfur-containing amino acid, comes into contact with the enzyme alliinase when raw garlic is chopped, crushed, or chewed (Lawson and Hughes, 1992). Enzyme allinase is activated upon injuring the garlic bulb. The formation of allicin is completed in 0.2 to 0.5 minutes at room temperature. Allicin is an unstable product and undergo additional reactions to form different derivatives, depending on environmental conditions. This process requires hours at room temperature and minutes during cooking. Allicin, which has antimicrobial effects against many viruses, bacteria, fungi and parasites, was first chemically isolated in the 1940's. (Block, 1985). Other thiosulfinates present in garlic are allyl methyl thiosulfate and trans-1 propenyl-thiosulfinate which are unstable in nature (Lawson *et al.*, 1991).

Allyl sulfide and sulfur dioxide are formed by the decomposition of allicin. The major organosulfur volatiles that are identified from disrupted garlic and garlic essential oil are diallylsulfide, diallyl disulfide, diallyl trisulfide, methyl allyl disulfide, methyl allyl trisulfide, vinyldithiins and ajoenes (Amagase, 2006). Vinyldithiins are the thermal degradation products of allicin. It is formed by a type of mechanism involving diels-alder dimerization of thioacrolein, which is obtained by the beta elimination of allicin. The oil macerate of raw garlic is a very rich source of 2-vinyl-4H-1,3 dithiin (Lawson & Gardner, 2005). Ajoene, an unsaturated disulfide, is formed from a chemical reaction involving two allicin molecules. There are mainly 2 isomers of ajoene commonly known as E-ajoene and Z-ajeone present in oil macerated extract of garlic (Yoshida *et al.*, 1999).

When garlic is extracted with an aqueous solution, the l-γ-Glutamyl-S-allyl-L-cysteines are converted into s-allyl cysteine (SAC) through an enzymatic transformation with δ-glutamyl transpeptidase.

Table 2.1: List of major compounds present in garlic with their approximate concentration (Omar and Al-Wabel, 2010)

Sl. No.	Compounds in garlic	Concentration in bulb
1.	Allicin	1,500 - 27,800 ppm
2.	Alliin	5,000 - 10,000 ppm
3.	L-γ-glutamyl-S-allyl-cysteine	5000-16000 ppm
4.	Ajoene	268 ppm
5.	Allixin	1400 ppm
6.	Diallyl-sulfide	2 - 99 ppm
7.	Diallyl-disulfide	16 - 613 ppm
8.	Diallyl-trisulfide	10 - 1,061 ppm
9.	Dimethyl-disulfide	0.6 - 2.5 ppm
10.	Dimethyl-trisulfide	0.8 - 19 ppm
11.	Allyl-propyl-disulfide	36 - 216 ppm
12.	Dimethyl-difuran	5 - 30 ppm
13.	Ferulic-acid	27 ppm
14.	Isobutyl-isothiocyanate	25 ppm
15.	Kaempferol	83,200 ppm
16.	Apigenin	21,700 ppm
17.	P-coumaric-acid	58 ppm
18.	Quercetin	200 ppm
19.	Phloroglucinol	100 ppm
20.	Sinapic-acid	27 ppm
21.	S-allyl-mercapto-cysteine	2 ppm
22.	S-allyl-cysteine	10 ppm
23.	3-vinyl-4h-1,2-dithiin	0.34 - 10.65 ppm
24.	2-vinyl-4h-1,3-dithiin	2 - 29 ppm
25.	2-methyl-benzaldehyde	0.1 ppm

SAC is a water soluble compound which is predominantly found in the aqueous and alcoholic extracts of garlic (Kodera *et al.*, 2002). S-allyl mercapto-cysteine (SAMC) is another water soluble organosulfur compound produced in later transformation process. Further transformation of organosulfur compounds occurs after interaction with free sulfhydryl groups, including those present in cysteine, glutathione or proteins (Gilbert, 1990). Incubation of cysteine with DAS group produces allyl mercaptan.

2.3 Bioavailability and metabolism of organosulphur compounds

For any component to have physiological effects on the body, it needs to be absorbed by the body and the extent of absorption can be studied by following its bioavailability using *in vitro* or *in vivo* models.

The bioavailability of alliin was studied in a mouse murine model by oral administration with a dosage level of 10 mg/mouse. The results showed that alliin was present in stomach, intestine and liver to the extent of 7.2, 22.4 and 2.5 percent respectively. The bioavailability was said to be highly efficient since it did not result in the production of allicin or other degradation compounds such as DADS, vinyl dithiin and allyl conjugated compounds. In another animal study, bioavailability of alliin was found to be 16.5% with an oral dose of 60 mg/kg within a time span of 4 hours after ingestion, however, plasma concentration was found to be lesser (Guo *et al.*, 1990).

Pharmacokinetics study conducted by Lachmann *et al.* (1994) indicated the efficacy of absorption of synthesized s-labelled alliin. It was observed that the extent of absorption was about 60–70% in rats. It was also noticed that alliin as well as DADS (Diallyl-di-sulphides) could be detected in the perfusate after performing the isolated rat liver passage, but allicin was not found. This indicated that alliin itself is never converted to allicin in the body.

Egen-Schwind *et al.* (1992) reported the metabolic fate of allicin using isolated perfused rat liver. They stated that after the infusion of allicin, it was quickly

converted to DADS at a very low concentration. In later stages of metabolism it resulted in the formation of allylmercaptan in bile as well as in liver tissue. But allicin itself was not detected in the liver, only its metabolites were seen. Hence it was concluded that allicin is not a biologically active component of garlic. Vinyl dithiins could be detected in the serum, kidney and fat tissues after 24 hours of oral ingestion. No metabolites of vinyldithiins were present in either the perfusate or bile of the liver.

In a human study, Lawson and Wang (2005) demonstrated that when allicin was injected into the blood, it was found to be transformed very quickly into allyl mercaptan, but this compound was not seen in blood or urine of people who consumed garlic habitually. Allyl methyl mercaptan was predicted to be formed as an intermediary product immediately after consumption. Cooked whole garlic cloves do not produce allyl mercaptan because of the inactivation of allinase at cooking temperature, which is an essential component for conversion of alliin into allicin.

Rosen *et al.* (2000) demonstrated that after garlic consumption, allyl methyl sulfide (AMS), a metabolite of allicin, was the main volatile metabolite to be formed and it was exhaled through air, whereas DAS and DADS were detected at lower quantities. Using an animal model, Germain *et al.* (2002) demonstrated the after effect of DADS treatment. They stated that in urine the oxidized forms of AMS (Allyl Methyl sulphide) were present such as allyl methyl sulfoxide (AMSO) and allyl methyl sulfone ($AMSO_2$). From this it can be inferred that AMS or its derivative has the possibility of undergoing further transformation. The enzyme cytochrome P450 is implicated to be involved in metabolizing the organosulfur compounds in the body.

The pharmacokinetic property of SAC in animal studies have suggested that it was highly dependent on oral doses of SAC given. N-acetyl-S-allyl-L-cysteine, which is a metabolite of SAC was detected in the urine of dogs and

humans. The bioavailability of SAC was found to be 103% in mice, 98.2% in rats and 87.2% in dogs (Nagae *et al.*, 1994).

2.4 Garlic phytocompounds in therapeutics

2.4.1 Garlic in cancer

Cancer, also known as a malignant tumor or malignant neoplasm, is a group of diseases involving abnormal cell growth with the potential to invade or spread to other parts of the body. Production of a new lump involving any organ of the body along with abnormal bleeding and unexplained weight loss are the primary symptoms of cancer and in the later stages turn into a vulnerable disease leading to death, when not given proper treatment to the patient. The great majority of cancers, some 90–95% of cases, are due to environmental factors. Lifestyle, economic and behavioural factors are serious factors for cause of cancer. Common environmental factors that contribute to cancer death include tobacco (25–30%), diet and obesity (30–35%), infections (15–20%), radiation (both ionizing and non-ionizing, up to 10%), stress, lack of physical activity, and environmental pollutants. The most common types of cancer in males are lung cancer, prostate cancer, colorectal cancer, and stomach cancer, while in females, the most common types are breast cancer, colorectal cancer, lung cancer, and cervical cancer (Anand *et al.*, 2008).

Garlic contains compounds, which inhibit the initiation of carcinogenesis at a variety of sites in many animal species and with variety of carcinogens. The association between consumption of *Allium* vegetables and risk for cancer has been assessed in several experimental and epidemiological case–control studies in human and animals according to the reviews of Fleischauer, A. T, and Arab, L. (2001) and Khanum *et al.* (2004).

The preventive effects of garlic on human gastric cancer have been reported by Mei *et al.* (1982) in China Gangshan country, where residents had the lowest gastric cancer death rates (3.45/100000) because they consumed a large

amount of garlic (on average 20 g garlic) daily whereas Quixia county had highest death rates (40/100000), where little garlic is eaten (1 gm per day).

This hypothesis was further supported by results of another epidemiologic study in China conducted by You *et al.* (1989) where a significant reduction in stomach cancers associated with increasing consumption of *Allium* vegetables, including garlic, scallion, and chives has been reported.

There is evidence that at least part of the chemopreventive action of garlic in carcinogenesis is due to the induction of phase II detoxification enzymes including glutathione transferases, quinone reductase, epoxide hydrolase and glucuronosyl- transferase, which inactivate toxic substances and facilitate their excretion. These enzymes are highly inducible in animals and humans and a strong inverse relationship exists between tissue levels of phase II enzymes and susceptibility to chemical carcinogenesis. Glutathione-S-transferases (GSTs) are important detoxifying enzymes that remove harmful electrophiles by conjugating them with glutathione (Jakoby, 1978). Any substance that increases the levels and/or activity of GSTs has the potential to be chemopreventive. The effect of garlic-derived organosulfur compounds on GSTs and other detoxifying enzymes has been investigated in a number of laboratories.

Sparnins *et al.* (1986) first showed that allyl methyl tri sulphide (AMTS) increased the activity of GST in the forestomach, small-intestine, liver, and lung of mice and later again in 1988 showed that other allyl derivatives (allyl methyl disulfide, diallyl trisulfide, diallyl disulphide) also increased GST activity in these tissues. They observed that 96 h after oral administration of AMTS, GST activity was increased in all tissues and, in addition, benzo[a]pyrene induction of forestomach tumors was suppressed. They also noticed that propyl derivatives did not affect GST activity in these organs in mice indicating that allyl group is important for stimulation of GST activity. These studies suggest that stimulation of glutathione-s-transferase activity in the liver and other target organs of carcinogens by garlic derived organosulphur compounds may be responsible for

their protective effects in chemical carcinogenesis and on different stages of carcinogenesis. These results were confirmed by Sumiyoshi and Wargovich (1990), who found a greater effect of thioallyl than thiopropyl derivatives in inducing hepatic and colonic GST in mice. Derivatives with a propyl instead of an allyl group were found less active or inactive.

Imai and co-workers (1994) studied the antioxidant properties of three garlic preparations and organosulfur compounds in garlic. They observed that aged garlic extract, but not the fresh garlic extract, exhibited radical scavenging activity. Among the organosulfur compounds tested, the two major compounds in aged garlic extract, SAC and S-allylmercapto- L-cysteine had the highest radical scavenging activity.

Shirin *et al.* (2001) found that S allyl mercapto cysteine (SAMC) inhibited growth of colon cancer cell lines by inducing apoptosis and this was associated with increase in caspase-3- like activity. SAMC inhibited progression of cells at G2-M in the cell cycle.

Oommen *et al.* (2004) found that allicin inhibited the growth of cancer cells of murine and human origin. Allicin induced the formation of apoptotic bodies, nuclear condensation and a typical DNA ladder in cancer cells. They have observed that allicin activate the caspases-3,-8 and –9 and cleavage of poly (ADP-ribose) polymerase.

Wu *et al.* (2011) explored the anti-cancer effects of DAS in HeLa human cervical cancer cells. DAS treatment for 24-72 hours resulted in a marked decrease in cell viability time- and dose-dependently. Flow cytometric analysis showed that a 48-h treatment of 75 μM DAS induced G0/G1 cell cycle arrest and sub-G1 phase (apoptosis) in HeLa cells. Cells treated with different concentrations of DAS also showed changes typical of apoptosis such as morphological changes, DNA damage and fragmentation, dysfunction of mitochondria, cytochrome c release and increased expression of pro-caspase-3 and 9.

Ng *et al.* (2012) investigated the effect of SAC on the proliferation and metastasis of hepatocellular carcinoma (HCC). The result showed that the proliferation rate and colony-forming abilities of MHCC97L cells were suppressed by SAC. SAC significantly induced apoptosis and necrosis of MHCC97L cells through suppressing Bcl-xL and Bcl-2 as well as activating caspase-3 and caspase-9.

Liu *et al.* (2012) evaluated the anticancer effects of SAC on androgen-independent human prostate cancer (PC-3) cells. SAC suppressed the proliferation of PC-3 cells and led to cell cycle arrest at the G0/G1 phases, as well as inducing cell apoptosis which was accompanied by the decreased expression of Bcl-2 and increased expression of Bax and caspase 8. This study demonstrated the chemo-preventive activity of SAC *in vitro*, and that SAC may be a promising candidate for prostate cancer treatment.

Wallace *et al.* (2013) examined whether DATS could effectively reduce ectopic glioblastoma tumor burden. They found that a range of DATS doses (10μg/kg-10mg/kg) dose-dependently reduced tumor volume and number of mitotic cells within tumors after seven days.

A population-based case-control study has been conducted in a Chinese population from 2003 to 2010, with the aim to explore the association between raw garlic consumption and lung cancer (Jin *et al.*, 2013). Epidemiological data was collected by face-to-face interviews using a standard questionnaire among 1,424 lung cancer cases and 4,543 healthy controls. It was concluded that protective association between consumption of raw garlic and lung cancer has been observed with a clear dose-response pattern, suggesting that raw garlic consumption may potentially serve as a chemopreventive agent for lung cancer.

Xu *et al.* (2014) investigated the effects of S-allyl cysteine (SAC), a water-soluble garlic derivative, on human ovarian cancer cells *in vitro*. Human epithelial ovarian cancer cell line A2780 was used for the study. SAC (1−100 mmol/L) inhibited the proliferation of A2780 cells in dose- and time-dependent manners

(the IC50 value was approximately 25 mmol/L at 48 h, and less than 6.25 mmol/L at 96 h). Furthermore, SAC dose-dependently inhibited the colony formation of A2780 cells.

Dong *et al.* (2014) investigated the effects of aged black garlic extract (ABGE) on the proliferation and apoptosis of HT29 colon cancer cells. ABGE inhibited HT29 cell growth via the induction of apoptosis and cell cycle arrest through the inhibition of the PI3K/Akt pathway, suggesting that ABGE may be effective in the prevention and treatment of colon cancer in humans.

Zhang *et al.* (2014) found that SAMC exhibited an effective cell growth inhibition of human breast cancer cell lines MCF-7 (Estrogen Receptor positive) and MDA-MB-231 (Estrogen Receptor negative) in a dose- and time-dependent manner by inducing cell cycle arrested in G0/G1 phase. Furthermore, SAMC-mediated cell cycle arrest was accompanied with promotion of apoptosis. SAMC clearly triggered the mitochondrial apoptotic pathway as indicated by activation of Bax, decreased expression of Bcl-2 and Bcl-XL, and subsequent activation of caspase-9 and caspase-3. These results highlight the value of SAMC as a potential antitumor candidate for breast cancer.

2.4.1.1 Molecular targets identified for cancer

a. AKT/ Protein kinase B

Protein kinase B, also known as Akt, is a serine/threonine-specific protein kinase that plays a key role in multiple cellular processes such as glucose metabolism, apoptosis, cell proliferation, transcription and cell migration. AKT has 3 family members out of which 2 members, who are encoded by AKT1 and AKT2 genes, are putative oncogenes. Akt is involved in the PI3K/AKT/mTOR pathway and other signaling pathways. These signalling cascades are exclusively regulated by phosphatase and tensin homolog (PTEN). Mutation of any of the member or receptor or regulator of the cascade can lead to malignancy. Increased expression and activation of Akt is observed in many human cancers. Tumor cells

have been found to constantly activate Akt (Mahajan & Mahajan, 2012). Studies have identified gene amplification of the Akt isoforms in many types of cancer, including glioblastoma, ovarian, pancreatic, prostate and breast cancers. Overexpression also contributes to the malignant phenotype of human ductal pancreatic cancers.

b. Androgen receptor

Androgen receptor (AR) is a type of nuclear receptor that is activated by binding of androgenic hormone testosterone. Androgen regulated genes are critical for the development and maintenance of the male sexual phenotype and defects of AR cause phenotypic abnormalities of male sexual development. Alterations of AR function and expression have been characterized in clinical prostatic cancers. Approximately 80–90% of prostate cancers are dependent on androgen at initial diagnosis, and endocrine therapy of prostate cancer is directed toward the reduction of serum androgens and inhibition of AR (Denis and Griffiths, 2000). Prostate-specific antigen is up-regulated by androgens in the prostate. The different causes of AR alteration in prostate cancer are overexpression, mutation and hyper methylation of AR promoter regions (Suzuki *et al.*, 2003).

c. B cell lymphoma-2

B cell lymphoma-2 (Bcl-2), encoded in humans by the BCL2 gene, is a regulatory protein family that regulates cell death (apoptosis), by either inducing (pro-apoptotic) or inhibiting (anti-apoptotic) apoptosis. This family of proteins contains nearly 25 proteins such as Bcl-2 proper, Bcl X (Bcl X_L and Bcl X_S), Bcl w, Bax, Mcl-1, Bfl-1, Brag-1 etc. Simultaneous over-expression of Bcl-2 family anti apoptotic members and under-expression of pro-apoptotic members protects cell death and may produce aggressive B-cell malignancies including Follicular lymphoma, a type of blood cell tumours involving B cells. This will result in the lack of cell death that is characteristic of cancer. Evidence is found that Bcl X_L (Extra large isoform of Bcl X gene) along with Bcl-2 proper has protective effects

on anti-cancer drugs, whereas Bax and Bcl X_S (Extra small isoform of Bcl X gene) accelerate cell death induced by anti cancer drugs (Schmitt *et al.*, 1998). Thus the whole family of Bcl-2 genes has been implicated as the cause of a number of other cancers also such as breast, prostate, chronic lymphocytic leukemia, and lung cancer.

d. cMET

c-Met, also called as hepatocyte growth factor receptor (HGFR), is a protein found in humans and encoded by MET proto-oncogene (Comoglio *et al.*, 2008) . The protein possesses tyrosine kinase activity. It has also been found to be aberrantly activated in human cancers such as lung, breast, ovary, kidney, colon, thyroid, liver, and gastric carcinomas.

Deregulation and the consequent aberrant signaling of c-MET may occur by different mechanisms including gene amplification, overexpression, activating mutations, increased autocrine or paracrine ligand-mediated stimulation, and interaction with other active cell-surface receptors (Sierra and Tsao, 2011). Abnormal c-MET activation triggers tumor growth, formation of new blood vessels (angiogenesis) that supply the tumor with nutrients, and cancer spread to other organs.

e. Cyclin dependent kinase

Cyclin-dependent kinases (CDKs) are a family of heterodimeric protein kinases known as "master regulators" of cell cycle progression, molecular engines that drive cell cycle transitions. CDK binds a regulatory protein called cyclin. Constitutive or deregulated hyperactivity of these kinases due to amplification, overexpression or mutation of cyclins or CDK, contributes to proliferation of cancer cells. Mutations can inactivate checkpoint regulators, and tumour suppressor genes resulting in loss of cell cycle inhibition.

These kinases therefore constitute biomarkers of proliferation and attractive pharmacological targets for development of anticancer therapeutics. If it

is possible to selectively interrupt the cell cycle regulation in cancer cells by interfering with CDK action, the cell will die. There are about 20 different CDKs discovered and all of them being found to be responsible in causing of various types of human cancers such as ovary, brain, lung, cervix, breast, pancreas, prostate, bladder, colon, colorectal, endometrial, blood, ovary, thyroid etc. cancers (Morris *et al.*, 2015).

f. DNA Topoisomerase

DNA Topoisomerases are enzymes that regulate the overwinding or underwinding of DNA. Topoisomerases are classified as type I and type II. Type I enzyme cleaves one DNA strand at a time and type II cleaves both strands at a time (Champoux, 2001). They have grown into targets for cancer prevention.

Topoisomerase inhibitors such as topotecan, camptothecin, doxorubicin etc. interferes the work of topoisomerase and induces breaks in the DNA that ultimately lead to apoptosis. They prevent DNA religation and induce lethal DNA strand breaks (Pommier, 2009). Cancer cells are selectively sensitive to the generation of these DNA lesions.

g. Epidermal growth factor receptor (EGFR)

Epidermal growth factor receptor is a cell-surface receptor which exists on the cell surface and is activated by binding of its specific ligands, including epidermal growth factor and transforming growth factor α. Upon activation by its growth factor ligands, EGFR undergoes a transition from an inactive monomeric form to an active homodimer. Mutation in EGFR results in the formation of a number of cancers, including lung, breast, ovarian, prostate, anal cancers and glioblastoma (Walker *et al.* 2009; Liffers *et al.*, 2015). These somatic mutations involving EGFR lead to its constant activation, which produces uncontrolled cell division.

Mutations, amplifications or misregulations of EGFR or family members are implicated in about 30% of all epithelial cancers. Aberrant EGFR signalling

has also been implicated in psoriasis, eczema and atherosclerosis (Dreux *et al.*, 2006).

h. Estrogen receptor (ER)

Estrogen receptors are receptors that are activated by the hormone estrogen. Once activated by estrogen, the ER is able to translocate into the nucleus and bind to DNA to regulate the activity of different genes. Estrogen receptor is mainly involved in the development and maintenance of female sexual phenotype (Omoto and Iwase, 2015). Alteration of function of estrogen receptors due to mutation or other reasons lead to over-expression in around 80% of breast cancer cases (Lumachi *et al*, 2015).

i. Heat shock protein 90

Heat shock proteins (HSP) are a family of proteins that are produced by cells in response to exposure to stressful conditions. Numerous studies speculated that over-expression of HSP is in part responsible for resistance to many anti-tumor agents and chemotherapeutics (Khalil *et al.*, 2011). The ATPase activity of Hsp90 drives the chaperone cycle and releases its client proteins. Its client proteins include kinases, steroid hormone receptors and transcription factors that are directly involved in malignancy, and also mutated oncogenic proteins required for the transformed phenotype i.e. Her2, Raf-1, Akt, Cdk4, Polo-1 kinase, cMet, mutant B-Raf, mutant p53, AR, ER, Bcr-Abl, HIF-1 alpha, and hTERT. Thus HSP90 has been implicated as a promising target for cancer therapy (Jego *et al.*, 2013).

j. Mast/Stem cell growth factor receptor (SCFR)/ CD117/ KIT)

SCFR/CD117 is a receptor tyrosine kinase encoded in humans by proto oncogene KIT gene. Altered mutated forms of this receptor are associated with different types of cancers such as gastrointestinal, acute myeloid leukaemia, melanoma, mast cell cancer, nasal T-cell lymphoma, seminoma (a type of germ cell tumor) etc. Mutations often occur in membrane proximal immunoglobulin-

like domain (D5, exon 8 and exon 9), near membrane domain (exon 11) and the tyrosine kinase domain (exon 17). These mutations include missing, point mutation, duplication and insertion that can lead to c-kit receptor activation (Liang *et al.*, 2013).

k. Matrix metalloproteinase

Matrix metalloproteinases (MMPs) are zinc-dependent endopeptidases capable of degrading all kinds of extracellular matrix proteins. When their function or expression goes abnormal, they can contribute to virtually all steps of tumor progression (Kessenbrock *et al.*, 2015). MMPs have the ability to regulate the tumour micro-environment and their expression and activation is increased in almost all human cancers compared to normal tissue, when overproduction of MMPs occur (Yadav *et al.*, 2014).

There is compelling scientific evidence that MMPs play important roles in the development of cardiovascular diseases such as diabetes, atherosclerosis, myocardial infarct, hypertension, cardiomyopathy etc. Inhibition of these enzymes is beneficial to cure these cardiovascular conditions (Azevedo *et al.*, 2014; Vacek *et al.*, 2015). High expression of MMPs is also well documented in various neurological disorders including Parkinson's disease, Alzheimer's disease and Glaucoma (Singh *et al.*, 2015).

l. Nuclear factor kappa light chain enhancer of activated B cells (NFkB)

Nuclear factor kappa light chain enhancer of activated B cells (NFkB) is a protein complex that controls transcription of DNA, cytokine production and cell survival. Incorrect regulation of Nf-kB has been linked to cancer, inflammation, and autoimmune diseases (Hoesel and Schmid, 2013; Pal *et al.*, 2014). The activation of NFκB pathway is dependent upon the stabilization and activation of the NFκB inducing kinase (NIK).

The family of NFkB also contains receptor activator of nuclear factor kappa-B ligand (RANKL) which also has been found to be responsible in 80%

cases of chronic lymphocytic leukemia (CLL) and multiple myeloma. Overproduction of RANKL is implicated in a variety of degenerative bone diseases, such as rheumatoid arthritis and psoriatic arthritis. Thus NFkB along with its family members NIK and RANKL has long been considered as therapeutic targets for cancer and arthritis (Thu and Richmond, 2010; Xia *et al.*, 2014).

m. Phosphoinositide 3-kinase Gamma (PI3K-γ)

PI3Ks are intracellular signal transducer enzymes involved in cellular functions such as cell growth, proliferation, differentiation, motility, survival and intracellular trafficking. This enzyme family is a part of PI3K/AKt/mTOR pathway, an important pathway for cancer cells. There are 3 classes PI3Ks available in animal kingdom- Class I, II and III. Class I PI3Ks have the unique ability to generate phosphoinositide 3,4,5 trisphosphate (PIP3), which is a critical PI3K product that links lipid kinase activity to a network of downstream signals originating in AKT. There are four Class I PI3K isoforms (PI3K α, β, γ, δ). PIP3 is a 'secondary messenger' used by many different cell surface receptors to control cell movement, growth, survival and differentiation. This mechanism is correlated with oncogenicity of PI3Ks, the requisite gain of function achieved by either mutation or by differential expression (Vogt *et al.*, 2007; Vogt *et al.*, 2011). Many of these mutations cause the enzyme to be more active. PI3Ks are the most mutated kinases in case of glioblastoma (malignant brain tumor) and hematologic malignancies (blood cancers) (Jabbour *et al.*, 2014).

Recent reports of patients with rheumatic diseases such as systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) have shown pathogenic evidence of involvement of PI3K/AKt/mTOR pathway (Tamura, 2012). Selective inhibition of PI3Kδ, γ or β has been shown to reduce the severity of inflammation in one or more models of autoimmune disease, respiratory disease or allergic inflammation (Hawkins and Stephens, 2015).

n. Progesterone receptor (PR)

Progesterone Receptor is a nuclear receptor activated by progesterone hormone. Like its similar counterparts, Estrogen receptor and androgen receptor, it is also involved in the development and maintenance of female sexual phenotype. Alterations of PR functions lead to breast cancer, endometrial cancer, ovary cancer and menstrual disorders (Conzen, 2008; Bertos and Park, 2011; Shao, 2013; Diep *et al.*, 2015).

o. Thymidylate synthase

Thymidylate synthase is an enzyme that catalyzes the conversion of deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP). Thus it is essential for DNA synthesis and repair. TS protein and its mRNA levels are found elevated in many human cancers, and high TS levels have been correlated with poor prognosis in patients with colorectal, breast, cervical, bladder, kidney, and non-small cell lung cancers (Rahman *et al.*, 2004; Lurje *et al.*, 2009).

2.4.2 Garlic in diabetes

Diabetes is a metabolic disturbance that gradually affects the function of various systems in the body. Poorly controlled blood glucose is believed to be the most important factor in the development of diabetic complications (American Association of Diabetes Educators, 2002).

According to a report by Ryan *et al.* (2001), one-third of diabetic patients take alternative medications that they consider efficacious, of which garlic is the most commonly used. In another similar study by Johnson *et al.* (2006) on the use of natural remedies for Type II diabetes mellitus treatment in a diabetic women group from United States, garlic appeared as the most used vegetable to cure diabetes.

Jain and co-workers (Jain and Vyas, 1975) investigated the effect of extracts of garlic with water or several different organic solvents on oral glucose

tolerance in normal and alloxan-induced diabetic rabbits. Oral administration of 0.25 gm/kg of ethanol, petroleum ether, ethyl ether extract of *Allium sativum* causes 18.9, 17.9, 26.2% reduction in blood sugar in alloxan-diabetic rabbits. Ethyl ether extract from garlic is the most potent and active principle producing maximum hypoglycemic activity ($P < 0.001$).

S-allyl-cysteine sulfoxide /alliin (SACS), a constituent of garlic was shown to have a hypoglycemic effect (Sheela and Augusti, 1992). Administration of it at a dose of 200 mg/kg body weight decreased significantly the concentration of serum lipids, blood glucose and activities of serum enzymes like alkaline phosphatase, acid phosphatase, lactate dehydrogenase and liver glucose-6-phosphatase.

In alloxan treated diabetic rats, SACS controlled lipid peroxidation better than glibenclamide and insulin and ameliorated diabetic condition almost to the same extent as they did. Furthermore, SACS significantly stimulated *in vitro* insulin secretion from β cells isolated from normal rats (Augusti and Sheela, 1996).

Patumraj *et al.* (2000) reported that daily oral feeding of garlic extract (100 mg/kg) increased cardiovascular functions in STZ rats, prevented abnormality in lipid profile and increased fibrinolytic activities with decreased platelet aggregation. Plasma insulin level increased with concomitant decrease in plasma glucose levels. In addition, daily oral feeding of the same dose for 16 weeks showed anti-atherosclerotic effects in STZ diabetic rats. Thus, garlic may prevent diabetic cardiovascular complications.

Srinivasan in 2005 proposed that the bioactive constituent from garlic such as S-allyl cysteine sulphoxide (SACS), exert anti-diabetic action by stimulating insulin production and secretion by β cells of pancreas in rats, interfering with dietary glucose absorption.

In a study by Shariatzadeh *et al.* (2008) male wistar rats were divided into four groups viz. control, control+garlic extract, diabetic and diabetic+garlic extract. Diabetes was induced by intraperitoneal injection of 200 mg of sugar. The diabetic rats were treated with extract for 4 weeks. The final blood sugar was reduced significantly in diabetic rats treated with aqueous ethanolic extract of garlic.

Sivaraman *et al.* (2013) studied the effect of aqueous garlic extract on insulin resistance, inflammation and oxidative stress in male wistar rats fed with high fructose diet. Diabetes was induced in male albino wistar rats by feeding 60% fructose rich diet. The diabetic rats showed a significant increase in plasma fasting glucose, insulin, insulin resistance, tumour necrosis factor alpha and malondialdehyde level, decreased levels of total antioxidant status, reduced glutathione, catalase and glutathione peroxidase. Treatment with garlic extract restored all these biochemical changes.

2.4.2.1 Molecular targets identified for diabetes

a. Aldose reductase

Aldose reductase (or aldehyde reductase) is a cytosolic NADPH-dependent oxidoreductase that catalyzes the reduction of monosaccharides. It catalyzes reduction of glucose to sorbitol, the first step in polyol pathway of glucose metabolism (Petrash, 2004). The cells of the retina, kidney, and nervous tissues (myelin sheath) are insulin-independent, so glucose moves freely across their cell membrane, regardless of the action of insulin, where polyol pathway comes in action (Gabbay *et al.*, 1966). When blood glucose level in those organs is normal, aldose reductase has low affinity for glucose. In a hyperglycemic state, the affinity of aldose reductase for glucose rises, causing much sorbitol to accumulate.

Excessive activation of the polyol pathway increases intracellular and extracellular sorbitol concentrations and cause increased concentrations of

reactive oxygen species and decreased concentrations of glutathione. These imbalances can damage cells of those organs. Thus aldose reductase has long been believed to be responsible for diabetic complications such as diabetic retinopathy and diabetic neuropathy (Brownlee, 2001).

b. Dipeptidyl peptidase-4

Dipeptidyl peptidase-4 (DPP-4) is an enzyme which plays an important role in glucose metabolism. The enzyme protein encoded by the DPP4 gene is an intrinsic membrane glycoprotein and is responsible for the degradation of incretin such as glucagon-like peptide-1(GLP-1). GLP-1 is an incretin hormone which stimulate a decrease in blood glucose levels by increasing the amount of insulin released from pancreatic beta cells of the islets of Langerhans (Presswala and Shubrook, 2015). Thus by inhibiting DPP-4, the level of GLP-1 increases in blood and thereby releases adequate insulin which regulates the metabolism of glucose. Hence DPP-4 can be considered as a promising target for the treatment of type 2 diabetes (Vijayakumari *et al.*, 2011).

c. Glucokinase

Glucokinase is a hexokinase enzyme coded by the GCK gene. It facilitates phosphorylation of glucose to glucose-6-phosphate. Glucokinase occurs in cells in the liver, pancreas, gut, and brain. In each of these organs it plays an important role in the regulation of carbohydrate metabolism by acting as a glucose sensor, triggering shifts in metabolism or cell function in response to rising or falling levels of glucose (Iynedjian, 2009). Glucokinase in the liver is regulated by a glucokinase regulatory protein, which prevents glucokinase from becoming activated and available until glucose is metabolized, such as after meals, when insulin must be released to normalize blood glucose.

Glucokinase activity in the cytoplasm rises and falls with available glucose. Insulin acts directly or indirectly on glucokinase expression and activity

in the liver. Mutations of the gene for this enzyme can cause unusual forms of diabetes or hypoglycaemia (Gidh-Jain *et al.*, 1993; Shen *et al.*, 2006).

d. Glycogen synthase kinase 3 (GSK3)

Glycogen synthase kinase 3 is a serine/threonine protein kinase that mediates the addition of phosphate molecules onto serine and threonine amino acid residues. In mammals GSK-3 is encoded by two known genes, GSK-3 alpha (GSK3A) and GSK-3 beta (GSK3B). GSK-3 is active in a number of central intracellular signaling pathways, including cellular proliferation, migration, inflammation and immune responses, glucose regulation, and apoptosis. Due to its involvement in a great number of signaling pathways, GSK-3 has been associated with many diseases. GSK-3 inhibitors are currently being tested for therapeutic effects in Type II diabetes, inflammation, Alzheimer's disease, and bipolar disorder (Jope *et al.*, 2007; MacAulay and Woodgett, 2008).

GSK-3 play positive roles in cell proliferation and its aberrant expression as a tumor promoter. GSK-3 is overexpressed in various tumor types including colon, liver, ovarian and pancreatic tumors (McCubrey *et al.*, 2014).

e. Insulin receptor

Insulin receptor (IR), encoded by a single gene INSR, is a transmembrane receptor that is activated by insulin and insulin like growth factors and belongs to the large class of tyrosine kinase receptors. Activation of the insulin receptor is occurred for inducing of glucose uptake. Mutations in the insulin-receptor gene have been identified in patients with genetic syndromes of extreme insulin resistance i.e. a decrease in insulin receptor signalling (Taylor *et al.*, 1990). Thus, insulin insensitivity may lead to diabetes mellitus.

f. Peroxisome proliferator-activated receptor gamma (PPAR-γ)

Peroxisome proliferator-activated receptor gamma (PPAR-γ or PPARG) is a type II nuclear receptor that in humans is encoded by the PPARG gene. PPARG is mainly present in adipose tissue, colon and macrophages. PPARG regulates

fatty acid storage and glucose metabolism. The genes activated by PPARG stimulate lipid uptake and adipogenesis by fat cells. There is a constant flux of FFA (Free Fatty Acids) entering and leaving adipose tissue. If insulin is elevated there is a net inward flux of FFA and when insulin is low FFA leave adipose tissue.

Evidence suggests that PPAR- γ activation causes insulin sensitization (Haluzík and Haluzík, 2006). Increased levels of circulating free fatty acids and lipid accumulation in adipose tissue have been implicated in the development of insulin resistance. PPARγ is known to be implicated in various human chronic diseases such as diabetes mellitus, atherosclerosis, pulmonary hypertension, rheumatoid arthritis, inflammatory bowel disease, and Alzheimer's disease (Huang *et al.*, 2012; Usuda and Kanda, 2014).

2.4.3 Garlic in cardiovascular diseases

Cardiovascular disease (CVD) is a class of disease that involves the heart or blood vessels. Stroke, hypertensive heart disease, rheumatic heart disease, cardiomyopathy, atrial fibrillation, congenital heart disease, endocarditis, aortic aneurysms, and peripheral artery disease are different forms of CVD. This may be caused by high blood pressure, smoking, diabetes, lack of exercise, obesity, high blood cholesterol, poor diet, and excessive alcohol consumption, among others. High blood pressure results in 13% of CVD deaths, while tobacco results in 9%, diabetes 6%, lack of exercise 6% and obesity 5%. It is estimated that 90% of CVD is preventable. Prevention of atherosclerosis is by decreasing risk factors through: healthy eating, exercise, avoidance of tobacco smoke and limiting alcohol intake. (McGill *et al.*, 2008).

Garlic is a popular supplement well-perceived as a healthy choice among people to increase cardiovascular wellness. Approximately 30% of cardiovascular patients who use herbal supplements take garlic (Yeh *et al.* 2006).

2.4.3.1 Hypotensive effect of garlic

Hypertension is associated with structural changes in the heart and blood vessels which may lead to cardiovascular mortality and morbidity (i.e. cardiovascular disease, stroke, peripheral vascular disease, and renal disease). Hypertension is typically defined as having a systolic blood pressure (SBP) > 140 mmHg and a diastolic blood pressure (DBP) > 90 mm Hg (National High Blood Pressure Education Programme Coordinating Committee, 2003). Worldwide, approximately 1 billion people are affected by hypertension and seven million deaths per year may be attributed to hypertension (WHO, 2003).

The hypotensive effect of garlic was recognized by Loeper & Debray (1921). For centuries garlic has been used against hypertension in China and Japan and is recognized officially for this treatment by the Japanese food and drug administration (Bolton *et al.*, 1982).

Damrau (1941) gave two Allimin tablets containing 4.75gm of garlic concentrate (equivalent to about 0.31 g of desiccated garlic and 2.375gm of desiccated parsley) to 26 hypertensive patients three times daily for three days. Blood pressure reduction was observed in 85% of the patients: the average decline in systolic and diastolic blood pressure was 12.3mm Hg and 6.5mm Hg respectively.

The effect of oral administration of N^{ω}-nitro-L-arginine-methyl-ester (L-NAME), which induces arterial hypertension was checked for 4 weeks on control and garlic-fed rats by Pedraza-Chaverri *et al.* (1998). It was found that LNAME induced arterial hypertension on week's l-4 in control rats, but not in garlic-fed rats, whose blood pressure remained essentially at the basal values. Also, during this time period, blood pressure remained unchanged in garlic-fed rats without L-NAME treatment. It was concluded that garlic blocks LNAME- induced hypertension.

Garlic is considered one of the most popular complementary therapies for blood pressure control and is thought to be used by 50% of patients with hypertension (Bongiorno, 2008). According to a survey of 900 people conducted by the US Food and Drug Administration in 2002, garlic is the second most utilized complementary therapy (after *Echinacea*) with 17% of the population using this supplement within the preceding 12 month period (Timbo, 2006).

Blood pressure reducing properties of garlic have been linked to its hydrogen sulphide production (Benavides *et al.*, 2007) and aqueous garlic extract has been shown *in vivo* to inhibit angiotensin-converting enzyme (which aids in the production of angiotensin, a potent vasoconstrictor) and therefore reduces blood pressure in individuals with hypertension (Ali *et al.*, 2000).

Mousa and Mousa (2007) carried out an investigation to determine the *in vivo* effects of garlic and antioxidants on marginally high blood pressure in human subjects. The antioxidant vitamin C alone (2.0 g/d), garlic alone (2.5 g/d), or a combination was administered for 10 days in human subjects with marginally high blood pressure. Vitamin C alone did not result in any changes in systolic or diastolic blood pressure. In contrast, garlic resulted in a significant lowering ($P < 0.05$) of mean systolic but not diastolic blood pressure. In contrast, garlic plus vitamin C resulted in a distinct lowering of mean systolic and diastolic blood pressures to reference ranges.

The potential role of garlic for blood pressure lowering was supported by a meta-analysis of ten clinical trials by Reinhart *et al.* (2008). This formal meta-analysis found that garlic significantly lowers blood pressure in patients with hypertension by approximately 16.3 mm Hg systolic and 9.3 mm Hg diastolic compared with placebo in patients with elevated systolic blood pressure (SBP). This meta-analysis suggested that garlic is associated with blood pressure reductions in patients with an elevated SBP.

A review by Ried *et al.* (2008) that included 25 studies, eleven of which were eligible for meta-analysis (503 patients included in the SBP analysis and 565

in the DBP analysis), concluded that garlic preparations are, in fact, superior to placebo for lowering blood pressure in a mixed population of normotensive and hypertensive patients.

Effects of processed garlic (PG) on the systolic blood pressure (SBP) and the diastolic blood pressure (DBP) of spontaneously hypertensive rats (SHR) was evaluated by a study in 2011 by Han *et al.* The active compound SAC was analysed and calculated as 75.3 mg/100 g in PG. A placebo-controlled trial was conducted to test the efficacy of PG on lowering SBP and DBP of 44 hypertensive subjects over a period of 8 weeks. PG significantly lowered SBP after only 2 weeks ($p<0.01$), while a significant reduction in DBP ($p<0.05$) took after 8 weeks. After 8 weeks, PG lowered SBP by 8.05mm Hg. The study concluded that taking two 500 mg capsules of PG for 8 weeks can significantly lower blood pressure in hypertensive subjects.

In a study by Nwokocha *et al.* (2011), they investigated the cardiovascular effects of aqueous garlic extracts (AGE) on normotensive and hypertensive rats using the two-kidney one-clip (2K1C) model. Mean arterial blood pressure (MAP) and heart rate (HR) were measured in normotensive and 2K1C rat models. Intravenous injection of AGE (5-20 mg/kg) caused a significant ($p<0.05$) decrease in both MAP and HR in a dose-dependent manner in both the normotensive and 2K1C models, with more effects on normotensive than 2K1C rat model. The dose of 20mg/kg of AGE significantly ($p<0.05$) reduced systolic (16.7 ± 2.0%), diastolic (26.7 ± 5.2%), MAP (23.1 ± 3.6%) and HR (38.4 ± 4.3%) in normotensive rats. In 2K1C group, it significantly ($p<0.05$) reduced systolic (22.2 ± 2.1 %), diastolic (30.6 ± 3.2%), MAP (28.2 ± 3.1%) and HR (45.2 ± 3.5%) from basal levels. The study concluded that aqueous garlic extract reduces blood pressure and heart rate in both hypertensive and normotensive rats in a dose-dependent manner.

In another study in 2013 by Ried *et al.*, a total of 79 general practice patients with uncontrolled systolic hypertension participated in a double-blind

randomised placebo-controlled dose–response trial of 12 weeks. Participants were allocated to one of three garlic groups with either of one, two or four capsules daily of aged garlic extract (240/480/960 mg containing 0.6/1.2/2.4 mg of S-allylcysteine) or placebo. Blood pressure was assessed at 4, 8 and 12 weeks and compared with baseline using a mixed-model approach. Mean systolic blood pressure was significantly reduced by 11.8±5.4 mm Hg in the 2-capsule group over 12 weeks compared with placebo (P=0.006), and reached borderline significant reduction in the 4-capsule group at 8 weeks (−7.4±4.1 mm Hg, P=0.07). Changes in systolic blood pressure in the garlic-1-capsule group and diastolic blood pressure were not significantly different to placebo. The trial suggests that aged garlic extract is an effective and tolerable treatment in uncontrolled hypertension, and may be considered as a safe adjunct treatment to conventional antihypertensive therapy.

2.4.3.1.1 Molecular targets identified for hypertension

a. Adrenergic receptor

Adrenergic receptors are a class of G protein-coupled receptors that are targets of the catecholamines, especially norepinephrine (noradrenaline) and epinephrine (adrenaline). There are two main groups of adrenergic receptors, α and β, with several subtypes. Alpha adrenergic receptors evoke vasoconstriction, whereas beta receptors evoke vasodilation. The more the binding of catecholamines on these receptors, the more these receptors get involved in cardiac stimulation leading to hypertension, asthma, coronary heart disease and bradycardia. Permanent stimulation with catecholamines induce detrimental alterations of the cardiac function and thus increases blood pressure (Ciccarelli *et al.*, 2013).

b. Angiotensin Converting enzyme (ACE)

Angiotensin-converting enzyme (ACE) is a zinc-metallopeptidase enzyme which indirectly increases blood pressure by causing blood vessels to constrict. It

does that by cleaving the carboxy terminal His-Leu dipeptide of angiotensin I to produce a potent vasopressor octapeptide, angiotensin II. Angiotensin II is a potent vaso-active peptide that causes blood vessels to constrict, resulting in increased blood pressure (Weir and Dzau, 1999). ACE also degrades bradykinin, a potent vasodilator, and other vasoactive peptides.

ACE is a part of Renin-angiotensin-aldosterone system (RAAS) which is a hormone system that regulates blood pressure. If the renin–angiotensin–system is abnormally active, blood pressure will be too high (Baudin, 2002). As a part of RAAS, these above actions make ACE inhibition necessary in the treatment of conditions such as high blood pressure, heart failure and diabetic nephropathy.

c. Carbonic anhydrase (CA)

Carbonic anhydrase are a family of enzymes that catalyse the rapid interconversion of carbon dioxide and water to bicarbonate ions and protons (or vice versa). In humans, 15 CA isoforms are expressed. Carbonic anhydrase isoforms are highly expressed in pathological conditions like glaucoma and ocular hypertension (Swenson, 2014). They also associate with epilepsy, mountain sickness and different types of cancer (Potter and Harris, 2003).

2.4.3.2 Anticholesterol action of garlic

The presence of high levels of cholesterol in the blood is known as hypercholesterolemia. It is a form of hyperlipidemia i.e. elevated levels of lipids in the blood. Since cholesterol is insoluble in water, it is transported in the blood plasma within protein particles (lipoproteins). Lipoproteins are classified by their density: very low density lipoprotein (VLDL), low density lipoprotein (LDL), intermediate density lipoprotein (IDL) and high density lipoprotein (HDL) (Biggerstaff and Wooten, 2004).

All lipoproteins carry cholesterol, but elevated levels of lipoproteins other than HDL, particularly LDL-cholesterol are associated with an increased risk of atherosclerosis and coronary heart disease (Carmena *et al.*, 2004). Intake of high-

fat meals causes a significant increase in serum triglyceride and cholesterol levels (Groot & Scheck, 1984).

A study by Bordia (1981) was done to compare the effect of garlic on blood lipids in patients with coronary heart disease and healthy humans. The study was conducted on two groups of individuals divided into Group A and Group B. Group A consisted of 20 healthy volunteers, who were fed garlic for 6 months and then followed for another 2 months without garlic. Garlic administration significantly lowered the serum cholesterol and triglycerides while raising the high-density lipoproteins. Group B consisted of 62 patients with coronary heart disease with elevated serum cholesterol. They were randomly divided into two subgroups: B1 was fed garlic for 10 months while B2 served as a control. Garlic decreased the serum cholesterol ($p < 0.05$), triglycerides ($p < 0.05$) and low-density hipoprotein ($p < 0.05$) while increasing the high density cholesterol ($p < 0.001$). The change reached statistically significant levels at the end of 8 months and persisted for the next 2 months of follow-up. The study showed that essential oil of garlic shows distinct hypolipidemic action in both healthy individuals and patients with coronary heart disease.

Effects of garlic on lipid metabolism were studied by Chi *et al.* (1982) in three experiments using different aged male Sprague-Dawley rats fed a diet containing 1% cholesterol or 15% lard. Lyophilized garlic was supplemented at 2% and 4% of the diet. Rats fed with cholesterol and lard diets increased plasma cholesterol and triglycerides compared to controls. Garlic decreased plasma cholesterol in cholesterol- and lard-fed rats, but decreased plasma triglycerides only in the lard-fed group. Garlic supplementation decreased very low density lipoprotein (VLDL) cholesterol and increased high density lipoprotein (HDL) cholesterol. The liver weight, total liver lipid and cholesterol were increased in rats fed with a cholesterol diet but a supplementation of garlic decreased those parameters by about 30%.

Effects of garlic extract supplementation on blood lipid profile and oxidant/antioxidant status were investigated in volunteer subjects with high blood cholesterol by Durak *et al.* (2004). A total of 23 volunteer subjects with high blood cholesterol (>5.98 mmol/L) participated in the study. Of them, 13 patients were evaluated as a hypertensive group and the others a normotensive group. Subjects ingested aqueous garlic extract at the dose of 1 mL per kg of body weight per day (~10 g garlic/day) for 4 months. Serum total cholesterol, low-density lipoprotein (LDL) and very–low-density lipoprotein (VLDL) cholesterols, and triglyceride levels were found to be significantly lowered. The total: HDL cholesterol ratio was also found to be significantly decreased after the extract use. Systolic and diastolic blood pressure values were also found to be significantly lowered after extract supplementation in the hypertensive group, but no similar changes were observed in the normotensive group.

In 2007, a study was conducted by Kojuri *et al.* on the effects of *Anethum graveolens* and garlic on lipid profile in hyperlipidemic patients. Lipid profiles of 150 hyperlipidemic patients were checked at same conditions. They were divided into three equal groups randomly (each comprising of 50 patients) and were given enteric-coated garlic powder tablet (equal to 400 mg garlic, 1 mg allicin) twice daily, *Anethum* tablet (650 mg) twice daily, and placebo tablet. In garlic group, the total cholesterol (decreased by 26.82 mg/dl and 12.1% reduction) and LDL-cholesterol (decreased by 22.18 mg/dl and 17.3% reduction) dropped and HDL-cholesterol (increased by 10.02 mg/dl and 15.7% increases) increased, while in *Anethum* group the result was not significant.

In a study in 2014, Mohammadi and Oshaghi investigated the effect of garlic on lipid profile, glucose as well as liver X receptor α (LXRα) expression in intestine and liver of mice. LXRα is an important regulator of cholesterol, triglyceride and glucose homeostasis that belongs to the nuclear receptor superfamily. Forty male N-Mary mice were randomly divided into 3 groups (n = 8): group1 received chow + 2% cholesterol + 0.5% cholic acid, group 2: chow + 4% (w/w) garlic extract + 2% cholesterol + 0.5% cholic acid, and group 3:

chow only. Treatment ran for 1 month. Compared with hypercholesterolemic mice, treatment with garlic extract significantly decreased total cholesterol, low-density lipoprotein cholesterol (LDL-C), triglycerides, very low density lipoprotein-cholesterol (VLDL-C), alanine aminotransferease (ALT) and aspartate aminotransferase (AST) (all of them $P < 0.05$). Garlic extract also reduced LXRα expression in the liver and increased its expression in the intestine. These effects probably have an important role in reducing serum triglyceride and cholesterol.

2.4.3.2.1 Molecular target identified for cholesterol

a. HMG CoA reductase

3-hydroxy-3-methyl-glutaryl-CoA reductase (HMG CoA reductase) is an enzyme of the mevalonate pathway that produces cholesterol and other isoprenoids. About 70 percent of total cholesterol in the body is made by HMG CoA reductase. High cholesterol levels have been associated with cardiovascular disease. HMG-CoA reductase is active when blood glucose is high. This enzyme is thus the target of the widely available cholesterol-lowering drugs (Yeganeh *et al.*, 2014).

Statins are a class of drugs used to lower cholesterol levels by inhibiting the enzyme HMG-CoA reductase (Sharpe and Brown, 2013). Statins have been found to prevent cardiovascular disease and mortality in those who are at high risk.

2.4.4 Garlic in arthritis

Arthritis is a form of joint disorder that involves inflammation of one or more joints. The most common form of arthritis is osteoarthritis (degenerative joint disease), a result of trauma to the joint, infection of the joint, or age. Another important type is rheumatoid arthritis (RA), which is a long lasting autoimmune disorder. Pain, which can vary in severity, is a common symptom in virtually all types of arthritis. Other symptoms include swelling, joint stiffness and aching around the joints. While the cause of rheumatoid arthritis is not clear, it is

believed to involve a combination of genetic and environmental factors. The underlying mechanism involves the body's immune system attacking the joints.

Denisov *et al.* (1999) performed clinical trial of alisate--a garlic preparation produced in Russia. An open controlled trial of alisate enrolled 30 patients with rheumatoid arthritis (RA). 15 patients with RA of varying clinical form, stage and activity were given alisate in a dose of 300 mg (1 tablet) twice a day for 4-6 weeks, whereas rest 15 control RA patients received conventional antirheumatic therapy. The alisate group achieved a good and partial response in 86.5% of cases and the drug was well tolerated and had no side effects. Alisate was thus recommended for treatment of RA patients in combined and monotherapy.

Hussein and Sharara (2007) performed a population based study to compare the effect of combined garlic therapy and comprehensive rehabilitation program versus comprehensive rehabilitation program in controlling the clinical manifestations and quality of life in patients with knee osteoarthritis. 43 patients with knee osteoarthritis randomized to group I (comprehensive rehabilitation) (n=15) and group II (combined garlic therapy and comprehensive rehabilitation) (n=28). All patients had diet modification, electrotherapy, resistance and flexibility exercises for legs 3 times weekly for 8 weeks. Group II patients specially received garlic capsules 900mg daily with breakfast for 8 weeks. As a result, knee pain significantly decreased in group II mean ± standard deviation (-51.77 ± 11.17%) more than in group I (-22.92 ±5.31%) (P=.00001) and synovial inflammatory mediators significantly reduced only in group II (interleukin1β (-89.67% ± 3.73) (P=.00001), interleukin 6 (-92.98% ± 5.02) (P=.00001), tumor necrosis factor ά (-83.20% ± 8.52) (P=.00001). Thus it was concluded that garlic improves rehabilitation outcome of knee osteoarthritis.

Ban *et al.* (2009) reported that thiacremonone, a novel sulphur compound isolated from garlic exerted anti-inflammatory and arthritic effects. Topical application of thiacremonone suppressed inflammatory and arthritic responses as

well as expression of iNOS and COX-2. Thiacremonone (2.5-10 μg/ml) also inhibited lipopolysaccharide (LPS, 1 μg/ml)-induced nitric oxide (NO) production, NF-κB transcriptional and DNA binding activity in a dose dependent manner.

Lee *et al.* (2012) found that four sulfur-containing compounds from garlic namely Z- and E-ajoene and 2 oxidized sulfonyl derivatives of ajoene inhibited the production of nitric oxide (NO), prostaglandin E2 (PGE2) and the expression of pro-inflammatory cytokines tumor necrosis factor, interleukin-1b, and interleukin-6 in lipopolysaccharide (LPS)-activated macrophages. Western blotting and reverse transcription– polymerase chain reaction analysis demonstrated that these sulfur compounds attenuated the LPS-induced expression of the inducible NO synthase (iNOS) and cyclooxygenase-2 (COX-2) proteins and mRNA.

Shin *et al.* (2013) evaluated the effects of DADS on airway inflammation using an ovalbumin-induced model of allergic asthma and RAW264.7 cells. DADS decreased nitric oxide production with a reduction in the levels of interleukins (IL)-1b and IL-6 in RAW264.7 cells stimulated with lipopolysaccharide (LPS). DADS also reduced the expression of proinflammatory proteins including inducible nitric oxide synthase (iNOS), NF-kB, and matrix metalloproteinase (MMP)-9, and it enhanced the expression of antioxidant proteins including Nrf-2 and hemeoxygenase (HO)-1.

Kim *et al.* (2013) investigated whether caffeic acid, S-allyl cysteine, and uracil, which were isolated from garlic, modulate UVB-induced wrinkle formation and effect the expression of matrixmetalloproteinase (MMP) and NF-κB signaling. The results obtained showed that all three compounds significantly inhibited the degradation of type I procollagen and the expressions of MMPs *in vivo* and attenuated the histological collagen fiber disorder and oxidative stress *in vivo*. Furthermore, caffeic acid and S-allyl cysteine were found to decrease oxidative stress and inflammation by modulating the activities of NF-kB and AP-

1, and uracil exhibited an indirect anti-oxidant effect by suppressing cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) expressions levels.

Ho and Su (2014) evaluated the anti-neuroinflammatory capacity of raw and steamed garlic in lipopolysaccharide (LPS)-stimulated BV2 microglia. Raw garlic dose-dependently attenuated the production of LPS-induced nitric oxide (NO), interleukin-1β (IL-1β), tumor necrosis factor (TNF)-α, and monocyte chemo-attractant protein-1 (MCP-1), whereas steamed garlic could not provide much effect.

2.4.4.1 Molecular targets identified for arthritis and inflammation

a. Cyclooxygenase 2 (Cox2)

Cyclooxygenase is an enzyme that is responsible for formation of prostanoids, including prostaglandins such as prostacyclin and thromboxane. Prostaglandins are involved in various pathophysiological processes like inflammatory responses, carcinogenesis and cardiovascular events. Two isoforms of the protein COX are known: COX-1, which is constitutively expressed in most tissues and is responsible for the physiological production of prostaglandins; and COX-2, which is induced by tumor promoters, cytokines, mitogens and endotoxins, growth factors and hypoxia in inflammatory cells and is responsible for the elevated production of prostaglandins during inflammation (Gately, 2000). COX-2 is unexpressed under normal conditions in most cells, but elevated levels are found during inflammation (Elder and Paraskeva, 1999).Thus it was seen that pharmacological inhibition of COX2 can provide relief from the symptoms of inflammation and pain.

The overexpression of COX-2 along with increased angiogenesis is significantly associated with many carcinomas such as colon, intestine, pancreas, cervical, endometrial, breast, thyroid, laryngeal, bone, lung, ovary, prostate, blood

cancers etc. (Challa *et al.*, 2010 and Sonawane *et al.*, 2011). Consequently inhibiting COX-2 may benefit the prevention and treatment of cancers.

b. Glucocorticoid receptor (GR)

The glucocorticoid receptor is the receptor to which cortisol and other glucocorticoids bind. Cortisol and corticoids are steroid hormones and is produced in humans by adrenal cortex within the adrenal gland. It is released in response to stress and low blood glucose. They function to increase blood sugar through gluconeogenesis, to suppress the immune system, and to aid in the metabolism of fat, protein, and carbohydrate. The GR is expressed ubiquitously in almost every cell in the body and regulates genes controlling the development, metabolism, and immune response.

GR interacts with signalling pathways such as PI3K, JNK proteins and components of the T cell receptor (TCR) signalling complex and thereby modulate pro-inflammatory gene expression. GR regulates inflammation both through direct transcriptional action on target genes and by indirectly inhibiting transcriptional activities of transcriptional factors such as NF-kB, AP-1 or interferon regulatory factors (Herrero *et al.*, 2015). Thus GRs have been implicated in causing of inflammatory diseases and cardiovascular diseases such as atherosclerosis and hypertension (Kadmiel and Cidlowski, 2013).

c. Mineralocorticoid receptor

Mineralocorticoid receptor, also known as aldosterone receptor is encoded in humans by NR3C2 gene. These receptors have equal affinity for mineralocorticoids (aldosterone and deoxycorticosterone) and glucocorticoids (cortisol and corticoids) and are activated by these hormones. Its counterpart glucocorticoid receptor is expressed ubiquitously in all tissues but this one is expressed in selected tissues such as colon, salivary and sweat glands, distal nephron and vascular endothelium. Aldosterone is largely responsible for long term regulation of blood pressure by acting on distal tubules and ducts of nephron,

increasing reabsorption of ions and water in kidney (Gomez-Sanchez and Gomez-Sanchez, 2014). Aldosterone is a part of RAAS (Renin-angiotensin-aldosterone System), the activation of which is one of the reasons of established pathogenesis of hypertension (McGraw *et al.*, 2013).

Increase in aldosterone secretion by adrenal cortex because of stress conditions, increases the chance of activation of MR, which is implicated in cases of cancer like adrenal cancer, inflammation, atherosclerosis and hypertension (Xanthakis and Vasan, 2013; Moss and Jaffe, 2015).

d. Nitric Oxide Synthase

Nitric oxide synthases (NOSs) are a family of enzymes catalyzing the production of nitric oxide (NO) from L-arginine. NO is an important cellular signalling molecule and functions as a neurotransmitter. There are three known isoforms of NOS in mammals such as neuronal NOS (nNOS), endothelial NOS (eNOS) and inducible (iNOS).

iNOS is involved in immune defence against pathogens. Induction of the high-output iNOS usually occurs in an oxidative environment, and thus high levels of NO have the opportunity to react with superoxide leading to peroxynitrite formation and cell toxicity. iNOS produces large quantities of NO upon high stimulation, along with proinflammatory cytokines (eg. IL-1, TNF-α and Interferon δ). Increased levels of NO activity have been found in the synovial fluid of patients with rheumatoid arthritis (Nagy *et al.*, 2010). Overall, NO plays a critical role during inflammation and therefore, remains a potential target for developing therapeutics for inflammatory diseases (Kobayashi, 2010). Aberrant expression of iNOS has been documented in development of different types of cancers such as of colon, breast, prostate, lung, brain, bladder and skin (Janakiram and Rao, 2012; Jahani-Asl and Bonni, 2013).

e. p38 kinase /MAPK 14

Mitogen activated protein kinase 14 (MAPK14) is an enzyme encoded by MAPK 14 gene. These enzymes are stress activated serine threonine kinases. Cellular stress produced due to heavy network of signal transduction leads to over expression of MAPKs. There are 4 types of p38 kinase isozymes detected in mammalians which are p38α, p38β, p38γ and p38δ.

A large body of evidences indicate that p38 α activity is critical for normal immune and inflammatory response. The p38 MAPK pathway is a key regulator of pro-inflammatory cytokines biosynthesis at the transcriptional and translational levels, which makes this pathway a potential target for the treatment of autoimmune and inflammatory diseases (Cuenda and Rousseau, 2007; Clark and Dean, 2012). Inflammatory stimuli such as lipopolysaccharide (LPS), TNF and interleukin-1 (IL1) are the major inducers of p38α. Dysregulation of p38 MAPK levels in patients are also associated with advanced stages of cancers like prostate, breast, bladder, liver, and lung cancer (Koul *et al.*, 2013).

f. TNF Alpha

TNF Alpha is a cell signaling protein (cytokine) involved in systemic inflammation. The primary role of TNF is in the regulation of immune cells. Dysregulation of TNF production has been implicated in a variety of human diseases including Alzheimer's disease and cancer (Chu, 2013).

TNF alpha promotes the inflammatory response, which, in turn, causes many clinical problems associated with autoimmune disorders such as rheumatoid arthritis, inflammatory bowel disease and psoriasis (Mewar and Wilson, 2011).

g. VEGFR1

Vascular endothelial growth factor (VEGF)-A, is a tyrosine kinase which is a major regulator for angiogenesis, binds and activates two tyrosine kinase receptors, VEGFR1 (Flt-1) and VEGFR2 (Flk-1). These receptors regulate physiological as well as pathological angiogenesis. VEGFR2 is a direct signal

transducer for pathological angiogenesis including cancer and diabetic retinopathy, whereas VEGFR1 promotes tumor growth, metastasis, inflammation, rheumatoid arthritis and atherosclerosis (Shibuya, 2006).VEGFs and its ligands are highly expressed in synovial fluid in rheumatoid arthritis (Murakami *et al.*, 2006). Immuno-histo-chemical studies have indicated frequent VEGFR-1 expression in multiple human malignancies, including breast, nonsmall cell lung, squamous cell head and neck, prostate, pancreas, ovarian, colon, bladder, hematologic, and other cancers (Schwartz, 2010).

2.5 *In silico* docking studies

Millions of dollars and man-hours are devoted to the process of drug discovery and development (Ooms, 2000). Considering both the potential benefits to human health and the enormous costs in time and money of drug discovery, any tool or technique that increases the efficiency of any stage of the drug discovery enterprise will be highly prized. Computer-aided drug discovery (CADD) is a method which can be used to increase efficiency of the drug discovery process. The main benefit of CADD is cost effectiveness and reduced time in research and development. Its exploitation in drug development helps in the selection of only a potent lead molecule and thus avoids late stage clinical study failures (Wadood *et al.*, 2013). Molecular docking is a computational tool in CADD which determines binding affinity between molecules (protein structure and ligand) (Mukesh and Rakesh, 2011).

Singh and Singh (2010) performed *insilico* studies of 19 compounds in garlic regarding bioavailability using Spartan 06 programme. They found that S-propyl cysteine sulfoxide is potent to become a successful drug. It passed Lipinski's rule and Veber's protocol effectively, was well soluble in plasma and slightly soluble in water. Therefore, it is likely to bind most of the plasma proteins. Oral LD50 tool considered it to be slightly toxic.

Pany *et al.* (2013) performed molecular docking of 12 natural molecules using Autodock Vina against Cyclooxygenase which is implicated in

inflammatory responses. Naringin showed highest binding affinity (8.6 Kcal/mol) followed by rutin (8.3 Kcal/mol). Thus it was found that natural molecules can be potential anti-inflammatory agents.

Priya and Nazeem (2014) performed molecular docking of 16 phytocompounds from *Boerhaavia diffusa* against Bcl-2 family of proteins using Discovery Studio 3.5. *Boerhaavia diffusa* is an important medicinal plant used in various human ailments, including cancer, diabetes, hepatoprotective and anti-inflammatory effects. However, most of the reports in traditional medicine are yet to be validated through scientific studies. The study reported that phytocompound boeravinone F exhibited good interaction energy and has an inhibitory effect on receptor molecule Bcl-2. It was concluded that boeravinone F exhibits potent anticancer activity and might be considered as a lead compound for the development of potentially useful drugs that inhibit the target Bcl2 protein their by regulating apoptosis.

Nazeem *et al.* (2014) performed molecular docking of thirty five phytocompounds having anti-cancer properties with PI3K and NFkB, the signalling proteins involved in regulating Matrix metalloproteinase 9 expression, using Discovery studio programme 4.0. This signalling pathway for its upregulation and downregulation has long been implicated for causing cancer. It was found that five compounds namely allixin, capsaicin, eugenol, niazimicin and piperine passed ADMET filtering and so they were docked with the signalling proteins, out of which only niazimicin, a phytocompound of *Moringa Oleifera*, showed better interaction with the proteins.

Dhananjayan *et al.* (2014) studied the binding affinity of some selective bioflavonoids towards HMGCoA reducatse, an enzyme responsible for rate limiting step in mevalonate pathway. Insilico docking studies were performed using Autodock 4.2, to evaluate the binding affinity of bio-flavonoids like -(-) epiafzelechin, 2',3,5,6',7-Pentahydroxyflavanone, 2,3-Dehydrokievitone, 2'-Hydroxygenistein, 3,7-ODiacetylpinobanksin, 3-O-Acetylpinobanksin, 4'-

Hydroxywogonin, Acacetin on crystal structure of 3-hydroxy-3- methyl-glutaryl-CoA reductase. Among them 2, 3-Dehydrokievitone shows lowest binding energy (-8.29 kcal/mol). Binding site analysis showed interactions with amino acid residues like LYS691, ASP690, VAL805, ASN658, ILE762, ALA768, ASP767, GLY808, MET655, ASP767, and GLY656. Analysing binding sites and free energy of binding produced, explains the importance of bioflavonoids in targeting 3-hydroxy-3-methyl-glutaryl-CoA reductase inhibition in the treatment of hyperlipidemia next to statins and other drugs in future.

Mala *et al.* (2015) performed large scale molecular docking of 574 phytochemicals from ten medicinal plants (*Catharanthus roseus*, *Aegle marmelos*, *Aloe vera*, *Boerhaavia diffusa*, *Phyllanthus amarus*, *Pseurdarthira viscida*, *Pterocarpus marsupium*, *Sida rhombifolia*, *Trigonella foenum*, *Wrightia tinctoria*) and five spice crops (*Allivum sativum*, *Curcuma longa*, *Murraya koenigii*, *Piper nigrum*, and *Zingiber officinale*) of Kerala against anti-apoptotic target receptors Bcl 2 and Bcl XL. The study aimed at computational screening of potent lead molecules from these selected plants. It was found that around 20 phytocompounds had potential to be further developed into therapeutic drugs against these target proteins.

The accuracy of computational tools used in drug discovery and molecular interaction studies was assessed by Vipin and co-workers in 2015, in a study where Discovery Studio 3.5 programme was used for molecular docking purposes of 50 commercially available diabetes mellitus type 2 drugs against Dipeptidyl peptidase 4 (DPP4) enzyme. This enzyme is involved in Diabetes mellitus type 2 disease. The analysis showed that out of the fifty selected drugs, 33 drugs passed the Lipinski's rule and commercially prescribed drugs namely sulfonylurea, pregabalin and metaformin were found to have maximum interaction with the target. This study confirmed the efficacy of these drugs, druggability of the target DPP4 as well as the accuracy of the tool used.

All these studies reported earlier were found to be of general type and only few of them were concerned with the activity of special phyto-compounds from garlic (*Allium sativum* L.). Though the medicinal effect of garlic is well known, the true compounds which have ability to cure the disease is not much experimented. Garlic contains many organo-sulphur compounds and polyphenolic compounds which warrants novel research to exploit their potential. Garlic and other related *Allium*s are efficient in providing plant based drugs in the coming century. But bringing a drug in market needs plenty of years' research with huge skilful manpower. Computer aided drug discovery has the potential to help researchers in finding the right compound easily which has ability to inhibit targets involved in various diseases. In order to confirm the potential of computer aided drug discovery, wet lab studies could be performed extensively to confirm the ability of specific compounds in curing diseases. Based on the available literature, it could be highlighted that much more efforts are to be put for exploiting garlic based phytocompounds and developing novel drugs through computational tools.

Materials and Methods

3. Materials and methods

The study entitled 'Detection of novel metabolites in garlic (*Allium sativum* L.) through *in silico* analysis and its validation' was carried out in two parts viz. dry lab studies and wet lab studies.

Dry lab studies (*in silico* work) were carried out at Distributed Information Centre (DIC), Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture, Kerala Agricultural University during 2013-2015 with the objective to identify novel metabolites in garlic by molecular docking studies.

Validation of the outcome of computational interaction study was performed at Amala Cancer Research Centre, Thrissur and at CPBMB, KAU through wet lab studies.

3.1 Materials

3.1.1 *Insilico* study materials

3.1.1.1 Work Station and software

For performing *in silico* studies work station viz. a computer with suitable software installed for molecular docking studies was used. Distributed Information Centre (DIC) at KAU maintains work stations with high computing facilities installed with high graphics softwares. All the work stations are of 4 GB RAM and runs WINDOWS 7 in them. The commercially licensed software used for research purpose on molecular docking was Discovery Studio 4.0 developed and distributed by Accelrys, USA.

3.1.1.2 Internet and online databases and tools

Internet connectivity provided by KAU under National Knowledge Network (NKN) was extensively used for collecting information and accessing various biological databases for literature survey, target identification and ligand selection. For literature survey, MEDLINE (Medical Literature Analysis and

Retrieval System Online) database, which contain references and abstracts on life sciences and biomedical topics was accessed. Compiled by the United States National Library of Medicine (NLM), MEDLINE is freely available on the Internet and searchable via PubMed and NLM's National Center for Biotechnology Information's Entrez system.

For molecular docking work, a druggable target and a ligand is necessary. A target is commonly a protein which may be of different origin inside human body and may attempt for different physiological function. There are several such working proteins present in humans. They include enzymes, ion channels, receptors etc (Overington *et al.*, 2006). These targets sometimes function abnormally due to some external stimuli or due to mutation or genetic disorder and cause severe diseases (Imming *et al.*, 2006). So these targets are directed with ligands where the latter can bind at an active site/binding site of the former. A ligand was a signal triggering small molecule derived from any chemical or herbal source and has the ability to prevent a target from working abnormally. If the ligand can prove itself to successfully inhibit the abnormal working of a target, it can thus serve as a candidate drug and can be developed as a commercial one in later stages (Landry and Gies, 2008).

For target identification, Therapeutic Target Database (TTD-http://bidd.nus.edu.sg/group/cjttd/) provided by Bioinformatics and Drug Design Group in National University of Singapore was accessed and for retrieval of targets, the crystallographic database for three-dimensional structural data of protein such as Protein Data Bank (PDB) was used.

For ligand selection, Dr. Duke's database (http://www.ars-grin.gov/duke/), a database on active compounds present in different medicinal plants, was accessed. Forty eight compounds from garlic were selected and the three dimensional structures of these compounds were retrieved from chemical molecule databases such as Pubchem (maintained by National Centre for

Biotechnology Information) and Chemspider (maintained by Royal Society of Chemistry).

3.1.2 Wet lab study materials

3.1.2.1 Cell line

Cancer cell line HCT 15 (originated from human colon adenocarcinoma), L 929 (originated from murine fibro sarcoma) and Raw 264.7 (originated from murine leukaemic monocyte/macrophage) were procured from National Centre for Cell Sciences, Pune. All cells were of adherent nature.

3.1.2.2 Laboratory chemicals, glass wares and equipments

The chemicals and glass wares used for the work are of good quality and were procured from Sigma Aldrich, Life Technologies, Himedia, SISCO research laboratories (SRL), Bangalore Genie, Takara Bioindia Pvt.Ltd. and Tarson India Ltd. The list of chemicals and items procured for the work is mentioned in Annexure I. The equipments and machineries used were centrifuge, incubator, thermal cycler, laminar air flow, inverted microscope, ELISA reader and Applied Biosystems 7300 Real time PCR (Annexure II).

3.1.2.3 Primers

Forward and reverse primers specific to human gene EGFR was procured from SigmaAldrich. The sequences are as follows:

Forward-5′ GAGACGAGAACTGCCAGAA 3′

Reverse- 5 ′ GTAGCATTTATGGAGAGTC 3 ′

3.2 Methods

3.2.1 *In silico* studies

3.2.1.1 Identification of phytocompounds in garlic

Research papers, review papers and books related to garlic and its phytocompounds' action over various human diseases such as cancer, diabetes, arthritis, blood pressure and cholesterol were searched online in MEDLINE database via its free search engine Pubmed. Also journals and books from library were collected and studied thoroughly. Mechanism of action of proteins and their pathways involved in causing different life style diseases were also thoroughly studied.

3.2.1.2 Characterization and retrieval of phytocompounds present in garlic

Garlic (*Allium sativum*) contains a wide range of pharmacologically active compounds which has been used from historical period for curing of several diseases. Activities of these phytocompounds were obtained from literature survey and then their three dimensional chemical structures were retrieved from chemical structure databases. A total of 48 ligands/phytocompounds were identified and structures were retrieved from 2 databases. 46 ligand compound structures retieved from PubChem database (https://pubchem.ncbi.nlm.nih.gov/) in .sdf format, whereas rest 2 were retrieved from Chemspider database (http://www.chemspider.com/) in .mol format (Bolton *et al.*, 2008, Stephen *et al.*, 2009, Pence and Wiliams, 2010). The homepage of both these two databases were opened in a computer and the names of the compounds were typed in the search box provided, so as to download the three dimensional structures of the compounds selected. Molecular properties of the ligands such as molecular weight, molecular formula, partition coefficient, no. of hydrogen bond donors and acceptors were also recorded.

3.2.1.3 Preparation of ligand molecules and filtration

The retrieved molecules were prepared using 'Prepare ligand' protocol in Discovery Studio 4.0. The steps followed for performing the work was as follows: Open DS 4.0-> Click on file-> Click Open-> Add a ligand-> Click on small molecule-> Click on prepare ligands-> Click Run on the new window of the protocol. The finished work gets saved in a jobs window automatically. The

molecules were prepared by removing duplicates, enumerating tautomers/isomers, adding hydrogen bonds and the energy was minimized using CHARMm (Chemistry at Harvard Macromolecular Mechanics) force field (Brooks *et al.*, 1983 and Brooks *et al.*, 2009). The prepared molecules were filtered by Lipinski's Rule of five and Vebers protocol that sets the criteria for drug like properties and focuses on the drugs bioavailability. Lipinski's rule stated that compounds which tend to have drug like properties should have molecular mass less than 500 daltons, no. of hydrogen bond donors (total no. of nitrogen-hydrogen and oxygen-hydrogen bonds) should not exceed 5, no. of hydrogen bond acceptors (total no. of nitrogen or oxygen atoms) should not exceed 10 and logP value (octanol water partition coefficient) should not exceed 5 (Lipinski *et al.*, 2001 and Lipinski, 2004). Veber's protocol stated that value of polar surface area should not exceed 140 $Å^2$, no. of rotatable bonds should not exceed 10, and total no. of hydrogen bond acceptors and donors should not exceed 12 (Veber *et al.*, 2002). From saved jobs, the work of ligand preparation was opened and then subjected to "Filter by Lipinski and Veber rules" coming under the section of small molecules in DS 4.0. The filtered compounds were further forwarded for molecular docking.

3.2.1.4 ADMET analysis

The increase in the number of new compounds discovered each year has not resulted in the expected increase in the number of marketed new drugs. This has been attributed to poor pharmacokinetic (PK) properties of the candidate molecules (Kennedy, 1997). ADMET refers to absorption, distribution, metabolism, excretion and toxicity. These are the pharmacokinetic parameters which need to be screened preclinical in case of a potential candidate molecule before developing it as a drug i.e. these parameters sets for drug like properties (Modi, 2003 and Balani *et al.*, 2005). ADMET screening was performed by *in silico* method using "ADMET descriptors" protocol coming under the section of small molecules in DS 4.0. Preclinical ADMET screening facilitates early elimination of weak candidates and directs the entire focus of the drug development program towards fewer potential lead candidates. Hence, it is

mandatory that the pre-clinical candidates are subjected to as many possible reality checks (Singh, 2006 and Honorio *et al.*, 2013). The calculations were used to eliminate compounds with unfavourable ADMET characteristics. These parameter filtering can be done both before and after molecular docking to check bioavailability.

The drug when administered in the body needs to be easily absorbed in the small intestine, optimally soluble in body fluid, easily transported to target site, non-toxic to the body and easily excreted from body before its accumulation (Norinder and Bergstrom, 2006). ADMET parameters of best ligands were analysed by the 'ADMET descriptors' provided by Discovery Studio 4.0 (Shen *et al.*, 2010). There were 7 parameters calculated by this descriptor and they included human intestinal absorption, aqueous solubility, blood brain barrier, hepatotoxity, CYP2D6 inhibition, plasma protein binding and AlogP (Table 3.1). All these parameters were calculated in mathematical values (Reddy *et al.*, 2012).

Absorption level model predicts human intestinal absorption after oral administration and reports a classification of absorption level. It was calculated on the basis of a training set of 199 well absorbed molecules, their logP value and polar surface area. Absorption level was measured from 0 to 3. Rankings of 0 and 1 are acceptable only. Ranking higher or lower than these are not acceptable and thus show poor pharmacokinetic results.

Solubility level model predicts the solubility of each compound in water at 25°C and reports the predicted solubility and ranking relative to the solubility of a training set of 784 drug molecules with experimentally measured solubilities. Solubility level of any compound or drug was measured in values ranging from 0 to 5. Rankings from 2 to 4 indicate acceptable values.

Hepatotoxicity prediction model predicts the occurrence of hepatoxicity. It is totally dose dependent and increases as the dose increases. Compounds were classified as either toxic (read as true) or non-toxic (read as false). This parameter

Table 3.1: Description of ADMET parameters on the basis of rankings

Sl. No.	ADMET Descriptors	Rankings					
1.	Solubility level	0-Extremely low	1-Very low	**2-Low**	**3-Good**	**4-Optimal**	5-Too soluble
2.	BBB level	0-Very High penetrant	1-High	**2-Medium**	**3-Low**	**4-Very low**	
3.	CYP2D6 Prediction	**False-Non Inhibitor**	True-Inhibitor				
4.	Hepatotoxic Prediction	**False-Non toxic (0)**	True-Toxic (1)				
5.	Absorption level	**0-Good**	**1-Moderate**	2-Poor	3-Very poor		
6.	PPB Prediction	**False- poorly bounded**	True- Highly bounded				
7.	Alog P98 Value	**<4**					

was calculated on the basis of 382 training compounds which are known to exhibit severe liver toxicity in human body.

CYP2D6 prediction model predicts cytochrome P450 2D6 enzyme inhibition and reports whether or not a compound is an inhibitor of that enzyme. It was calculated on the basis of a training set of 100 compounds which are known inhibitors of Cytochrome P450 2D6. Cytochrome P450 2D6 is an enzyme which helps in metabolism of drugs by introducing hydrophilic functionaries into the drug molecule to facilitate easy excretion. Compounds are classified as either inhibitor (read as true) or non-inhibitor (read as false).

BBB level model predicts the blood brain barrier penetration of a molecule in CNS, defined as the ratio of the concentrations of solute (compound) on both sides of the membrane after oral administration. It was calculated on the basis of a confidence ellipse model derived from 800 CNS (Central Nervous System) therapeutic compounds and a robust regression model based on 120 CNS penetrating compounds. Blood Brain Barrier (BBB) level was measured in values ranging from 0 to 4. Rankings from 2 to 4 indicate acceptable values.

Plasma Protein Binding (PPB) was predicted using PPB prediction model to check whether or not a compound is likely to be highly bound to carrier proteins in the blood. Predictions were based on the similarity between the candidate molecule and two sets of marker molecules; one used to flag binding at a level of 90 percent or greater and the other at 95 percent or greater. Thus candidate molecules were classified as highly bounded and poorly bounded. The rankings in ADMET analysis and their interpretation are as follows:

3.2.1.5 Target protein Identification

The identification of targets is a significant step in the drug discovery process. The reported successful molecular targets responsible for diseases like cancer, diabetes, arthritis, high blood pressure and cholesterol were selected from Therapeutic Target Database (TTD- http://bidd.nus.edu.sg/group/cjttd/) (Liu *et al.*,

2011 and Qin *et al.*, 2014). The homepage of this database was opened and the names of the selected diseases were typed in the search box provided, so as get information about target proteins involved in causing the diseases.

The three dimensional crystal structures of these target proteins were retrieved from Protein Data Bank(PDB- http://www.rcsb.org/pdb/home/home.do), the major protein repository maintained by RCSB (Research Collaboratory for Structural Bioinformatics) and saved in .pdb format (Berman, 2000). The homepage of this database was opened and the names of the selected targets were typed in the search box provided, so as to download the three dimensional structures of those targets. The structures retrieved were selected on the basis of the following parameters:

i) structures are obtained through X ray diffraction process
ii) must contain one or more active site for binding to ligands
iii) must contain a natural inhibitor to understand its nature of binding
iv) must have a high active side residue count
v) resolution of the structure should be as less as possible to understand the position of amino acids on a Ramachandran plot.

Along with the 3D structures, the amino acid sequences of all the target protein structures were also retrieved in .fasta format, which is needed for checking the physico-chemical properties of the target proteins.

3.2.1.6 Checking of Physico-chemical properties

The physico-chemical properties of the retrieved protein molecules from PDB were checked by sequence analysis using ProtParam tool (http://web.expasy.org/protparam/) available in ExPASy server. For this work, the homepage of this tool was opened and the amino acid sequences of the target proteins were pasted in .fasta format in the empty box provided for computing the various parameters. This tool checks for instability index (stability in nature), aliphatic index (thermal stability), theoretical pI (acidic or basic character) and GRAVY values (hydrophilic or hydrophobic character) of the proteins which are

important factors for effective binding of ligands with the targets (Gasteiger *et al.*, 2005).

Instability index is a measure of unreactive nature of any concerned protein trying to maintain a static equilibrium at adverse conditions. It is measured in scale of above and below scores of 40. Score lesser than 40 indicates stable nature, whereas more than 40 indicates instability may because of complexity of the protein or maybe due to their involvemen in cell signalling.

The aliphatic index of a protein is defined as the relative volume occupied by aliphatic side chains (alanine, valine, isoleucine, and leucine). It is regarded as a positive factor for the increase of thermo- stability of proteins. Thermo-stability is the property of any protein to resist any change in its structure at a high temperature. Higher the aliphatic index, higher is the stability of the protein at a high temperature. Thermo-stability of a protein is attributed to several factors like hydrophobicity, compactness of structure, increased occurrence of proline residues in amino acid sequence, increased polar surface area, increased hydrogen bonding and many others. Score varying from 75 to 90 indicates a high thermal stability of the proteins.

Theoretical pI or isoelectric point is the pH at which a protein molecule does not carry any electrical charge. The net charge on protein molecule is affected by pH of its surrounding environment and can become more positively or negatively charged due to the gain or loss. Isoelectric point mostly depends on seven charged amino acids: glutamate (δ-carboxyl group), aspartate (ß-carboxyl group), cysteine (thiol group), tyrosine (phenol group), histidine (imidazole side chains), lysine (ε-ammonium group) and arginine (guanidinium group). At a pH below their pI, proteins carry a net positive charge; above their pI they carry a net negative charge. Protein pI is calculated using pKa values of amino acids. The pKa value of amino acids depends on its side chain. In an amino acid with only one amine (basic side chain) and one carboxyl group (acidic side chain), the pI can be calculated from the mean of the pKas of this molecule as shown below:

$$pI = \frac{pK_{a1} + pK_{a2}}{2}$$

Thus, this property has an important role in defining the pH dependent characteristics of a protein. pI value lesser than 7 indicates the acidic nature of the protein with an acidic environment whereas more than 7 indicates basic nature of the protein with a basic environment.

The Grand average of hydropathy (GRAVY) value of a protein is the measure of hydrophilic or hydrophobic nature of the protein. It is calculated as the sum of hydropathy values of all the amino acids, divided by the number of residues in the sequence. Increasing positive score indicates a greater hydrophobicity. A good negative value of GRAVY index indicates hydrophilic nature of protein for which they may get tendency to interact more with water than ligands, the reason for which water molecules are being removed during protein preparation.

3.2.1.7 Preparation of protein molecules

The PDB retrieved protein molecules (targets) contain impurities such as water molecules, hetero atoms, DNA structures, co factors and metal ions. So, they were prepared using 'prepare protein' protocol of Discovery Studio 4.0 using CHARMM force-field. The steps followed for performing the work was as follows: Open DS 4.0-> Click on file-> Click open-> Add a protein molecule-> Click on macromolecules-> Click prepare protein-> click on run on the new window of the protocol. This protocol corrects the protein structures by inserting missing atoms, modelling loop regions and side chains, adding hydrogen atoms, removing water molecules, natural ligands and hetero atoms, minimizing energy to avail a stable conformation. The finished work gets saved in a jobs window automatically.

3.2.1.8 Active site identification

Identification of binding site/ active site was crucial in the process of molecular docking. Active sites are the pockets in a protein receptor where amino acids join hands with any incoming ligand with the help of hydrogen bonds and hydrophobic bonds. Several active sites can be there in a single protein structure. The active sites were identified using 'PDB site records' method of DS 4.0. For this process, the prepared protein was displayed on the DS 4.0 screen, after which "Receptor ligand interactions" section was accessed to define an active site through PDB site records. In this process, probable binding sites are located looking at their natural ligands i.e. the inhibitors provided along with the crystal structures. The critical residues present in the active sites were identified through literature search.

3.2.1.9 Molecular docking

The protein molecules were then subjected to Molecular Dynamic (MD) simulation to identify the conformational involvement in biological activity (Duraant and McCammon, 2011). Structure based molecular docking was performed between the prepared target protein molecules and the identified *Allium* phytocompounds by 'CDOCKER' protocol of DS 4.0 to find the best pose and know the binding affinity of each phytocompound (including their isomers/tautomers) with each target molecule (Wu *et al.*, 2003). The steps followed for performing the work was as follows: Open DS 4.0-> click on Receptor ligand interactions-> Click on Dock ligands-> select CDOCKER protocol-> select input receptor as the visible prepared protein structure-> select input ligands as the visible filtered compound structure-> Click on Run. CDOCKER is a grid based molecular docking algorithm used for identifying the best ligand molecule based on their CDOCKER energy, CDOCKER interaction energy, and binding energy. With CDOCKER, ligand conformations are sampled via high temperature molecular dynamics (MD) and are allowed to flex during the

refinement via simulated annealing MD. CDOCKER algorithm has been shown to give highly accurate docked poses (Erickson *et al.*, 2004).

A maximum of ten poses were allowed to be analysed. The first pose which contains the least difference between CDOCKER energy and CDOCKER interaction energy (difference should not be more than 10) was considered as the best interaction. Also the pose which represents a high CDOCKER energy than the CDOCKER interaction energy is rejected. The scoring function was analysed using binding energy calculation. The calculation was performed first on the target receptor, then on the ligand and finally on the interacting complex. The energy difference is then calculated using the equation: $\Delta E = E_{complex} - E_{ligand} - E_{protein}$ (ΔE is the ligand binding energy). All the energy values were considered negative as the basis of molecular docking is an exothermic reaction, where any positive values are rejected indicating endothermic reaction. Number of hydrogen bonds between the target and ligand were also calculated, where more the number of bonds, stronger the affinity considered. Hydrogen bond with the lowest length was selected as the best attraction.

3.2.1.10 Comparative studies

Molecular docking studies between the target proteins and *Allium* phytocompounds were checked for their interaction efficiency by interacting the same targets with FDA (Food and Drug Administration) approved drugs. Information about the approved drugs were derived from Drug databank (http://www.drugbank.ca/) (Wishart *et al.*, 2006, Wishart *et al.*, 2008). For doing this work, the homepage of this database was opened and the name of protein target was typed in the search box provided, so as to gain information about drugs available in market against those disease causing proteins.

3.2.2 Wet lab studies

Wet lab studies were performed to validate some of the results obtained in *in silico* analysis. The studies were taken up to ascertain the anti-cancerous

activity of four selected phyto-compounds from garlic through cell line studies. The phytocompounds identified included s-allyl cysteine (SAC), alliin/ s-allyl cysteine sulfoxide (SACS), ferulic acid and p-coumaric acid. Details of cell line used is provided in section 3.1.2.1.

3.2.2.1 Preparation of media

RPMI (Roswell Park Memorial Institute medium) is a form of medium used in cell culture and tissue culture work. The RPMI 1640 liquid media procured from Sigma Aldrich (composition provided in annexure no. III) was enriched with sodium pyruvate, foetal bovine serum, glutamic acid and glucose as an additional source of energy. Sodium pyruvate and glutamic acid were added at a quantity of 10ml/lt of media, whereas glucose was added at a quantity of 4.5 gm/lt of media. Foetal bovine serum is the most widely used serum-supplement for the *in vitro* animal cell culture and it is added at heat inactivated form at a quantity of 100ml/lt of media.

HEPES buffer was also added to the media to maintain physiological pH. The RPMI medium was then filtered twice by an autoclaved filtering apparatus to avoid any contamination of bacteria and other micro-organisms, using 0.45 micron filter paper for positive filtering and 0.22 micron filter for negative filtering. After filtering, antibiotics such as penicillin and streptomycin were added to it at 100 International Unit per ml of media.

3.2.2.2 Cell line culturing and sub culturing

Cancer cell line cultures procured from National Centre for Cell Sciences (NCCS), Pune were maintained in an asceptic animal tissue culture lab at 37° C in RPMI 1640 media kept in a 25 cm^2 tissue culture flask (T flask) in an incubator and media was changed when the culture was confluent (Plate 8). Confluency was detected by a change in colour of red coloured media to yellow and vapours of carbon dioxide seen on the upper surface of flask indicated that the cells were

breathing. Further, the cell lines were also checked under an inverted microscope to check any possibility of contamination.

For sub-culturing, the used media was decanted and the lower surface of the flask was washed twice with Phosphate Buffer Saline (PBS) of pH 7.4, the composition of which is given in annexure no IV. PBS is used normally for dilution purposes of any drug and also washing purposes of dead cells. After washing with PBS, 1 ml of diluted trypsin in EDTA was added to detach the adherent cells on the surface of the tissue culture flasks and then kept for 1 minute incubation in the incubator at 37° C. 1-2 ml media was then added in the flask and slightly shaken, so that all the cells of the flask come suspended in that media. Then the media containing the cells was divided into 2 parts and kept in 2 flasks for further use.

3.2.2.3 Drug stock preparation

Stock of 10 mg/ml was prepared from phytocompounds such as alliin and s-allyl cysteine which are readily dissolved in water. For ferulic acid and p-coumaric acid, 10 mg/ml stock was made by dissolving in 400 µl ethanol and made upto 1 ml by PBS.

3.2.2.3 Plating and MTT assay

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay is a colorimetric assay for assessing cell survival and proliferation activity (Mosmann, 1983). This assay detects living, but not dead cells and the signal generated is dependent on the degree of activation of the cells. MTT is a yellow colour dye which attaches with live cells by forming an insoluble purple formazan. The proliferation activity of cell populations under different treatment conditions were determined based on the detection of mitochondrial dehydrogenase enzyme activity in living cells (Berridge and Tan, 1993; Berridge *et al.*, 2005). For MTT assay, 3 cell lines were plated in twelve (3x4) sterile 96 well plates (100 µl in each well) at a density of $1x10^6$ cells/ml and kept for

overnight incubation at 37° C. Next day the cells were treated with 4 phytocompounds seperately in all the twelve 96 well plates at a concentration of 10-250 µg/ml. For alliin and s-allyl cysteine, the concentrations used to treat cells were 10, 30, 50, 70, 90 and 100 µg/ml, whereas for ferulic acid and p-coumaric acid, the concentrations used to treat cells were 100, 130, 150, 180, 200, 230 and 250µg/ml. For each concentration, six wells of the 96-well microplate were used. A row of 6 wells was kept as control without any treatment of drugs.

After 48 hours incubation with the phytocompounds, 10 µl of MTT (5mg/ml in PBS) was added to each well in twelve 96 well plates and then kept for incubation at 37° C for 4 hours. The insoluble formazan formed by MTT with mitochondrial dehydrogenase, was dissolved by adding100 µl DMSO (Dimethyl sulfoxide) to each well. The absorbance was measured at 570 nm at 37° C using a monochromatic ELISA Microplate reader run with the help of VersaMax™ software. From the readings of absorbance values, IC_{50} values of all 4 phytocompounds were calculated and thus the optimum dose was computed. The concentration needed for 50% cell death was calculated as IC_{50} value (half maximal inhibitory concentration). For knowing the IC_{50} value, percentage of cell death was calculated by the following formula:

Percentage of cell death = (mean of control−mean of specific concentration)/ (mean of control) × 100.

3.2.2.4 cDNA synthesis and RT-qPCR analysis

Effect of selected garlic phytocompounds on the expression of EGFR gene was studied using HCT-15 cell line through RT-qPCR assay. For real time PCR (RT-qPCR) analysis, HCT 15 cell line at a volume of 2 ml was plated in 6 well plates at 37° C in RPMI 1640 media and overnight incubated in a CO_2 incubator for approximately 24 hours. Phytocompounds from *Allium* were used to treat the cell lines after 24 hours. They were applied at 2 concentrations up and down of approximate IC50 value (for Alliin and S allyl cysteine 80 and 120 µg/ml, for p-Coumaric acid 200 and 250 µg/ml and for ferulic acid 125 and 175 µg/ml). After

adding the phytocompounds drug, the cell lines were again incubated for more 24 hours to proceed for cDNA synthesis in order to check the effect of those phytocompounds on cancer cells by help of RT-qPCR analysis using gene specific primers.

For cDNA extraction, the cells were washed twice with PBS and then trypsinized to allow detaching the cells from the surface of the wells. When all the cells were detached, serum containing RPMI media was added to inactivate the trypsin. Then the cells were pipetted gently up and down to mix properly with the fresh media and transferred finally to 1 ml centrifuge tubes. The cell suspension was centrifuged at 2000 rpm for 5 minutes to pellet. The supernatant was aspirated and the precipitated pellet was washed with 500µl of cold PBS. The same was again centrifuged and aspirated to collect a fresh pellet which actually is a lump of cells. This pellet was again resuspended in 500µl of cold PBS and verified to be in a concentration of 1-10,000 cells / µl using a hemacytometer chamber. Cell density was adjusted using PBS. After cell counting, 1 µl of cells were transferred to small 0.2 ml thin walled PCR tubes as according to the no. of samples. 1µl of lysis enhancer was added over the cell suspensions of different samples. Incubation of 10 minutes was done in a thermal cycler preheated to 75° C. All the tubes were kept on ice for 2 minutes. Then the cell lysate was treated with DNase I (5µl) and DNase buffer (1.6µl) to degrade any contaminating DNA. A small spin was given to gently mix all the cell suspension and reagents together and then incubated for 5 minutes at 37° C. 1.2 µl of 25mM EDTA was added on each sample and gently pipetted up and down followed by another incubation at 70° C in a thermal cycler for 5 minutes. After this incubation, OligodT and dNTP mix were added at a volume of 2 µl and 1 µl respectively and again kept for incubation at 70° C for 5 minutes. Subsequently the PCR tubes were placed in ice for 2 minutes and the following were added-

I) RT buffer- 6 µl

II) RNaseOUT- 1 µl

III) Superscript RT- 1 µl

IV) DTT- 1 µl

By gently pipetting up and down the cell solution was mixed and then incubated for 50 minutes in a thermocycler preheated to 50° C. The reaction was inactivated at 85° C for 5 minutes. The single stranded cDNA produced at this step was stored at -80° C overnight. Next day the protocol was proceeded for second strand cDNA synthesis in a real time PCR.

For RT-qPCR, where we can actually know the amount of amplicon being produced after each cycle by the help of fluorescent dye, SYBR Premix Ex Taq II was used which was a readymade mastermix of the SYBR green fluorescent dye, Mg^{2+}, dNTP Mixture, RNase H and Taq polymerase. The experiment was actually performed to study the gene expression profile of EGFR. The reliability of any relative qPCR experiment can be improved by including an invariant endogenous gene control/ reference gene in the assay to correct sample variation. B actin was used as a reference gene in the present analysis.

For making a volume of 25 µl reaction mixture for performing PCR, 12.5 µl of SYBR Premix Ex Taq II, 1 µl each of forward and reverse primers of EGFR, 0.5 µl of reference dye (ROX), 5 µl of DEPC treated nuclease free water and 5 µl of template cDNA were added.

The PCR protocol was carried out in Applied Biosystems 7300 Real-Time PCR System. The 7300 system SDS software was used to make the following PCR programme:-

Stage 1: Initial denaturation. Reps 1, 95° C for 30 seconds

Stage 2: PCR. Reps 40, 95° C for 5 seconds and 60° C for 34 seconds

Stage 3: Dissociation stage. Reps 1, 95° C for 15 seconds, 60° C for 1 minute and 95° C for 15 seconds.

RT-qPCR results were calculated in terms of Ct values. The expression of target gene EGFR (Epidermal Growth Factor Receptor) was analysed using the

ΔΔCt method as prescribed by Livak and Schmittgen (2001). The formula for calculation was as follows:

$$\Delta\Delta Ct = (\Delta Ct_{target} - \Delta Ct_{actin})^{Test} - (\Delta Ct_{target} - \Delta Ct_{actin})^{Control}$$

A positive ΔΔCT value indicates down-regulation (decreased expression) and a negative ΔΔCT value indicates upregulation (increased expression) (Goni *et al.*, 2009). Compared to the endogenous house-keeping gene β- actin, the extent of target gene expression was showed as fold difference (increase or decrease).

Results

4. Results

The results of the study entitled 'Detection of novel metabolites in garlic (*Allium sativum* L.) through *in silico* analysis and its validation' are presented in this chapter under 2 main headings i.e. *in silico* analysis and wet lab analyis.

4.1 *In silico* analysis

4.1.1 Retrieval of phytocompounds from garlic and FDA approved commercial drugs

Forty-eight phytocompounds from garlic with medicinal properties were identified through literature survey and their three dimensional structures were retrieved from chemical structure databases of Pubchem and Chemspider in .sdf and .mol format respectively. Out of forty eight, thirty six compounds were exclusive *Allium* compounds, whereas twelve of them (not exclusive to *Allium*) were found present in all major plant families. For comparing their activity against several lifestyle diseases, 28 FDA approved commercial drugs were also identified and retrieved from Pubchem. The details of selected phytocompounds in garlic are presented in Table 4.1. Details of commercial drugs which are true inhibitors of common target proteins causing cancer, diabetes, blood pressure, cholesterol and arthritis are presented in Table 4.2.

4.1.2 Preparation of ligand molecules and filtration

Preparation of ligands was done by "prepare ligand" protocol of DS 4.0. On preparation of selected 48 ligands from *Allium sativum*, 67 conformations were obtained. The increase in number of ligands was due to the formation of isomers in some of the original ligands (Table 4.3). After preparation, all the 67 ligands were filtered through Lipinksi's rule of five and Veber's protocol, which allowed all 67 of them to pass. The filtering parameters as per Lipinski's rule of 5 and Veber's protocol for all ligands/ phytocompounds are presented in Table 4.4.

Table 4.1: Details of garlic compounds selected for *in silico* study from Pubchem/ Chemspider database

Sl. No.	Compound name	Source ID*	Chemical formula	Molecular Weight (g/mol)
1.	Alliin	87310	$C_6H_{11}NO_3S$	177.22
2.	Allicin	65036	$C_6H_{10}OS_2$	162.27
3.	Allixin	86374	$C_{12}H_{18}O_4$	226.26
4.	Allyl alcohol	7858	C_3H_6O	58.07
5.	Allyl Mercaptan	13367	C_3H_6S	74.14
6.	Allyl Methyl Sulfide	66282	C_4H_8S	88.17
7.	Allyl Methyl Disulfide	62434	$C_4H_8S_2$	120.23
8.	Allyl Methyl Trisulfide	61926	$C_4H_8S_3$	152.30
9.	Allyl Propyl Disulfide	16591	$C_6H_{12}S_2$	148.28
10.	Cycloalliin	193294	$C_6H_{11}NO_3S$	177.22
11.	Diallyl Sulfide	11617	$C_6H_{10}S$	114.20
12.	Diallyl Disulfide	16590	$C_6H_{10}S_2$	146.27
13.	Diallyl Trisulfide	16315	$C_6H_{10}S_3$	178.33
14.	Diallyl Tetrasulfide	75552	$C_6H_{10}S_4$	210.40
15.	Dimethyl Sulfide	1068	C_2H_6S	62.13
16.	Dimethyl Disulfide	12232	$C_2H_6S_2$	94.19
17.	Dimethyl Trisulfide	19310	$C_2H_6S_3$	126.26
18.	Dimethyl Tetrasulfide	79828	$C_2H_6S_4$	158.32
19.	Dimethyl Difuran	236769	$C_{18}H_{18}O_6$	330.33
20.	E-Ajoene	5386591	$C_9H_{14}OS_3$	234.40
21.	L-γ-Glutamyl-S-allyl-L-cysteine	9521749	$C_{11}H_{18}N_2O_5S$	290.33
22.	Methyl Propyl Disulfide	16592	$C_4H_{10}S_2$	122.25
23.	S-Allyl-D Cysteine	10313252	$C_6H_{11}NO_2S$	161.22
24.	S-Allyl-L- Cysteine	98280	$C_6H_{11}NO_2S$	161.22
25.	S-allyl mercapto cysteine	21375384	$C_6H_{11}NO_2S_2$	193.28
26.	S-Ethyl-L-cysteine	92185	$C_5H_{11}NO_2S$	149.21
27.	S-Ethyl-L-Cysteine Sulfoxide	25202	$C_5H_{11}NO_3S$	165.21
28.	S-Methyl-L-cysteine	24417	$C_4H_9NO_2S$	135.18
29.	S-Methyl-L- Cysteine Sulfoxide	182092	$C_4H_9NO_3S$	151.18

Table 4.1 contd.

Sl. No.	Compound name	Source ID*	Chemical formula	Molecular Weight (g/mol)
30.	S-Propyl-L-Cysteine	101975	$C_6H_{13}NO_2S$	163.23
31.	S-Propyl-L-Cysteine Sulfoxide	25202184	$C_6H_{13}NO_3S$	179.23
32.	Thiacremonone	539170	$C_6H_8O_3S$	160.19
33.	Z-Ajoene	9881148	$C_9H_{14}OS_3$	234.40
34.	2-Methylbenzaldehyde	10722	C_8H_8O	120.14
35.	2-Vinyl-4H-1,3-Dithiin	133337	$C_6H_8S_2$	144.25
36.	3-Vinyl-4H-1,2-Dithiin	150636	$C_6H_8S_2$	144.25
37.	Apigenin	5280443	$C_{15}H_{10}O_5$	270.23
38.	Cyanidol	68247	$C_{15}H_{11}ClO_6$	322.69
39.	Campesterol	173183	$C_{28}H_{48}O$	400.68
40.	Ferulic acid	445858	$C_{10}H_{10}O_4$	194.18
41.	Isobutyl isothiocyanate	68960	C_5H_9NS	115.19
42.	Kaempferol	5280863	$C_{15}H_{10}O_6$	286.23
43.	Myricetin	5281672	$C_{15}H_{10}O_8$	318.23
44.	P-Coumaric Acid	637542	$C_9H_8O_3$	164.15
45.	Phloroglucinol	359	$C_6H_6O_3$	126.11
46.	Quercetin	5280343	$C_{15}H_{10}O_7$	302.23
47.	Sinapinic acid	637775	$C_{11}H_{12}O_5$	224.20
48.	Taurine	1123	$C_2H_7NO_3S$	125.14

*Source ID- Source of compounds was from 2 databases- Pubchem and Chemspider. From group A, only compound no. 21 and 25 were from Chemspider database, rest all (including group B) were from Pubchem database.

Table 4.2: Details of FDA approved commercial drugs selected for *in silico* study

Sl. No.	Drug name	Drug databank ID	Pubchem ID	Chemical formula	Target protein inhibited	Disease involved
1.	Alvocidib	DB03496	5287969	$C_{21}H_{20}ClNO_5$	Cyclin-dependent kinase	Cancer
2.	Arsenic trioxide	DB01169	518740	As_2O_3	AKT /Protein kinase B	Cancer
3.	Bevacizumab+ Rituximab	DB00112	24801581	$C_{14}H_{19}IN_2O_3$	B cell lymphoma-2	Cancer
4.	Brinzolamide	DB01194	68844	$C_{12}H_{21}N_3O_5S_3$	Carbonic anhydrase	Blood pressure
5.	Cabozantinib	DB08875	25102847	$C_{28}H_{24}FN_3O_5$	c-Met kinase	Cancer
6.	Captopril	DB01197	44093	$C_9H_{15}NO_3S$	Angiotensin Converting enzyme	Blood pressure
7.	Cyproterone	DB04839	5284537	$C_{22}H_{27}ClO_3$	Androgen receptor	Cancer
8.	Dasatinib	DB01254	3062316	$C_{22}H_{26}ClN_7O_2S$	Mast/stem cell receptor	Cancer
9.	Dexamethasone	DB01234	5743	$C_{22}H_{29}FO_5$	Glucocorticoid receptor	Cancer and blood pressure
10.	Diclofenac	DB00586	3033	$C_{14}H_{11}Cl_2NO_2$	Cyclooxygenase-2	Arthritis and cancer
11.	Doxycycline	DB00254	54671203	$C_{22}H_{24}N_2O_8$	Matrix metalloproteinase	Cancer, blood pressure, diabetes and arthritis
12.	Epalrestat	DB02383	1549120	$C_{15}H_{13}NO_3S_2$	Aldose reductase	Diabetes
13.	Fluorouracil	DB00544	3385	$C_4H_3FN_2O_2$	Thymidilate synthase	Cancer
14.	Gefitinib	DB00317	123631	$C_{22}H_{24}ClFN_4O_3$	Endothelial Growth Factor Receptor	Cancer

Table 4.2 contd.

Sl. No.	Drug name	Drug databank ID	Pubchem ID	Chemical formula	Target protein inhibited	Disease involved
15.	Geldanamycin	DB02424	5288382	$C_{29}H_{40}N_2O_9$	Heat shock protein 90	Cancer
16.	Humalog	DB00046	16132438	$C_{257}H_{389}N_{65}O_{77}S_6$	Insulin receptor	Diabetes
17.	Idelalisib	DB09054	11625818	$C_{22}H_{18}FN_7O$	PI3K-gamma	Cancer
18.	Imatinib	DB00619	5291	$C_{29}H_{31}N_7O$	p38 MAP kinase	Cancer and arthritis
19.	Lithium carbonate	DB01356	767	CLi_2O_3	Glycogen synthase kinase 3	Diabetes
20.	Lovastatin	DB00227	53232	$C_{24}H_{36}O_5$	HMG CoA reductase	Cholesterol
21.	Megestrol acetate	DB00351	11683	$C_{24}H_{32}O_4$	Progesterone receptor	Cancer
22.	Pioglitazone	DB01132	4829	$C_{19}H_{20}N_2O_3S$	Peroxisome proliferator-activated receptor gamma	Diabetes
23.	Propranolol	DB00571	4946	$C_{16}H_{21}NO_2$	Adrenergic receptor	Blood pressure
24.	Regorafenib	DB08896	11167602	$C_{21}H_{15}ClF_4N_4O_3$	Vascular endothelial growth factor receptor 1	Cancer and arthritis
25.	Spironolactone	DB00421	5833	$C_{24}H_{32}O_4S$	Mineralocorticoid Receptor	Cancer and blood pressure
26.	Tamoxifen	DB00675	2733526	$C_{26}H_{29}NO$	Estrogen receptor	Cancer
27.	Topetecan	DB01030	60700	$C_{23}H_{24}ClN_3O_5$	DNA topoisomerase	Cancer
28.	Vildagliptin	DB04876	6918537	$C_{17}H_{25}N_3O_2$	Dipeptidyl peptidase-4	Diabetes

Table 4.3: List of phytocompound isomers observed on ligand preparation

Sl. No.	Ligand name	No. of isomers produced
1.	Alliin or S-Allyl L-Cysteine sulfoxide	2
2.	Apigenin	5
3.	Cyanidin Chloride or cyanidol	2
4.	Ethiin or S-Ethyl Cysteine Sulfoxide	2
5.	Kaempferol	5
6.	Methiin or S-Methyl Cysteine Sulfoxide	2
7.	Myricetin	3
8.	Propiin or S-Propyl L-Cysteine Sulfoxide	2
9.	Phloroglucinol or 1 3 5-benzenetriol	2
10.	Quercetin	2
11.	S-Allyl-Mercapto Cysteine	2
12.	Thiacremonone	2
13.	Rest of 36 compounds produced no isomers	0
	Total	67

Table 4.4: Characteristics of selected phytocompounds as per Lipinski's rule and Veber's protocol

Sl. No.	Ligand name	No. of H bond donors (<5)	No. of H bond acceptors (<10)	AlogP (<5)	No. of rotatable bonds (<10)	Polar surface area (<140 Å^2)
A. Compounds exclusive to *Allium*						
1.	Alliin	3	4	-3.39	5	104.05
2.	Allicin	0	1	2.01	5	61.58
3.	Allixin	1	4	1.87	5	55.76
4.	Allyl alcohol	1	1	0.25	1	20.23
5.	Allyl Mercaptan	0	0	1.23	1	38.79
6.	Allyl Methyl Sulfide	0	0	1.41	2	25.3
7.	Allyl Methyl Disulfide	0	0	2.00	3	50.6
8.	Allyl Methyl Trisulfide	0	0	2.59	4	75.9
9.	Allyl Propyl Disulfide	0	0	2.88	5	50.6
10.	Cycloalliin	2	4	-3.61	1	93.02
11.	Diallyl Sulfide	0	0	2.03	4	25.3
12.	Diallyl Disulfide	0	0	2.62	5	50.6
13.	Diallyl Trisulfide	0	0	3.21	6	75.9
14.	Diallyl Tetrasulfide	0	0	2.57	3	101.2
15.	Dimethyl Sulfide	0	0	0.79	0	25.3
16.	Dimethyl Disulfide	0	0	1.39	1	50.6
17.	Dimethyl Trisulfide	0	0	1.98	2	75.9
18.	Dimethyl Tetrasulfide	0	0	2.57	3	101.2

Table 4.4 contd.

Sl. No.	Ligand name	No. of H bond donors (<5)	No. of H bond acceptors (<10)	AlogP (<5)	No. of rotatable bonds (<10)	Polar surface area (<140 Å^2)
19.	Dimethyl Difuran	0	6	3.14	6	78.88
20.	E-Ajoene	0	1	2.06	8	86.88
21.	L-γ-Glutamyl-S-allyl-L-cysteine	4	7	-4.51	10	162.3
22.	Methyl Propyl Disulfide	0	0	2.26	3	50.6
23.	S-Allyl-D Cysteine	3	3	-2.28	5	93.07
24.	S-Allyl-L- Cysteine	3	3	-2.28	5	93.07
25.	S-allyl mercaptocysteine	0	3	-0.48	5	120.97
26.	S-Ethyl-L-cysteine	2	4	-2.65	4	102.43
27.	S-Ethyl-L-Cysteine Sulfoxide	3	4	-3.66	4	104.05
28.	S-Methyl-L-cysteine	3	3	-2.89	3	93.07
29.	S-Methyl-L- Cysteine Sulfoxide	3	4	-4.01	3	104.05
30.	S-Propyl-L-Cysteine	3	3	-2.02	5	93.07
31.	S-Propyl-L-Cysteine Sulfoxide	3	4	-3.14	5	104.05
32.	Thiacremonone	1	4	-0.703	0	85.66
33.	Z-Ajoene	0	1	2.06	8	86.88
34.	2-Methylbenzaldehyde	0	1	2.08	1	17.07
35.	2-Vinyl-4H-1,3-Dithiin	0	0	1.98	1	50.6
36.	3-Vinyl-4H-1,2-Dithiin	0	0	2.11	1	50.6
B. Compounds not exclusive to *Allium*						
37.	Apigenin	2	5	1.71	1	89.82
38.	Cyanidol	5	6	3.04	1	112.45
39.	Campesterol	1	1	7.63	5	20.23

Table 4.4 contd.

Sl. No.	Ligand name	No. of H bond donors (<5)	No. of H bond acceptors (<10)	AlogP (<5)	No. of rotatable bonds (<10)	Polar surface area (<140 Å^2)
40.	Ferulic acid	1	4	0.19	3	69.59
41.	Isobutyl isothiocyanate	1	1	2.10	2	46.06
42.	Kaempferol	2	6	0.47	1	112.88
43.	Myricetin	4	8	-0.02	1	153.34
44.	P-Coumaric Acid	1	3	0.21	2	60.36
45.	Phloroglucinol	3	3	1.10	0	60.69
46.	Quercetin	2	7	-0.48	1	135.94
47.	Sinapinic acid	1	5	0.18	4	78.81
48.	Taurine	3	4	-4.49	2	93.22

Similarly the same protocol was followed for FDA approved commercial drugs. On preparation of selected 28 FDA approved drugs, 108 isomers were produced. Only 99 of them passed Lipinksi's rule of five and Veber's protocol. The best isomer for each drug which satisfied the rule of 5 was selected for further analysis. Details of such isomers selected is provided in Table 4.5.

4.1.3 ADMET results

ADMET descriptors as provided by DS 4.0 use seven parameters for estimating the bio-availability of the concerned drug in human body. These 7 parameters have filtered the 48 phytocompounds and 28 commercial FDA approved drugs on the basis of bioavailability. The parameters are solubility level, absorption level, BBB level, PPB prediction, CYP2D6 prediction, hepatotoxicity prediction and AlogP. There are mathematical values for these parameters which ranges from 0 to 5 and the compounds screened have to fall in acceptable range. Results of ADMET evaluation of *Allium* phytocompounds are given in Table 4.6 and that of commercial drugs in Table 4.7.

All the phytocompounds and commercial drugs were classified into 3 categories: Acceptable, Highly acceptable and Not acceptable. Highly acceptable are those compounds which fall strictly in the acceptable limits of the screened parameters. Acceptables are those which show upto 2 parameters not falling in the acceptable range and non-acceptables are those which have 3 or more parameters not falling in the acceptable range. Out of 48 phytocompounds, it was found that only 4 compounds were highly acceptable as they strictly came under unobjectionable range. They were allixin, allyl methyl sulfide, s-allyl mercapto cysteine and p-coumaric acid. Non-acceptable group contains only 2 compounds i.e. campesterol and taurine. Rest 42 compounds came under acceptable range.

Out of 28 commercial drugs, only one drug i.e. dexamethasone strictly came under highly acceptable group, whereas 5 of them such as cabozantinib, gefitinib, pioglitazone, regorafenib and tamoxifen were found highly non-acceptable. Rest 22 drugs came under acceptable range.

Table 4.5: Characteristics of commercial drugs (selected isomers) on ligand preparation and filtration as per Lipinski's rule and Veber's protocol

Sl. No.	Drug name	Isomer details		Characteristics					
		Nos. observed	Nos. passed	Molecular Weight (g/mol)	No. of H bond donors (<5)	No. of H bond acceptors (<10)	AlogP (<5)	No. of rotatable bonds (<10)	Polar surface area (<140 $Å^2$)
1.	Alvocidib	11	11	401.84	4	6	3.11	2	93.06
2.	Arsenic trioxide	1	1	197.84	0	3	0	0	27.69
3.	Bevacizumab+Rituximab	1	1	390.21	2	5	-0.95	7	73.66
4.	Brinzolamide	2	2	383.50	4	8	-2.00	7	168.38
5.	Cabozantinib	8	2	501.50	3	8	4.76	8	100.03
6.	Captopril	1	1	217.28	0	4	-0.81	3	99.24
7.	Cyproterone	1	1	374.90	1	3	2.94	1	54.37
8.	Dasatinib	18	18	488.00	4	9	4.12	7	139.44
9.	Dexamethasone	1	1	392.46	3	5	1.71	2	94.83
10.	Diclofenac	1	1	296.14	1	3	2.89	4	52.16
11.	Doxycycline	7	0	444.43	7	10	-3.13	2	188.48

Table 4.5 contd.

Sl. No.	Drug name	Isomer details		Characteristics					
		Nos. observed	Nos. passed	Molecular Weight (g/mol)	No. of H bond donors (<5)	No. of H bond acceptors (<10)	AlogP (<5)	No. of rotatable bonds (<10)	Polar surface area (<140 Å^2)
12.	Epalrestat	1	1	319.39	0	4	1.97	4	117.83
13.	Fluorouracil	9	9	130.07	2	4	0.83	0	66.24
14.	Gefitinib	5	5	446.90	2	7	4.20	8	69.99
15.	Geldanamycin	3	0	560.63	4	11	3.04	5	166.97
16.	Idelalisib	8	8	415.42	2	8	3.62	5	99.16
17.	Imatinib	10	10	493.60	4	8	4.90	7	92.06
18.	Lithium carbonate	1	1	60.01	0	3	-1.97	0	63.19
19..	Lovastatin	1	1	404.53	1	5	4.22	7	72.83
20.	Megestrol acetate	1	1	384.50	0	4	3.51	3	60.44
21.	Pioglitazone	4	4	356.43	1	5	4.59	7	97.08
22.	Propranolol	1	1	259.34	3	3	1.31	1	46.07
23.	Regorafenib	3	3	482.81	3	7	5.06	7	95.84
24.	Repaglinide	2	2	452.59	2	4	2.18	10	82.9

Table 4.5 contd.

Sl. No.	Drug name	Isomer details		Characteristics					
		Nos. observed	Nos. passed	Molecular Weight (g/mol)	No. of H bond donors (<5)	No. of H bond acceptors (<10)	AlogP (<5)	No. of rotatable bonds (<10)	Polar surface area (<140 $Å^2$)
25.	Spironolactone	1	1	416.57	0	4	3.73	2	85.74
26.	Tamoxifen	2	2	371.51	1	2	4.76	8	13.67
27.	Topetecan	2	2	457.90	3	8	1.15	3	108.47
28.	Vildagliptin	2	2	303.39	3	5	0.18	3	80.94

Table 4.6: ADMET properties of phytocompounds in garlic*

Sl. No.	Ligand	Solubility level (2-4)	BBB level (2-4)	CYP2D6 Prediction (False-non inhibitor)	Hepatotoxic Prediction (False-non toxic)	Absorption level (0-1)	PPB Prediction (False-poorly bound)	Alog P98 Value (<4)	Remarks
	A. Compounds exclusive to *Allium*								
1.	Alliin	5	4	FALSE	FALSE	3	FALSE	-3.39	A
2.	Allicin	4	1	FALSE	FALSE	0	FALSE	1.51	A
3.	Allixin	3	2	FALSE	FALSE	0	FALSE	1.87	HA
4.	Allyl alcohol	5	2	FALSE	TRUE	1	FALSE	0.26	A
5.	Allyl mercaptan	4	4	FALSE	TRUE	1	FALSE	1.23	A
6.	Allyl Methyl Sulfide	4	4	FALSE	FALSE	1	FALSE	1.41	HA
7.	Allyl Methyl Disulfide	4	1	FALSE	FALSE	1	FALSE	2.00	A
8.	Allyl Methyl Trisulfide	3	1	FALSE	FALSE	1	FALSE	2.59	A
9.	Allyl Propyl Disulfide	3	0	FALSE	FALSE	0	FALSE	2.88	A
10.	Cycloalliin	5	4	FALSE	FALSE	3	FALSE	-3.61	A
11.	Diallyl Sulfide	4	1	FALSE	FALSE	1	FALSE	2.02	A

Table 4.6 contd.

Sl. No.	Ligand	Solubility level (2-4)	BBB level (2-4)	CYP2D6 Prediction (False-non inhibitor)	Hepatotoxic Prediction (False-non toxic)	Absorption level (0-1)	PPB Prediction (False-poorly bound)	Alog P98 Value (<4)	Remarks
12.	Diallyl Disulfide	3	1	FALSE	TRUE	1	FALSE	2.62	A
13.	Diallyl Trisulfide	3	0	FALSE	TRUE	0	FALSE	3.21	A
14.	Diallyl Tetrasulfide	3	0	FALSE	TRUE	0	FALSE	3.8	A
15.	Dimethyl Sulfide	4	4	FALSE	TRUE	2	FALSE	0.79	A
16.	Dimethyl Disulfide	4	4	FALSE	TRUE	1	FALSE	1.39	A
17.	Dimethyl Trisulfide	4	1	FALSE	TRUE	1	FALSE	1.98	A
18.	Dimethyl Tetrasulfide	3	1	FALSE	TRUE	1	FALSE	2.57	A
19.	Dimethyl Difuran	2	2	FALSE	TRUE	0	TRUE	3.14	A
20.	E-Ajoene	4	1	FALSE	FALSE	0	FALSE	2.06	A
21.	L-γ-Glutamyl-S-allyl-L-cysteine	5	4	FALSE	FALSE	3	FALSE	-4.51	A
22.	Methyl Propyl Disulfide	4	1	FALSE	FALSE	1	FALSE	2.26	A

Table 4.6 contd.

Sl. No.	Ligand	Solubility level (2-4)	BBB level (2-4)	CYP2D6 Prediction (False-non inhibitor)	Hepatotoxic Prediction (False-non toxic)	Absorption level (0-1)	PPB Prediction (False-poorly bound)	Alog P98 Value (<4)	Remarks
23.	S-Allyl-D Cysteine	5	4	FALSE	FALSE	3	FALSE	-2.28	A
24.	S-Allyl-L- Cysteine	5	4	FALSE	FALSE	3	FALSE	-2.28	A
25.	S-allyl mercapto cysteine	4	3	FALSE	FALSE	0	FALSE	-0.09	HA
26.	S-Ethyl-L-cysteine	5	4	FALSE	FALSE	3	FALSE	-2.55	A
27.	S-Ethyl-L-Cysteine Sulfoxide	5	4	FALSE	FALSE	3	FALSE	-3.66	A
28.	S-Methyl-L-cysteine	5	4	FALSE	FALSE	3	FALSE	-2.89	A
29.	S-Methyl-L- Cysteine Sulfoxide	5	4	FALSE	FALSE	3	FALSE	-4.01	A
30.	S-Propyl-L-Cysteine	5	4	FALSE	FALSE	2	FALSE	-2.02	A
31.	S-Propyl-L-Cysteine Sulfoxide	5	4	FALSE	FALSE	3	FALSE	-3.14	A

Table 4.6 contd.

Sl. No.	Ligand	Solubility level (2-4)	BBB level (2-4)	CYP2D6 Prediction (False-non inhibitor)	Hepatotoxic Prediction (False-non toxic)	Absorption level (0-1)	PPB Prediction (False-poorly bound)	Alog P98 Value (<4)	Remarks
32.	Thiacremonone	4	3	FALSE	TRUE	0	FALSE	-0.70	A
33.	Z-Ajoene	4	1	FALSE	FALSE	0	FALSE	2.06	A
34.	2-Methylbenzaldehyde	3	1	FALSE	FALSE	0	TRUE	2.08	A
35.	2-Vinyl-4H-1,3-Dithiin	3	1	FALSE	TRUE	1	FALSE	1.98	A
36.	3-Vinyl-4H-1,2-Dithiin	3	1	FALSE	FALSE	1	FALSE	2.11	A
	B. Compounds not exclusive to *Allium*								
37.	Apigenin	4	3	FALSE	TRUE	0	FALSE	0.34	A
38.	Cyanidol	3	4	FALSE	TRUE	0	FALSE	2.95	A
39.	Campesterol	1	4	FALSE	FALSE	3	TRUE	7.63	NA
40.	Ferulic acid	4	3	FALSE	FALSE	0	TRUE	0.19	A
41.	Isobutyl isothiocyanate	4	1	FALSE	TRUE	0	FALSE	1.97	A
42.	Kaempferol	4	3	FALSE	TRUE	0	FALSE	-0.31	A
43.	Myricetin	4	4	FALSE	TRUE	2	FALSE	-1.38	A

Table 4.6 contd.

Sl. No.	Ligand	Solubility level (2-4)	BBB level (2-4)	CYP2D6 Prediction (False-non inhibitor)	Hepatotoxic Prediction (False-non toxic)	Absorption level (0-1)	PPB Prediction (False-poorly bound)	Alog P98 Value (<4)	Remarks
44.	P-Coumaric Acid	4	3	FALSE	FALSE	0	FALSE	0.21	HA
45.	Phloroglucinol	4	3	FALSE	TRUE	0	FALSE	1.10	A
46.	Quercetin	4	4	FALSE	TRUE	1	TRUE	-0.48	A
47.	Sinapinic acid	4	3	FALSE	TRUE	0	TRUE	0.18	A
48.	Taurine	5	4	FALSE	TRUE	3	FALSE	-2.95	NA

A- Acceptable, HA- Highly acceptable, NA- Not acceptable

*Desirable values provided along with header

Table 4.7: ADMET properties of FDA approved commercial drugs*

Sl. No.	Ligand	Solubility level (2-4)	BBB level (2-4)	CYP2D6 Prediction (False-non inhibitor)	Hepatotoxic Prediction (False-non toxic)	Absorption level (0-1)	PPB Prediction (False-poorly bound)	Alog P98 Value (<4)	Remarks
1.	Alvocidib	3	3	FALSE	TRUE	0	TRUE	2.41	A
2.	Arsenic trioxide	4	4	FASLE	TRUE	1	FALSE	-0.46	A
3.	Bevacizumab + Rituximab	4	3	FALSE	TRUE	0	FALSE	-0.95	A
4.	Brinzolamide	4	4	FALSE	TRUE	1	FALSE	-1.54	A
5.	Cabozantinib	2	4	FALSE	TRUE	1	TRUE	4.24	NA
6.	Captopril	4	3	FALSE	TRUE	1	FALSE	-0.81	A
7.	Cyproterone	2	2	FALSE	TRUE	0	FALSE	2.94	A
8.	Dasatinib	2	3	FALSE	TRUE	0	FALSE	2.21	A
9.	Dexamethasone	3	3	FALSE	FALSE	0	FALSE	1.71	HA
10.	Diclofenac	2	2	TRUE	FALSE	0	TRUE	2.89	A

Table 4.7 contd.

Sl. No.	Ligand	Solubility level (2-4)	BBB level (2-4)	CYP2D6 Prediction (False-non inhibitor)	Hepatotoxic Prediction (False-non toxic)	Absorption level (0-1)	PPB Prediction (False-poorly bound)	Alog P98 Value (<4)	Remarks
11.	Doxycycline	4	4	FALSE	TRUE	3	FALSE	-2.27	A
12.	Epalrestat	3	2	FALSE	FALSE	0	TRUE	1.97	A
13.	Fluorouracil	4	3	FALSE	TRUE	0	FALSE	0.83	A
14.	Gefitinib	2	1	TRUE	TRUE	0	TRUE	4.20	NA
15.	Geldanamycin	2	4	FALSE	TRUE	3	FALSE	3.04	A
16.	Idelalisib	2	3	FALSE	TRUE	0	FALSE	3.49	A
17.	Imatinib	3	3	FALSE	TRUE	0	FALSE	1.29	A
18.	Lithium carbonate	5	4	FALSE	TRUE	2	FALSE	-1.97	A
19.	Lovastatin	2	2	FALSE	FALSE	0	TRUE	4.22	A
20.	Megestrol acetate	2	2	FALSE	FALSE	0	TRUE	3.51	A
21.	Pioglitazone	2	1	FALSE	FALSE	0	TRUE	4.52	NA
22.	Propranolol	4	2	FALSE	TRUE	0	FALSE	1.31	A

Table 4.7 contd.

Sl. No.	Ligand	Solubility level (2-4)	BBB level (2-4)	CYP2D6 Prediction (False-non inhibitor)	Hepatotoxic Prediction (False-non toxic)	Absorption level (0-1)	PPB Prediction (False-poorly bound)	Alog P98 Value (<4)	Remarks
23.	Regorafenib	1	4	FALSE	TRUE	0	TRUE	4.38	NA
24.	Repaglinide	5	4	FALSE	FALSE	3	FALSE	-3.19	A
25.	Spironolactone	2	1	FALSE	FALSE	0	TRUE	3.73	A
26.	Tamoxifen	2	0	FALSE	TRUE	0	TRUE	4.76	NA
27.	Topetecan	4	4	FALSE	TRUE	0	FALSE	-0.79	A
28.	Vildagliptin	4	3	FALSE	TRUE	1	FALSE	-1.05	A

A- Acceptable, HA- Highly acceptable, NA- Not acceptable

*Desirable values provided along with header

4.1.4 Target protein Identification

Thirty two targets which were found responsible for causing serious lifestyle diseases such as cancer, diabetes, blood pressure, cholesterol and arthritis were identified. Fifteen targets were selected for cancer studies, six for diabetes, three for blood pressure, one for cholesterol, and seven for arthritis and inflammation. The three dimensional crystal structures of these protein targets were retrieved from Protein Data Bank in .pdb format on the basis of X ray diffraction, presence of active site for ligand binding, high number of active residue count in that active site and presence of a natural inhibitor. The list of targets selected is presented in Table 4.8 along with their PDB IDs.

4.1.5 Checking of Physico-chemical properties of identified targets

Using ProtParam tool provided by Expasy server, physico-chemical properties of the targets were evaluated. The properties included instability index (stability in nature), aliphatic index (thermal stability), theoritical pI (acidic or basic) and GRAVY values (hydrophilic or hydrophobic). These attributes are important elements for effective binding of ligands with the targets.

ProtParam analysis on all the thirty two target proteins revealed that seventeen target proteins were unstable in nature indicating a value >40, three were low in thermo stability at high temperature indicating a value <75, ten target proteins were with theoretical pI exceeding 7, twenty nine target proteins were very hydrophilic (negative GRAVY value) whereas the rest two were slightly hydrophobic. Details are provided in Table 4.9.

4.1.6 Preparation of protein molecules

The 'Prepare protein' protocol of Discovery Studio 4.0 using CHARMM force-field, corrected the thirty-two target protein structures by inserting missing atoms, modelling loop regions and side chains, adding hydrogen atoms, removing water molecules, natural ligands and hetero atoms so as to minimize the energy to avail a stable conformation (Plate 1). The energy status of the protein structures

Table 4.8: List of protein targets involved in various human diseases selected for *in silico* molecular docking studies

Sl. No.	Target name	PDB ID
A. Cancer		
1.	AKT /Protein kinase B	3E8D
2.	Androgen receptor	1E3G
3.	B cell lymphoma-2	3ZK6
4.	cMET	3VW8
5.	Cyclin dependent kinase	2A4L
6.	DNA Topoisomerase	1K4T
7.	Epidermal growth factor receptor	3UG2
8.	Estrogen receptor	1ERR
9.	Heat shock protein	2FWZ
10.	Mast/Stem cell growth factor receptor	4U0I
11.	Matrix metalloproteinase	3TT4
12.	Nuclear factor kappa light chain enhancer of activated B cells (NFkB)	4DN5
13.	Phosphoinositide 3-kinase Gamma (Pi3k-γ)	3APD
14.	Progesterone receptor	3D90
15.	Thymidilate synthase	1I00
B. Diabetes		
16.	Aldose reductase	1IEI
17.	Dipeptidyl peptidase-4	3W2T
18.	Glucokinase	3FGU
19.	Glycogen synthase kinase 3	1Q41
20.	Insulin receptor	3ETA
21.	Peroxisome proliferator-activated receptor gamma (PPAR γ)	4EMA
C. Blood pressure		
22.	Adrenergic receptor	3D4S
23.	Angiotensin Converting enzyme	1O86

Table 4.8 contd.

Sl. No.	Target name	PDB ID
24.	Carbonic anhydrase	3CAJ
D. Cholesterol		
25.	HMG CoA reductase	1HWL
E. Arthritis		
26.	Cyclooxygenase	4COX
27.	Glucocorticoid receptor	1M2Z
28.	Mineralocorticoid receptor	2AB2
29.	Nitric Oxide Synthase	4NOS
30.	p38 kinase /Mitogen activated protein kinase 14 (MAPK 14)	3HEC
31.	Tumor Necrosis Factor Alpha (TNF α)	2AZ5
32.	Vascular endothelial growth factor receptor 1 (VEGFR1)	3HNG

Table 4.9: Physico chemical properties of the target proteins (ProtParam studies)*

Sl. No.	Target Protein name	Instability index (<40)	Aliphatic index (<75)	Theoritical pI (<7)	Grand average of hydropathy (GRAVY)
1.	Adrenergic receptor	36.87	100.49	8.90	0.15
2.	AKT/Protein kinase B	30.20	84.09	5.92	-0.35
3.	Aldose reductase	36.58	93.13	6.52	-0.25
4.	Androgen receptor	44.32	93.73	8.69	-0.05
5.	Angiotensin Converting enzyme	39.46	78.86	5.82	-0.44
6.	B cell lymphoma-2	37.64	70.61	5.21	-0.48
7.	Carbonic anhydrase	21.68	76.46	6.87	-0.58
8.	cMET	42.78	96.19	6.98	-0.06
9.	Cyclin dependent kinase	33.25	100.74	8.80	-0.08
10.	Cyclooxygenase	39.20	79.20	6.86	-0.36
11.	Dipeptidyl peptidase-4	44.29	76.00	5.73	-0.43
12.	DNA Topoisomerase	42.61	67.89	9.38	-0.97
13.	Epidermal growth factor receptor	44.71	95.15	5.71	-0.20
14.	Estrogen receptor	38.98	113.32	6.34	0.09
15.	Glucocorticoid receptor	51.54	98.25	6.58	-0.09
16.	Glucokinase	39.54	77.11	5.33	-0.37
17.	Glycogen synthase kinase 3	29.27	80.35	8.77	-0.34
18.	Heat shock protein	37.06	78.87	5.03	-0.53
19.	HMG CoA reductase	53.06	90.92	7.56	-0.04
20.	Insulin receptor	39.00	75.62	5.65	-0.41
21.	Mast/Stem cell growth factor receptor	46.88	88.89	7.16	-0.09
22.	Matrix metalloproteinase	27.56	70.13	4.64	-0.51
23.	Mineralocorticoid receptor	52.07	92.18	7.17	-0.16

Table 4.9 contd.

Sl. No.	Target Protein name	Instability index (<40)	Aliphatic index (<75)	Theoritical pI (<7)	Grand average of hydropathy (GRAVY)
24.	Nitric Oxide Synthase	48.66	75.34	7.47	-0.44
25.	Nuclear factor kappa light chain enhancer of activated B cells	44.31	81.91	6.42	-0.33
26.	Peroxisome proliferator-activated receptor gamma (PPAR γ)	43.64	104.25	5.85	-0.11
27.	Phosphoinositide 3-kinase Gamma (Pi3k-γ)	42.93	92.43	6.36	-0.28
28.	Progesterone receptor	55.55	109.15	8.35	0.02
29.	p38 kinase /Mitogen activated protein kinase 14 (MAPK 14)	40.39	97.24	5.96	-0.22
30.	Thymidilate synthase	43.38	86.07	6.45	-0.32
31.	Tumor Necrosis Factor Alpha (TNF α)	26.40	100.81	5.91	-0.15
32.	Vascular endothelial growth factor receptor 1 (VEGFR1)	41.84	78.28	6.16	-0.46

*Desirable values provided along with header

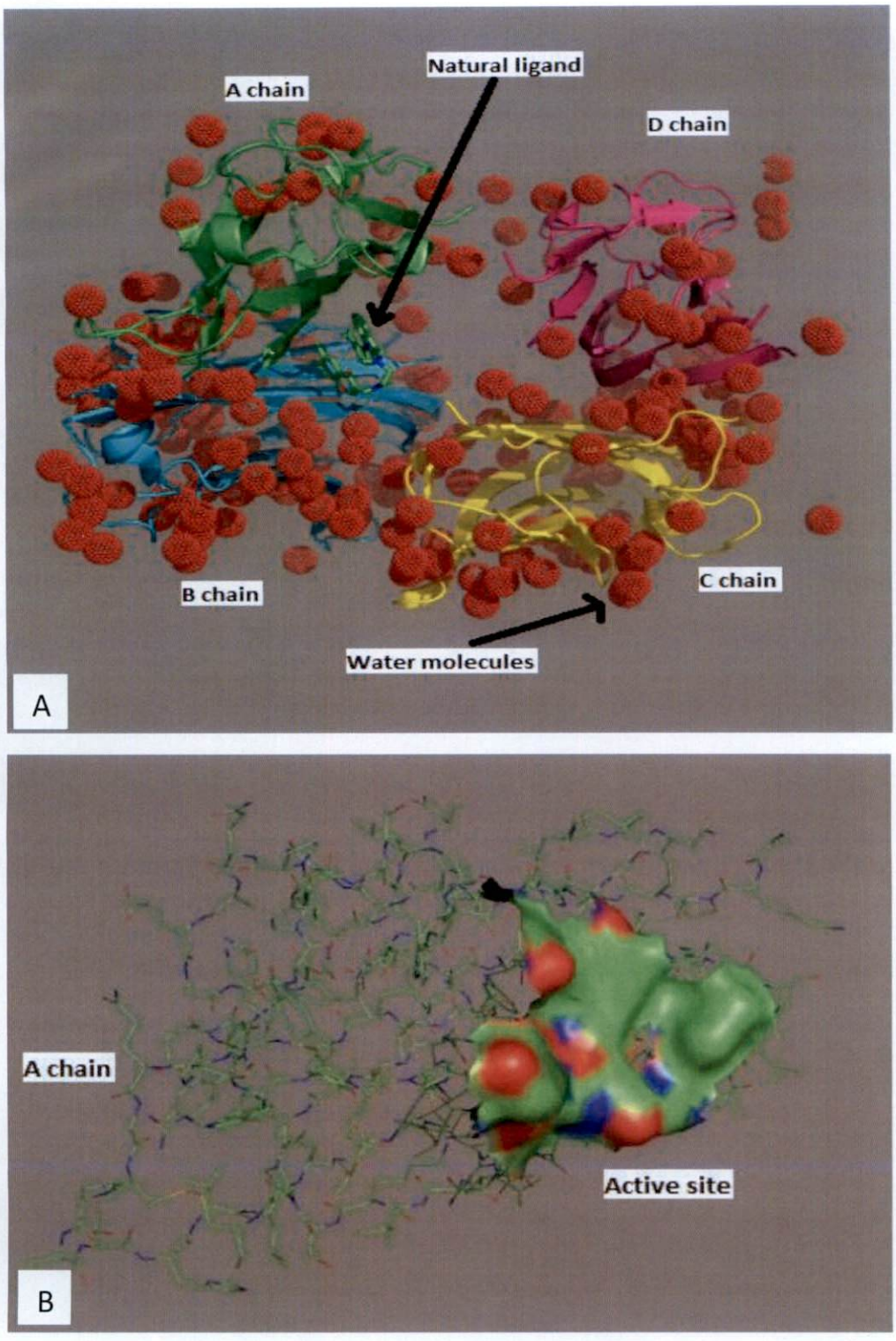


Plate 1: 3D structure of Tumor Necrosis Factor alpha (a gene for arthritis and inflammation) before (A) and after (B) preparation

before and after preparation is provided in Table 4.10. The energy status was found to decrease after energy minimization using CHARMM forcefield. All the prepared target protein structures were saved in .dsv format for conducting further studies.

4.1.7 Active site identification

Active sites of the target proteins were identified from 'PDB site records' and critical amino acid residues in the active sites which can interact with incoming ligands were also identified. Five target proteins were found to have only a single active site, whereas rest twenty seven targets were found to have more than one active site. Cyclooxygenase (4COX), the target for arthritis and inflammation was found to have the maximum number of active sites i.e. twenty four, followed by seventeen active sites found in both Dipeptidyl peptidase 4 (3W2T) and Nitric oxide synthase (4NOS). But only one active site, which had maximum number of amino acid residues binding with the natural ligand provided with the target, was selected for each target protein for docking purposes (Plate 2). Details of active sites along with the amino acid residues are given in Table 4.11.

4.1.8 Molecular docking

Molecular docking was carried out by "CDOCKER" protocol between the targets and the selected phytocompounds. The same was done for FDA approved drugs also to have a comparative study. The medicinal effects of garlic against cancer, diabetes, cholesterol, blood pressure and arthritis were thus assessed by the above mentioned protocol of molecular docking. Active inhibitors of the target proteins involved in the above mentioned various lifestyle diseases were identified based on lowest binding energy. The ligands from garlic when docked were also screened on criteria of CDOCKER and CDOCKER interaction energy. The best interaction was measured on the difference between CDOCKER and CDOCKER Interaction energy. The lesser the difference, the better is the interaction. If some ligand showed CDOCKER and CDOCKER Interaction energy as same, then that was considered as the best interaction.

Table 4.10: Details of energy status of the target proteins before and after energy minimization

Sl. No.	Protein name	Energy status before minimization			Energy status after minimization		
		Potential Energy (kcal/mol)	Van der Waals Energy (kcal/mol)	Electrostatic Energy (kcal/mol)	Potential Energy (kcal/mol)	Van der Waals Energy (kcal/mol)	Electrostatic Energy (kcal/mol)
1.	Adrenergic receptor	-11952.90	-2661.95	-13915.36	-16314.23	-3800.02	-14640.32
2.	AKT /Protein kinase B	-20070.79	-2036.24	-24481.01	-13759.76	-2639.16	-12657.19
3.	Aldose reductase	432487.82	436542.04	-11764.09	-8378.82	-1712.09	-11714.44
4.	Androgen receptor	-3242.06	2120.37	-8316.32	-8721.73	-1747.47	-8658.04
5.	Angiotensin Converting enzyme	-6260.15	6963.04	-23425.73	-25267.99	-4655.69	-23753.79
6.	B cell lymphoma-2	16481.97	23335.18	-10164.55	-12471.71	-2029.85	-12033.79
7.	Carbonic anhydrase	-1074.43	4593.85	-10572.59	-10884.55	-1936.05	-10301.54
8.	cMET	-5893.58	377.52	-10458.02	-12921.87	-2512.67	-12157.22
9.	Cyclin dependent kinase	3487.95	9191.88	-9784.23	-10655.26	-2090.16	-10960.65
10.	Cyclooxygenase	7260.05	65374.99	-84077.11	-20248.85	-3238.83	-21267.34
11.	Dipeptidyl peptidase-4	-15991.51	16758.35	-56704.77	-30980.15	-5921.49	-29099.08
12.	DNA Topoisomerase	0.61E+18	0.61E+18	-0.21E+05	-22008.09	-4424.95	-20706.69

Table 4.10 contd.

Sl. No.	Protein name	Energy status before minimization			Energy status after minimization		
		Potential Energy (kcal/mol)	Van der Waals Energy (kcal/mol)	Electrostatic Energy (kcal/mol)	Potential Energy (kcal/mol)	Van der Waals Energy (kcal/mol)	Electrostatic Energy (kcal/mol)
13.	EGFR	-1074.43	4593.85	-10572.59	-10884.55	-1936.05	-10301.54
14.	Estrogen receptor	6741.26	16846.51	-16254.54	-8293.41	-1318.94	-8954.09
15.	Glucocorticoid receptor	-2047.65	9205.95	-18750.59	-9800.19	-1829.89	-9439.52
16.	Glucokinase	-11571.34	-255.59	-17191.94	-18882.03	-3669.15	-17584.31
17.	Glycogen synthase kinase 3	-4522.13	10278.75	-22969.41	-13507.64	-2559.77	-12996.01
18.	Heat shock protein	16336.67	20476.53	-8598.73	-9119.10	-1480.49	-8582.64
19.	HMG CoA reductase	-44160.99	-2828.88	-57847.25	-30153.47	-5550.24	-30027.84
20.	Insulin receptor	-10020.48	4495.96	-21503.34	-12305.29	-2346.94	-11472.64
21.	KIT	-6238.93	-15.54	-10346.70	-12250.08	-2212.15	-11761.34
22.	Matrix metalloproteinase	14737.29	18392.74	-7216.36	-5457.54	-1115.37	-5553.69
23.	Mineralocorticoid receptor	-1976.17	8364.04	-18638.89	-10129.43	-2022.75	-9439.38
24.	Nitric Oxide Synthase	220406.08	258752.75	-68131.96	-17096.98	-3050.14	-16643.95
25.	NFkB	-6260.15	6963.04	-23425.73	-25267.99	-4655.69	-23753.79

Table 4.10 contd.

Sl. No.	Protein name	Energy status before minimization			Energy status after minimization		
		Potential Energy (kcal/mol)	Van der Waals Energy (kcal/mol)	Electrostatic Energy (kcal/mol)	Potential Energy (kcal/mol)	Van der Waals Energy (kcal/mol)	Electrostatic Energy (kcal/mol)
26.	PPAR-γ	-44160.99	-2828.88	-57847.25	-30153.47	-5550.24	-30027.84
27.	Pi3k-γ	-17964.31	3721.42	-29494.02	-35530.95	-6453.85	-33935.28
28.	Progesterone receptor	-1563.43	7191.07	-16926.61	-9579.53	-1836.41	-9309.97
29.	MAPK 14	10658.04	17618.32	-11611.36	-11898.46	-2085.26	-13048.43
30.	Thymidilate synthase	-14007.29	2488.65	-22830.53	-11125.37	-1796.55	-10903.76
31.	TNF α	-2794.09	10288.14	-20249.63	-11089.68	-1588.38	-11991.26
32.	VEGFR1	-6119.77	770.75	-10123.26	-9718.07	-1435.21	-10274.78

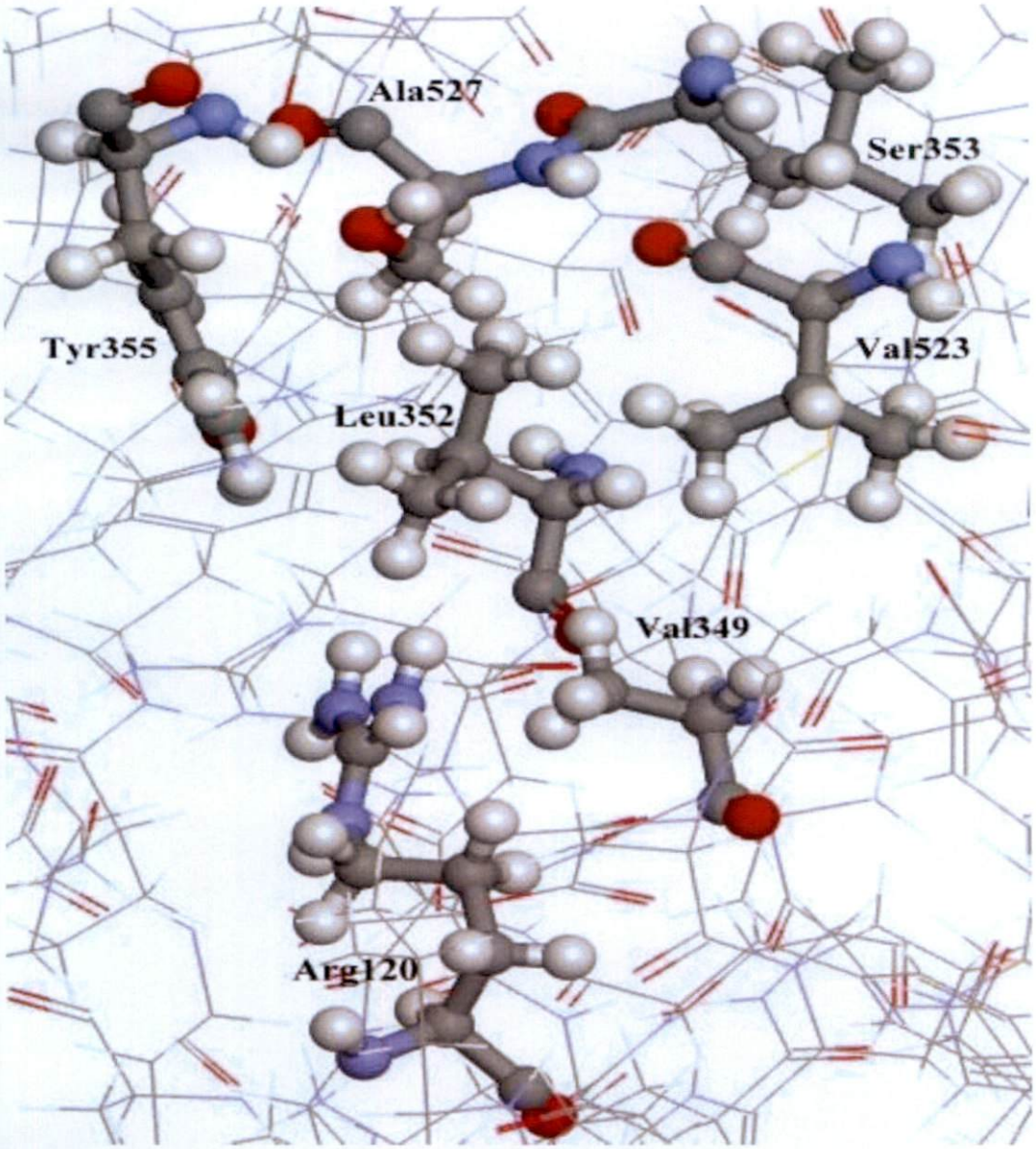


Plate 2: Active site of Cyclooxygenase2 (COX2) with its active site residues

Table 4.11: Details of active site identified for target proteins

Sl. No.	Target Protein name	Total no. of Active sites	Best active site	Critical amino acid residues at selected active site
1.	Adrenergic receptor	6 (AC 1 to 6)	AC 1	Asp113, Asn312, Asn293, Ser203, Ser204, Ser207, Thr118, Tyr316, Tyr308
2.	AKT /Protein kinase B	2 (AC 1 to 2)	AC 1	Asp293, Ser9, Glu200, Val166, Thr292, Phe294, Ala232, Phe163, Glu236, Asn280
3.	Aldose reductase	2 (AC 1 to 2)	AC 1	Tyr48, Trp111, Trp20, Phe122, Leu300,Ser 214, Ser 210, Tyr209, Gln183, Lys262, Ser263, Thr265, Arg268, Glu271, Asn272, Leu212
4.	Androgen receptor	1 (AC 1)	AC 1	Arg752, Asn705, Thr877, Met745, Gln711
5.	Angiotensin Converting enzyme	5 (AC 1 to 5)	AC 5	Ala354, Glu384, His353, Tyr523, Tyr 520, Gln281, Lys511, Tyr523, Ser355, Val518
6.	B-cell lymphoma-2	2 (AC 1 to 2)	AC 1	Asn136, Phe105, Arg139, Phe97, Gly138, Arg102, Phe146, Ala104, Leu130
7.	Carbonic anhydrase	5 (AC 1 to 5)	AC 3	His94, His96, His199, Leu198, Thr199, Thr200, Pro202, Pro201, Val121, Gln92, Phe131
8.	cMET kinase	2 (AC 1 to 2)	AC 1	Ile892, Val898, Cys919, Val916, Leu1019, Phe1047, Ile1044, Met1160, Asp1222, Phe1223, Glu1127, Leu1157, Met1211, Ala1108, Phe1200, Ile1130, Ala1221, Val1092, Ile1084, Phe1134

Table 4.11 contd.

Sl. No.	Target Protein name	Total no. of Active sites	Best active site	Critical amino acid residues at selected active site
9.	Cyclin dependent kinase	1 (AC 1)	AC 1	Glu8, Glu12, Ile10, Gly11, Thr14, Tyr15, Gly13, Lys33, Asp86, Lys89, Leu83, Val18, Ala31, Val64, Glu81,His84, Lys129, Leu134, Phe80, Phe82, Ala144, Glu131, Asn132, Asp145
10.	Cyclooxygenase	24 (CAT, ACE, HEM, SUB, AC 1 to 9, BC 1 to 9, CC 1 to 2)	BC 5	Tyr355, Arg120, Val349, Ala527, Ser353, Val523, Leu352
11.	Dipeptidyl peptidase-4	17 (AC 1 to 9 and BC 1 to 8)	AC 1	Glu206, Asn710, Glu205, Tyr547, Tyr631, Tyr662, Phe357, Asn710, Ser209, His126, His740, Ser630, Arg125, Trp629, Arg358, Val207
12.	DNA Topoisomerase	4 (AC 1 to 4)	AC 1	Lys532, Asp533, Leu530, His511, Asn722, Tyr723, Arg364
13.	Epidermal growth factor receptor	2 (AC 1 to 2)	AC 1	Leu777, Met766, Phe856, His835, Lys879, Ile878, Met790, Met793, Gln791, Asp855, Lys745, Thr790, Phe723, Arg858, Tyr891
14.	Estrogen receptor	1 (AC 1 to 2)	AC 1	Arg394, Phe404, Glu353, Asp353, His524, Leu387, Ala350, Trp383, Leu525, Met 421, Thr347, Leu354, Met388
15.	Glucocorticoid receptor	5 (AC 1 to 5)	AC 4	Gln642, Asn564, Gln570, Met601, Met646, Asp687
16.	Glucokinase	4 (AC 1 to 4)	AC 1	Thr168, Glu290, Asp205, Lys169, Glu256, Asn 231, Asn204, Ser336, Ser411, Thr228, Gly229, Arg333

Sl. No.	Target Protein name	Total no. of Active sites	Best active site	Critical amino acid residues at selected active site
17.	Glycogen synthase kinase 3	2 (AC 1 to 2)	AC 1	Val135, Asp133, Thr138, Cys199, Asp200, Leu188, Gln185, Arg141, Tyr134, Leu132, Val110, Lys85, Ala83, Val70, Asn64, Gly63, Ile62
18.	Heat shock protein	1 (AC 1)	AC 1	Phe138, Thr184, Asp93, Leu107, Met98
19.	HMG CoA reductase	7 (AC 1 to 7)	AC 4	Ser565, Lys735, Arg590, Asp690, Ser684, Asn755, Lys691, Glu599, His752
20.	Insulin receptor	2 (AC 1 to 2)	AC 1	Glu1077, Met1079, Glu1047, Asp1150, Leu1002, Val1010, Phe1151, Met1139, Phe1121, Met1051, Val1059, Ala1028
21.	Mast/Stem cell growth factor receptor	3 (AC 1 to 3)	AC 1	Glu640, Asp810, Cys672, His790, Ile789, Tyr672, Leu799, Leu595, His790, Thr 670, Asp810, Lys623
22.	Matrix metalloproteinase 8	6 (AC 1 to 6)	AC 2	Asp115, Phe192, Leu160, Glu198, Tyr219, Leu 214, His197
23.	Mineralocorticoid receptor	3 (AC 1 to 3)	AC 2	Arg817, Gln776, Ala773, Leu769, Met845, Met807, Leu810
24.	Nitric Oxide Synthase	17 (COF, SUB, ZNB, AC 1 to 9, BC 1 to 5)	AC 3	Cys200, Glu377, Trp372, Trp463, Arg381, Phe476, Ser118, Ile462
25.	Nuclear factor kappa light chain enhancer of activated B cells	11 (AC 1 to 9, BC 1 to 2)	AC 8	Asp534, Asp535, Phe411, Gly412, Glu470, Asp515, Asp519, Asn520, Lys 517, Lys 429, Leu522, Val414, Glu440, Thr559

(Table 4.11 contd.)

Table 4.11 contd.

Sl. No.	Target Protein name	Total no. of Active sites	Best active site	Critical amino acid residues at selected active site
26.	Peroxisome proliferator-activated receptor gamma (PPAR γ)	1 (AC 1)	AC 1	Ser289, His449, Gly284, Ile341, Cys285, Leu330
27.	Phosphoinositide 3-kinase Gamma (Pi3k-γ)	3 (AC 1 to 3)	AC 1	Asp836, Lys833, Met804, Ile963, Ile879, Met953, Asp841, Tyr867, Val882
28.	Progesterone receptor	2 (AC 1 to 2)	AC 1	Gln725, Cys 891, Leu718, Met756, Arg766
29.	p38 kinase /Mitogen activated protein kinase 14 (MAPK 14)	2 (AC 1 to 2)	AC 1	Asp168, Glu71, Met109, Phe169, Thr106, Lys53, Leu74, Asp168, Leu75, Lys53, Thr106, Leu108
30.	Thymidilate synthase (TS)	4 (AC 1 to 4)	AC 1	His256, Ser216, Arg215, Arg176, Arg175, Arg50, Asp218, Asn226, His196, Tyr 258, Cys190, Phe80, Gly83, Asn112, Tyr135, Cys195, Leu221, Gly222, Phe225
31.	Tumor Necrosis Factor Alpha (TNF α)	2 (AC 1 to 2)	AC 1	Leu57, Ser60, Tyr 59, Tyr119, Leu120, Gly121, Gly122, Tyr151
32.	Vascular endothelial growth factor receptor 1 (VEGFR1)	2 (AC 1 to 2)	AC 1	Glu878, Asp1040, Cys912, Lys861, Leu882, Val892, Ala859, Leu1029, Val841, Val909

On the other hand, if the difference between the two exceeds more than 10, then the interaction was considered unstable. Interactions showing difference of CDOCKER and CDOCKER Interaction energy more than 10 were thus rejected. CDOCKER Interaction energy should also be greater than CDOCKER energy. Also number of hydrogen bonds and their distance was noted. The more the no. of hydrogen bonds formed between target and ligand, the better is the affinity of the ligand towards the target protein. Shorter the length of hydrogen bond, nearer is the distance of the target from the ligand. Results of molecular docking involving *Allium* phytocompounds are presented hereunder. The phytocompounds with best interaction (<-50 kcal/mol) and with less deviation between CDOCKER and CDOCKER interaction energy (<10 kcal/mol) are highlited in the results.

4.1.8.1 Docking results of target proteins involved in cancer

4.1.8.1.1 AKT /Protein kinase B

Five compounds exclusive to *Allium* showed better dock scores with AKT /Protein kinase B in comparison with the commercial FDA approved drug Arsenic trioxide for cancer. The lowest binding energy was shown by s-allyl d-cysteine (-169.96 Kcal/mol). The interacting active site residue was Glu236 for which the hydrogen bond length was 1.99 Å. The difference of CDOCKER energy and CDOCKER interaction energy was 0.34 Kcal/mol. The least deviation in CDOCKER and CDOCKER interaction energies was observed for cycloalliin (-0.06 Kcal/mol), which also interacted with AKT /Protein kinase B via same active site residue Glu236 making a single hydrogen bond of length 2.13 Å. The highest no. of hydrogen bonds (2 nos.) was recorded for alliin interacting with the same residue Glu236 twice.

Only taurine from the non-*Allium* specific group showed good dock score with a binding energy as low as -214.09 Kcal/mol interacting with same active site residue Glu236, with a deviation of over 3.00 Kcal/mol. The commercial drug arsenic trioxide showed a positive CDOCKER energy while interacting and so the

binding energy was not calculated. Detailed results of dock scores of *Allium* phytocompounds and AKT /Protein kinase B are presented in Table 4.12.

4.1.8.1.2 Androgen Receptor (Cancer target)

During molecular docking with Androgen Receptor (AR), best dock score was observed for cycloalliin among exclusive *Allium* phytocompounds. Lowest binding energy of -142.12 Kcal/mol was recorded, with a deviation of 0.57 Kcal/mol among the CDOCKER energies (Table 4.13). The interacting active site residues were Leu704 and Arg752 with hydrogen bond distances of 2.21 Å and 2.36 Å respectively. However, Leu704 was found as a non-critical residue. In contrast, s-allyl-mercapto-cysteine showed the least deviation between CDOCKER energies (0.09 Kcal/mol) interacting with residues Leu704, Arg752, Met745 and Gln711 making 5 hydrogen bonds.

Among non-*Allium* specific group, phloroglucinol showed the least binding energy (-157.08 Kcal/mol) with a low difference of 0.91 Kcal/mol between CDOCKER energy and CDOCKER interaction energy. The interacting active site residues were the same as the ones mentioned above. However, isobutyl isothiocyanate recorded the least difference of CDOCKER energies as 0.62 Kcal/mol, interacting with active side residue Asn705 forming a hydrogen bond of length 2.12 Å. The commercial drug cyproterone showed a positive CDOCKER energy while interacting and hence the binding energy was not calculated.

4.1.8.1.3 B cell lymphoma-2 (Cancer target)

Detailed results of dock scores of *Allium* phytocompounds with B cell lymphoma-2 are presented in Table 4.14. Molecular docking studies of *Allium* compounds with target protein B cell lymphoma-2 revealed that almost all the compounds with least binding energies docked with a critical active site residue Arg139. The least binding energy was shown by s-allyl-mercapto-cysteine (-92.59 Kcal/mol) at a difference of 0.43 Kcal/mol for the CDOCKER energies. The

Table 4.12: Dock scores for the cancer target AKT /Protein kinase B with selected ligands

Type of compound	Sl. No.	Ligand	(-) CDOCKER energy (Kcal/mol)	(-) CDOCKER Interaction energy (Kcal/mol)	No. of H bonds	H bond	Distance (Å)	Binding Energy (Kcal/mol)
Allium-specific compounds	1.	S-Allyl D-Cysteine	34.30	34.64	1	GLU236*	1.99	-169.96
	2.	Alliin	35.46	38.29	2	GLU236 (2)*	2.25	-164.39
	3.	S-Allyl L-Cysteine	34.15	34.69	1	GLU236*	1.97	-153.19
	4.	Cycloalliin	34.51	34.57	1	GLU236*	2.13	-96.82
	5.	L-γ-Glutamyl-S-allyl-L-cysteine	46.24	50.33	2	GLU236 (2)*	2.18	-60.44
Non-*Allium* specific compounds	6.	Taurine	28.95	31.99	1	GLU236*	1.83	-214.09
Commercial drug	7.	Arsenic trioxide	Positive CDOCKER energy					

* amino acid residues present in active site

Table 4.13: Dock scores for the cancer target Androgen receptor with selected ligands

Type of compound	Sl. No.	Ligand	(-) CDOCKER energy (Kcal/mol)	(-) CDOCKER Interaction energy (Kcal/mol)	No. of H bonds	H bond	Distance (Å)	Binding Energy (Kcal/mol)
Allium-specific compounds	1.	Cycloalliin	37.22	37.79	2	LEU704 ARG752*	2.21 2.36	-142.12
	2.	Alliin	33.48	35.95	1	LEU704	2.07	-124.56
	3.	S-Allyl Mercapto Cysteine	35.47	35.56	5	LEU704 ARG752 (2)* MET745* GLN711*	1.95 2.08 2.26 2.40	-114.88
	4.	S-Allyl D-Cysteine	31.02	31.44	3	GLN711 (2)* ARG752*	2.06 2.07	-101.26
	5.	S-Allyl L-Cysteine	32.58	33.38	4	MET745* GLN711 (2)* ARG752*	2.17 2.28 2.32	-65.99
Non-*Allium* specific compounds	6.	Phloroglucinol	39.25	40.16	2	ARG752* LEU704	1.71 2.20	-157.08
	7.	Isobutyl isothiocyanate	20.84	21.46	1	ASN705*	2.12	-117.64
	8.	Kaempferol	40.85	41.51	1	THR877*	2.25	-88.97
Commercial drug	9.	Cyproterone	Positive CDOCKER energy					

* amino acid residues present in active site

Table 4.14: Dock scores for the cancer target B cell lymphoma-2 with selected ligands

Type of compound	Sl. No.	Ligand	(-) CDOCKER energy (Kcal/mol)	(-) CDOCKER Interaction energy (Kcal/mol)	No. of H bonds	H bond	Distance (Å)	Binding Energy (Kcal/mol)
Allium-specific compounds	1.	S-Allyl Mercapto Cysteine	36.40	36.83	5	ARG139 (4)* ASN136*	2.08 2.09	-92.59
	2.	L-γ-Glutamyl-S-allyl-L-cysteine	31.46	33.57	2	ARG139* ASN136*	2.07 2.43	-89.30
	3.	Alliin	24.83	26.16	3	ARG139 (2)* ASN136*	2.00 2.10	-85.97
	4.	Cycloalliin	24.53	24.99	2	ARG139 (2)*	2.29	-80.33
Non-*Allium* specific compounds	5.	Quercetin	54.61	56.13	2	ARG139 (2)*	1.83	-206.18
	6.	Myricetin	42.16	44.86	2	ARG139 (2)*	1.76	-184.04
	7.	Phloroglucinol	28.34	28.92	2	ARG139 (2)*	1.85	-128.10
Commercial drug	8.	Bevacizumab+Rituximab	29.37	44.12	2	ARG139* ASN136*	2.00 2.18	-77.60

* amino acid residues present in active site

compound made 5 hydrogen bonds in total with same residue Arg139 (4 bonds) and one more with Asn136.

On the other hand, from the group of non-*Allium* specific compounds quercetin exhibited the least binding energy (-206.18 Kcal/mol) at a difference of 1.52 Kcal/mol. This compound interacted with the same critical amino acid residue Arg139 from active site AC1. The smallest hydrogen bonding of length 1.76 Å was formed by myricetin. Another compound phloroglucinol displayed a less deviation of CDOCKER energies (0.58 Kcal/mol) and interacted with the same active site residue Arg139 making 2 hydrogen bonds. The commercial drug bevacizumab+rituximab interacted with B cell lymphoma-2 with a good binding energy, but was considered unstable because of a deviation of CDOCKER energies which surpassed the limit of 10 Kcal/mol.

4.1.8.1.4 cMET (Cancer target)

During molecular docking with cMET, the lowest binding energy (-82.54 Kcal/mol) was shown by s-allyl d-cysteine among exclusive *Allium* phytocompounds. The interacting residue for the compound was Lys1110 which was discovered as non-critical. Compounds like s-allyl l-cysteine and alliin interacted with active site essential amino acid residue Asp1222.

Phloroglucinol was the only compound from the non-*Allium* specific group, which showed a decent low binding energy (-110.56 Kcal/mol) with a difference of 1.03 Kcal/mol between CDOCKER energy and CDOCKER interaction energy. The interacting active site residue was the same non-essential amino acid residue mentioned in the above paragraph. The commercial drug cabozantinib depicted a high binding energy of -104.80 Kcal/mol and interacted with critical active site residue Asp1222 making a single hydrogen bond, but was considered unstable because of an immense deviation of CDOCKER energies which surpassed the limit of 10 Kcal/mol. Detailed results of dock scores of *Allium* phytocompounds against cMET are presented in Table 4.15.

Table 4.15: Dock scores for the cancer target cMET with selected ligands

Type of compound	Sl. No.	Ligand	(-) CDOCKER energy (Kcal/mol)	(-) CDOCKER Interaction energy (Kcal/mol)	No. of H bonds	H bond	Distance (Å)	Binding Energy (Kcal/mol)
Allium-specific compounds	1.	S-Allyl D-Cysteine	33.37	34.79	1	LYS1110	1.86	-82.54
	2.	S-Allyl L-Cysteine	33.90	34.60	2	LYS1110 ASP1222*	1.80 1.89	-76.27
	3.	Cycloalliin	25.97	26.08	2	LYS1110 (2)	1.82	-60.31
	4.	Alliin	36.22	37.24	3	LYS1110 (2) ASP1222*	1.88 1.88	-56.04
Non-*Allium* specific compounds	5.	Phloroglucinol	28.42	29.45	1	LYS1110	2.24	-110.56
Commercial drug	6.	Cabozantinib	22.28	65.16	1	ASP1222*	2.17	-104.80

* amino acid residues present in active site

4.1.8.1.5 Cyclin dependent kinase (Cancer target)

Molecular docking studies of *Allium* compounds with target protein Cyclin Dependent Kinase (CDK) revealed seven potent compounds from *Allium* (4 exclusive to *Allium* and 3 other compounds) against CDK. Detailed dock scores of *Allium* phytocompounds against CDK are presented in Table 4.16. The binding energy of compounds ranged from -51.01 Kcal/mol to -143.29 Kcal/mol. Among the exclusive group, alliin showed the best dock score with the lowest binding energy of -77.23 Kcal/mol and made 4 hydrogen bonds while connecting active site residues, Lys33 (thrice) and Thr14, displaying a difference of CDOCKER energies as 1.25 Kcal/mol.

Among non-*Allium* specific group, kaempferol displayed the lowest binding energy of -143.29 Kcal/mol by joining with residues such as Lys89 and Thr14, the hydrogen bond lengths (2 nos.) being 1.64 Å and 2.21 Å respectively. The deviation of CDOCKER energies observed for kaempferol (1.18 Kcal/mol) was found higher than that of phloroglucinol (0.45 Kcal/mol). The smallest hydrogen bond of length 1.61 Å, was formed by phloroglucinol while joining with active site residue Lys33, yielding a low binding energy of -138.74 Kcal/mol. The commercial FDA approved drug alvocidib also interacted with critical active site residues such as Lys89, Asp145, Leu83 and Gln131 with a decent binding energy, making five hydrogen bonds with CDK, but was evaluated unstable due to huge difference between CDOCKER energy and CDOCKER interaction energy surpassing the limit of 10 Kcal/mol.

4.1.8.1.6 DNA Topoisomerase (Cancer target)

Molecular docking scores for the selected *Allium* phytocompounds with DNA Topoisomerase are presented in Table 4.17. Five compounds exclusive to *Allium* showed better dock scores with DNA Topoisomerase in comparison with the commercial FDA approved drug topetecan for cancer. The lowest binding energy was observed for s-allyl mercapto cysteine as -300.14 Kcal/ mol (Plate 3). The difference of CDOCKER and CDOCKER interaction energy was as low as

Table 4.16: Dock scores for the cancer target Cyclin dependent kinase with selected ligands

Type of compound	Sl. No.	Ligand	(-) CDOCKER energy (Kcal/mol)	(-) CDOCKER Interaction energy (Kcal/mol)	No. of H bonds	H bond	Distance (Å)	Binding Energy (Kcal/mol)
Allium-specific compounds	1.	Alliin	35.27	36.52	4	LYS33 (3)* THR14*	1.99 2.14	-77.23
	2.	S-Allyl D-Cysteine	30.97	31.16	2	LYS89 (2)*	1.88	-76.29
	3.	S-Allyl Mercapto Cysteine	28.57	29.85	2	LYS33* THR14*	1.86 2.30	-65.85
	4.	S-Allyl L-Cysteine	32.81	33.48	3	LYS89 (2)* ASP86*	1.88 2.30	-51.01
Non-*Allium* specific compounds	5.	Kaempferol	51.72	52.90	2	LYS89* THR14*	1.64 2.21	-143.29
	6.	Phloroglucinol	35.09	35.54	1	LYS33*	1.61	-138.74
	7.	P-Coumaric Acid	29.86	32.59	2	LYS33* GLU81*	1.82 1.94	-71.76
Commercial drug	8.	Alvocidib	20.26	50.63	5	LYS89* ASP145 (2)* LEU83* GLN131*	1.94 2.00 2.01 2.39	-65.95

* amino acid residues present in active site

Table 4.17: Dock scores for the cancer target DNA Topoisomerase with selected ligands

Type of compound	Sl. No.	Ligand	(-) CDOCKER energy (Kcal/mol)	(-) CDOCKER Interaction energy (Kcal/mol)	No. of H bonds	H bond	Distance (Å)	Binding Energy (Kcal/mol)
Allium-specific compounds	1.	S-Allyl Mercapto Cysteine	37.76	38.03	1	ASP533*	2.29	-300.14
	2.	Alliin	35.05	37.17	1	ARG488	1.89	-300.14
	3.	S-Allyl D-Cysteine	29.05	29.26	2	ASP533* THR718*	1.81 2.29	-150.46
	4.	Cycloalliin	32.67	33.01	3	ASP533* ARG488 THR718*	1.91 2.05 2.20	-136.26
	5.	S-Allyl L-Cysteine	29.06	29.51	2	ASP533* ARG488	2.14 2.32	-112.08
Non-*Allium* specific compounds	6.	Kaempferol	62.19	65.62	2	LYS532* ARG488	1.71 1.79	-575.04
	7.	Myricetin	56.15	59.24	5	LYS532 (2)* ARG364* ARG488 HIS632	1.70 1.70 2.09 2.25	-455.71
	8.	Phloroglucinol	41.62	41.65	1	LYS532*	1.61	-318.78
	9.	Sinapic Acid	39.99	41.88	3	LYS532* ARG488 (2)	1.85 1.92	-291.86
	10.	Apigenin	46.79	47.85	5	ARG488 (2) ARG364 (2)* LYS532*	1.78 2.19 2.22	-264.19
Commercial drug	11.	Topetecan	Positive CDOCKER energy					

* amino acid residues present in active site

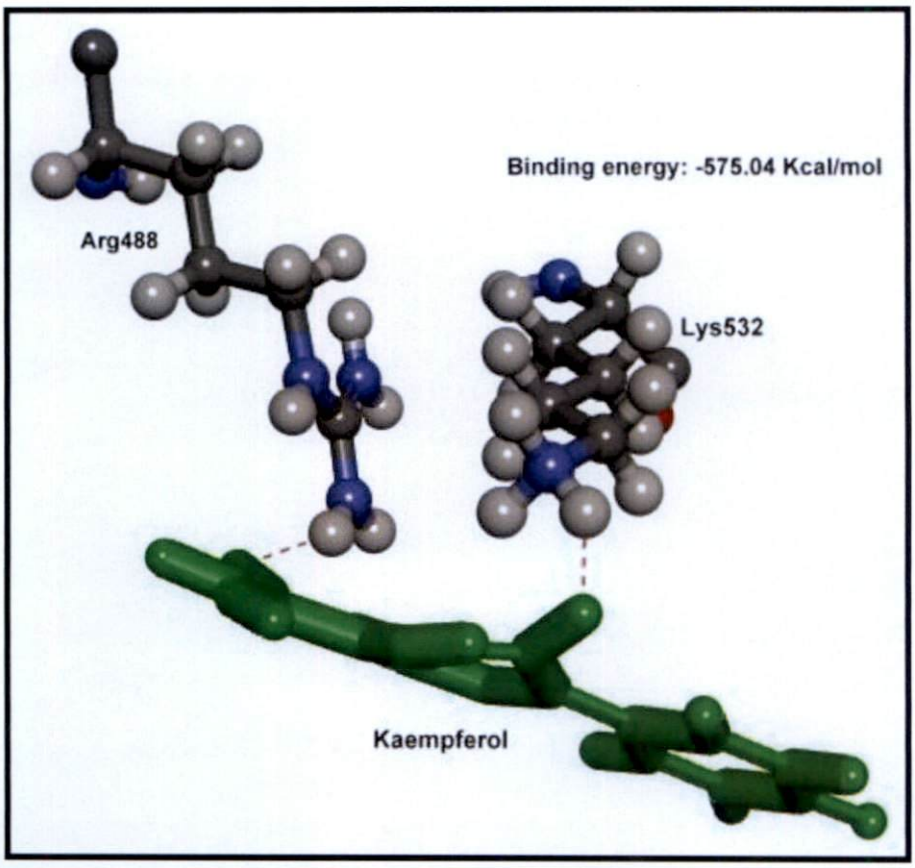
0.27 Kcal/mol. The interacting active site residue was Asp533, the hydrogen bond distance being 2.29 Å. However, the least difference of CDOCKER energies was displayed by s-allyl d-cysteine (0.21 Kcal/mol) which made 2 hydrogen bonds by joining with active site residues such as Asp533 and Thr718. The highest no. of hydrogen bonds (3 nos.) was formed by the compound cycloalliin.

Among the non-*Allium* specific group, kaempferol showed the least binding energy of -575.04 Kcal/mol, for which the interacting active site residues were Lys532 and Arg488, out of which Arg488 was observed as a non-critical residue (Plate 3). The deviation of CDOCKER energies for kaempferol (3.43 Kcal/mol) was quite higher than that of phloroglucinol (0.03 Kcal/mol), which made only one hydrogen bond with active site residue Lys532, yielding a very good binding energy of -318.78 Kcal/mol. The commercial drug topetecan showed a positive CDOCKER energy while interacting and so the binding energy was not calculated.

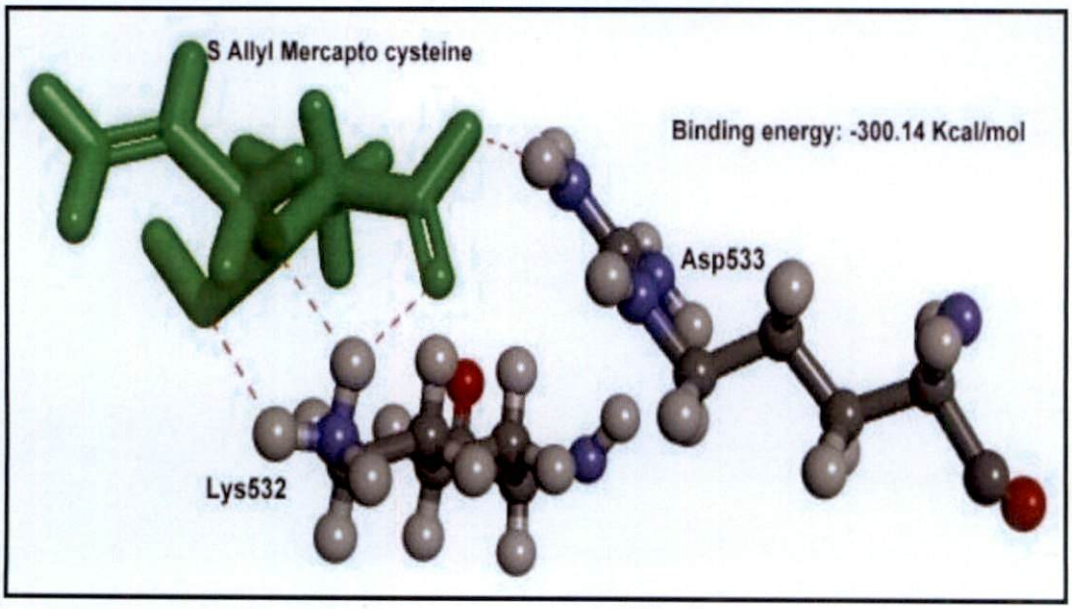
4.1.8.1.7 Epidermal growth factor receptor (EGFR) (Cancer target)

Molecular docking studies of *Allium* compounds with the target protein EGFR revealed 11 potent compounds from *Allium* (3 exclusive to *Allium* and 8 others) against EGFR. Detailed results of dock scores are presented in Table 4.18. The binding energy of compounds ranged from -141.83 Kcal/ mol to -347.13 Kcal/mol. Among the exclusive group, alliin showed the lowest binding energy of -244.97 Kcal/mol displaying a difference of CDOCKER energies as 2.27 Kcal/mol. The compound made 2 hydrogen bonds while connecting the critical amino acid residue Lys745.

Among non-*Allium* specific group, myricetin displayed the lowest binding energy of -347.13 Kcal/mol by joining with critical amino acid residues such as Lys745 and Met793. The deviation of CDOCKER energies observed for myricetin was 1.78 Kcal/mol. The highest no. of hydrogen bonds (5 nos.) was formed by quercetin joining with the same residues mentioned above. The smallest hydrogen bond of length 1.60 Å, was formed by quercetin while joining



A. DNA topoisomerase vs Kaempferol



B. DNA topoisomerase vs S-allyl mercapto cysteine

Plate 3: Hydrogen bond interaction of cancer target DNA topoisomerase with Kaempferol (A) and S-allyl mercapto cysteine (B)

Table 4.18: Dock scores for the cancer target EGFR with selected ligands

Type of compound	Sl. No.	Ligand	(-) CDOCKER energy (Kcal/mol)	(-) CDOCKER Interaction energy (Kcal/mol)	No. of H bonds	H bond	Distance (Å)	Binding Energy (Kcal/mol)
Allium-specific compounds	1.	Alliin	30.93	33.20	2	LYS745 (2)*	2.32	-244.97
	2.	S-Allyl L-Cysteine	29.25	29.66	1	THR854	2.42	-201.46
	3.	S-Allyl Mercapto Cysteine	24.01	25.01	1	LYS745*	1.82	-182.92
Non-*Allium* specific compounds	4.	Myricetin	52.29	54.07	3	LYS745* MET793 (2)*	1.60 2.45	-347.13
	5.	Quercetin	47.47	50.62	5	LYS745* MET793 (2)* THR854 (2)	1.60 1.98 2.11	-322.36
	6.	Apigenin	45.44	47.46	1	LYS745*	1.64	-301.84
	7.	P-Coumaric Acid	26.23	29.50	2	LYS745* GLN791*	1.84 1.87	-271.90
	8.	Ferulic acid	26.19	28.86	1	MET793*	2.23	-266.25
	9.	Phloroglucinol	23.08	23.53	2	THR854 LYS745*	1.80 1.81	-225.63
	10.	Sinapic Acid	26.11	27.08	1	LYS745*	1.88	-193.44
	11.	Taurine	22.62	24.67	2	ASP855* GLU762	1.83 1.91	-141.83
Commercial drug	12.	Gefitinib	Positive CDOCKER energy					

* amino acid residues present in active site

with active site residue Lys745, yielding a low binding energy of -322.36 Kcal/mol. The commercial FDA approved drug gefitinib showed a positive CDOCKER energy while interacting and hence binding energy was not calculated.

4.1.8.1.8 Estrogen Receptor (Cancer target)

While molecular docking with Estrogen Receptor (ER), it was observed that no compound exclusive to *Allium* performed molecular interactions in the identified active site. Only three compounds from the non-*Allium* specific group such as p-coumaric acid, isobutyl isothiocyanate and taurine showed better dock scores against ER as in case of FDA approved commercial drug tamoxifen. The least binding energy was shown by p-coumaric acid (-83.45 Kcal/mol) with a deviation of 3.57 Kcal/mol for the CDOCKER energies. But this compound interacted with a non-critical residue Lys529. The compound isobutyl isothiocyanate displayed a better interaction as it was having the least difference of 0.59 Kcal/mol for the CDOCKER energies and the compound interacted with active site amino acid residues Glu353 and Arg394. The FDA approved commercial drug tamoxifen interacted with a non-essential amino acid residue Asp351, and was also evaluated as an unstable interaction due to a huge difference between CDOCKER energies. Detailed results of dock scores of *Allium* phytocompounds and ER are presented in Table 4.19.

4.1.8.1.9 Heat shock protein (Cancer target)

Average dock scores were found for *Allium* compounds when docked with Heat Shock Protein (HSP). It was observed that only six compounds from garlic showed mediocre dock scores against the mentioned target for cancer in between -54.27 Kcal/mol to -131.02 Kcal/mol. Among exclusive *Allium* compounds, only 3 compounds such as alliin, s-allyl d-cysteine and s-allyl l-cysteine displayed somewhat good docking results. Alliin displayed the lowest binding energy of -65.89 Kcal/mol, making a single hydrogen bond of length 2.05 Å with amino acid residue Asp93. The deviation for CDOCKER energies for this compound was 1.8

Table 4.19: Dock scores for the cancer target Estrogen receptor with selected ligands

Type of compound	Sl. No.	Ligand	(-) CDOCKER energy (Kcal/mol)	(-) CDOCKER Interaction energy (Kcal/mol)	No. of H bonds	H bond	Distance (Å)	Binding Energy (Kcal/mol)
Allium-specific compounds	1.	Nil						
Non-*Allium* specific compounds	2.	P-Coumaric Acid	31.94	35.51	2	LYS529 (2)	1.91	-83.45
	3.	Isobutyl isothiocyanate	20.53	21.12	2	GLU353* ARG394*	2.21 2.35	-74.73
	4.	Taurine	26.52	29.79	1	GLU353*	1.82	-61.12
Commercial drug	5.	Tamoxifen	15.73	53.39	1	ASP351*	1.97	-90.30

* amino acid residues present in active site

Kcal/mol. However, the deviation was quite low for another compound s-allyl l-cysteine (0.01 Kcal/mol), which interacted with amino acid residues such as Asp93 and Thr184, the hydrogen bond distances being 1.84 Å and 2.10 Å respectively. The maximum no. of hydrogen bonds (3 nos.) was formed by the compound s-allyl d-cysteine.

Among the non-*Allium* specific compounds, kaempferol recorded least binding energy (-131.02 Kcal/mol) while binding with the amino acid residue Thr184. Compounds such as apigenin and isobutyl isothiocyanate also interacted with critical amino acid residues Thr184 and Asp93. The commercial FDA approved drug geldanamycin could not pass Lipinski's filter of five and Veber's protocol. Detailed results of dock scores of *Allium* phytocompounds and HSP are presented in Table 4.20.

4.1.8.1.10 Mast/Stem cell growth factor receptor (KIT) (Cancer target)

Detailed results of dock scores of *Allium* phytocompounds and KIT are presented in Table 4.21. Only six compounds from garlic showed mediocre dock scores against the mentioned target with a binding energy ranging from -51.27 Kcal/mol to -91.33 Kcal/mol. Among the exclusive *Allium* compounds, only 4 compounds such as s-allyl l-cysteine, s-allyl d-cysteine, l-γ-glutamyl-s-allyl-l-cysteine and alliin displayed somewhat good docking results. s-allyl l-cysteine displayed the lowest binding energy of -89.46 Kcal/mol, making two hydrogen bonds with active site amino acid residue Thr670. The difference for the CDOCKER energies for this compound was 0.95 Kcal/mol. However, the value was quite low for the compound's dextrorotatory isomer s-allyl d-cysteine (0.22 Kcal/mol), which interacted with amino acid residues such as Glu640 and Asp810. The smallest hydrogen bond of length 1.79 Å was formed by alliin, while interacting with active site residue Glu640.

Among the non-*Allium* specific compounds, taurine recorded least binding energy (-65.92Kcal/mol) while binding with the active site amino acid residues Glu640 and Lys623. Another compound p-coumaric acid also interacted with

Table 4.20: Dock scores for the cancer target Heat shock protein with selected ligands

Type of compound	Sl. No.	Ligand	(-) CDOCKER energy (Kcal/mol)	(-) CDOCKER Interaction energy (Kcal/mol)	No. of H bonds	H bond	Distance (Å)	Binding Energy (Kcal/mol)
Allium-specific compounds	1.	Alliin	26.46	28.29	1	ASP93*	2.05	-65.89
	2.	S-Allyl D-Cysteine	26.22	26.43	3	ASP93* THR184* SER52	1.87 2.05 2.37	-59.26
	3.	S-Allyl L-Cysteine	26.05	26.06	2	ASP93* THR184*	1.84 2.10	-54.27
Non-*Allium* specific compounds	4.	Kaempferol	38.25	40.23	1	THR184*	1.98	-131.02
	5.	Apigenin	42.82	43.93	2	THR184* ASP93*	1.83 2.32	-122.28
	6.	Isobutyl isothiocyanate	20.54	22.02	1	ASP93*	2.09	-97.77
Commercial drug	7.	Geldanamycin	Geldanamycin not passed Lipinski's filter					

* amino acid residues present in active site

Table 4.21: Dock scores for the cancer target Mast/Stem cell growth factor receptor with selected ligands

Type of compound	Sl. No.	Ligand	(-) CDOCKER energy (Kcal/mol)	(-) CDOCKER Interaction energy (Kcal/mol)	No. of H bonds	H bond	Distance (Å)	Binding Energy (Kcal/mol)
Allium-specific compounds	1.	S-Allyl L-Cysteine	28.72	29.67	2	THR670 (2)*	2.08	-89.46
	2.	S-Allyl D-Cysteine	28.76	28.98	3	GLU640* ASP810 (2)*	1.84 1.89	-64.92
	3.	L-γ-Glutamyl-S-allyl-L-cysteine	49.12	52.01	2	THR670* ASP810*	1.97 2.41	-59.43
	4.	Alliin	29.53	32.08	3	GLU640* ASP810 (2)*	1.79 1.84	-51.27
Non-*Allium* specific compounds	5.	Taurine	26.51	29.49	2	GLU640* LYS623*	1.90 2.39	-65.92
	6.	P-Coumaric Acid	26.28	29.03	2	LYS623* GLU640*	2.23 2.48	-59.44
Commercial drug	7.	Dasatinib	25.57	47.49	2	LYS623* ARG791	2.01 2.38	-91.33

* amino acid residues present in active site

the same amino acid essential residues. The FDA approved commercial drug dasatinib interacted with a non-essential amino acid residue Arg791, but was evaluated as an unstable interaction due to a huge difference between CDOCKER energies exceeding the limit of 10 Kcal/mol.

4.1.8.1.11 Matrix Metallo Proteinase (Cancer target)

While molecular docking with Matrix Metallo Proteinase (MMP), it was observed that only 3 compounds exclusive to *Allium* peformed molecular interactions in the identified active sites. They were cycloalliin, s-allyl l-cysteine and s-allyl d-cysteine. The binding energy of those compounds with MMP ranged between -80.54 Kcal/mol and -90.81 Kcal/mol. The lowest binding energy was displayed by cycloalliin with a deviation of only 0.5 Kcal/mol between the CDOCKER energies. The compound connected with 2 residues Pro217 and Glu198 for making hydrogen bonding, out of which Pro217 was identified as a non-critical residue.

Only three compounds from the non-*Allium* specific group such as taurine, isobutyl isothiocyanate and phloroglucinol showed better dock scores against MMP as in case of FDA approved commercial drug doxycycline. The least binding energy (-120.92 Kcal/mol) was shown by taurine with a deviation of 3.26 Kcal/mol for the CDOCKER energies. The compound isobutyl isothiocyanate displayed a better interaction as it was having the least difference of 0.61 Kcal/mol for the CDOCKER energies. The FDA approved commercial drug doxycycline could not pass Lipinski's rule and so it was not forwarded for molecular docking studies. Detailed results of dock scores of *Allium* phytocompounds and MMP are presented in Table 4.22.

4.1.8.1.12 Nuclear factor kappa light chain enhancer of activated B cells (NFkB) (Cancer target)

Ten potent compounds from *Allium* (4 exclusive *Allium* and 6 others) interacted with NFkB. Detailed results of dock scores of *Allium* phytocompounds

Table 4.22: Dock scores for the cancer target Matrix metalloproteinase with selected ligands

Type of compound	Sl. No.	Ligand	(-) CDOCKER energy (Kcal/mol)	(-) CDOCKER Interaction energy (Kcal/mol)	No. of H bonds	H bond	Distance (Å)	Binding Energy (Kcal/mol)
Allium-specific compounds	1.	Cycloalliin	28.95	29.45	3	PRO217 (2) GLU198*	2.24 2.39	-90.81
	2.	S-Allyl L-Cysteine	28.08	28.87	2	HIS197* PRO217	1.98 2.07	-86.94
	3.	S-Allyl D-Cysteine	30.74	31.50	2	PRO217 HIS197*	1.97 2.00	-80.54
Non-*Allium* specific compounds	4.	Taurine	20.87	24.13	2	PRO217 HIS197*	1.83 1.99	-120.92
	5.	Isobutyl isothiocyanate	22.51	23.12	1	HIS197*	2.09	-86.92
	6.	Phloroglucinol	21.51	22.41	4	ALA161 (2) LEU160* TYR219*	1.98 2.05 2.24	-68.14
Commercial drug	7.	Doxycycline	Doxycycline could not pass Lipinski's rule					

* amino acid residues present in active site

against NFkB are presented in Table 4.23. Among the exclusive group, l-γ-glutamyl-s-allyl-l-cysteine showed the best dock score with the lowest binding energy of -144.20 Kcal/ mol, which made 5 hydrogen bonds with the residues Lys429, Asp534 (2) and Asn520 (2), displaying a difference of CDOCKER energies as 1.77 Kcal/mol. However, the least difference in CDOCKER energies was displayed by s-allyl d-cysteine (0.53 Kcal/ mol), which made 4 hydrogen bonds via joining with active site residues Asp534, Lys429 and Asn520 (2). The highest no. of hydrogen bonds (6 nos.) was recorded for alliin, which also interacted with the same active site residues as l-γ-glutamyl-s-allyl-l-cysteine.

Among the non-*Allium* specific group, kaempferol displayed the lowest binding energy of -264.32 Kcal/mol, which made 2 hydrogen bonds with Lys517 and Lys429, and the hydrogen bond lengths being 1.59 Å and 1.62 Å respectively. The deviation of CDOCKER energies observed for kaempferol was 2.81 Kcal/mol. This value was quite low for myricetin (0.72 Kcal/mol) which displayed a decent binding energy of -197.69 Kcal/mol.

4.1.8.1.13 Phosphoinositide 3-kinase Gamma (Pi3k-γ) (Cancer target)

During molecular docking with Pi3k-γ, it was observed that 10 compounds from *Allium* displayed very good dock scores. The best dock score (-243.90 Kcal/mol) was shown by l-γ-glutamyl-s-allyl-l-cysteine among the exclusive *Allium* phytocompounds, with a difference of 1.09 Kcal/mol among the CDOCKER energies. The interacting residues for the compound were Lys833, Asp964, Tyr867 and Asp836. Compounds like s-allyl d-cysteine and cycloalliin showed a lesser deviation of 0.27 Kcal/mol and 0.50 Kcal/mol respectively and interacted with Lys833 more than once.

Quercetin was the compound from the non-*Allium* specific group, which showed a least binding energy (-399.59 Kcal/mol) with a difference of 2.82 Kcal/mol between CDOCKER energy and CDOCKER interaction energy. The

Table 4.23: Dock scores for the cancer target NFkB with selected ligands

Type of compound	Sl. No.	Ligand	(-) CDOCKER energy (Kcal/mol)	(-) CDOCKER Interaction energy (Kcal/mol)	No. of H bonds	H bond	Distance (Å)	Binding Energy (Kcal/mol)
Allium-specific compounds	1.	L-γ-Glutamyl-S-allyl-L-cysteine	61.13	62.90	5	LYS429* ASP534 (2)* ASN520 (2)*	1.80 1.91 1.94	-144.20
	2.	S-Allyl L-Cysteine	38.19	38.77	2	ASP534* LYS429*	1.81 2.06	-93.56
	3.	S-Allyl D-Cysteine	37.54	38.07	4	ASP534* LYS429* ASN520 (2)*	1.82 1.90 2.10	-72.35
	4.	Alliin	38.08	42.13	6	LYS429 (3)* ASP534* ASN520 (2)*	1.88 1.94 2.06	-64.18
Non-*Allium* specific compounds	5.	Kaempferol	60.95	63.76	2	LYS517* LYS429*	1.59 1.62	-264.32
	6.	Myricetin	66.03	66.75	2	LYS517* LYS429*	1.62 1.69	-197.69
	7.	P-Coumaric Acid	32.64	35.83	5	ASP519* LYS429* SER410 PHE411* GLY412*	1.95 2.07 2.19 2.19 2.40	-104.56
	8.	Phloroglucinol	33.64	35.42	2	ASP519* LYS429*	1.99 1.67	-65.99
	9.	Isobutyl isothiocyanate	23.03	23.52	2	ASN520* ASP534*	1.97 2.18	-65.68
	10.	Taurine	20.84	23.57	2	ASN520* ASP519*	1.79 1.81	-62.27

* amino acid residues present in active site

interacting active site residues were Lys833 and Lys890. Apigenin showed a very less deviation of 0.15 Kcal/mol between the CDOCKER energies. The commercial drug idelalisib showed a poor binding energy and was also considered to form an unstable interaction with the target protein because of a big deviation of CDOCKER energies which surpassed the limit of 10 Kcal/mol. Detailed results of dock scores of *Allium* phytocompounds against Pi3k-γ are presented in Table 4.24.

4.1.8.1.14 Progesterone Receptor (Cancer target)

Three compounds exclusive to *Allium* showed better dock scores with Progesterone Receptor (PR) in comparison with the commercial FDA approved drug megestrol acetate for breast cancer. The lowest binding energy was shown by cycloalliin (-85.07 Kcal/mol). The interacting active site residues were Leu718 and Gln725, for which the hydrogen bond length were 1.90 Å and 2.11 Å respectively. The difference of CDOCKER energy and CDOCKER interaction energy was 0.05 Kcal/mol. The highest no. of hydrogen bonds (3 nos.) was formed by s-allyl l-cysteine interacting with the active site residues Gln725 (2) and Arg766.

Among the non-*Allium* specific group, kaempferol showed a good binding energy of -168.57 Kcal/mol interacting with active site residue Gln725, with a deviation of only 0.27 Kcal/mol. The other compounds like quercetin, phloroglucinol and apigenin also showed good binding energy connecting with the active site residues Gln725, Cys891, Leu718 and Arg766. The commercial drug megestrol acetate showed a positive CDOCKER energy while interacting and hence the binding energy was not calculated. Detailed results of molecular docking of *Allium* phytocompounds against PR are presented in Table 4.25.

Table 4.24: Dock scores for the cancer target Pi3k-γ with selected ligands

Type of compound	Sl. No.	Ligand	(-) CDOCKER energy (Kcal/mol)	(-) CDOCKER Interaction energy (Kcal/mol)	No. of H bonds	H bond	Distance (Å)	Binding Energy (Kcal/mol)
Allium-specific compounds	1.	L-γ-Glutamyl-S-allyl-L-cysteine	57.42	58.51	6	LYS833 (2)* ASP964 (2) TYR867* ASP836*	1.94 2.17 2.19 2.45	-243.90
	2.	S-Allyl Mercapto Cysteine	31.35	33.07	2	LYS833 (2)*	1.91	-185.45
	3.	S-Allyl D-Cysteine	38.38	38.65	3	ASP964 LYS833 (2)*	1.76 2.12	-153.60
	4.	Cycloalliin	29.26	29.76	3	LYS833 (3)*	1.97	-146.25
	5.	S-Allyl L-Cysteine	37.36	38.04	1	LYS833*	1.88	-142.62
Non-*Allium* specific compounds	6.	Quercetin	68.53	71.35	2	LYS833* LYS890	1.69 1.79	-399.59
	7.	Apigenin	65.57	65.72	3	LYS890 (2) LYS833*	1.69 1.71	-333.99
	8.	Myricetin	53.14	55.16	3	LYS890 (2) LYS833*	1.66 1.85	-188.73
	9.	Phloroglucinol	35.81	36.37	1	LYS833*	1.63	-136.15
	10.	Kaempferol	46.15	47.00	3	LYS833* LYS890 (2)	1.63 1.95	-112.41
Commercial drug	11.	Idelalisib	20.74	38.66	3	LYS890 (2) LYS833*	2.16 2.24	-50.58

* amino acid residues present in active site

Table 4.25: Dock scores for the cancer target Progesterone receptor with selected ligands

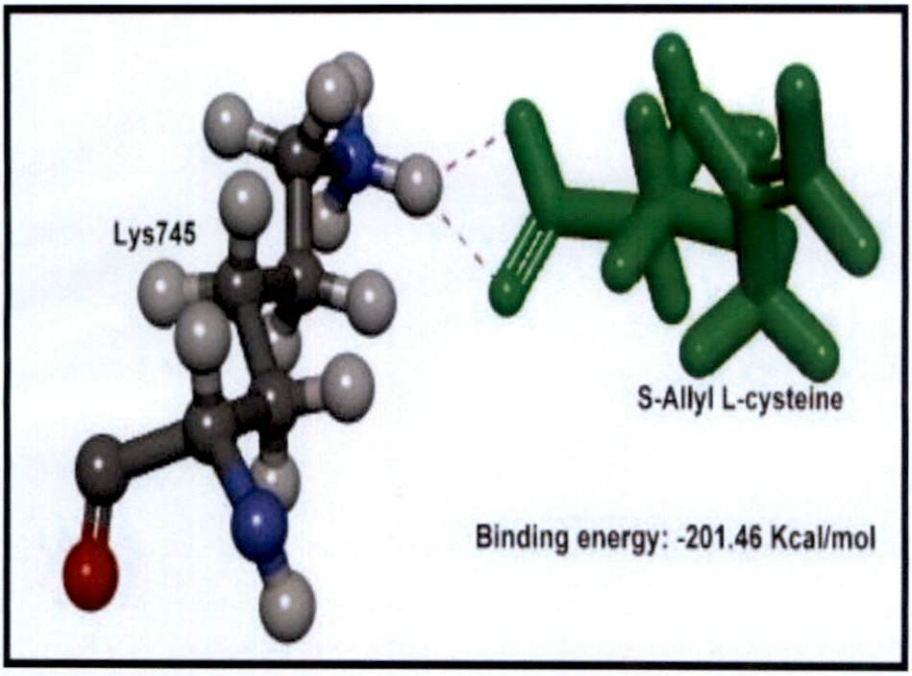
Type of compound	Sl. No.	Ligand	(-) CDOCKER energy (Kcal/mol)	(-) CDOCKER Interaction energy (Kcal/mol)	No. of H bonds	H bond	Distance (Å)	Binding Energy (Kcal/mol)
Allium-specific compounds	1.	Cycloalliin	32.80	32.85	2	LEU718* GLN725*	1.90 2.11	-85.07
	2.	S-Allyl L-Cysteine	28.82	29.05	3	GLN725 (2)* ARG766*	1.89 2.15	-73.84
	3.	2 Methyl Benzaldehyde	21.14	24.52	2	ARG766* GLN725*	2.03 2.23	-50.07
Non-*Allium* specific compounds	4.	Kaempferol	41.24	41.51	1	GLN725*	1.99	-168.57
	5.	Quercetin	46.31	48.68	2	GLN725* CYS891*	1.96 2.34	-154.66
	6.	Phloroglucinol	36.24	37.03	3	ARG766* LEU718* GLN725*	1.84 1.94 1.98	-149.31
	7.	Apigenin	50.50	51.99	3	GLN725* ARG766* CYS891*	1.92 1.93 2.01	-114.45
	8.	P-Coumaric Acid	25.96	28.77	2	ARG766* GLN725*	1.93 2.19	-104.83
Commercial drug	9.	Megestrol acetate	Positive CDOCKER energy					

*amino acid residues present in active site

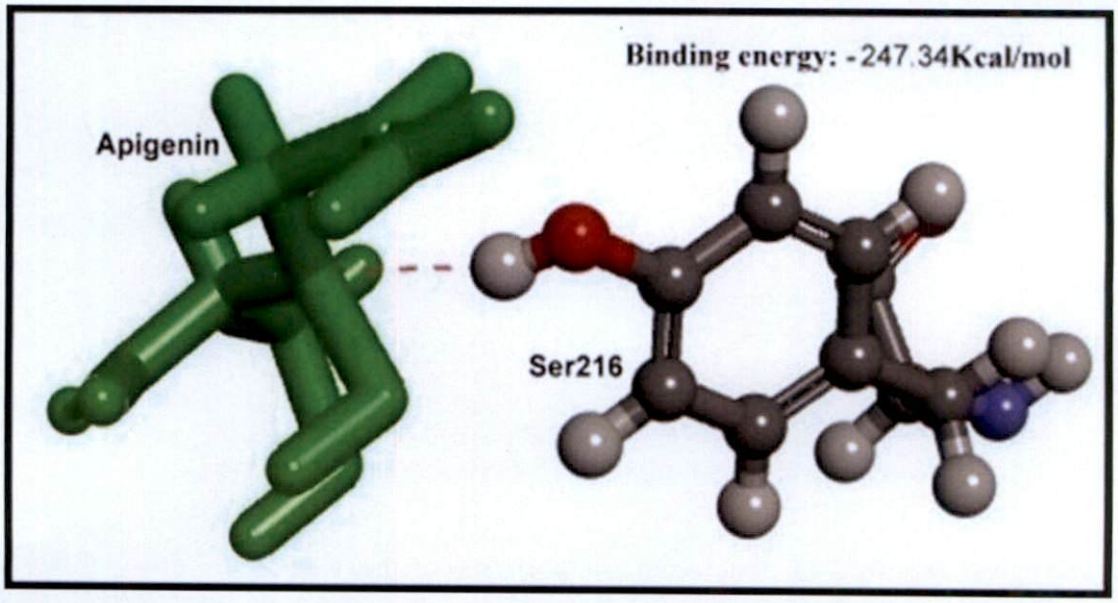
4.1.8.1.15 Thymidilate synthase (Cancer target)

Molecular docking studies of *Allium* compounds with target protein Thymidilate Synthase (TS) revealed six potent compounds from *Allium* (4 exclusive *Allium* and 2 others). Detailed results of dock scores of *Allium* phytocompounds against TS are presented in Table 36. The binding energy of compounds ranged between -86.15 Kcal/mol and -251.29 Kcal/mol. Among the exclusive group, s-allyl mercapto cysteine recorded the best dock score with the lowest binding energy of -233.09 Kcal/mol, which made only one hydrogen bond with the active site residue Ser216 displaying a difference of CDOCKER energies as 1.69 Kcal/mol. s-allyl l-cysteine made maximum no. of hydrogen bonds (3 nos.) by joining with active site critical residues Asn226 and His196 (2), but with a lower dock score of -146.05 Kcal/mol.

Among the other group of compounds, p-coumaric acid and apigenin displayed lowest binding energy of -251.29 Kcal/mol and -247.34 Kcal/mol. The smallest hydrogen bond of length 1.85 Å, was formed by apigenin while joining with active site residue Ser216 (Plate 4). The commercial FDA approved drug fluorouracil interacted with critical active site residues such as Arg215, His256, Arg50, Ser216 and Cys195, making five hydrogen bonds with TS, but yielded a very poor binding energy of only -0.41 Kcal/mol. Detailed results of molecular docking of *Allium* phytocompounds against TS are presented in Table 4.26.



A. EGFR vs S Allyl L cysteine



B. Thymidilate synthase vs Apigenin

Plate 4: Hydrogen bond interaction of cancer targets EGFR and Thymidilate synthase with S Allyl L cysteine (A) and Apigenin (B)

Table 4.26: Dock scores for the cancer target Thymidilate synthase with selected ligands

Type of compound	Sl. No.	Ligand	(-) CDOCKER energy (Kcal/mol)	(-) CDOCKER Interaction energy (Kcal/mol)	No. of H bonds	H bond	Distance (Å)	Binding Energy (Kcal/mol)
Allium-specific compounds	1.	S-Allyl Mercapto Cysteine	37.41	39.10	1	SER216*	1.98	-233.09
	2.	S-Allyl L-Cysteine	31.13	31.35	3	ASN226* HIS196 (2)*	2.14 2.22	-146.05
	3.	Cycloalliin	34.74	35.30	1	SER216*	2.21	-134.79
	4.	S-Allyl D-Cysteine	30.80	31.66	1	SER216*	1.99	-86.15
Non-*Allium* specific compounds	5.	P-Coumaric Acid	37.04	40.10	1	ARG50*	2.08	-251.29
	6.	Apigenin	52.79	55.35	1	SER216*	1.85	-247.34
Commercial drug	7.	Fluorouracil	18.44	21.38	5	ARG215* HIS256* ARG50* SER216* CYS195*	1.86 2.08 2.25 2.30 2.34	-0.41

* amino acid residues present in active site

4.1.8.2 Docking results of target proteins involved in diabetes disease

4.1.8.2.1 Aldose Reductase

Detailed results of molecular docking between *Allium* phytocompounds with Aldose Reductase (AR) are presented in Table 4.27. Six phytocompounds (4 *Allium* specific and 2 others) recorded good dock scores. L-γ-glutamyl-s-allyl-l-cysteine showed the best dock score with AR with a binding energy of -290.54 Kcal/ mol, but with a CDOCKER energy deviation of 1.22 Kcal/ mol, whereas the least difference of CDOCKER energy and CDOCKER interaction energy was shown by s-allyl d-cysteine (0.84 Kcal/mol). The interacting active site residue for l-γ-glutamyl-s-allyl-l-cysteine was Ser210 with a hydrogen bond distance of 2.21 Å. S-allyl d-cysteine also interacted with other residues such as Ile260 and Ser214, from which Ile260 was recognized as a non-critical active site residue.

Among non-*Allium* specific group, apigenin showed the least binding energy of -285.09 Kcal/mol with the best interaction of a minimum difference of 0.34 Kcal/mol between CDOCKER and CDOCKER interaction energy. Apigenin interacted with residues such as Asp43 and Tyr48 out of which Asp43 was discovered as non-critical active site residue. The commercial drug epalrestat interacted with a very good binding energy, but was considered unstable because of huge deviation of CDOCKER energies more than 10 Kcal/mol.

4.1.8.2.2 Dipeptidyl Peptidase-4

During molecular docking with dipeptidyl peptidase-4 (DPP4), best dock score was shown by l-γ-glutamyl-s-allyl-l-cysteine among the exclusive *Allium* phytocompounds. A least binding energy of -220.91 Kcal/mol was shown by the mentioned compound, with the lowest difference of 0.25 Kcal/mol among the CDOCKER energies. The same compound also formed maximum number (7) of hydrogen bonds with DPP4 while interacting. The interacting active site residues were GLU206, GLU205, ARG125, TYR547 and HIS740 with hydrogen bond distances of 1.93 Å, 1.95 Å, 2.05 Å, 2.13 Å and 2.19 Å respectively. The smallest

Table 4.27: Dock scores for the diabetes target aldose reductase with selected ligands

Type of compound	Sl. No.	Ligand	(-) CDOCKER energy (Kcal/mol)	(-) CDOCKER Interaction energy (Kcal/mol)	No. of H bonds	H bond	Distance (Å)	Binding Energy (Kcal/mol)
Allium-specific compounds	1.	L-γ-Glutamyl-S-allyl-L-cysteine	66.94	68.16	1	SER210*	2.21	-290.54
	2.	Cycloalliin	35.47	38.12	5	LYS21 SER210 (2)* SER214 (2)*	1.88 1.98 1.99	-102.99
	3.	S-Allyl D-Cysteine	36.48	37.32	3	ILE260 SER210* SER214*	1.78 2.04 2.07	-79.82
	4.	S-Allyl L-Cysteine	33.77	35.99	4	ILE260 LYS21 SER210* SER214*	1.98 2.23 2.35 2.37	-57.64
Non-*Allium* specific compounds	5.	Apigenin	52.48	52.82	2	ASP43 TYR48*.	2.21 2.26	-285.09
	6.	Phloroglucinol	38.02	40.61	3	ILE260 SER210* LYS262*	1.83 1.91 2.09	-211.94
Commercial drug	7.	Epalrestat	19.65	52.36	2	LYS262* ASP216	1.86 2.26	-230.35

* amino acid residues present in active site

interacting hydrogen bond of 1.73 Å was formed by alliin with an active site residue Tyr662.

Among non-*Allium* specific group, apigenin showed the least binding energy (-242.16 Kcal/mol) with a low difference of 0.44 Kcal/mol between CDOCKER energy and CDOCKER interaction energy. The interacting active site residue was His740 with a hydrogen bond of length 1.88 Å. However, kaempferol showed a more low difference of CDOCKER energies as 0.03 Kcal/mol, interacting with active side residue Arg125 forming a hydrogen bond of length 1.79 Å. The commercial drug vildagliptin showed a positive CDOCKER energy while interacting and so the binding energy was not calculated. Detailed results of dock scores of *Allium* phytocompounds and DPP4 are presented in Table 4.28.

4.1.8.2.3 Glucokinase (diabetes target)

Molecular docking studies of *Allium* compounds with target protein Glucokinase (GK) revealed 8 potent compounds from *Allium* (six exclusive *Allium* and two other compounds) against GK (Table 4.29). The binding energy of compounds ranged from -147.00 Kcal/mol to -261.03 Kcal/mol. L-γ-glutamyl-s-allyl-l-cysteine showed the best dock score with lowest binding energy (-261.03 Kcal/mol) and made 6 hydrogen bonds while connecting residues Asp205, Lys169, Ser411, Thr228 and Ser151, displaying a difference of CDOCKER energies as low as 0.38 Kcal/mol (Plate 5).

Among the non-*Allium* specific group, p-coumaric acid displayed the lowest binding energy of -182.77 Kcal/mol by connecting with residues such as Lys169, Gly229 and Asp409, the hydrogen bond lengths (3 nos.) being 1.88 Å, 2.26 Å and 1.96 Å respectively. However, Asp409 was observed as a non-critical residue. The deviation of CDOCKER energies observed for p-coumaric acid (2.75 Kcal/mol) was little higher than that of phloroglucinol (2.00 Kcal/mol). Phloroglucinol recorded making 5 hydrogen bonds while connecting with the same active site residues mentioned above, out of which the smallest hydrogen bond length of 1.80 Å was formed with residue Thr228.

Table 4.28: Dock scores for the diabetes target Dipeptidyl Peptidase-4 with selected ligands

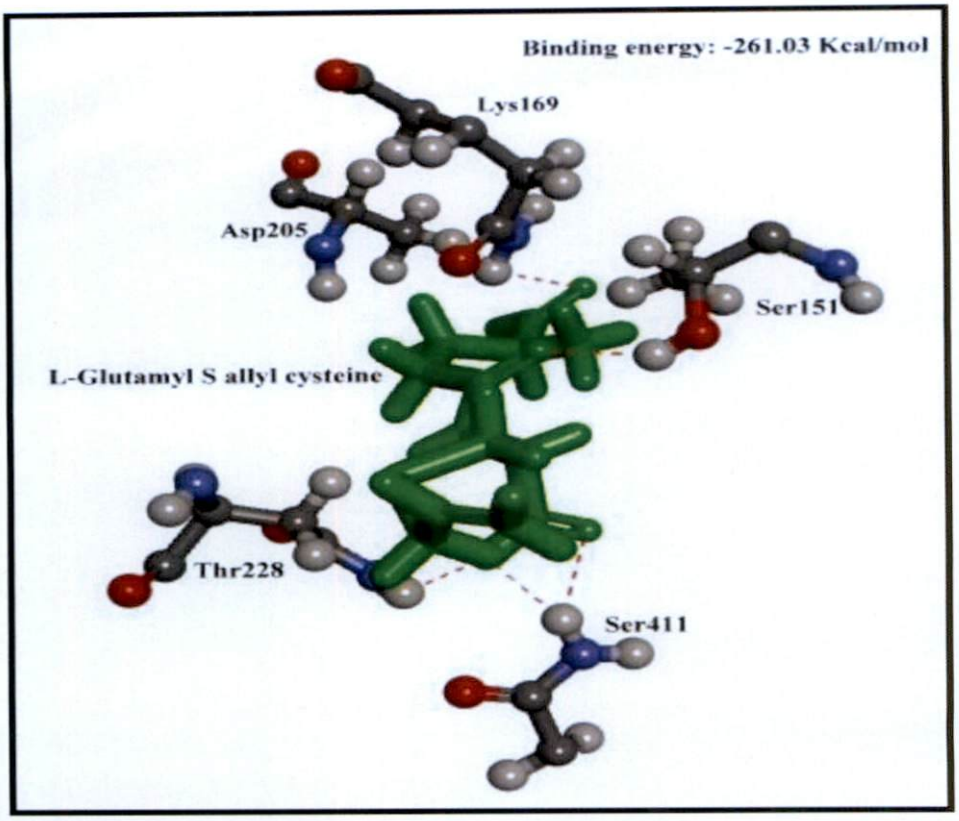
Type of compound	Sl. No.	Ligand	(-) CDOCKER energy (Kcal/mol)	(-) CDOCKER Interaction energy (Kcal/mol)	No. of H bonds	H bond	Distance (Å)	Binding Energy (Kcal/mol)
Allium-specific compounds	1.	L-γ-Glutamyl-S-allyl-L-cysteine	56.19	56.44	7	GLU206 (2)* GLU205* ARG125 (2)* TYR547* HIS740*	1.93 1.95 2.05 2.13 2.19	-220.91
	2.	Alliin	35.20	37.49	5	TYR662* GLU205* GLU206 (2)* ASN710*	1.73 1.87 2.16 2.25	-154.09
	3.	S-Allyl D-Cysteine	34.26	34.73	3	TYR662* GLU205* ARG125*	1.79 1.89 2.14	-133.89
	5.	S-Allyl L-Cysteine	35.19	35.78	3	TYR662* GLU205* ARG125*	1.78 1.94 2.11	-124.46
	6.	Cycloalliin	33.48	33.99	4	GLU205* GLU206* TYR547* SER630*	2.01 2.21 2.22 2.36	-117.97
Non-*Allium* specific compounds	7.	Apigenin	59.73	60.17	1	HIS740*	1.88	-242.16
	8.	Kaempferol	44.59	44.62	1	ARG125*	1.79	-237.58
	9.	Myricetin	43.48	43.52	5	TYR547* ASN710 (2)* TYR662* SER630*	1.98 2.18 2.31 2.41	-222.59
Commercial drug	12.	Vildagliptin	Positive CDOCKER energy					

* amino acid residues present in active site

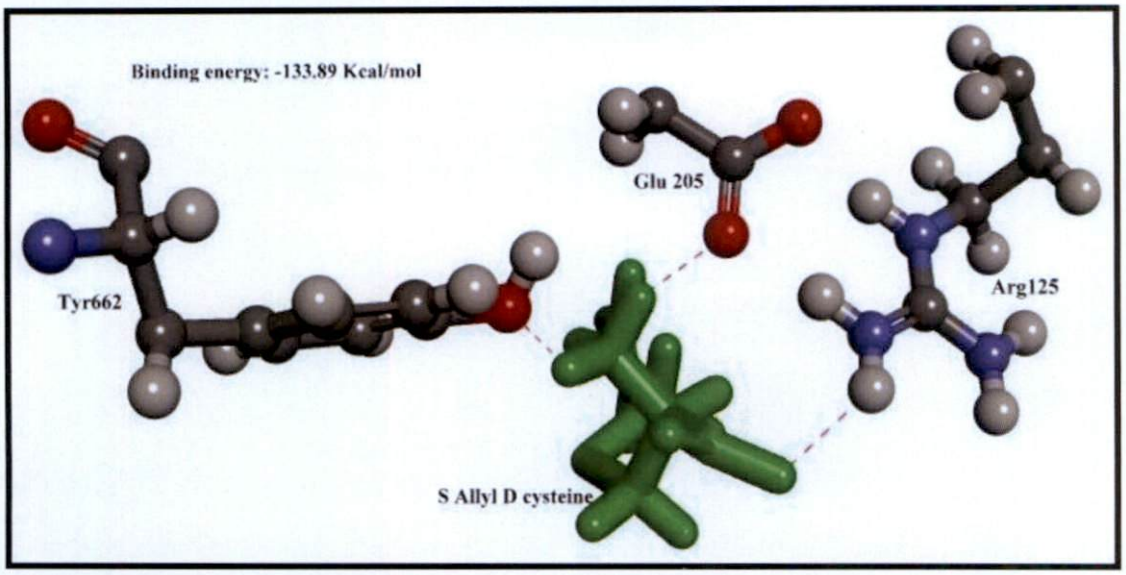
Table 4.29: Dock scores for the diabetes target Glucokinase with selected ligands

Type of compound	Sl. No.	Ligand	(-) CDOCKER energy (Kcal/mol)	(-) CDOCKER Interaction energy (Kcal/mol)	No. of H bonds	H bond	Distance (Å)	Binding Energy (Kcal/mol)
Allium-specific compounds	1.	L-γ-Glutamyl-S-allyl-L-cysteine	66.33	66.71	6	ASP205* LYS169* SER411 (2)* SER151* THR228*	1.81 1.99 2.00 2.16 2.35	-261.03
	2.	Alliin	37.63	41.84	6	LYS169 (2)* GLY229* ASP205* THR228 (2)*	2.09 2.11 2.29 2.44	-186.26
	3.	S-Allyl Mercapto Cysteine	31.81	35.65	3	LYS169* GLY229* ASP78*	1.94 2.31 2.31	-171.28
	4.	Cycloalliin	44.07	45.15	4	LYS169 (2)* ASP205* THR228*	1.92 2.00 2.48	-161.81
	5.	S-Allyl D-Cysteine	38.07	39.09	4	ASP205* LYS169 (2)* THR228*	1.88 1.96 2.36	-150.09
	6.	S-Allyl L-Cysteine	37.52	38.36	2	LYS169* ASP205*	1.83 1.85	-147.00
Non-*Allium* specific compounds	7.	P-Coumaric Acid	35.39	38.14	3	LYS169* ASP409 GLY229*	1.88 1.96 2.26	-182.77
	8.	Phloroglucinol	44.01	46.01	5	THR228 (2)* LYS169* ASP205* GLY81	1.80 1.82 1.97 2.41	-148.67

* amino acid residues present in active site



A. Glucokinase vs L-γ-Glutamyl-S-allyl-L-cysteine



B. DPP4 vs S Allyl D cysteine

Plate 5: Hydrogen bond interaction of diabetes targets Glucokinase and DPP4 with L-γ-Glutamyl-S-allyl-L-cysteine (A) and S-allyl D-cysteine (B)

4.1.8.2.4 Glycogen Synthase Kinase 3 (diabetes target)

Detailed results of dock scores of *Allium* phytocompounds with Glycogen synthase kinase 3 (GSK3) are presented in Table 4.30. Among the exclusive *Allium* phytocompounds, s-allyl mercapto cysteine showed the least binding energy as -145.60 Kcal/mol, but the remainder of CDOCKER energies (2.93 Kcal/ mol) is more than that of energy difference showed by s-allyl l-cysteine (0.01 Kcal/ mol). The active site residues interacting with s-allyl l-cysteine were Val135, Tyr134 and Pro136, out of which only Tyr134 made hydrogen bonds with s-allyl mercapto cysteine. Almost all the compounds formed very less number of hydrogen bonds while interacting. Alliin and s-allyl l-cysteine both made 3 hydrogen bonds with the same active site amino acid residues mentioned above.

Three compounds from the other distinct group also interacted with GSK3 making one hydrogen bond each, with active site residues such as Val135, Tyr134 and Ile62. Myricetin showed the least binding energy (-239.37 Kcal/mol) but with a remainder of CDOCKER energies as around 3.77 Kcal/mol. The lowest deviation (0.95 Kcal/mol) was recorded by phloroglucinol at a binding energy of -152.87 Kcal/mol. The shortest hydrogen bond of 1.79 Å was also formed by phloroglucinol. The commercial drug lithium carbonate also interacted with active site residue Tyr134 with a very good binding energy and a minimum remainder of 0.71 Kcal/mol between the CDOCKER energies.

4.1.8.2.5 Insulin receptor (diabetes target)

Two compounds exclusive to *Allium* showed best dock scores with Insulin receptor (IR) in comparison with the commercial FDA approved drug repaglinide for diabetes. The lowest binding energy was shown by s-allyl l-cysteine (-99.49 Kcal/mol). The interacting active site residues were Glu1047 and Asp1150 for which the hydrogen bond distances were 1.94 Å and 2.15 Å respectively. But the difference of CDOCKER energy and CDOCKER interaction energy was 2.13 Kcal/mol. However, the least difference of CDOCKER energies was shown by s-

Table 4.30: Dock scores for the diabetes target Glycogen synthase kinase 3 with selected ligands

Type of compound	Sl. No.	Ligand	(-) CDOCKER energy (Kcal/mol)	(-) CDOCKER Interaction energy (Kcal/mol)	No. of H bonds	H bond	Distance (Å)	Binding Energy (Kcal/mol)
Allium-specific compounds	1.	S-Allyl Mercapto Cysteine	21.03	23.96	2	TYR134 (2)*	2.21	-145.60
	2.	Alliin	27.71	31.20	3	TYR134* VAL135* PRO136*	1.97 2.12 2.32	-111.60
	3.	Cycloalliin	29.04	29.45	2	VAL135* TYR134*	1.97 2.07	-108.29
	4.	S-Allyl L-Cysteine	31.23	31.24	3	VAL135* TYR134* PRO136*	1.88 2.01 2.25	-67.05
Non-*Allium* specific compounds	5.	Myricetin	46.91	50.68	1	VAL135*	2.01	-239.37
	6.	Phloroglucinol	26.63	27.69	1	TYR134*	1.79	-152.87
	7.	P-Coumaric Acid	26.74	29.95	1	ILE62*	2.47	-152.78
Commercial drug	8.	Lithium carbonate	20.24	20.95	1	TYR134*	1.99	-190.31

* amino acid residues present in active site

allyl d-cysteine (1.25 Kcal/mol) interacting with the same active site residues mentioned above (Table 4.31).

Among non-*Allium* specific group, taurine showed least binding energy (-107.30 Kcal/mol) while interacting with same active site residues Asp1150 and Glu1047, making hydrogen bonds of length 1.78 Å and 2.06 Å respectively, out of which Asp1150 was found to form the hydrogen bond of shortest length (1.78 Å). The commercial drug repaglinide also had interaction with same active site residue but with a very poor binding energy, making only one hydrogen bond with IR, with huge remainder between CDOCKER energy and CDOCKER interaction energy surpassing the limit of 10 Kcal/mol.

4.1.8.2.6 Peroxisome proliferator-activated receptor gamma (PPARγ) (diabetes target)

Molecular docking scores for the selected *Allium* phytocompounds and PPARγ are presented in Table 4.32. Among exclusive *Allium* phytocompounds, alliin showed the least binding energy (-103.06 Kcal/mol) while interacting with PPARγ, but the remainder of CDOCKER energies was found to be over 3.00 Kcal/mol.

The interacting active site residue was Ser289, the hydrogen bond distance being 2.34 Å. However, s-allyl mercapto cysteine exhibited the lowest difference between CDOCKER energy and CDOCKER interaction energy. Both the energies were found to be nearly same with a difference of only 0.1 Kcal/mol. But the interacting residue Tyr327, with which s-allyl mercapto cysteine formed hydrogen bonding, was found to be a non-critical residue.

From the non-*Allium* specific group, apigenin displayed the best dock score with a binding energy of -160.95 Kcal/mol and the least difference of 1.22 Kcal/mol between the CDOCKER energies. The active site residues from active site 1 of PPARγ, connecting with apigenin via hydrogen bonds were Ser289, His449 and Tyr473, the lengths of those bonds being 2.18 Å, 2.37 Å and 2.38 Å

Table 4.31: Dock scores for the diabetes target Insulin receptor with selected ligands

Type of compound	Sl. No.	Ligand	(-) CDOCKER energy (Kcal/mol)	(-) CDOCKER Interaction energy (Kcal/mol)	No. of H bonds	H bond	Distance (Å)	Binding Energy (Kcal/mol)
Allium-specific compounds	1.	S-Allyl L-Cysteine	28.19	30.32	2	GLU1047* ASP1150*	1.94 2.15	-99.49
	2.	S-Allyl D-Cysteine	30.96	32.21	2	ASP1150* GLU1047*	1.81 1.83	-93.22
Non-*Allium* specific compounds	3.	Taurine	21.83	24.22	2	ASP1150* GLU1047*	1.78 2.06	-107.30
	4.	Isobutyl isothiocyanate	23.35	24.06	1	GLU1047*	2.13	-96.19
Commercial drug	5.	Repaglinide	4.31	46.76	1	ASP1150*	1.93	-19.40

* amino acid residues present in active site

Table 4.32: Dock scores for the diabetes target PPAR γ with selected ligands

Type of compound	Sl. No.	Ligand	(-) CDOCKER energy (Kcal/mol)	(-) CDOCKER Interaction energy (Kcal/mol)	No. of H bonds	H bond	Distance (Å)	Binding Energy (Kcal/mol)
Allium-specific compounds	1.	Alliin	30.54	33.93	1	SER289*	2.34	-103.06
	2.	S-Allyl Mercapto Cysteine	26.89	26.99	1	TYR327	2.00	-81.44
Non-*Allium* specific compounds	3.	Apigenin	39.65	40.87	3	SER289* HIS449* TYR473*	2.18 2.37 2.38	-160.95
	4.	Kaempferol	33.75	37.77	1	MET364	2.88	-100.58
Commercial drug	5.	Pioglitazone	33.70	42.63	1	CYS285*	2.49	-58.89

* amino acid residues present in active site

respectively. Apigenin also made the highest no. of hydrogen bonds (3 nos.) among both exclusive *Allium* and other compounds. Pioglitazone, the commercial FDA approved drug also interacted with target protein PPARγ via amino acid residue Cys285 making only one hydrogen bond with a difference of CDOCKER energies lesser than 10 Kcal/mol, but with a binding energy higher than the *Allium* compounds (-58.89 Kcal/mol).

4.1.8.3 Docking results of target proteins involved in blood pressure

4.1.8.3.1 Adrenergic Receptor

Molecular docking scores for the selected *Allium* phytocompounds with Adrenergic Receptor are presented in Table 4.33. Five compounds exclusive to *Allium* showed better dock scores with Adrenergic receptor in comparison with the commercial FDA approved drug propranolol for blood pressure. The lowest binding energy was observed for s-allyl d-cysteine (-89.04 Kcal/mol). The best interaction was also shown by the same compound, for which the difference of CDOCKER and CDOCKER interaction energy was as low as 0.26 Kcal/mol. The interacting active site residues were Ser203 and Ser204, the hydrogen bond distance being 1.88 Å and 2.31 Å respectively.

Among the non-*Allium* specific group, p-coumaric acid showed the least binding energy (-101.31 Kcal/mol), for which the interacting active site residue was Asp113 with the hydrogen bond distance 2.16 Å. However, the difference of CDOCKER and CDOCKER interaction energy was 3.36 Kcal/mol. Highest no. of hydrogen bonds was observed for alliin, which made three hydrogen bonds with Adrenergic Receptor while interacting via amino acid residues Ser203, Ser204 and Asn293, which included the smallest hydrogen bond length of 1.78 Å with Ser203 as its active site residue. The commercial drug propranolol had interactions with same active site residues, making 4 hydrogen bonds with Adrenergic Receptor, but with huge difference of CDOCKER energy and CDOCKER interaction energy exceeding the limit of 10 Kcal/mol.

Table 4.33: Dock scores for the blood pressure target Adrenergic receptor with selected ligands

Type of compound	Sl. No.	Ligand	(-) CDOCKER energy (Kcal/mol)	(-) CDOCKER Interaction energy (Kcal/mol)	No. of H bonds	H bond	Distance (Å)	Binding Energy (Kcal/mol)
Allium-specific compounds	1.	S-Allyl D-Cysteine	30.64	30.90	2	SER203* SER204*	1.88 2.31	-89.04
	2.	Alliin	31.04	34.29	3	SER203* SER204* ASN293*	1.78 2.31 2.47	-88.89
	3.	S-Allyl L-Cysteine	30.60	31.50	2	SER203* SER204*	1.79 2.37	-83.29
	4.	Cycloalliin	24.89	25.15	1	SER203*	2.41	-78.46
	5.	S-Allyl Mercapto Cysteine	25.06	25.73	1	SER204*	2.27	-73.55
Non-*Allium* specific compounds	6.	P-Coumaric Acid	26.59	29.95	1	ASP113*	2.16	-101.31
	7.	Isobutyl isothiocyanate	19.30	20.30	1	ASP113*	2.02	-88.43
	8.	Ferulic acid	29.46	33.28	1	SER204*	2.29	-80.35
Commercial drug	9.	Propranolol	26.93	45.20	4	ASN312 (2)* ASP113 (2)*	1.88 1.93	-67.52

* amino acid residues present in active site

4.1.8.3.2 Angiotensin Converting enzyme (blood pressure target)

Five compounds exclusive to *Allium* showed better dock scores with Angiotensin Converting Enzyme (ACE) in comparison with the commercial FDA approved drug captopril for blood pressure. The lowest binding energy was shown by l-γ-glutamyl-s-allyl-l-cysteine (-256.81 Kcal/mol). The interacting active site residue were Lys511, Tyr520, Gln281 and Glu162 for which the hydrogen bond distances were 1.91 Å, 2.06 Å, 2.26 Å and 2.48 Å respectively. This compound was observed to form highest no. of hydrogen bonds (7 nos.) with ACE during interaction. However the deviation in CDOCKER energy was 2.49 Kcal/mol. The least difference in CDOCKER and CDOCKER interaction energy was observed for s-allyl l-cysteine (0.45 Kcal/mol), which had the smallest interacting hydrogen bond length of 1.86 Å with Lys511 as its active site residue (Table 4.34). The other interacting active site residues were the same as with l-γ-glutamyl-s-allyl-l-cysteine.

Among the non-*Allium* specific group, p-coumaric acid showed the least binding energy (-144.56 Kcal/mol), with a deviation of 3.05 Kcal/mol interacting with active site residues Tyr520 and Gln281. The commercial drug captopril also had interactions with same active site residues with a good binding energy, making 5 hydrogen bonds with ACE, but with huge difference of CDOCKER energy and CDOCKER interaction energy, exceeding the limit of 10 Kcal/mol.

4.1.8.3.3 Carbonic anhydrase (blood pressure target)

During molecular docking with Carbonic Anhydrase (CA), best dock score was shown by s-allyl l-cysteine among the exclusive *Allium* phytocompounds. It recorded showing the least binding energy (-107.71 Kcal/mol) and least difference of CDOCKER and CDOCKER interaction energy (0.51 Kcal/mol). Highest no. of hydrogen bonds (5 nos.) was also recorded for the same compound. The interacting active site residues were His94, His96, His119 and Thr199, the hydrogen bond distances being 1.99 Å, 2.13 Å, 2.36 Å and 2.44 Å respectively.

Table 4.34: Dock scores for the blood pressure target Angiotensin Converting enzyme with selected ligands

Type of compound	Sl. No.	Ligand	(-) CDOCKER energy (Kcal/mol)	(-) CDOCKER Interaction energy (Kcal/mol)	No. of H bonds	H bond	Distance (Å)	Binding Energy (Kcal/mol)
Allium-specific compounds	1.	L-γ-Glutamyl-S-allyl-L-cysteine	62.92	65.41	7	LYS511 (2)* TYR520 (2)* GLU162 (2)* GLN281*	1.91 2.06 2.26 2.48	-256.81
	2.	S-Allyl D-Cysteine	35.98	36.19	2	GLU162* LYS511*	1.86 1.92	-131.63
	3.	Cycloalliin	32.76	33.51	4	LYS511 (2)* GLN281* TYR520*	1.87 2.39 2.49	-121.59
	4.	Alliin	30.26	34.14	3	LYS511* TYR520* GLN281*	1.87 1.96 2.19	-104.79
	5.	S-Allyl L-Cysteine	32.52	32.97	5	LYS511 (2)* TYR520* GLN281 (2)*	1.86 2.40 2.41	-99.87
Non-*Allium* specific compounds	6.	P-Coumaric Acid	29.73	32.78	3	TYR520 (2)* GLN281*	2.15 2.17	-144.56
	7.	Phloroglucinol	38.21	39.25	2	LYS511* GLN281*	1.59 1.95	-127.55
	8.	Ferulic acid	31.54	36.08	3	GLN281* TYR520* LYS511*	2.02 2.08 2.09	-104.92
Commercial drug	9.	Captopril	28.43	41.13	5	LYS511* GLN281 (2)* TYR520* GLU384*	1.84 2.28 2.36 2.49	-121.88

* amino acid residues present in active site

However, the smallest interacting hydrogen bond length of 1.74 Å was shown by its dextro-rotatory isomer with Thr199 as its active site residue.

Among the non-*Allium* specific group, p-coumaric acid showed the least binding energy as -87.47 Kcal/mol, however the difference of CDOCKER and CDOCKER interaction energy for this compound was 2.86 Kcal/mol. The interacting active site residue was His64, which was found as a non-critical active site residue having a hydrogen bond distance of 2.06 Å. The difference of CDOCKER and CDOCKER interaction energy was observed the least for isobutyl isothiocyanate (0.9 Kcal/mol). The interacting active site residues were the same as s-allyl l-cysteine. The commercial drug brinzolamide showed no interactions with carbonic anhydrase. Detailed results of dock scores of *Allium* phytocompounds and carbonic anhydrase are presented in Table 4.35.

4.1.8.4 Docking results of target proteins involved in cholesterol

4.1.8.4.1 HMG CoA reductase

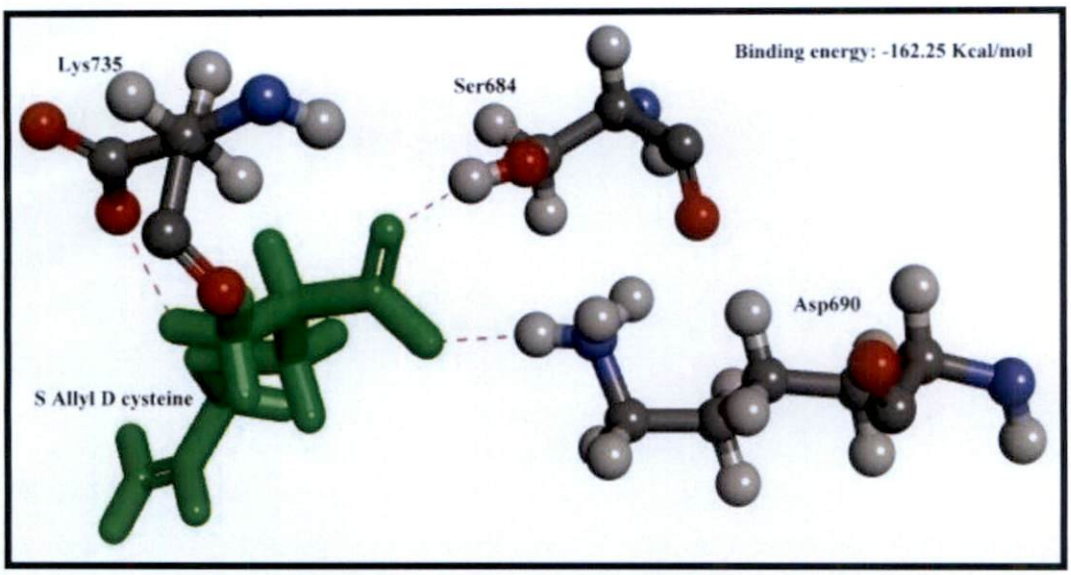
Four compounds exclusive to *Allium* showed better dock scores with HMG CoA reductase in comparison with the commercial FDA approved drug lovastatin for blood pressure. The lowest binding energy was observed for cycloalliin (-180.93 Kcal/mol). The interacting active site residues were Lys735, Ser684, Lys692, Asp690 and Arg590. The difference of CDOCKER and CDOCKER interaction energy was found quite less (0.75 Kcal/mol). Highest no. of hydrogen bonds (5 nos.) was recorded for the same compound with HMG CoA reductase while interacting. However, s-allyl d-cysteine, showed the least difference of CDOCKER and CDOCKER interaction energy (0.32 Kcal/mol) with a low binding energy of -154.39 kcal/mol (Plate 6).

Among non-*Allium* specific group, apigenin showed least binding energy (-294.40 Kcal/mol), with a low deviation in CDOCKER energy (1.34 Kcal/mol), which was recorded as the best interaction in that group. The interacting active site residues were the same as exclusive *Allium* compounds. Detailed results of

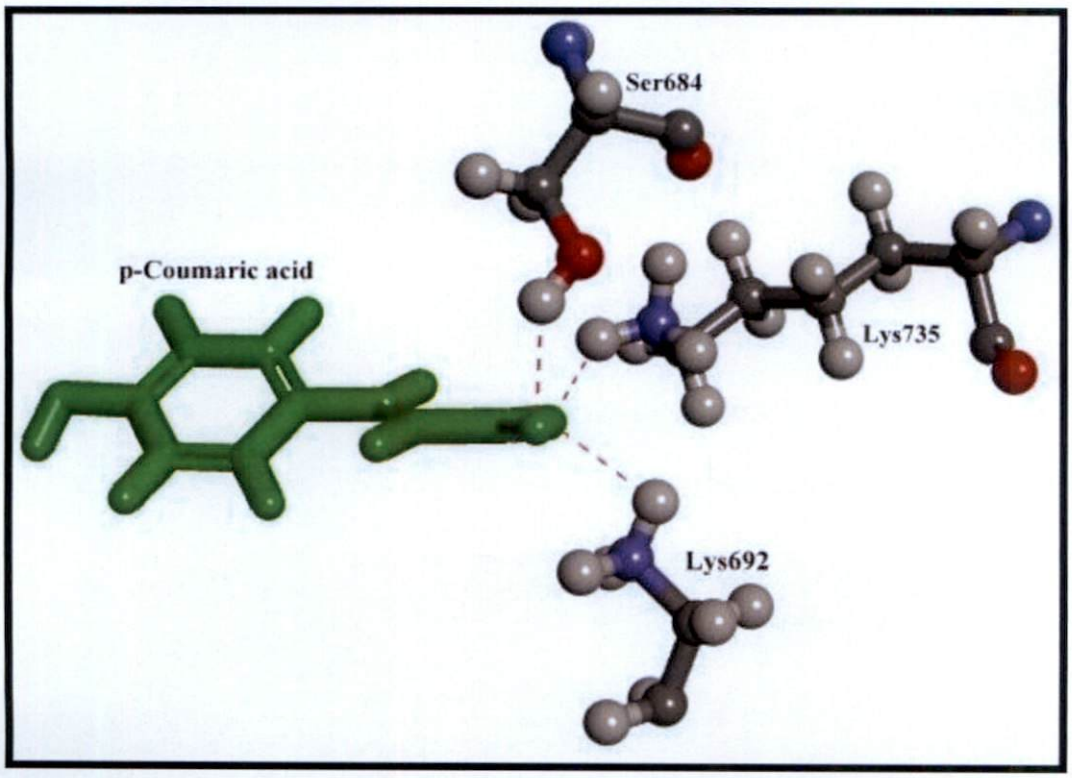
Table 4.35: Dock scores for blood pressure target carbonic anhydrase with selected ligands

Type of compound	Sl. No.	Ligand	(-) CDOCKER energy (Kcal/mol)	(-) CDOCKER Interaction energy (Kcal/mol)	No. of H bonds	H bond	Distance (Å)	Binding Energy (Kcal/mol)
Allium-specific compounds	1.	S-Allyl L-Cysteine	35.00	35.52	5	HIS94* HIS96* HIS119 (2)* THR199*	1.99 2.13 2.36 2.44	-107.71
	2.	Alliin	35.06	39.41	4	THR199 (3)* HIS96*	2.04 2.47	-102.18
	3.	S-Allyl D-Cysteine	35.41	36.15	4	THR199 (2)* HIS119* HIS96*	1.74 2.03 2.19	-94.13
	4.	S-Allyl Mercapto Cysteine	23.33	25.69	3	HIS64 (2) THR200*	2.09 2.40	-76.02
Non-*Allium* specific compounds	5.	P-Coumaric Acid	27.33	30.19	2	HIS64 (2)	2.06	-87.47
	6.	Taurine	27.58	30.74	4	HIS94* HIS119* HIS96* THR199*	1.96 2.04 2.26 2.27	-72.64
	7.	Isobutyl isothiocyanate	26.15	27.05	2	HIS119* HIS96*	2.38 2.41	-56.89
Commercial drug	8.	Brinzolamide			No hydrogen bonding			

* amino acid residues present in active site



A. HMG CoA reductase vs S-allyl D cysteine



B. HMG CoA reductase vs p-Coumaric acid

Plate 6: Hydrogen bond interaction of cholesterol target HMG CoA reductase with S-allyl D cysteine (A) and p-Coumaric acid (B)

dock scores of *Allium* phytocompounds and HMG CoA reductase are presented in Table 4.36.

4.1.8.5 Docking results of target proteins involved in arthritis and inflammation disease

4.1.8.5.1 Cyclooxygenase 2

Five compounds exclusive to *Allium* showed better dock scores with Cyclooxygenase 2 in comparison with the commercial FDA approved drug diclofenac for arthritis and inflammation. The lowest binding energy was observed for s-allyl mercapto cysteine (-117.28 Kcal/mol). The interacting active site residue was Arg120. The difference of CDOCKER and CDOCKER interaction energy was found quite high (3.32 Kcal/mol). Highest no. of hydrogen bonds (4 nos.) was recorded for the compound s-allyl d-cysteine with Cyclooxygenase 2 while interacting and it recorded a binding energy of -107.54 Kcal/mol. However, l-γ-glutamyl-s-allyl-l-cysteine showed the least difference of CDOCKER and CDOCKER interaction energy (0.04 Kcal/mol) with a low binding energy of -111.66 kcal/mol.

Among non-*Allium* specific group, kaempferol showed least binding energy (-226.95 Kcal/mol), with a high deviation in CDOCKER energy (3.77 Kcal/mol) and connected with the active site residue Arg120. However, the compound phloroglucinol exhibited the lowest difference (0.51 Kcal/mol) among the CDOCKER energies and a low binding energy (-126.19 Kcal/mol), while connecting with active site residues Arg120 and Tyr355. Detailed results of dock scores of *Allium* phytocompounds and Cyclooxygenase 2 are presented in Table 4.37.

Table 4.36: Dock scores for the cholesterol target HMG CoA reductase with selected ligands

Type of compound	Sl. No.	Ligand	(-) CDOCKER energy (Kcal/mol)	(-) CDOCKER Interaction energy (Kcal/mol)	No. of H bonds	H bond	Distance (Å)	Binding Energy (Kcal/mol)
Allium-specific compounds	1.	Cycloalliin	45.86	46.61	5	LYS735* SER684* LYS692* ASP690* ARG590*	1.86 2.07 2.44 2.47 2.48	-180.93
	2.	S-Allyl D-Cysteine	44.29	44.85	3	LYS735* SER684* ASP690*	1.92 2.09 2.12	-162.25
	3.	S-Allyl L-Cysteine	44.18	44.49	3	ASP690* LYS735* SER684*	1.95 1.95 2.02	-154.39
Non-*Allium* specific compounds	5.	Apigenin	66.01	67.35	2	LYS735* SER684*	1.73 1.99	-294.40
	6.	Phloroglucinol	49.93	51.36	3	LYS735* ASP690* SER684*	1.66 1.92 1.99	-260.67
	7.	P-Coumaric Acid	42.93	46.33	3	LYS735* LYS692* SER684*	1.86 2.39 2.43	-229.41
Commercial drug	8.	Lovastatin	4.04	39.94	3	ALA751 LYS691* ASN755*	1.83 1.85 2.17	-67.97

* amino acid residues present in active site

Table 4.37: Dock scores for the arthritis and inflammation target Cyclooxygenase with selected ligands

Type of compound	Sl. No.	Ligand	(-) CDOCKER energy (Kcal/mol)	(-) CDOCKER Interaction energy (Kcal/mol)	No. of H bonds	H bond	Distance (Å)	Binding Energy (Kcal/mol)
Allium-specific compounds	1.	S-Allyl Mercapto Cysteine	28.63	31.95	1	ARG120*	1.97	-117.28
	2.	Alliin	31.20	35.00	3	ARG120 (2)* TYR355*	1.92 2.34	-112.86
	3.	L-γ-Glutamyl-S-allyl-L-cysteine	50.25	50.29	1	ARG120*	2.00	-111.66
	4.	S-Allyl D-Cysteine	31.75	31.86	4	TYR355 (2)* ARG120 (2)*	1.89 1.95	-107.54
	5.	S-Allyl L-Cysteine	27.83	28.94	1	ARG120*	2.14	-102.84
Non-*Allium* specific compounds	6.	Kaempferol	46.22	49.99	1	ARG120*	2.47	-226.95
	7.	P-Coumaric Acid	31.57	34.35	1	ARG120*	2.18	-150.54
	8.	Phloroglucinol	36.08	36.59	2	ARG120* TYR355*	1.79 2.31	-126.19
Commercial drug	9.	Diclofenac			No hydrogen bonding			

* amino acid residues present in active site

4.1.8.5.2 Glucocorticoid Receptor (arthritis and inflammation target)

While molecular docking with Glucocorticoid Receptor (GR), it was observed that only 2 compounds exclusive to *Allium* peformed molecular interactions in the identified active site. They were s-allyl d-cysteine and cycloalliin. The lowest binding energy was observed for s-allyl d-cysteine with a deviation of 1.38 Kcal/mol between the CDOCKER energies. The compound made a single hydrogen bond with active site residue Asn564, the hydrogen bond length being 1.85 Å. Another compound cycloalliin also made 1 hydrogen bond with the same active site residue, displaying a binding energy of -71.24 Kcal/mol with a deviation of 0.68 Kcal/mol between the CDOCKER energies.

From the non-*Allium* specific group, no compound was found to interact with the arthritis and inflammation protein target GR. The FDA approved commercial drug dexamethasone interacted with GR with a good binding energy, but was considered unstable because of a huge deviation of CDOCKER energies which surpassed the limit of 10 Kcal/mol. Detailed results of dock scores of *Allium* phytocompounds and GR are presented in Table 4.38.

4.1.8.5.3 Mineralocorticoid Receptor (arthritis and inflammation target)

Average dock scores were found for *Allium* compounds when docked with Mineralocorticoid Receptor (MR). It was observed that only 4 compounds from garlic showed mediocre dock scores against the mentioned target for arthritis and inflammation in between -57.704.27 Kcal/mol to -97.13 Kcal/mol. Among exclusive *Allium* compounds, only 2 compounds such as l-γ-glutamyl-s-allyl-l-cysteine and alliin displayed somewhat good docking results. L-γ-glutamyl-s-allyl-l-cysteine displayed the lowest binding energy of -91.56 Kcal/mol, making 4 hydrogen bonds with amino acid residues Arg817 (2), Gln776 and Leu810. The maximum no. of hydrogen bonds (4 nos.) was formed by the same compound l-γ-glutamyl-s-allyl-l-cysteine.

Table 4.38: Dock scores for the arthritis and inflammation target Glucocorticoid receptor with selected ligands

Type of compound	Sl. No.	Ligand	(-) CDOCKER energy (Kcal/mol)	(-) CDOCKER Interaction energy (Kcal/mol)	No. of H bonds	H bond	Distance (Å)	Binding Energy (Kcal/mol)
Allium-specific compounds	1.	S-Allyl D-Cysteine	24.89	26.27	1	ASN564*	1.85	-73.06
	2.	Cycloalliin	27.55	28.23	1	ASN564*	1.89	-71.24
Non-*Allium* specific compounds	3.	No						
Commercial drug	4.	Dexamethasone	1.08	66.45	4	GLN642 (2)* ASN564* ARG611	2.01 2.22 2.44	-70.72

* amino acid residues present in active site

Among non-*Allium* specific compounds, p-coumaric acid recorded least binding energy (-97.13 Kcal/mol) and made hydrogen bonding with the same amino acid residues as of l-γ-glutamyl-s-allyl-l-cysteine. The deviation of CDOCKER energies for that compound was 2.84 Kcal/mol. The commercial FDA approved drug spironolactone showed a positive CDOCKER energy while interacting, hence the binding energy was not calculated. Detailed results of dock scores of *Allium* phytocompounds and MR are presented in Table 4.39.

4.1.8.5.4 Nitric Oxide Synthase (arthritis and inflammation target)

Molecular docking studies of *Allium* compounds with target protein Nitric Oxide Synthase (NOS) revealed that almost all the compounds with least binding energies docked with a critical active site residue Cys200. The least binding energy was shown by cycloalliin (-131.03 Kcal/mol) at a difference of 0.72 Kcal/mol for the CDOCKER energies. The compound made 2 hydrogen bonds with the same residue Cys200 for a length of 2.13 Å. However, the compound s-allyl l-cysteine showed a lesser difference of CDOCKER energies (0.56 Kcal/mol) while interacting with the protein target NOS (Plate 7).

From the group of non-*Allium* specific compounds, quercetin exhibited the least binding energy (-153.62 Kcal/ mol) at a difference of 3.27 Kcal/mol between the CDOCKER energies by connecting with active site residue Tyr491. Another compound isobutyl isothiocyanate displayed a lesser deviation of CDOCKER energies (0.3 Kcal/mol) and interacted with NOS via active site residue Cys200 making a hydrogen bond of length 2.04 Å. Detailed results of dock scores of *Allium* phytocompounds with NOS are presented in Table 4.40.

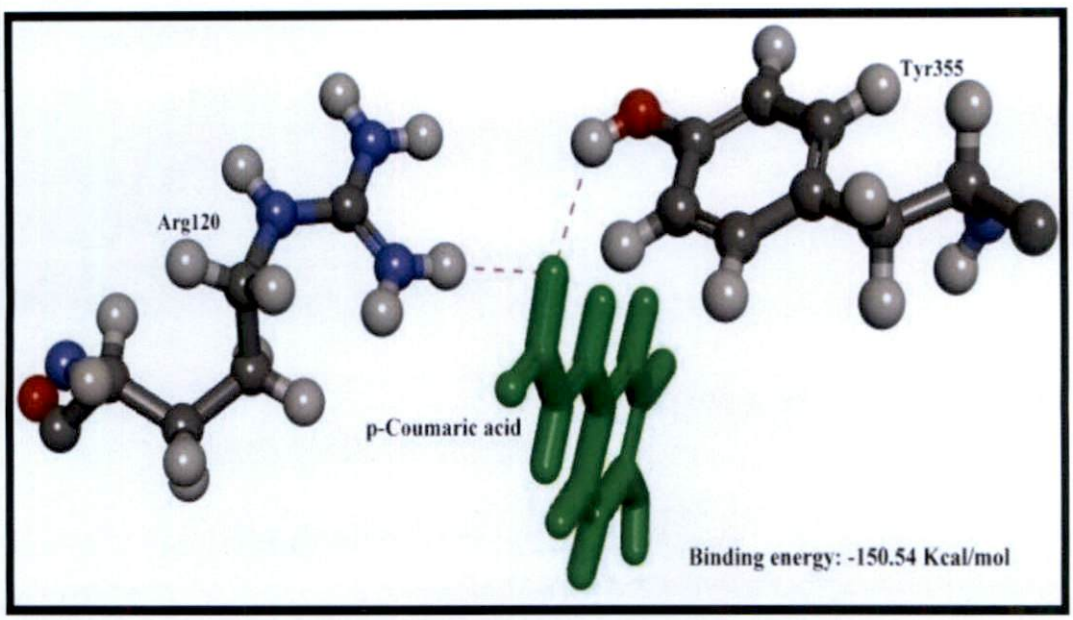
4.1.8.5.5 p38 kinase /Mitogen activated protein kinase 14 (arthritis and inflammation target)

Detailed results of dock scores of *Allium* phytocompounds with Mitogen activated protein kinase 14 (MAPK14) are presented in Table 4.41. Among the exclusive *Allium* phytocompounds, s-allyl d-cysteine showed the least binding

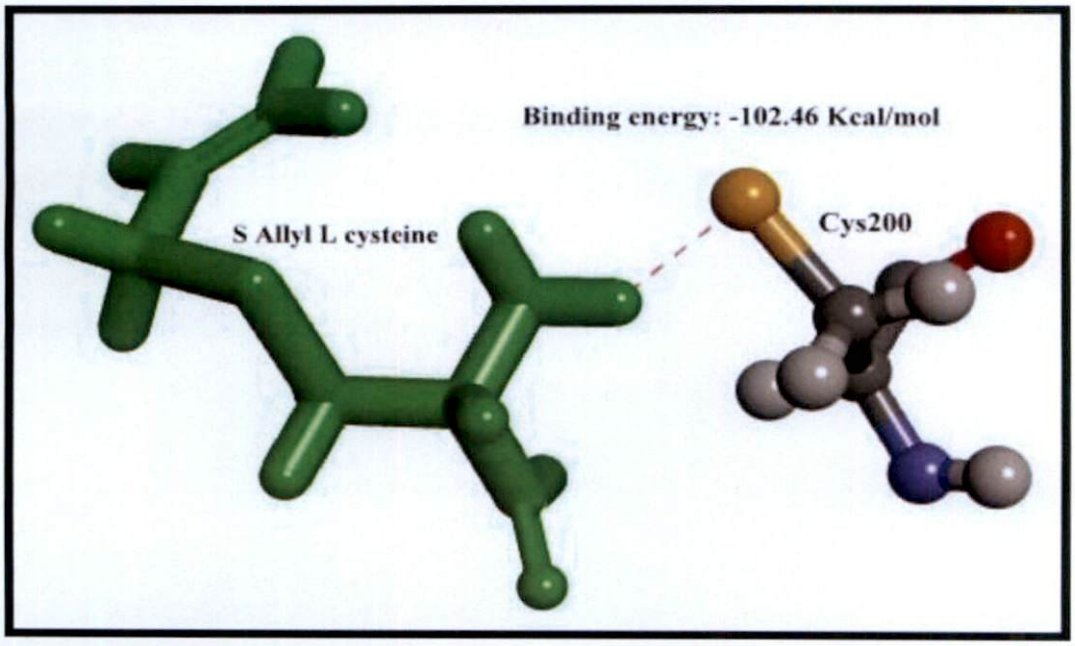
Table 4.39: Dock scores for the arthritis and inflammation target Mineralocorticoid receptor with selected ligands

Type of compound	Sl. No.	Ligand	(-) CDOCKER energy (Kcal/mol)	(-) CDOCKER Interaction energy (Kcal/mol)	No. of H bonds	H bond	Distance (Å)	Binding Energy (Kcal/mol)
Allium-specific compounds	1.	L-γ-Glutamyl-S-allyl-L-cysteine	49.19	51.53	4	ARG817 (2)* GLN776* LEU810*	1.83 2.17 2.43	-91.56
	2.	Alliin	28.57	32.36	3	ARG817* GLN776* LEU810*	1.92 2.33 2.36	-57.70
Non-*Allium* specific compounds	3.	P-Coumaric Acid	28.05	30.89	3	ARG817 (2)* GLN776*	1.84 2.08	-97.13
	4.	Ferulic acid	30.61	34.31	2	ARG817* GLN776*	1.83 2.19	-96.95
Commercial drug	5.	Spironolactone	Positive CDOCKER energy					

* amino acid residues present in active site



A. COX2 vs p-Coumaric acid



B. NOS vs S-allyl L cysteine

Plate 7: Hydrogen bond interaction of arthritis targets COX2 and NOS with p-coumaric acid (A) and S-allyl L cysteine (B)

Table 4.40: Dock scores for the arthritis and inflammation target Nitric Oxide Synthase with selected ligands

Type of compound	Sl. No.	Ligand	(-) CDOCKER energy (Kcal/mol)	(-) CDOCKER Interaction energy (Kcal/mol)	No. of H bonds	H bond	Distance (Å)	Binding Energy (Kcal/mol)
Allium-specific compounds	1.	Cycloalliin	28.37	29.09	2	CYS200 (2)*	2.13	-131.03
	2.	S-Allyl L-Cysteine	25.79	26.31	1	CYS200*	1.89	-102.46
	3.	S-Allyl D-Cysteine	26.20	26.76	1	CYS200*	1.90	-102.25
	4.	Alliin	27.73	31.76	1	CYS200*	1.85	-97.13
Non-*Allium* specific compounds	5.	Quercetin	38.63	41.90	1	TYR491*	1.76	-153.62
	6.	P-Coumaric Acid	22.04	25.19	1	CYS200*	2.09	-93.44
	7.	Isobutyl isothiocyanate	22.59	22.89	1	CYS200*	2.04	-91.95
	8.	Taurine	20.95	24.22	1	CYS200*	1.83	-85.63
	9.	Apigenin	37.99	39.33	2	TYR491* TRP372*	1.82 2.08	-79.83

* amino acid residues present in active site

Table 4.41: Dock scores for the arthritis and inflammation target p38 kinase /Mitogen activated protein kinase 14 with selected ligands

Type of compound	Sl. No.	Ligand	(-) CDOCKER energy (Kcal/mol)	(-) CDOCKER Interaction energy (Kcal/mol)	No. of H bonds	H bond	Distance (Å)	Binding Energy (Kcal/mol)
Allium-specific compounds	1.	S-Allyl D-Cysteine	29.22	31.51	1	ASP168*	2.28	-109.57
	2.	S-Allyl L-Cysteine	33.00	33.95	1	ALA172	1.88	-73.23
	3.	Alliin	28.13	31.74	1	MET78	2.46	-69.07
	4.	2 Methyl Benzaldehyde	20.27	23.63	1	ASP168*	2.35	-55.55
Non-*Allium* specific compounds	5.	Phloroglucinol	34.88	35.91	3	THR106* ALA172 GLU71*	1.92 1.97 2.39	-122.83
	6.	Taurine	23.35	26.39	1	ALA172	1.74	-75.74
Commercial drug	7.	Imatinib	Positive CDOCKER energy					

* amino acid residues present in active site

energy (-109.57 Kcal/mol), with a deviation of 2.29 Kcal/mol among the CDOCKER energies. The active site residue interacting with s-allyl d-cysteine was Asp168. Compounds like s-allyl l-cysteine and alliin displayed a mediocre binding energy through connecting with non-critical amino acids Ala172 and Met78 respectively.

From the group of non-*Allium* specific compounds, only phloroglucinol interacted with MAPK14 with a decent binding energy of -122.83 Kcal/mol, displaying a deviation of 1.03 Kcal/mol among the CDOCKER energies. The compound made 3 hydrogen bonds with residues Thr106, Glu71 and Ala172. The commercial drug imatinib showed a positive CDOCKER energy while interacting and so the binding energy was not calculated.

4.1.8.5.6 Tumor Necrosis Factor Alpha (arthritis and inflammation target)

Four compounds exclusive to *Allium* showed mediocre dock scores with Tumor Necrosis Factor Alpha (TNFα). The lowest binding energy was shown by s-allyl mercapto cysteine (-71.07 Kcal/mol). The interacting active site residue was Leu120 for which the hydrogen bond length was 2.09 Å. The difference of CDOCKER energy and CDOCKER interaction energy was 1.46 Kcal/mol. The least difference in CDOCKER and CDOCKER interaction energies was observed for s-allyl d-cysteine (0.86 Kcal/mol), which interacted with TNFα via active site residues Tyr151 and Gly121 making 2 hydrogen bond of length 1.90 Å and 2.03 Å respectively.

From the group of non-*Allium* specific compounds, quercetin showed good dock score with a binding energy as low as -254. 25 Kcal/mol interacting with active site residue Tyr151, with a deviation of over 3.00 Kcal/mol. Detailed results of dock scores of *Allium* phytocompounds and TNFα are presented in Table 4.42.

Table 4.42: Dock scores for the arthritis and inflammation target Tumor Necrosis Factor Alpha with selected ligands

Type of compound	Sl. No.	Ligand	(-) CDOCKER energy (Kcal/mol)	(-) CDOCKER Interaction energy (Kcal/mol)	No. of H bonds	H bond	Distance (Å)	Binding Energy (Kcal/mol)
Allium-specific compounds	1.	S-Allyl Mercapto Cysteine (SAMC)	20.71	22.17	1	LEU120*	2.09	-71.07
	2.	L-γ-Glutamyl-S-allyl-L-cysteine	37.64	40.90	1	GLY121*	1.85	-62.94
	3.	S-Allyl L-Cysteine	23.55	24.58	1	GLY121*	2.16	-58.16
	4.	S-Allyl D-Cysteine	25.35	26.21	2	TYR151* GLY121*	1.90 2.03	-52.73
Non-*Allium* specific compounds	5.	Quercetin	29.65	33.01	1	TYR151*	2.35	-254. 25
	6.	Myricetin	33.73	36.50	2	TYR151* GLY121*	1.91 2.46	-132.73
	7.	P-Coumaric Acid	20.65	24.72	1	TYR151*	1.92	-69.67

* amino acid residues present in active site

4.1.8.5.7 Vascular endothelial growth factor receptor 1 (VEGFR1) (arthritis and inflammation target)

Average dock scores were found for *Allium* compounds when docked with VEGFR1. It was observed that only six compounds from garlic showed mediocre dock scores against the mentioned target for arthritis and inflammation in between -62.03 Kcal/mol to -102.76 Kcal/mol. Among exclusive *Allium* compounds, only 3 compounds such as alliin, l-γ-glutamyl-s-allyl-l-cysteine and s-allyl l-cysteine displayed somewhat good docking results. Alliin displayed the lowest binding energy of -102.76 Kcal/mol, making a single hydrogen bond of length 1.84 Å with amino acid residue Glu878. The deviation for CDOCKER energies for this compound was 3.35 Kcal/mol. However, the deviation was quite low for another compound s-allyl l-cysteine (0.67 Kcal/mol), which interacted with amino acid residue Cys912, the hydrogen bond distances being 2.07 Å.

Among the group of non-*Allium* specific compounds, p-coumaric acid recorded least binding energy (-95.95 Kcal/mol) while binding with the amino acid residue Glu878. However, the least deviation among this group was shown by phloroglucinol (0.34 Kcal/mol). The commercial FDA approved drug regorafenib interacted with VEGFR1 with a poor binding energy by connecting with 3 active site residues Glu878, Cys912 and Asp1040 for 2 times each. However, the interaction was considered unstable because of a huge difference of CDOCKER energies which surpassed the limit of 10 Kcal/mol. Detailed results of dock scores of *Allium* phytocompounds and VEGFR1 are presented in Table 4.43.

Table 4.43: Dock scores for the arthritis and inflammation target Vascular endothelial growth factor receptor 1 with selected ligands

Type of compound	Sl. No.	Ligand	(-) CDOCKER energy (Kcal/mol)	(-) CDOCKER Interaction energy (Kcal/mol)	No. of H bonds	H bond	Distance (Å)	Binding Energy (Kcal/mol)
Allium-specific compounds	1.	Alliin	29.83	33.18	1	GLU878*	1.84	-102.76
	2.	L-γ-Glutamyl-S-allyl-L-cysteine	47.80	50.48	1	ASP1040*	2.43	-64.60
	3.	S-Allyl L-Cysteine	30.53	31.20	1	CYS912*	2.07	-62.03
Non-*Allium* specific compounds	4.	P-Coumaric Acid	28.33	33.10	1	GLU878*	2.07	-95.95
	5.	Apigenin	37.18	39.78	1	ASP1040*	2.33	-82.99
	6.	Phloroglucinol	28.47	28.81	2	ASP1040* GLU878*	1.89 2.41	-73.96
Commercial drug	7.	Regorafenib	36.50	63.16	6	GLU878 (2)* CYS912 (2)* ASP1040 (2)*	1.96 2.06 2.08	-57.99

* amino acid residues present in active site

5.1 Wet lab analysis

5.1.1 *In vitro* cytotoxicity assay

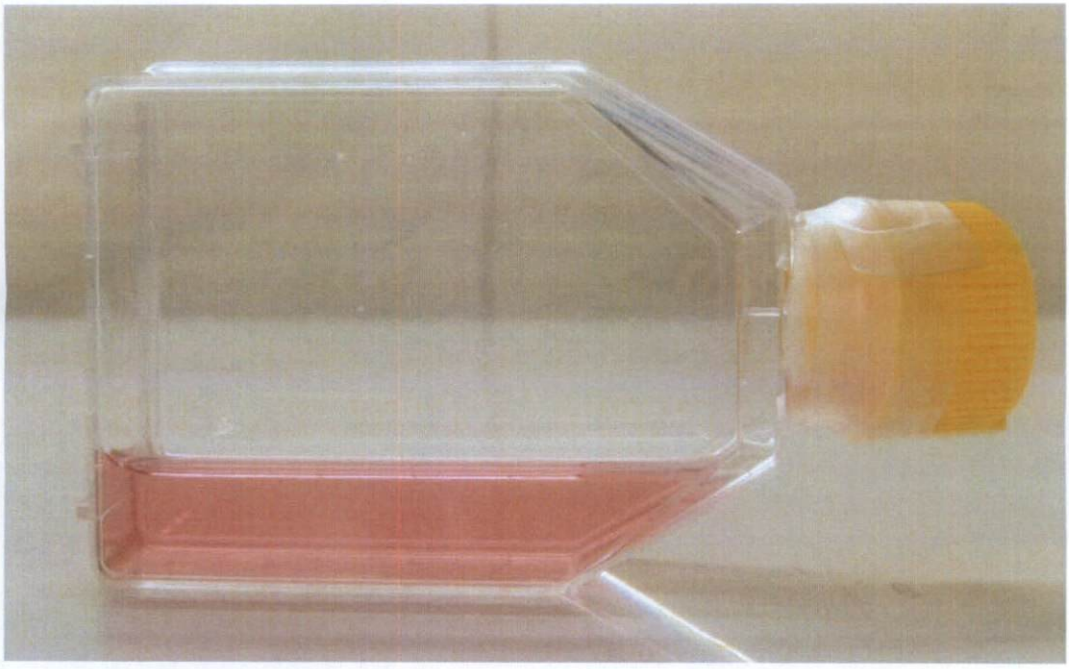
On the basis of good dock scores as evaluted in *in silico* studies, four phytocompounds from garlic were selected for further evaluation of their anticancerous properties using three different mammalian cell lines. The compounds selected were alliin/s-allyl cysteine sulfoxide (SACS), s-allyl cysteine, p-coumaric acid and ferulic acid. Three cell lines were selected for the cytotoxicity assay (1 human cell line-HCT 15 and 2 murine cell lines L929 and Raw 264.7). MTT assay was performed as a long term (48 hours incubation) cytotoxicity assay to determine the toxic effects of all the 4 compounds on the above mentioned cancer cell lines.

5.1.1.1 Effect of the phytocompounds on different cell lines

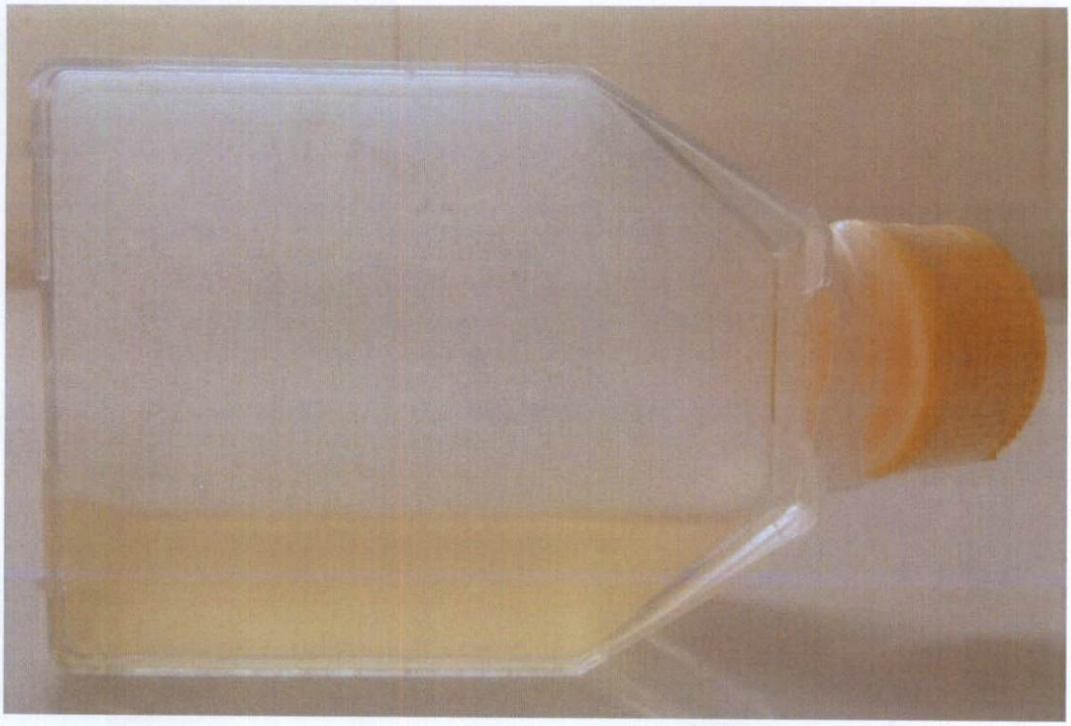
The viability of cancer cells decreased significantly by all four phyto-compounds in a dose dependent manner. The compounds were found to be toxic to cancer cell lines HCT 15, L929 and Raw 264.7. IC_{50} values obtained after 48 hours of treatment are provided in Table 4.44 (Plate 9). It was observed that with the increase in concentration of phytocompounds, the percentage of cell death was increasing. Cytotoxicity of all four compounds on different cell lines are shown in Fig 17, 18, 19 and 20. The IC_{50} value of ferulic acid on L929 and Raw 264.7 was found non significant.

5.1.2 cDNA synthesis and RT-qPCR analysis

For real time PCR (RT-qPCR) analysis, HCT 15 cell line was treated with the phytocompounds from *Allium* for 24 hours. They were applied at two concentrations above and below the IC_{50} value (for alliin and s-allyl cysteine 80 and 120 µg/ml, for p-coumaric acid 225 and 250 µg/ml and for ferulic acid 125 and 175 µg/ml). cDNA was extracted from untreated and treated cells. The expression of target gene EGFR (Epidermal Growth Factor Receptor) was quantified using the ΔΔCT method. A positive ΔΔCT value indicates down-regul-



A. Suspension of cell lines in fresh RPMI media



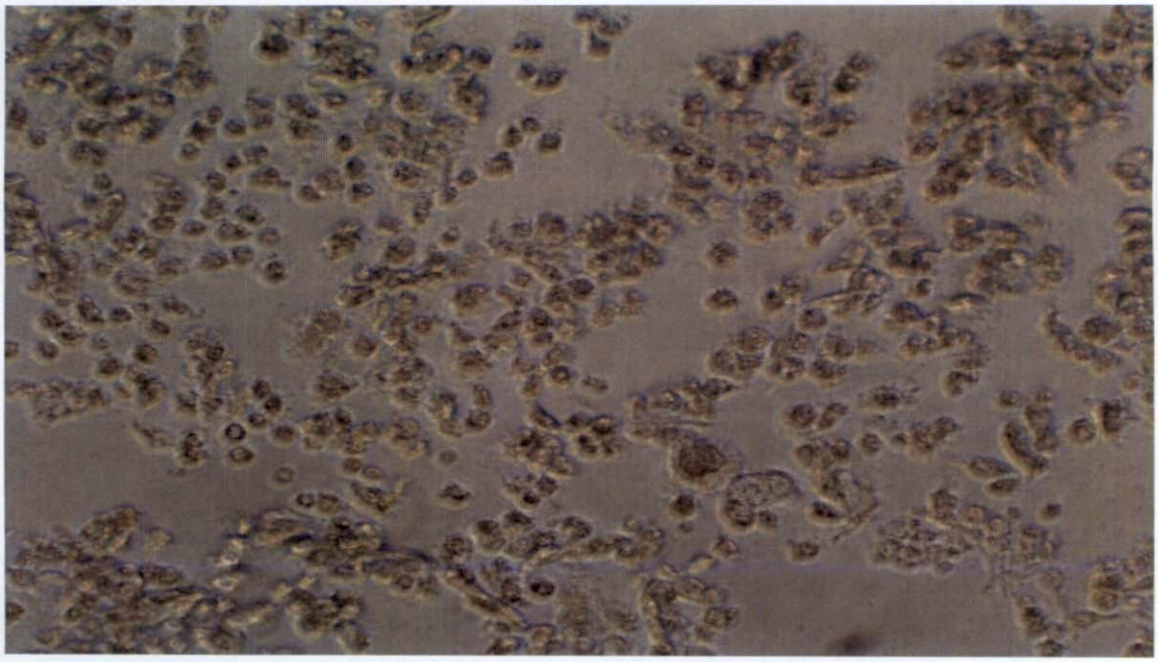
B. Suspension of growing cell lines in used RPMI media after 48 hours

Plate 8: Maintenance of cell lines in Tissue culture flasks 25 cm^2

Table 4.44: IC_{50} values of the four phytocopounds on different cell lines

Phytocompounds	Cancer cell lines		
	HCT 15	**L 929**	**Raw 264.7**
Alliin	99 µg/ml	79 µg/ml	71 µg/ml
SAC	100 µg/ml	78 µg/ml	90 µg/ml
P Coumaric acid	222 µg/ml	185 µg/ml	145 µg/ml
Ferulic acid	154 µg/ml	>250 µg/ml	>250 µg/ml

A. HCT 15 cells after 48 hours incubation (before treatment)



B. HCT 15 cells after 48 hours incubation (after treatment)

Plate 9: Effect of Alliin on HCT 15 cancer cell line

-ation (decreased expression) and a negative ΔΔCT value indicates upregulation (increased expression). Compared to the untreated control, the extent of target gene expression in treated cells is shown as fold difference increase or decrease. After RT-qPCR analysis the following results (Table 4.45) were obtained:

i) The ΔΔCT values obtained for EGFR with a high and low dose of Alliin were -0.34 and 3.45 respectively. From these values it was understood that for higher dose of alliin (120 μg/ml), the expression of target gene EGFR was increased, whereas for the lower dose (80 μg/ml) the expression was decreased.

ii) The ΔΔCT values of EGFR gene when treated with s-allyl cysteine revealed that, for higher dose (120 μg/ml) there was 1.28 fold decrease in expression of the target gene, whereas for a lower dose (80 μg/ml) it is 3.85 fold decrease.

iii) The ΔΔCT values for both higher (250 μg/ml) and lower doses (225 μg/ml) of p-coumaric acid was quite poor, which revealed only a slight decrease of 0.46 fold and 0.28 fold respectively for the gene expression.

iv) The ΔΔCT values obtained for EGFR with a high and low dose of ferulic acid were 4.28 and 3.89 respectively, which indicated that there was quite good amount of decrease (down regulation) of the target gene for both the concentrations. Best result was shown by ferulic acid, as it displayed a 4.28 fold decrease of the target gene EGFR.

Amplification plot obtained from RT-qPCR analysis for the EGFR gene against actin as a reference gene is shown in plate 10. Fold changes of EGFR gene expression by treatment of 4 different test compounds on HCT 15 cell line is shown in Fig 1.

Table 4.45: ΔΔCt values as observed on Real Time PCR analysis of EGFR gene for the four selected phytocompounds on a high dose and low dose

Sl. No.	Test compound	Gene				{ΔCt (Target)- ΔCt (reference)}		ΔCt (Control)- ΔCt (Test)
		Control		Test		ΔCt (Control)	ΔCt (Test)	ΔΔCt
		Target (EGFR)	Reference (b actin)	Target (EGFR)	Reference (b actin)			
1.	FA HD	24.559	26.943	28.836	26.943	-2.384	1.893	4.277
2.	FA LD	24.9	26.943	28.79	26.943	-2.043	1.847	3.89
3.	pCA HD	27.72	26.943	28.18	26.943	0.777	1.237	0.46
4.	pCA LD	27.736	26.943	28.02	26.943	0.793	1.077	0.284
5.	sACS HD	28.635	26.943	28.3	26.943	1.692	1.357	-0.335
6.	sACS LD	24.576	26.943	28.02	26.943	-2.367	1.077	3.444
7.	sAC HD	28.428	26.943	29.71	26.943	1.485	2.767	1.282
8.	sAC LD	28.219	26.943	32.07	26.943	1.276	5.127	3.851

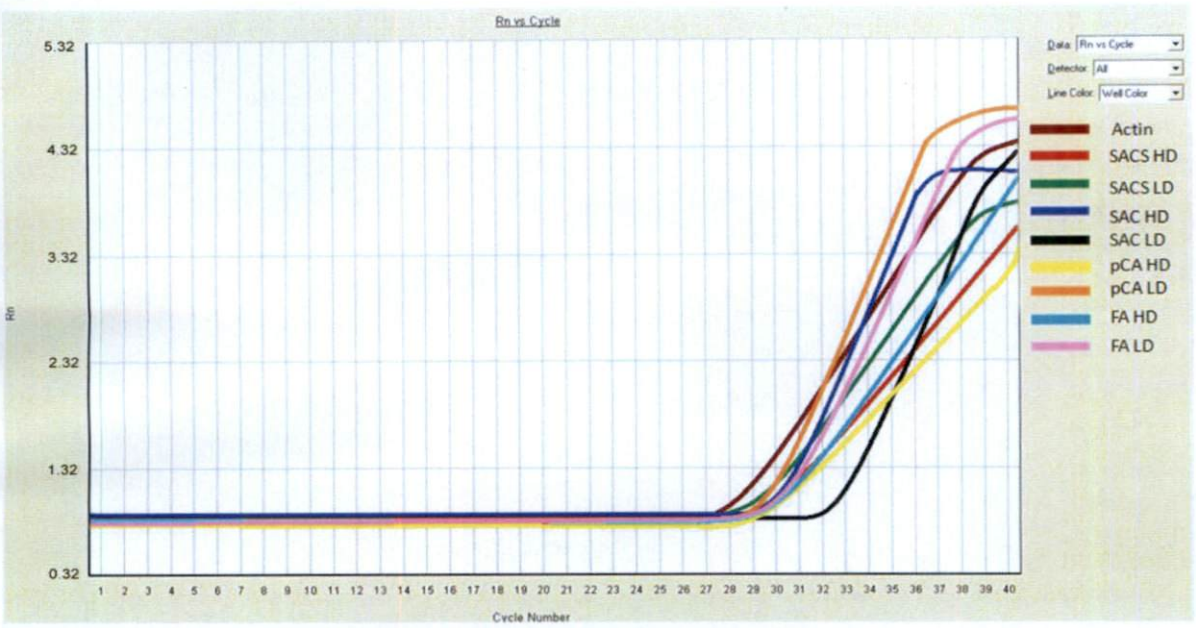


Plate 10: Amplification plot obtained from RT-qPCR analysis for the EGFR gene against actin as reference gene

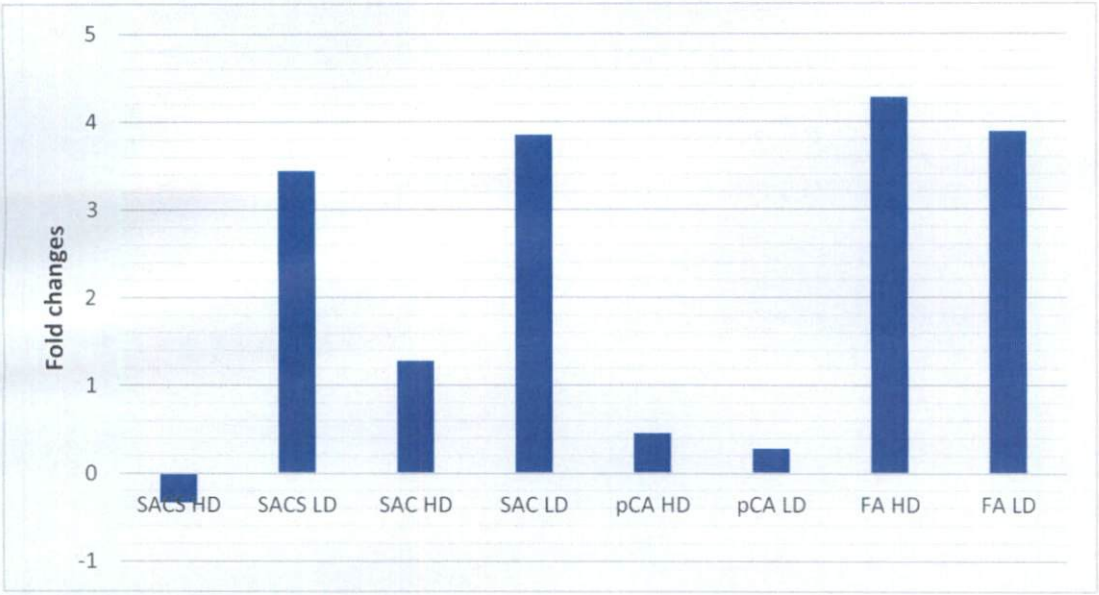


Fig 1: Changes in EGFR gene expression by treatment with four different garlic phytocompounds on HCT 15 cell line

Discussion

5. Discussion

Garlic is one of the earliest documented example of plants employed for treatment of diseases and maintenance of health. Almost 25 centuries ago, Hippocrates, the Father of Medicine, stated "let food be thy medicine and let medicine be thy food". Supporting this statement, he himself prescribed garlic for a variety of health related pathological conditions. During the last century, lots of scientific research and clinical trials have been done to determine the effects of garlic consumption on human health. Its potential in combating lifestyle related disorders like cancer, hypercholesterolemia, dyslipidemia, and high blood pressure has been the focus of major research interventions. Though the medicinal properties of garlic are well known and are being exploited for quite a long time, the exact phytocompounds of garlic behind the action of curing diseases by interacting with protein targets is not much studied or reported. Wet lab analysis and clinical trials to identify such phytocompounds and their activity will be highly time consuming and laborious.

Use of bioinformatics (*in silico*) tools such as molecular docking will help to screen out potential phytocompounds that could be later developed as useful drug to cure several diseases. The present study entitled "Detection of novel metabolites in garlic (*Allium sativum* L.) through *in silico* analysis and its validation" was taken up to analyze the interaction of 48 phytocompounds in garlic against 32 protein targets involved in various lifestyle diseases such as cancer, diabetes, arthritis, blood pressure and cholesterol. The same was done for FDA approved drugs also for a comparative analysis. The 48 selected phytocompounds from garlic included 36 compounds exclusive to *Allium* species and the other 12 were present in other plant species.

5.1 *In silico* analysis

5.1.1 Preparation of protein targets and ligands

The three dimensional structure of the 48 phytocompounds were retrieved from reliable and most commonly used databases such as Pubchem and

Chemspider and prepared following the protocol prescribed in Discovery Studio version 4.0, developed and distributed by Accelrys, USA. They were further filtered using Lipinski's Rule of 5 and Veber's protocol, which sets the criteria for drug likeliness. The 48 compounds on preparation yielded 67 ligand isomers and all those 67 passed the rule of drug likeliness. The 28 FDA approved commercial drugs on preparation yielded 119 isomers, out of which only 96 passed the Lipinski's rule of 5 and Veber's protocol. The reasons behind 23 drug isomers not passing the filtration process were their high molecular weight, increased no. of hydrogen bond acceptors and donors, rotatable bonds than standard value, high polar surface area etc.

The protein targets were analyzed for their physicochemical properties by Protparam tool provided by Expasy server and then prepared to minimize the energy for better interaction with ligand molecules via the process of molecular docking. Protparam analysis (Table 4.9) revealed that seventeen target proteins were unstable in nature indicating a value >40. Instability may result because of complexity of the protein or maybe they are involved in cell signaling. Most of the seventeen proteins were found to be involved in cancer and it is well known that due to high rate of uncontrolled cell division and quick transfer of cancer cells all over the body, they are considered unstable in nature (Mobahat *et al.*, 2014). Instability of proteins is also possibly determined by the order of certain amino acids in its sequence and foldings. Location of a protein in the cells, presence of disulphide bridges, ligand binding, protease recognition mechanisms, etc., also influence protein stability (Rechsteiner *et al.*, 1987). Three targets i.e. B cell lymphoma 2, DNA topoisomerase and Matrix metalloproteinase were observed to be low in thermo stability at high temperature indicating a value <75. Sequence and structural factors such as packing, oligomerization, insertions and deletions, proline substitution, helical content, polar surface area, hydrogen bonds and salt bridges contribute to greater stability of thermophilic proteins (Kumar *et al.*, 2000). Ten target proteins were found with theoretical pI exceeding 7 indicating that they are basic in nature. These included Adrenergic Receptor, Androgen

Receptor, CDK, DNA topoisomerase, GSK3, HMG CoA Reductase, KIT, Mineralocorticoid Receptor, NOS and Progesterone Receptor. The negative GRAVY value indicated that twenty nine target proteins were very hydrophilic in nature (Table 4.9), for which they may have interaction more with water than ligands (Ahmed *et al.*, 2013). This was the reason for which water molecules were removed during protein preparation. The rest 3 proteins such as Adrenergic Receptor, Estrogen Receptor and Progesterone Receptor were found to be hydrophobic because of a positive GRAVY value.

The active sites in the selected target protein structures were identified with important amino acid residues in the active sites. These amino acid residues were the building blocks inside a protein molecule which allow forming hydrogen bonds with incoming ligand to block the abnormal activity of concerned protein, so as to cure diseases. Finding the appropriate active site inside the protein structure was the essential requirement so that the ligand can easily form a bonding with the amino acid residue. It was performed through accessing the receptor ligand interaction section of DS 4.0 to define an appropriate active site through PDB site records.

5.1.2 ADMET analysis of the ligands

Before studying molecular docking interactions, the phyto compounds and commercial drugs were subjected to ADMET analysis. Seven parameters such as solubility level, absorption level, BBB level, PPB prediction, CYP2D6 prediction, hepatotoxicity prediction and AlogP were thoroughly checked so that the compounds can be finely screened for better bioavailability inside a human body. ADMET screening was performed by *in silico* method using "ADMET descriptors" protocol in DS 4.0. All these parameters were calculated in mathematical values (Reddy *et al.*, 2012). Based on the values, all the phytocompounds and commercial drugs were classified into 3 categories: Acceptable (A), Highly Acceptable (HA) and Not Acceptable (NA). Highly acceptable are those compounds which fall strictly in the acceptable limits of the

screened parameters. Acceptables are those which shows upto 2 parameters not falling in the acceptable range and non-acceptables are those which have 3 or more parameters not falling in the acceptable range.

Out of 48 phytocompounds evaluated (Table 4.6), it was found that only 4 compounds were highly acceptable as they strictly come under unobjectionable range. They were allixin, allyl methyl sulfide and s-allyl mercapto cysteine exclusive to *Allium* sp. and p-coumaric acid from the non-*Allium* specific group. These compounds were evaluated to be easily soluble in body fluid, easily absorbed in the intestine, low penetration to Central Nervous System, easily carried away with plasma proteins to the target site, non-inhibitor of CYP2D6 drug metabolizing enzyme, possess no hepatotoxicity, and easily excretable from the body. Non-acceptable group included only 2 compounds i.e. campesterol and taurine. They were either found to be very high or very low soluble in body fluid with a very poor absorption, hepatotoxic to the liver, poorly bound to plasma proteins and with poor excretability. Rest of the 42 compounds were brought under acceptable range i.e. they dissatisfied any 2 parameters out of the seven parameters checked in ADMET analysis. Out of 28 commercial drugs, only one drug i.e. dexamethasone used against Glucocorticoid Receptor, a target for cancer and blood pressure, strictly comes under highly acceptable group, whereas 5 of them such as cabozantinib, gefitinib, pioglitazone, regorafenib and tamoxifen were found to be non-acceptable due to undesirable qualities like very high or very low solubility in body fluid with very poor absorption, hepatotoxicity to the liver, poorly bound to plasma proteins and with poor excretability (Table 4.7). Rest 22 drugs came under the acceptable range.

5.1.3 Molecular docking

Molecular docking was performed using the "CDOCKER" protocol of the commercial software Discovery Studio Version 4.0. The interaction between the targets and the phytocompounds was analysed based on different dock scores. The purpose of the present study was thus to identify potent phytocompounds in garlic which can suppress important human disorders like cancer, diabetes, arthritis,

blood pressure and cholesterol. Active inhibitors of the target proteins involved in the selected lifestyle diseases were identified based on lowest binding energy (Ramakanth *et al.*, 2012). The highly negative (low) binding energy of a protein-ligand complex indicated that it released more free energy and moves to a lower, more thermodynamically stable energy state (Ahmed *et al.*, 2013). The ligands were also screened based on difference in CDOCKER energy and CDOCKER interaction energy. The lesser the difference, the better is the interaction. This mechanism basically relies on lock and key model where protein is the lock and ligand is the key. CDOCKER energy is the combined energy produced by the sum of internal ligand strain energy and receptor-ligand interaction energy whereas CDOCKER interaction energy is the interaction energy between the protein and ligand and the values of these two parameters indicate the strength of interaction between the proteins and the ligands. On the other hand, if the difference between the two exceeds more than 10, then the interaction was considered unstable (Chilom *et al.*, 2006). Interactions showing difference of CDOCKER and CDOCKER Interaction energy more than 10 were thus rejected. The interactions of garlic phytocompounds with targets identified for various lifestyle diseases are discussed here under.

5.2.1 Interaction of garlic phytocompounds with cancer inducing targets

The interaction of 48 phytocompounds in garlic with 15 protein targets involved in various types of cancer was analyzed using molecular docking studies by CDOCKER protocol. The protein targets were AKT /Protein kinase B, Androgen Receptor, B Cell Lymphoma-2, cMET, Cyclin Dependent Kinase, DNA Topoisomerase, Epidermal Growth Factor Receptor, Estrogen Receptor, Heat Shock Protein, Mast/Stem cell Growth Factor Receptor, Matrix Metallo Proteinase, NFkB, Pi3k-γ, Progesterone Receptor and Thymidilate Synthase (Table 4.8). Out of the 48 phytocompounds, only 17 (7 exclusive to *Allium* and 10 others) showed good interaction with low binding energy.

All the seventeen phytocompounds identified were found to be interacting with the 15 targets identified for cancer. However, the intensity of interaction varied with respect to different targets. Good interaction of phytocompounds for inhibition was observed for targets such as DNA Topoisomerase, EGFR, PI3Kγ and Thymidilate synthase (Table 4.17, 4.18, 4.24 and 4.26) having an average binding energy of >150 (Table 5.1). The exclusive *Allium* compounds s-allyl L cysteine and s-allyl mercapto cysteine were observed in common to interact with the above mentioned 4 targets with a very low binding energy (Fig. 2). From the non-*Allium* group, apigenin was found to be highly interacting with the above mentioned 4 targets with a good dock score (Fig. 4). Other compounds like s-allyl-d-cysteine, alliin, cycloalliin, phloroglucinol, kaempferol, taurine, and p-coumaric acid also interacted invariably with all the selected targets for cancer upto at least 7 targets out of 15 numbers (Table 5.2). Kaempferol was observed to score the lowest binding energy (-575.04 Kcal/mol) against DNA Topoisomerase (Fig 3. A) with a difference of 3.43 Kcal/mol via amino acid residue Lys532 of active site no.1 (Table 4.17). A maximum deviation of approximately 4 Kcal/mol was observed between CDOCKER and CDOCKER interaction energies (Fig. 3 and Fig. 5). Thus it could be assumed that the combined action of such compounds might be contributing to the anti-cancerous properties of garlic.

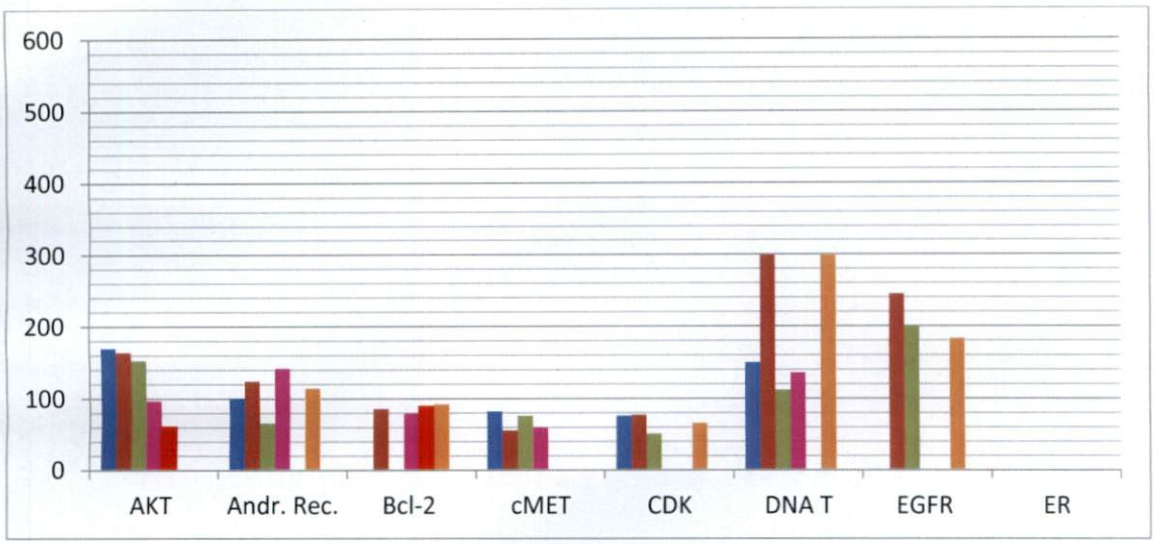
DNA topoisomerase is an important enzyme involved in releasing tension in supercoiled DNA and is currently considered as an important target for cancer prevention. If this enzyme can be inhibited, it is possible to induce lethal breaks in the cancer cell DNAs, which ultimately lead to apoptosis (Pommier, 2009). Epidermal Growth Factor Receptor (EGFR) is a cell-surface receptor which exists on the cell surface and is activated by binding of its specific ligands, including epidermal growth factor and transforming growth factor α. Mutation in EGFR results in the formation of a number of cancers, including lung, breast, ovarian, prostate, anal cancers and glioblastoma (Liffers *et al.*, 2015). PI3Ks are intracellular signal transducer enzymes involved in cellular functions such as cell

Table 5.1: Average binding energy recorded by the interacting compounds for the selected targets of cancer disease

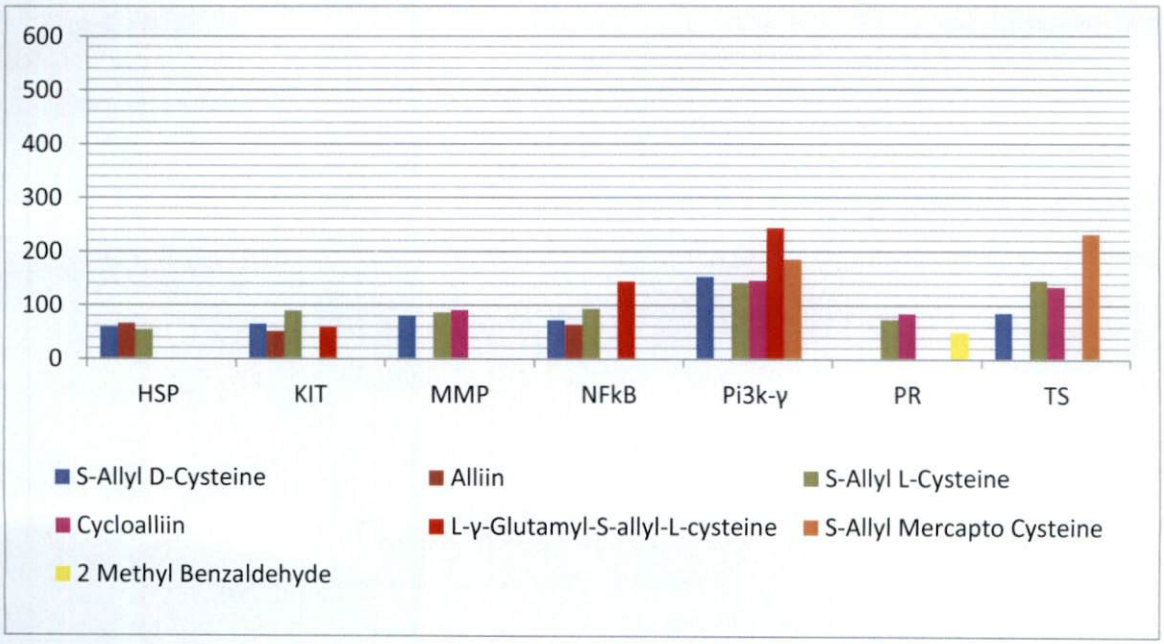
Sl. No.	Target	No. of phytocompounds interacting with each target	Average (-) binding energy (Kcal/mol)
1.	AKT /Protein kinase B	6	143.14
2.	Androgen receptor	8	114.06
3.	B cell lymphoma-2	7	123.78
4.	cMET kinase	5	77.14
5.	Cyclin dependent kinase	7	89.17
6.	DNA Topoisomerase	10	290.47
7.	EGFR	11	245.43
8.	Estrogen receptor	3	73.1
9.	Heat shock protein	6	88.42
10.	KIT	6	65.07
11.	Matrix metalloproteinase	6	89.05
12.	NFkB	10	113.48
13.	Pi3k-γ	10	204.27
14.	Progesterone receptor	8	112.6
15.	Thymidilate synthase	6	183.12

Table 5.2: Important garlic phytocompounds identified for their inhibitory effect on cancer

Sl. No.	Garlic phytocompounds	Targets interacted out of 15 nos.
1.	S-allyl L cysteine	13
2.	S-allyl D cysteine	11
3.	Alliin/ allyl cysteine sulfoxide	10
4.	Phloroglucinol	10
5.	Cycloalliin	8
6.	S allyl mercapto cysteine	7
7.	p-coumaric acid	7
8.	Kaempferol	7
9.	Apigenin	6
10.	Taurine	6

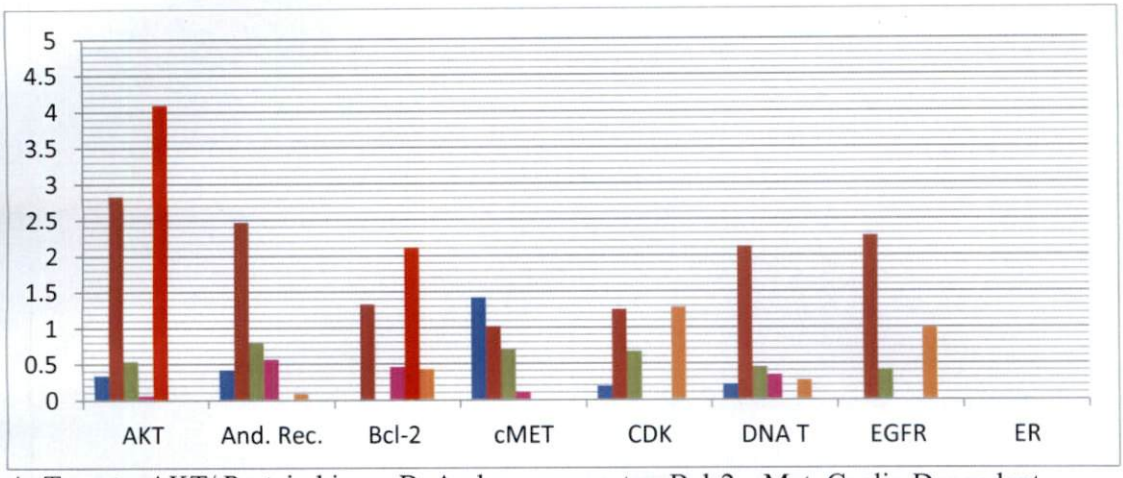


A. Targets: AKT/ Protein kinase B, Androgen receptor, Bcl-2, cMet, Cyclin Dependent kinase, DNA Topoisomerase, EGFR and Estrogen receptor (ER)

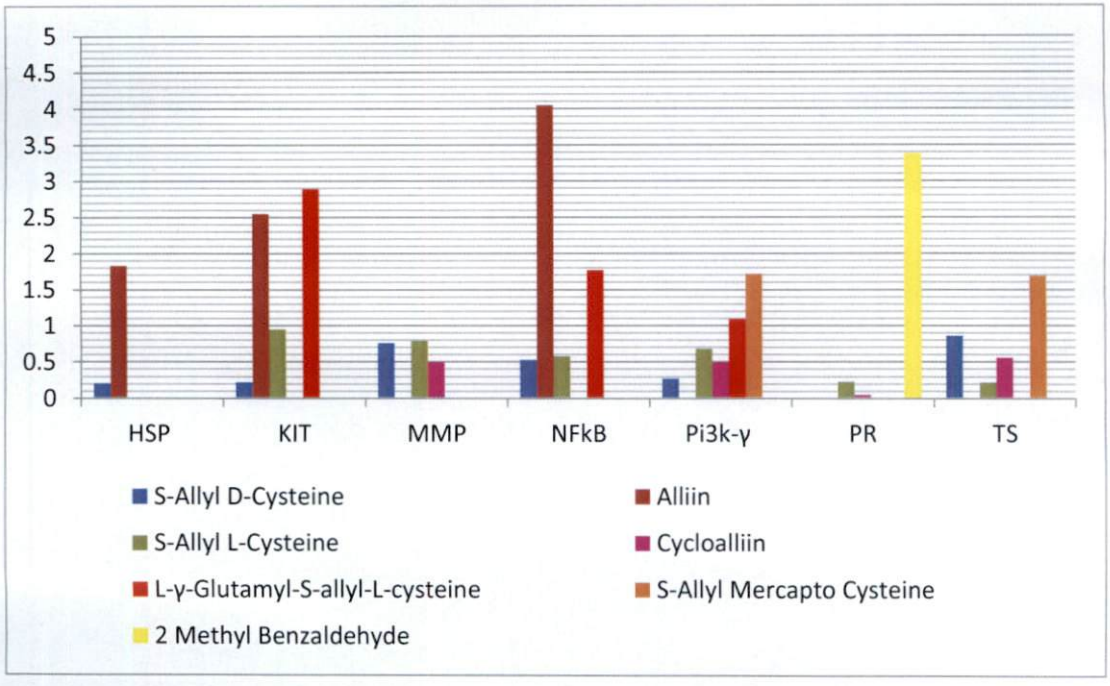


B. Targets: Heat Shock Protein, Mast/ Stem cell receptor (KIT), Matrix Metalloproteinase (MMP), NFkB, Pi3K-γ, Progesterone receptor (PR) and Thymidilate synthase (TS)

Fig 2: Negative binding energy (Kcal/mol) displayed by *Allium* specific compounds against different cancer protein targets

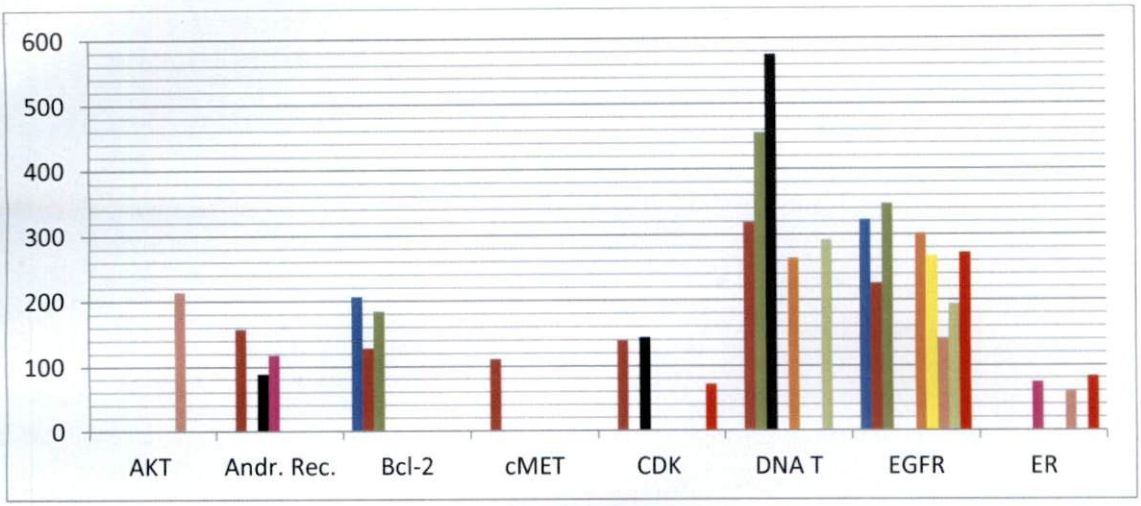


A. Targets: AKT/ Protein kinase B, Androgen receptor, Bcl-2, cMet, Cyclin Dependent kinase, DNA Topoisomerase, EGFR and Estrogen receptor (ER)

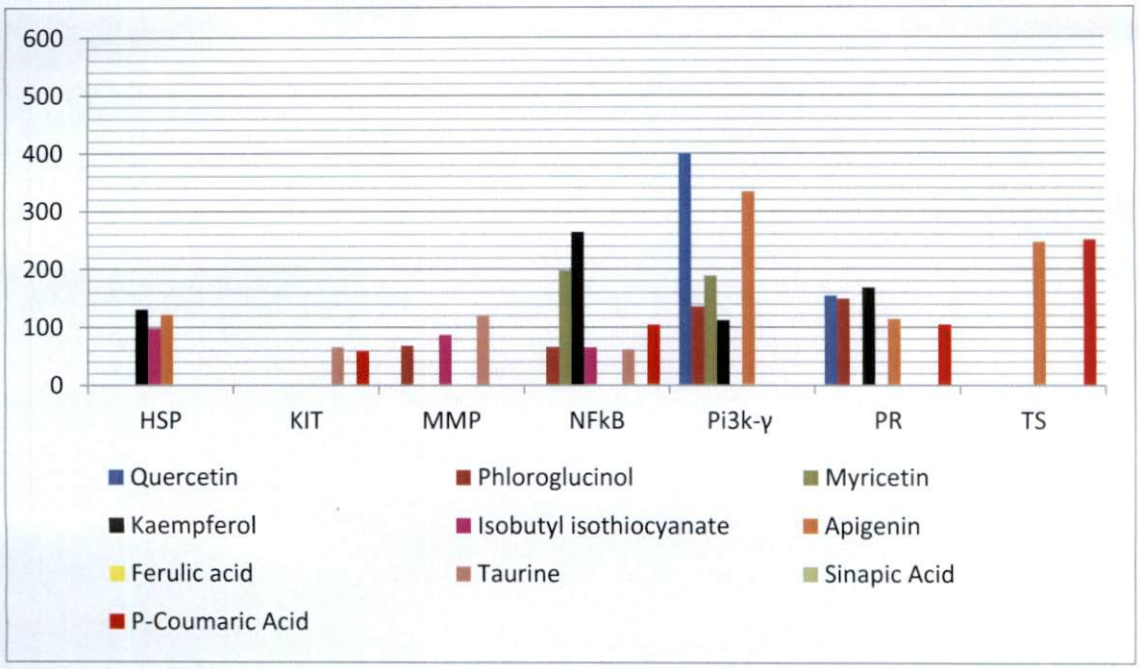


B. Targets: Heat Shock Protein, Mast/ Stem cell receptor (KIT), Matrix Metalloproteinase (MMP), NFkB, Pi3K-γ, Progesterone receptor (PR) and Thymidilate synthase (TS)

Fig 3: Deviation of CDOCKER and CDOCKER interaction energies observed for *Allium* specific phytocompounds while interacting with different cancer targets

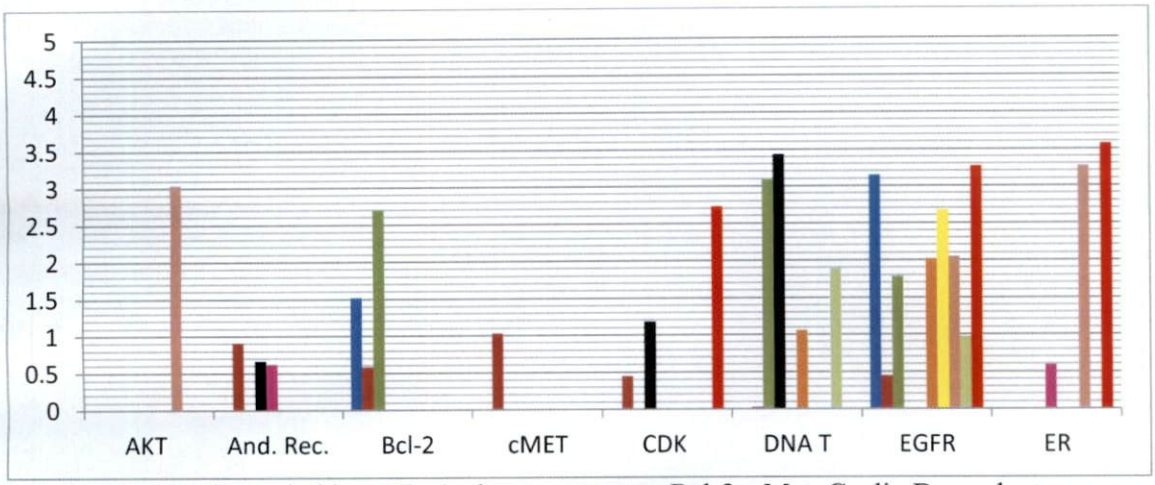


A. Targets: AKT/ Protein kinase B, Androgen receptor, Bcl-2, cMet, Cyclin Dependent kinase, DNA Topoisomerase, EGFR and Estrogen receptor (ER)

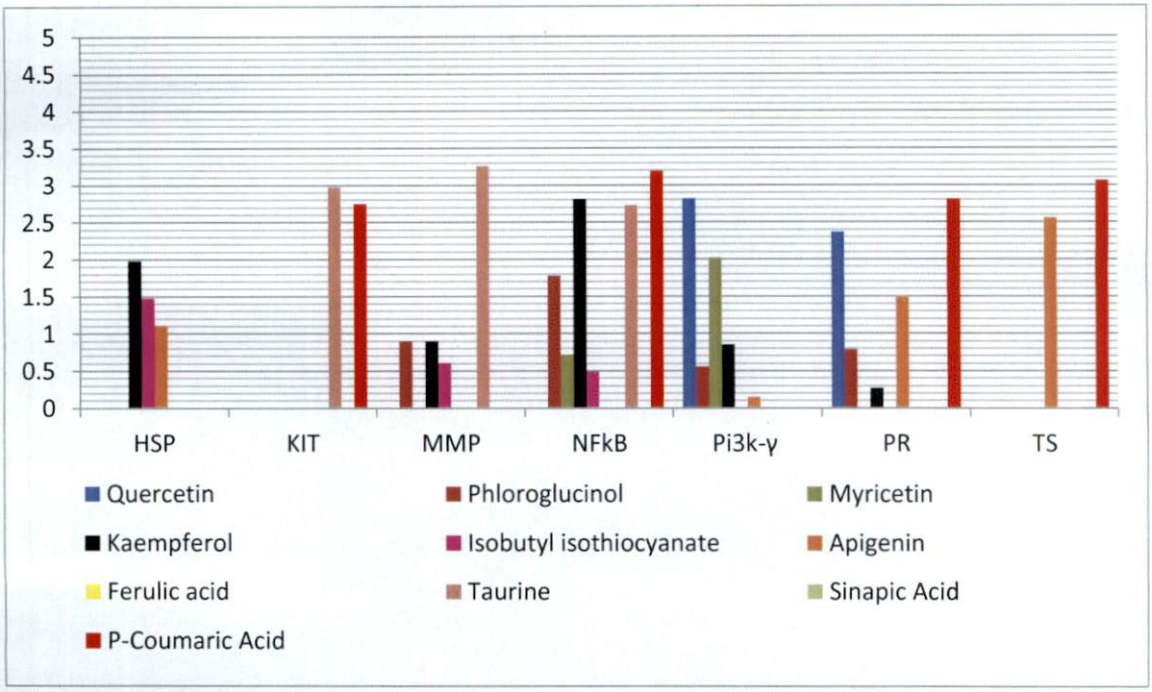


B. Targets: Heat Shock Protein, Mast/ Stem cell receptor (KIT), Matrix Metalloproteinase (MMP), NFkB, Pi3K-γ, Progesterone receptor (PR) and Thymidilate synthase (TS)

Fig 4: Negative binding energy (Kcal/mol) displayed by non-*Allium* specific compounds in *Allium* against different cancer protein targets



A. Targets: AKT/ Protein kinase B, Androgen receptor, Bcl-2, cMet, Cyclin Dependent kinase, DNA Topoisomerase, EGFR and Estrogen receptor (ER)



B. Targets: Heat Shock Protein, Mast/ Stem cell receptor (KIT), Matrix Metalloproteinase (MMP), NFkB, Pi3K-γ, Progesterone receptor (PR) and Thymidilate synthase (TS)

Fig 5: Deviation of CDOCKER and CDOCKER interaction energies observed for non-*Allium* specific compounds while interacting with different cancer protein targets

growth, proliferation, differentiation, motility, survival and intracellular trafficking. This enzyme family is a part of PI3K/AKt/mTOR pathway, an important pathway for growth progression of cancer cells. PI3Ks are the most mutated kinases in case of glioblastoma (malignant brain tumor) and hematologic malignancies (blood cancers) (Jabbour *et al.*, 2014). Thymidylate Synthase (TS) is an enzyme that catalyzes the conversion of deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP). This enzyme is essential for DNA synthesis and repair. TS protein and its mRNA levels are found elevated in many human cancers, and high TS levels have been correlated with poor prognosis in patients with colorectal, breast, cervical, bladder, kidney, and non-small cell lung cancers (Rahman *et al.*, 2004; Lurje *et al.*, 2009).

Few research reports exist in garlic for its action against cancer. This can be better explained through the *in silico* results in the present study. Pandrangi (2015) have reviewed the usefulness of garlic organosulfur compounds on prevention of cancer by decreasing the activity of pro tumor markers such as Bcl-2, EGFR, VEGF etc. It was reported that DNA topoisomerase inhibitor irinotecan is involved in curing colorectal cancer (Gilbert *et al.*, 2012). Liang *et al.*, (2011) reported that s-allyl mercaptocysteine effectively inhibits the proliferation of colorectal cancer cells under in vitro and in vivo conditions. Thus garlic compounds are found effective for inducing DNA lesions in cancer cells through targeting DNA topoisomerase. Lee *et al.* (2013) reported that N-Acetyl Cysteine (NAC), a hydrophilic organosulfur compound found in *Allium* decreased EGFR/Akt signaling in EGFR-overexpressing oral cancer. Zhang and co-workers (2015) also reported that garlic oil suppressed Nitrosodiethylamine-induced hepatocarcinoma in rats by inhibiting PI3K-AKT-NF-κB pathway. These researches thus highlight the effects of garlic on the expression of these cancer inducing targets.

Most of garlic phytocompounds formed hydrogen bonding with active site residues Asp533 and Lys532 of DNA topoisomerase, which indicated the importance of these 2 amino acids in defining the active site of the target protein

(Table 4.17). Similarly, Lys745 and Met793 (for EGFR), Lys833 (PI3K-γ) and Ser216 (TS) were identified as the most important active site residues for the selected targets (Table 4.18, 4.24 and 4.26). It was found that during the comparative study, the DNA Topoisomerase poison topetecan scored positive dock scores and made no hydrogen bonds with the target. Positive dock scores can happen when the mode of action of drug is different from the phytocompounds taken as ligands or when the active site for interaction selected is different for commercial drugs and phytocompounds. Topotecan kills cells by trapping cleavage complexes formed by topoisomerases while acting on DNA, rather than inhibiting the enzymes' catalytic activity (Pommier *et al.*, 1998). So, it can be said that the reason behind getting a positive dock score for topotecan is the case of not inhibiting the enzyme. Similarly, gefitinib, the inhibitor of EGFR was also found to display a positive CDOCKER energy. Gefitinib acts upon EGFR by way of binding to the adenosine triphosphate (ATP)-binding site of the enzyme (Lynch *et al.*, 2004). Thus, it can be said that the active site selected for docking of garlic compounds on EGFR may not be the same active site for ATP binding, where gefitinib competitively binds to the protein. This may be the reason behind the positive CDOCKER interaction of gefitinib on EGFR. Commercial drugs like idelalisib and fluorouracil acting against PI3K and TS respectively were found to dock with the concerned proteins properly, but with lesser dock scores than the garlic phytocompounds.

In the present study, molecular docking of garlic phytocompounds with 2 breast cancer targets Estrogen Receptor (ER) and Progesterone Receptor (PR) gave varying results. None of the *Allium* specific compounds showed interaction with ER (Fig 1), while few other compounds like p- Coumaric acid, isobutyl isothiocyanate and taurine gave dock scores below 100 for interaction with ER (Fig 2). The interaction of *Allium* specific compounds with PR was also low (less than 100), while few other compounds like kaempferol, quercetin, phloroglucinol and apigenin gave little better dock scores of more than 100.

Garlic compounds like s-allyl d-cysteine, alliin and s-allyl l-cysteine showed good dock scores over 100 when docked with protein target AKT /Protein kinase B. Protein kinase B, also known as AKT, is a serine/threonine-specific protein kinase that plays a key role in multiple cellular processes such as glucose metabolism, apoptosis, cell proliferation, transcription and cell migration. Studies have identified gene amplification of the AKT isoforms in many types of cancer, including glioblastoma, ovarian, pancreatic, prostate and breast cancers (Mahajan & Mahajan, 2012). Xiao and Singh (2006) reported that constituents in garlic inactivates AKT to trigger mitochondrial translocation of BCL-2-associated death promoter and caspase-mediated apoptosis in human prostate cancer cells. The amino acid residue from active site 1 of AKT/Protein kinase B commonly interacting with all the phytocompounds was Glu236, which depicted the importance of this amino acid in defining the active site of the target protein. The only compound specific to non-*Allium* specific group observed to perform better against AKT was taurine which was found to interact with the same amino acid residue and gave a dock score of over 200.

Howard *et al.* (2007) reported that s-allyl mercapto cysteine is a novel antimetastatic agent for prostate cancer. Approximately 80 to 90 % of prostate cancer are dependent on androgens at initial diagnosis, and endocrine therapy of prostate cancer is directed toward the reduction of serum androgens and inhibition of AR (Denis and Griffiths, 2000). Garlic compounds also performed well in docking studies against the most important prostate cancer target Androgen Receptor (AR) with a binding energy of more than 100. The compounds were phloroglucinol, cycloalliin, isobutyl isothiocyanate, s-allyl mercapto cysteine and s-allyl d-cysteine. Arg752 was identified as an important active site residue thus making it the most important building block in the target protein to interact with ligands via formation of hydrogen bonds.

The polyphenolic compounds present in garlic such as quercetin, phloroglucinol, myricetin, kaempferol, apigenin were found to interact nicely with cancer targets such as BCL-2, Cyclin Dependent Kinase, Heat Shock Protein and

NFkB with dock scores near about 150, whereas the exclusive *Allium* compounds failed to perform good against the same targets as mentioned above. Dock scores for both exclusive *Allium* and other compounds were observed to be less for protein targets like Mast/ stem cell receptor (KIT) and Matrix metalloproteinase (MMP).

The results of molecular docking of *Allium* phytocompounds on several protein targets involved in cancer revealed some interesting facts. Garlic compounds might be acting together in a complementary and synergistic way to inhibit the action of target proteins. Ten to eleven compounds were found interacting with most of the protein targets. The inhibiting action as predicted by binding energy though not high (<100) for many of the compounds, might be having a complementary and synergistic action in suppressing the protein targets. However, some of the compounds like kaempferol, quercetin and apigenin recorded very good dock scores of over 300. Similarly, s-allyl cysteine and s-allyl mercapto cysteine were found to interact with cancer target proteins with lower dock scores than the other *Allium* specific compounds. Thus the anticancerous properties of garlic can be attributed to the combined action of different phytocompounds on a specific target.

5.2.2 Interaction of garlic phytocompounds with diabetes inducing targets

The protein targets selected for diabetes were Aldose reductase, Dipeptidyl peptidase-4, Glucokinase, Glycogen synthase kinase 3, Insulin receptor and PPARγ (Table 4.8). Out of the 48 phytocompounds studied in garlic, only 13 (6 exclusive to *Allium* and 7 others) showed good interaction with low binding energy. However, the intensity of interactions of these compounds on diabetes targets were much different (Fig. 6 and Fig. 8). Good interaction was observed with targets such as DPP4, Glucokinase (GK) and Aldose reductase (Table 4.28, Table 4.29 and Table 4.27), having an average binding energy of >150 (Table 5.3) The compounds such as s-allyl d-cysteine, s-allyl l-cysteine, cycloalliin and l-γ-glutamyl-s-allyl-l-cysteine, which were exclusive to *Allium* were observed to

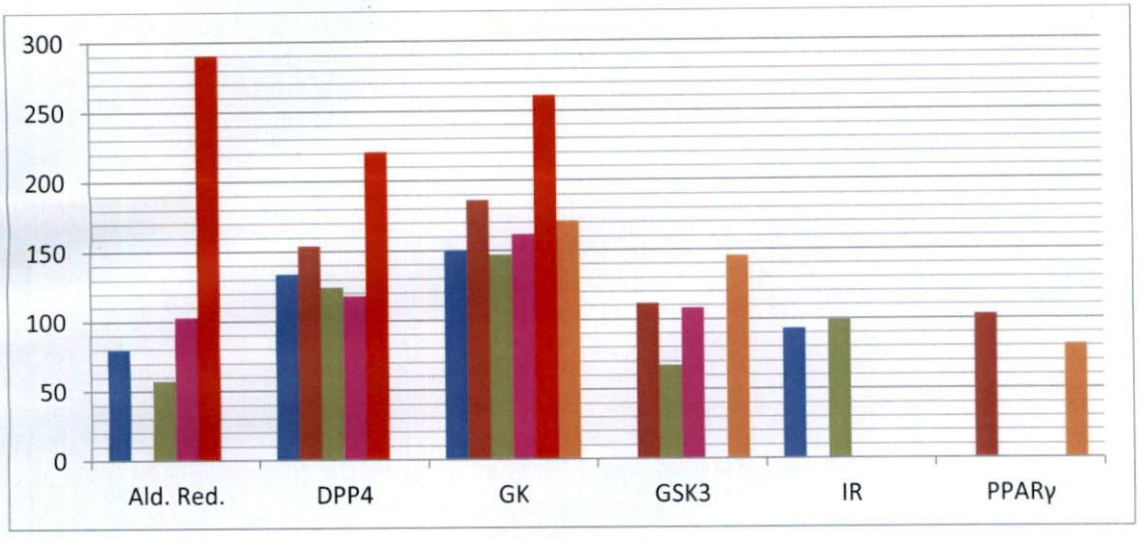


Fig 6: Negative binding energy (Kcal/mol) displayed by *Allium* specific compounds against different diabetes protein targets

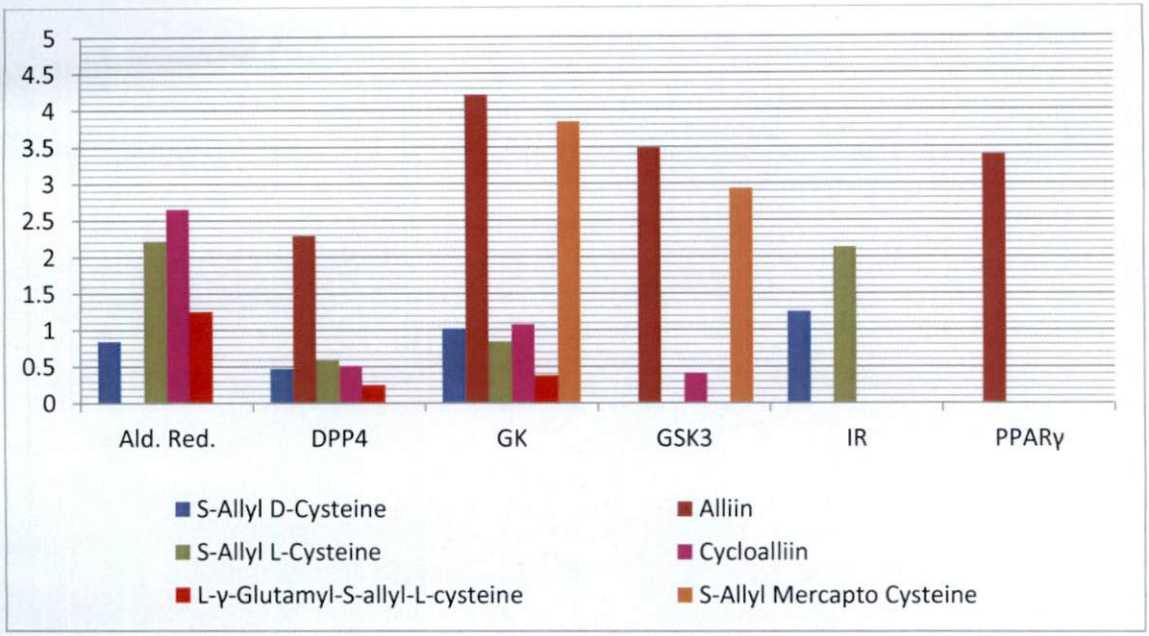


Fig 7: Deviation of CDOCKER and CDOCKER interaction energies observed for *Allium* specific phytocompounds while interacting with different diabetes targets

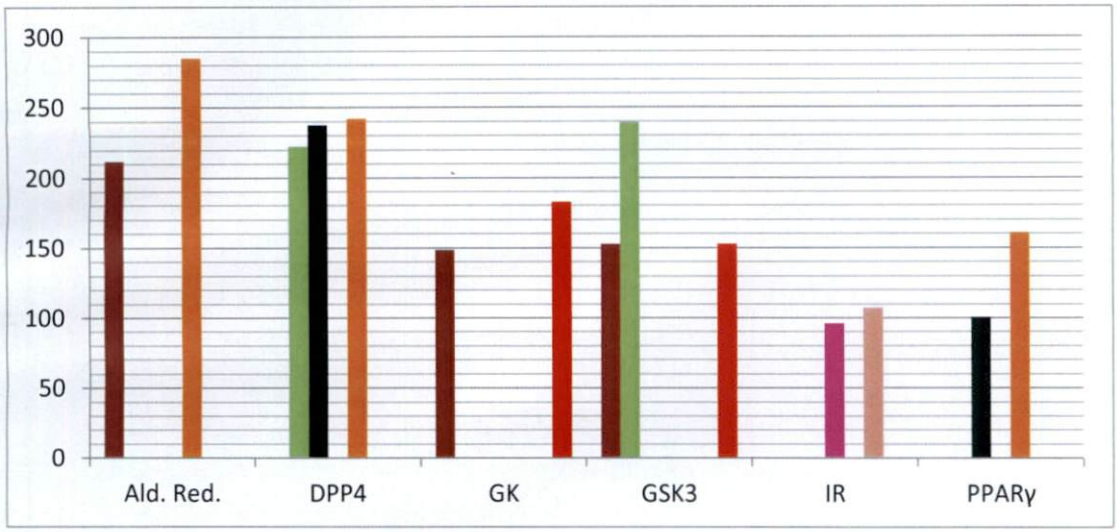


Fig 8: Negative binding energy (Kcal/mol) displayed by non- *Allium* specific compounds against different diabetes protein targets

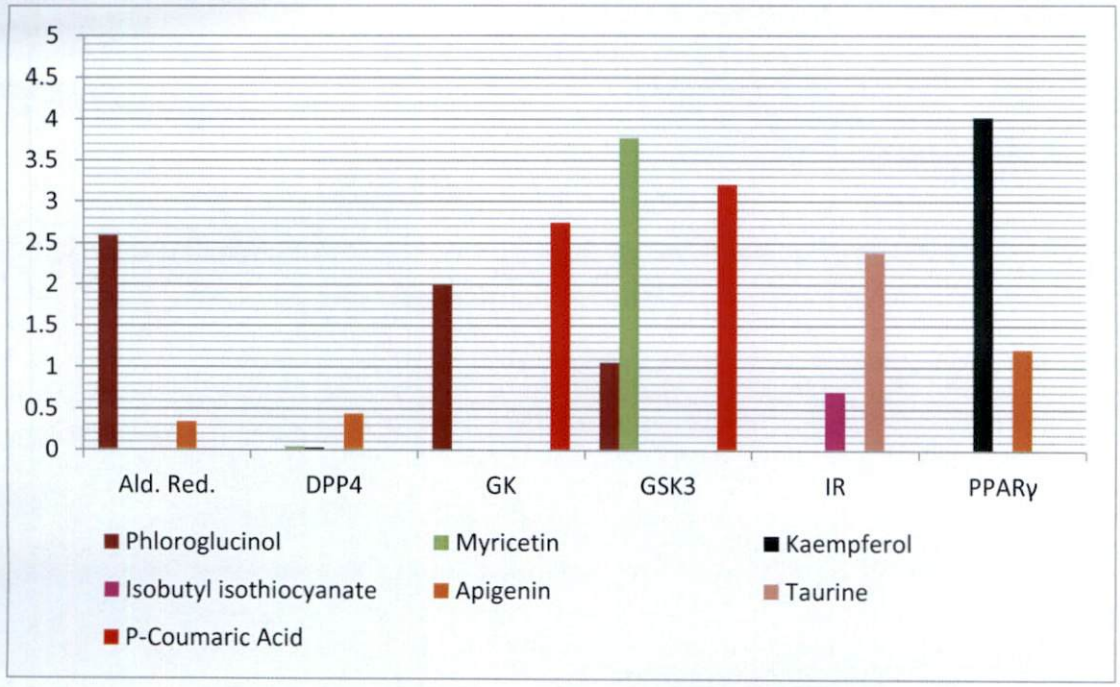


Fig 9: Deviation of CDOCKER and CDOCKER interaction energies observed for non- *Allium* specific compounds while interacting with different diabetes protein targets

interact with Glucokinase (GK), DPP4 and Aldose reductase involved in diabetes with a low binding energy. Other compounds like alliin, s-allyl mercapto cysteine, apigenin and phloroglucinol also interacted invariably with all the selected targets for diabetes upto at least 3 targets out of 6 numbers (Table 5.4). L-γ-Glutamyl-S-allyl-L-cysteine recorded the lowest binding energy (-290.54 Kcal/mol) with a difference of 1.25 Kcal/mol via amino acid residue Ser210 of active site no.1 (Table 4.27). The maximum deviation observed was only 4.21 Kcal/mol between CDOCKER and CDOCKER interaction energies (Fig. 7 and Fig. 9) indicating stable interactions.

DPP4 is an important target for diabetes mellitus, the inhibition of which allows adequate release of insulin to regulate the metabolism of glucose (Vijayakumari *et al.*, 2011). Noor *et al.*, (2013) reported the mechanism through which organosulfur compounds from garlic and onion prevents diabetes through the inhibition of DPP4 and thus allowing smooth insulin flow in the system. Srinivasan (2005) proposed that s-allyl cysteine sulfoxide (Alliin) exert anti diabetic action through insulin production and secretion by the pancreas interfering with dietary glucose absorption. In the present study, the compounds exclusive to *Allium* (Table 4.28) such as s-allyl l-cysteine, alliin, s-allyl d-cysteine, cycloalliin and l-γ-glutamyl-s-allyl-l-cysteine were found to interact with DPP4 with a low binding energy ranging from -117.97 Kcal/mol to -220.91 Kcal/mol. Alliin was observed to form 5 hydrogen bonds with the target protein effectively yielding a low binding energy of -154.09 Kcal/mol (Table 4.28). From the non-garlic group, apigenin was observed to interact with DPP4 with a good dock score (-242.16 Kcal/mol) even better than that observed for exclusive *Allium* compounds.

Panda and Kar (2007) reported that apigenin, has the potential to regulate hyperglycaemia. Administration of 0.78 mg/kg of apigenin for 10 consecutive days in alloxan treated diabetec animal increased the level of insulin and thyroid hormones with a parallel decrease in glucose concentration. Compounds such as

Table 5.3: Average binding energy recorded by the interacting compounds for the selected targets of diabetes disease

Sl. No.	Target	No. of phytocompounds interacting with each target	Average (-) binding energy (Kcal/mol)
1.	Aldose reductase	6	171.34
2.	DPP4	8	181.71
3.	Glucokinase	8	176.11
4.	GSK3	7	139.65
5.	Insulin receptor	4	99.05
6.	PPAR γ	4	111.51

Table 5.4: Important garlic phytocompounds identified for their inhibitory effect on diabetes

Sl. No.	Garlic phytocompound name	Targets interacted out of 6 nos.
1.	S-allyl L cysteine	5
2.	Alliin	4
3.	S-allyl D cysteine	4
4.	Cycloalliin	4
5.	S allyl mercapto cysteine	3
6.	L-γ-Glutamyl-S-allyl-L-cysteine	3
7.	Apigenin	3
8.	Phloroglucinol	3

kaempferol and myricetin also interacted effectively with the target DPP4. In the present study, the garlic phytocompounds mostly formed hydrogen bonding with the amino acid residues Glu205, Glu206, Tyr662, Arg125 and Tyr547 which are reported in the active site 1 (Table 4.11).

The DPP4 inhibitor (commercial drug) selected in the study was vildagliptin which recorded positive dock scores. Vildagliptin actually increases incretin levels of glucagon-like peptide-1 (GLP1) which inhibits glucagon release and increases insulin secretion, thus reducing the blood glucose level (Presswala and Shubrook, 2015). Thus, vildagliptin does not directly inhibit DPP4 and instead increases the level of GLP1. This may be the reason, why there was a positive CDOCKER relation between vildagliptin and DPP4.

Glycolysis and gluconeogenesis are the two primary complementary events balancing the glucose load in our body. Insulin prevents hyperglycemia, in part, by suppressing hepatic gluconeogenesis and glycogenolysis and facilitates hepatic glycogen synthesis. Glucokinase has a major role in the control of blood glucose homeostasis because it is the predominant hexokinase expressed in the liver, and has a very high control on hepatic glucose disposal and is the glucose sensor for insulin secretion in pancreatic β-cells. Glucokinase is currently considered a strong candidate target for antihyperglycemic drugs for type 2 diabetes (Agius, 2009). In the present study, the exclusive *Allium* compounds interacted with Glucokinase with a low binding energy ranging from -147 Kcal/mol to -261.03 Kcal/mol (Table 4.29). Vijayakumar *et al.* (2015) have reported that oral administration of p-coumaric acid (100 mg/kg b.wt) for a period of 30 days to lower blood glucose and improve the level of insulin, enzymatic and non-enzymatic antioxidant activities in streptozotocin induced diabetic rats. It was suggested that the decrease in blood glucose and increase in the level of insulin in diabetic rats treated with p-coumaric acid might be due to enhanced insulin secretion and increased utilization of glucose. In the present study, from the non-garlic group, p-coumaric acid showed good interaction (-182.77 Kcal/mol) with Glucokinase (Fig. 7). All these compounds formed hydrogen bonding commonly

with active site amino acid residues Asp205, Lys169, Thr228 and Gly229 of Active site no.1 in Glucokinase which indicated the correct interaction of garlic phytocompounds with the target (Table 4.29).

Aldose Reductase is an enzyme of polyol pathway and catalyzes reduction of glucose to sorbitol. In a hyperglycemic state, the affinity of aldose reductase for glucose rises, causing much sorbitol to accumulate and these imbalances damage cells of retina, kidney, and nervous tissues (Brownlee, 2001). L-γ-Glutamyl-S-allyl-L-cysteine recorded the least binding energy among all the phytocompounds interacting with Aldose Reductase (Table 4.27). L-γ-glutamyl-s-allyl-l-cysteine is the parent compound of all sulfoxides available in garlic. It is not much bioavailable in its original form, but is converted to s-allyl cysteine and s-allyl cysteine sulfoxides easily (Kodera *et al.*, 2002). S-allyl cysteine is reported to be bioavailable upto high amounts in mice and rats (Nagae *et al.*, 1994). In a study by Tsai *et al.* (2011), s-allyl cysteine was found to reduce the mRNA expression of Aldose Reductase and Sorbitol Dehydrogenase which are responsible for sorbitol accumulation. Thus, s-allyl cysteine can be considered as anti glycative. In the present study, s-allyl d-cysteine was found to interact with aldose reductase with a good dock score of -79.82 Kcal/mol (Table 4.27). From a group of non-garlic specific phytocompounds, apigenin was recorded to interact with aldose reductase with a good dock score equally similar to that of l-γ-glutamyl-s-allyl-l-cysteine and with a lesser difference of CDOCKER energies (0.34 Kcal/mol). Most of the the compounds interacting with Aldose Reductase formed hydrogen bonding mostly with active site residues Tyr48, Ser210, Ser214 and Lys262, which revealed the correct interaction of garlic compounds with the target *al*dose reductase. The commercial drug epalrestat against Aldose Reductase showed a very good binding energy of around -230 Kcal/mol (Table 4.27). However, it indicated unstable interaction with the target protein because of a high deviation of CDOCKER energies which surpassed the limit of 10 Kcal/mol.

Glycogen synthase kinase 3 (GSK3) is a serine/threonine protein kinase that mediates the addition of phosphate molecules onto serine and threonine

amino acid residues. Due to its involvement in a great number of signaling pathways, GSK-3 has been associated with many diseases. GSK-3 inhibitors are currently being tested for therapeutic effects in Type II diabetes, inflammation, Alzheimer's disease, and bipolar disorder (MacAulay and Woodgett, 2008). When garlic compounds were docked with GSK3, it was observed that the non-garlic compounds such as myricetin, phloroglucinol and p-coumaric acid interacted with GSK-3 with a better dock score than that of exclusive *Allium* compounds such as s-allyl mercapto cysteine, alliin, cycloalliin and s-allyl l cysteine (Table 4.30). Most of these phytocompounds were bound with Tyr134 and Val135 indicating a correct stable interaction at active site no. 1. The commercial drug used as GSK3 inhibitor is lithium carbonate and it performed equally good as all the garlic compounds with a low binding energy of -190.31 Kcal/mol and a very low deviation of 0.71 Kcal among CDOCKER energies connecting with the active site residue Tyr134 (Table 4.30).

Very few compounds from both *Allium* and non-*Allium* group interacted with the diabetes protein targets PPARγ and insulin receptor (IR) (Fig. 5 and Fig. 7). The compounds that interacted with PPARγ were alliin, s-allyl mercapto cysteine, kaempferol and apigenin (Table 4.32), whereas s-allyl d cysteine, s-allyl l-cysteine, isobutyl isothiocyanate and taurine were the compounds interacting with IR (Table 4.31). For the target PPARγ, the interactions of s-allyl mercapto cysteine and kaempferol was found insignificant as they connected residues from other active sites (Table 4.32). PPARγ regulates fatty acid storage and glucose metabolism. The genes activated by PPARγ stimulate lipid uptake and adipogenesis by fat cells. PPARγ is known to be implicated in various human chronic diseases such as diabetes mellitus, atherosclerosis, pulmonary hypertension etc. (Huang *et al.*, 2012). The second target IR is a transmembrane receptor that is activated by insulin and insulin like growth factors. Mutations in the insulin-receptor gene have been identified in patients with genetic syndromes of extreme insulin resistance i.e. a decrease in insulin receptor signalling (Taylor *et al.*, 1990). Thus insulin insensitivity may lead to diabetes mellitus. Keophiphath *et al.* (2009) reported that garlic compounds significantly reduced gene expression

of PPARγ. In the present study, the amino acid residues Ser289, His449 and Tyr473 in PPARγ was found to be most important in interacting with the garlic phytocompounds (Table 4.32). Similarly, for the protein target IR, the amino acid residues such as Glu1047 and Asp1150 were found to form hydrogen bonds with garlic phytocompounds (Table 4.31). Commercial drugs for these targets i.e. pioglitazone and repaglinide yielded poor binding energies in comparison to the garlic phytocompounds.

5.2.3 Interaction of garlic phytocompounds with blood pressure inducing targets

Three protein targets involved in blood pressure were analyzed for their interation with phytocompounds identified in garlic. The protein targets were adrenergic receptor, angiotensin converting enzyme and carbonic anhydrase. Out of the 48 phytocompounds, only 11 (6 exclusive to *Allium* and 5 others) showed good interaction with low binding energy. However, the intensity of interaction varied with respect to different targets (Fig. 10 and Fig. 12). Good interaction of phytocompounds for inhibition was observed for target Angiotensin Converting Enzyme (ACE) (Table 4.34), having an average binding energy of >100 (Table 5.5). Compounds like s-allyl l-cysteine, alliin, s-allyl d-cysteine, p-coumaric acid, cycloalliin, s-allyl mercapto cysteine, isobutyl isothiocyanate and ferulic acid interacted invariably with all the selected targets for blood pressure upto at least 2 targets out of 3 numbers (Table 5.6). The deviation observed for all the 11 compounds interacting with all the targets for blood pressure and cholesterol, was <4.5 Kcal/mol between CDOCKER and CDOCKER interaction energies (Fig. 11 and Fig. 13), indicating a good interaction of garlic phytocompounds with blood pressure and cholesterol inducing targets.

ACE is a part of Renin-angiotensin-aldosterone system (RAAS) which is a hormone system that regulates blood pressure. If the renin–angiotensin–system is abnormally active, blood pressure will be too high (Baudin, 2002). Asdaq and Inamdar (2010) reported that S allyl cysteine (SAC), a water soluble compound of

Table 5.5: Average binding energy recorded by the interacting compounds for the selected targets of blood pressure disease

Sl. No.	Target	No. of phytocompounds interacting with each target	Average (-) binding energy (Kcal/mol)
1.	Adrenergic receptor	8	85.42
2.	ACE	8	136.47
3.	Carbonic anhydrase	7	85.29

Table 5.6: Important garlic phytocompounds identified for their inhibitory effect on blood pressure

Sl. No.	Garlic phytocompound name	Targets interacted out of 3 nos.
1.	S-allyl L cysteine	3
2.	Alliin	3
3.	S-allyl D cysteine	3
4.	p-Coumaric acid	3
5.	Cycloalliin	2
6.	S allyl mercapto cysteine	2
7.	Isobutyl isothiocyanate	2
8.	Ferulic acid	2

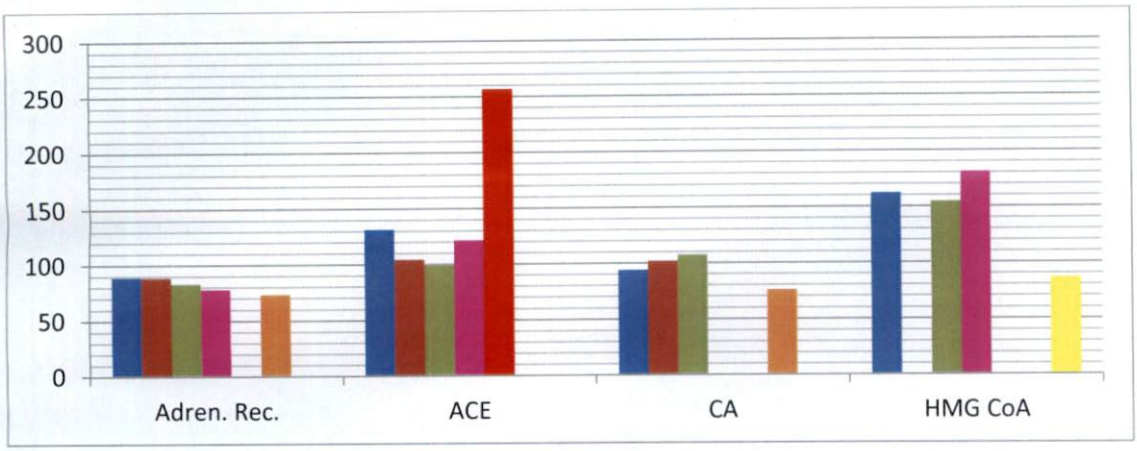


Fig 10: Negative binding energy (Kcal/mol) displayed by *Allium* specific compounds against different blood pressure and cholesterol protein targets

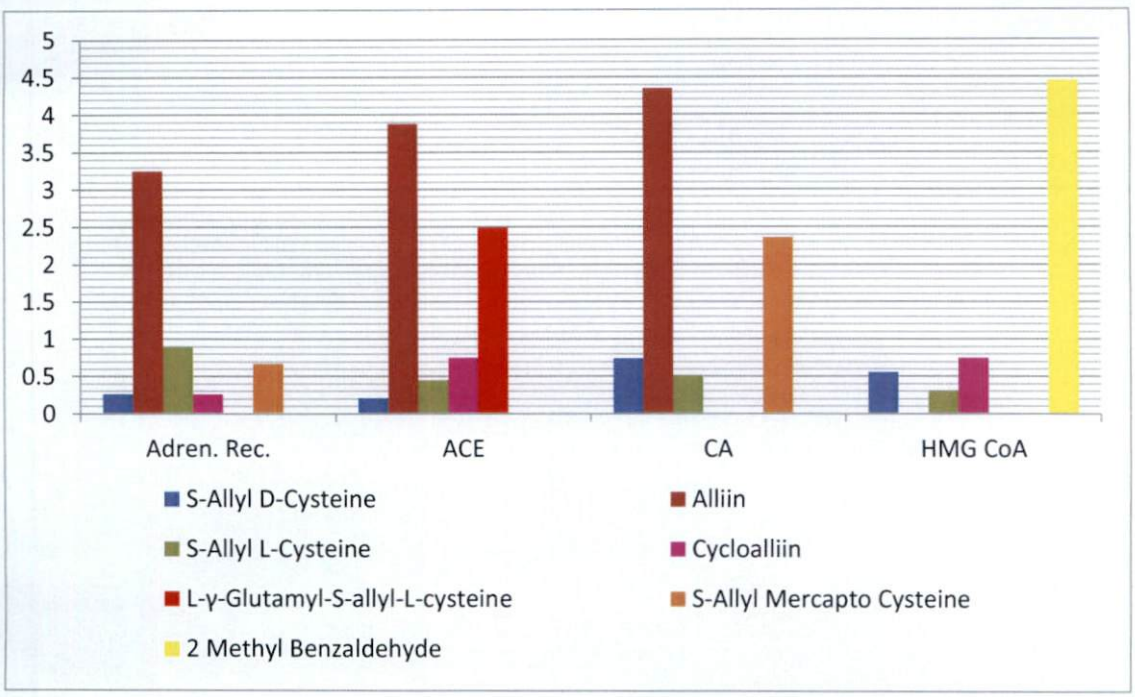


Fig 11: Deviation of CDOCKER and CDOCKER interaction energies observed for *Allium* specific phytocompounds while interacting with different blood pressure and cholesterol protein targets

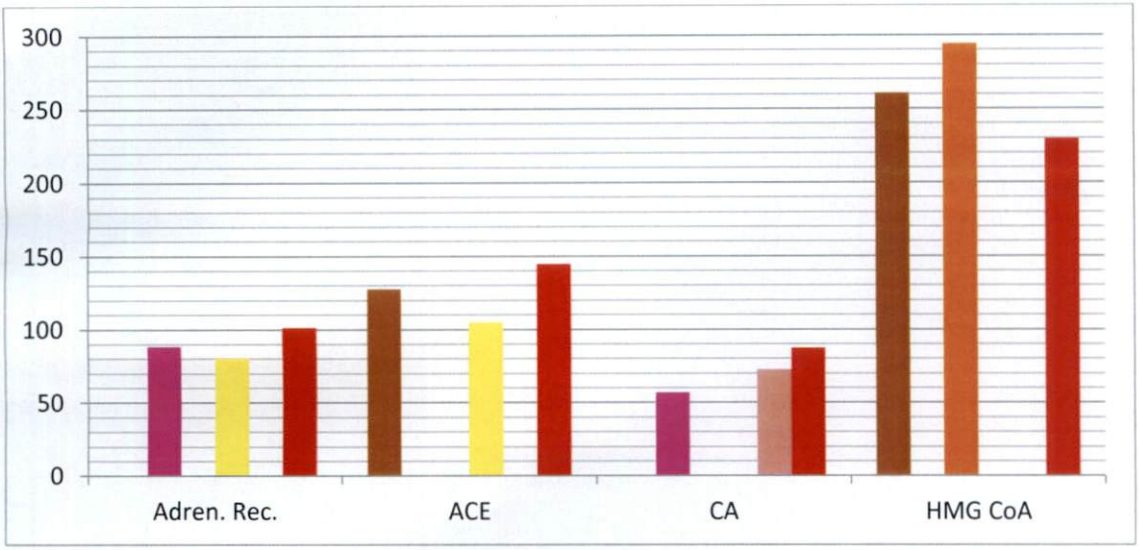


Fig 12: Negative binding energy (Kcal/mol) displayed by non- *Allium* compounds in *Allium* against different blood pressure and cholesterol protein targets

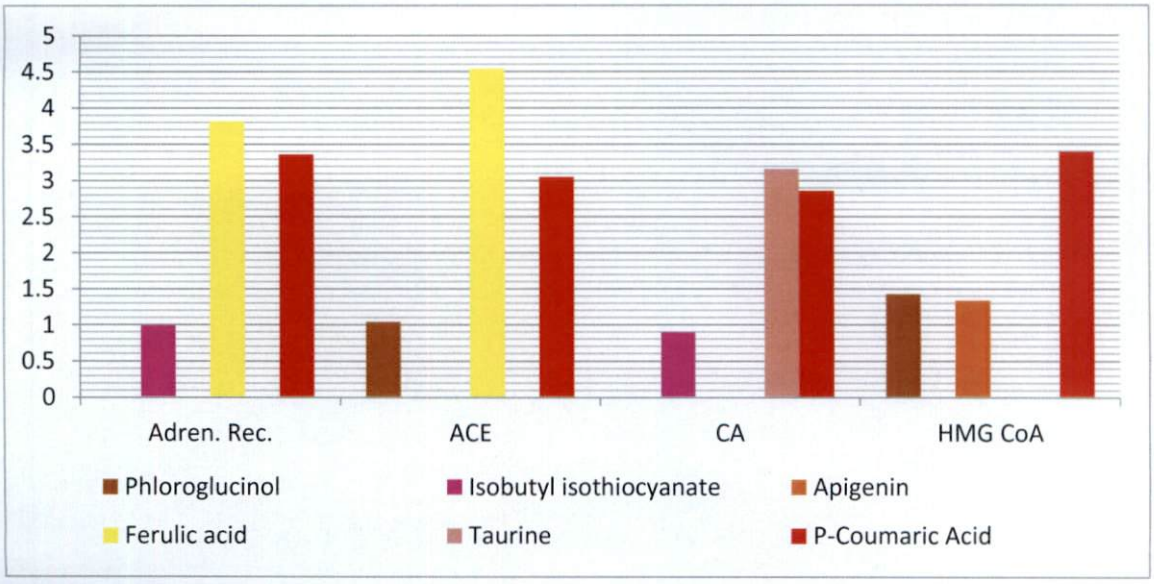


Fig 13: Deviation of CDOCKER and CDOCKER interaction energies observed for non- *Allium* specific in *Allium* while interacting with different blood pressure and cholesterol protein targets

garlic acts synergistically with captopril to inhibit ACE and decrease blood pressure in guinea pigs and rats.

This observation could, at least in part, constitute an underlying mechanism for the hypotensive effect of garlic. In the present study, exclusive *Allium* compounds like l-γ-glutamyl-s-allyl-l-cysteine, s-allyl-d- cysteine, s-allyl l-cysteine, cycloalliin and alliin showed good dock scores against ACE. Similarly non-*Allium* compounds such as p-coumaric acid, phloroglucinol and ferulic acid also displayed good dock scores equivalently like *Allium* specific compounds. The binding energy ranged from -99.87 Kcal/mol to -256.81 Kcal/mol (Table 4.34). The most common amino acid residues from ACE interacting with the phytocompounds were Glu162, Lys511, Gln281, and Tyr520 of active site no. 5. When FDA approved commercial drugs were taken for a comparison against garlic phytocompounds (e.g. captopril for ACE) by *in silico* studies, it was found that commercial drugs displayed a good dock score of -121.88 Kcal/mol, but was considered to form an unstable interaction with the target protein because of a big deviation of CDOCKER energies which surpassed the limit of 10 Kcal/mol (Table 4.34).

Allium phytocompounds were observed to give a mediocre performance in molecular docking interactions against Carbonic Anhydrase (CA) and Adrenergic Receptor blood pressure protein targets with low binding energy. Compounds like s-allyl d-cysteine, alliin, s-allyl l-cysteine, s-allyl mercapto cysteine, p-coumaric acid and isobutyl isothiocyanate were found in common to interact with CA and Adrenergic Receptor with dock scores mostly below <100 (Fig: 9).

Carbonic Anhydrase are a family of enzymes that catalyse the rapid inter conversion of carbon dioxide and water to bicarbonate ions and protons (or vice versa). CA isoforms are highly expressed in pathological conditions like glaucoma and ocular hypertension (Swenson, 2014). In the present study, it was observed that garlic compounds interacted with carbonic anhydrase with binding energy ranging from -56.59 Kcal/mol to -107.71 Kcal/mol. The most important

active site residues from CA found interacting with garlic compounds were His94, His96, His119 and Thr199 (Table 4.35).

Adrenergic Receptors are a class of G protein-coupled receptors that are targets of the catecholamines, especially norepinephrine (noradrenaline) and epinephrine (adrenaline). The more the binding of catecholamines on these receptors, the more these receptors get involved in cardiac stimulation leading to hypertension, asthma, coronary heart disease and bradycardia (Ciccarelli *et al.*, 2013). Fehri *et al.*, (2011) studied the relaxant effect of garlic bulb aqueous extract on isolated smooth muscle of trachea of rats precontracted with acetylcholine (10^{-5} M). It was found that garlic bulb aqueous extract induced a dose dependent relaxation i.e. at low doses (10^{-6} to 10^{-4} gm /ml) the extract induced contractile effect and at high doses (10^{-3} to 3.10^{-3} gm/ml) it induced relaxation effect. It was suggested that the resulted relaxation was due to the release of prostaglandins E1 and E2 consecutively after α and β adrenergic receptor stimulation. In the present study, the garlic compounds found interacting against adrenergic receptor with a binding energy ranging from -73.55 Kcal/mol to -101.31 Kcal/mol (Table 4.33). The most important active site residues were Ser203, Ser204 and Asp113.

5.2.4 Interaction of garlic phytocompounds with cholesterol inducing target

Only one protein target involved in cholesterol was analyzed for its interation with phytocompounds identified in garlic. The protein targets selected was HMG CoA reductase. Out of the 48 phytocompounds, only 7 (4 exclusive to *Allium* and 3 others) showed good interaction with low binding energy. About 70 percent of total cholesterol in the body is made by HMG CoA reductase and high cholesterol levels have been associated with cardiovascular disease. This enzyme is thus the target of widely available cholesterol-lowering drugs (Yeganeh *et al.*, 2014). SAC has been reported to inhibit cholesterol synthesis by deactivating HMG-CoA Reductase in cultured rat hepatocytes by increasing sulfhydryl oxidation of the enzyme (Liu and Yeh, 2002).

In the present study, garlic specific compounds such as cycloalliin, s-allyl d-cysteine and s-allyl l-cysteine was found to interact with HMG CoA Reductase with very good dock scores (Fig. 10). The non-*Allium* specific compounds such as apigenin, phloroglucinol and p-coumaric acid were found to be more interacting with HMG CoA reductase than that of garlic specific compounds (Fig. 12). Apigenin was observed to score the lowest binding energy (-294.4 Kcal/mol) against HMG CoA reductase with a difference of 1.34 Kcal/mol via amino acid residue Lys735 and Ser684 of active site no.4 (Table 4.36). Lys735, Ser684 and Asp690 of active site no. 4 were the most common active site residues in HMG CoA Reductase found interacting with all these garlic phytocompounds (Table 4.36).

5.2.5 Interaction of garlic phytocompounds with arthritis and inflammation inducing targets

Seven protein targets involved in arthritis and inflammation were analyzed using the CDOCKER protocol. The protein targets were Cyclooxygenase, Glucocorticoid Receptor, Mineralocorticoid Receptor, Nitric Oxide Synthase, MAPK 14, Tumor Necrosis Factor Alpha (TNF α) and VEGFR1. Out of the 48 phytocompounds, 16 (7 exclusive to *Allium* and 9 others) showed good interaction with low binding energy. However, all the interactions of these compounds on diabetes targets were found to be divergent (Fig 14 and Fig. 16). Good interaction of phytocompounds for inhibition was observed for the targets Cyclooxygenase 2 (COX2), Nitric Oxide Synthase (NOS) and TNF alpha (Table 4.37, Table 4.40 and Table 4.42) having an average binding energy of >100 (Table 5.7). The exclusive garlic compounds s-allyl l-cysteine and s-allyl d-cysteine were observed in common to interact with COX 2, iNOS and TNFα with a low binding energy (Fig. 13). From the non-*Allium* group, p-coumaric acid was the only compound found in common to be interacting with the above mentioned 3 targets with a good dock score (Fig. 16). Other compounds like alliin, l-γ-glutamyl-s-allyl-l-cysteine and phloroglucinol also interacted invariably with all the selected targets for diabetes upto at least 3 targets out of 7 numbers (Table 5.8). Quercetin was

Table 5.7: Average binding energy recorded by the interacting compounds for the selected targets of arthritis and inflammation disease

Sl. No.	Target	No. of phytocompounds interacting with each target	Average (-) binding energy (Kcal/mol)
1.	Cyclooxygenase	8	131.98
2.	Glucocorticoid receptor	2	72.15
3.	Mineralocorticoid receptor	4	85.84
4.	Nitric Oxide Synthase	9	104.15
5.	MAPK 14	6	79.38
6.	TNF α	7	100.22
7.	VEGFR1	6	80.38

Table 5.8: Important garlic phytocompounds identified for their inhibitory effect on arthritis and inflammation

Sl. No.	Garlic phytocompound name	Targets interacted out of 7 nos.
1.	S-allyl L cysteine	5
2.	Alliin	5
3.	S-allyl D cysteine	5
4.	p-coumaric acid	5
5.	L-γ-Glutamyl-S-allyl-L-cysteine	4
6.	Phloroglucinol	3

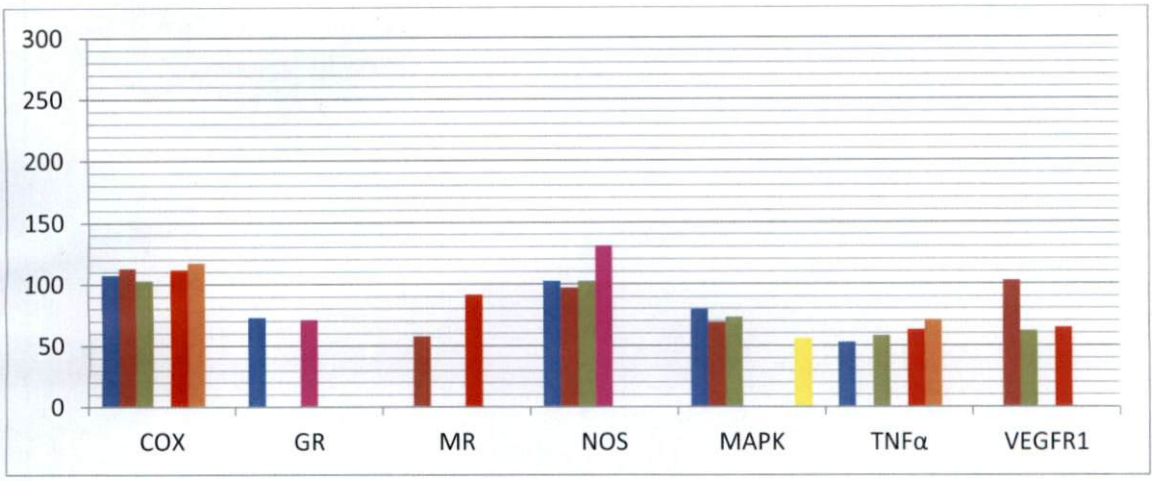


Fig 14: Negative binding energy (Kcal/mol) displayed by *Allium* specific compounds against different arthritis and inflammation protein targets

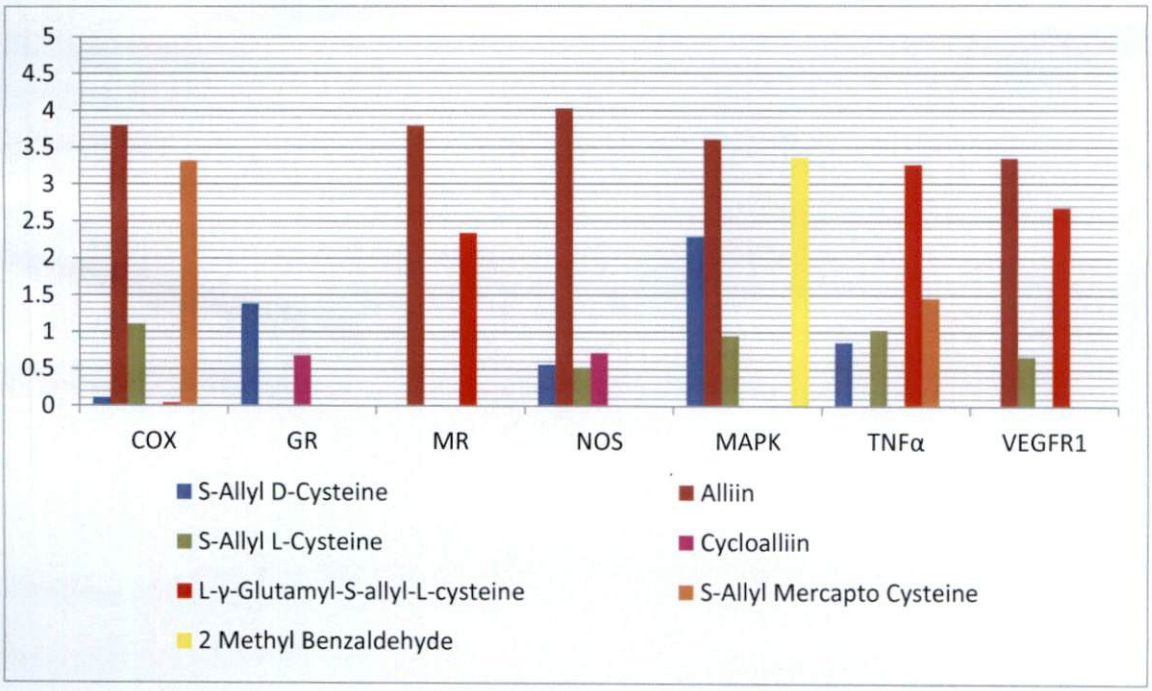


Fig 15: Deviation of CDOCKER and CDOCKER interaction energies observed for *Allium* specific phytocompounds while interacting with different arthritis and inflammation protein targets

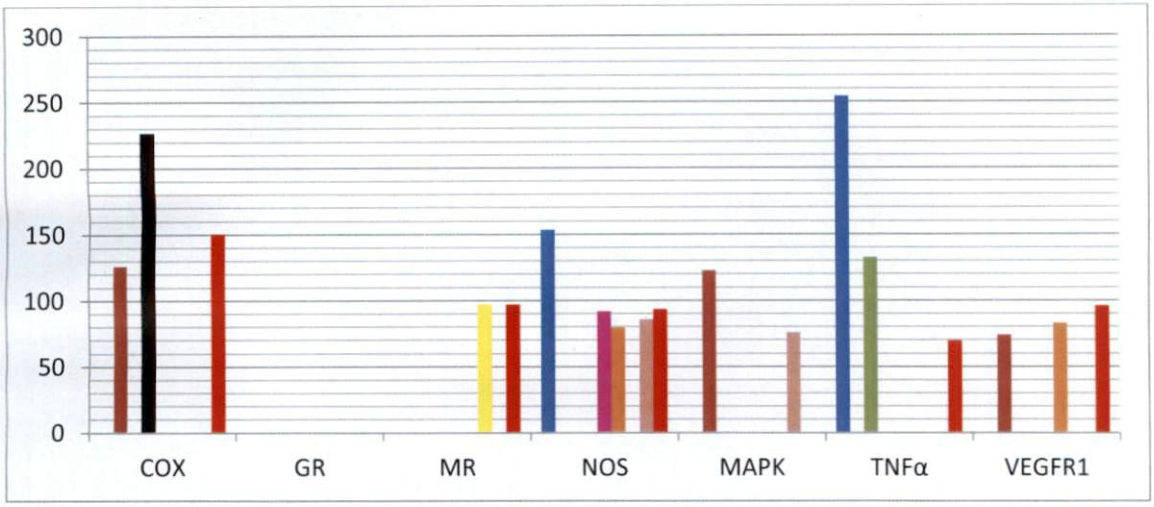


Fig 16: Negative binding energy (Kcal/mol) displayed by non- *Allium* specific compounds against different arthritis and inflammation protein targets

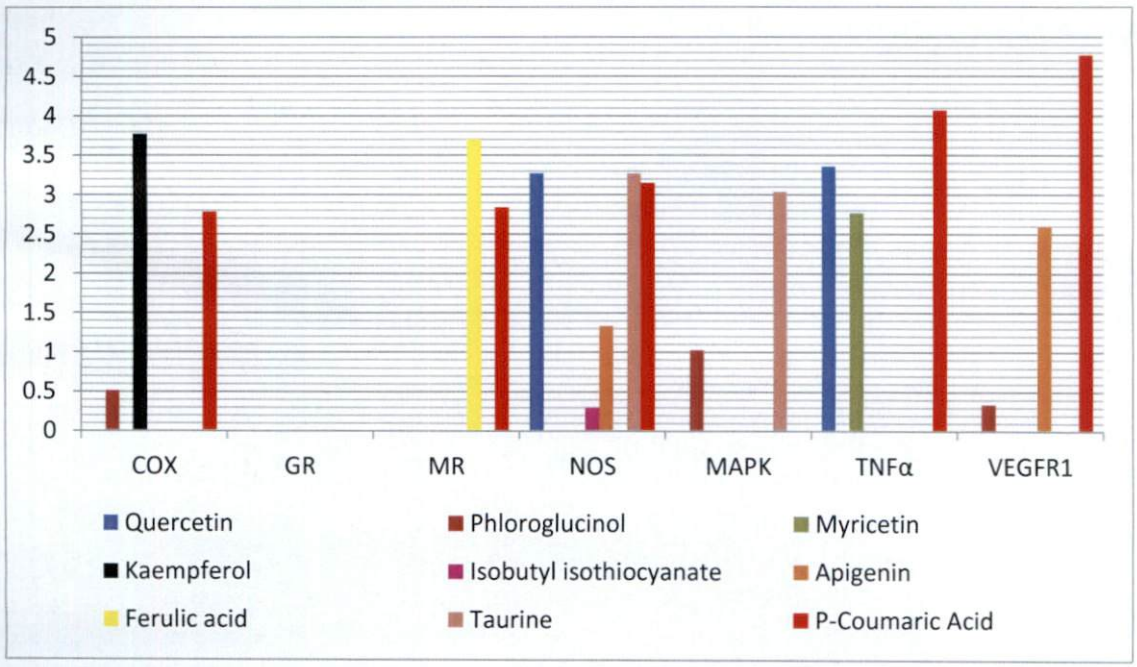


Fig 17: Deviation of CDOCKER and CDOCKER interaction energies observed for non- *Allium* specific compounds while interacting with different arthritis and inflammation protein targets

observed to score the lowest binding energy (-254.25 Kcal/mol) against target TNF α with a deviation of 3.36 Kcal/mol connecting with the active site residue Tyr151 (Table 4.42). The maximum deviation observed was below 5.0 Kcal/mol between CDOCKER and CDOCKER interaction energies for all the arthritis and inflammation targets (Fig. 15 and Fig. 17).

COX-2 is an enzyme which is found unexpressed under normal conditions in most cells, but elevated levels are found during inflammation (Elder and Paraskeva, 1999). Nitric oxide synthase (NOS) is an enzyme catalyzing the production of Nitric Oxide (NO) along with proinflammatory cytokines (eg. IL-1, TNF-α and Interferon δ) and is involved in immune defence against pathogens. Increased levels of NO activity have been found in the synovial fluid of patients with rheumatoid arthritis (Nagy *et al.*, 2010). Overall, NO plays a critical role during inflammation and therefore, remains a potential target for developing therapeutics for inflammatory diseases (Kobayashi, 2010). Lee *et al.* (2012) found that sulfur-containing compounds from garlic attenuated the LPS-induced expression of NO synthase (NOS) and cyclooxygenase-2 (COX-2) proteins and mRNA. Furthermore, it was also observed that they markedly inhibited the LPS-induced phosphorylations of p38 mitogen-activated protein kinases.

Most of the interacting garlic phytocompounds formed hydrogen bonding with active site residues Arg120 and Tyr355 of COX2, which indicated a correct interaction (Table 4.37). It was found that during the comparative study, the commercial drug diclofenac used as COX2 inhibitor made no hydrogen bonds with the target. Diclofenac is a commonly prescribed non-steroidal anti-inflammatory drug (NSAID) that acts via inhibition of COX1 and COX2 enzymes. However, extensive research showed that sometimes the pharmacological activity of diclofenac goes beyond COX inhibition and include different modes of action. Research suggested that diclofenac can inhibit the thromboxane prostanoid receptor, affect arachidonic acid release and uptake, inhibit lipoxygenase enzymes, PPAR gamma, N-methyl D aspartate receptor (NMDA) and block acid sensing ion channels (Gan, 2010). So, it can be inferred

that instead of inhibiting COX, diclofenac would be inhibiting the above mentioned targets, because of which it may have not shown any hydrogen bonding with COX2. Similarly, Cys200 and Tyr491 were identified as the most important active site residues for the target NOS (Table 4.40). As there is no commercial drug available for NOS in market, no comparative study has been done.

TNF Alpha is a cell signaling protein (cytokine) involved in systemic inflammation. The primary role of TNF is in the regulation of immune cells. Dysregulation of TNF alpha promotes inflammatory response, which, in turn, causes many clinical problems associated with autoimmune disorders such as rheumatoid arthritis, inflammatory bowel disease and psoriasis (Mewar and Wilson, 2011). Ho and Su (2014) evaluated the anti-neuroinflammatory capacity of raw garlic on lipopolysaccharide (LPS)-stimulated BV2 microglia, in which they found that raw garlic dose-dependently attenuated the production of tumor necrosis factor (TNF)-α and suggested that the anti-neuroinflammatory capacity of raw garlic is due to alliin-derived organo sulfur compounds. Other compounds such as myricetin, s-allyl mercapto cysteine, p-coumaric acid, l-γ-glutamyl-s-allyl-l-cysteine, s-allyl l-cysteine and s-allyl d-cysteine also showed interaction with amino acid residues Tyr151, Leu120 and Gly121 of active site no. 1 in TNFα, but yielded poor binding energies.

P38 Mitogen activated protein kinase 14 (MAPK14) is a stress activated enzyme. Cellular stress produced due to heavy network of signal transduction leads to over expression of MAPKs. The p38 MAPK pathway is a key regulator of pro-inflammatory cytokines biosynthesis at the transcriptional and translational levels, which makes this pathway a potential target for the treatment of autoimmune and inflammatory diseases (Clark and Dean, 2012). In the present study, it was found that *Allium* compounds interacted with MAPK14 with mediocre binding energies. Phloroglucinol is the compound which showed the least binding energy (-122.83 Kcal/mol) with a deviation of 1.03 Kcal/mol connecting with residues Thr106, Ala172 and Glu71, out of which Ala172 was found to be a non-critical residue (Table 4.41). Other compounds like s-allyl d-

cysteine and 2-methyl benzaldehyde showed interactions with amino acid residue Asp168 yielding poor binding energies. The compounds s-allyl l-cysteine, alliin and taurine interacted with non-essential amino acid residues.

Docking results of *Allium* compounds with target proteins like Glucocorticoid Receptor, Mineralocorticoid Receptor and Vascular Endothelial Growth Factor Receptor 1 was found not satisfactory due to poor binding energy.

Thus it was found that the *Allium* specific compounds such as s-allyl cysteine, alliin and s-allyl mercapto cysteine have a profound effect on all the lifestyle diseases studied. Similarly, good effect of curing these diseases was also found for p-coumaric acid, kaempfeol and apigenin. These compounds are found in abundant quantities in garlic (Table 2.1) and are potent phytocompounds to be developed as drugs.

5.2 Wet lab analysis

In silico studies of the interaction of 32 different targets involved in various lifestyle diseases such as cancer, diabetes, arthritis, blood pressure and cholesterol with several *Allium* and non-*Allium* based compounds present in garlic revealed the medicinal effects of garlic on lifestyle related diseases. These studies had to be evaluated by wet lab analysis. On the basis of good dock scores as evaluated in *in silico* studies, 4 phytocompounds from garlic were selected for further evaluation through laboratory assys. The compounds selected were alliin/ s-allyl cysteine sulfoxide (SACS), s-allyl cysteine, p-coumaric acid and ferulic acid. Three cell lines were selected for cytotoxicity assay (1 human cell line-HCT 15 and 2 murine cell lines L929 and Raw 264.7). MTT assay was performed as a long term (48 hours incubation) cytotoxicity assay to determine the toxic effects of all the 4 compounds on the above mentioned cancer cell lines.

5.2.1 *In vitro* cytotoxicity assay

In the present study, as observed from the results of *in silico* studies, 4 phytocompounds from garlic viz. alliin, s-allyl cysteine, p-coumaric acid and

ferulic acid were used for cytotoxicity assays of 3 cell lines: HCT 15 (human cell line), L 929 (murine cell line) and Raw 264.7 (murine cell line). Alliin and s-allyl cysteine are exclusively allyl origin, whereas p-coumaric acid and ferulic acid are available in almost all plant species along with garlic in quite a high quantity. When all these phyto-compounds were checked for cytotoxicity analysis on the above mentioned cell lines, it was observed that all these compounds were able to kill cancer cells in a dose dependent manner. All the phytocompounds were shown to be toxic to cancer cells at a higher dose. The pure *Allium* compounds such as alliin and s-allyl cysteine recorded showing half maximal inhibitory concentration (IC_{50} value) at a range between 70 to 100 µg/ml, whereas for polyphenolic organic acids such as p-coumaric acid and ferulic acid the IC50 values were from 145 µg/ml to over 250 µg/ml. Organic acids upto a concentration of 100 µg/ml had shown no effect on all the cancer cells.

HCT 15, the human colon cancer cell line was found to be less sensitive to both exclusive *Allium* compounds alliin and s-allyl cysteine (IC50 value 99 µg/ml and 100 µg/ml respectively) at the maximum experimental concentration (100 µg/ml) (Fig. 18 and Fig. 19). But with the same allyl compounds, both the 2 murine cell lines showed half maximal inhibition at a concentration quite lower than that for HCT-15. L 929 showed IC_{50} value at a concentration of 79 µg/ml and 78 µg/ml for alliin and s-allyl cysteine respectively. In case of p-coumaric acid, all the 3 cell lines showed a huge variation of IC_{50} value (Fig 20). Here also, HCT 15 was found to be less sensitive to p-coumaric acid and found a half maximal inhibition at a concentration of 222 µg/ml. Raw 264.7 showed IC_{50} at much lower conc. (145 µg/ml) of p-coumaric acid as compared to the response of HCT 15. L 929 was found half minimal inhibited at a conc. of 185 µg/ml of p-coumaric acid. In case of ferulic acid, HCT 15 showed a steepy curve of linear death at a dose dependent manner (Fig 21). The IC50 value was obtained at 150 µg/ml, whereas the same compound was found less effective to kill L 929 and Raw 264.7 upto a conc. of 250 µg/ml (Table 4.44).

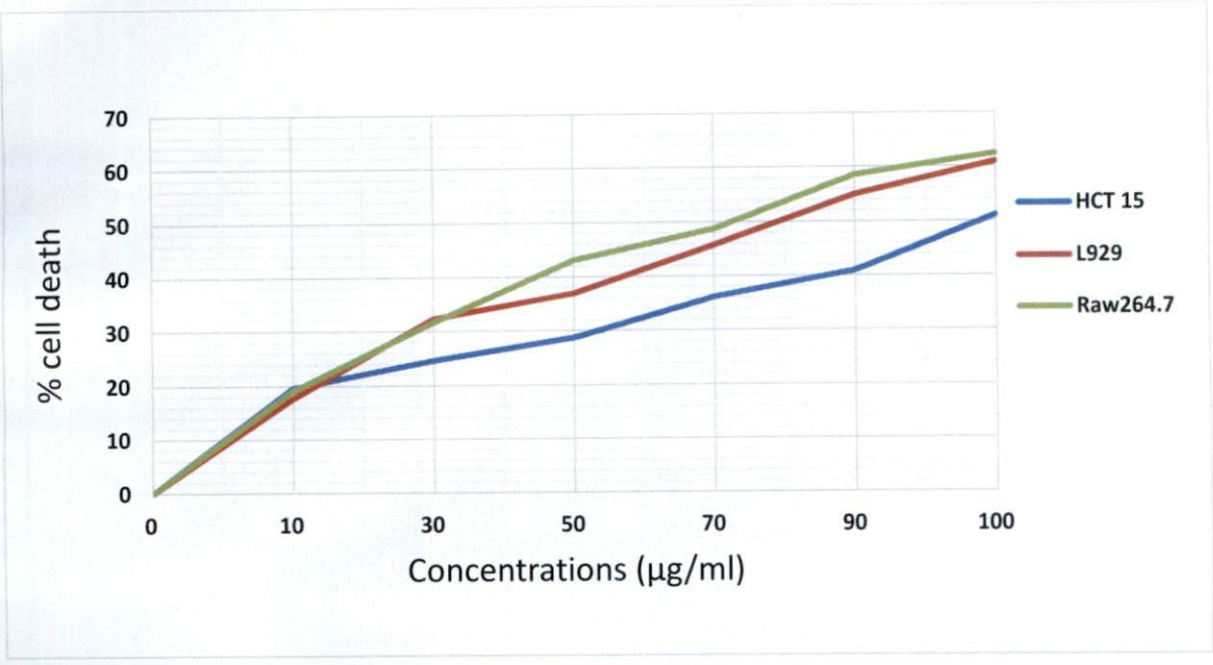


Fig 18: Effect of Alliin on different cancer cell lines

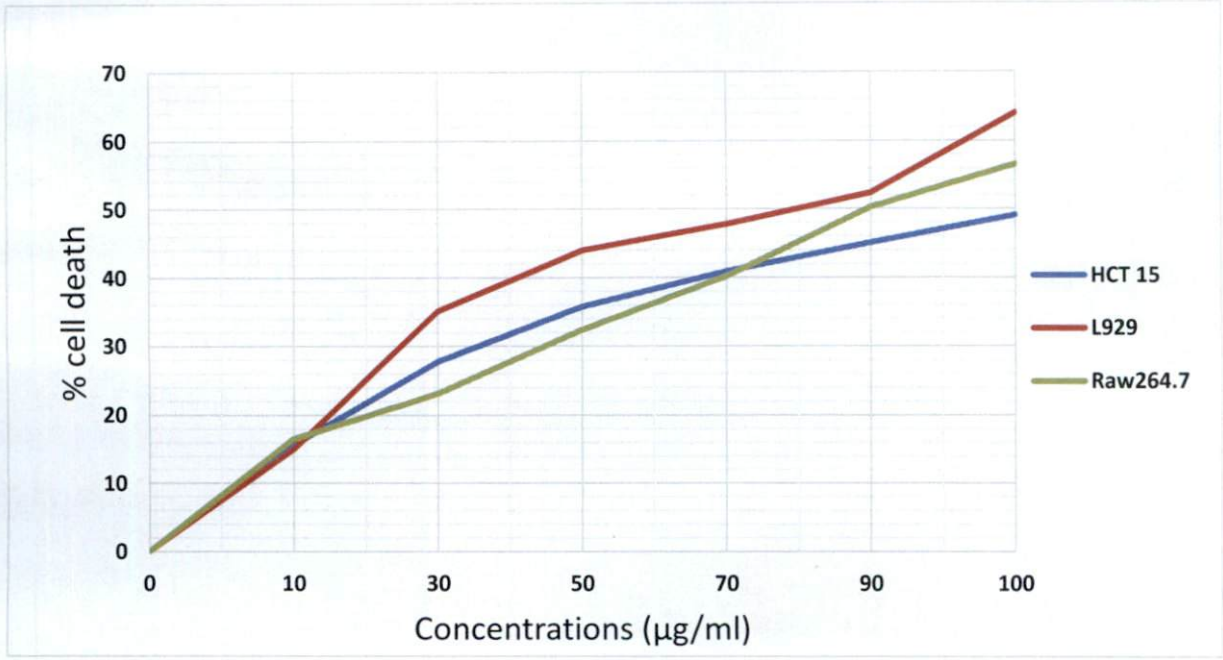


Fig 19: Effect of SAC on different cancer cell lines

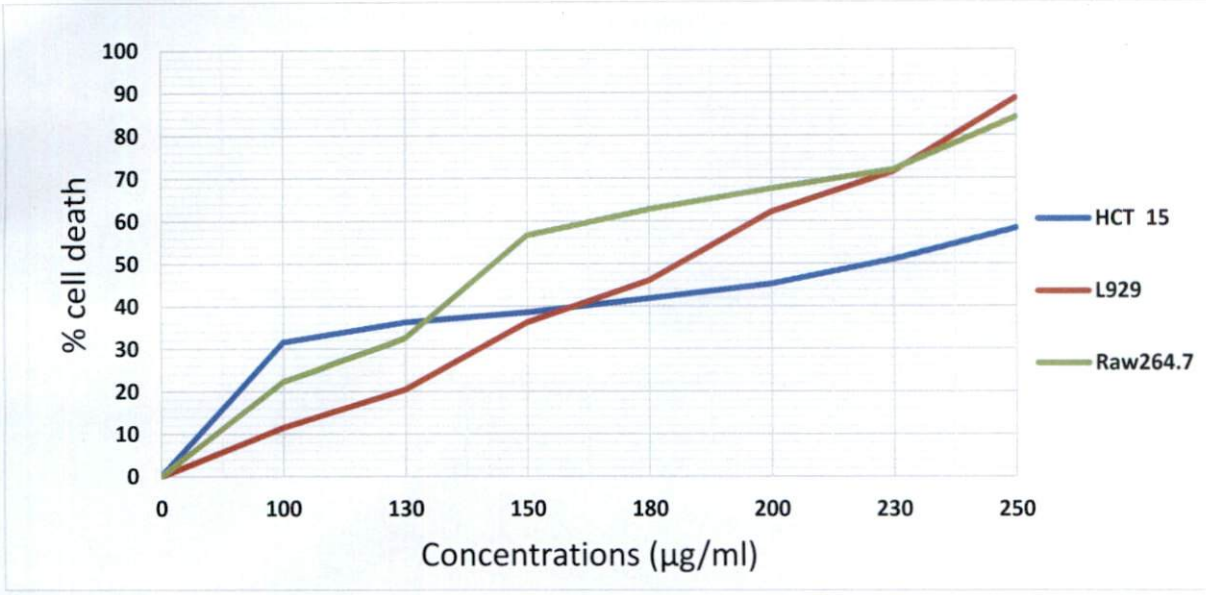


Fig 20: Effect of p-Coumaric acid on different cancer cell lines

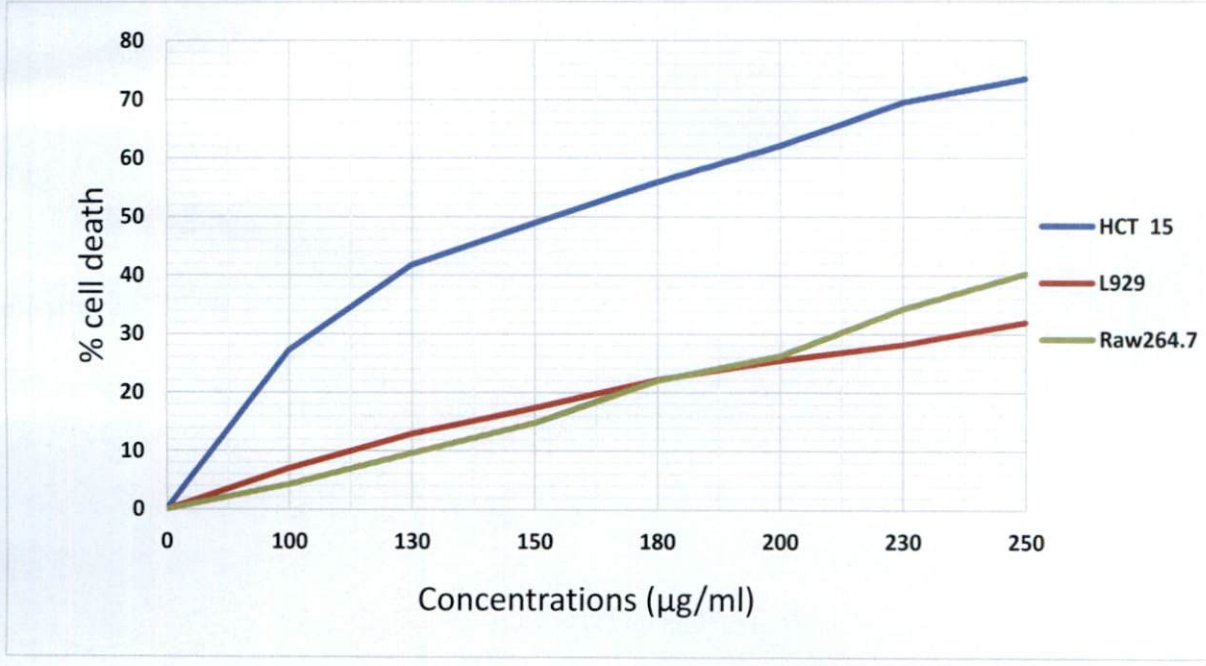


Fig 21: Effect of Ferulic acid on different cancer cell lines

Garlic contains compounds of sulphur origin and polyphenolic organic acids (hydroxycinnamic acids) which have the ability to cure cancer. Garlic in total acts as an antioxidant by increasing antioxidant enzyme activity, reducing reactive oxygen species generation, and protecting proteins and lipids from oxidation. Basically, when cytotoxicity assays of some drugs over some cancer lines are done, there may be several mechanisms working behind killing of the cells (Dvořáková *et al.*, 2015).

The anti-proliferative effects of garlic compounds such as alliin and s-allyl cysteine over the cell lines may be mediated by several mechanisms. A large amount of studies have confirmed the ability of garlic to induce cell cycle arrest, which could be a mechanism of carcinogenesis inhibition. Yuan *et al.* (2004) reported that di-allyl disulfide, an organosulfur compound from garlic induced G2/M arrest of human gastric cancer MGC803 cells via activation of p38 MAPK kinase oxidative stress pathway. There was a decreased expression of Cdc25 protein expression by p38 MAPK found out by flow cytometry analysis, which played a crucial role in cell cycle arrest. A number of reports have described activation of p38 MAPK pathway during apoptosis. This pathway is activated in response to cytokines and stress signals leading to oxidative stress. c-Jun NH2 terminal kinase (JNK) is another partner of p38 MAPK in promoting apoptosis (Zhang *et al.*, 2013). Garlic polysulfides induce oxidative stress in cancer cells through increased production of ROS. Enhanced oxidative stress triggers apoptotic signalling pathway. Filomeni *et al.* (2003) reported that DADS enhances ROS generation and therefore activates the c-Jun N-terminal kinase (JNK) pathway, which triggers cell death in neuroblastoma cells. Human nasopharyngeal carcinoma CNE2 cells when treated with garlic compound DADS, it induced apoptosis by transient increase of phosphorylated MAPKs. A potent increase of nearly 9 fold of apoptotic cells had been seen in a time and concentration dependent manner. Garlic organosulfur compounds can also induce cell cycle arrest in prometaphase, which is connected with hypermethylation of key subunits of the anaphase-promoting complex/ cyclosome (APC/C) (Herman-Antosiewicz *et*

al., 2007). Shin *et al.* (2014) reported that diallyl-trisulfide (DATS) induced apoptosis of bladder cancer cells T24 by activation of caspase-8 and caspase-9, the respective initiator caspases of the extrinsic and intrinsic apoptotic pathways. Caspases are a family of cysteine proteases that play an essential role in apoptosis. Caspases are synthesized as inactive pro caspases that consist of death effector domain which enables clustering of death initiator caspases to activate executioner caspases to start apoptotic pathway. Bat-Chen *et al.*, (2010) reported that allicin exerts cytotoxic effects on HCT116 and induces apoptosis via a mechanism associated with transactivation of the transcription factor Nrf2. Nrf2 (Nuclear factor erythroid derived 2) is a transcription factor that regulates the expression of anti-oxidant proteins that protect against oxidative damage. Under normal conditions, Nf2 is kept in cytoplasm by a cluster of proteins that degrade it quickly, whereas under stress conditions Nrf2 is not degraded and travels to nucleus where it binds to DNA promoters and initiate transcription of antioxidative genes and their protiens such as NADPH dehydrogenase and Glutathione S transferase. These anti oxidative proteins help in killing cancer cells by acting as detoxification agents. Ng *et al.*, (2012) reported that SAC has anti-proliferation activity on HCC cell line MHCC97L of hepatocellular carcinoma. SAC significantly down regulated antiapotoptic proteins (BclxL and Bcl2) and induced caspase3 and caspase9 mediated apoptosis and necrosis. Additionally SAC down regulated cdc25c, cdc2 and cyclin B1 inducing the S phase arrest of MHCC97L cells. These are the several mechanisms via which garlic may act in killing cancer cells.

Similarly, there may be several mechanisms acting for the anti-proliferative effect of these organic acids on cancer cells. Rocha *et al.* (2012) in a review described about several mechanisms involved in the anti-cancer properties of hydroxycinnamic acids. Hydroxycinnamic acids are important antioxidants associated with oxidative stress such as cancer and cardiovascular diseases. The most important hydroxycinnamic acids prevalent in nature are caffeic acid, p-coumaric acid, ferulic acid, vannilic acid, gallic acid, syringic acid, sinapinic acid

etc. Puangpraphant *et al.* (2011) reported that these organic acids inhibited the proliferation of RKO and HT 29 human colon cancer cell lines by inducing apoptosis rather than arresting cell cycle. Apoptosis occured through induction of the ratio of Bax: Bcl2 protein expression and induced the cleavage of procaspase 3 to active caspase 3, which is a key step of apoptosis. Wang *et al.* (2005) reported that when HCT 116 cell line was exposed to hydroxycinnamic acid such as caffeic acid at different concentrations, caffeic acid displayed a strong growth inhibitory effect in a dose dependent manner against HCT 116. Flow cytometry analysis showed that the ratio of G0/G1 phase cells increased and S phase ratio decreased. G_0 / G_1 phase is the quiescent phase or resting phase of cell cycle where the cells do not divide, whereas S phase is the phase in which DNA start replicating. Janicke *et al.* (2011) investigated the effects of ferulic acid and p-coumaric acid in the gene expressions in Caco2 colon cancer cells. It was found that treatment with FA and pCA delayed cell cycle progression and regulated the expression of a number of genes involved in centrosome assembly. FA treatment downregulated the expression of CCNB1 gene, the product of which is Cyclin B1 and this protein is required for G_2 phase progression and G_2/M transition. FA treatment upregulated the expression of WEE 1 gene which negatively regulates the entry of cell cycle into mitosis phase. Thus, the upregulation and downregulation of these genes prevented the proliferation of Caco2 colon cancer cells. pCA treatment on Caco2 cell lines also did a significant modulation of mRNA levels of genes involved in cell cycle progression. pCA treatment upregulated the expression of genes such as CDKN1A and CCNG2 which have inhibitory role in cell cycle progression, whereas pCA treatment downregulated gene expressions of MYC, CDK4, CCNB1, CDC25A which have a stimulatory role on cell cycle progression. Thus, it can be told that in the present study when FA and pCA treatment was given on HCT 15, the compounds might have acted in the above mentioned way. Sudheer *et al.* (2007) demonstrated that FA at a concentration of 150 µM counteracted nicotine induced lipid peroxidation and glutathione depletion in rat lymphocytes. Nicotine had been shown to significantly impair the antioxidant cell defense system, but administration of FA

counteracted nicotine induced decrease in superoxide dismutase catalase and gluthatione peroxidase.

5.2.2 RT-qPCR analysis for gene expression

Real time PCR analysis was done for doing gene expression studies. EGFR was the gene selected for human colon cancer cell line HCT 15. Epidermal growth factor receptor (EGFR) is a tyrosine kinase receptor which is always found over expressed on the membrane of epithelial cancer cells and counts for nearly 60-80 % of colon cancer cases. EGFR activated signal cascade lead to increased proliferation of cancer cell and provides resistance to pro apoptotic stimuli. In the present study, this gene was selected for expression studies by use of real time PCR. Phytocompounds from garlic were applied at 2 doses: one at higher dose and another at lower dose of the IC50 value (for alliin and s-allyl cysteine 80 and 120 µg/ml, for p-coumaric acid 200 and 250 µg/ml and for ferulic acid 125 and 175 µg/ml). The expression of target gene EGFR was quantified using a ΔΔCT method while comparing to endogenous housekeeping gene β actin.

The ΔΔCT values obtained for EGFR with alliin (80 and 120 µg/ml) were found to be 3.45 and -0.34 respectively which indicates that with lower dose of alliin, the expression of EGFR was downregulated by 3.45 fold and for higher dose it was upregulated by 0.34 fold (Table 4.45). Currently the reason behind the effect of alliin in this way cannot be explained but it can be said that as a cytotoxic drug alliin might have tried to upregulate the expression of EGFR to enhance their susceptibility to lymphokine activated killer (LAK) mediated antibody dependent cell mediated toxicity (ADCC). Correale *et al.* (2010) reported this mechanism for a cytotoxic drug cetuximab, which is a monoclonal antibody used against EGFR for treatment of colon, neck, head and non-small cell lung cancer. In the study, the colon carcinoma cell lines were assessed in cytotoxic assays for their sensitivity to cetuximab mediated ADCC for which interleukin 2 activated LAK cells were used as immune effectors. The results showed the ability of cytotoxic drug combinations to upregulate EGFR expression

on the surface of colon carcinoma cells, enhancing their sensitivity to cetuximab mediated ADCC by LAK cells. The enhanced expression of EGFR made the tumour cells more sensitive to EGFR immune targeting strategy.

The ΔΔCT values obtained for EGFR with SAC (80 and 120 µg/ml) were found to be 3.85 and 1.28 respectively which indicates that lower dose of SAC could effectively downregulate the action of EGFR gene expression on HCT 15 by 3.85 folds, whereas for a higher dose of SAC, the expression of EGFR gene was decreased by only 1.28 fold (Table 4.45). Currently the reason behind this contradicton cannot be explained, but it may be said that the preliminary studies conducted only once on *Allium* compounds were not sufficient to give a proper conclusion. As the fund for procuring costly phyto-chemicals from garlic was limited, it is suggested that the mechanism of action of these *Allium* compounds on cancer has to be further evaluated through repeated assays.

The ΔΔCT values obtained for EGFR with p-coumaric acid (200 and 250 µg/ml) were found to be 0.28 and 0.46 respectively which indicated that dose dependently p-coumaric acid was able to downregulate the expression of EGFR gene on HCT 15 cancer cell line. Similarly, in case of ferulic acid (125 and 175 µg/ml) the ΔΔCT values obtained for EGFR were 3.89 and 4.28 respectively, which inferred that ferulic acid dose dependently down regulated the expression of EGFR gene on HCT 15 (Table 4.45). The effect of ferulic acid on HCT 15 was found to be higher on HCT 15 compared to p-coumaric acid as ferulic acid at a concentration of 175 µg/ml could decrease the expression of EGFR by 4.28 folds, whereas p-coumaric acid at a concentration upto 250 µg/ml could decrease the expression of EGFR gene upto 0.46 fold only. Repeated assays was done for both ferulic acid and p-coumaric acid which revealed the anti-oxidant property of these compounds from garlic on cancer cells.

Results of the present study could screen out the important phytocompounds in garlic having inhibitory action on lifestyle diseases such as cancer, diabetes, blood pressure, cholesterol and arthritis. Molecular docking could be suggested as

an effective tool for screening out important phytocompounds since the *in silico* results were validated through wet lab analysis. Much more research is needed for a fruitful outcome and the future line of work suggested include:

I. Better validation of degree of regulation of EGFR through repeated assays
II. Validation of in silico results on other target proteins through cell line studies and animal models
III. Analysis of effect of garlic phytocompounds on expression of other genes involved in metabolic disorders
IV. Development of garlic based pharmaceutical drugs

Summary

6. SUMMARY

The study entitled "Detection of novel metabolites in garlic (*Allium sativum* L.) through *in silico* analysis and its validation" was carried out in 2 stages viz. *in silico* analysis and wet lab validation. *In silico* analysis was carried out at Distributed Information Centre (DIC), CPBMB, KAU, whereas the wet lab experiments were carried out at Amala Cancer Research Centre, Thrissur.

In silico work was based on molecular docking, where phyto compounds from garlic were interacted with various targets involved in lifestyle diseases such as cancer, diabetes, arthritis, blood pressure and cholesterol. This work was done using a commercial software "Discovery Studio version 4.0" following the protocol of CDOCKER. Four compounds with positive interactions were selected for validation through wet lab experiments such as MTT cytotoxicity assay and Real time PCR gene expression assay.

The findings of the study are as follows:

i) Fourty eight phytocompounds with medicinal properties were identified in garlic through Pubmed survey.

ii) Thirty two proteins responsible for causing different lifestyle diseases such as cancer, diabetes, blood pressure, cholesterol and arthritis were identified along with their mechanism of action in causing the diseases.

iii) Three dimensional structure of both proteins and phytocompounds were retrieved from biological databases such as PDB, Pubchem amd Chemspider.

iv) Active site region for interaction were identified for all the protein targets.

v) Phytocompounds were filtered through Lipinski's rule of 5 and Veber's protocol and all of them passed the screening, indicating their drug likeliness properties.

vi) ADMET screening was done to check the bioavailibilty of all the 48 phytocompounds, and all except taurine and campesterol qualified five out of seven parameters.

vii) The results of molecular docking revealed the combined action of many compounds present in it, towards curing of various life style diseases by interacting with various targets of these diseases.

viii) Seventeen garlic phytocompounds were found to inhibit all the fifteen protein targets identified for cancer. Maximum inhibition was observed with protein targets such as DNA Topoisomerase, EGFR, PI3Kγ and Thymidilate synthase. The compounds like S allyl L cysteine, S allyl mercapto cysteine and Apigenin were observed in common to interact with the above mentioned 4 targets with a very low binding energy. S-allyl l –cysteine was found to inhibit 13 out of 15 targets identified for cancer.

ix) Thirteen phytocompounds in garlic were observed to have inhibitory effect on six protein targets identified for diabetes out of which 8 compounds interacted with at least 3 targets of the 6 studied. Good interaction was observed with targets such as Glucokinase, DPP4 and Aldose reductase. S-allyl l-cysteine was found to inhibit 5 out of 6 targets studied.

x) Eleven phytocompounds in garlic were found to inhibit three target proteins (adrenergic receptor, angiotensin converting enzyme and carbonic anhydrase) involved in enhancing the blood pressure. Best interaction was observed with Angiotensin Converting Enzyme (ACE). Alliin derivatives along with phloroglucinol and p-coumaric acid recorded good interaction.

xi) Phytocompounds of garlic such as s-allyl l-cysteine, s-allyl d-cysteine, cycloalliin, phloroglucinol, apigenin and p-coumaric acid showed good interaction with HMG CoA reductase, the important target involved in cholesterol biosynthesis.

xii) Sixteen garlic phytocompounds were found to have inhibitory action on seven protein targets involved in arthritis and inflammation were used for the docking study, out of which best dock scores were obtained for Cyclooxygenase 2 (COX2) and Nitric Oxide Synthase (NOS). Compounds such as s-allyl l-cysteine, s-allyl d-cysteine, alliin and p-coumaric acid recorded good interaction.

xiii) Based on the *in silico* analysis, two *Allium* specific organosulfur compounds s-allyl cysteine and alliin and two anti-oxidants ferulic acid and p-coumaric acid were used for wet lab validation.

xiv) Cytotoxicity assays (MTT) were conducted on 3 cell lines HCT 15, Raw 264.7 and L929. All the four compounds in garlic are efficient in killing the cells. The IC_{50} values of the *Allium* specific compounds were low in a range of 70 to 100 µg/ml, whereas for non-*Allium* compounds (anti-oxidants) it was high and found in a range of 145 µg/ml to over 250 µg/ml.

xv) As a part of gene expression studies, EGFR was selected to be checked for upregulation or downregulation by the 4 test drugs (phyto-compounds) from garlic via Real Time PCR technique.

xvi) The ΔΔCT values were calculated in gene expression analysis through RT-qPCR for EGFR with *Allium* specific compounds such as alliin (80 and 120 µg/ml) and SAC (80 and 120 µg/ml). Results indicated that lower doses downregulated the gene's activity more than that of the higher doses. Repeated assays are suggested for the work.

xvii) The ΔΔCT values obtained for EGFR with non-*Allium* compounds such as p-coumaric acid (200 and 250 µg/ml) and ferulic acid (125 and 175 µg/ml) indicated that the higher doses downregulated the gene activity.

xviii) Results of the present study highlight the inhibitory effect of garlic phytocompounds on important protein targets involved in lifestyle diseases such as cancer, diabetes, blood pressure, cholesterol and arthritis. Wet lab studies confirmed the *in silico* results. Medicinal

properties of garlic could be attributed to the combined action of more than fifteen phytocompounds rather than the superior effect of any specific compound. However, alliin and five other derivatives found in garlic had inhibitory action on all the protein targets studied.

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Annexures

ANNEXURE I

List of chemicals and other items used for wet lab studies

Sl. No.	Product Name	Company name
1.	RPMI (Roswell Park Memorial Institute) 1640 Liquid media	Sigma Aldrich
2.	HEPES buffer	Himedia
3.	Fetal Bovine Serum (FBS)	Life Technologies
4.	Sodium Pyruvate	Himedia
5.	Thiazolyl Blue Extrapure (MTT)	SISCO research laboratories
6.	Dimethyl sulfoxide	Himedia
7.	Antibiotics (Penicillin and Streptamycin)	Sigma Aldrich
8.	L-Alliin	Sigma Aldrich
9.	S-Allyl cysteine	Sigma Aldrich
10.	Ferulic acid	Sigma Aldrich
11.	p Coumaric acid	Sigma Aldrich
12.	cDNA synthesis kit	Invitrogen
13.	SYBR premix ex Taq	Takara Bioindia
14.	25 cm^2 sterile tissue culture flask	Tarson
15.	96 sterile well plate	Tarson
16.	6 sterile well plate	Tarson

ANNEXURE II

List of laboratory equipment/ software and machineries used for the study

Sl. No.	Items	Procured from
1.	Discovery Studio 4.0	Accelrys, USA
2.	Centrifuge	Eppendorf, Germany
3.	Incubator	JEIO Tceh, Korea
4.	Thermal cycler	Bio-Rad, USA
5.	Laminar air flow	Labline industries, India
6.	Inverted microscope	Nikon, Japan
7.	ELISA reader	Molecular devices, USA
8.	7300 real time PCR	Applied Biosystems, USA

ANNEXURE III

Chemical composition of medium used for cell culture studies

Sl. No.	Reagents	Quantity
1.	RPMI liquid media	1000 ml
2.	Fetal Bovine Serum	100 ml/lt of media
3.	Sodium pyruvate	10 ml/lt of media
4.	Glutamic acid	10 ml/lt of media
5.	Glucose	4.5 gm/lt of media
6.	HEPES buffer	10 ml/lt of media
7.	Penicilium and Streptomycin	100 IU/lt of media

Annexure IV

Composition of Phosphate Buffer Saline (PBS) used for cell culture studies

Sl. No.	Reagents	Quantity
1.	Nacl	8 gm
2.	$Na_2HPO_4.2H_2O$	1.44 gm
3.	KH_2PO_4	0.2 gm
4.	KCl	0.2 gm
5.	Distilled water	1000 ml

Abstract

DETECTION OF NOVEL METABOLITES IN GARLIC (*Allium sativum* L.) THROUGH IN SILICO ANALYSIS AND ITS VALIDATION

By

NABARUN ROY

(2013-11-106)

ABSTRACT OF THE THESIS

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Abstract

Garlic belonging to Alliaceae family, is an important spice crop used from long back in Ayurveda and other forms of medicine for treatment of various ailments such as cancer, diabetes, cardiovascular diseases etc. The medicinal properties of garlic are mainly due to the presence of organosulfur compounds and several polyphenolic compounds. The major organosulfur compounds in garlic include alliin, allicin, l-γ-glutamyl-s-allyl-l-cysteine, s-allyl cysteine, s-allyl mercapto cysteine, allyl mercaptan, diallyl disulfide, diallyl trisulfide, allyl propyl disulfide, vinyldithiin etc. L-γ-Glutamyl-S-allyl-L-cysteine and alliin are the precursor molecules of all other compounds, the breakdown of which produce other compounds by several reactions with the help of enzyme allinase. Polyphenols like apigenin, quercetin, phloroglucinol, p-coumaric-acid, ferulic-acid, sinapic acid etc. also contribute to the medicinal effects of garlic. Though the medical literature for garlic is well established, the exact compounds from garlic against various diseases is not much experimented.

The study entitled "Detection of novel metabolites in garlic (*Allium sativum* L.) through *in silico* analysis and its validation" was taken up to analyze the medicinal effects of important compounds in garlic by inhibiting the targets involved in lifestyle diseases such as cancerous, cardiovascular, arthritic, hypercholesterol and diabetic complications through *in silico* molecular docking analysis and validation through wet lab analysis. The experiment was performed using a commercial software Discovery Studio version 4.0 at Distributed Information Centre (DIC), CPBMB, KAU. For the study, 32 protein targets involved in different diseases were selected. Wet lab experiments such as *in vitro* cell line cytotoxicity test was conducted at Amala Cancer Research Centre, Thrissur and at CPBMB, KAU. Gene expression studies were conducted for validating the anti- cancerous properties of garlic compounds through RT-qPCR.

Seventeen compounds from garlic both of allyl nature and polyphenols exhibited positive interaction with the targets selected for the lifestyle diseases. The important targets most inhibited by garlic phytocompounds were DNA

topoisomerase, Epidermal growth factor receptor (EGFR), Phosphoinositide 3-kinase Gamma (PI3Kγ) and Thymidilate synthase for cancer; Glucokinase (GK) and Dipeptidyl peptidase (DPP4) for diabetes; Cyclooxygenase 2 (COX2) and Nitric Oxide Synthase (NOS) for arthritis and inflammation; and Angiotensin Converting Enzyme (ACE) and HMG CoA reductase for blood pressure and cholesterol. It was found from the dock scores obtained, that s-allyl cysteine (SAC), alliin/ s-allyl cysteine sulfoxide (SACS), ferulic acid (FA) and p-coumaric acid (pCA) were found superior to other compounds and were found to inhibit most of the targets.

These compounds (SAC, SACS, FA and pCA) which gave positive interaction in docking studies were procured in their pure form (upto 98% HPLC grade) from Sigma Aldrich and used for cell line studies. The three cancer cell lines HCT-15, Raw264.7 and L929 exhibited dose dependent cytotoxicity for all the four test compounds from garlic. The IC_{50} value for true *Allium* compounds such as SAC and SACS were found in the range of 71-100 µg/ml, whereas for polyphenolic compounds such as FA and pCA, was found in the range of 145-222 µg/ml. HCT-15, the human colon cancer cell line showed more sensitivity to all the compounds and so it was forwarded for gene expression studies. EGFR was the gene selected for the study, which is most commonly expressed in human colon cancer. All the compounds from garlic showed down regulation of the EGFR gene dose dependently, except for SACS.

Overall results from both *in silico* and wet lab studies indicated the medicinal effects of different compounds in garlic on various lifestyle diseases. Thus the present study gives a strong scientific background to highlight the health benefits of garlic.