SCREENNG GINGER (*Zingiber officinale* Rosc.) SOMACLONES FOR GINGEROL CONTENT AND VALIDATION OF ANTICANCEROUS PROPERTIES OF GINGEROL

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KERALA, INDIA
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DECLARATION

I hereby declare that the thesis entitled **"Screening ginger (*Zingiber officinale* Rosc.) somaclones for gingerol content and validation of anticancerous properties of gingerol"** 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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Date: 07-08-2015 (2013-11-102)

CERTIFICATE

Certified that the thesis entitled **"Screening ginger (*Zingiber officinale* Rosc.) somaclones for gingerol content and validation of anticancerous properties of gingerol"** is a record of research work done independently by **Mr. Manikesh Kumar (2013-11-102)** under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, associateship, fellowship to him.

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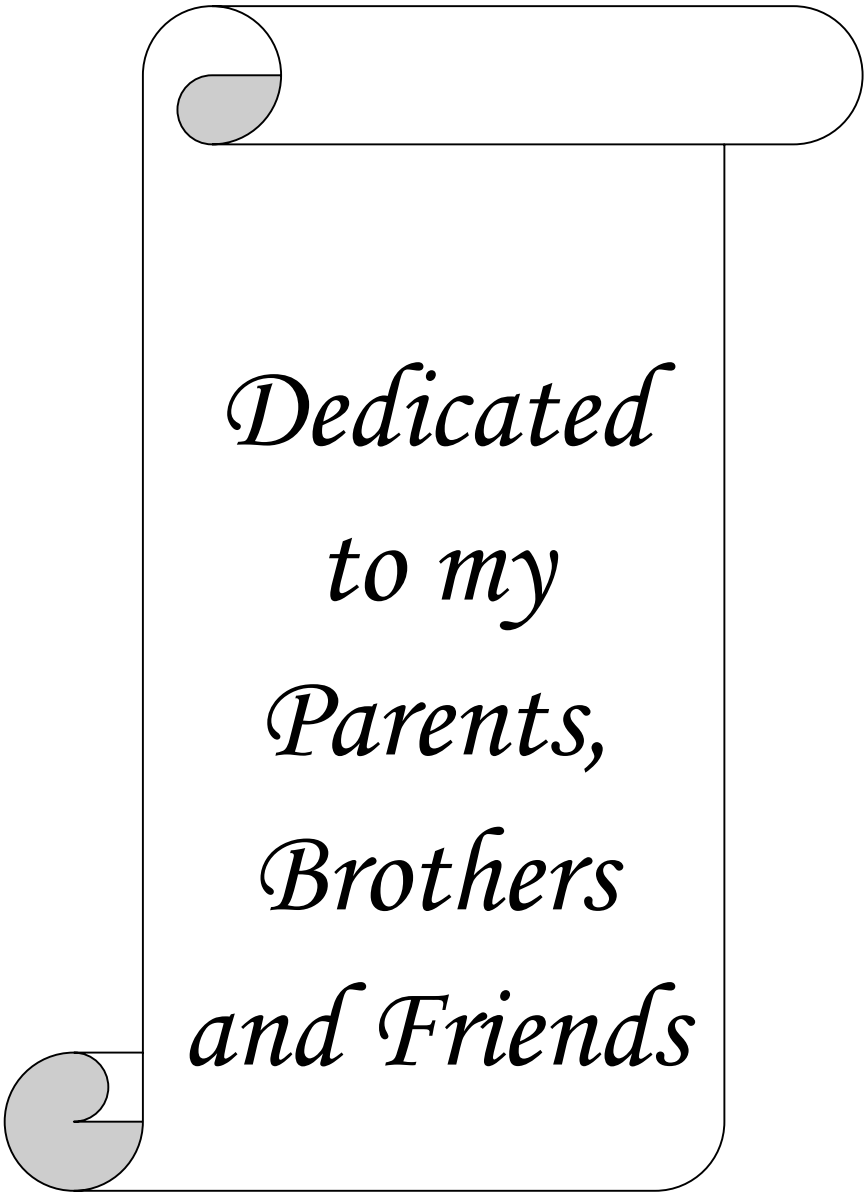
Dedicated
to my
Parents,
Brothers
and Friends

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ABBREVATIONS

%	Percentage
µg	Microgram
AFLP	Amplified Fragment Length Polymorphism
AP-PCR	Arbitrarily Primed Polymerase Chain Reaction
bp	Base pair
cc	cubic centimetre
cm	Centimetre
CPBMB	Centre for Plant Biotechnology and Molecular Biology
CTAB	CetylTrimethyl Ammonium Bromide
DNA	Deoxyribonucleic Acid
DAF	DNA Amplification Fingerprinting
DNase	Deoxyribonuclease
dNTPs	Deoxyribo Nucleoside Triphosphate
EDTA	Ethylene Diamine Tetra Acetic acid
EMS	Ethyl methane sulphonate
EST	Expressed sequence tags
g	Gram
Gy	Gamma ray
ha	Hectare
ISSR	Inter Simple Sequence Repeat
Kb	Kilo basepairs
l	Litre
M	Molar
mg	Milligram
ml	Millilitre
ME	Milling Energy
mM	Milli mole
NC	Neem Cake
ng	Nanogram

NAA	1-Naphthaleneacetic acid
ºC	Degree Celsius
OD	Optical Density
PCR	Polymerase Chain Reaction
pH	Hydrogen ion concentration
PIC	Polymorphic Information Content
pM	Pico molar
PVP	Poly vinyl pyrolidone
QTL	Quantitative Trait Loci
RAPD	Random Amplified Polymorphic DNA
RED	Recommended Dose Fertilizers
RFLP	Restriction Fragment Length Polymorphism
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Revolutions per minute
SCAR	Sequence Characterized Amplified Region
SCMV	Sugercane Mosaic Virus
SNP	Single Nucleotide Polymorphism
SSR	Simple Sequence Repeats
U	Unit
UV	Ultra violet
V	Volts
WSMV	*Wheat streak mosaic virus*
YVMV	Yellow vein mosaic virus
β	Beta
µl	Microlitre

Introduction

1. INTRODUCTION

Ginger (*Zingiber officinale* Rosc.*)* is an important spice crop with immense medicinal properties and health benefits. It is cultivated in many tropical and subtropical countries including India, China, Nigeria, Australia, Jamaica and Thailand. India is the world's leading producer with a production of 6, 83, 160 t. from an area of 1, 38,200 ha. as per 2013-2014 statistics (www.indianspices.com). During the year 2013-2014, 23,300 t. of ginger was exported, fetching a foreign exchange earning of Rs. 25,614.27 lakhs (www.indianspices.com). Ginger finds wide range of applications in traditional medicine and also in nutraceutical and health food industries. Carminative, stimulant and digestive properties are widely utilized in traditional medicine while anti-oxidant, anti-inflammatory, anti-diabetic and anti-hypercholesterolemic properties are being utilized in nutraceutical industries.

Due to its health beneficial effects ginger is exported to almost all countries and extensively consumed in fresh form and also added in many kinds of food additives and dietary supplements. The U.S. Food and Drug Administration (FDA) regards ginger as ''generally recognised as safe'' (GRAS) (Kubra & Rao, 2011). The biological activities of ginger arise from its active chemical components. Of the various compounds present in ginger, gingerols are the most potent and pharmacologically active compounds and posses anti-inflammatory, analgesic, antipyretic, gastro protective, cardiotonic and antihepatotoxic activities. Of the gingerols, the most potent and pharmacologically bioactive compound is 6-gingerol and is now a target for drug development. The 6- gingerol can induce stress in cancer cells and cause apoptotic cell death and is effective for the treatment of ovarian, gastric, postrate and colorectal cancer. Gingerol is also a therapeutic agent for motion sickness, allergic asthma, arthritis related problems and cardiovascular diseases. Gingerols are thermally labile due to the presence of a β-hydroxy keto group in the structure and undergo dehydration readily to form the corresponding shogaols.

Elaborate studies on exploitation of somaclonal variation in ginger was conducted at Kerala Agricultural University. Somaclones derived from two cultivars viz. Maran and Rio-de-Janeiro were evaluated for yield, quality and reaction to pests and diseases (Paul and Shylaja, 2009; Paul *et al.,* 2009; Paul *et al.,* 2011; Paul and shylaja, 2012). After conducting on farm evaluation and multilocational trials, three high yielding high quality somaclones were released as varieties under the names "Athira", "Karthika" and "Aswathy" (Shylaja *et al.,* 2010; Shylaja *et al.,* 2014). DNA finger printing of three released varieties and selected superior somaclones were done at CPBMB using two molecular marker systems viz. RAPD and ISSR (Pujaita, 2013). The variability of somaclones regenerated through various mode of regeneration and *in vitro* mutagenesis was analysed using molecular markers like RAPD and ISSR (Sharda, 2013).

Centre for Plant Biotechnology and Molecular Biology maintains a good collection of germplasm of ginger somaclones regenerated through direct/ indirect organogenesis/ embryogenesis and *in vitro* mutagenesis.

Bioinformatics tools are now-a-days employed to identify targets for different ligands or to identify ligands for different targets. Targets are proteins which include enzymes, ion channels, and receptors. Ligand is a substance that include substrates, inhibitors, activators and neurotransmitters forms a complex with a biomolecule to serve a biological purpose. Pharmacological investigations have revealed that ginger and its major pungent ingredients have chemopreventive and chemotherapeutic effects on a variety of cancer cell lines and on animal models (Shukla and Singh, 2007). Induction of apoptosis in human leukemia (HL-60) cells by 6-gingerol and 6-paradol was reported by Lee and Surh (1998). Kim *et al.* (2011) observed apoptotic effects of 6-gingerol in LNCaP human cancer cells based on the evaluation on prostate cancer cells and found maximum inhibition of cell viability at 0.03mM of gingerol, 48 hours after treatment.

Considering the importance of gingerol, the present study focuses to screen fifty somaclones of ginger derived from the cultivar Maran for gingerol content, to identify cancer targets for gingerols and shogoal using *in silico* tools and to validate anticancerous properties of gingerol. The study will help to locate high gingerol yielding clones, gather more information on drug development through *in silico* docking studies and assess the effectiveness of gingerol on different types of cancer cells.

Review of Literature

2. REVIEW OF LITERATURE

Ginger (*Zingiber officinale* Rosc.) is an important medicinal and culinary herb, known worldwide for its health promoting properties. Ginger rhizome is generally consumed as a fresh paste, dried powder, slices preserved in syrup, candy (crystallized ginger). In India and China fresh ginger is used to prepare vegetable and meat dishes. Ginger has been used traditionally for varied human ailments, to treat stomach upset, arthritis, rheumatism, sprains, muscular aches, pains, sore throats, cramps, constipation, indigestion, vomiting, hypertension, dementia, fever, infectious diseases helminthiasis, diarrhoea and nausea (Ali *et al.,* 2008).

Ginger is a monocotyledon, belonging to the family Zingiberaceae in the order Zingiberales. It is a herbaceous rhizomatous perennial with aromatic thick lobed pale yellowish rhizomes differing in shape and size in different cultivated types. The diploid chromosome number of ginger is 2n= 22.

Ginger has several chemical constituents present in rhizome in which 6-gingerol is important as a major active component. Shukla and singh (2007) reported 6-gingerol has strong anti-inflammatory, anti-oxidative, anti-cancerous and anti-mutagenic activities. The objectives of the present study were to screen ginger somaclones for gingerol content, to identify potential cancer targets for gingerols and shogaol and to validate of anticancerous properties of gingerol. The relevant literature regarding various aspects of this study are reviewed in this chapter.

2.1 Chemistry of ginger

The constituents of ginger are numerous and vary depending upon the place of origin and whether the rhizomes are fresh or dry. The ginger rhizome contains steam volatile oil, non-volatile pungent principles, resins, proteins, cellulose, starch and mineral elements. Of these, starch is the most abundant and comprises 40–60 per cent of the rhizome on dry weight basis (Parthasarathy *et al.,* 2008).

Natarajan *et al.* (1972) reported quality parameters of ginger rhizomes from Kerala (India) with respect to essential oil (1 to 2.7 percent), acetone extract (3.9 to 9.3 percent), crude fiber (4.8 to 9.8 percent) and starch (40.4 to 59 percent). The odour of ginger depends mainly on its volatile oil and the yield of which varied from 1 percent to 3 percent (Ali *et al*., 2008). Langner *et al*. (1998) and Evans (2002) reported that over 50 components of the oil were characterized and they were mainly monoterpenoids (b-phellandrene, camphene, cineole, geraniol, curcumene, citral, terpineol, borneol) and sesquiterpenoids (a-zingiberene (30–70 percent), b-sesquiphellandrene (15–20 percent), b-bisabolene (10–15 percent), a-farnesene, arcurcumene, zingiberol).

A typical analysis of green ginger sample has the following values in per cent moisture 80.9, protein 2.3, fat 0.9, carbohydrates 12.3, fibre 2.4 and minerals 1.2. The principal minerals and vitamins in mg/100 g are Ca 20, P 60 and Fe 2.6, the vitamins and thiamine 0.06, riboflavin 0.03, niacin 0.6 and ascorbic acid 6.0. The rhizome contains 7.6 per cent pentoses on a dry weight basis and small quantities of the free sugars, glucose, fructose and sucrose. Ginger contains 1.6–2.4 per cent nitrogen on a dry weight basis, of which non-protein nitrogen is roughly one third. About 18.6 per cent of the protein remains unextracted; the extracted proteins contain 35.6 per cent albumin, 16.9 per cent globulin, 11.0 per cent prolamine and 17.9 per cent glutelin of total proteins (Govindarajan, 1982).

The oleoresin contains volatile oil and pungent principles, together with fatty oil, palmitic acid and some other free fatty acids, resins and carbohydrates. The yield and the relative abundance of the components of the oleoresin are dependent, however, on the raw material solvent used and on the extraction conditions. Commercial dried ginger was reported to contain oleoresins 3.5–10 per cent and volatile 1.5–30 per cent (Govindarajan, 1982).

The non-volatile pungent compounds contain biologically active constituents such as gingerols, shogaols, paradols and zingerone that produce a ‘‘hot’’ sensation in the mouth. The primary pungent agent of ginger are phenylalkylketones or vanillyl ketones which included 6-gingerol, 8-gingerol and 10-gingerol, 6- shogaol, 8-shogaol and 10-shogaol and zingerone, 6-paradol, 6- dehydrogingerdione and 10-dehydrogingerdione, 6- gingerdione and 10-gingerdione (Chrubasik *et al*., 2007).

2.2 Somaclonal variation and its exploitation for improving quality

Somaclonal variation is defined as variation originating in cell and tissue cultures (Larkin and Scowcroft, 1981). The causes of somaclonal variation are thought to be due to a combination of factors. Some of the variability is due to pre-existing mutations in cells of the explant material (Lorz, 1984; orton, 1984). A large part of the variation is induced during the culture cycle and this variation is attributed to chromosomal abnormalities commonly observed in cultured cells. Ploidy changes and changes inducing translocations, deletions, amplification and point mutations occur in culture cycle (Larkin *et al.,* 1984). Also, changes occur both in single gene and in polygenic traits (Evans and Sharp, 1983; Evans *et al.,* 1984; Larkin *et al.,* 1984) and in both organelle and nuclear genomes (McNay *et al.,* 1984). Some other factors were also found to influence the rate of somaclonal variation. They include growth regulators (Evans, 1988; Griesbach *et al.,* 1988; Shoemaker *et al.,* 1991), length of time *in vitro* (Skirvin *et al.,* 1994), proliferation rate (Smith and Drew, 1990) and culture conditions (Skirvin *et al.,* 1994).

Somaclonal variation was effectively utilized for upgrading in quality several crop species. Booij *et al.* (1993) reported deglet nour dates showed variability in level of sugar and amino acids composition regenerated through indirect somatic embryogenesis.

Sudharshan and Srikrishna (1998) reported in cardamom that micropropagated plants exhibited 30 per cent yield increase as compared to open

pollinated plants. The essential oil content was higher in micropropagated plants (7.2 per cent) when compared to open pollinated seedlings (6.9 per cent).

Sanchu (2000) studied variability in morphological, yield and quality parameters of black pepper cultivar Cheriakaniyakkadan derived through indirect organogenesis. She observed variability in leaf area, number of lateral branches, number of spikes per branch, spike length, number of berries per spike and recovery of essential oil and piperine. She could isolate five calliclones of black pepper tolerant to P*hytophthora* foot rot disease and a superior somaclone having high yield, quality and tolerance to P*hytophthora* foot rot from the study.

Kukreja *et al.* (1992) reported performance and stability behaviour of six *in vitro* derived somaclones (Sc 59, Sc 93, Sc 114, Sc 121, Sc 124, Sc 179) of Japanese mint (*Mentha arvensis*) estimated for oil yield and yield attributes at two different environments (Lucknow and Pantnagar) over a period of three years. Among six somaclones, Sc 93 and Sc 179 maintained their significant superiority over the parent for oil yield.

Rao *et al.* (2000) observed no significant variation in morphological characters in adventitious bud regenerates of Jamaican ginger but quality wise somaclones were superior to the local ginger cultivar Kuruppapady in terms of oil and oleoresin recovery. Elaborate studies on exploitation of somaclonal variation in ginger was conducted at Kerala Agricultural University. Somaclones derived from two cultivars viz. Maran and Rio-de-Janeiro were evaluated for yield, quality and reaction to pests and diseases (Paul and Shylaja, 2009; Paul *et al.,* 2009; Paul *et al.,* 2011; Paul and shylaja, 2012). After conducting on farm evaluation and multilocational trials, three high yielding high quality clones were released as varieties under the names “Athira”, “Karthika” and “Aswathy” (Shylaja *et al.,* 2010; Shylaja *et al.,* 2014).

Anu *et al.* (2002) reported somaclonal variation in *Capsicum annuum* for its improvement. Five genotypes (Round ornamental, PBC 535, PBC 375, PBC 385 and PBC 066) of *C. annuum* were used to evaluate their morphological, yield and colour characters. They found variation in quality characters like fruit colour and it was also found that the variations were more among the seedling progenies of the somaclones.

Gupta *et al.* (2002) reported somaclones of geranium showed variability in content of essential oil. Ravindra *et al.* (2004) and Saxena *et al.* (2008) also reported variability in geranium with respect to content of essential oil.

2.3 Chromatographic analyses of quality components in ginger

Jaffery *et al*. (2003) reported that quantity and quality of polyphenols present in ginger vary significantly due to different factors, such as plant genetics, cultivar, soil composition, growing conditions, maturity state and post-harvest conditions. They quantified 6-gingerol in twelve different ginger cultivars using RP-HPLC to evaluate the antioxidant activity of each cultivar by the DPPH (2, 2-diphenyl-1-picrylhydrazyl) and FRAP (ferric-reducing antioxidant power) methods in order to compare and differentiate cultivars as valuable sources of antioxidant compounds.

Ma and Gang (2006) reported metabolic profiling using GC/MS and LC-ESI-MS to determine chemical differences between greenhouses grown or *in vitro* micropropagated ginger plants. Three different ginger lines were analyzed. The constituent of gingerols and gingerol-related compounds, diarylheptanoids and methyl ether derivatives of these compounds as well as mono- and sesquiterpenoids were identified. Principal component analysis and hierarchical cluster analysis revealed chemical differences between lines (yellow ginger vs. white ginger and blue ring ginger) and tissues (rhizome, root, leaf and shoot). The analysis indicated no significant differences between growth treatments. The biochemical mechanisms to

produce large array of compounds found in ginger were not affected by *in vitro* propagation.

Jiang *et al.* (2006) reported phylogenetic analysis and metabolic profiling to investigate the diversity of plant material within the ginger species and closely related species in the genus Zingiber. Phylogenetic analysis demonstrated that all *Zingiber officinale* samples from different geographical origins were genetically indistinguishable. The other Zingiber species were significantly divergent. In the metabolic profiling analysis, *Z. officinale* samples derived from different origins showed no qualitative differences in major volatile compounds, although they did show some significant quantitative differences in non-volatile composition which included 6-gingerol, 8-gingerol and 10-gingerols. The differences in gingerol content were analyzed by HPLC. Comparative DNA sequence/chemotaxonomic phylogenetic trees showed that the chemical characters of the investigated species were able to generate essentially the same phylogenetic relationships as the DNA sequences.

Schwertner and Rios (2007) reported that HPLC method is a simple, reproducible and accurate method and applicable to analyze a wide variety of ginger-containing products. 6-gingerol, 6-shogaol, 8-gingerol and 10-gingerol were extracted from various ginger-containing products with ethyl acetate and analyzed by HPLC on a C-8 reversed phase column at 282 nm. The recovery of 6- gingerol, 8-gingerol, 10-gingerol and 6-shogaol from the ginger dietary supplements and ginger-containing products were 94.7±4.1, 93.6±3.4, 94.9±4.0, 97.1±3.8 per cent, respectively. The coefficients of variation for 6-gingerol, 6-shogaol, 8-gingerol and 10-gingerol standards at 50.0g/ml were 2.54, 2.38, 2.55, and 2.31 percent respectively. The variation (CV's) in the 6-gingerol, 6-shogaol, 8-gingerol and 10-gingerol concentrations of nine different ginger root dietary supplements were 115.2, 45.7, 72.3, and 141.7 per cent.

Sanwal *et al.* (2010) reported that total gingerol content in the tetraploid ginger was higher than diploid. Both diploid and tetraploid types of the cv. Meghalaya Local contained the highest level of gingerol. At both ploidy levels, the genotypes showed significant differences in phenols and antioxidant activities. Correlation of total phenolic compound with different antioxidant assay at both diploid and tetraploid level was linear.

Zick *et al.* (2010) reported a method to determine the main pungent ginger constituents, 6-gingerol, 8-gingerol, 10-gingerols and 6-shogaol in human plasma. Quantitation was achieved using a reverse-phase C18 column using High Performance Liquid Chromatography with electrochemical detection. High Performance Liquid Chromatography method allows detection of all four of ginger's pungent constituents simultaneously in a relatively short run time of 25 minutes. This method can be used to determine plasma levels of 6-gingerol, 8-gingerol, 10-gingerol and 6-shogaol in phase I clinical trials.

Pawar *et al.* (2011) reported the antioxidant capacity and phenolic content from the rhizomes of 12 ginger cultivars from different agro climatic zones of India. The quantity of phenolic compound, 6-gingerol was determined with reverse phase High Performance Liquid Chromatography (RP-HPLC) which ranged from 0.1 to 0.2 per cent. The antioxidant capacity was determined using DPPH (1, 1-diphenyl- 2-picrylhydrazyl) and FRAP (ferric-reducing antioxidant power) assays. The content of 6-gingerol of ginger cultivars ranged from 0.117 per cent in Udaipur sample to 0.208 per cent in Rajasthan sample. Pawar *et al.* (2015) reported rhizome derived from callus culture produced lowest amount of 6-gingerol as compared to conventionally grown plants.

Wang *et al.* (2011) reported a clean-up step on silica column and high-speed counter-current chromatography (HSCCC) to purify gingerols from an extract of the dried rhizome of ginger. They found 132 mg of 6-gingerol, 31 mg of 8-gingerol and

61 mg of 10-gingerol from 360 mg of pre-purified sample. The purity of each compound was over 98 per cent as determined by HPLC. Cheng *et al.* (2011) also used HPLC method with TOF/MS and DAD to analyse the chemical constituents in ginger.

Zhan *et al.* (2011) reported a novel method of purifying gingerols from ginger using a high-speed counter-current chromatography (HSCCC). The two-phase solvent system was used to separate and purify 6-gingerol, 8-gingerol and 10-gingerol from a crude extract of ginger. The experiment yielded 30.2 mg of 6-gingerol, 40.5 mg of 8-gingerol, 50.5 mg of 10-gingerol from 200 mg of crude extract in one-step separation. The purity of these compounds was 99.9, 99.9 and 99.2 per cent determined by High Performance Liquid Chromatography.

Salmon *et al.* (2012) reported that high performance thin layer chromatography (HPTLC) technique showed good resolution of the pungent compounds of ginger viz. 6-gingerol, 8-gingerol, 10-gingerol and 6-shogaol. Quantification of the compounds by high-performance liquid chromatography (HPLC) revealed significant differences in total pungency among the four cultivars of Jamaican ginger. Essential oil yields from the cultivars were also varied significantly.

Yudthavorasit *et al.* (2014) studied Chromatographic fingerprints of ginger from five different ginger-producing countries (China, India, Malaysia, Thailand and Vietnam) to discriminate the origin of ginger. The pungent bioactive principles of ginger, gingerols and six other gingerol-related compounds were determined and identified. The study suggested that 8-gingerol and 6-gingerol were significant markers for specifying ginger from India and Thailand, while 8-gingerdion, 10-gingerol and 8-gingerdion employed as a marker for Chinese ginger.

Ghosh and Mandi (2015) reported variation in content of 6-gingerol in land races collected from different locations and they correlated the content with gene expression studies. They observed high Chalcone Synthase gene (Chalcone Synthase

is the rate limiting enzyme of 6-gingerol biosynthesis pathway) expression in high 6-gingerol containing landraces. They suggested that a specific amino acid change viz. Asparagine to serine was the cause for difference in expression.

2.4 Medicinal and pharmacological properties of ginger

Ginger has been used as medicine from Vedic period and is called "maha aushadh", means the great medicine. It is widely used in Chinese, Ayurvedic and Tibb-Unani herbal medicines in all over the world. The important medicinal part of the ginger plant is rhizome. The importance of ginger has been increased recently because of its low toxicity and its broad spectrum of biological and pharmacological applications, viz. antitumor, antioxidant, anti-inflammatory, antiapoptotic, cytotoxic, anti-proliferative and anti-platelet activities (Sekiwa *et al.,* 2000; Shukla & Singh, 2007; Wei *et al*., 2005; Young *et al*., 2005; Rahmani *et al.,* 2014). Mishra *et al.* (2012) also reported pharmocological properties of ginger which included, anti-cancer, anti-coagulant, anti-emetic, anti-inflammatory, anti-nociceptive, antioxidant, cardiovascular, gastrointestinal, antitussive, immune modulatory effects, antimicrobial and anti-genotoxic activity. The natural polyphenolic alkanone 6-gingerol, a major pharmacologically active component of ginger was reported to have antibacterial, antioxidant, anti-inflammatory and anti-tumour properties (Park *et al.,* 2006; Shukla and Singh, 2007; Jeong, 2009; Rahmani *et al.,* 2014).

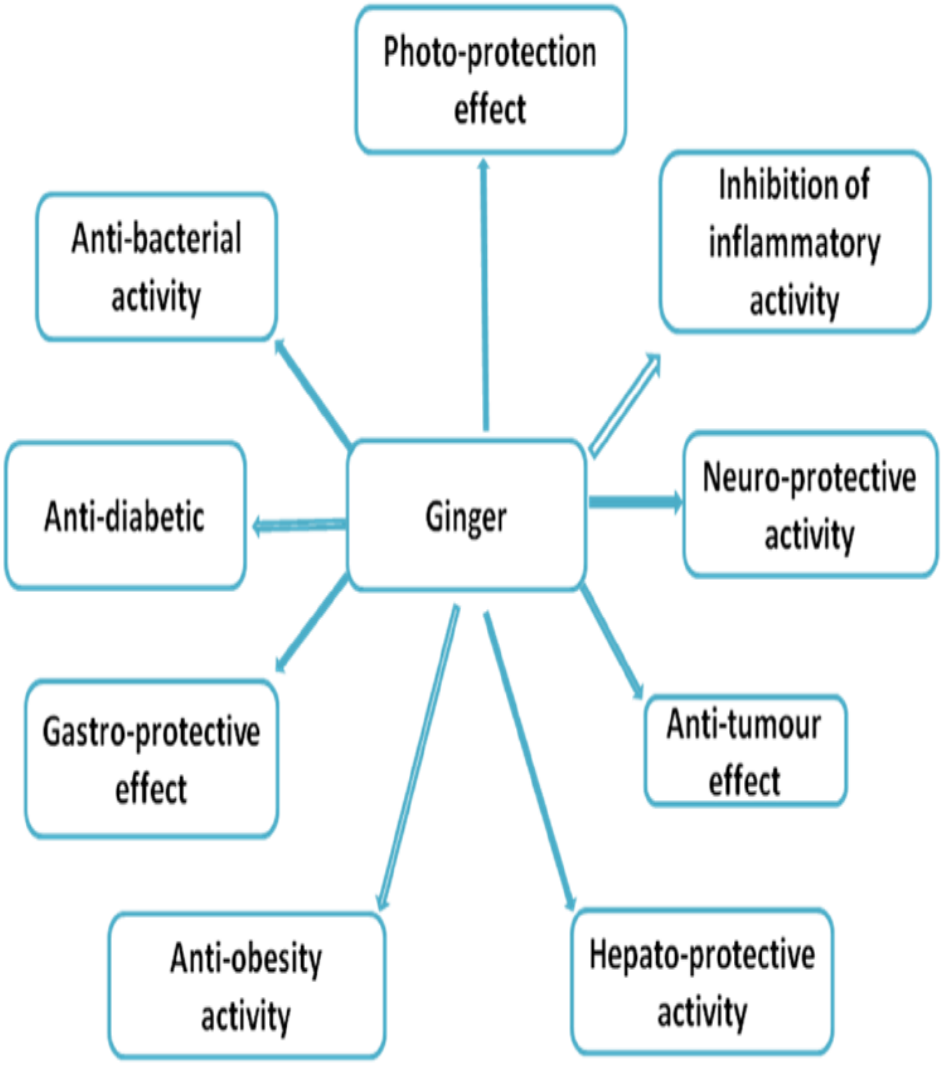


Figure 1. Health beneficial effects of ginger (Rahmani *et al.*, 2014)

3.5 Cancer

Cancer is one of the most deadly diseases in the world, which is caused due to uncontrolled growth of cells or malfunction of genes that control normal cell growth and division. Normally, cells divide to produce more cells only when the body needs them. If cells keep dividing when new cells are not needed, a mass of tissue forms which is called a growth or tumor which can be benign or malignant. Benign tumors are not cancer. They can usually be removed and in most cases they do not come back. Benign tumors are rarely a threat to life. Malignant tumors are cancer. Cancer cells can invade and damage tissues and organs near the tumor. The spread of cancer is called metastasis (Ghosh, 2011).

Most cancer cells have similar characteristics present in a normal cell except that in some of the cells signaling processes are highly up regulated while in some they are heavily down regulated. Causes of cancer can be inherited through genes or can be adapted due to several activities like exposure to carcinogens. As biomarkers are defined as molecular, cellular, functional measurable parameters indicative of a particular genetic, epigenetic or functional status of a biological system (Ludwig and Weinstein 2005), cancer cell biomarkers, which are the property of tumor cells can be used for diagnosis, prognosis, staging and treatment.

Characteristics of cancer cells can be listed in six different catagories as shown in figure 2.

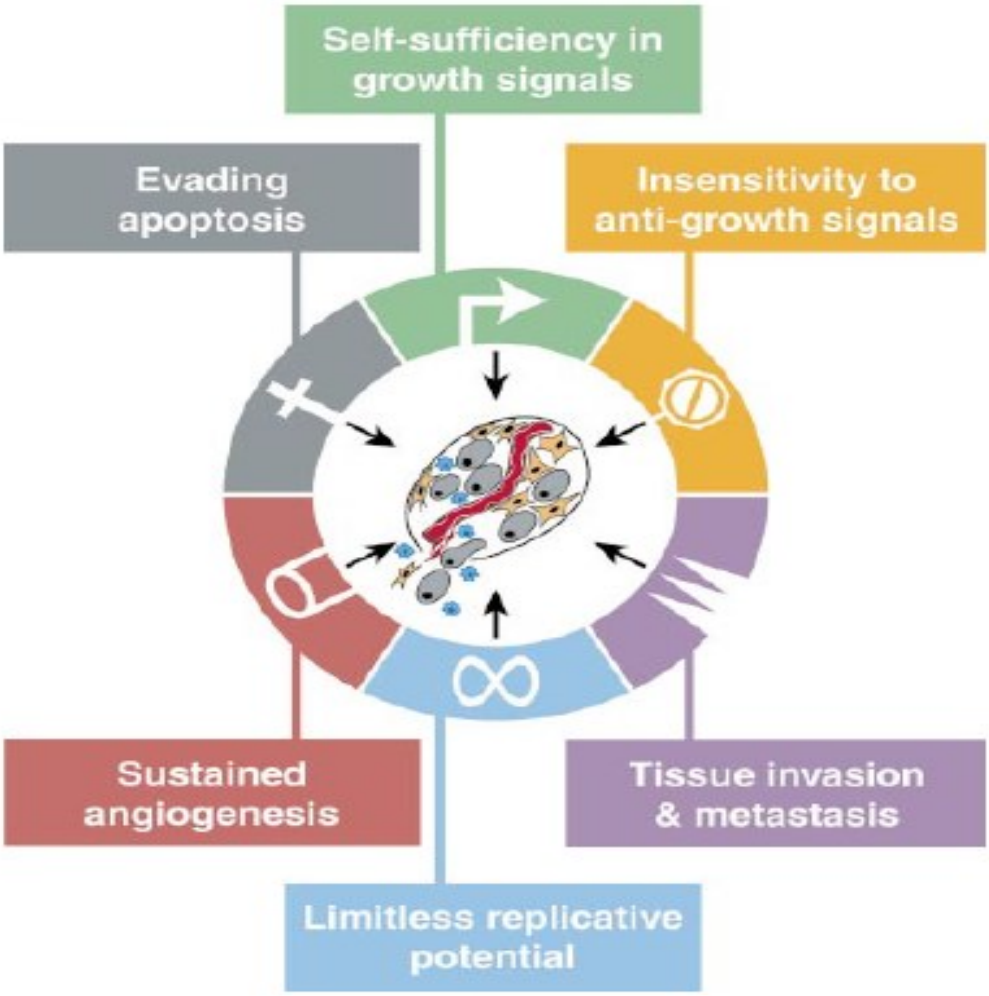


Figure 2. Six hallmarks of cancer (Hanahan and Weinberg, 2000)

3.6 Growth signals and cancer

There are various soluble growth factors that are present in a cell, based on which cell function normally. A normal cell usually recognizes different kinds of growth signals and responds accordingly but cancer cells do not require those exogenous growth signals and can grow independent. Cancer cells are able to synthesize most soluble mitogen growth factors and positively respond to the signals. Some of the growth factors produced by cancer cells are platelet derived growth factor (PDGF) and tumor growth factor (TGF). Cancer cell being self-sufficient in growth signal is explained by higher expression of the receptors. Due to the high level of receptors present in cancer cell, even very low level of growth factors are also detected, which usually would not be recognized in normal cells. For example in breast cancer cells and oesophageal cancer cell, epidermal growth factor receptor (EGF-R) and Her2/*neu* receptor are overexpressed (Slamon *et al*., 1987; Kawaguchi *et al.,* 2007).

Normal cells have the property of obeying anti-growth signal whereas cancer cells resist to anti-growth signals. Normally when cells receive anti-growth signal, they get into quescient stage or permanently lose their ability to proliferate, but cancer cells deregulate this phenomena by disrupting pRb proteins and mutating various proteins like p15 and p21 (Zuo *et al.*, 1996).

3.7 Apoptosis and cancer

Apoptosis is also called programmed cell death. It is a normal process that occurs during development of cell. When stimuli for death are received, cells undergo various morphological changes and die in a regular and controlled manner. When signals are received by cells there are various pathways that are active in order to cause apoptosis. During early phase of apoptosis, there are families of proteins called caspases that are activated. These groups of proteins create a cascade that leads to

cleavage or change in many structural proteins, nuclear proteins or enzymes eventually causing cell death.

But in case of cancer, the process of apoptosis is uncontrolled. Cancer cell gain the ability of escaping apoptosis by mutating tumor suppressor genes like p53, overexpressing apoptotic oncogenes like bcl-2, c-myc and inhibiting pro-apoptotic proteins like Bax and cytochrome-C.

3.8 Anticancerous properties of ginger

Lee and Surh (1998) reported, 6-gingerol and 6-paradol has a strong anti-inflammatory activity which is considered to be closely associated with its cancer chemo preventive potential.

Wang *et al.* (2001) reported 6-gingerol and 6-paradol found to exert inhibitory effects on the viability and DNA synthesis of human promyelocytic leukemia (HL-60) cells. The cytotoxic and anti-proliferative effects of both compounds were associated with apoptotic cell death. The antioxidative effects of 6-gingerol were detected by DPPH and DCFH assays and 6-gingerol predicted as an antioxidant to protect HL-60 cells from oxidative stress.

Rhode *et al.* (2007) reported the effect of ginger and the major ginger components on cell growth, determined in a panel of epithelial ovarian cancer cell lines. SKOV3, CaOV3, and ES-2 three cancer cells were harvested from patients with recurrent ovarian cancer. Ovarian cancer cells were maintained in DMEM supplemented with 10 per cent fetal bovine serum, 100 units/ml penicillin and 100 mg/ml streptomycin Human ovarian surface epithelial cells were obtained from patients undergoing surgery for non-ovarian cancer gynecologic indication. Cells were initially cultured in Medium 199/105 (1:1) supplemented with 10 per cent fetal bovine serum, 100 units/ml penicillin and 100 mg/ml streptomycin and EGF 10 ng/ml during primary culture. After establishing adequate growth, cells were cultured with

the above media, excluding EGF prior to use in assays. CaOV3 and SKOV3 cell lines were transfected with the indicated expression plasmid using LipofectA. They found continuous exposure to ginger extract resulted in a marked reduction in cell growth after 1–5 days of exposure in A2780, CaOV3 and SKOV3 ovarian cancer cells.

Lee *et al.* (2008) reported effects of 6-gingerol on adhesion, invasion, motility, activity and the amount of MMP-2 or -9 in the MDA-MB-231 human breast cancer cell line. They cultured MDA-MB-231 cells in the presence of various concentrations of 6-gingerol (0, 2.5, 5 and 10 μM) found that 6-gingerol had no effect on cell adhesion up to 5 μM, but resulted in a 16 per cent reduction at 10 μM. The activities of MMP-2 or MMP-9 in MDA-MB-231 cells were decreased by treatment with 6- gingerol and occurred in a dose-dependent manner. The amount of MMP-2 protein was decreased in a dose-dependent manner, although there was no change in the MMP-9 protein levels following treatment with 6-gingerol. MMP-2 and MMP-9 mRNA expression were decreased by 6-gingerol treatment.

Chen and Li (2012) reported signaling effects of 10 - gingerol on human colorectal cancer cells. The 10-gingerol caused a slow and sustained rise of Ca^{2+} in a concentration-dependent manner. Induced a Ca^{2+} rise when extra cellular Ca^{2+} was removed but the magnitude was reduced by 38 per cent. In a Ca^{2+} free medium the 10-gingerol induced Ca^{2+} raise was partially abolished by depleting stored Ca^{2+} with thapsigargin. The 10-gingerol killed cells in a concentration-dependent manner.

Ling *et al*. (2010) reported that shogaols (6-, 8- and 10-shogaol) inhibited PMA-stimulated MDA-MB-231 cell invasion with an accompanying decrease in MMP-9 secretion. 6-Shogaol was identified to display the greatest anti-invasive effect in association with a dose-dependent reduction in MMP-9 gene activation, protein expression and secretion. The NF-kB transcriptional activity was decreased by 6-shogaol, an effect mediated by inhibition of IkB phosphorylation and degradation that subsequently led to suppression of NF-kB p65 phosphorylation and

nuclear translocation. In addition, 6-shogaol was found to inhibit JNK activation with no resulting reduction in activator protein-1 transcriptional activity.

Lin *et al.* (2012) investigated the anti-tumor effects of 6-gingerol on colon cancer cells. The 6-gingerol treatment significantly reduced the cell viability of human colon cancer cell LoVo in a dose-dependent manner. Further flow cytometric analysis showed that 6-gingerol induced significant G2/M phase arrest and had slight influence on sub-G1 phase in LoVo cells. Therefore levels of cyclins, cyclin-dependent kinases (CDKs) and their regulatory proteins involved in S-G2/M transition were investigated. The levels of cyclin A, cyclin B1 and CDK1 were diminished in contrast levels of the negative cell cycle regulators p27Kip1 and p21Cip1 were increased in response to 6-gingerol treatment. In addition, 6- gingerol treatment elevated intracellular reactive oxygen species (ROS) and phosphorylation level of p53. These findings indicated that exposure of 6-gingerol induced intracellular ROS and upregulated p53, p27Kip1 and p21Cip1 levels leading to consequent decrease of CDK1, cyclin A and cyclin B1 as result of cell cycle arrest in LoVo cells.

Brahmbhatt *et al.* (2013) reported the effectiveness of whole extract of ginger on prostate cancer. They used human prostate cancer; PC-3 cells obtained from American Type Culture Collection (ATCC), cultured in RPMI-1640 medium supplemented with 10 per cent heat inactivated fetal bovine serum (FBS) and one per cent antibiotic (penicillin/ streptomycin). The MTT dye (Thiazolyl Blue Tetrazolium Bromide, 98 per cent TLC) and Dimethyl Sulfoxide (DMSO) were from Sigma-Aldrich. Cells were cultured at 37°C with 5 per cent CO2. The ginger phytochemicals, 6-gingerol (6G), 8-gingerol (8G), 10-gingerol (10G), and 6-shogoal (6S) were purchased from Chromadex, Inc. their purity was confirmed by HPLC analysis. MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay was employed to evaluate the proliferative capacity of cells. A 96-well format was used to seed 100 µl medium containing cells at a density of 5×10^3 cells per

well. After 24h. of incubation, cells were treated with gradient concentration of ginger extract, gingerols, and shogaol which were dissolved in DMSO. The final concentration of DMSO in the culture medium was maintained at 0.1 per cent. After 48h of incubation, the spent medium was removed and the wells were washed twice with PBS. 100 µl of fresh medium and 10 µl of MTT (5 mg/ml in PBS) were added to the wells and cells were incubated at 37°C in dark for 4h. The formazan product was dissolved by adding 100 µl of 100 per cent DMSO after removing the medium from each well. The absorbance was measured at 570 nm using a Spectra Max Plus multi-well plate reader. The IC50 values were obtained at 75 µM (22.07 µg/ml) for 6-gingerol, 10 µM (3.22 µg/ml) for 8-gingerol, 50 µM (17.53 µg/ml) for 10-gingerol, 4 µM (1.12 µg/ml) for 6-shagaol and 250 µg/ml for ginger extract.

Kim and Kim (2013) reported the effect of 6-gingerol on pancreas cancer cell. They performed cytotoxicity of 6-gingerol on PANC-1cancer cell line of pancreas cancer using MTT assay. They used 96 well plates at 1 x 10^4 cells/well of plate. Different concentration of 6-gingerol (0, 5, 10, 20 µM) were used. Observation was recorded 24 h. of incubation at 570 nm wave length and they found no effect on cell growth at concentration less than 20 µM.

Rhadhakrishnan *et al*. (2014) also reported that the anticancer and chemopreventive efficacy of 6-gingerol is the major active principle of the medicinal plant ginger (*Zingiber officinale*) in colon cancer cells. The compound was evaluated in two human colon cancer cell lines for its cytotoxic effect. The most sensitive cell line SW-480 was selected for the mechanistic evaluation of its anticancer and chemo-preventive efficacies. The non-toxic nature of 6-gingerol was confirmed by viability assays on rapidly dividing normal mouse colon cells. The 6-gingerol inhibited cell proliferation and induced apoptosis as evident by externalization of phosphatidyl serine in SW-480 while the normal colon cells were unaffected. Sensitivity to 6-gingerol in SW-480 cells was associated with activation of caspases 8, 9, 3 &7 and cleavage of PARP which attests induction of apoptotic cell death.

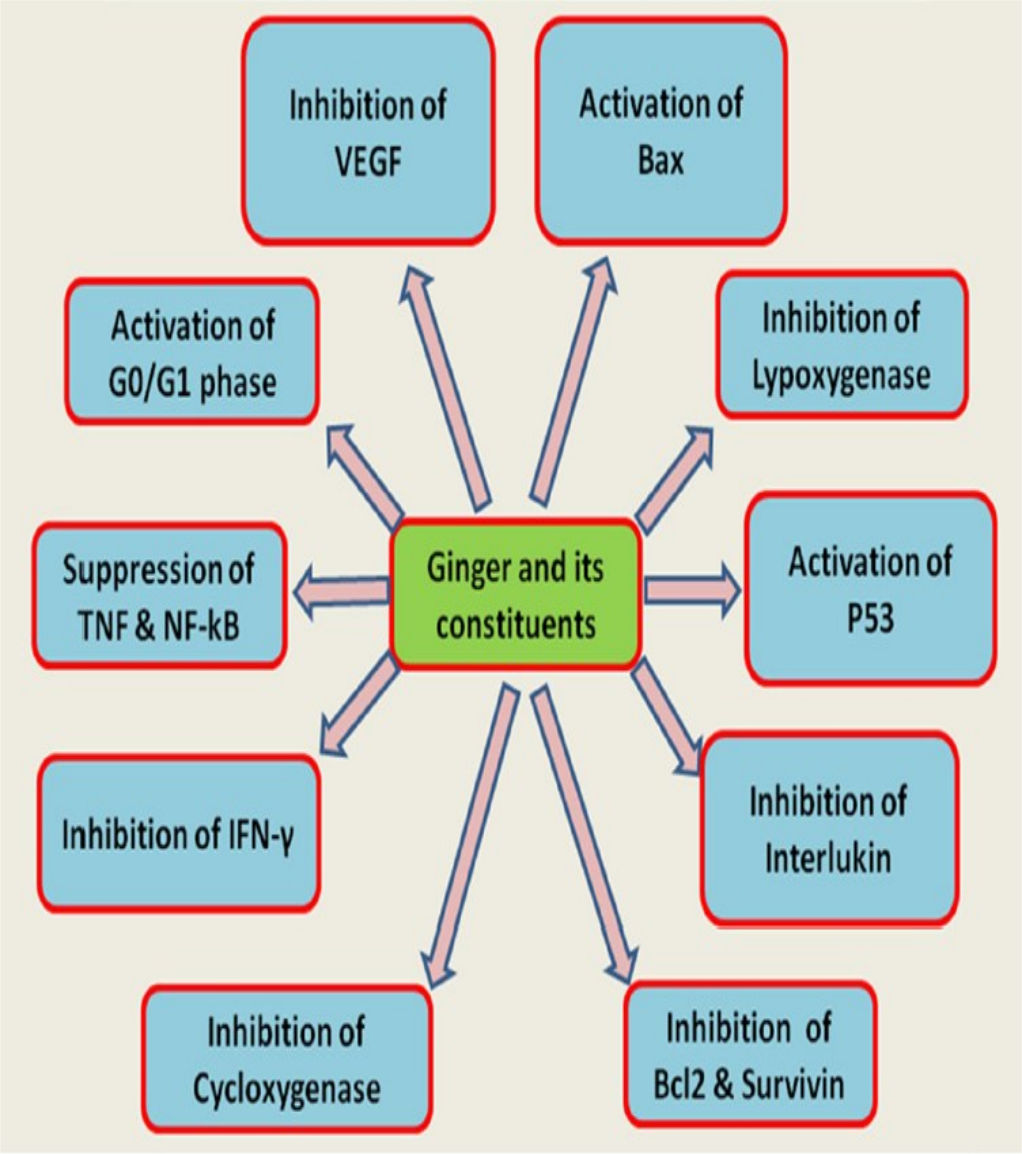


Figure 3. Pharmacological effects of ginger and its constituents in cancer management via modulation of molecular mechanisms (Rahmani *et al.*, 2014)

The 6-shogaol effectively reduced survival and induced apoptosis of cultured human (LNCaP, DU145, and PC3) and mouse (HMVP2) prostate cancer cells (Saha *et al.,* 2014). Saha *et al.* (2014) also reported 6-shagaol inhibited activity of constitutive and TNF induced NF-kB. The 6-shogaol decreased the level of several STAT3 and NF-kB regulated target genes at the protein level including cyclin D1, survivin and cMyc and modulated mRNA levels of chemokine, cytokine, cell cycle and apoptosis regulatory genes (IL-7, CCL5, BAX, BCL2, p21, and p27). The 6-shogaol was found more effective than two other compounds found in ginger 6-gingerol and 6-paradol at reducing survival of prostate cancer cells and reducing STAT3 and NF-kB signaling. The 6-shogaol also showed significant tumor growth inhibitory activity in an allograft model using HMVP2 cells.

Prasad and Tyagi (2015) reported anticancer activity of ginger to modulate several signaling molecules like NF-κB, STAT3, MAPK, PI3K, ERK1/2, Akt, TNF-α, COX-2, cyclin D1, cdk, MMP-9, survivin, cIAP-1, XIAP, Bcl-2, caspases, and other cell growth regulatory proteins and 6-gingerol and 6-shogaol exert anticancer activities against gastro intestine cancer.

3.9 *In silico* docking studies

Jeong *et al.* (2009) reported that 6-gingerol, a natural component of ginger exhibited anti-inflammatory and anti-tumorigenic activities. The leukotriene A4 hydrolase (LTA4H) protein is regarded as a relevant target for cancer therapy. The *in silico* prediction using a reverse-docking approach revealed that LTA4H was a potential target of 6-gingerol. They predicted that 6-gingerol suppressed anchorage-independent cancer cell growth by inhibiting LTA4H activity in HCT116 colorectal cancer cells. They showed that 6-gingerol effectively suppressed tumor growth *in vivo* in nude mice, an effect that was mediated by inhibition of LTA4H activity.

Ittiyavirah and Paul (2013) reported *in silico* approach used to assess the use of constituents of ginger as potential agents that could act as antidepressant agents.

Docking studies of 6-gingerol and shogaol were carried out using Argus lab 4.0.1. Analysis of the results of the docking software suggested that 6-gingerol and shogaol can act as potent antidepressants. For the binding analysis, 5HT1A receptor protein was taken as it is considered being a potential target for treatment of depression. The standard drug, imipramine was subjected to docking analysis for comparative study.

Mahto *et al.* (2013) performed docking study with seven different oncoprotein with 22 quinoline based inhibitors. The stability of docking between ligand and the target protein depends on the binding interactions. The hydrogen bonding interaction which was vital parameter for the stability of *drug-protein complex* is found in all the best scoring molecules as a result of docking. The binding interaction of highest scoring molecule with active site amino acids of each protein used as target was depicted in the docking. Based on docking score they found five ligands have best score.

Shankar *et al.* (2013) reported *in silico* molecular docking of cancer biomarkers with bioactive compounds of *Tribulus terrestris*. The target biomarkers selected for analysis included NSE (lung cancer), Follistatin (prostate cancer), GGT (hepatocellular carcinoma) and Human Prostasin (ovarian cancer). The total number of ligands selected were nine and all bioactive compounds of *Tribulus terrestris* was found minimum binding energy with Follistatin.

Nazeem *et al.* (2014) reported phosphatidylinositide 3-kinase/ Nuclear Factor - kappa B signaling pathways known to be involved in regulating MMP-9 expression. Synergistic targeting of these pathways using NF-κB and PI3K inhibitors may have great potential for cancer treatment. They used thirty five phytocompounds with anticancer properties, screening out using Lipinski rule of five and ADMET, five compounds namely allixin, capsaicin, eugenol, niazimicin and piperine were docked with PI3K and NF-κB proteins. Niazimicin exhibited interaction for PI3K and NF-κB with residues CYS 633, ASP 632, GLN 392 and LYS 145 respectively. Niazimicin, a

phytocompound of *Moringa Oleifera* which is an underexploited vegetable crop with medicinal properties showed maximum interaction with the targets.

Antony and Nazeem (2014) performed molecular docking in Bcl-2 family of protein with phytocompounds of *Boerhaavia diffusa.* There were total sixteen phytocompounds of *Boerhaavia diffusa* with 42 ligands of various conformations selected for docking. They found that phytocompounds Boeravinone D, Boeravinone E, Boeravinone F showed good interaction with the target Bcl-2 family of protein.

Shruthy and Yusuf (2014) reported *in silico* molecular modelling of molecules by various softwares which included ACD chemsketch 12.1, Molinspiration, PASS online and Schrodinger (9.3) software. Fifty derivatives were selected for screening out of which five derivatives were selected for synthesis. The analogue with *para* methoxy group (CSB2) got the maximum docking score for anticancer and anti-inflammatory activity.

James *et al.* (2015) reported computational functional annotation of the available ESTs and identification of genes which play a significant role in gingerol biosynthesis. Ginger transcriptome was analyzed using EST dataset (38169 totals) from NCBI. ESTs were clustered and assembled, resulting in 8624 contigs and 8821 singletons. Assembled dataset of EST annotation workflow included blast, gene ontology (go) analysis, and pathway enrichment by kyoto encyclopedia of genes and genomes (kegg) and interproscan. The unigene datasets were further exploited to identify simple sequence repeats that enable linkage mapping. The hypothetical miRNA is warranted to play an important role in controlling genes involved in gingerol biosynthesis.

Materials and Methods

3. MATERIALS AND METHODS

The study entitled "Screening ginger (*Zingiber officinale* Rosc.) somaclones for gingerol content and validation of anticancerous properties of gingerol" was carried out at the Centre for Plant Biotechnology and Molecular Biology (CPBMB) and Distributed Information Centre (DIC) College of Horticulture, Kerala Agricultural University during 2013-2015. The objectives of the study were to screen ginger somaclones for gingerol content, to identify potential cancer targets for gingerols and shogaol using *in silico* tools and to validate the anticancerous properties of gingerol.

3.1 MATERIALS

3.1.1 Plant materials

Centre for Plant Biotechnology and Molecular Biology maintains a good collection of germplasm of ginger somaclones regenerated through direct/ indirect methods of organogenesis/embryogenesis and *in vitro* mutagenesis.

Fifty somaclones of Maran regenerated through direct/ indirect methods of organogenesis/ embryogenesis/ *in vitro* mutagenesis and one control (Conventionally propagated) were used in this study. The details of somaclones studied are presented in Table 1

Table 1. Details of ginger somaclones studied

Sl. No.	Code No. for groups of somaclones	Methods of regeneration	No. of plants in the group
1	M B	*In vitro* adventitious bud regeneration	10
2	MC	Indirect organogenesis	08
3	MSe	Indirect embryogenesis	06
4	MC 10Gy	Indirect organogenesis – Irradiated with γ rays (10Gy)	12
5	MSe 10Gy	Indirect embryogenesis – Irradiated with γ rays (10Gy)	02
5	MSe 20Gy	Indirect embryogenesis – Irradiated with γ rays (20Gy)	12
6	M	Control (Conventionally propagated)	01
Total			(50 + 1control) = **51**

3.1.2 Laboratory chemicals and glasswares

The chemicals used for oleoresin extraction and for screening somaclones for gingerol content were of analytical grade procured from Merck India Ltd. Chemicals for HPLC analysis were procured from Sigma Aldrich India Ltd. Acetone, Acetonitrile, Orthophosphoric acid, Methanol were procured from Merck India Ltd. and standards of 6-gingerol, 8-gingerol, 10-gingerol and 6-shagaol from Sigma Aldrich Ltd. The chemicals used for cell culture studies such as HEPES buffer, RPMI – 1640 Medium, Sodium pyruvate, Thiazolyl Blue exptrapure AR (MTT) and FBS were procured from Sigma Aldrich India Ltd. Life Technology, Himedia Laboratories Pvt. Ltd. and SISCO Research Laboratories Pvt. Ltd. The glass wares used were borosilicate, plastic wares used were procured from Tarsons Products Pvt. Ltd. and syringe filters from SPINCO Biotech Pvt. Ltd.

3.1.3 Equipment and machinery

The equipment items available at the Centre for Plant Biotechnology and Molecular Biology (CPBMB) and computer facility and software available at Distributed Information Centre (DIC) of College of Horticulture were used for the study. The oleoresin extraction was carried out using Soxhlet apparatus. High Pressure Liquid Chromatography (HPLC) Shimadzu LC20AD with LC solution software with PDA detector was used to separate gingerols and shogaols from ginger oleoresin. Software, Accelry Discovery Studio 4.0 (USA) available at DIC of College of Horticulture was used for docking studies. For cell culture studies, Laminar Air Flow (LAF), shaker incubator, ELISA reader (VERSA max microplate reader) and inverted microscope available at CPBMB were used. Details of laboratory equipment items used for the study is provided in Annexure I.

3.2 Methods

3.2.1 Raising of somaclones in field

Fifty somaclones were raised in mounds and evaluated as per (Paul, 1996) in the field attached to Centre for Plant Biotechnology and Molecular Biology (CPBMB) during 2013-14 as a part of an externally aided project ongoing in the department (Plate 1). Seed bits 15-20g with one or two viable buds were used for planting. The clones were managed as per PoP recommendations of Kerala Agricultural University. The clones planted in last week of May were harvested in January 2014.

3.2.2 Preparation of dry ginger

The harvested ginger rhizomes after removing mud and roots were thoroughly washed in water and the peel is removed from flat surface of the rhizome with a bamboo splinter and dried for seven to ten days till the rhizome broke easily.

Plate 1. Raising of ginger somaclones in field

3.2.3 Extraction of oleoresin from ginger rhizomes of selected somaclones

The extraction of oleoresin from the selected fifty Maran somaclones was done using Soxhlet apparatus which worked on the principle of solvent extraction.

3.2.3.1 Procedure

The content of oleoresin in the sample was estimated using the soxhlet method of extraction as per AOAC (1980). After grinding the dry ginger rhizome, five gram of the powder was wrapped in coarse filter paper and placed in the extraction chamber of the apparatus. Two replications were kept per sample. Extraction was carried out in the apparatus with 100 percent acetone till the solvent became colorless. After removing the clear solvent, the extract of the sample was transferred to pre – weighed beaker and the final traces of acetone evaporated and the weight of the beaker was recorded. The percentage of the oleoresin was calculated as

$$\frac{\text{Weight of oleoresin}}{\text{Weight of dry ginger powder}} \times 100$$

3.2.4 Screening somaclones for gingerol content

Gingerol content in the somaclones was estimated using HPLC analytical platform. High Pressure Liquid Chromatography (HPLC) is a separation technique that involves the injection of a small volume of liquid sample into a tube packed with tiny particles (3 to 5 micron (μm) in diameter) called the stationary phase where individual components of the sample are moved down the column with a liquid (mobile phase) forced through by high pressure delivered by a pump (Figure 4).

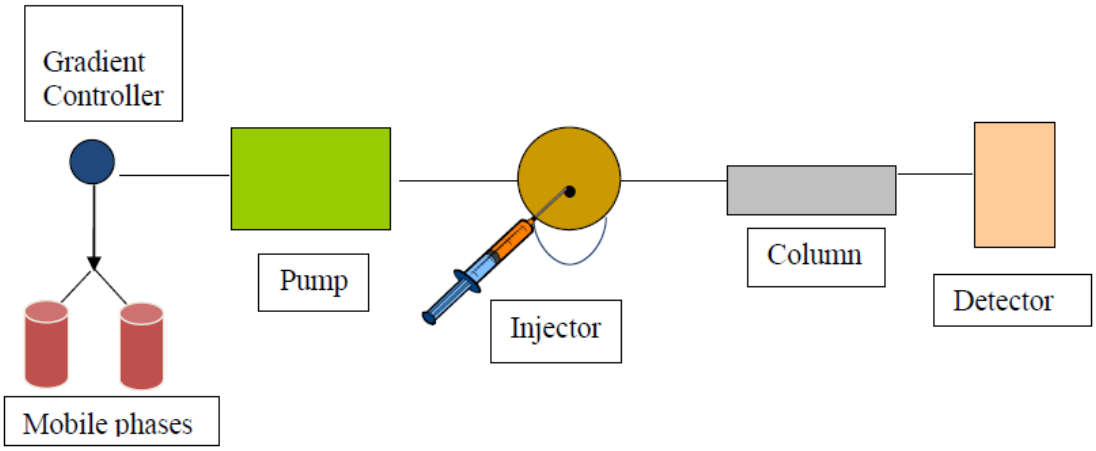


Figure 4. Working principle of HPLC

3.2.4.1 Reagents

Acetonitrile, Orthophosphoric acid (0.1 %) and Methanol in the proportion 55:44:1 respectively were used as mobile phase. Methanol: Water (50:50) was used as a washing buffer. Standards of 6-gingerol (98 % pure), 8-gingerol (95 % pure), 10-gingerol (95 % pure) and 6-shogaol (90 % pure) were used for comparing the samples.

3.2.4.2 Preparation of standards of 6-gingerol, 8-gingerol, 10-gingerol and 6-shogaol

Five milligram of 6-gingerol, 8-gingerol, 10-gingerol and 10 milligram of 6 shogaol were dissolved in 1.0 ml methanol to make standard stock of gingerols at concentration 5mg/ml and shogaol at 10mg/ml. Further serial dilutions were made from each to produce 250, 500, 750, 1000, 1250 µg/ml working standards. All ginger standards were capped and stored at 4±4 ˚C until used.

3.2.4.3 Preparation of sample

The extracted oleoresins from somaclones were dissolved in methanol to obtain sample at concentration 5mg/ml. The prepared samples were filtered using syringe filter and transferred to ependorff tube and samples were analysed.

3.2.4.5 HPLC conditions

The mobile phase consists of Acetonitrile, Orthophosphoric acid (0.1 %) and Methanol in the proportion 55:44:1 respectively. Membrane filter (SPINCP TECH.) was used for filtration of mobile phase which was degassed using sonicator prior to use.

The experiment was performed in an isocratic mode. The mobile phase was pumped at flow rate of 1.3 ml per minute with 20 µl injection volume. The column

temperature was set at ambient temperature. Detection was performed with Photo Diode Array (PDA) detector at 282 nm and the content of 6-gingerol, 8-gingerol, 10-gingerol and 6-shogaol were calculated.

3.2.5 *In silico* screening of potential ligands and targets for molecular docking studies against cancer

Biological targets are proteins which include enzymes, ion channels, and receptors. A ligand is a substance that include substrates, inhibitors, activators, and neurotransmitters and form a complex with a biomolecule to serve a biological purpose. The ligand is usually a signal-triggering molecule binding to an active site of a target protein. Ligand binding alters the chemical conformation (three-dimensional shape) of a receptor.

Of the various bioactive compounds present in ginger, gingerols and shogaols are pharmacologically more active. Hence in the present molecular docking study for validation of anticancerous properties, gingerols and shogaols were selected as ligands. Approved drugs for different types of cancer were also selected from Drug Bank to compare with gingerols and shogaols in molecular docking studies. The targets for molecular docking were selected through literature survey and from Potential Drug Target Database (PDTD).

3.2.5.1 Retrieval of structure of gingerols, shogaol and approved drugs

Structure of gingerols, shogaols and selected approved drugs were retrieved from Pubchem online database. Homepage of PubChem database was opened (http://www.ncbi.nlm.nih.gov/pccompound) (Figure 5). Compound name/compound ID was entered into the search box. 3D conformer of the compounds were saved in sdf. Format. Molecular properties of ligands such as molecular weight, partition coefficient, number of H-bond donor and H-bond acceptor were recorded from PubChem data base (Table 2).

Figure 5. Homepage of pubchem

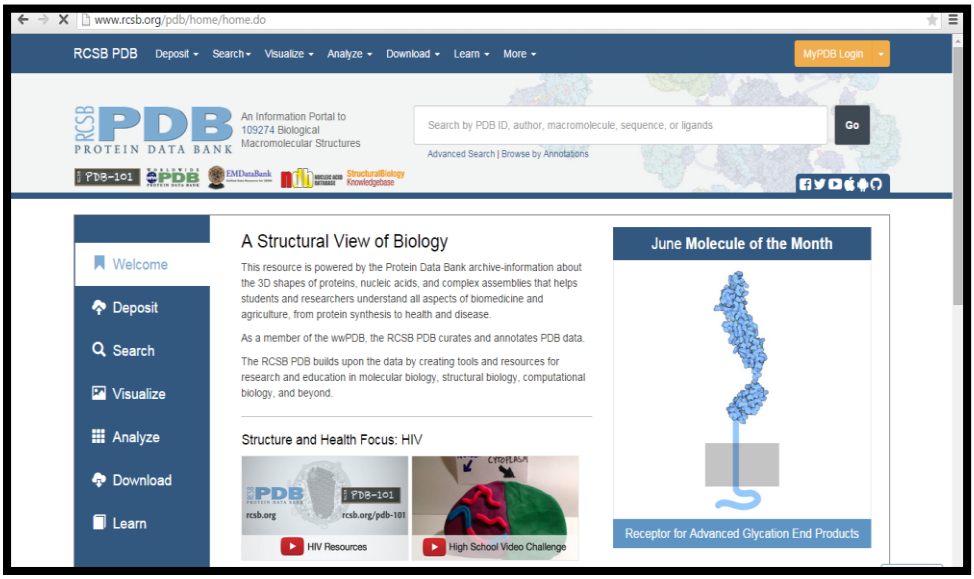


Figure 6. Homepage of Protein Data Bank (PDB)

3.2.5.2 Identification of targets for different types of cancer

The targets for different types of cancer were identified using literature survey and from Potential Drug Target Database (PDTD). The target name, effectiveness in different types of cancer, mode of action and scientist reported were noted (Table 3).

.2.5.2 Retrieval of structure of different targets

Structure of different targets were retrieved from Protein Data Bank (PDB) online database. Homepage of PDB was opened (http://www.rcsb.org/pdb/home/home.do) (Figure 6). Targets name/Targets ID were entered into the search box. 3D format of the targets was downloaded and saved in files in PDB File (Text). The structure of targets was retrieved based on X-ray diffraction method and resolution power and some were retrieved based on literature survey.

3.2.5.4 Prediction of active sites for the targets

Active sites are the binding region of the targets to the ligand. Binding site of the processed protein was identified by using Discovery studio 4.0. The active sites were selected for docking based on number of amino acid residues present in the binding site. The Receptor cavity and current selection tools of Discovery studio 4.0 were used to analyse the binding mode of ligands in the selected region.

Table 2. Molecular properties of ligands from ginger and approved drugs for cancer treatment

Sl. No.	Compound name	Compound ID	Molecular Formula	Molecular weight (g/mol)	Partition coefficient (XLogP3)	H - bond Donor	H – bond Acceptor
Ligands from ginger							
1	6-gingerol	442793	$C_{17}H_{26}O_4$	294.38594	2.5	2	4
2	8-gingerol	168114	$C_{19}H_{30}O_4$	322.4391	4.2	2	4
3	10-gingerol	168115	$C_{21}H_{34}O_4$	350.49226	5.3	2	4
4	6-shogaol	5281794	$C_{17}H_{24}O_3$	276.37066	3.7	1	3
Approved drugs							
1	Tazarotene	5381	$C_{21}H_{21}NO_2S$	351.46194	4.9	0	4
2	Lapatinib	208908	$C_{29}H_{26}ClFN_4O_4S$	581.057543	5.1	2	9
3	Quinestrol	9046	$C_{25}H_{32}O_2$	364.52038	5.3	1	2
4	Raltitrexed	104758	$C_{21}H_{22}N_4O_6S$	458.48758	1.4	4	8
5	Fulvestrant	104741	$C_{31}H_{47}F_5O_3S$	606.770796	9.2	2	9
6	Gycodiazine	9565	$C_{13}H_{15}N_3O_4S$	309.3409	0.9	1	7
7	Amifostine	2141	$C_5H_{15}N_2O_3PS$	214.222962	-4.5	4	6
8	Gefitinib	123631	$C_{22}H_{24}ClFN_4O_3$	446.902363	4.1	1	8
9	Celebrex	2662	$C_{17}H_{14}F_3N_3O_2S$	381.37217	3.4	1	7
10	Disulfiram	3117	$C_{10}H_{20}N_2S_4$	296.5392	3.9	0	4
11	Quercitrin	5280459	$C_{21}H_{20}O_{11}$	448.3769	0.9	7	11

Table 3. Targets selected for different types of cancer

Sl. no.	Targets	Types of cancer	References
1.	RSK2 (PDB ID: 3G51)	Skin cancer	Chen *et al.,* 2012
2.	EGFR (PDB ID: 1XKK)	All types of cancer	PDTD
3.	Oestrogen receptor (PDB ID: 1ERE)	Breast cancer	Chavez *et al.,* 2010
4.	EGFRK (PDB ID: 1M17)	All types of cancer	Mendelsohn and Baselga, 2006
5.	NAT 2 receptor (PDB ID: 2PFR)	Colorectal cancer	PDTD
6.	c-MET (PDB ID: 4GG7)	All types of cancer	Christensen *et al.,* 2004
7.	PI3K (PDB ID: 1E8W)	All types of cancer	PDTD
8.	Follistatin (PDB ID: 2BOU)	Prostate cancer	Shankar *et al.,* 2013
9.	Neuron-specific enolase (PDB ID: 1TE6)	Lung cancer	Shankar *et al.,* 2013
10.	17-Beta HSD (PDB ID: 1FDT)	Breasts cancer	Gunnarsson *et al.,* 2005
11.	Cyclooxygenase-2 (COX-2) (PDB ID: 4HMY)	All types of cancer	PDTD
12.	Nuclear factor-kappa B (NF-*k*B) (PDB ID: 2V2T)	All types of cancer	Kim and Kim, 2013; Saha*et al.,* 2014
13.	Activator protein-1 (AP-1) (PDB ID: 3LN1)	All types of cancer	Eferl and Wagner, 2003

4.2.1 Description of selected cancer targets

4.2.1.1 RSK2

RSK2 is a p90 ribosomal S6 kinase family (p90RSK) member regulating cell proliferation and transformation induced by tumor promoters such as epithelial growth factor (EGF) and 12-*O*-tetradecanoylphorbol-13-acetate. This family of p90RSK is classified as a serine/threonine kinase that respond to many growth factors, peptide hormones, neurotransmitters and environmental stresses such as ultraviolet (UV) light. Activation of RSK2 by EGF and UV through extracellular-

activated protein kinases signaling pathway induces cell cycle progression, cell proliferation and anchorage-independent cell transformation.

Activated and total RSK2 protein levels are highly detected in human skin cancer tissues including squamous cell carcinoma, basal-cell carcinoma and malignant melanoma. Kaempferol and eriodictyol are natural substances to inhibit kinase activity of the RSK2 N-terminal kinase domain, which is a critical kinase domain to transduce their activation signals to the substrates by phosphorylation.

4.2.1.2 Epidermal Growth Factor Receptor

The Epidermal Growth Factor Receptor (EGFR; ErbB-1; HER1 in humans) is the cell-surface receptor for members of the epidermal growth factor family (EGF-family) of extracellular protein ligands. The epidermal growth factor receptor is a member of the ErbB family of receptors, a sub family of four closely related receptor tyrosine kinases: EGFR (ErbB-1), HER2/c-neu (ErbB-2), Her 3 (ErbB-3) and Her 4 (ErbB-4). Mutations affecting EGFR expression or activity could result in cancer.

Mutations that lead to EGFR overexpression (known as up regulation) or over activity have been associated with a number of cancers, including lung , anal, colon and glioblastoma multiforme. These somatic mutations involving EGFR lead to its constant activation which produces uncontrolled cell division.

4.2.1.3 Oestrogen receptor

Oestrogens are best known for their effects upon the female reproductive system where they play a major role in ovulation, implantation, pregnancy maintenance, child birth and lactation. However, these steroid hormones are also essential for sperm production in males and have important physiological functions in the cardiovascular system, immune system, central nervous system and in bone, where they support several diverse physiological processes such as cardiovascular protection, humoral immune response, neuroprotection and bone remodelling.

The biological actions of oestrogens are only found in cells expressing oestrogen receptors. Oestrogen receptors (ERs) act as transcription factors, either activating or inhibiting the expression of a wide array of genes. Cells can respond to oestrogen in different, often opposing ways, because of the presence of two functionally distinct oestrogen receptors and their ability to interact with a number of different co-factors and signalling proteins.

The ER is also capable of ligand-independent activity via a variety of intracellular signalling pathways, including the mitogen-activated protein kinase (MAPK) pathway. These signalling pathways exert their effects through the phosphorylation of the ER by protein kinases, or indirectly through the regulation of co-factors bound to the ER. The overexpression of various cofactors such as NCO3, NCO6 and PPRB have been found in both breast and ovarian cancers.

4.2.1.4 N-acetyltransferase 2

N-acetyltransferase 2 (arylamine N-acetyltransferase) also known as NAT2 is an enzyme encoded by the *NAT2* gene in human. This gene encodes a type of N-acetyltransferase. Polymorphisms in NAT2 are associated with higher incidences of cancer and drug toxicity. The distribution and levels of NAT expression in humans are tissue specific. NAT1 is present in most tissues throughout the body whereas NAT2 is expressed predominantly in liver and gastrointestinal tracts.

4.2.1.5 c-MET

c-Met also called MET and hepatocyte growth factor receptor (HGFR) is a protein encoded by the *MET* gene (MET proto-oncogene, receptor tyrosine kinase) in humans. MET is a membrane receptor that is essential for embryonic development and wound healing. MET is normally expressed by cells of epithelial origin, while expression of HGF is restricted to cells of mesenchyme origin.

Abnormal MET activation in cancer correlates with poor prognosis where aberrantly active MET triggers tumor growth, formation of new blood vessels (angiogenesis) that supply the tumor with nutrients and cancer spread to other organs (metastasis). MET is deregulated in many types of human malignancies including cancers of kidney, liver, stomach, breast, and brain.

4.2.1.6 Phosphoinositide 3-kinase

Phosphoinositide 3-kinase is a family of enzymes involved in cellular functions such as cell growth, proliferation, differentiation, motility, survival and intracellular trafficking which in turn are involved in cancer.

The phosphoinositol-3-kinase family is divided into four different classes: Class I, Class II, Class III, and Class IV. The classifications are based on primary structure, regulation and *in vitro* lipid substrate specificity.

The class IA PI 3-kinase p110α is mutated in many cancers. Many of these mutations cause the kinase to be more active. It is the single most mutated kinase in glioblastoma, the most malignant primary brain tumor. In addition, the epidermal growth factor receptor EGFR that functions upstream of PI 3-kinase is mutationally activated or overexpressed in cancer.

4.2.1.7 Follistatin

Follistatin also known as activin-binding protein that in humans is encoded by the *FST* gene. Follistatin (FST) an autocrine single chain glycoprotein is expressed in nearly all human tissues such as kidney, brain, uterus, and breast with the highest concentration found in human ovarian tissue. Secretion of FST in immortalized ovarian surface epithelial cells derived from either normal human ovaries or ovaries of an ovarian cancer patient carrying a mutation in BRCA1 gene leads to breast cancer.

4.2.1.8 Neuron-specific enolase

Gamma-enolase, also known as enolase 2 (ENO2) or neuron specific enolase (NSE) is an enzyme that is encoded by the *ENO2* gene in human. Gamma-enolase is a phosphopyruvate hydratase, one of the three enolase isoenzymes found in mammals. This isoenzyme is a homodimer found in mature neurons and cells of neuronal origin. It is produced by small cell carcinomas which are neuroendocrine in origin and hence a useful tumor marker for lung cancer patients.

4.2.1.9 17Beta Hydroxysteroid dehydrogenase

17β-Hydroxysteroid dehydrogenase is a group of dehydrogenation of 17 hydroxysteroids in steroidogenesis. Isozyme 3 is responsible for 17-beta-hydroxysteroid dehydrogenase deficiency. 17β-Hydroxysteroid dehydrogenase III deficiency is a rare disorder of sexual development affecting testosterone biosynthesis by17β-hydroxysteroid dehydrogenase III (17β-HSD III), which can produce impaired virilization (traditionally termed male pseudo hermaphrodite).

Adrenal androgens have to be converted to estrogen to stimulate breast carcinoma cells. Several enzymes such as aromatase, steroid sulfatase and 17beta-hydroxysteroid dehydrogenases (17beta-HSDs) are involved in the production of estrogens. The reaction related to 17beta-HSDs activity is one of the last steps of estradiol biosynthesis and 14 isozymes of 17beta-HSD have been identified at this juncture. The balance of the relative expression levels of 17beta-HSD isozymes in human breast carcinomas is thought to play a pivotal role in supply of estradiol to estrogen receptor positive carcinoma cells.

4.2.1.10 Cyclooxygenase-2

Cyclooxygenase (COX) also known as prostaglandin-endoperoxide synthase (PTGS) is an enzyme that is responsible for formation of prostanoids including prostaglandins such as prostacyclin and thromboxane. Cyclooxygenases are enzymes that take part in a complex biosynthetic cascade that results in the

conversion of polyunsaturated fatty acids to prostaglandins and thromboxane(s). Their main role is to catalyze the transformation of arachidonic acid into the intermediate prostaglandin H2 which is the precursor of a variety of prostanoids with diverse and potent biological actions.

Cyclooxygenases have two main isoforms that are called COX-1 and COX-2 (as well as a COX-3). COX-1 is responsible for the synthesis of prostaglandin and thromboxane in many types of cells including the gastro-intestinal tract and blood platelets. COX-2 plays a major role in prostaglandin biosynthesis in inflammatory cells and in the central nervous system. Prostaglandin synthesis in these sites is a key factor in the development of inflammation and hyperalgesia. COX-2 inhibitors have analgesic and anti-inflammatory activity by blocking the transformation of arachidonic acid into prostaglandin H2 selectively.

4.2.1.11 Nuclear factor-kappa B

NF-κB is a protein complex that controls transcription of DNA. NF-κB is found in almost all animal cell types and is involved in cellular responses to stimuli such as stress, cytokines, free radicals, ultraviolet irradiation, oxidized LDL and bacterial or viral antigens. NF-κB plays a key role in regulating the immune response to infection. Disregulation of NF-κB has been linked to cancer, inflammation and autoimmune diseases, septic shock, viral infection, and improper immune development. NF-κB has also been implicated in processes of synaptic plasticity and memory.

4.2.1.12 Activator protein-1

The activator protein 1 (AP-1) is a transcription factor which is a heterodimeric protein composed of proteins belonging to the c-Fos, c-Jun, ATF and JDP families. It regulates gene expression in response to a variety of stimuli including cytokines, growth factors, stress and bacterial and viral

infections. AP-1 in turn controls a number of cellular processes including differentiation, proliferation and apoptosis.

3.2.5.5 Molecular docking and analyses

3.2.5.5.1 Preparation of protein structure

Preparation of the retrieved protein was performed by using protein Preparation Wizard of software "Accelry Discovery studio 4.0" (USA). The protein was prepared by removing extra chain of target protein, internal ligand, crystallographic water molecules and hetero atoms. Hydrogen atoms were added to correct the chemistry of protein. Energy minimization was performed by employing CHARMm force field.

3.2.5.5.2 Preparation of ligand structure

Preparation of ligands was performed by using ligand preparation wizard of software "Accelry Discovery studio 4.0" USA. Preparation was carried out by energy optimization and by adding hydrogen atoms.

3.2.5.5.3 Filtration of ligands

Filtration of ligands was done for identifying the drug likeliness using Lipinski's and Veber rules. To pass Lipinski's and Veber rules, a compound should have molecular weight <500 daltons, number of hydrogen bond donors <5 number of hydrogen bond acceptors < 10 and partition coefficient (LogP) < 5. Based on the criteria the ligands were classified.

3.2.5.5.4 Protein – ligand docking

Molecular docking analyses were carried out using C-DOCKER docking protocol of Discovery studio 4.0. All target proteins were docked against the ligands from ginger and approved drugs to find out the binding geometries and protein ligand

interactions. Docking of the protein – ligand complex was targeted to the predicted active site. Docking simulations were performed using "Accelry Discovery studio 4.0" as the docking engine. The selected residues of the receptor were defined to be a part of the binding site. A maximum of ten poses were allowed to be analyzed and minimum difference between –C-DOCKER and –C-DOCKER interaction energy was identified for the best pose of the ligand.

The scoring function was analyzed using binding energy calculation. The calculation was performed first on the receptor, then on the ligand, and finally on the complex. The energy difference was then calculated using the equation: $\Delta E = E_{complex} - E_{ligand} - E_{protein}$ (ΔE is the ligand binding energy).

3.2.5.5.5 ADME & Toxicity Studies

Absorption, Distribution, Metabolism, Excretion and Toxicity (ADME/T) studies were performed *in silico* using ADME Descriptor algorithm of Accelrys Discovery studio 4.0 (USA) in which various pharmacokinetic parameters like Aq. solubility, Human Intestinal Absorption, Blood-Brain-Barrier (BBB) penetration, cytochrome P450 inhibition and Hepatotoxicity levels were estimated. Standard levels of various ADMET parameters are presented in Table 4.

Table 4 Standard levels of ADMET descriptors from Discovery studio 4.0

Human Intestinal Absorption level		BBB Level		Aq. Solubility level		Hepatotoxicity prediction		CPY2D6 prediction	
Level	Intensity	Level	Intensity	Level	Drug-likeness	Level	Value	Level	Value
0	Good	0	Very high penetration	0	Extremly low	0	Nontoxic (False)	0	Non-inhibitor (False)
1	Moderate	1	High	1	No, very low, but possible	1	Toxic (True)	1	Inhibitor (True)
2	Poor	2	Medium	2	Yes, low				
3	Very poor	3	Low	3	Yes, good				
		4	Undefined	4	Yes, optimal				
				5	No, too soluble				

3.2.6 Validation of anticancerous properties of gingerols using different cell lines

The major pharmacologically active compound of ginger is 6-gingerol. Validation of anticancerous properties of 6-gingerol was done in three cancer cell lines which included, HCT15 (colon cancer), Raw 264.7 (mouse leukaemic monocyte macrophage) and L929 (murine fibro sarcoma).

3.2.6.*1 In vitro* cytotoxicity analysis of 6-gingerol using different cancer cell lines

Three cancer cell lines, Human colon cancer (HCT15), mouse leukaemic monocyte macrophage (Raw 264.7) and murine fibro sarcoma (L929) cells were received from Amala Cancer Research Centre, Thrissur were used to study *in vitro* cytotoxicity of 6-gingerol and effect of 6-gingerol on cytotoxicity was observed at 24, 48 and 72 h intervals. 6-gingerol was added at different levels (17, 34, 68, 102, 136

and 170 μM) to different cell lines. The experiment was replicated thrice for HCT15, five times for Raw 264.7 and L929.

3.2.6.3 Maintenance of cell culture

The cells were cultured in RPMI-1640 (Appendix II)medium supplemented with 10 per cent fetal bovine serum (FBS), 4.5 g glucose, 1 per cent each HEPES buffer, sodium pyruvate and antibiotic (penicillin and streptomycin) at 37°C. Trypsin and EDTA were used for detaching cancer cells from the bottom of tissue culture flask during plating or sub-culturing. PBS buffer was used to wash the culture for removing dead cells. Details of media composition is provided in Annexure II and III.

3.2.6.4 *In vitro* cytotoxicity assay

MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay was done to evaluate the proliferative capacity of cells. A 96 well plate was used with 100 μl medium containing cells. After 48 h of incubation, the cells were treated with gradient concentration (17, 34, 68, 102, 136 and 170 μM) of 6-gingerol which was dissolved in ethanol. Observations were recorded at 24, 48 and 72 h intervals. The spent medium was removed and 100 μl of fresh medium and 10 μl of MTT (5 mg/ml in PBS) were added to the wells and cells were incubated at 37°C in dark for 4 h. The formazan product was dissolved by adding 100 μl of DMSO. The absorbance was measured at 570 nm using monochromatic ELISA reader (VERSA max microplate reader).

3.2.7 Statistical analysis

Statistical analyses of data were conducted using Statistical Package for the Social Sciences (SPSS) software. Frequency distribution was fitted to know the frequency of each character in somaclones. Principle Component Analysis (PCA) was performed to cluster ginger somaclones based on quality.

Results

4. Results

The results of the investigations on "Screening ginger (*Zingiber officinale* Rosc.) somaclones for gingerol content and validation of anticancerous properties of gingerol" carried out at the Centre for Plant Biotechnology and Molecular Biology (CPBMB) and Distributed Information Centre (DIC), College of Horticulture, Vellanikkara during August 2013 to June 2015 are presented in this chapter under different sub headings.

4.1 Screening somaclones for gingerol content

4.1.1 Raising of somaclones in filed and evaluation of yield

The somaclones planted in last week of May 2013 as a part of an externally aided project on going at CPBMB were harvested in January 2014 after drying the pseudostem. The fresh yield per plant from the fifty somaclones were recorded and frequency distribution of yield in somaclones is presented in Table 5a. The yield ranged from 91.5 to 574.83 g/plant in the somaclones studied. The frequency distribution presented in Table 5a showed the variability in fresh yield in the clones. Majority of the clones (37.25 percent) recorded per plant yield from 301 to 400 g/plant. It was observed that 7.84 percent of clones were high yielders recording yield of 501 to 600 g/plant and 1.96 percent of clones were low yielders.

When yield was analyzed in groups of somaclones (Table 5b), clones in the group (M Se) were high yielders followed by MC 10Gy and M Se 20Gy. The highest variability in yield was observed in MC 10Gy group of clones.

Table 5a. Frequency distribution of yield realized in somaclones of ginger

Class No.	Fresh yield (g/plant)	No. of clones in the group	% of clones	Mean (g/plant)
1	<100	1	1.96	91.5
2	100 – 200	7	13.73	144.36
3	201 – 300	14	27.45	266.5
4	301 – 400	19	37.25	344.89
5	401 – 500	6	11.76	436
6	501 – 600	4	7.84	531.33

Grand Mean = 316.22 Standard deviation = 110.96

Table 5b. Per plant yield in different groups of somaclones of ginger

Sl No.	Groups of clones	No. of clones	Fresh yield (g/plant) Range	Mean (g/plant)
1	MB	10	137.5 - 337.5	286.3
2	MC	08	91.5 – 393	225.41
3	M Se	06	251.5 – 513	380.92
4	MC 10Gy	12	126 - 574.83	366.24
5	M Se 10Gy	02	132 - 323.5	227.75
5	M Se 20Gy	12	100.5 - 502.5	318.54
6	Control	01	198.52	198.52

4.1.2 Dry ginger recovery

The quantity of fresh ginger available for each clone was sun dried after peeling the skin from flat surface for seven to ten days to make dry ginger. The recovery of dry ginger in the fifty somaclones is presented in Table 6a. The frequency distribution presented in the Table 6a showed the variability in dry ginger. In majority of clones the recovery was in between 25-30 percent. Groupwise analysis of dry ginger recovery (Table 6b) showed that the highest recovery was in clones of MC

10Gy group followed by M Se and MB. High variability in dry ginger recovery was observed in clones of MC 10Gy group.

Table 6a. Frequency distribution of dry ginger recovery in somaclones of ginger

Class No.	Dry recovery (%)	No. of clones in the group	% of clones	Mean (%)
1	15 – 20	3	5.88	19.55
2	20.01 – 25	21	41.18	22.70
3	25.01 – 30	27	52.94	26.78

Grand Mean = 24.67 Standard deviation = 2.70

Table 6b. Dry ginger recovery in different groups of somaclones of ginger

Sl No.	Groups of clones	No. of clones	Dry recovery (%) Range	Mean (%)
1	MB	10	23.20 - 27.41	25.50
2	MC	08	20.87 - 26.33	24.16
3	MSe	06	21.16 - 28.91	25.63
4	MC 10Gy	12	19.62 - 29.03	25.76
5	MSe 10Gy	02	20.23 - 25.32	22.78
5	MSe 20Gy	12	19.46 - 26.5	23.12
6	Control	01	23.88	23.88

4.1.3 Extraction of oleoresin

Extraction of oleoresin was done using Soxhlet apparatus. Oleoresin yield in the somaclones studied is presented in Table 7a. Oleoresin content varied from 4.45 to 12.78 per cent in the somaclones studied. In majority of somaclones (58.82 per cent), content of oleoresin ranged from 5.01-7.0 per cent. The groupwise distribution presented in Table 7b showed the variability in oleoresin yield in the group of clones.

The highest yield of oleoresin (8.06 per cent) was found in clones of Mse 20Gy groups followed by M Se group of clones. The highest variability in oleoresin yield was observed in M Se 20Gy group of clones followed by MC 10Gy.

Table 7a. Frequency distribution of oleoresin yield in ginger somaclones

Class No.	Oleoresin yield (%)	No. of clones in the group	% of clones	Mean (%)
1	3.0 - 5.0	3	5.88	4.69
2	5.01 - 7.0	30	58.82	5.94
3	7.01 - 9.0	11	21.57	7.61
4	9.01 - 11.0	6	11.76	10.21
5	11.01 - 13.0	1	1.96	12.78

Grand Mean = 6.86 Standard deviation = 1.79

Table 7b. Oleoresin yield in different groups of somaclones of ginger

Sl No.	Groups of clones	No. of clones	Recovery of oleoresin (%) Range	Mean (%)
1	MB	10	4.74 - 7.7	5.72
2	MC	08	5.07 - 7.50	5.82
3	MSe	06	7.19 - 10.79	7.75
4	MC 10Gy	12	5.17 - 10.85	6.86
5	MSe 10Gy	02	7.45 - 7.81	7.28
6	MSe 20Gy	12	5.17 - 12.78	8.06
7	Control	01	5.31	5.31

4.1.4 Screening somaclones for gingerol content

Somaclones (50Nos.) were screened for gingerol content using HPLC analytical platform. Chromatographic analysis showed variations in the content of 6-gingerol, 8-gingerol, 10-gingerol and 6-shogaol in somaclones studied.

4.1.4.1 Screening somaclones for 6-gingerol content

The content of 6-gingerol in the selected somaclones along with the control is presented in Table 8a. The content of 6-gingerol varied from 3.33 to 13.83 g/kg dry ginger powder in the selected somaclones studied. The frequency distribution presented in Table 8a showed that maximum number of clones (43.14 percent) contain 6-gingerol in the range of 7.01 to 9 g/kg dry ginger powder. Groupwise analysis of 6-gingerol content (Table 8b) showed highest variability in M Se 20Gy followed by MC 10Gy group of somaclones.

Table 8a. Frequency distribution of 6-gingerol content in somaclones of ginger

Class No.	6-gingerol (g/kg dry ginger powder)	No. of clones in the group	% of clones	Mean (g/kg dry ginger powder)
1	3.0 - 5.0	3	5.88	3.72
2	5.01 - 7.0	16	31.37	5.83
3	7.01 - 9.0	22	43.14	7.81
4	9.01 - 11.0	5	9.80	9.88
5	11.01 - 13.0	4	7.84	11.63
6	>13.0	1	1.96	13.83

Grand Mean = 7.69 Standard deviation = 2.07

Table 8b. The content of 6-gingerol in different groups of somaclones of ginger

Sl No.	Groups of clones	No. of clones	6-gingerol (g/kg dry ginger powder) Range	Mean (g/kg dry ginger powder)
1	MB	10	3.33 - 4.33	3.72
2	MC	08	5.80 - 9.81	7.30
3	MSe	06	6.11 - 10.29	8.13
4	MC 10Gy	12	5.89 - 12.42	8.32
5	MSe 10Gy	02	7.73 - 8.03	7.88
5	MSe 20Gy	12	6.13 - 13.83	8.48
6	Control	01	7.2	7.2

4.1.4.2 Screening somaclones for 8-gingerol content

The content of 8-gingerol in somaclones is presented in Table 9a. The 8-gingerol content varied from 0.25 to 0.94 g/kg dry ginger powder in the selected somaclones. The frequency distribution presented in Table 9a showed the variability in 8-gingerol content in the clones. In majority of somaclones (24 percent), the content of 8-gingerol ranged from 0.26 to 0.50 g/kg dry ginger powder. When the groups of somaclones were examined, high variability was observed in M Se 20Gy group of somaclones followed by MC 10Gy group of somaclones (Table 9b). The groupwise analysis also showed the variability in 8-gingerol content in groups of clones. The highest mean recovery of 8-gingerol (0.65) was observed in M Se 10Gy group of clones.

Table 9a. Frequency distribution of 8-gingerol content in somaclones of ginger

Class No.	8-gingerol (g/kg dry ginger powder)	No. of clones in the group	% of clones	Mean (g/kg dry ginger powder)
1	0 - 0.25	1	1.96	0.25
2	0.26 - 0.50	24	47.06	0.42
3	0.51 - 0.75	18	35.29	0.60
4	0.76 - 1.0	8	15.69	0.60

Grand Mean = 0.55 Standard deviation = 0.17

Table 9b. The content of 8-gingerol in different groups of somaclones of ginger

Sl No.	Groups of clones	No. of clones	8-gingerol (g/kg dry ginger powder) Range	Mean (g/kg dry ginger powder)
1	MB	10	0.25 - 0.67	0.43
2	MC	08	0.41 - 0.79	0.58
3	MSe	06	0.45 - 0.72	0.55
4	MC 10Gy	12	0.33 - 0.90	0.59
5	MSe 10Gy	02	0.46 - 0.83	0.65
6	MSe 20Gy	12	0.28 - 0.94	0.56
7	Control	01	0.60	0.60

4.1.4.3 Screening somaclones for 10-gingerol content

The content of 10-gingerol in the somaclones studied is presented in Table 10a. The 10-gingerol content varied from 0.17 to 1.15 g/kg dry ginger powder in the selected somaclones. In majority of somaclones (25 percent), the content of 10-gingerol ranged from 0.26 to 0.50 g/kg dry ginger powder. The frequency distribution presented in Table 10a showed the variability in 10-gingerol content in the somaclones. When the groups of somaclones were examined, the higher mean recovery of 10-gingerol (0.65 percent) was found in clones of group MC. However, the recovery was found highest in the conventionally propagated control plant. The groupwise analysis presented in Table 10b showed the variability in 10-gingerol content in the groups of clones. The highest variability among the groups of somaclones was observed in M Se 20Gy group followed by MC 10Gy group of somaclones.

Table 10a. Frequency distribution of 10-gingerol content in somaclones of ginger

Class No.	10-gingerol (g/kg dry ginger powder)	No. of clones in the group	% of clones	Mean (g/kg dry ginger powder)
1	0 - 0.25	6	11.76	0.21
2	0.26 - 0.50	25	49.02	0.41
3	0.51 - 0.75	12	23.53	0.63
4	0.76 - 1.0	4	7.84	0.88
5	>1.0	4	7.84	1.06

Grand Mean = 0.53 Standard deviation = 0.24

Table 10b. The content of 10-gingerol in different groups of somaclones of ginger

Sl No.	Groups of clones	No. of clones	10-gingerol (g/kg dry ginger powder) Range	Mean (g/kg dry ginger powder)
1	MB	10	0.25 - 0.78	0.42
2	MC	08	0.39 - 0.98	0.65
3	MSe	06	0.29 - 0.70	0.50
4	MC 10Gy	12	0.19 - 1.06	0.57
5	MSe 10Gy	02	0.25 - 1.03	0.64
5	MSe 20Gy	12	0.17 - 1.15	0.47
6	Control	01	0.66	0.66

4.1.4.4 Screening somaclones for 6-shogaol content

The content of 6-shogaol in the somaclones is presented in Table 11a. The 6-shogaol content varied from 0.61 to 2.33 g/kg dry ginger powder in the somaclones studied. In majority of somaclones (64.71 percent), the content of 6-shogaol ranged from 1.01 to 1.50 g/kg dry ginger powder. The groupwise analysis presented in Table 11b showed the variability in 6-shogaol content in the groups of clones. The higher

mean recovery of 6-shogaol (1.60 g/kg dry ginger powder) was found in MSe 10Gy groups of clones. The highest variability in 6-shogaol content was observed in clones of MC 10Gy group followed by M Se 20Gy group.

Table 11a. Frequency distribution of 6-Shogaol content in somaclones of ginger

Class No.	6-Shogaol (g/kg dry ginger powder)	No. of clones in the group	% of clones	Mean (g/kg dry ginger powder)
1	0.51 - 1.0	9	17.65	0.85
2	1.01 - 1.50	33	64.71	1.24
3	1.51 - 2.0	7	13,73	1.72
4	>2.0	2	3.92	2.32

Grand Mean = 1.28 Standard deviation = 0.35

Table 11b. The content of 6-shogaol in different groups of somaclones of ginger

Sl No.	Groups of clones	No. of clones	6-shogaol (g/kg ginger powder) Range	Mean (g/kg ginger powder)
1	MB	10	0.61 - 1.29	1.05
2	MC	08	0.82 - 1.59	1.15
3	MSe	06	0.8 - 1.44	1.22
4	MC 10Gy	12	0.96 - 2.33	1.34
5	MSe 10Gy	02	1.43 - 1.76	1.60
5	MSe 20Gy	12	0.98 - 2.30	1.49
6	Control	01	0.97	0.97

4.1.4.5 Screening somaclones for total gingerol content

The content of total gingerol (6-gingerol + 8-gingerol + 10-gingerol) in the somaclones is presented in Table 12a. Total gingerol content varied from 3.88 to 15.12 g/kg dry ginger powder in the somaclones studied. In majority of somaclones (23 percent), content of total gingerol ranged from 7.01 to 9.0 g/kg dry ginger

powder. The groupwise analysis presented in Table 12b showed the variability in total gingerol content in groups of clones. The higher content of total gingerol (10.21 g/kg dry ginger powder) was found in M Se 20Gy groups of clones. The variability in total gingerol content was more in clones of the group M Se 20Gy followed by MC 10Gy.

Total 12a. Frequency distribution of total gingerol content in selected somaclones of ginger

class No.	Total gingerol (g/kg dry ginger powder)	No. of clones in the group	% of clones	Mean (g/kg dry ginger powder)
1	3.0 - 5.0	2	3.92	3.90
2	5.01 - 7.0	7	13.73	6.44
3	7.01 - 9.0	23	45.10	8.01
4	9.01 - 11.0	11	21.60	9.64
5	11.01 - 13.0	4	7.84	11.85
6	>13.0	4	7.84	14.10

Grand Mean = 8.76 Standard deviation = 2.34

Total 12b. The content of total gingerol in different groups of somaclones of ginger

Sl No.	Groups of clones	No. of clones	Total gingerol (g/kg dry ginger powder) Range	Mean (g/kg dry ginger powder)
1	MB	10	3.88 - 10.6	6.91
2	MC	08	6.6 - 11.47	8.5
3	M Se	06	7.04 - 11.71	9.18
4	MC 10Gy	12	6.41 - 14.19	9.49
5	M Se 10Gy	02	8.44 - 9.89	9.17
5	M Se 20Gy	12	6.59 - 15.12	10.21
6	Control	01	8.46	8.46

4.1.4.6 Clustering of somaclones based on quality parameters

Principle component analysis was done to cluster the somaclones based on oleoresin yield and content of 6-gingerol, 8-gingerol, 10-gingerol and 6-shogaol and total gingerol. When all the quality parameters were considered, five clones viz. 49 (M Se 2Kr 418), 43 (M Se 2Kr 175), 39 (M Se 2Kr 862), 28 (MC 1Kr 330) and 22 (MC 1Kr 168) were rated as superior (Figure 7). . Details of selected ginger somaclones used for the study is provided in Annexure IV. The chromatogram of selected somaclones are presented in Figure 8, 9, 10, 11 and 12.

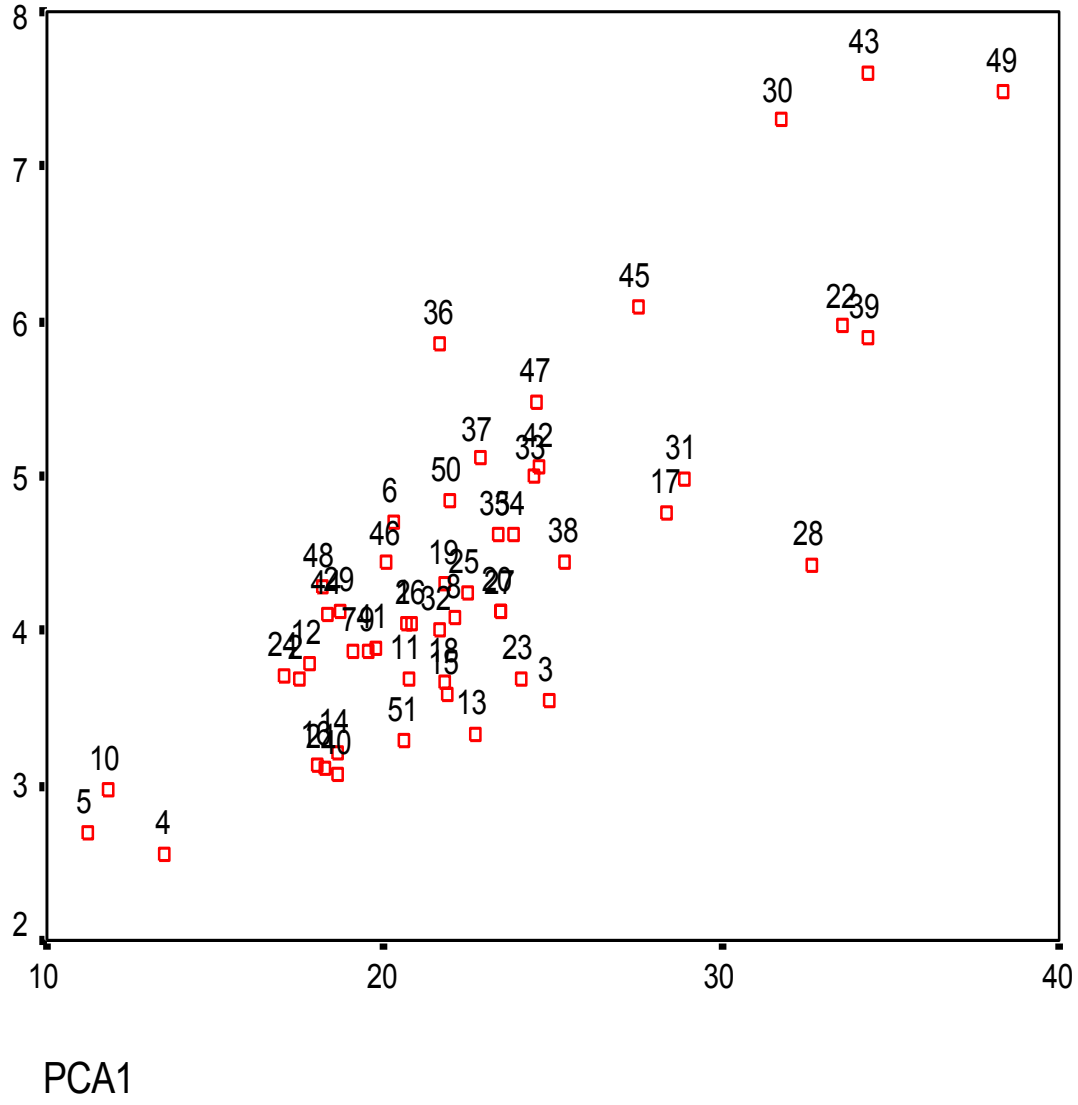


Figure 7. Principle Component Analysis for clustering of ginger somaclones based on quality parameters

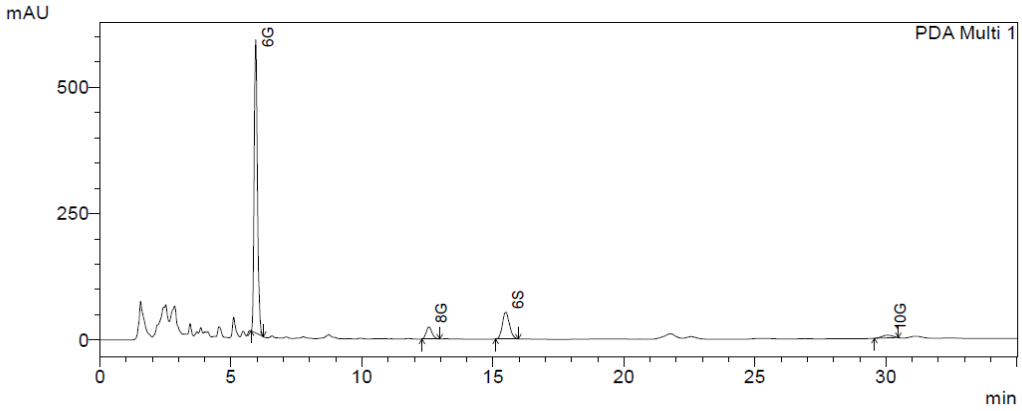


Figure 8. High Performance Liquid Chromatogram of M Se 20GY 418

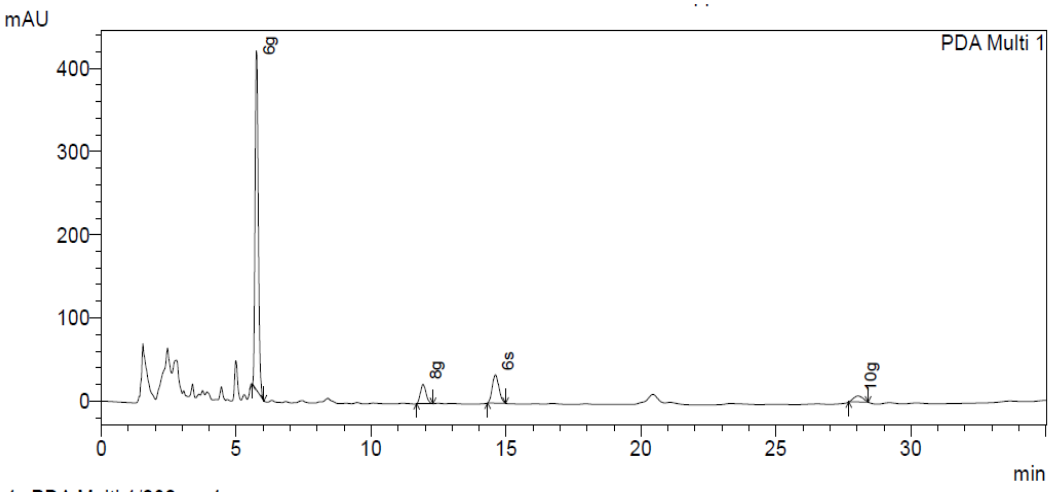


Figure 9. High Performance Liquid Chromatogram of M Se 20Ky 175

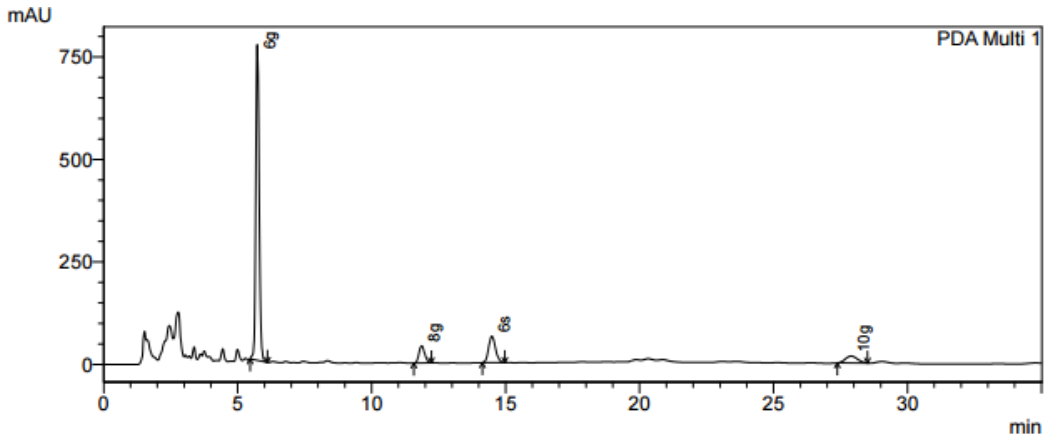


Figure 10. High Performance Liquid Chromatogram of M Se 20Gy 862

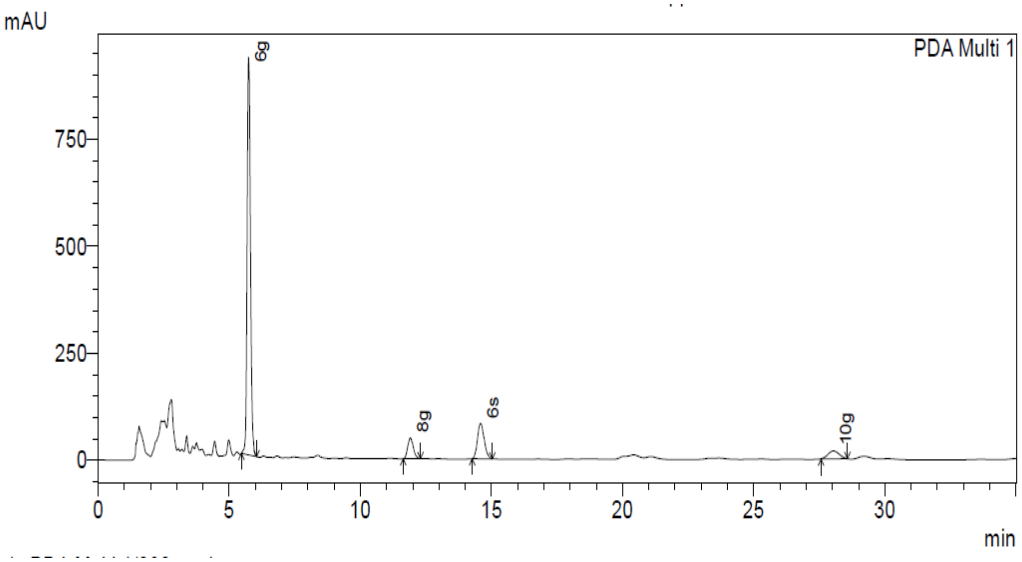


Figure 11. High Performance Liquid Chromatogram of MC 10Gy 330

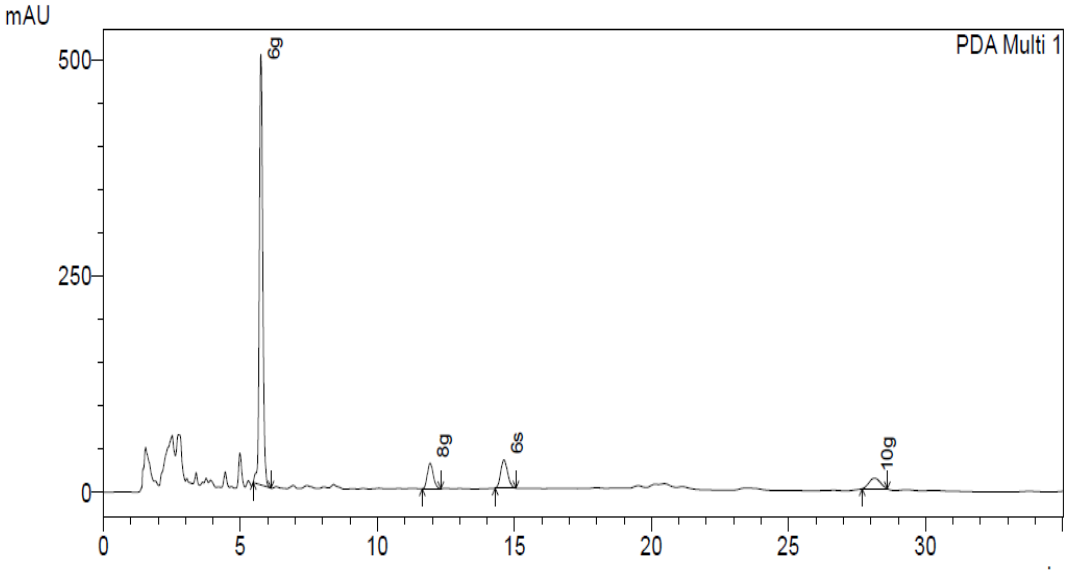


Figure 12. High Performance Liquid Chromatogram of MC 10Gy 168

4.2 *In silico* analysis of potential ligands and cancer targets for molecular docking against cancer

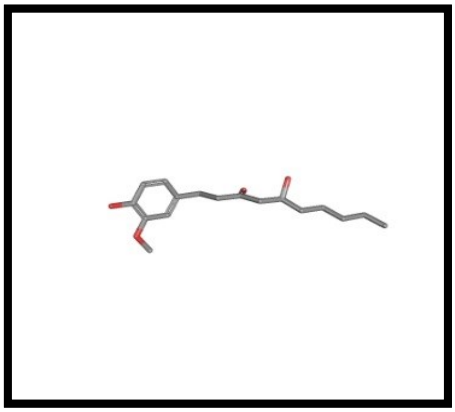
The four ginger ligands and eleven approved drugs were selected for molecular docking. The structure of ligands were retrieved from PubChem online database and molecular properties of ligands were recorded from the database. Table 2 shows the molecular properties of ginger ligands and approved drugs. The compound ID, molecular formula, molecular weight, partition coefficient and number of H-bond donor and acceptor are dealt in Table 2. The partition coefficient decides the distribution of drug molecules inside the human body. The H-bond donor indicated the electron donor and H-bond acceptor indicated the strength of the bond.

Total thirteen targets were identified through literature survey and from Potential Drug Targets Database. The list of selected targets is presented in Table 3. Of the targets selected seven were for all types of cancer, two for breast cancer and one each for skin, colorectal and lung cancer.

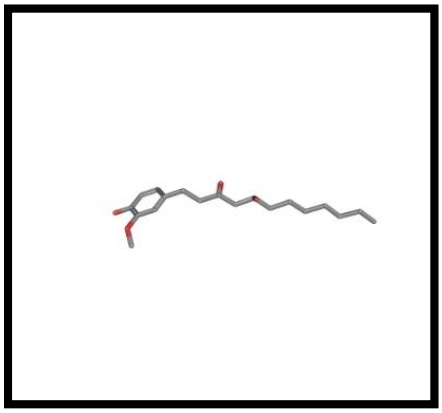
4.2.2 Retrieval of structure of different ligands

Structure of four ligands from ginger viz. 6-gingerol (CID: 442793), 8-gingerol (CID: 168114), 10-gingerol (CID: 168115), 6-shogaol (CID: 5281794) and commonly used drugs for cancer treatment like amifostine (CID: 2141), tazarotene (CID: 5381), quinestrol (CID: 9046), glycodiazine (CID: 9565), fulvestrant (CID: 104741), raltitrexed (CID: 104758), lapatinib (CID: 208908), celecoxib (CID: 2662), quercitrin (CID: 5280459) and disulfiram (CID: 3117) were downloaded in 3D form from PubChem in sdf. Format. The 3D structures of different ligands are presented in Figure 13. All the ginger ligands and approved drugs have similarity in presence of benzene ring except absent in amifostine and disulfiram.

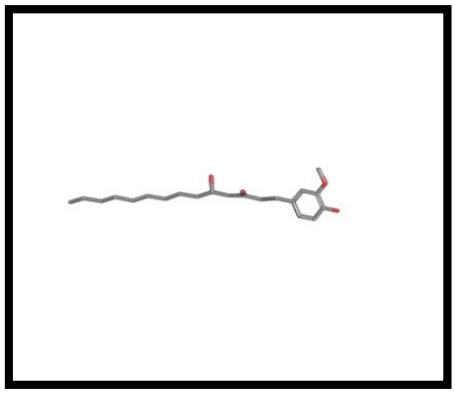
Preparation of the ligands was done by using ligand Preparation wizard of software Discovery studio 4.0 to change ionization and generate tautomer, isomers and 3D coordinates. Ligands were filtered after analysis using “Lipinski’s and Veber



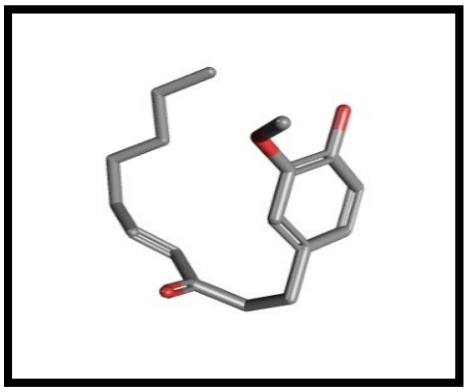
6-gingerol



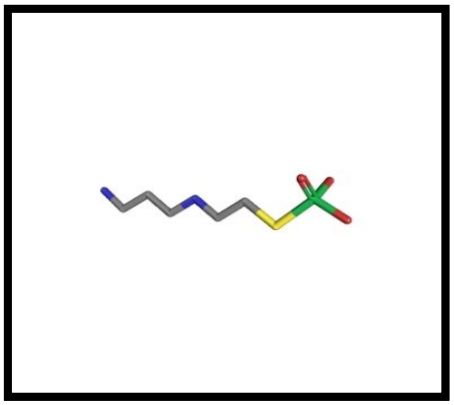
8-gingerol



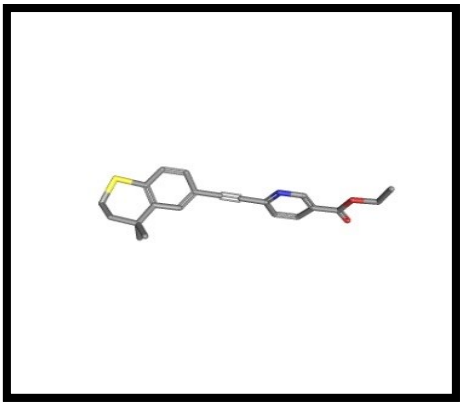
10-gingerol



6- shogaol

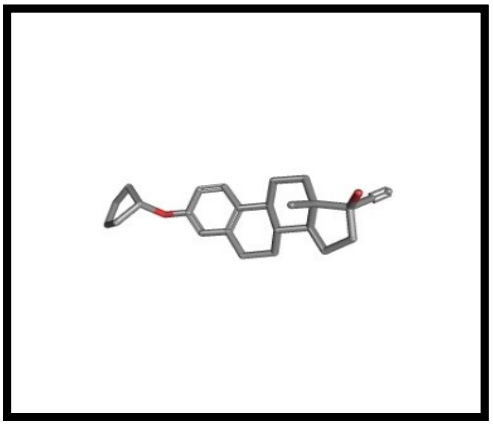


Amifostine

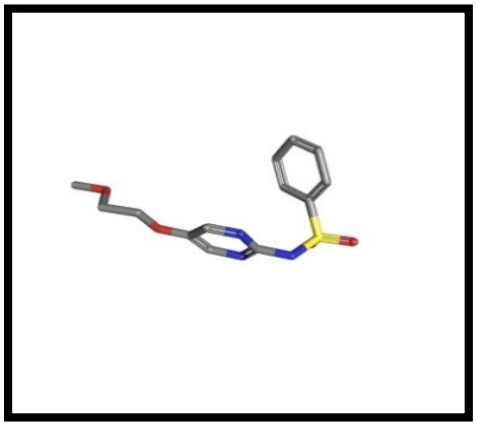


Tazarotene

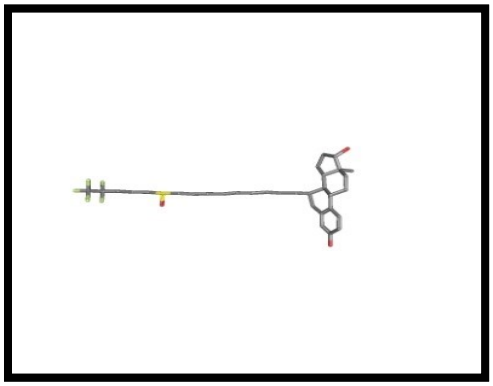
Figure 13. 3D structure of ginger ligands and approved drugs for cancer



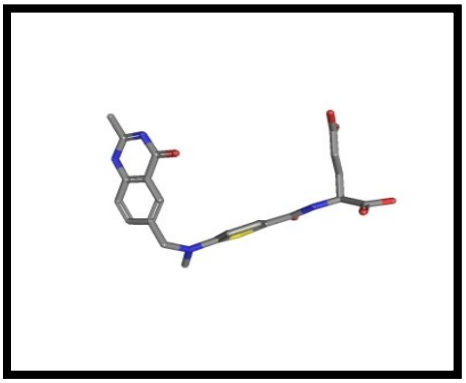
Quinestrol



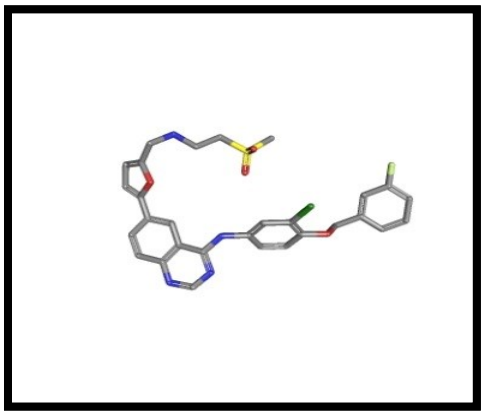
Glycodiazine



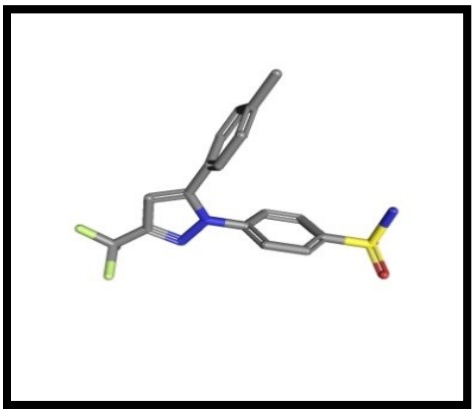
Fulvestrant



Raltitrexed

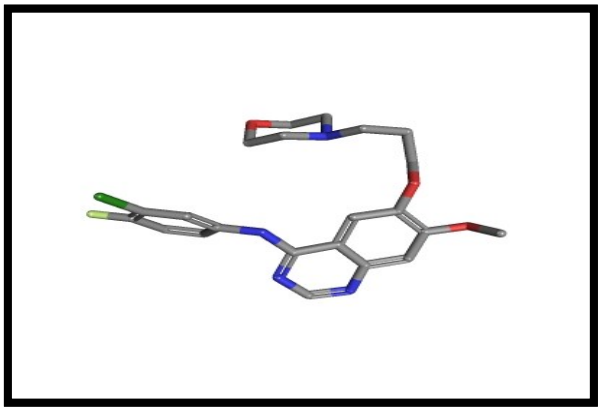


Lapatinib

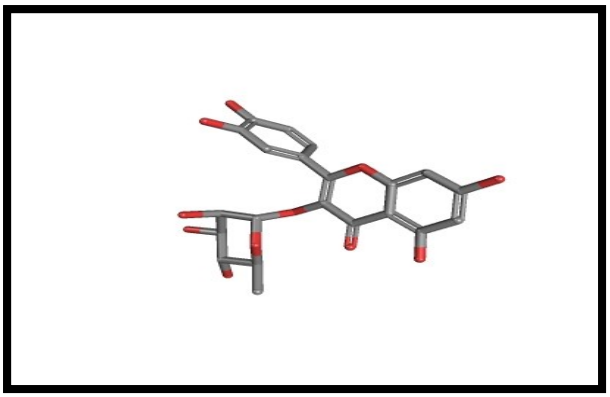


Celecoxib

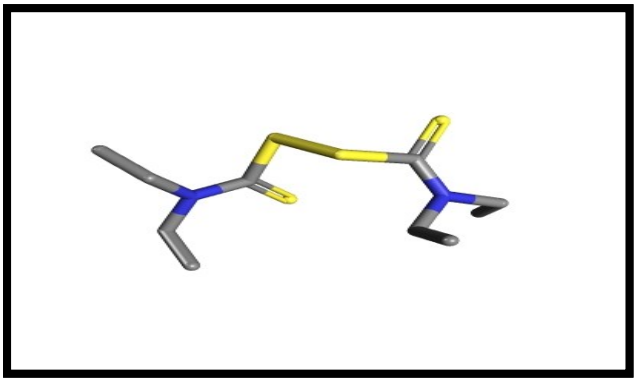
Figure 13. 3D structure of ginger ligands and approved drugs for cancer



gefitinib



quercitrin



disulfiram

Figure 13. 3D structure of ginger ligands and approved drugs for cancer

rules' (Lipinski 1997) and the details are presented in Table 13. Of the four ligands from ginger filtered using lipinski's and veber rules, two viz. 6-gingerol and 6-shogaol passed while 8-gingerol and 10-gingerol failed. Of the eleven approved drugs, two failed and nine passed the rules.

Table 13. Filtration of ginger ligands and approved drugs using Lipinki's and Veber rules

Sl. No.	Compound name	Partition coefficient (XLogP3)	Hydrogen Bond Donar (No.)	Hydrogen Bond Acceptor (No.)	No. of rotation bonds (No.)	Lipinski's and Veber rules
Ligands from ginger						
1	6-gingerol	<5	<5	<10	10	PASS
2	8-gingerol	<5	<5	<10	12	FAIL
3	10-gingerol	>5	<5	<10	14	FAIL
4	6-shogaol	<5	<5	<10	09	PASS
	Approved drugs					
1	Tazarotene	<5	<5	<10	05	PASS
2	Lapatinib	>5	<5	<10	11	PASS
3	Quinestrol	>5	<5	<10	03	PASS
4	Raltitrexed	<5	<5	<10	09	PASS
5	Fulvestrant	>5	<5	<10	14	FAIL
6	Gycodiazine	<5	<5	<10	07	PASS
7	Amifostine	<5	<5	<10	07	PASS
8	Gefitinib	<5	<5	<10	08	PASS
9	Celebrex	<5	<5	<10	03	PASS
10	Disulfiram	<5	<5	<10	07	PASS
11	Quercitrin	<5	>5	>10	03	FAIL

4.2.3 Retrieval of structure of different cancer targets

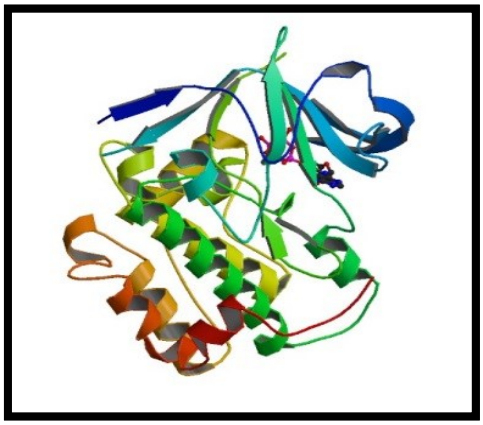
The cancer targets identified for different types of cancer through literature survey and Potential Drug Target Database included Ribosomal S6 kinase (skin cancer), Epidermal Growth Factor Receptor (all types of cancer), oestrogen receptor

(breast cancer), Epidermal Growth Factor Receptor kinase (all types of cancer), N-acetyltransferase 2 receptor (colorectal cancer), c-MET (all types of cancer), Phosphoinositide 3-kinase (all types of cancer), follistatin (prostate cancer), Neuron-specific enolase (lung cancer), 17-Beta Hydroxysteroid dehydrogenase (breast cancer), cyclooxygenase-2 (all types of cancer), Nuclear factor-kappa B (all types of cancer) and Activator protein-1 (all types of cancer).

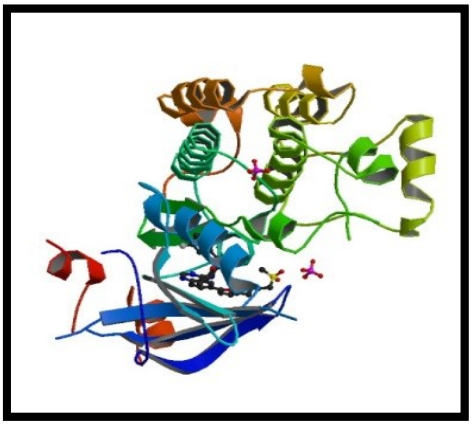
The structure of targets retrieved from Protein Data Bank (http://www.rcsb.org/pdb/).are presented in Figure 14. Preparation of the retrieved protein was performed using protein preparation wizard of software Discovery studio 4.0.

4.2.4 Prediction of active sites of the cancer targets

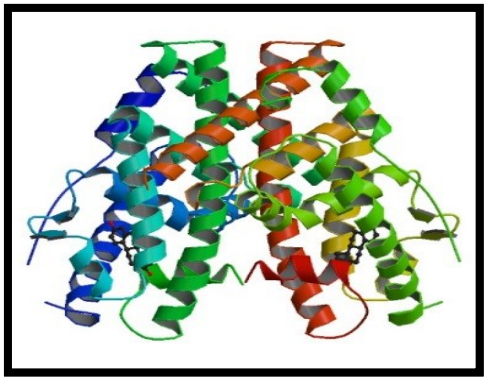
Prediction of active sites of the targets was done based on "Receptor cavity" and "Current selection" method of Accelry Discovery studio 4.0 (USA). The maximum number of active sites were twenty five in target N- acetyltransferase 2 receptor (2PFR) followed by nine in cyclooxygenase-2 (3LN1) and five in Follistatin (2BOU). Only one active site was selected from each target for docking which had maximum number of amino acid residues in their active site. The list of active sites and site used for docking is presented in Table 14. The maximum number of amino acid residues was eight in Ribosomal S6 kinase followed by seven in N-acetyltransferase 2 receptor and five in Activator protein-1.



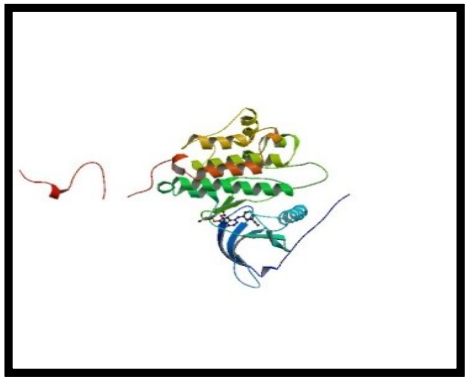
3G51 (ribosomal S6 kinase)



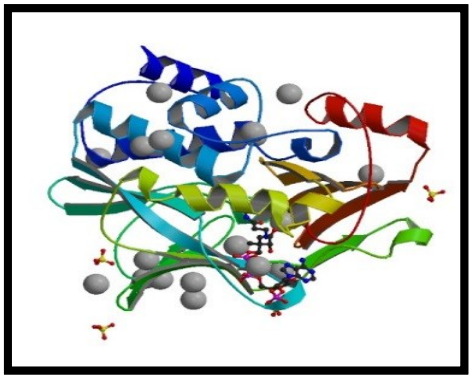
1XKK (Epidermal Growth Factor Receptor)



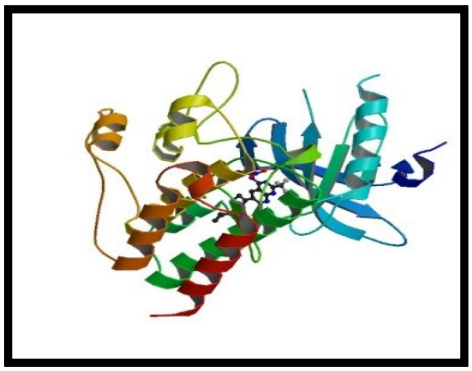
1ERE (oestrogen receptor)



1M17 (Epidermal Growth Factor Receptor kinase

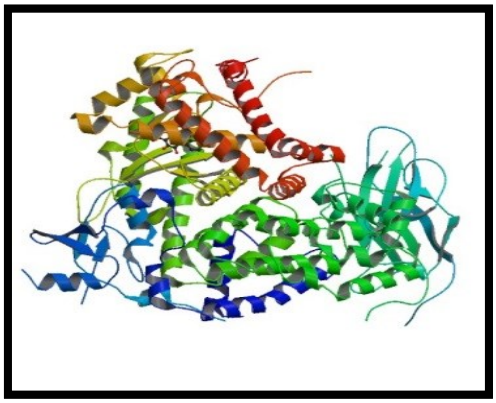


2PFR (N- acetyltransferase 2 receptor)

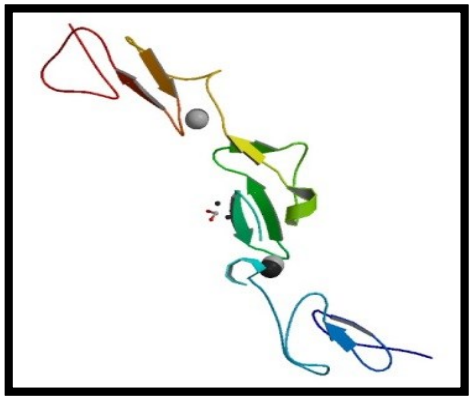


4GG7 (c-MET)

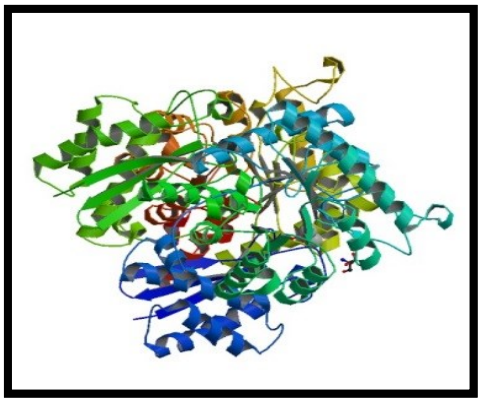
Figure 14. Structure of targets retrieved from Protein Data Bank



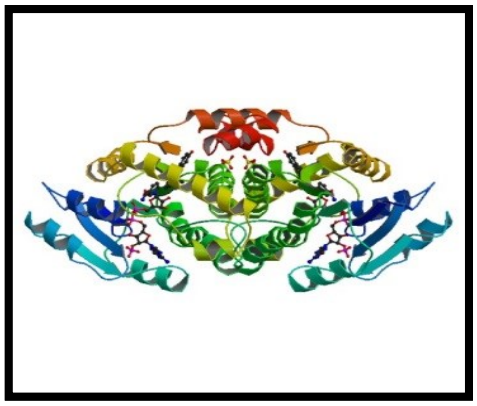
1E8W (Phosphoinositide 3-kinase)



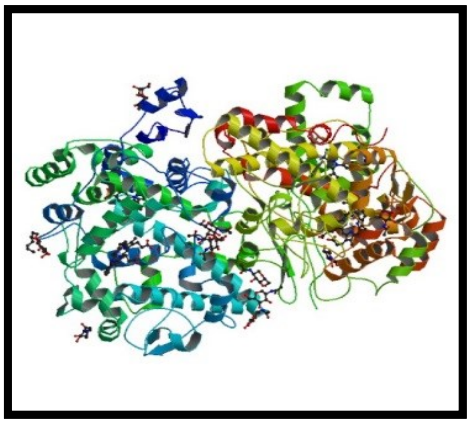
2BOU (follistatin)



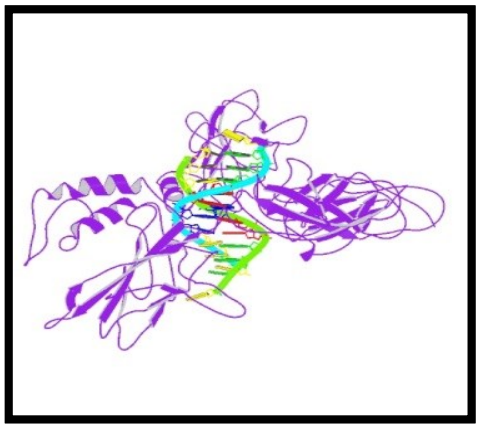
1TE6 (Neuron-specific enolase)



1FDT (17-Beta Hydroxysteroid dehydrogenase)

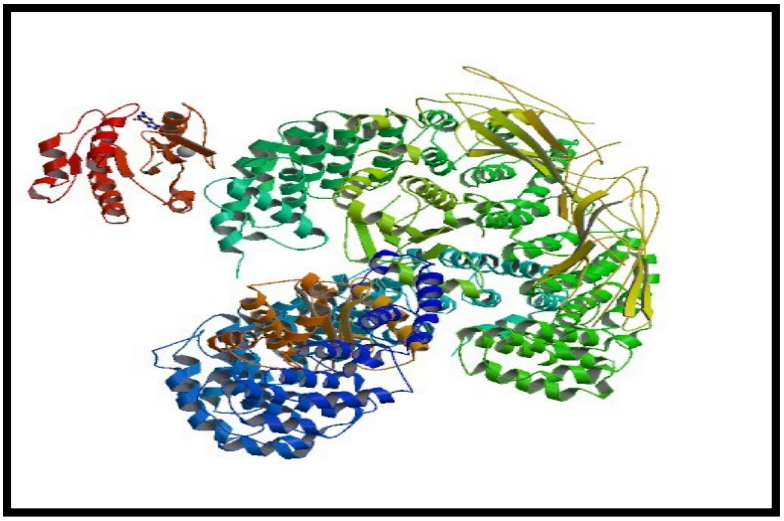


3LN1 (Cyclooxygenase-2)



2V2T (Nuclear factor-kappa B)

Figure 14. Structure of targets retrieved from Protein Data Bank



4HMY (Activator protein-1)

Figure 14. Structure of targets retrieved from Protein Data Bank

Table 14. Predicted active sites for the targets identified for cancer

Sl. No.	Targets	Name of active sites	No. of active sites	Total no. of amino acid residues	Details of amino acid residues	Active site with maximum no. of amino acid residues used for docking
1	Ribosomal S6 kinase (3G51)	AC1	1	8	Ser78, Asn198, Lys216, Lys100, Thr210, Glu197, Leu150, Asp148	AC1
2	Epidermal Growth Factor Receptor (1XKK)	AC1, AC2 and AC3	3	3	Met793, Asp800, Phe856	AC3
3	Oestrogen receptor (1ERE)	AC1	1	3	Glu353, His524, Arg394	AC1
4	Epidermal Growth Factor Receptor kinase (1M17)	AC1	1	1	Met769	AC1
5	N-acetyltransferase 2 receptor (2PFR)	AC1, AC2, AC3, AC4, AC5, AC6, AC7, AC8, BC1, BC2, BC3, BC4, BC6, BC9, CC1, CC4, CC7, CC8, DC1, DC2, DC3, DC4, DC5, DC6 and DC7	25	7	Ser216, Thr103, Gly 104, Cys68, Ser287, Tyr208, Thr214	AC8
6	c-MET (4GG7)	AC1	1	3	Met1160 Tyr1230 Asp1222	AC1
7	Phosphoinositide 3-kinase (1E8W)	AC1	1	4	Val882, Glu880, Asp964, Lys833	AC1
8	Follistatin (2BOU)	AC1, AC2, AC3, AC4 and AC5	5	1	Ser59	AC5
9	Neuron-specific enolase (1TE6)	AC1, AC2, AC3 and AC6	4	3	Gln297, Glu249 Thr40	AC6
10	17-Beta Hydroxysteroid dehydrogenase (1FDT)	AC1, AC2 and AC3	3	3	His221, Tyr155 Ser142	AC3
11	Cyclooxygenase-2 (3LN1)	AC1, AC2, AC3, AC4, AC5, AC6, AC7, AC8 and BC2	9	4	Glu165, Leu338, Ser339, Tyr40	AC7
12	Nuclear factor-kappa B (2V2T)	-	-	2	Thr45, Asn20	Receptor cavity
13	Activator protein-1 (4HMY)	AC1 and AC2	2	5	Thr32, Thr45, Thr46, Lys127, Ile46	AC1

4.2.5 Molecular docking and analyses

Molecular docking with the active binding site of targets was performed with 6-gingerol, 8-gingerol, 10-gingerol and 6-shogaol. The analyses of docking was done based on parameters like energy values, number of hydrogen bonds, bond length, number of amino acids and binding energy. The energy value includes -C-Docker and -C-Docker interaction energy. The difference between -C-Docker and -C-Docker interaction energy if minimum, target and ligands had more intermolecular interaction (pose). If number of hydrogen bond was more and bond length was less, ligand had good binding affinity with targets. Binding energy is the energy required to dis-assemble a whole system into separate parts. Binding energy of targets and ligands should be minimum for better interaction of ligands and target.

4.2.5.1 Molecular docking and analyses of 6-gingerol

Docking of 6-gingerol with binding sites of selected targets are presented in Table 15. The difference between -C-Docker and –C-Docker interaction energy of docked 6-gingerol ranged from 2.2870 to 8.4489. Among the selected targets, activator protein-1 recorded minimum binding energy (-138.2092) followed by Epidermal Growth Factor Receptor (-107.9914) and Phosphoinositide 3-kinase (-83.9303). The number of amino acid residues ranged from 1 to 8. The maximum number of amino acid residues was recorded in Ribosomal S6 kinase (8) followed by N- acetyltransferase 2 receptor (7) and the least value was recorded in Follistatin (1).

The number of hydrogen bonds in selected targets and 6-gingerol varied from 1 to 5. The maximum hydrogen bond (5) was observed in Follistatin with amino acid Ser59, Tyr76 (Fig. 15) followed by 17β hydroxysteroid dehydrogenase (4) with amino acid Lys159, Thr140, Gly141 (Fig. 16) and cyclooxygenase-2 (4) with amino acid His200, Thr369 (Fig. 17). The mean bond length ranged from 1.8039 to 2.3823. The minimum bond length was observed in Cyclooxygienase-2 (1.8039).

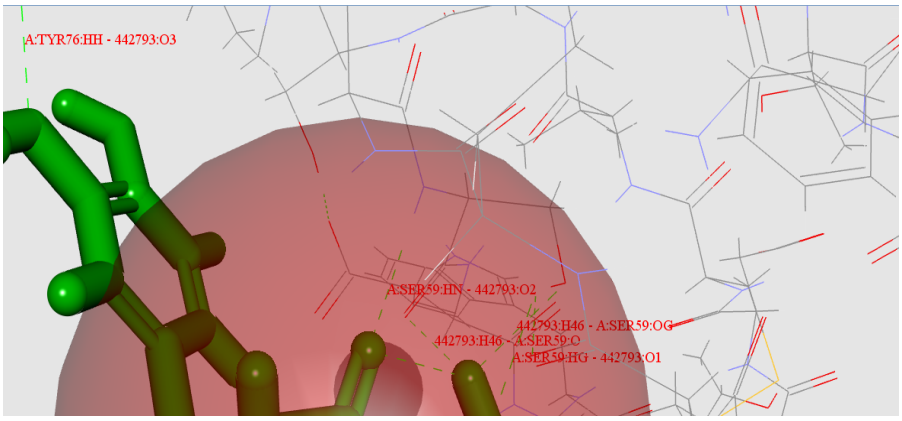


Figure 15. Docking of 6-gingerol with Follistatin (2BOU) with five hydrogen bonds

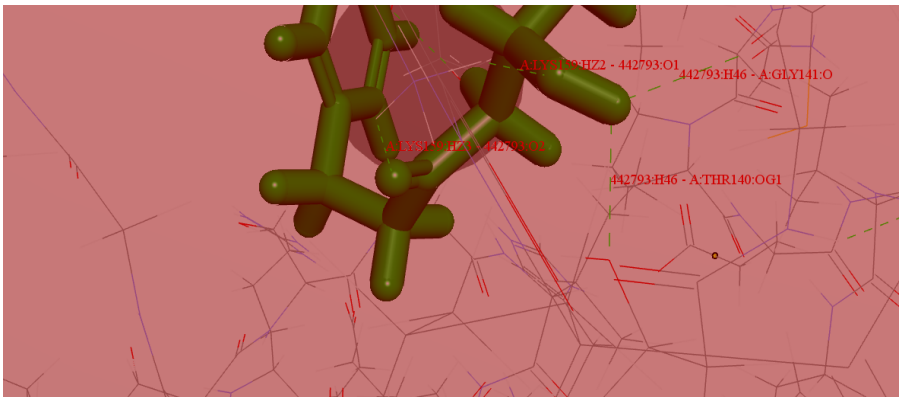


Figure 16. Docking of 6-gingerol with 17β hydroxysteroid dehydrogenase (1FDT) with four hydrogen bonds

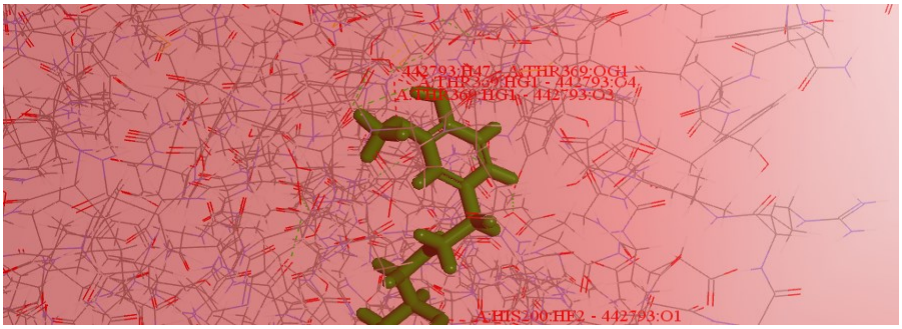


Figure 17. Docking of 6-gingerol with cyclooxygenase-2 (3LN1) with four hydrogen bonds

4.2.5.2 Molecular docking and analyses of 8-gingerol

Docking of 8-gingerol with the binding sites of selected targets are presented in Table 16. The difference between -C-Docker and –C-Docker interaction energy of docked 8-gingerol ranged from -0.4741 to 10.5945. Among the selected targets, Activator protein-1 recorded minimum binding energy (-140.5949) followed by Oestrogen receptor (-100.0471), Neuron-specific enolase (-87.694), 17β hydroxysteroid dehydrogenase (-85.5495) and phosphoinositide 3-kinase (-77.5847).

The number of hydrogen bonds in the selected targets and 8-gingerol varied from 1 to 5. The maximum hydrogen bond (5) was observed in Cyclooxygenase-2 with amino acids Thr198, Asn368, Gln440 (Figure 18) followed by Ribosomal S6 kinase (4) with amino acids Lys100, Asn198, Thr210, Leu74 (Figure 19). The mean bond length ranged from 1.91287 to 2.4401. The minimum bond length was observed in N- acetyltransferase 2 receptor (1.91287).

4.2.5.3 Molecular docking and analyses of 10-gingerol

Docking of 10-gingerol with the binding sites of selected targets are presented in Table 17. The difference between -C-Docker and –C-Docker interaction energy of docked 10-gingerol ranged from -0.2416 to 10.7149. Among the selected targets, Epidermal Growth Factor Receptor recorded minimum binding energy (-131.1699) followed by Ribosomal S6 kinase (3G51) (-102.6721), Activator protein-1 (-95.172), Cyclooxygenase-2 (-89.9435) and Phosphoinositide 3-kinase (-87.5317).

The number of hydrogen bonds in selected targets and 10-gingerol varied from 1 to 5. The maximum hydrogen bond (5) was observed in Phosphoinositide 3-kinase with amino acids Ser806, Lys833, Lys890, Asp964 (Figure 20) followed by Ribosomal S6 kinase (4) with amino acids Leu150, Asn198, Glu197 (Figure 21). The mean bond length ranged from 1.89998 to 2.18913. The minimum bond length was observed in 17β hydroxysteroid dehydrogenase (1.89998).

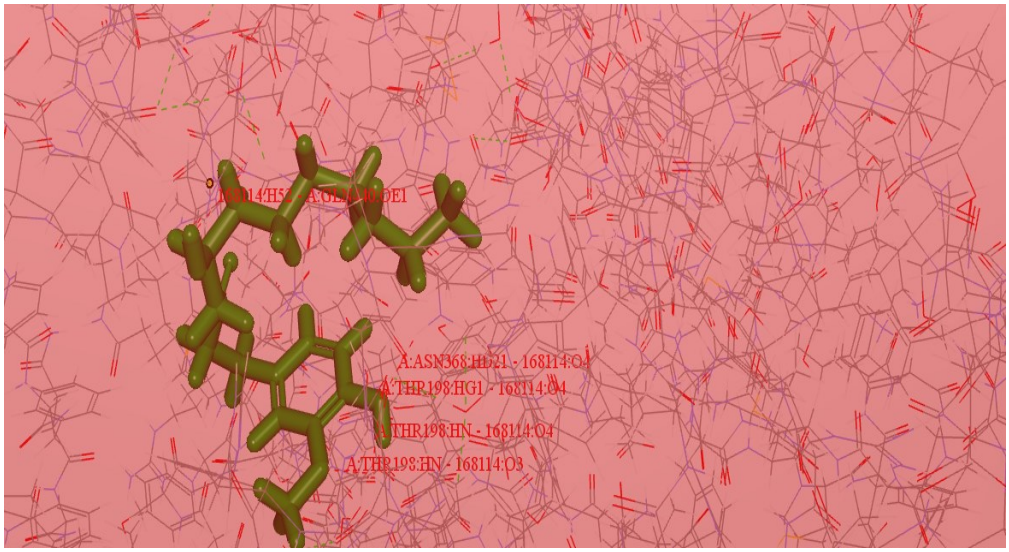


Figure 18. Docking of 8-gingerol with cyclooxygenase-2 (3LN1) with five hydrogen bonds

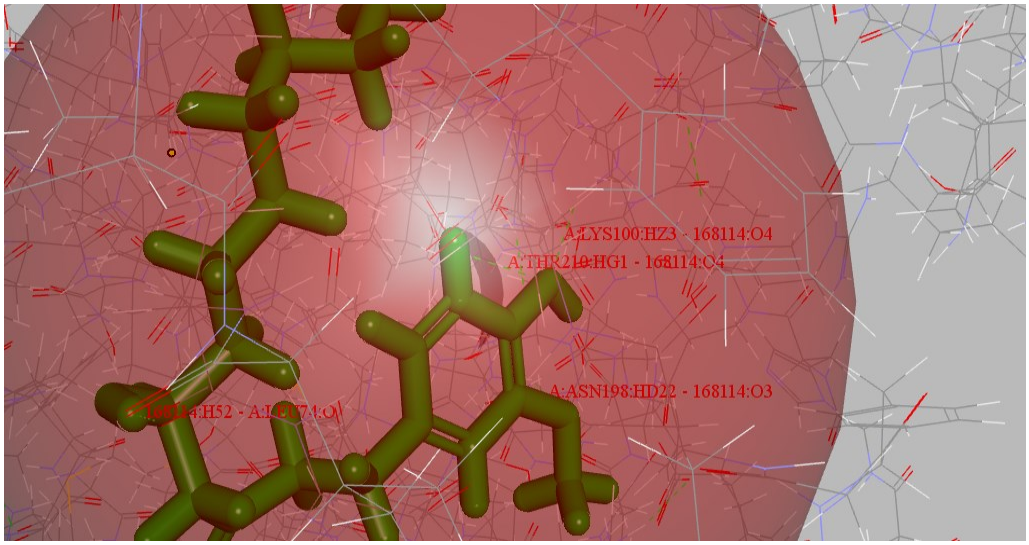


Figure 19. Docking of 8-gingerol with Ribosomal S6 kinase (3G51) with four hydrogen bonds

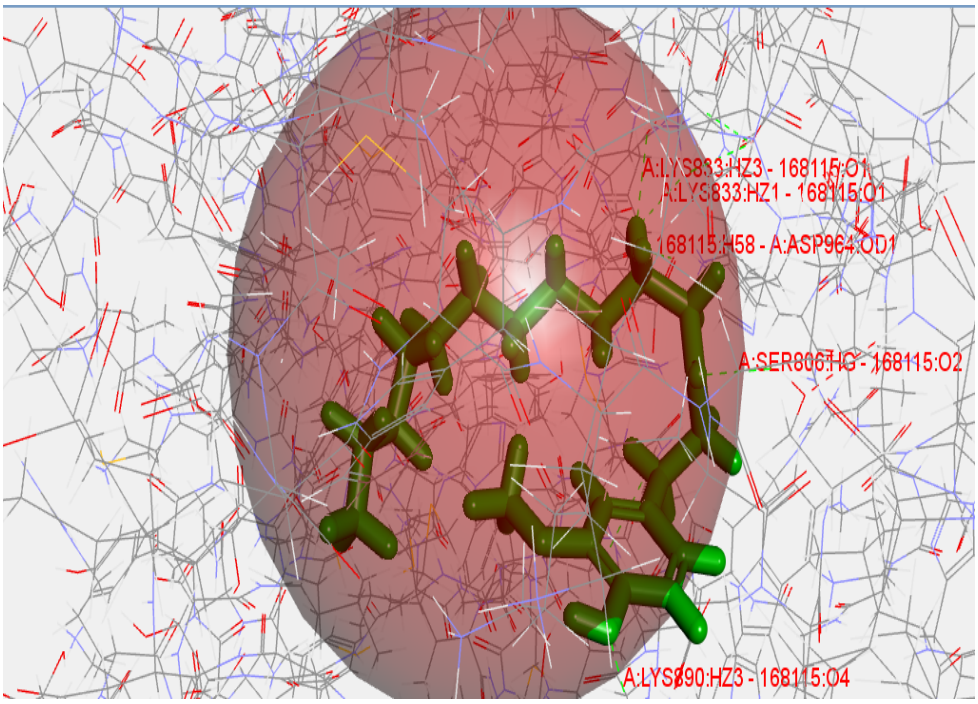


Figure 20. Docking of 10-gingerol with Phosphoinositide 3-kinase (1E8W) with five hydrogen bonds

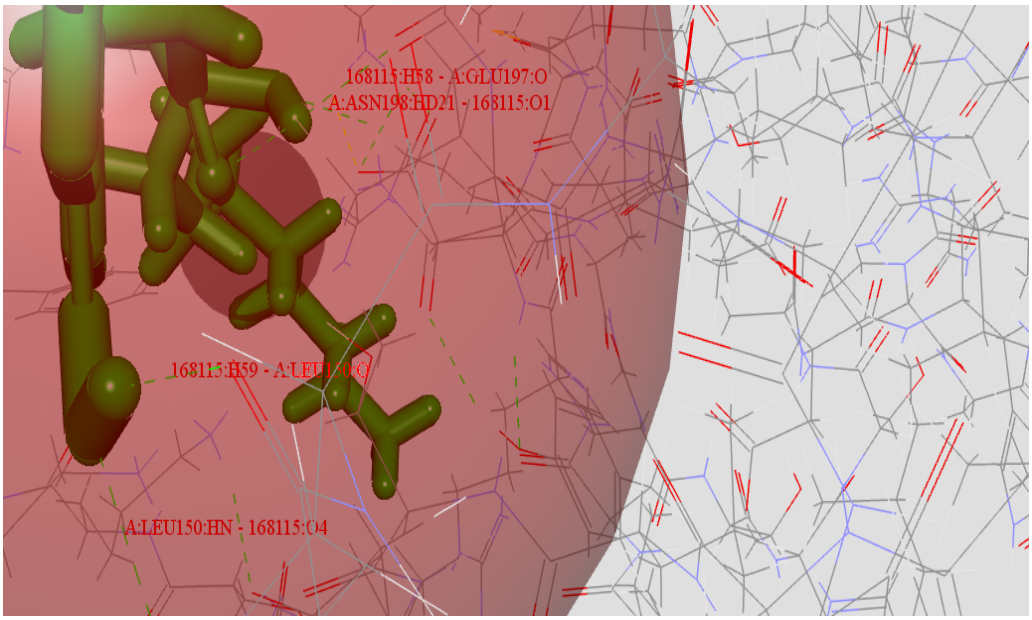


Figure 21. Docking of 10-gingerol with Ribosomal S6 kinase (3G51) with four hydrogen

4.2.5.4 Molecular docking and analyses of 6-shogaol

Docking of 6-shogaol with the binding sites of selected targets are presented in Table 18. The difference between -C-Docker and –C-Docker interaction energy of docked 6-shogaol ranged from 5.8666 to 16.8936. Among the selected targets, Activator protein-1 recorded minimum binding energy (-117.683) followed by Epidermal Growth Factor Receptor (-107.9644) and Oestrogen receptor (-91.9994).

The number of hydrogen bonds in selected targets and 6-shogaol varied from 1 to 4. The maximum hydrogen bond (4) was observed in Epidermal Growth Factor Receptor with amino acids Lys745, Asp855 (Figure 22) and Cyclooxygenase-2 with amino acids Thr198, Asn368 (Figure 23) followed by 17β hydroxysteroid dehydrogenase (3) with amino acids Thr140, Lys159 (Figure 24). The mean bond length ranged from 1.8540 to 2.4062. The minimum bond length was observed in Activator protein-1 (1.8540).

4.2.5.5 Molecular docking and analyses of approved drug

Docking of 6-gingerol, 8-gingerol, 10-gingerol and 6-shogaol were compared with the results of approved drugs, amifostine (CID: 2141), tazarotene (CID: 5381), quinestrol (CID: 9046), glycodiazine (CID: 9565), fulvestrant (CID: 104741), raltitrexed (CID: 104758), lapatinib (CID: 208908), celecoxib (CID: 2662), quercitrin (CID: 5280459) and disulfiram (CID: 3117). The docking results of approved drugs are presented in Table 19. Among the approved drugs, minimum energy difference between -C-Docker and –C-Docker interaction energy (6.3462) was recorded in quercitrin. The minimum binding energy was -478.0884 (quercitrin) with activator protein-1 followed by -283.2001 (raltitrexed) with N- acetyltransferase 2 receptor, -143.8459 (raltitrexed) with Epidermal Growth Factor Receptor, -115.0021 (lapatinib), with Epidermal Growth Factor Receptor, -107.871 (fulvestrant) with 17β hydroxysteroid dehydrogenase and -101.2196 (lapatinib) with neuron-specific enolase.

Figure 22. Docking of 6-shogaol with Epidermal Growth Factor Receptor (1XKK) with four hydrogen bonds

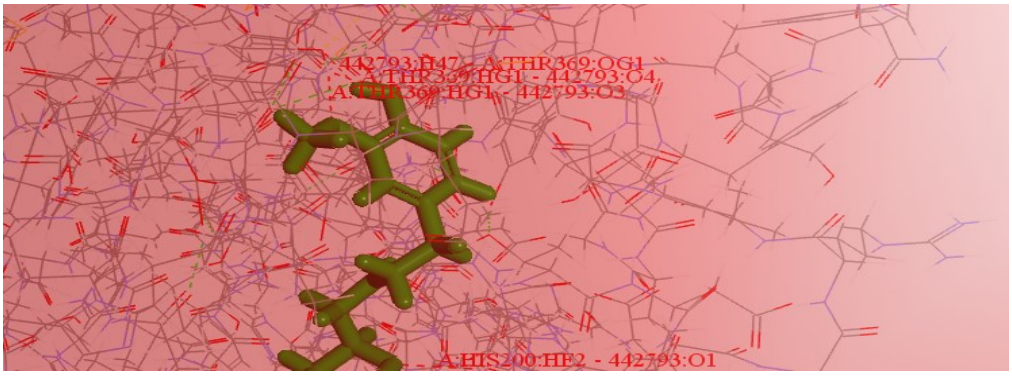


Figure 23. Docking of 6-shogaol with Cyclooxygenase-2 (3LN1) with four hydrogen bonds

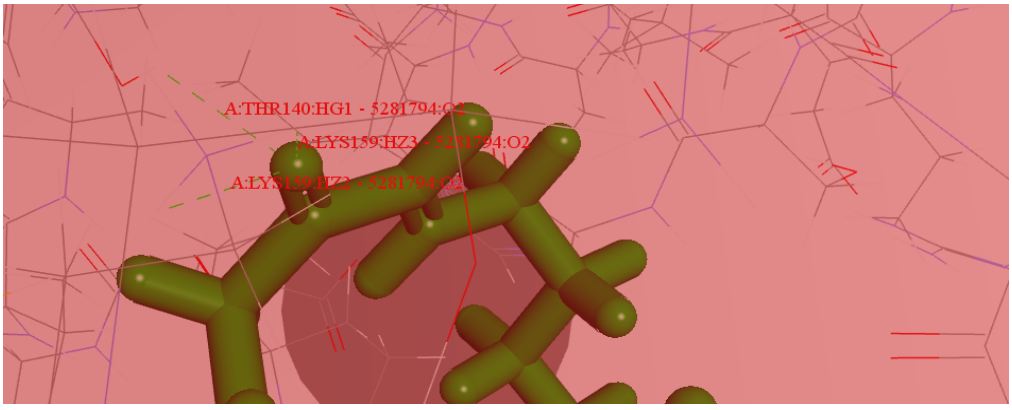


Figure 24. Docking of 6-shogaol with 17β hydroxysteroid dehydrogenase (1FDT) with three hydrogen bonds

The number of hydrogen bonds formed with selected cancer targets and approved drugs varied from 1 to 6. The maximum hydrogen bond (6) of raltitrexed was observed in N- acetyltransferase 2 receptor with amino acids Lys100, Thr103, UNX1013 (Figure 25) followed by amifostine (5) with Epidermal Growth Factor Receptor with amino acids Lys745, Asp855, Asn842 (Figure 26). The mean bond length ranged from 1.89696 to 2.45454. The minimum bond length (1.89696) was observed between Neuron-specific enolase and Lapatinib.

The compound 10-gingerol showed minimum binding energy as compared to tazarotene with ribosomal S6 kinase target, while with Epidermal Growth Factor Receptor, 6-gingerol, 10-gingerol and 6-shogaol showed minimum binding energy as compared to amifostine, glycodiazine, fulvestrant, raltitrexed and lapatinib.

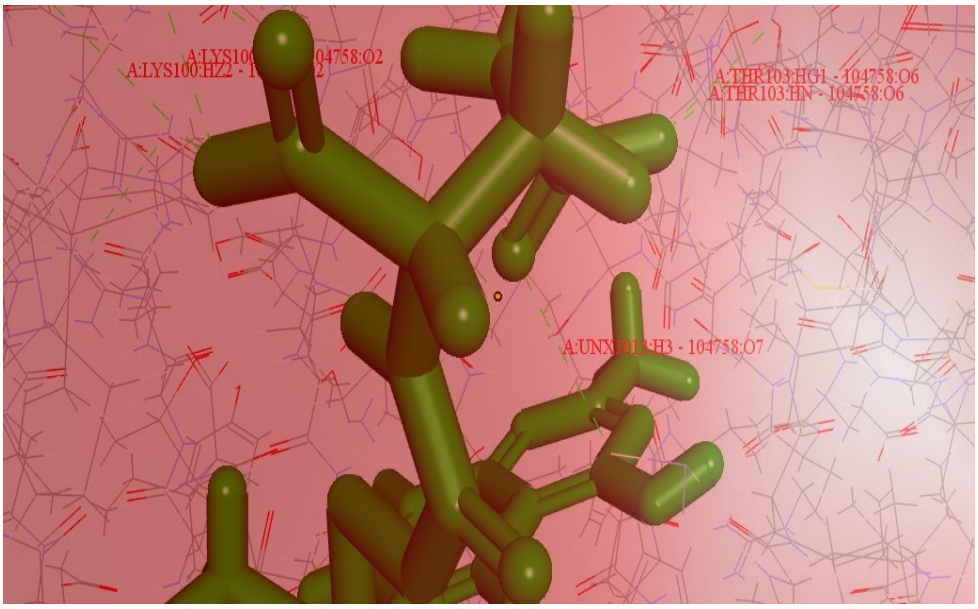


Figure 25. Docking of raltitrexed with N- acetyltransferase 2 receptor (2PFR) with six hydrogen bonds

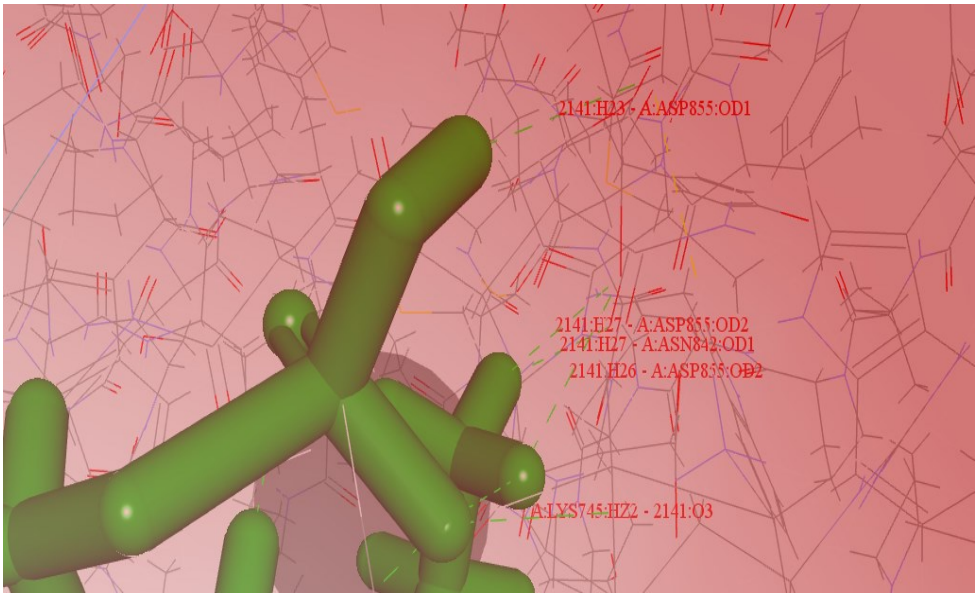


Figure 26. Docking of amifostine with Epidermal Growth Factor Receptor (1XKK) with five hydrogen bonds

Table 15. Docking of 6-gingerol with the binding sites of selected cancer targets

Targets	-C-Docker energy	-C-Docker interaction energy	Diff. between -C-Docker interaction energy and -C-Docker energy	Binding energy (Kcal/mol)	Amino acids bound to H-bond	No. H-bonds	Bond length
RSK2 (Ribosomal S6 kinase) (3G51)	39.7448	46.4526	6.7078	-65.2251	Thr 210 Glu 197	2	2.15183 2.20881
EGFR (Epidermal Growth Factor Receptor) (1XKK)	40.8626	45.0524	4.1898	-107.9914	Lys745 Met793	2	1.8848 2.05818
ER (Oestrogen receptor) (1ERE)	44.4051	48.3683	3.9632	-53.3912	Glu353 His524 Arg394	3	2.41211 2.41211 2.32282
EGFRK (Epidermal Growth Factor Receptor kinase) (1M17)	32.2661	37.3921	5.126	-20.0505	Thr830 Asp831	2	2.33538 1.95684
NAT 2 receptor (N-acetyltransferase 2 receptor) (2PFR)	32.7894	36.4335	3.6441	-	-	-	-
c-MET (4GG7)	36.7507	39.0377	2.287	-66.7825	Met1160 Pro1158	2	1.98413 2.34384
PI3K (Phosphoinositide 3-kinase) (1E8W)	39.8259	43.7252	3.8993	-83.9303	Lys833 Asp964 Asp836	3	1.90325 2.26741 2.47363
Follistatin (2BOU)	30.1246	33.3156	3.191	-35.1916	Ser59 Tyr76	5	1.90515 2.48491 2.38663 2.27386 2.2635
NSD (Neuron-specific enolase) (1TE6)	26.7131	30.1803	3.4672	-28.7656	Thr40	1	1.94515
17β HSD (17-Beta Hydroxysteroid dehydrogenase) (1FDT)	41.3192	43.8971	2.5779	-77.9543	Lys159 Thr140 Gly141	4	1.9379 2.28067 2.39452 2.14466
COX-2 (Cyclooxygenase-2) (3LN1)	35.1914	40.4706	5.2792	-61.3134	His200 Thr369	4	2.46257 1.537 1.316 1.9002
NF-kB (Nuclear factor-kappa B) (2V2T)	30.7019	35.3497	4.6478	-76.0004	Asn202	1	2.20704
AP-1 (Activator protein-1) (4HMY)	42.6345	51.0834	8.4489	-138.2092	Thr32 Thr45 Ile46	3	2.20704 1.83896 2.20685

Table 16. Docking of 8-gingerol with the binding sites of selected cancer targets

Targets	-C-Docker energy	-C-Docker interaction energy	Diff. between -C-Docker interaction energy and -C-Docker energy	Binding energy (Kcal/mol)	Amino acids bound to H-bonds	No. of H-bonds	Bond length
RSK2 (Ribosomal S6 kinase) (3G51)	43.5732	53.0121	9.4389	-43.4718	Lys100 Asn198 Thr210 Leu74	4	1.78695 2.20203 2.20805 1.89988
EGFR (Epidermal Growth Factor Receptor) (1XKK)	42.2719	47.0784	4.8065	-55.2001	Lys745 Asn842 Asp855	3	1.81925 2.09659 2.01724
ER (Oestrogen receptor) (1ERE)	44.4404	55.0349	10.5945	-100.0471	His524 Glu353	2	2.44895 2.43124
EGFRK (Epidermal Growth Factor Receptor kinase) (1M17)	35.4982	38.335	2.8368	-45.8829	Asp831	1	1.96874
NAT 2 receptor (N-acetyltransferase 2 receptor) (2PFR)	31.215	30.7409	-0.4741	-19.2055	Ser216 Thr219	2	1.87646 1.94928
c-MET (4GG7)	39.1134	41.0177	1.9043	-21.9807	Met1160	1	2.12443
PI3K (Phosphoinositide 3-kinase) (1E8W)	46.1988	49.6355	3.4367	-77.5847	Lys833 Lys890	2	2.46582 1.83638
Follistatin (2BOU)	32.2591	32.9137	0.6546	-38.1175	Cys55 Ser54	2	2.00299 2.31456
NSD (Neuron-specific enolase) (1TE6)	29.4513	35.4584	6.0071	-87.694	Gln297	1	2.10578
17β HSD (17-Beta Hydroxysteroid dehydrogenase) (1FDT)	43.7523	47.1068	3.3545	-85.5495	Thr140 Lys159 Thr190	3	2.33358 1.89973 2.0263
COX-2 (Cyclooxygenase-2) (3LN1)	40.9833	48.2271	7.2438	-48.4629	Thr198 Asn368 Gln440	5	2.0672 2.43983 1.89462 2.12429 2.38492
NF-kB (Nuclear factor-kappa B) (2V2T)	23.7329	31.2246	7.4917	-37.3553	Asn202	1	2.16265
AP-1 (Activator protein-1) (4HMY)	47.2985	53.279	5.9805	-140.5949	Lys127 Thr45 Ile46	3	1.80785 2.08323 2.20406

Table 17. Docking of 10-gingerol with the binding sites of selected cancer targets

Targets	-C-Docker energy	-C-Docker interaction energy	Diff. between -C-Docker interaction energy and -C-Docker energy	Binding energy (Kcal/mol)	Amino acids bound to H-bond	No. Of H-bonds	Bond length
RSK2 (Ribosomal S6 kinase) (3G51)	51.9996	58.9577	6.9581	-102.6721	Leu150 Asn198 Glu197	4	2.41716 2.14849 2.14401 2.00586
EGFR (Epidermal Growth Factor Receptor) (1XKK)	47.6207	51.7268	10.1061	-131.1699	Lys745 Asp855	2	1.91366 2.01802
ER (Oestrogen receptor) (1ERE)	39.3802	50.0951	10.7149	-82.4287	His524 Gly521	2	1.92286 2.12617
EGFRK (Epidermal Growth Factor Receptor kinase) (1M17)	41.3329	47.7457	6.4128	-	-	-	-
NAT 2 receptor (N-acetyltransferase 2 receptor) (2PFR)	40.8429	47.8001	6.9572	-51.1845	His107	1	2.05888
c-MET (4GG7)	40.0831	38.3251	-1.758	-	-	-	-
PI3K (Phosphoinositide 3-kinase) (1E8W)	51.8771	56.129	4.2519	-87.5317	Ser806 Lys833 Lys890 Asp964	5	2.27926 1.89662 2.46017 2.22526 2.08436
Follistatin (2BOU)	34.2619	37.3391	3.0772	-43.5775	Cys47	1	1.97126
NSD (Neuron-specific enolase) (1TE6)	32.3855	32.1439	-0.2416	-50.901	Gln297	1	2.19995
17β HSD (17-Beta Hydroxysteroid dehydrogenase) (1FDT)	48.6991	50.6379	1.9388	-76.0992	Lys159 Thr190	2	1.75254 2.04742
COX-2 (Cyclooxygenase-2) (3LN1)	39.1249	44.6087	5.4838	-89.9435	His200 Asn368	3	1.93091 2.14657 2.43644
NF-kB (Nuclear factor-kappa B) (2V2T)	-	-	-	-	-	-	-
AP-1 (Activator protein-1) (4HMY)	42.8941	41.2684	-1.6257	-95.172	Thr45	1	2.17047

Table 18. Docking of 6-shogaol with the binding sites of selected cancer targets

Targets	-C-Docker energy	-C-Docker interaction energy	Diff. between -C-Docker interaction energy and -C-Docker energy	Binding energy (Kcal/mol)	Amino acids bound to H-bond	No. H-bonds	Bond length
RSK2 (Ribosomal S6 kinase) (3G51)	34.1662	43.791	9.6248	-50.7224	Leu150 Asp148	2	2.40025 2.29644
EGFR (Epidermal Growth Factor Receptor) (1XKK)	28.2694	42.1514	13.882	-107.9644	Lys745 Asp855	4	2.21415 1.80567 2.29606 2.03871
ER (Oestrogen receptor) (1ERE)	35.8663	44.9657	9.0994	-91.9994	Arg394 Glu353	2	2.4923 2.32018
EGFRK (Epidermal Growth Factor Receptor kinase) (1M17)	23.7508	30.9037	7.1529	-67.0454	Met769	1	2.31129
NAT 2 receptor (N-acetyltransferase 2 receptor) (2PFR)	31.0656	38.7042	7.6386	-65.4309	UNX1013	1	2.09532
c-MET (4GG7)	26.9714	37.2637	10.2923	-46.9104	Met1160	1	2.14521
PI3K (Phosphoinositide 3-kinase) (1E8W)	33.5313	39.3979	5.8666	-42.7721	Ser806	1	1.854
Follistatin (2BOU)	18.6625	29.406	10.7435	-22.0862	Cys47	1	1.9039
NSD (Neuron-specific enolase) (1TE6)	19.6133	28.2799	8.6666	-65.3447	Gln297	1	2.06867
17β HSD (17-Beta Hydroxysteroid dehydrogenase) (1FDT)	33.4016	39.4871	6.0855	-43.5263	Thr140 Lys159	3	2.42071 2.291 1.95395
COX-2 (Cyclooxygenase-2) (3LN1)	30.9843	42.787	11.8027	-38.7325	Thr198 Asn368	4	2.07547 2.35934 1.90532 2.17221
NF-kB (Nuclear factor-kappa B) (2V2T)	19.2346	33.0365	13.8019	-40.7234	Ile205	2	2.35441 1.84261
AP-1 (Activator protein-1) (4HMY)	30.7921	47.6857	16.8936	-117.683	Thr45	2	2.16736 1.78828

Table 19. Docking of approved drugs with the binding sites of selected cancer targets

Targets	Ligands	-C-Docker energy	-C-Docker interaction energy	Diff. between -C-Docker interaction energy and -C-Docker energy	Binding energy (Kcal/mol)	Amino acids bound to H-bond	No. of H-bonds	Bond length
RSK2 (Ribosomal S6 kinase) (3G51)	Tazarotene	36.7661	51.3317	14.5656	-98.736	Lys100 Lys195 Thr210 Lys216	4	2.01716 2.20241 2.39174 1.71972
EGFR (Epidermal Growth Factor Receptor) (1XKK)	Amifostine	63.5207	38.1966	-25.3241	-90.6521	Lys745 Asp855 Asn842	5	1.8456 2.0403 2.07766 1.92342 2.39566
	Tazarotene	30.2562	42.9466	12.6904	-	-	-	-
	Quinestrol	-13.753	46.4672	60.2202	-	-	-	-
	Glycodiazine	29.9806	40.763	9.2564	-96.2369	Lys745 Thr854	3	2.44496 2.16707 2.04224
	Fulvestrant	25.0484	72.2071	47.1587	-90.872	Lys745 Thr854 Asp855	4	1.95292 2.18109 2.29333 1.93432
	Raltitrexed	57.5369	65.0138	7.4769	-143.8459	Asp855	1	2.45454
	Lapatinib	60.8761	79.301	18.4249	-115.0021	Phe856 Ser720	2	2.28476 1.98742
	Gefitinib	20.2496	57.3799	37.1303	-	-	-	-
ER (Oestrogen receptor) (1ERE)	Fulvestrant	-86.4926	15.367	101.8596	0.6642	Trp393 Thr347 Met421	3	2.12457 1.96639 2.00926
NAT 2 receptor (N-acetyltransferase 2 receptor) (2PFR)	Raltitrexed	57.1072	61.9439	4.8367	-283.2001	Lys100 Thr103 UNX1013	6	2.02849 1.80405 2.46607 2.08738 2.02519 2.45478
Follistatin (2BOU)	Quinestrol	-29.0541	31.1443	60.1984	-6.082	Cys55	1	2.02301
NSD (Neuron-specific enolase) (1TE6)	Lapatinib	30.1876	48.8092	18.6216	-101.2196	Thr40	1	1.89696
17β HSD (17-Beta Hydroxysteroid dehydrogenase) (1FDT)	Fulvestrant	22.5343	73.4188	50.8845	-107.871	Thr140Lys159 His221 Gly141	4	2.47455 1.78892 2.34353 2.15645
COX-2 (Cyclooxygenase-2) (3LN1)	Celebrex	-0.33315	31.3191	31.65225	-	-	-	-
NF-kB (Nuclear factor-kappa B) (2V2T)	Disulfiram	17.5971	24.1089	6.5118	-23.2973	Ser126	1	2.23638
AP-1 (Activator protein-1) (4HMY)	Quercitrin	62.7811	69.1273	6.3462	-478.0884	Gly29 Thr45 Lys127 Thr32	4	2.11572 1.88631 2.36784 2.00863

4.2.6 ADME/T prediction

The various pharmacokinetic properties of selected ligands were analyzed. Pharmacokinetic properties include, Solubility, Human Intestinal Absorption, Blood-Brain-Barrier (BBB) penetration, Cytochrome P450 inhibition and hepatotoxicity level. Adsorption, Distribution, Metabolism, Excretion and Toxicity (ADME/T) descriptor levels of the analogs were obtained from the ADME Descriptors protocol of Discovery studio 4.0 which is presented in Table 20. Among the ginger ligands, 6-gingerol, 8-gingerol and 10-gingerol recorded good solubility (3) while 6-shogaol showed low solubility (2). The solubility level in approved drugs varied from 1 to 4. The good solubility (3) was observed with gycodiazine and quercitrin. All ginger ligands showed good absorption (0) while the approved drugs, tazarotene, lapatinib, gycodiazine, gefitinib, celebrex and disulfiram showed good absorption (0), quinestrol showed moderate (1), raltitrexed showed poor (2) and fulvestrant, amifostine and quercitrin showed very poor absorption (3).

Among the ginger ligands, 6-gingerol showed medium level of Blood Brain Barrier (BBB) while other ginger ligands, 8-gingerol, 10-gingerol and 6-shogaol showed high level of Blood Brain Barrier (BBB). Blood Brain Barrier (BBB) of approved drugs varied from 0 to 4. Very low (4) Blood Brain Barrier (BBB) was observed in lapatinib, raltitrexed, fulvestrant, amifostine and quercitrin and very high Blood Brain Barrier (BBB) was observed in tazarotene, quinestrol and disulfiram. All ginger ligands were found non-toxic while in the approved drugs, only tazarotene, quinestrol and fulvestrant were found non-toxic. All ginger ligands were found as non-inhibitor (false) of the enzyme CYP2D6 in metabolism of xenobiotic in the body while the approved drugs are similar to ginger ligands except gefitinib which was found inhibitory (true) in metabolism of xenobiotic in the body.

Table 20. ADME/T properties of ginger ligands and approved drugs

Sl. No.	Compounds name	ADMET Solubility level	ADMET Absorption level	ADMET BBB Level	Hepatotoxic prediction	CYP2D6 Prediction
1	6-gingerol	3	0	2	FALSE	FALSE
2	8-gingerol	3	0	1	FALSE	FALSE
3	10-gingerol	3	0	1	FALSE	FALSE
4	6-shogaol	2	0	1	FALSE	FALSE
5	Tazarotene	2	0	0	FALSE	FALSE
6	Lapatinib	2	0	4	TRUE	FALSE
7	Quinestrol	1	1	0	FALSE	FALSE
8	Raltitrexed	4	2	4	TRUE	FALSE
9	Fulvestrant	1	3	4	FALSE	FALSE
10	Gycodiazine	3	0	3	TRUE	FALSE
11	Amifostine	5	3	4	TRUE	FALSE
12	Gefitinib	2	0	1	TRUE	TRUE
13	Celebrex	1	0	2	TRUE	FALSE
14	Disulfiram	2	0	0	TRUE	FALSE
15	Quercitrin	3	3	4	TRUE	FALSE

4.1 Validation of anticancerous properties of gingerols using different tumour cell lines.

4.1.1 *In vitro* cytotoxicity of gingerols

MTT assay was performed to determine the cytotoxicity of 6-gingerol on HCT15, L929 and Raw 264.7 cells with 17, 34, 68, 102, 136 and 170 μM concentrations. 6-gingerol was found to inhibit the cell growth in all cells.

4.1.2 Effect of 6-gingerol on cell cytotoxicity in different cancer cell lines

The viability of the cells decreased significantly by 6-gingerol in a dose dependent manner. Cytotoxicity of 6-gingerol on different cancer cell lines at different concentrations 24 h. after treatment is shown in Figure 27. The IC_{50} value of 6-gingerol on different cancer cell lines includes, HCT15, L929 and Raw 264.7 was observed at 100 μM, 102 μM and 102 μM respectively 24 h. after treatment.

Cytotoxicity of 6-gingerol on different cancer cell lines at different concentrations 48 h. after treatment is shown in Figure 28. The IC_{50} value of 6-

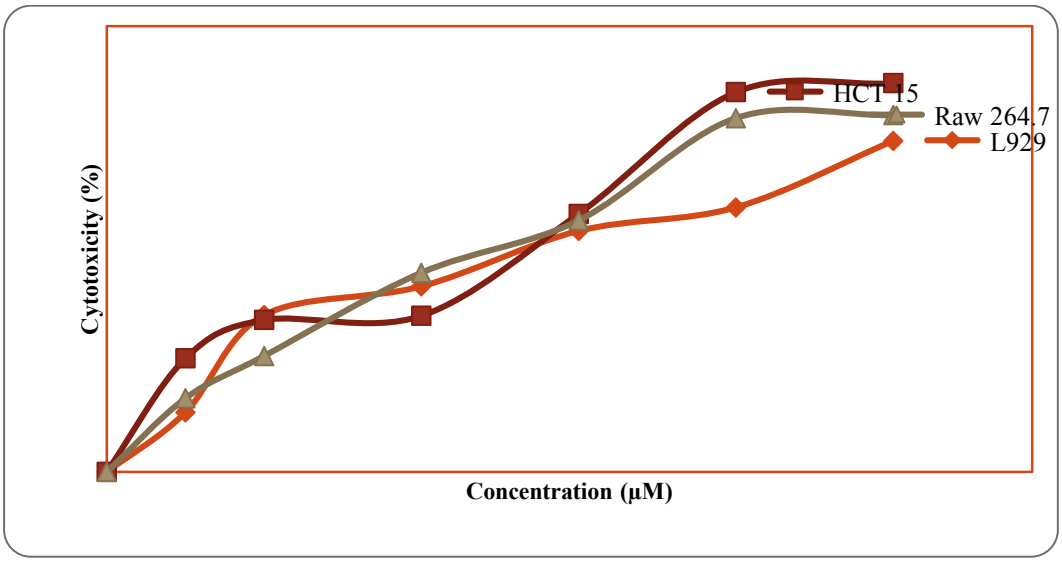


Figure 27. Cytotoxicity of 6-gingerol in different cell lines 24 hours after treatment

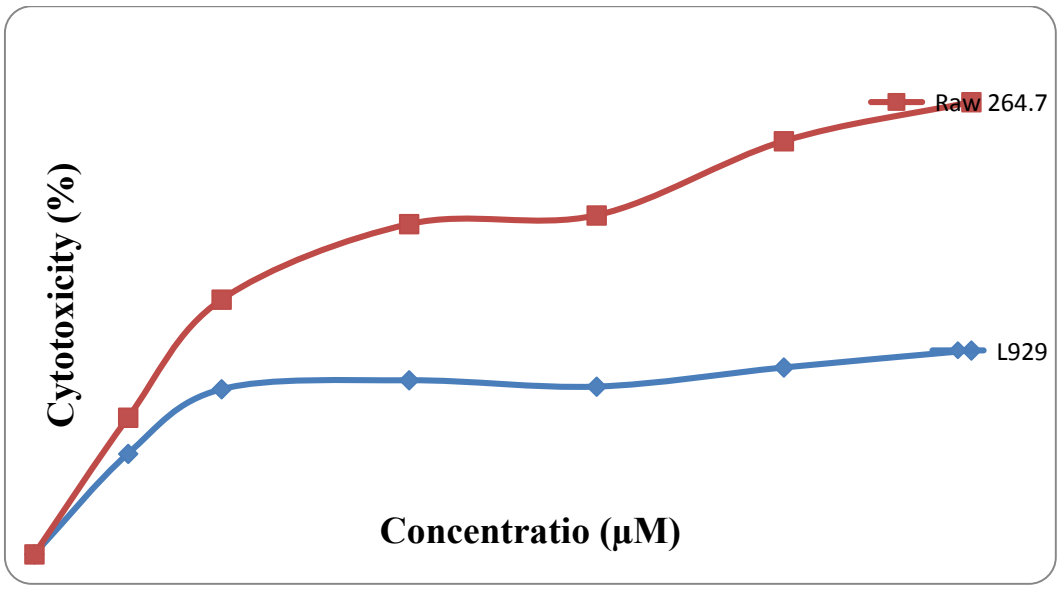


Figure 28. Cytotoxicity of 6-gingerol in different cell lines 48 hours after treatment

gingerol on cancer cell lines, L929 and Raw 264.7 was observed at 208.54 μM and 68 μM 48 h. after treatment.

Cytotoxicity of 6-gingerol on different cancer cell lines at different concentrations 72 h. after treatment is shown in Figure 29. The IC_{50} value of 6-gingerol on cancer cell lines, L929 and Raw 264.7 was observed at 432.94 μM and 34 μM 72 h. after treatment.

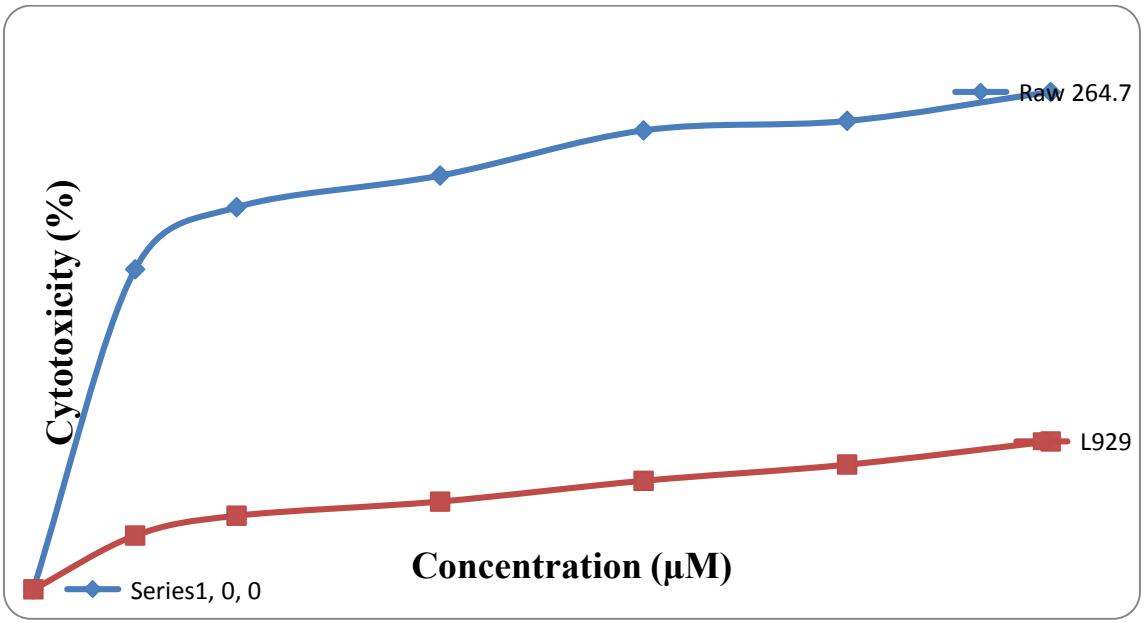


Figure 29. Cytotoxicity of 6-gingerol in different cell lines 72 hours after treatment

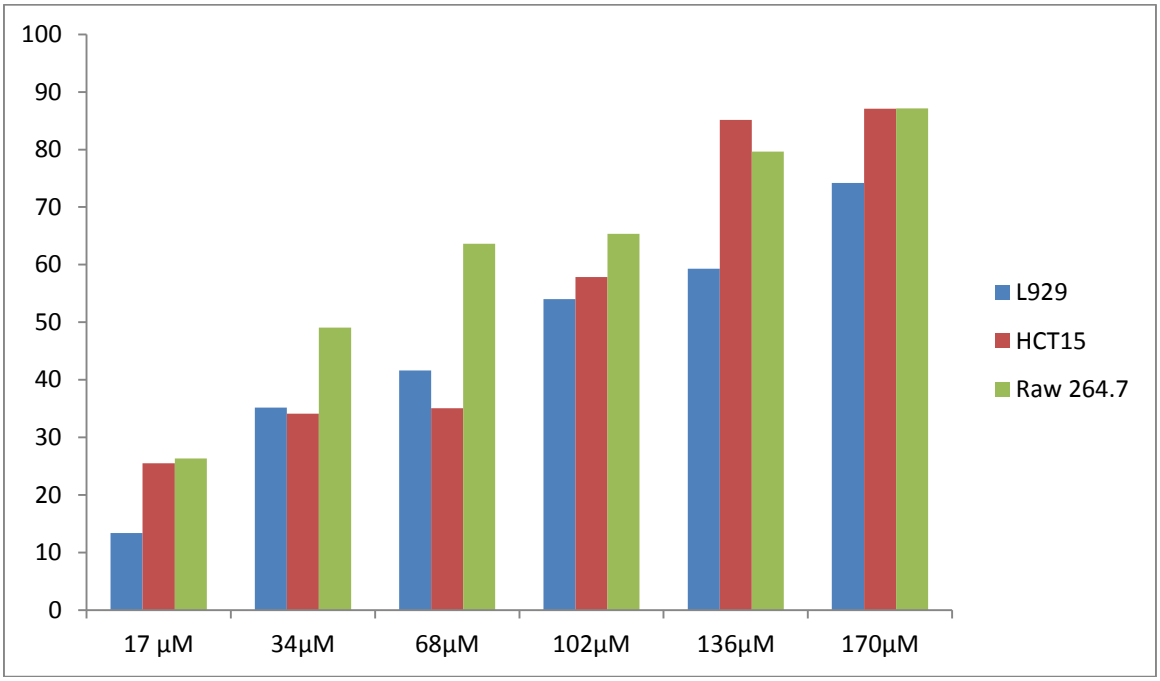


Figure 31. Effect of 6-ginerol on cytotoxicity in cancer cell lines 24 hours after treatment

Discussion

5. DISCUSSION

The present investigations on "Screening ginger (*Zingiber officinale* Rosc.) somaclones for gingerol content and validation of anticancerous properties of gingerol" were undertaken to screen fifty ginger somaclones derived from cultivar Maran for gingerol content, to identify potential cancer targets for gingerols and shogaol using *in silico* tools and to validate the anticancerous properties of gingerol. The results of the study are discussed in this chapter.

5.1 Screening somaclones for gingerol content

5.1.1 Raising of somaclones in filed, preparation of dry ginger and extraction oleoresin

The fifty somaclones evaluated were grouped under six sets according to mode of regeneration. The somaclones evaluated exhibited variability in yield. Fresh yield/ plant varied from 91.5 to 574.83 g. Somaclones of M Se group were observed as high yielders. Variability in yield was more in somaclones clones of MC 10Gy group. Somaclones in all the six groups registered higher yield than the conventionally propagated control plant (Table 5b).

Ma and Gang (2006) reported difference in morphological characters between *in vitro* propagated plants and traditionally propagated clonal siblings. Ginger plants derived *in vitro* grew better (were larger and greener) and produced more rhizomes than plants derived from rhizomes that had not undergone *in vitro* propagation. In present study also, ginger somaclones regenerated through direct/ indirect organogenesis/embryogenesis and *in vitro* mutagenesis recorded higher yield. Variability in yield was more in somaclones subjected to *in vitro* mutagenesis. Paul (2006) also reported higher yield for somaclones of ginger than conventionally propagated plants. Higher variability in yield in regenerates clones derived after *in vitro* mutagenesis was reported by Shylaja (2010).

Dry ginger recovery

The recovery of dry ginger ranged from 19.46 to 29.03 percent. Fifty three per cent of somaclones registered dry recovery of 25 to 30 per cent. Somaclones of MC 10Gy group showed more variability in dry ginger recovery and the group recorded higher mean recovery (Table 6b). Sanchu (2000) and Paul (2006) also observed higher dry recovery in somaclones of black pepper and ginger respectively.

Extraction of oleoresin

Oleoresin yield in fifty somaclones varied from 4.45 to 12.78 per cent. The oleoresin yield ranged from 5.01 to 7.0 per cent in 59 per cent of somaclones studied. Higher recovery of oleoresin and high variability in oleoresin yield were observed in somaclones of M Se 20Gy (Table 7b). Similar variation in oleoresin yield in different ginger cultivars was reported by Lewis *et al.* (1972), Mathai (1972), Muralidharan (1973), Nair (1975) and Nybe (1978).

5.1.2 Screening somaclones for total gingerol and shogaol content

Screening of somaclones (50 Nos.) for gingerol and shogaol content was performed using HPLC analytical platform. The content of 6-gingerol varied from 3.33 to 13.83 g/kg dry ginger powder in the somaclones studied. In twenty two percent of somaclones, 6-gingerol ranged from 7.01 to 9.0g/kg ginger powder. In groupwise analysis, the content of 6-gingerol showed higher recovery and also high variability in M Se 20Gy group of somaclones (Table 8b).

The content of 8-gingerol varied from 0.25 to 0.94 g/kg dry ginger powder in the somaclones studied. In 24 per cent of somaclones, it ranged from 0.26 to 0.50 g/kg dry ginger powder. In groupwise analysis, the content of 8-gingerol was higher in M Se 10Gy group of somaclones and high variability was seen in M Se 20Gy group of somaclones (Table 9b).

The content of 10-gingerol varied from 0.17 to 1.15 g/kg dry ginger powder in the somaclones studied. In twenty four per cent of clones it ranged from 0.26 to 0.50 g/kg dry ginger powder. In groupwise analysis, the content of 10-gingerol showed high variability in M Se 20Gy group of somaclones. High mean yield was recorded for the control (0.66) followed by clones of the group MC (Table 10b).

The content of total gingerol varied from 3.88 to 15.12 g/kg dry ginger powder in the somaclones studied. In twenty three per cent of somaclones, the content of total gingerol ranged from 7.01 to 9.0 g/kg dry ginger powder. The higher content of total gingerol (10.21 g/kg dry ginger powder) and high variability in total gingerol was found in M Se 20Gy group of somaclones (Table 11b).

Ashraf *et al.* (2014) reported geographical variation in the content of gingerols in ginger rhizomes from different regions of India. The concentration of gingerols varied with environmental conditions. Yudthavorasit *et al.* (2014) reported the use of pungency principles as markers for classification of geography of ginger samples. They analyzed eighty ginger samples from China, India, Thailand, Malaysia and Vietnam. They suggested that 6-gingerol and 8-gingerol are significant markers for specifying ginger from India and Thailand while methyl diacetoxy 8-gingerdiol, 10-gingerol and diacetoxy 8-gingerdion employed as a marker for Chinese ginger. In the present investigation also, 6-gingerol and 6-shagaol showed significant variations in the somaclones analyzed and somaclones which had undergone *in vitro* mutagenesis recorded high content of their pungent principles. The conventionally propagated control plant recorded lower values for the parameters studied.

Cultivar variation in the content of pungency principles in ginger was reported by several workers. The 6-gingerol content showed high variability in Rio De Janero and Rajasthan genotypes (Pawar *et al.,* 2011). Salmon *et al.* (2012) also reported significant variation in 6-gingerol, 8-gingerol, 10-gingerol and 6-shogaol in HPTLC fingerprints. They studied ginger cultivars which showed chemical homogeneity with

small qualitatively observed differences in the intensities of gingerol and shogaol zones. Quantification of these compounds by high-performance liquid chromatography (HPLC) revealed significant differences in total pungency among the cultivars. Essential oil yields from the cultivars also showed variation significantly. Sanwal *et al.* (2010) reported tetraploid clones of ginger somaclones have high content of total gingerol as compared to diploid clones of ginger.

The content of 6-shogaol varied from 0.61 to 2.33 g/kg dry ginger powder in the somaclones studied. In sixty per cent of clones the content of 6-shogaol ranged from 1.01 to 1.50 g/kg ginger powder. The highest mean recovery of 6-shogaol (1.60 g/kg ginger powder) was found in MSe 10Gy group and high variability was observed in MC 10Gy group of somaclones (Table 12b).

Pawar *et al.* (2015) reported that callus and micropropagated rhizome produced lesser amount of secondary metabolites as compared to conventionally grown rhizome. The concentration of 6-gingerol was thrice more (0.16%) in conventionally grown ginger rhizome than callus (0.056) and about half (0.078) in micropropagated plant rhizome. But in the present investigations the micropropagated plants produced higher recovery of secondary metabolites. Different groups of somaclones showed variability in content of 6-gingerol. In groupwise analysis, the content of 6-gingerol was high in M Se 20Gy followed by MC 10Gy, M Se, M Se 10Gy and MC. Higher piperine content in micropropagated black pepper was reported by (Sanchu 2000). Similarly variability in pungency principles in micropropagated in ginger was reported by shylaja (2010). From the study, high gingerol yielding ginger variety Karthika was released (Shylaja *et al.,* 2010).

5.2 *In silico* analysis of potential ligands and targets for cancer and molecular docking

The interaction of fifteen ligands (four ginger phenolic compounds + eleven approved drugs) with thirteen targets identified for cancer were evaluated using

commercial software Accelry Discovery studio version 4.0. The structure of ginger ligands and approved drugs were retrieved from PubChem and preparation of ligands was done using ligand preparation wizard of the software. Ligands were filtered after analysis using Lipinski's and veber rules (Lipinski, 1997).

Among the ginger ligands, 8-gingerol and 10-gingerol failed during filtration due to presence of high number of rotation bonds in 8-gingerol and 10-gingerol. Among the approved drugs, quercitrin and fulvestrant failed during filtration. Quercitrin was failed since the number of H-bond donar was more than five and number of H-bond acceptor was more than ten. In case of fulvestrant, the value of partition coefficient was more than five (Table 13).

Thirteen cancer targets were selected through literature survey and from Potential Drug Targets Database. 3D Structure of selected targets were retrieved from Protein Data Bank. Preparation of the retrieved protein was performed using protein preparation wizard of the software. Prepared protein and ligands were docked using CDOCKER and binding energy was calculated.

Many experimental studies showed that ginger and its active components including 6-gingerol and 6-shogaol exert anticancer activities against gastro intestinal cancer (Prasad and Tyagi, 2015). The anticancer activity of ginger is attributed to its ability to modulate several signaling molecules like NF-κB, STAT3, MAPK, PI3K, ERK1/2, Akt, TNF-α,COX-2, cyclin D1, cdk, MMP-9, survivin, cIAP-1, XIAP, Bcl-2, caspases, and other cell growth regulatory proteins (Prasad and Tyagi, 2015). Shankar *et al.* (2013) used Neuron-specific enolase (PDB ID: 1TE6) for Lung cancer and Follistatin (PDB ID: 2BOU) for Prostate cancer as targets when *in silico* molecular docking analysis of cancer biomarkers with bioactive compounds of *Tribulus terrestris* was attempted.

The detection of ligand-binding sites to target protein is often the starting point for protein function identification and drug discovery. The active site of 5HT1A

comprises amino acid residues such as LEU 453, PHE 454, and TYR 457 (Ittiyavirah and Paul, 2013). In present investigations, maximum number of active sites was twenty five as seen in Table 14 for N-acetyltransferase 2 receptor (2PFR) with amino acid residues such as Ser216, Thr103, Gly104, Cys68, Ser287, Tyr208 and Thr214.

5.2.1 Molecular docking and analysis of gingerols and shogaol

In the present investigations, molecular docking was attempted with four ginger ligands and eleven approved drugs with thirteen cancer targets. Results were analyzed based on interaction energy, binding energy, number of hydrogen bonds, number of amino acid residues and bond length (Table 15,16,17,18 and 19).

It was considered that the difference between -C-Docker interaction energy and -C-Docker energy if minimum, target and ligands had more intermolecular interaction (pose). Binding energy of targets – ligands complex should be minimum for better interaction between ligands and target. Archana *et al.* (2010) reported curcumin interacts with FTO (obesity) protein at GLN 86, LYS 107 and GLU 325 forming three hydrogen bonds with high binding affinity and minimum binding energy (-6.57 Kcal/mol). Shrivastava *et al.* (2013) and Shruthy and Yusuf (2014) also reported that if the binding energy was minimum ligands and targets showed good interaction.

Molecular docking of ginger ligands, 6-gingerol, 8-gingerol, 10-gingerol and 6-shogaol with ribosomal S6 kinase showed good interaction with the minimum difference between -C-Docker interaction energy and -C-Docker energy and minimum binding energy. The minimum difference between -C-Docker interaction energy and -C-Docker energy and minimum binding energy were recorded with 10-gingerol for the target ribosomal S6 kinase. RSK2 was selected as a molecular target

as it is activated by ERKs and phosphoinositide-dependent kinase 1 protein and plays an important role in human skin cancer development and progression (Cho *et al.,* 2012).

Docking of ginger ligands, 6-gingerol, 8-gingerol and 10-gingerol with Epidermal Growth Factor Receptor showed good interaction. The minimum difference between -C-Docker interaction energy and -C-Docker energy with minimum binding energy were recorded with 6-gingerol for the target Epidermal Growth Factor Receptor. Epidermal Growth Factor Receptor was seen overexpressed in a variety of cancer like NSCLC (Hirsch *et al.,* 2009; Gazdar, 2010), colon cancer (Ooi *et al.,* 2004), breast cancer (Munagala *et al*., 2011; Harrison *et al.,* 2013), pancreatic cancer, prostate cancer (Lorenzo *et al.,* 2002**)**, ovarian cancer and esophageal cancer (Kawaguchi *et al*., 2007).

Ginger ligands, 6-gingerol and 6-shogaol showed good interaction with oestrogen receptor. The minimum difference between -C-Docker interaction energy and -C-Docker energy with minimum binding energy were recorded for 6-gingerol with the target oestrogen receptor. Oestrogen receptor is a hormone playing a major role in ovulation, implantation, pregnancy maintenance, childbirth and lactation. Oestrogen receptor was used as a molecular cancer target in this study and it showed positive in breast cancer (Kelly and Carroll, 2007; Cuzick *et al*., 2013; Harrison *et al.,* 2013; Li *et al*., 2015).

With the cancer target Epidermal Growth Factor Receptor kinase, 6-gingerol, 8-gingerol and 6-shogaol showed good interaction. The minimum difference between -C-Docker interaction energy and -C-Docker energy with minimum binding energy were recorded with 8-gingerol for the target Epidermal Growth Factor Receptor kinase. Epidermal Growth Factor Receptor kinase was seen overexpressed in colon cancer (Ooi *et al.,* 2004)

With the target N-acetyltransferase 2 receptor, 6-gingerol, 10-gingerol and 6-shogaol showed good interaction. The minimum difference between -C-Docker interaction energy and -C-Docker energy with binding energy were recorded with 10-gingerol for the target N-acetyltransferase 2 receptor. Zhang *et al*. (2011)

investigated the effects of the diet, N-acetyltransferase (NAT) 2 acetylation status and their interaction on gastric cancer risk and they found that an inherited deficiency in NAT2 metabolic capacity may be an important modifier of gastric cancer risk.

Ginger ligands, 6-gingerol and 8-gingerol showed good interaction with the target c-MET. The minimum difference between -C-Docker interaction energy and -C-Docker energy and minimum binding energy were recorded with 8-gingerol for the target c-Met. The hepatocyte growth factor receptor (c-Met) is a receptor tyrosine kinase that plays an important role in tumor growth by activating mitogenic signaling pathways. c-Met is found overexpressed in variety of cancers like, breast cancer (Lengyel *et al*., 2005), ovarian cancer (Sawada *et al*., 2007), gastric cancer (Yu *et al.*, 2013), Lung cancer (Knowles *et al*., 2009) and colon cancer (Tsao *et al*., 1998).

Phosphoinositide 3-kinase receptor showed good interaction with the four ginger ligands. The minimum difference between -C-Docker interaction energy and -C-Docker energy was recorded with 6-gingerol for the target phosphoinositide 3-kinase. Phosphoinositide 3-kinase is a signaling molecule that plays a critical role in regulating apoptosis. Mutated phosphoinositide 3-kinase causes cancer development. PI3K is highly activated in variety of cancer like, gastric, colon, breast, pancreatic, prostate, cervical, ovarian, skin and lung cancer (Liu *et al.,* 2010; Kim et al., 2011; Liu et al., 2011; Kim *et al*., 2014; Yip *et al.*, 2014; Prasad and Tyagi, 2015; Yip, 2015)

With the target Follistatin, 6-gingerol, 8-gingerol and 10-gingerol showed good interaction. The minimum difference between -C-Docker interaction energy and -C-Docker energy with minimum binding energy was recorded with 8-gingerol for the target Follistatin. Sepporta *et al.* (2013) reported high level follistatin in prostate cancer and follistatin is good molecular target for studies on prostate cancer.

Among the ginger ligands, 6-gingerol, 8-gingerol, and 6-shogaol showed good interaction with Neuron-specific enolase. The minimum difference between -C-

Docker interaction energy and -C-Docker energy and minimum binding energy were recorded with 6-gingerol with the target Neuron specific enolase. Revee *et al.* (1986) reported that the value of neuron specific enolase (NSE) immune-reactivity can be used as a marker for small cell lung cancer (SCLC), assessed using a monoclonal antibody (MCAB) against NSE. Hong *et al.* (2013) also reported that neuron specific enolase (NSE) is an effective marker for small cell lung cancer.

With the target 17-β Hydroxysteroid dehydrogenase, ginger ligands showed good interaction. The minimum difference between -C-Docker interaction energy and -C-Docker energy with minimum binding energy were recorded with 10-gingerol for the target 17-β Hydroxysteroid dehydrogenase. Sevik *et al*. (2012) reported that an oestrogen positive tumour in breast cancer has high 17bHSD14 protein expression. Gunnarsson *et al.* (2005) also reported that 17Beta-Hydroxysteroid dehydrogenases involved in oestrogen synthesis in breast cancer.

Among the ginger ligands, 6-gingerol, 8-gingerol and 10-gingerol showed good interaction with the cancer target Cyclooxygenase-2. The minimum difference between -C-Docker interaction energy and -C-Docker energy with minimum binding energy were recorded with 10-gingerol for the target Cyclooxygenase-2. COX-2 is overexpressed in every premalignant and malignant condition colon, liver, pancreas, breast, lung, bladder, skin, stomach, head and neck and esophagus (Backlund *et al.,* 2003; Subbaramaiah and Dannenberg, 2003; Dannenberg and Subbaramaiah, 2003).

Saptarini *et al.* (2013) reported that 6-gingerol showed good interaction with COX-1 and COX-2. In the present study also 6-gingerol showed good interaction with COX-2. The binding energy (-61.3134) with minimum energy difference (5.2792) and four number of hydrogen bonds were recorded in their study.

Among the ginger ligands, 6-gingerol and 8-gingerol showed good interaction with the target nuclear factor – kappa B. The minimum difference between -C-Docker interaction energy and -C-Docker energy with minimum binding energy was

recorded with 6-gingerol for the target nuclear factor – kappa B. Nuclear factor-kappa B is a transcription factor and mutation leads to a variety of cancers. NF-kappa B activated in different types of solid tumors like prostate, breast, cervical, pancreatic, gastric, ovarian and lung cancer (Karin *et al*., 2005; Li *et al.,* 2009; Liu et al., 2010; Chen *et al*., 2012; Pal *et al*., 2014; Rhode et al., 2007; Chen *et al*., 2012).

With the target Activator protein-1, 6-gingerol and 8-gingerol showed good interaction. The minimum difference between -C-Docker interaction energy and -C-Docker energy with minimum binding energy were recorded with 8-gingerol for the target Activator protein-1. Activated protein-1 is also a transcription factor in regulation of genes involved in apoptosis and proliferation and may promote cell proliferation by activating the cyclin D1 gene and repressing tumor-suppressor genes, such as p53, p21cip1/waf1 and p16.

Among all ginger ligands, 6-gingerol showed good interaction with all the selected targets except N-acetyltransferase 2 receptor. Shanker *et al.* (2013) reported *in silico* molecular docking studies with methanolic extract of *Tribulus terrestris* and reported considerably low binding energy to follistatin (2BOU). But in case of neuron-specific enolase (1TE6) high binding energy was observed. In the present study all the ginger ligands showed good interaction with minimum binding energy. Among the ginger ligands 10-gingerol showed minimum binding energy with follistatin (2BOU) and in the case of neuron specific enolase (1TE6), 8-gingerol showed minimum binding energy.

Dock score (difference between C-Docker interaction energy and C-Docker energy) of ginger ligands with the selected targets for cancer are shown in Figure 30.

5.2.3 Molecular docking and analysis of approved drugs

Approved drugs used for molecular docking analysis were amifostine, tazarotene, quinestrol, glycodiazine, fulvestrant, raltitrexed, lapatinib, celecoxib,

quercitrin and disulfiram. The minimum difference between –C-Docker interaction energy and –C-Docker energy was with raltitrexed which showed good interaction with the target N- acetyltransferase 2 receptor (Table 19).

Among the approved drugs, quercitrin showed minimum difference between –C-Docker interaction energy and –C-Docker energy with minimum binding energy with the target Activator protein-1. Approved drug raltitrexed showed maximum hydrogen bonds with the target N- acetyltransferase 2 receptor.

Among the approved drugs, glycodiazine and raltitrexed showed good interaction with the target Epidermal Growth Factor Receptor. Disulfiram showed minimum difference between –C-Docker interaction energy and –C-Docker energy with the target nuclear factor kappa B.

5.2.4 ADME/Toxicity prediction

ADME/Toxicity is the computer based method for testing drug metabolism, pharmacokinetics and toxicity. Various medium and high-throughput *in vitro* ADMET screens are available to screen large number of compounds to know drug like properties of the compounds. ADME/Toxicity prediction is used to design stage of new compounds and compound libraries so as to reduce the risk of late-stage attrition and to optimize the screening and testing of the most promising compounds.

ADME/T prediction was done to know the Absorption, Distribution, Metabolism, Excretion and Toxicity of the ligands molecules. Based on ADME/ Toxicity parameter 6-gingerol was found superior when compared to all ginger ligands (Table 20). The Absorption and Distribution were found good, neurotoxicity of 6-gingerol showed medium, and it was also found non-toxic and non-inhibitor for

PY2D6. 6-shogaol also recorded good ADME/ Toxicity but drawbacks observed were the high neurotoxic effect and low solubility levels. All the approved drugs recorded some amount of toxicity and less solubility and absorption levels. Based on docking result and ADME/Toxicity analysis, 6-gingerol was found superior among

ginger ligands and approved drugs selected to validate the anticancerous properties through cell culture.

5.3 Validation of anticancerous properties of 6-gingerol using different tumour cell lines.

In the present investigations based on molecular docking and ADME/ Toxicity prediction 6-gingerol was used to validate anticancerous properties with three cancer cell lines (HCT15, L929 and Raw 264.7). with six different concentration of 6-gingerol (Figure 27).

5.3.1 *In vitro* cytotoxicity of 6-gingerol

The MTT assay is a colorimetric assay for assessing cell metabolic activity and measures the cell proliferation rate. NAD (P) H-dependent cellular oxidoreductase enzymes reflect the number of viable cells present. These enzymes are capable of reducing the tetrazolium dye MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to its insoluble formazan, which has a purple color. MTT assays are usually done in the dark since the MTT reagent is sensitive to light.

MTT assay was performed to determine the cytotoxicity of 6-gingerol on HCT15, L929 and Raw 264.7 cells with different concentration of 6-gingerol viz. 17, 34, 68, 102, 136 and 170 μM. 6-gingerol showed cell cytotoxicity in a dose dependent manor and IC_{50} value of 6-gingerol was observed at 100 μM in HCT15 (colon cancer), 102 μM in Raw 264.7 (mouse leukaemic monocyte macrophage) and 102 μM in L929 (murine fibro sarcoma).

Lee *et al.* (2008) observed sixteen per cent reduction in cell viability at 10 μM concentration of 6-gingerol and 6-paradol. Anticancerous effect was studied in MDA-MB-231 cells (breast cancer). In this investigations 13 per cent reduction in cell viability was observed in L929 (murine fibro sarcoma cell), 25 per cent in HCT15

(colon cancer) and 26 per cent in Raw 264.7 (mouse leukaemic monocyte macrophage cell) at 17 µM concentration of 6-gingerol (Figure 31 and Table 21).

Brahmabhatt *et al.* (2013) also reported the effect of 6-gingerol, 8-gingerol, 10-gingerol and 6-shogaol on cytotoxicity of cancer cell (PC-3) at different concentrations 75 µM, 10 µM, 50 µM and 4 µM.

Table 21. Effect of 6-ginerol on cytotoxicity in cancer cell line 24 hour after treatment

Concentration of 6-gingerol (µM)	Percentage of dead cells*		
	L929	HCT15	Raw 264.7
17	13.35404	25.46778	26.30303
34	35.15528	34.09563	49.09091
68	41.61491	35.03119	63.63636
102	54.03727	57.86556	65.33333
136	59.31677	85.16979	79.63636
170	74.2236	87.11019	87.15152

*Percentage of dead cells calculated over control

Percentage of dead cells in control = 0

Control is the cell line without 6-ginerol and percentage of dead cells observed in control is zero.

An overview of the results obtained from the present investigations

Natural products provide a vast source for discovery of useful therapeutics. Because the molecular targets of many natural products remain unknown, unraveling the targets of a natural compounds provide insights into its molecular mechanism and help in optimizing its potency and selectivity. The phytocompounds present in ginger especially the phenolics have vast amount of therapeutic value. Hence, the present study focused on screening the clones for gingerol content, molecular docking with gingerol and studying the anticancerous properties of 6-gingerol. The study could bring out valuable informations on selection

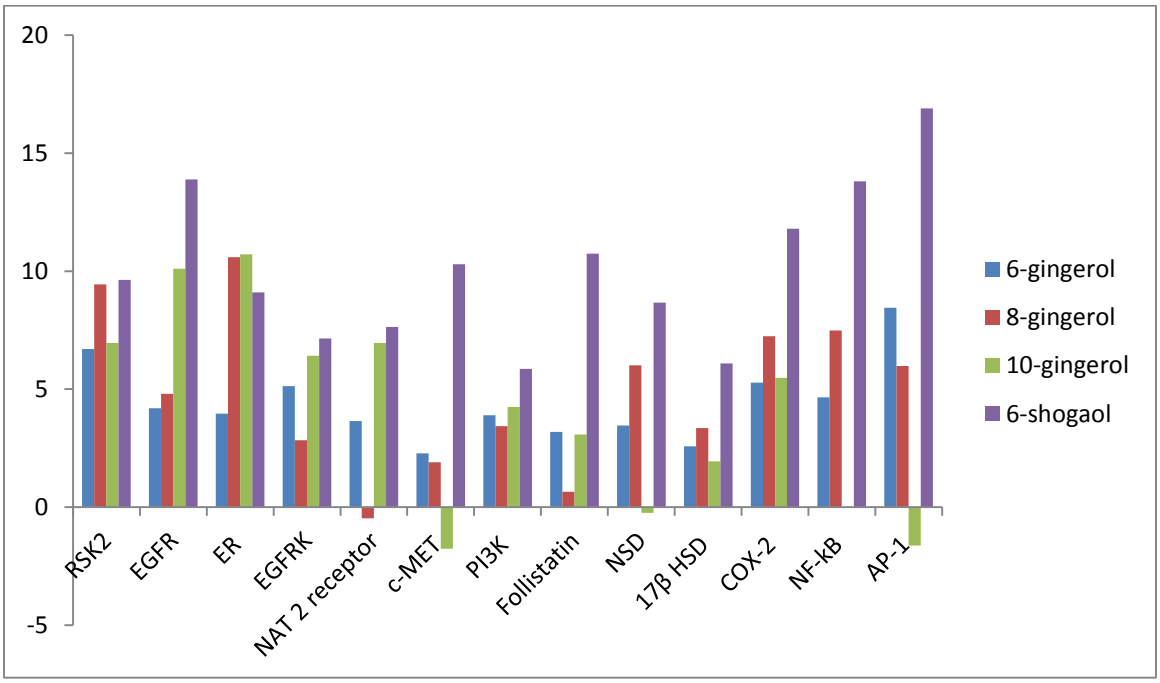


Figure 30. Dock score of ginger ligands with the selected targets for cancer

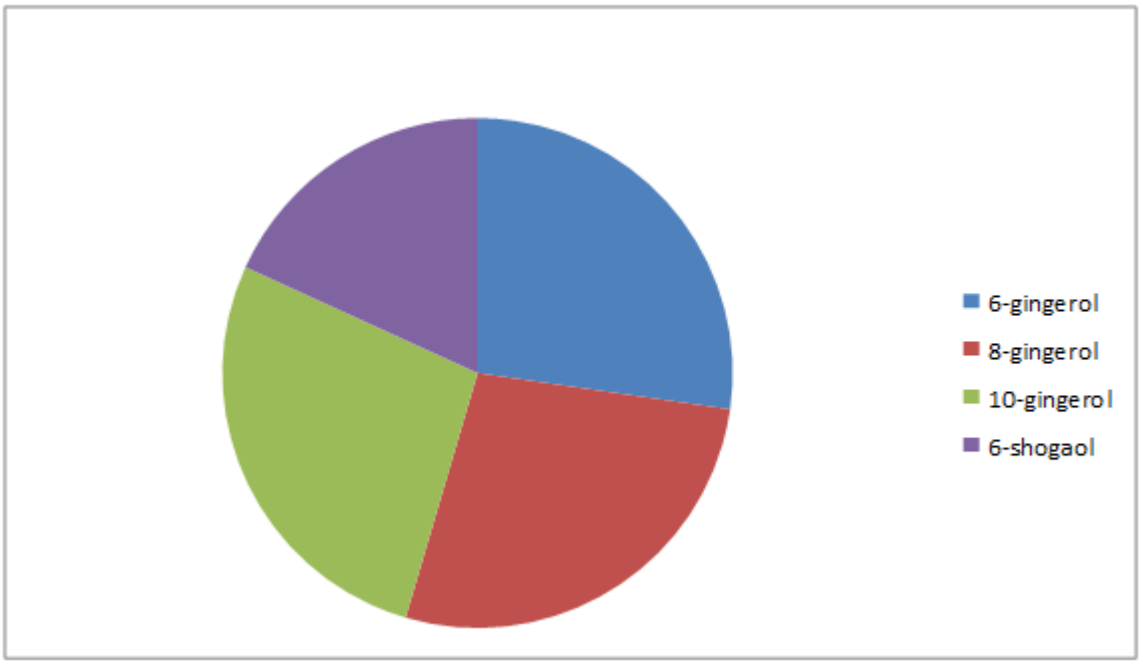


Figure 32. ADME Solubility levels of ginger ligands

of high gingerol yielding somaclones, molecular docking of ginger ligands and approved drugs against cancer targets and anticancerous activities of 6-gingerol.

From the present investigations, it was found that somaclones exhibited high variability in the content of bioactive compounds like 6-gingerol, 8-gingerol, 10-gingerol and 6-shogaol. High yield of bioactive compounds and high variability in the content were registered by plants regenerated after *in vitro* mutagenesis. From the study, five high gingerol yielding somaclones (Mse 20Gy 418, Mse 20Gy 175, Mse 20Gy 862, Mc 10Gy 330 and Mc 10Gy 168) could be selected (Table 22).

Table 22. Yield and quality parameters of selected ginger somaclones

Sample ID	Per Plant Yield (g/Plant)	Dry Recovery (%)	Oleoresin Yield (%)	6-gingerol (g/Kg dry ginger powder)	8-gingerol (g/Kg dry ginger powder)	10-gingerol (g/Kg dry ginger powder)	Total gingerol (g/Kg dry ginger powder)	6-shogaol (g/Kg dry ginger powder)
Mc 10Gy 168	574.83	25.49	10.2	11.4	0.93	1.01	13.35	1.25
Mc 10Gy 330	453	27.27	5.83	12.42	0.87	0.90	14.19	1.84
Mse 20Gy 175	346	19.46	12.78	11.06	0.89	0.63	12.59	1.59
Mse 20Gy 418	310.5	25.28	10.69	13.83	0.81	0.48	15.12	2.3
Mse 20Gy 862	361	25.68	9.67	11.64	0.94	1.15	13.73	1.78

Molecular docking with ginger ligands and approved drugs with selected cancer targets was attempted in the present investigations. Four ginger ligands viz. 6-gingerol, 8-gingerol, 10-gingerol and 6-shogaol and eleven approved drugs were docked with thirteen targets identified for cancer.

The difference between –C-Docker interaction energy and –C-Docker energy if minimum, targets and ligands had more intermolecular interaction. In the present study, minimum difference between –C-Docker interaction energy and –C-Docker

energy was observed when 6-gingerol was docked with targets c-Met, 8-gingerol with

follistatin, 10-gingerol with Hydroxysteroid dehydrogenase and 6-shogaol with Phosphoinositide 3-kinase. Among the approved drugs, raltitrexed showed minimum difference between -C-Docker interaction energy and C-Docker energy with the target, N- acetyltransferase 2 receptor (Table 15,16,17,18).

Binding energy of the targets and ligands should be minimum for better interaction of ligands and targets. Minimum binding energy was recorded for 6-gingerol and 6-shogaol with targets Activator protein-1 and Epidermal Growth Factor Receptor. Similary, minimum binding energy was recorded for 8-gingerol with Activator protein-1 and Oestrogen receptor and for 10-gingerol, minimum binding energy with Epidermal Growth Factor Receptor and Ribosomal S6 kinase. Among the approved drugs, quercitrin showed minimum binding energy with the target Activator protein-1.

The other parameter which was found important in molecular docking is the number of hydrogen bond and bond length. If number of hydrogen bond is more and bond length less, ligands have good binding affinity with the targets. In the present investigations, number of hydrogen bonds was more for the target follistatin when 6-gingerol and 8-gingerol were docked. Similary, for the target Phosphoinositide 3-kinase with 10-gingerol and for the targets Epidermal Growth Factor Receptor and Cyclooxygenase – 2 for 6-shogaol, the number of hydrogen bond was more. Among the approved drugs, raltitrexed showed maximum number of hydrogen bonds.

A number of drugs are withdrawn from the market due to the underlying ADME/Toxicity issues. Hence early screening for ADME/ Toxicity properties using *in silico* tools has been widely appreciated (Valerio, 2009 and Butina *et al.,* 2002).

In the present investigations ADME/ Toxicity screening of the four ginger ligands and eleven approved drugs was done. ADME/Toxicity screening revealed that solubility level was good for ginger ligands, 6-gingerol, 8-gingerol, 10-gingerol and 6-shogaol and also for the approved drugs, quercitrin and glycodiazine. ADME/

Toxicity solubility level was low for approved drugs, celebrex, fulvestrant and quinestrol and very low for tarazarotein, lapatinib and gefitinib.

ADME/ Toxicity absorption level was good for ginger ligands, 6-gingerol, 8-gingerol, 10-gingerol and 6-shogaol and also for the approved drugs, tazarotene, lapatinib, gycodiazine, gefitinib, Celebrex. For quinestrol, absorption level was moderate, poor for raltitrexed and very poor absorption was observed for fulvestrant, amifostine and quercitrin approved drugs.

Metabolic bio transformation of a new chemical entity is of high interest. P450 (CYPs) are the major enzymes involved in the process responsible for the metabolism of ~90% FDA approved drugs (Wilkinson, 2005). CYP2D6 prediction in the present study showed that all the ginger ligands were found non – inhibitor of CYP2D6 in metabolism. Among the approved drugs, quercitrin, disulfiram, celebrex, amifostine, gycodiazine, fulvestrant, raltitrexed, quinestrol, lapatinib and tazarotene were found non-inhibitor of CYP2D6 in metabolism while gefitinib showed inhibitory effect on CYP2D6 in metabolism.

Blood Brain Barrier partitioning is an important parameter determining the neurotoxicity of the drugs. In the present study, 6-gingerol showed medium levels of BBB while other ginger ligands, 8-gingerol, 10-gingerol and 6-shogaol showed high level of Blood Brain Barrier. Among the approved drugs, gycodiazine showed low level of Blood Brain Barrier while tazarotene, quinestrol and disulfiram showed very high levels of Blood Brain Barrier.

All the ginger ligands were found non – toxic with respect to hepatotoxicity while among the approved drugs only tazarotene, quinestrol and fulvestrant were found non – toxic and other approved drugs, tazarotene, quinestrol, disulfiram and gefitinib was found toxic.

When the four ginger ligands were compared, 6-gingerol was found superior in terms of minimum difference between –C-Docker interaction energy and –C-Docker energy, minimum binding energy and maximum number of hydrogen bonds and good ADME/ Toxicity parameters. 6-shogaol even through exhibiting minimum binding energy and more number of hydrogen bonds, the difference between –C-Docker interaction energy and –C-Docker energy was found high. In ADME/ Toxicity studies the solubility level was low and Blood Brain Barrier was high for 6-shogaol (Table 18, 20 and 32, 33).

When all the eleven approved drugs were compared, raltitrexed and quercitrin were found superior based on docking study but based on ADME/Toxicity analysis approved drugs had several demerits. ADME/ Toxicity solubility level was low for celebrex, fulvestrant and quinestrol. ADME/ Toxicity absorption level was very poor for fulvestrant, amifostine and quercitrin and poor for raltitrexed. Blood Brain Barrier level was very high for quinestrol and tazarotene, high for gefitinib. Hepatoxicity prediction was toxic for lapatinib, raltitrexed, gycodiazine, amifostine, celebrex, disulfiram and quercitrin. CYP2D6 prediction was showed inhibitor effect for gefitinib in ADME/ Toxicity analysis Quercitrin even through had biding energy it has got high Blood Brain Barrier level.

When the ginger ligands and approved drugs were considered together, 6-gingerol was found more effective than the approved drugs based on the docking results and ADME/ Toxicity analysis (Figure 34).

Another significant observation in the present investigations is the effectiveness of 6-gingerol on selected cancer targets involved in diseases other than cancer. To site an example Cyclooxygenase enzymes play an important role in inflammatory response and in the present study all the four ginger ligands were observed as COX-2 inhibitors.

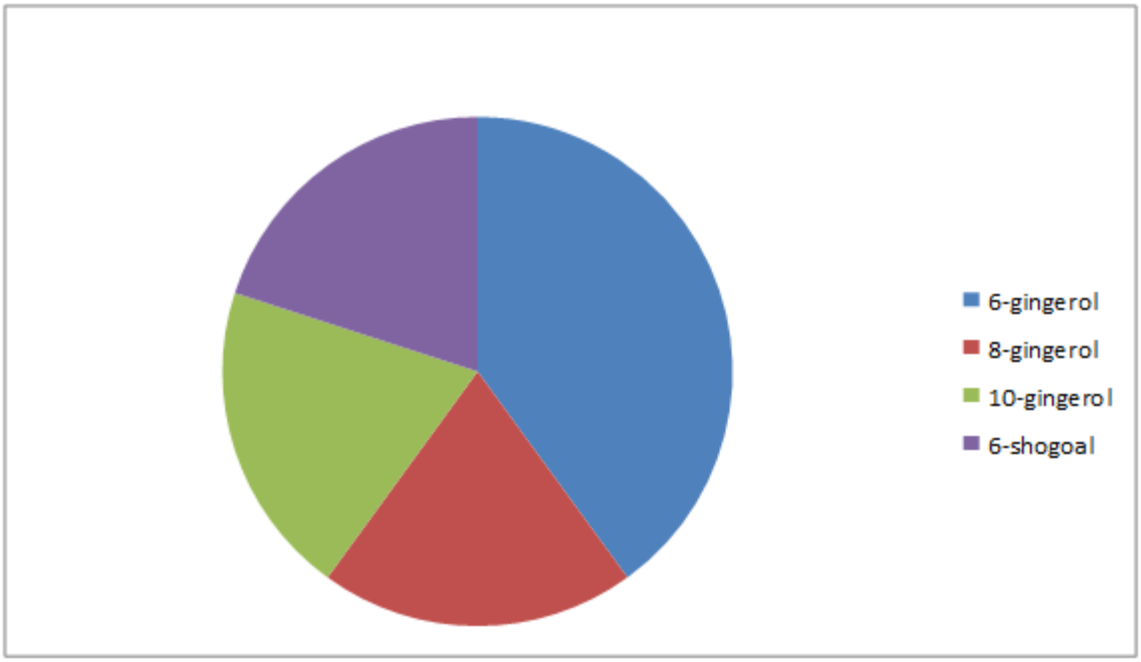


Figure 33. ADME BBB levels of ginger ligands

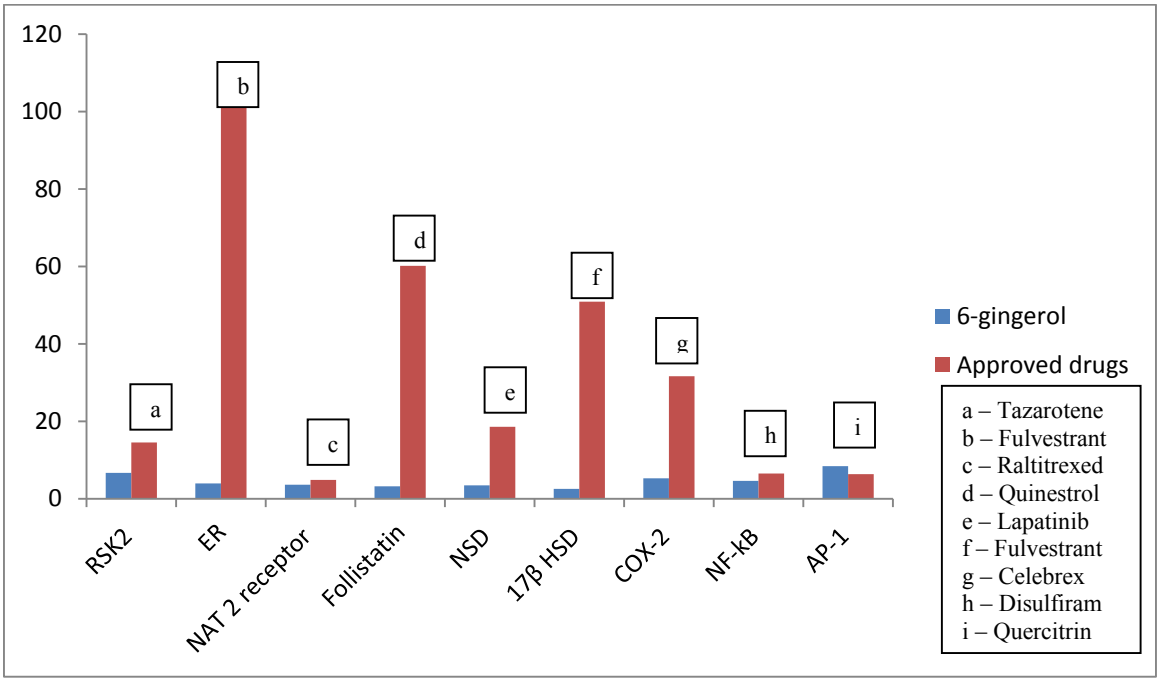


Figure 34. Dock score of 6-gingerol and approved drugs with the selected cancer targets

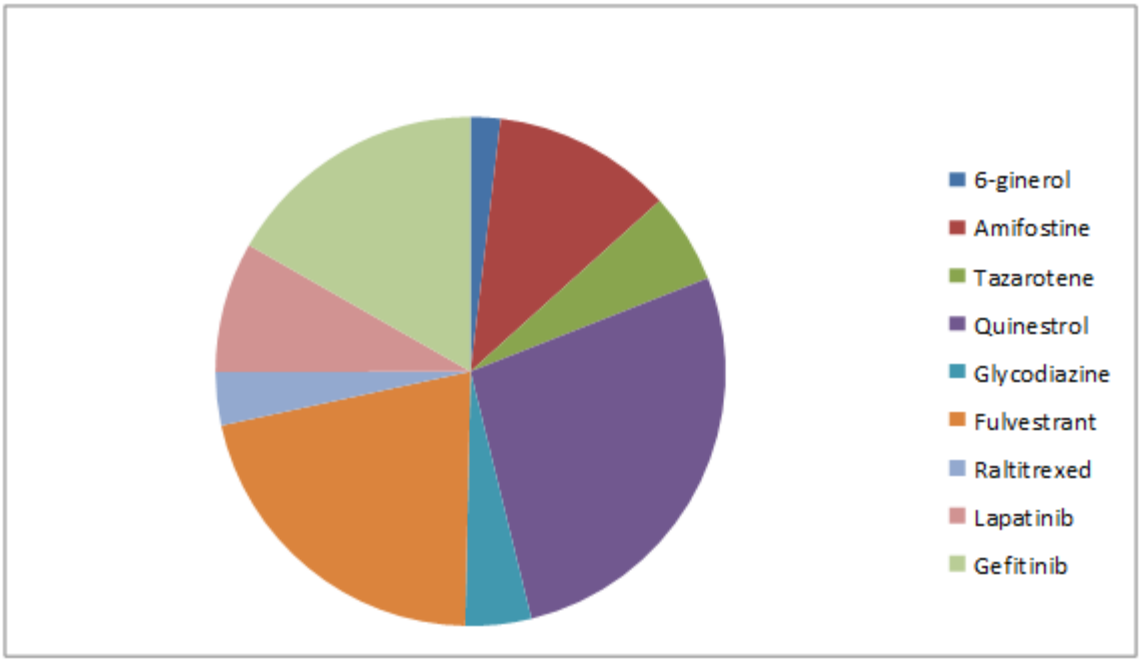


Figure 35. Dock score of 6-gingerol and approved drugs with the cancer target EGFR

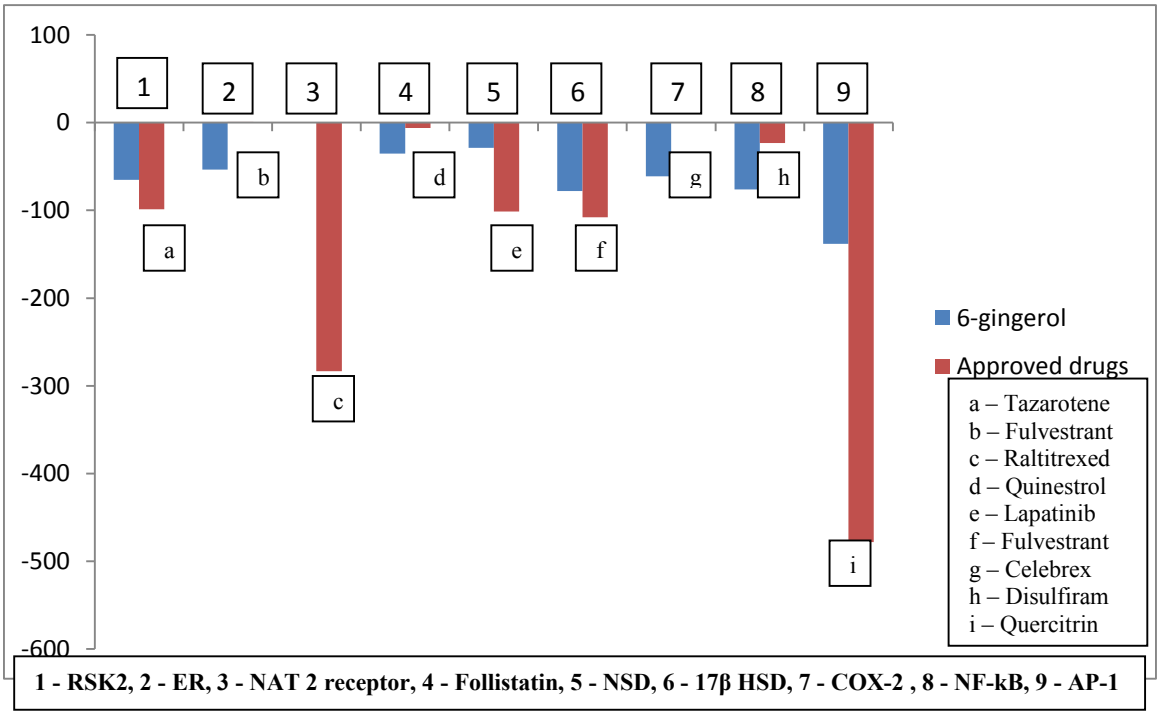


Figure 36. Binding energy of 6-gingerol and approved drugs with the selected cancer targets

In order to validate the anticancerous properties, the effect of 6-gingerol on three cancer cell lines was studied. 6-gingerol was found cytotoxic to the three cell lines investigated showing its anticancerous activity. The study also showed the increase in cell death with increase in concentration of 6-gingerol. The IC_{50} value observed in the three cancer cell lines were (100 μM in HCT15, 102 μM in L929 and Raw 264.7) showing the uniform activity in the three cancer cell lines.

Conclusion and future line

The investigations paved way to locate high gingerol yielding somaclones, to prove the effectiveness of 6-gingerol as an anticancerous phytochemical through molecular docking and cell culture studies and to highlight the potential of 6-gingerol for drug development. The study also gave an insight into the use of pungent phenolic compounds studied in ginger for other medicinal applications. Multiplication and evaluation of high gingerol yielding somaclones, indepth study on the effect of 6-gingerol and ginger extracts in different cancer cell lines and effect of ginger polyphenols on other diseases are the future research areas to be focused. As 6-gingerol is identified as a very good phytocompound compared to other ginger ligands and approved drugs, research thrust may be focused on drug development using 6-gingerol.

Summary

6. SUMMARY

The study entitled "Screening ginger (*Zingiber officinale* Rosc.) somaclones for gingerol content and validation of anticancerous properties of gingerol" was carried out at the Centre for Plant Biotechnology and Molecular Biology and Distributed Information Centre, College of Horticulture during 2013 to 2015. The objectives of the study were to screen ginger somaclones for gingerol content, to identify potential cancer targets for gingerols and shogaol using *in silico* tools and to validate the anticancerous properties of gingerol. Fifty somaclones of ginger cultivar Maran regenerated through direct/ indirect methods of organogenesis/ embryogenesis/ *in vitro* mutagenesis and one control (conventionally propagated) were used as the experimental material for screening for gingerol content and to validate the anticancerous properties of gingerol.

The salient findings of the study are summarised in three different sub heads.

Screening ginger somaclones for gingerol content

- Somaclones studied showed variability in yield. Fresh yield per plant ranged from 91.5 to 574.83 g in the somaclones studied and highest variability in yield was observed in MC 10Gy group of somaclones.
- Dry ginger recovery varied from 19.5 to 29 per cent in the somaclones studied and high variability in dry ginger recovery was observed in somaclone of MC 10Gy group.
- The oleoresin content varied from 4.45 to 12.78 per cent in the somaclones studied.
- High recovery of oleoresin (8.06 per cent) and maximum variability for the oleoresin content was found in somaclons of M Se 20Gy.
- The somaclones showed variability in the content of pungency principles like 6-gingerol (3.33-13.83 g/kg dry ginger powder), 8-gingerol (0.25-0.94 g/kg), 10-gingerol (0.17- 1.15 g/kg) and 6-shogaol (0.61-2.33 g/kg).

- Somaclones regenerated after *in vitro* mutagenesis recorded higher content of gingerol.
- Principle Component Analysis to cluster somaclones based on quality parameters could locate five high gingerol yielding somaclones viz. Mse 20Gy 418, Mse 20Gy 175, Mse 20Gy 862, Mc 10Gy 330 and Mc 10Gy 168.

***In silico* analysis of potential ligands and targets for cancer and molecular docking**

- The targets identified for cancer includes, ribosomal S6 kinase, Epidermal Growth Factor Receptor, Epidermal Growth Factor Receptor kinase, Oestrogen receptor, N-acetyltransferase 2, c-Met, Phosphoinositide 3-kinase, Follistatin, Neuron-specific enolase, 17Beta Hydroxysteroid dehydrogenase, Cyclooxygenase-2, Nuclear factor-kappa B, Activator protein-1
- Eleven ligands includes, ginger ligands (6-gingerol, 8-gingerol, 10-gingerol and 6-shogaol) and approved drugs like, amifostine, tazarotene, quinestrol, glycodiazine, fulvestrant, raltitrexed, lapatinib, celecoxib, quercitrin and disulfiram were used for molecular docking.
- Difference between C-Docker interaction energy and C-Docker energy was found minimum for the target c-Met when 6-gingerol was docked.
- For the target follistatin with 8-gingerol, Hydroxysteroid dehydrogenase with 10-gingerol and Phosphoinositide 3-kinase with 6-shogaol, the difference between C-Docker interaction energy and C-Docker energy was found minimum.
- Among the approved drugs, raltitrexed showed minimum difference between C-Docker interaction energy and C-Docker energy for the target N-acetyltransferase 2 receptor.
- Minimum binding energy was recorded for 6-gingerol and 6-shogaol with targets Activator protein-1 and Epidermal Growth Factor Receptor.

- Minimum binding energy was recorded for 8-gingerol with Activator protein-1 and Oestrogen receptor
- Minimum binding energy was recorded for 10-gingerol with Epidermal Growth Factor Receptor and Ribosomal S6 kinase.
- Among the approved drugs, quercitrin showed minimum binding energy with the target Activator protein-1.
- Number of hydrogen bonds was more for the target follistatin when 6-gingerol and 8-gingerol were docked.
- For the target Phosphoinositide 3-kinase with 10-gingerol and for Epidermal Growth Factor Receptor and Cyclooxygenase – 2 with 6-shogaol, the number of hydrogen bond was more (5).
- Among the approved drugs, raltitrexed showed maximum number of hydrogen bonding (6) with the cancer target N-acetyltransferase 2.
- ADME/ Toxicity solubility level was good for ginger ligands - 6-gingerol, 8-gingerol, 10-gingerol and 6-shogaol.
- Among the approved drugs, ADME/ Toxicity solubility level was good for quercitrin and glycodiazine.
- ADME/Toxicity solubility level was very low for approved drugs celebrex, fulvestrant and quinestrol, low for tarazarotein, lapatinib and gefitinib.
- ADME/ Toxicity absorption level was good for ginger ligands - 6-gingerol, 8-gingerol, 10-gingerol and 6-shogaol.
- Among the approved drugs, ADME/ Toxicity absorption level was good for tazarotene, lapatinib, gycodiazine, gefitinib, celebrex and disulfiram.
- ADME/ Toxicity absorption level was moderate for drugs quinestrol, poor for raltitrexed and very poor for fulvestrant, amifostine and quercitrin.
- ADME/ Toxicity Blood Brain Barrier level was medium for 6-gingerol which shows the less neurotoxic effects.
- Among the approved drugs, ADME/ Toxicity Blood Brain Barrier level was low for gycodiazine.

- ADME/ Toxicity Blood Brain Barrier level was very high for drugs tazarotene, quinestrol and disulfiram, high for gefitinib.
- ADME/ Toxicity hepatotoxicity prediction was false (non-toxic) for ginger ligands, 6-gingerol, 8-gingerol, 10-gingerol and 6-shogaol.
- Among the approved drugs, ADME/ Toxicity hepatotoxicity prediction was false (non-toxic) for tazarotene, quinestrol and fulvestrant.
- ADME/ Toxicity hepatotoxicity prediction was true (toxic) for drugs lapatinib, raltitrexed, gycodiazine, amifostine, gefitinib, celebrex, disulfiram and quercitrin.
- ADME/ Toxicity CPY2D6 prediction was false (non-inhibitor) for ginger ligands, 6-gingerol, 8-gingerol, 10-gingerol and 6-shogaol.
- Among the approved drugs, ADME/ Toxicity CPY2D6 prediction was false (non-inhibitor) for quercitrin, disulfiram, celebrex, amifostine, gycodiazine, fulvestrant, raltitrexed, quinestrol, lapatinib and tazarotene.
- ADME/ Toxicity CPY2D6 prediction was true (inhibitor) for drug gefitinib.
- Considering the results of molecular docking and ADME/ Toxicity analysis, 6-gingerol was found superior as compared to other ginger ligands and approved drugs.

Validation of anticancerous properties of 6-gingerol using different tumour cell lines

- The phytocompound 6-gingerol was found cytotoxic to all the three cancer cells lines studied (HCT15 (colon cancer), Raw 264.7(mouse leukaemic monocyte macrophage cell) and L929 (murine fibro sarcoma cell)).
- The cytotoxicity increased with increase in concentration of 6-gingerol.

- The IC_{50} values recorded for different cancer cell lines, 24 h. after treatment (100 μM for HCT15, 102 μM for L929 and 102 μM for Raw 264.7) showed the uniform cytotoxicity of 6-gingerol in the three cancer cell lines studied.

References

Ali, B. H., Blunden, G., Tanira, M. O., and Nemmar, A. 2008. Some phytochemical, pharmacological and toxicological properties of ginger (*Zingiber officinale* Roscoe): A review of recent research. *Food. Chem. Toxicol.* **46(2):** 409–420.

Antony, P. P. and Nazeem, P. A. 2014. Boerhaavia diffusa in cancer therapy-an INSILICO analysis. *Inter. J. Pharma. Sci. Res.* **5:** 0975-9492.

Anu, A., Babu, K. N. and Peter, K. V. 2002. Evaluation of paprika genotypes in Kerala. *Indian*. *J. Plant. Genet. Resour.* **15:** 93–99.

AOAC. 1980. *Official Methods of Analysis of the Association of Official Analyticul chemists*. Thirteenth edition. Association of Official Analytical Chemists, Washington D. C., 525p.

Archana, P., Sathishkumar, N. and Bharthi, N. 2010. *In silico d*ocking analysis of curcumin – an inhibitor for obesity. *International Journal of Pharma and Bio Sciences*. **1 (4):** 224-235**.**

Ashraf, K., Ahmad, A., Chaudhary, A. Mujeeb, M., Ahmad, S., Amir, M., Mallick, N. 2014. Genetic diversity analysis of *Zingiber Officinale* Roscoe by RAPD collected from subcontinent of India. *Saudi J. Biol. Sci.* **21:** 159–165.

Backlund LM, Nilsson BR, Goike HM et al. Short postoperative survival for glioblastoma patients with a dysfunctional Rb1 pathway in combination with no wild-type PTEN. *Clinic Cancer Res.* **9**: 4151–8.

Booij, I., Piombo, G., Risterucci, J. M., Thomas, D., Ferry, M. 1993. Sugar and free amino acid composition of five cultivars of dates palm from offshoots or in vitro-plants in open field. *J. Agric. Food. Chem.* **41:** 1553–1557.

Brahmbhatt, M., Gundala, S. R. and Asif, G. 2013. Ginger phytochemicals exhibit synergy to inhibit porstate cancer cell proliferation. Nutr. Cancer. **65**: 263-72.

Butania, D., Segall, M. D. and Frankcombe, K. 2002. Predicting ADME properties in silico: methods and models. *Drug. Discov. Today*. **7(11):** 83-88.

C Gunnarsson, C., Hellqvist, E., Sta, O. and the Southeast Sweden Breast Cancer Group. 2005. 17b-Hydroxysteroid dehydrogenases involved in local oestrogen synthesis have prognostic significance in breast cancer. *British Journal of Cancer*. **92:** 547 – 552.

Chen, H. J., Mok, T. S., Chen, Z. H., Guo, A. L., Zhang, X. C., Su, J. 2012. Clinicopathologic and molecular features of epidermal growth factor receptor T790M mutation and c-METamplification in tyrosine kinase inhibitor-resistant Chinese non-small cell lung cancer. *Pathol. Oncol. Res.* **15:** 651-658.

Chen, W., Li, Z., Bai, L. and Lin, Y. 2012. NF-kappaB, a mediator for lung carcinogenesis and a target for lung cancer prevention and therapy. *Front Biosci.* **16:** 1172–1185.

Cheng, J., Yuan, C. And Graham, T. L. 2011. Potential defense-related prenylated isoflavones in lactofen-induced soyabean. *Phytochem.* **72:** 875-881.

Cho, Y. Y., Lee, M. H., Lee, C. J., Yao, K., Lee, H. S., Bode, A. M and Dong, Z. RSK2 as a key regulator in human skin cancer. *Carcinogenesis*. **33(12):** 2529–2537.

Christensen, J. G., Burrows, J. and Salgia, R. 2004. c-Met as a target for human cancer and characterization of inhibitors for therapeutic intervention. *Cancer Letters* **225**: 1–26.

Chrubasik, J. E., Roufogalis, B. D., and Chrubasik, D. 2007. Evidence of effectiveness of herbal anti-inflammatory drugs in the treatment of painful osteoarthritis and chronic low back pain. *Phyto. Res.* **21:** 675-683.

Cuzick, J., Sestak, I., Bonanni, B., Costantino, J.P., Cummings, S., Andrea DeCensi, Mitch Dowsett, John F Forbes, Leslie Ford, Andrea Z LaCroix, John Mershon, Bruce H Mitlak, Trevor Powles, Umberto Veronesi, Victor Vogel, D Lawrence Wickerham, for the SERM Chemoprevention of Breast Cancer Overview Group. 2013. Selective oestrogen receptor modulators in prevention of breast cancer: an updated meta-analysis of individual participant data. **381**: 1827–34. http://dx.doi.org/10.1016/ S0140-6736(13)60140-3.

Dannenberg, A. J. and Subbaramaiah, K. 2003. Cyclooxygenase 2: a molecular target for cancer prevention and treatment. *Pharmaco.l Sci*. **24(2):** 96-102.

Eferl, R. and Wagner, E. F. 2003. AP-1: a double-edged sword in tumorigenesis. *Nature Reviews Cancer* **3**: 859-868.

Evans, D. A. 1988. Application of somaclonal variation. Biotechnology in Agriculture (ed. Mizrachi, A. Z.) Alan R. Liss, New York, pp.203-223

Evans, D. A. 1989. Somaclonal variation- genetic basis and breeding applications. Trends *Genetics*. **5:** 46-50.

Evans, D. A., Sharpe, W. R. and Bravo, J. E. 1984 Cell culture methods for crop improvement. In *Handbook of Pla/11Cell Cul/ure,* Vol. 2, eds Sharp, W.R., Evans, D.A, Ammirato, p.y. and Yamada, Y pp. 393-441.

Evans, D.A. and W.R. Sharp. 1983. Single gene mutations in tomato plants regenerated from tissue culture. *Science* **221**:949–951.

Evans, RM. 1988. The steroid and thyroid hormone receptor superfamily. *Science. 240: 889- 895.*

Evans, W.C. 2002. Ginger. Trease and Evans Pharmacognosy, 15th ed. WB Saunders, Edinburgh, pp. 277–280.

Gazdar, A. F. 2010. Epidermal growth factor receptor inhibition in lung cancer: the evolving role of individualized therapy. *Cancer. Metastasis. Rev*. **29(1):** 37–48.

Ghosh, A. K. 2011. Gingerol might be a sword to defeat colon cancer. *Int. J. Pharma. Bio Sci.* **2(1):** 816-827.

Ghosh, S. and Mandi, S. 2015. SNP in Chalcone Synthase gene is associated with variation of 6-gingerol content in contrasting landraces of *Zingiber officinale*. Roscoe *Gene.* 566: 184-188.

Govindarajan, V., 1982. Ginger-chemistry technology and quality evaluation: Part-I CRC. *Critical. Reviews. Food. Sci. Nutrition*. **17:**1–96.

Griesbach, R. J., Semeniuk, P., Rob, M. and Lawson, R. H. 1988. Tissue culture in the, improvement of Eustioma. *Hort. Science*. **23:**791.

Gunnarsson, C., Hellqvist, E., Sta, O., and the Southeast Sweden Breast Cancer Group2. 2005 .17b-Hydroxysteroid dehydrogenases involved in local oestrogen synthesis have prognostic significance in breast cancer. *Br. J. Cancer*. 92. 547 – 552.

Gupta, R., Banerjee, S., Mallavarapu, G. R., Sharma, S., Khanuja, S. P. S., Shasam, A. K., Kumar, S., Gupta, R. and Kumar, S. 2002. Development of a superior somaclone of rose. scented geranium and a protocol for inducing variants. *Hort. Science*. **37(4):** 632-636.

Harrison, H., Simões, B. M., Rogerson, L., Howell, S. J., Landberg, G., and Clarke, R. B. 2013. Oestrogen increases the activity of oestrogen receptor negative breast cancer stem cells through paracrine EGFR and Notch signaling. *Cancer. Res.* **15(21):** 1-12.

Hirsch, F. R., Garcia, V. M. and Cappuzzo, F. 2009. Predictive value of EGFR and HER2 overexpression in advanced non-small-cell lung cancer. *Oncogene.* **28(1):** 32–37.

Ittiyavirah, S. P. and Paul, M. 2013. *In silico* docking analysis of constituents of *Zingiber officinale* as antidepressant. *Journal of Pharmacognosy and Phytotherapy*. **5 (6):** 101-105.

Jaffery, E. H., Brown, A. F., Kurilich, A. C., Keek, A. S., Matusheski, N., and Klein, B. P. 2003. Variation in content of bioactive components in broccoli. *J. Food. Composition. Analysis.* **16:** 323–330.

James, P., Baby, B., Charles, S., Nair, L. S., Nazeem, P. A. 2015. Computer aided gene mining for gingerol biosynthesis. *Biomed. Informatics*. **11(6):** 316-321.

Jeong, C. H., Bode, A. M., Pugliese. 2009. [6]-Gingerol suppresses colon cancer growth by targeting leukotriene A4 hydrolase. *Cancer. Res.* 69(13): 5584-5591.

Jiang, J., Xu, N., Wu, C., Deng, H., Lu, m., Xu, B., Li, M., Wang, M., Xu, J., and Nilsson-Ehle, P.2006. Treatment of advanced gastric cancer by chemotherapy combined with autologous cytokine-induced killer cells. *Anticancer Research **26*:** 2237-2242

Joeng, C. H., Bode, A. M., Pugliese, A., Cho, Y. Y., Kim, H. G., Shim, J. H., Jeon, Y. J., Li, H., Jinag, H., and Dong, Z. 2009. [6]-Gingerol Suppresses Colon Cancer Growth by Targeting Leukotriene A4 Hydrolase. *Cancer. Res.* **6:** 584-591.

Karin, M., and Greten F. R. 2005. NF-kappaB: linking inflammation and immunity to cancer development and progression. *Nat. Rev. Immunol*. **5(10):** 749–59.

Kathryn J. Chavez1, Sireesha V. Garimella1, and Stanley Lipkowitz. 2010. Triple Negative Breast Cancer Cell Lines: One Tool in the Search for Better Treatment of Triple Negative Breast Cancer. Breast Dis. **32(2):** 35–48.

Kawaguchi, Y., Kono, K., Mimura, K., Mitsui, F., Sugai, H., Akaike, H and Fujii, H. 2007. Targeting EGFR and HER-2 with cetuximab- and trastuzumabmediated immunotherapy in oesophageal squamous cell carcinoma. *Br. J. Cancer.* **97:** 494 – 501.

Kelly, A. G. and Carroll, J S. 2007. Oestrogen-receptor-mediated transcription and the influence of co-factors and chromatin state. *Nat. Rev. Cancer*. **7:** 713-722.

Kim, S. O. and Kim, M. R. 2013. [6]-Gingerol Prevents Disassembly of Cell Junctions andActivities of MMPs in Invasive Human Pancreas Cancer Cells through ERK/NF-*κ*B/Snail Signal Transduction Pathway. Evidence-Based Complementary and Alternative Medicine. 9pp.

Kim, S. O., Chun, K. S., Kundu, J. K., and Surh, Y. J. 2011. Inhibitory effects of [6]-gingerol on PMA-induced COX-2 expression and activation of NF-kappaB and p38 MAPK in mouse skin. *Biofactors*. **21(1–4):** 27–31.

Knowles, E. L., Hernandez, S., Malats, N., Kogevinas, M., Lloreta, J., Carrato, A., Tardon, A., Serra, C., Real, F. X. 2009. PIK3CA mutations are an early genetic alteration associated with FGFR3 mutations in superficial papillary bladder tumors. *Cancer. Res.* 2009. **66:** 7401–7404.

Kubra, I. R., and Rao, L. J. M. 2011. An impression on current developments in the technology, chemistry, and biological activities of ginger (*Zingiber officinale* Roscoe). Critic. *Reviews. Food. Sci. Nutri.* **52(8):** 651–688

Kukreja, A. K., Dhawan, O. P., Ahuja, P. S., Sharma, S., and Mathur, A. K. 1992 Genetic improvements of mints: On the qualitativetraits of essential oil of *in*

vitro derived clones of Japanese mint (*Mentha arvensis* var piperascens Holmes). *J. Essent. Oil Res.* **4:** 623–629.

Langner, E., Greifenberg, S. and Gruenwald, J. 1998. Ginger: history and use. *Adv. Ther*. **15:** 25–44.

Larkin, P. J., Ryan, S. A., Brettel, R. I. S. and Scowcroft, W. R . 1984. Heritable somaclonal variation in wheat. *Theor. Appl. Genet.* **67:** 443-455.

Larkins, P. and Scowcroft, W. R. 1981. Somaclonal variation, a novel source of variability from cell cultures for plant improvement. *Theor. Appl. Genet.* **60**: 197-214.

Lee, E., and Surh, Y. J. 1998. Induction of apoptosis in HL-60 cells by pungent vanilloids, [6]-gingerol and [6]-paradol. *Cancer. Lett.***134:** 163-168.

Lee, H. S., Seo, E. Y., Kang, N. E., Kim, W. K. 2008. [6]-Gingerol inhibits metastasis of MDA-MB-231human breast cancer cells. *J. Nutr. Biochem.* **19:** 313–319.

Lengyel, E., Prechtel, D., Resau, J. H., Gauger, K., Welk, A., Lindemann, K. 2005. C-Met overexpression in node-positive breast cancer identifies patients with poor clinical outcome independent of Her2/neu. *Int. J. Cancer*. **113:** 678-682.

Lewis, Y. S., Mathew, A. G., Nambudin, E. S., Krishnamarthy, N. 1972. Ginger Flavour. *J. Ind. Chem*. **3:** 78-79.

Li, A., Zhou1, S., Li, M., Xu, Y., Shui, R., Yu, B., and Yang, W. 2015. clinicopathologic characteristics of oestrogen receptor-positive/progesterone Receptor-Negative/Her2-Negative breast cancer according to a Novel definition of Negative progesterone receptor status: A large population-based study from China. *Plos one.* | DOI:10.1371/journal.pone.0125067

Li, J., Jia, H., Xie, L., Wang, X., He, H., Lin, Y., and Hu L. 2009. Association of constitutive nuclear factor-kappaB activation with aggressive aspects and poor prognosis in cervical cancer. *Int. J. Gynecol. Cancer*. **19(8):** 1421

Lin, C. B., Lin, C. C., and Tsay, G. J. 2012. 6-Gingerol Inhibits Growth of Colon Cancer Cell LoVo via Induction of G2/M Arrest. *Evid. Complement. Alternat. Med.* 326096.

Ling, H., Yang, H., Tan, S. H., Chui, W. K., and Chew, E. H. 2010. 6-Shogaol, an active constituent of ginger, inhibits breast cancer cell invasion by reducing matrix metalloproteinase-9 expression via blockade of nuclear factor-κB activation. *Br. J. Pharm.* **161:** 1763-77.

Lipinski, C. A., Lombardo, F., Dominy, B. W., Feeney, P. J. 1997. Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. *Adv. Drug. Deliv. Rev*. **23:** 3–25.

Liu, J., Qu, X., Xu, L., Zhang, Y., Qu, J., Hou, K., and Liu, Y. 2010. Phosphoinositide 3-kinase/Akt and nuclear factor κB pathways are involved in tumor necrosis factor-related apoptosis-inducing ligand resistance in human gastric cancer cells. *Mol. Med. Rep.* **3:** 491-496.

Liu, N., Furukawa, T., Kobari, M. and Tsao, M.S. 2011. Comparative phenotypic studies of duct epithelial cell lines derived from normal human pancreas and pancreatic carcinoma. *Am. J. Pathol.* **153:** 263-269.

Lorenzo, G. D., Tortora, G., Armiento, F. P. D., Rosa, G. D., Staibano, S., Autorino, R., Armiento, M. D., Laurentiis, M. D., Placido, S. D., Catalano, G., Bianco, A. R. and Ciardiello, F. 2002. Expression of Epidermal Growth Factor Receptor Correlates with Disease Relapse and Progression to Androgen-independence in Human Prostate Cancer. *Clinical Cancer Research*. **8:** 3438–3444.

Ludwig, J. A. and Weinstein, J. N. 2005. Biomarkers in cancer staging, prognosis and treatment selection. *Nat. Rev. Cancer*. **5(11):** 845-856.

Ma, X. and Gang, D. R. 2006. Metabolic profiling of in vitro micropropagated and conventionally greenhouse grown ginger (*Zingiber officinale*). *Epub.* **67(20):** 2239-55.

Mahto, K, M., Meraj, K., Eftekhari, K., Zeinab, N., Poojitha, K., and Bhaskar, M. 2013 Molecular Docking And ADMET Study Of Quinoline---based Derivatives for Anti---Cancer Activity. *VRI Bioinfo & Proteo.* **1** : 1-8.

Mathai, C. K. 1972. Variability in turmeric germplasm for essential oil and curcumin. *Pl. Foods. Human Nutr.* **25:** 227-230.

McNay, J. W., Chourey, P. and Pring, D. R. 1984. *Theor. Appl. Genet.* **67:** 433-437.

Mendelsohn, J. and Baselga, J. 2006. Epidermal Growth Factor Receptor Targeting in Cancer. *Oncology*. **33(4)**: 369–385.

Mishra, R. K., Kumar, A. and Kumar, A. 2012. Pharmacological Activity of *Zingiber Officinale. Int. J. Pharma. Chem. Sci.* **1(3):** 1073.

Munagala, R., Aqil, F., and. Gupta. R.C. 2011. Promising molecular targeted therapies in breast cancer. *Indian. J. Pharmacol*. **43(3):** 236–245.

Muralidharan, A. and Balakrishnan, S. 1973. Studies on the performance of some varieties of turmeric and its fertilizer requirements. Agricultural Research Journal of Kerela **10:** 112-115.

Nair, P. C. S. 1975. Agronomy of ginger and turmeric. Proceedings of the national seminar on ginger and turmeric, Calicut, April 8-9, 1980. Central Plantation Crops Research Institute, Kasargod, 63-68pp.

Natarajan, C. P., Bai, R. P., Krishnamurthy, M. N., Raghawan, B., Shankaracharya, N. B., Kuppuswamy, S., Govinbarajan, V. S. ans Lewis, Y. S. 1972. Chemical composition of ginger varieties and dehydration studies on ginger. *India. J. Fd. Sci. Technol.* **9:** 120-124.

Nazeem, P. A., Nair, L. S., Mohan, M., and Keshavachandran, R. 2014. *IN SILICO* Docking studies of phytocompounds on PI3K/NF-KB mediated signalling pathway. *Int. J. Pharm. Bio. Sci*. **5(1):** 721 – 729.

Nybe, E. V. 1978. Quality variation of ginger at different period of maturity. Proc. Seminar on post-harvest Technology. Kerala Agricultural University, Vellanikkara, India.

Ooi, A., Wong, J. C., Petillo, D., Roossien, D., Trudova, V. P., Whitten, D., Min, B. W., Tan, M. H., Zhang, Z., Yang, X. J. 2004. An antioxidant response phenotype shared between hereditary and sporadic type 2 papillary renal cell carcinoma. *Cancer. Cell.* **20:** 511–523.

Pal, S., Bhattacharjee, A., Ali, A., Mandal, N. C., Mandal, S. C., and Pal, M. 2014. Chronic inflammation and cancer: potential chemoprevention through nuclear factor kappa B and p53 mutual antagonism. *J. Inflammation.* **11:** 23.

Park, D., **Yun**, K., Park, H, J., Kim, j, H., Shon, C., Jeon, K, W., Kim, I, B., and Yoo, H, C. 2006. HER-2 /neu Amplification Is an Independent Prognostic Factor in Gastric Cancer. *Digestive Diseases and Sciences*. **51**: 1371-1379.

Parthasarathy, V. A., Chempakam, B. & Zachariah, T. J. 2008. Chemistry of Spices. Pp45.

Paul, R, Shylaja, M. R, Abraham, K. and Nybe, E.V. 2011. Screening somaclones of ginger for soft rot disease. *J. Mycopathol. Res*. **49(1):** 513-517.

Paul, R. 2006. Induction of variation *in vitro* and field evaluation of somaclones in ginger. Ph.D.thesis, Kerala Agricultural University, Vellanikkara, Thrissur, Kerala, India.

Paul, R. and Shylaja, M. R. 2009. Production of toxic metabolites in cultures by Pythium aphanidermatum andRalstonia solacearum and bioassay of metabolites. *J. Mycol. Pl. Path.* 39pp.

Paul, R. and Shylaja, M. R. 2012. Preliminary screening in regenerants of ginger (Zingiber officinale Rosc.)derived through gamma irradiation of embryogenic cultures to soft rot and bacterial wilt diseases. *J. Mycol. Pl. Path.* **41(4):** 592-596.

Paul, R., Shylaja, M.R. and Abraham, K. 2009. Screening somaclones of ginger for bacterial wilt disease. *Indian Phytopathology*. 62(4).

Paul, R., Shylaja, M.R., Abraham, K. and Nybe, E.V. 2011. Screening somaclones of ginger for soft rot disease. *J. Mycopath. Res.*49(1): 513-517.

Pawar, M., Jadhav, A, P., and Kadam, V, J. 2015. In-vitro antioxidant activity of selected indian medicinal plants. *IJPR*. 5(2):816-824.

Pawar, N., Pai, S., Nimbalkar, M., Dixit, G., 2011. RP-HPLC analysis of phenolic antioxidant compound 6-gingerol from different ginger cultivars. Food. Chemistry. **126(3):** 1330–1336.

PDB [Protein Data Bank]. 2015. PDB home page [on line]. Available: http:// http://www.rcsb.org/pdb/home/home.do.[25 june 2015]

Prasad, S. and Tyagi, A.K. 2015. Ginger and Its Constituents: Role in Prevention and Treatment of Gastrointestinal Cancer. Hindawi Publishing Corporation Gastroenterology Research and Practice Volume 2015, Article ID 142979, 11 pages http://dx.doi.org/10.1155/2015/142979

Pujaita, G. 2013. DNA fingerprinting of released varieties and selected superior somaclones of ginger (*Zingiber officinale* Rosc.). M. Sc. (Ag) thesis, Kerala Agriculture University, Thrissur, 69p.

Radhakrishnan, E. K., Bava, S. V., Narayanan, S. S., Nath, L. R., and Thulasidasan, A. K. T. 2014. [6]-Gingerol Induces Caspase-Dependent Apoptosis and Prevents PMA-Induced Proliferation in Colon Cancer Cells by Inhibiting MAPK/AP-1 Signaling. PLoS ONE 9(8): e104401. doi:10.1371/journal.pone.0104401

Rahmani, A. H., shabrmi, F. M. A. and Aly, S. M. 2014. Active ingredients of ginger as potential candidates in the prevention and treatment of diseases via modulation of biological activities. *Int J Physiol Pathophysiol Pharmacol*. **6(2):** 125-136.

Rao, Y. S., Mathew, K. M., Lakshnian, R., Potty, S. N. and George, C. K. 2000. Improvement of tissue culture technique and field evaluation of ginger. Recent Advances in Plantation Crops Research, pp. 24-17.

Ravindra, N. S., Kulkarni, R. N., Gayathri, M. C., and Ramesh, S. 2004. Somaclonal variation for some morphological traits, herb yield, essential oil content and essential oil composition in an Indian cultivar of rose-scented geranium. *Plant. Breed.* **123:** 84–86.

Reeve, J. G., Stewart, j., Watson, J. V., Wulfrank, D., Twentyman, P. R., and Bleehen, N. M. 1986. Neuron specific enolase expression in carcinoma of the lung. *Br. J. Cancer*. **53:** 519-528.

Rhode, J., Fogoros, S., Zick, S., Heather, W., Griffith, K.A., Huang, J., and Rebecca Liu, J. 2007. Ginger inhibits cell growth and modulates angiogenic factors in ovarian cancer cells. *BMC Complementary and Alternative Medicine*. **7:** 44.

Saha, A., Blando, J., Silver, E., Beltran, L., Sessler, J., and DiGiovanni, J. 2014. 6-Shogaol from Dried Ginger Inhibits Growth of Prostate Cancer Cells Both In Vitro and In Vivo through Inhibition of STAT3 and NF-kB Signaling. Am. Association for Cancer Res. DOI: 10.1158/1940-6207.CAPR-13-0420.

Salmon, H., Franciszkiewicz , K., Damotte, D., -Nosjean, C, M., Validire, p., Trautoman, A., and Donnadiue, E. 2012. Matrix architecture defines the preferential localization and migration of T cells into the stroma of human lung tumors. J Clin Invest. 122(3): 899–910.

Sanchu, C. R. 2000. Variability analysis in calliclones of black Peper (Peper nigrum L.) M.Sc thesis, Kerala Agricultural Universitv, thrissur, 96p.

Sanwal, S. K., Rai, N., Singh, J. and Buragohain, J. 2010. Antioxidant phytochemicals and gingerol content in diploid and tetraploid clones of ginger (*Zingiber officinale* Roscoe). *Sci. Horti.* **124:** 280-285.

Saptarini, N. M., Sitorus, E. Y. and Levita, J. 2013. Structure-Based in Silico Study of 6-Gingerol, 6-Ghogaol, and 6-Paradol, Active Compounds of Ginger (*Zingiber officinale*) as COX-2 Inhibitors. *Int. J. Chem.* **5(3):** 12-18.

Sawada, K., Radjabi, A. R., Shinomiya, N., Kistner, E., Kenny, H., and Becker, A. R. 2007. c-Met Overexpression Is a Prognostic Factor in Ovarian Cancer and an Effective Target for Inhibition of Peritoneal Dissemination and Invasion. *Cancer. Res*. **67(4):** 1670-1679.

Saxena, G., Rahman, L, u., Vermac, P, C., Banerjee, s., kumar, s. 2008. Field performance of somaclones of rose scented geranium (*Pelargonium graveolens* L'Her Ex Ait.) for evaluation of their essential oil yield and composition. Industrial crops and products. 2 7: 86–90.

Schwertner, H. A. and Rios, D. C. 2007. High-performance liquid chromatographic analysis of 6-gingerol, 8-gingerol, 10-gingerol, and 6-shogaol in ginger–containing dietary supplements, spices, teas, and beverages. J. Chromatography. **856:** 41-47.

Sekiwa, Y., Kubota, K. and Kobayashi, A. 2000. Isolation of novel glucosides related to gingerdiol from ginger and their antioxidative activities. J. Agric. Food. Chem. **48:** 373–377.

Sepporta, M. V., Tumminello, F. M., Flandina, C., Crescimanno, M., Giammanco, M., Guardia, M. L., Majo, D. D. and Leto, G. 2013. Follistatin as potential therapeutic target in prostate cancer. *Targ Oncol*. **8:** 215-223.

Sevcik, M. A., Ghilardi, J. R., Peters, C. M., Lindsay, T. H., Halvorson, K. G., Jonas, B. M. 2012. Anti-NGF therapy profoundly reduces bone cancer pain and the accompanying increase in markers of peripheral and central sensitization. *Pain.* **115:** 128-141.

Shankar, K. G., Fleming, A. T., Vidhya, R. and Nirmal S. 2013. In-silico molecular docking analysis of cancer biomarkers with bioactive compounds of *Tribulus terrestris.* International Journal of Novel Trends in Pharmaceutical Sciences. **3(4):** 111-117.

Sharda, G. S. 2013. Variability analysis in ginger (*Zingiber officinale* Rosc.) somaclones using molecular markers. M. Sc. (Ag) thesis, Kerala Agriculture University, Thrissur, 68p.

Shoemaker, R. C., Palmer, R. G., Oglesby, L. and Rauch, J. P. 1991. Effect of 2, 4-dichlorophenoxy acetic acid concentration on somatic embryogenesis and heritable variation in soyabean (*Glycine max* L. Mer. R.). *In Vitro Cell Dev. Biol.* **27:** 84-88.

Shrivastava, S. R. B. L., Shrivastava, P. S. and Shrivastava, J. R. 2013. Role of self-care in management of diabetes mellitus. J. Diabetes. Metabolic. Disorders. 12-14pp.

Shruthy, V. S. and Shakkeela, Y. 2014. *In silico* design, docking, synthesis and evaluation of thiazole schiff bases. *International Journal of Pharmacy and Pharmaceutical Sciences*. **6(3):** 271-275.

Shukla, Y. and Singh, M. 2007. Cancer preventive properties of ginger. A brief review. Food. Chem. Toxicol. **45:** 683–690.

Shylaja, M. R. 2010. Onfarm evaluation and characterization of somaclones in ginger (Ginger officinale Rose.)., project completion report submitted to KAU and DBT.

Shylaja, M. R. Paul, R., Nybe, E. V., Abraham, K., Nazeem, P. A, Valsala, P. A. and Krishnan, S. 2010. Two new ginger varieties from Kerala Agricultural University. *Indian. J. Arecanut. Spices. Med. Pl.* **12(2):** 3-4.

Shylaja, M. R., Nybe, E. V., Nazeem, P. A., Mathew, S. K., Krishnan, S and Paul, R. 2014. Aswathy, a new ginger variety from Kerala Agricultural University for green ginger. Indian J. of Arecanut spices and Medicinal Plants. **16 (2**): 18-20.

Skirvin, R. M., Mcpheeters, K. D. and Norton, M. 1994. Sources and frequency of somaclonal variation. *Hort. Sci*. **29:** 1232-1237.

Slamon, D. J., Clark, G. M., Wong, S. G., Levin, W. J., Ullrich, A., and McGuire, W. L. 1987. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. *Sci*. **235(4785):** 177-182.

Smith, M. K. and Drew, R. A. 1990. Current applications of tissue culture in plant propagation and improvement. *Aust. J. Plant. Physiol.* **17:** 267-289.

Spice Board. 2014. *Export of spices [online]*. Available: http://www.indianspices.com/pdf/spicewisearprd.xls. [20 July 2015].

Spice Board. 2014. *Spice Wise Area and Production [online]*. Available: http://www.indianspices.com/pdf/spicewisearprd.xls. [20 July 2013].

Subbaramaiah, K., and Dannenberg, A. J. 2003. Cyclooxygenase 2: a molecular target for cancer prevention and treatment. *Trends. Pharmacol. Sci*. **24(2):** 96–102.

Sudharshan, M. R. and Sreekrishna, S. B. 1998. Tissue cultured cardamom clones: A comparative study. Develop. Plantation. Crops. Research. Pp. 73-76.

T. J. Orton. 1984. Somaclonal Variation: Theoretical and Practical Considerations. *Gene manipulation in plant improvement.* Pp. 427-468.

Tsao, M. S., Liu, N., Chen, J. R., Pappas, J., and Ho, J., 1998. Differential expression of Met/hepatocyte growth factor receptor in subtypes of non-small cell lung cancers. *Lung. Cancer.* **20:** 1-16.

Valerio, L. G., Arvidson, K. B., Chanderbhan, R. F. and Contrera, J. F. 2009. Prediction of rodent carcinogenic potential of naturally occurring chemicals in the human diet using highthroughput QSAR predictive modeling. *Toxicol. Appl. Pharmacol*. **22(1):** 204-210.

Wang, C. C., Chen, L. G., Lee, L. T., and Yang, L. L. 2001. Effects of 6-gingerol, an antioxidant from ginger, on inducing apoptosis in human leukemic HL-60 cells. *In Vivo*. **17:** 641-645.

Wang, Y. D., Zhang, L., Cai, G. Y., Zhang, X. G., Lv, Y., Hong, Q., Shi, S. Z., Yin, Z., Liu, X. F., and Chen, X. M. 2011. Fasudi ameliorates rhabdomyolysis-induced acute kidney injury via inhibition of apoptosis. *Ren. Fail.* 33: 811-818.

Wei, Q. Y., Ma, J. P., Cai, Y. J., Yang, L., Liu, Z. L., 2005. Cytotoxic and apoptotic activities of diarylheptanoids and gingerol-related compounds from the rhizome of Chinese ginger. *J. Ethnopharmacol.* **102:** 177–184.

Yip, P. Y. 2015. Phosphatidylinositol 3-kinase-AKT-mammalian target of rapamycin (PI3K-Akt-mTOR) signaling pathway in non-small cell lung cancer. *Transl. Lung. Cancer. Res.* **4(2):** 165-176.

Yip, P. Y., Cooper, W. A., Kohonen-Corish, M. R. 2014. Phosphorylated Akt expression is a prognostic marker in early-stage non-small cell lung cancer. *J. Clin. Pathol.* **67:** 333-340.

Young, H. Y., Luo, Y. L., Cheng, H. Y., Hsieh, W. C., Liao, J. C., Peng, and W. H. 2005. Analgesic and anti-inflammatory activities of [6]- gingerol. J. Ethnopharmacol. **96:** 207–210.

Yu, J. W., Wu, S. H., Lu, R. Q., Wu, J. G. 2013. Expression and significances of contactin-1 in human gastric cancer. *Gastroenterol. Res. Pract.* **21:** 2-5.

Yudthavorasit, S., Wongravee, K. and Leepipatpiboon, N. 2014.Characteristic fingerprint based on gingrol derivative analysis for discrimination of ginger (*Zingiber officinale*) according to geographical origin using HPLC-DAD conbined with chemometrics. *Food. Chem.* 158: 101-111.

Zhang, Y. W., Eom, S., Kim, Y. D., Song, Y. J., Yun, H. Y., and Park, J. S. 2011. Effects of dietary factors and the NAT2 acetylator status on gastric cancer in Koreans. *Int. J. Cancer*. **125:** 139–145.

Zick, S. M., Ruffin, M. T., Djuric, Z., Normolle, D., and Brenner, D. E. 2010. Quantitation of 6-, 8- and 10-gingerols and 6-shogaol in human plasma by high-performance liquid chromatography with electrochemical detection. *Int. J. Biomed. Sci.* **6:** 233–240.

Zuo, L., Weger, J., Yang, Q., Goldstein, A. M., Tucker, M. A., Walker, G. J., Hayward, N., and Dracopoli, N. C. 1996. Germline mutations in the p16INK4a binding domain of CDK4 in familial melanoma. *Nat. Genet*. **12(1):** 97-99.

Annexures

ANNEXURE I

List of laboratory equipment/ software used for the studies

Soxhlet apparatus	:	Rotex
HPLC with LC solution software with PDA dector	:	Shimadzu LC20AD
Sonicator	:	PCI, Analytics
Discovery studio 4.0	:	Accelry, USA
Laminar air flow	:	Labline industries, Kochi
Shaker incubator	:	JEIO Tceh, Korea
ELISA reader	:	VERSA max, USA

ANNEXURE II

Chemical composition of medium used for cell culture studies

Reagents:

1. RPMI media - 1640

2. FBS – 10 %

3. Glucose - 4.5g/ lit

4. HEPES buffer - 10ml/ lit

5. Sodium pyruvate – 10ml/ lit

6. Penicilium and Streptomycin – 1%

Composition of Phosphate Buffer Saline (PBS)

1. Nacl - 8g

2. $Na_2HPo_4.2H_2o$ - 1.44g

3. KH_2Po_4 - 0.2g

4. Kcl - 0.2g

5. Distil water - 1000ml

ANNEXURE III

Composition of RPMI -1640 medium

Component	g/ l
Inorganic Salts	
Ca(NO3)2* 4H2O	0.1
MgSO4 (anhydrous)	0.04884
KCl	0.4
NaHCO3	2
NaCl	6
Na2HPO4 (Anhydrous)	0.8
L-Arginine * HCl	0.2
L-Asparagine	0.05
L-Aspartic Acid	0.02
L-Cystine * 2HCl * H2O	0.0652
L-Glutamic Acid	0.02
L-Glutamine	0.3
Glycine	0.01
L-Histidine * HCl * H2O	0.015
Hydroxy-L-Proline	0.02
L-Isoleucine	0.05
L-Leucine	0.05
L-Lysine * HCl	0.04
L-Methionine	0.015
L-Phenylalanine	0.015
L-Proline	0.02

L-Serine	0.03
L-Threonine	0.02
L-Tryptophan	0.005
L-Tyrosine	0.02184
L-Valine	0.02
D-Biotin	0.0002
Choline Chloride	0.003
Folic Acid	0.001
myo-Inositol	0.035
Niacinamide	0.001
p-Aminobenzoic Acid	0.001
D-Pantothenic Acid * ½Ca	0.00025
Pyridoxine * HCl	0.001
Riboflavin	0.0002
Thiamine * HCl	0.001
Vitamin B12	0.000005
Other	
D-Glucose	2
Glutathione (reduced)	0.001
Phenol Red * Na	0.0053

ANNEXURE IV

Details of selected ginger somaclones

Sl. No.	Groups of somaclones clones	Somaclones code
1	MB	M 278
2		M 271
3		M 204
4		B 24
5		B 13
6		668 M
7		79 M
8		99 M
9		918 M
10		436 M
11	MC	Mc 263
12		Mc 270
13		Mc 545
14		MC 262
15		Mc 338
16		Mc 297
17		Mc 314
18		Mc 320
19	MC 10Gy	Mc 10Gy 624
20		Mc 10Gy 47
21		Mc 10Gy 774
22		Mc 10Gy 110
23		Mc 10Gy 112
24		Mc 10Gy 322
25		Mc 10Gy 330
26		Mc 10Gy 102
27		Mc 10Gy 168
28		Mc 10Gy 138
29		Mc 10Gy 190
30		Mc 10Gy 1064
31		Mse 1074

	M Se	
32		Mse 19
33		Mse 8
34		Mse 21
35		Mse 24
36		Mse 27
37	M Se 10Gy	Mse 10Gy 42
38		Mse 10Gy 584
39	M Se 20Gy	Mse 20Gy 164
40		Mse 20Gy 239
41		Mse 20Gy 418
42		Mse 20Gy 246
43		Mse 20Gy 536
44		Mse 20Gy 1351
45		Mse 20Gy 383
46		Mse 20Gy 178
47		Mse 20Gy 260
48		Mse 20Gy 426
49		Mse 20Gy 175
50		Mse 20Gy 862
51		Control MVKA

SCREENNG GINGER (*Zingiber officinale* Rosc.) SOMACLONES FOR GINGEROL CONTENT AND VALIDATION OF ANTICANCEROUS PROPERTIES OF GINGEROL

By

MANIKESH KUMAR
(2013-11-102)

ABSTRACT OF THE THESIS

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(Plant Biotechnology)

Faculty of Agriculture

Kerala Agricultural University, Thrissur

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Abstract

ABSTRACT

Ginger (*Zingiber officinale* Rosc.) is an important spice crop with immense medicinal properties and health beneficial effects. Various bioactive compounds present in ginger are responsible for the medicinal properties. Gingerols are the most pharmacologically active compounds in ginger and posses anti-inflammatory, analgesic, antipyretic, gastro protective, cardiotonic and antihepatotoxic activities. Of the different gingerols, the most potent and pharmacologically bioactive compound is 6-gingerol and is now a target for drug development. Centre for Plant Biotechnoloy and Molecular Biology maintains a good collection of germplasm of ginger somaclones regenerated through direct/ indirect of organogenesis/ embryogenesis and *in vitro* mutagenesis.

The investigations on "Screening ginger (*Zingiber officinale* Rosc.) somaclones for gingerol content and validation of anticancerous properties of gingerol" were carried out at Centre for Plant Biotechnology and Molecular Biology and Distributed Information Centre, College of Horticulture, Kerala Agricultural University, Thrissur during August 2013 to June 2015. The objectives of the study were to screen ginger somaclones for gingerol content, to identify cancer targets for gingerols and shogoal using *in silico* tools and to validate anticancerous properties of gingerol.

Fifty somaclones of ginger derived from the cultivar Maran regenerated through direct /indirect methods of regeneration and *in vitro* mutagenesis were raised in field during 2013-14 season. Dry ginger was prepared from the harvested clones and oleoresin was extracted by solvent extraction. The content of pungent principles in fifty ginger somaclones were estimated using High Performance Liquid Chromatography. Accelry Discovery studio 4.0 software was used for molecular docking of four ginger ligands viz. 6-gingerol, 8-gingerol, 10-gingerol and 6–shogaol and other approved drugs with the selected targets for cancer. Different cell lines obtained from Amala Cancer Research Centre, Thrissur viz. HCT15 (colon cancer),

Raw 264.7 (mouse leukaemic monocyte macrophage) and L929 (murine fibro sarcoma) were used to study the anticancerous properties of 6-gingerol.

The somaclones studied showed variability in the content of pungency principles like 6-gingerol (3.33-13.83g/kg dry ginger powder), 8-gingerol (0.2 - 0.94g/kg), 10-gingerol (0.17-1.15g/kg) and 6-shogaol (0.61 - 2.33 g/kg) . Somaclones regenerated after *in vitro* mutagenesis recorded higher content of gingerol. Principle Component Analysis done for clustering somaclones based on quality parameters could locate five high gingerol yielding somaclones viz. Mse 20Gy 418, Mse 20Gy 175, Mse 20Gy 862, Mc 10Gy 330 and Mc 10Gy 168.

Molecular docking was attempted with four ginger ligands and eleven approved drugs with thirteen targets.In the molecular docking studies, ginger ligands showed good interactions with some of the cancer targets selected for all types of cancer. The Difference between C-Docker interaction energy and C-Docker energy was found minimum for the target c-Met (2.287) when 6-gingerol was docked. Minimum binding energy was recorded for 6-gingerol with targets Activator protein-1 (-138.2092) and Epidermal Growth Factor Receptor (-107.9914). Similary, for 8-gingerol, minimum binding energy was recorded with Activator protein-1 (-140.5949) and Oestrogen receptor (-100.0471), for 10-gingerol with Epidermal Growth Factor Receptor (-131.1699) and Ribosomal S6 kinase (-102.6721) and for 6-shogaol with Activator protein-1 (-117.683) and Epidermal Growth Factor Receptor (-107.9644). The maximum number of hydrogen bonds (5) for 6-gingerol were recorded with target follistatin. Studies on ADME/Toxicity properties showed that 6-gingerol was superior with respect to absorption, solubility and less neurotoxic effect as compared to other ginger ligands and approved drugs. Based on the results of docking and ADME/ Toxicity properties, 6-gingerol was selected for cell culture studies to validate the anticancerous properties.

The phytocompound 6-gingerol was found cytotoxic to all the three cancer cells lines studied. The cytotoxicity increased with increase in concentration of 6-

gingerol. The IC50 values recorded for different cancer cell lines, 24 h. after treatment (100 μM for HCT15, 102 μM for L929 and 102 μM for Raw 264.7) showed the uniform cytotoxicity in the three cell lines studied.

The investigations paved way to locate high gingerol yielding somaclones, to prove the effectiveness of 6-gingerol as an anticancerous phytochemical through molecular docking and cell culture studies and to highlight the potential of 6-gingerol for drug development. The study also gave an insight into the use of pungent phenolic compounds studied in ginger for other medicinal applications.