CHAPTER-1

Molecular Documentation and Evaluation of Medicinal Potential in Ginger
INTRODUCTION
General morphology of ginger plant:

Ginger is one of the earliest known oriental spices and is being cultivated in India both as a fresh vegetable and as a dried spice since time immemorial. Ginger is obtained from the rhizomes of *Zingiber officinale*. The word ginger is derived from a Sanskrit word *singabera* meaning 'shaped like a deer's antlers (horn).

Ginger is a slender herbaceous perennial herb belonging to Zingiberaceae family. It forms a spreading, tuberous, underground stem or rhizome. The plant produces erect, tall and dark green leafy shoots (pseudostems) 30-100 cm of high. The aerial pseudostems usually bear 8-12 distichous leaves. Leaves are alternate, lanceolate or linear-lanceolate, acute, smooth and with subsessile sheathing. They are 5-25 cm long and 1-3 cm wide. There is a broad, thin, glabrous ligule (about 5 mm long) and slightly bilobed.

Flowers are borne in a bracteal, imbricated spike, terminating in a leafless stem reaching to about 15-25 cm in height. They are situated in the axils of large, greenish-yellow obtuse bracts. They are small, frail, short lived, very a few, and usually arising one or two at a time. The evanescent flowers are yellowish and speckled with a purplish lip. Calyx is thin, tubular, spathaceous, 1-1.2 cm long and is three-toothed. Corolla tube is 2-2.5 cm long with three lobes. The dorsal lobe is 1.5-2.5 cm long, 8 mm wide and is curved over the anther and narrowed to the tip. The labellum or lip corresponds to three stamens, is nearly circular, dull purple with cream blotches at base. The perfect stamen has a short filament; the anther is cream colored and is prolonged into a beak-like
appendage. The rhizome, protruding just below the apex of the appendage, has a circular apical aperture surrounded by stiff hairs. There are two slender free styloids. The inferior ovary is trilobular with several ovules per lobule on axile placentation. Fruit is an oblong, thin walled, three-valved capsule but is rarely produced. The seeds are small, black and arillate. Rhizomes are fleshy sympodial, hard and thick, laterally compressed, often palmately branched with about 1.5-2.5 cm in diameter. The inner core is usually pale yellow while the outer is light yellow. They are covered with small distichous scales with an encircling insertion and fine fibrous roots in the top layer.

❖ Distribution:

The family Zingiberaceae consists of about 53 genera and more than 1200 species, distributed mainly in tropics and subtropics with the centre of distribution in the Indo-Malayan region, but extending through tropical Africa to Central and South America (Figure-1.1) (Kress et al., 2002). Ginger is believed to have originated in India or Southeast Asia and introduced to Europe by Arab traders (Mabberley, 1997 and Ravindran et al., 1994). Schumann, (1904) recognized the family Zingiberaceae as having 38 genera and 800 species, whereas, Bailey (1949) recognized 40 genera and 400 species, however, Dahlgren et al. (1985) categorized it in 50 genera and about 1000 species. Since flowering is rare and no viable seeds are produced; its propagation occurs naturally by rhizomes; for cultivation rhizome pieces having three to four buds (referred to as ‘seeds’ are used (Ikeda and Tanabe, 1989 and Malamug et al., 1991) It was well known in England as early as the 11th century and had become a major item of the spice trade in the 13th and 14th centuries (Evans, 2002 and Sutamo et al., 1999). Today, ginger is cultivated in many tropical and subtropical areas and the
main producers are India, China, Indonesia, and Nigeria (Sutarno et al., 1999). An estimated 40% of the world's confectionary ginger is grown in relatively small areas of eastern Queensland, Australia (Smith and Hamill, 1996). In India it is indigenous in Malabar coasts of Kerala. This plant is found wild in the western ghat region, but mostly cultivated in large scales in warm and moist regions mainly around Chennai, Cochin, Himachal Pradesh, Meghalaya, Orissa, Uttar Pradesh (Nainital), and West Bengal including Darjeeling hills. In addition to availability under cultivation, large populations of these plants are also available as land races growing in Eastern and North-Eastern India, a region representing one of the biodiversity hot spots of the world.

Figure 1.1- Global distribution of Zingiber sp
Traditional Uses of ginger:

Ginger is extensively used around the world in foods as a spice. For centuries, it has been an important ingredient in Chinese, Ayurvedic and Tibb-Unani herbal medicines. Ginger is as popular a home remedy in India today, as it was 2,000 years ago (Awang 1992, Bisset and Witchal 1994). In India the fresh and dried roots were measured distinct medicinal products. Fresh ginger has been used for cold-induced disease, nausea, asthma, cough, colic, heart palpitation, swellings, dyspepsia, loss of appetite, and rheumatism. To dispel nausea, fresh ginger was mixed with a little honey, topped off with a nip of burnt peacock feathers. One modern government health guide in India suggests 1-2 teaspoons of ginger juice with honey as a cough suppressant. A glue of powdered dried ginger was applied to mitigate headache.

The rhizomes of ginger are used as spice in food and beverages and in traditional medicine as carminative, antipyrexia and in treatment of waist pain rheumatism and bronchitis. It is also used for the treatment of gastrointestinal disorders and piles.
Chemistry:

The pungent taste of ginger is due to gingerols, an oily liquid consisting of homologous phenols. It is formed from phenylalanine, malonate and hexonate through phenylpropanoid pathway (Evans et al. 2002). The powdered rhizome contains 3-6% fatty oil, 9% protein, 60-70% carbohydrates, 3-8% crude fiber, about 8% ash, 9-12% water and 2-3% volatile oil. The volatile oil consists of mainly mono and sesquiterpenes; camphene, betaphellandrene, curcumene, cineole, geranyl acetate, terpineol, terpenes, borneol, geraniol, limonene, linalool, alpha-zingiberene (30-70%), sesquiterpenoids (β-sesquiphellandrene, bisabolene and famesene) and a small monoterpenoid fraction (β-phelladrene, cineol, and citral) have also been identified (McGee and Harold 2004). Amadaldehyde is a novel compound has been isolated from the ginger extract (Policegoudra et al. 2010). Other pungent principles of the rhizomes are paradols, gingerdiols, gingerdiacetates, gingerdiones, 6-gingersulfonic acid, gingerenones etc. It also contains acrid resinous substances (5-8%) (Ali.M 1998). Ginger contains up to three percent of a fragrant essential oil whose main constituents are sesquiterpenoids, with (-)-zingiberene as the main component. Smaller amounts of other sesquiterpenoids (β-sesquiphellandrene, bisabolene and famesene) and a small monoterpenoid fraction (β-phelladrene, cineol, and citral) have also been identified (McGee and Harold-2004). Amadaldehyde is a novel compound that has been isolated from the ginger extract (Policegoudra et al. 2010). Other pungent principles of the rhizomes are paradols, gingerdiols, gingerdiacetates, gingerdiones, 6-gingersulfonic acid, gingerenones etc. Other minor compounds are methylegingediol, gingeracetates, methylegingediacetates and C20 - dialdehyde (Evans et al 2002).
In the fresh ginger rhizome, the gingerols were identified as the major active components and 6-gingerol [5-hydroxy-1-(4-hydroxy-3-methoxy phenyl) decan-3-one] is the most abundant and medicinally most important constituent among them whereas dried ginger powder, shogaol, a dehydrated product of gingerol, is a predominant pungent constituent (Figure-1.2) (Awang 1992, Mustafa et al 1993, Kiuchi et al 1993).

![Chemical structure of two medicinally important compounds obtained from Ginger](image)

**Figure 1.2- Chemical structure of two medicinally important compounds obtained from Ginger**
Medicinal uses of ginger:

Ginger plants have been source of medicines as they are a reservoir of chemical agents having therapeutic properties. Herbal medicines are dietary supplements to relieve and treat many different human disorders and have been used for thousands of years to enhance the flavor, colour and aroma of food. In addition to boosting flavor, ginger is also known for their preservative and medicinal value (Nielsen, 2000). Yet it is only in recent years that modern science has started paying attention to the properties of this valuable spice.

- **Cold and Flu Prevention and Treatment**

Ginger contains nearly a dozen antiviral compounds. When nausea accompanies an illness, ginger root, warms chills, fights infection and alleviates stomach distress. Scientists have isolated several chemicals (sesquiterpenes) in ginger that have specific effects against the most common cold virus; the rhinoviruses. Some of these chemicals are remarkably potent in their anti-rhinovirus effects. Other constituents in ginger, gingerols and shogaols, help relieve cold symptoms because they reduce pain and fever, suppress coughing and have a mild sedative effect that encourages rest (Kalra et al, 2011)

- **Migraine Relief**

Ginger is reported in Ayurvedic and Tibb systems of medicine to be useful in neurological disorders. It is proposed that administration of ginger may exert abortive and prophylactic effects in migraine headache without any side-effects (Mustafa and
Srivastava, 1990). A case study presented ginger as a preventive agent for migraine headache. With 500-600 mg of powdered ginger mixed with water, the migraine headaches ceased within 30 minutes. In addition, after the cessation of the migraine attack, the subject did not experience any side effects (Muhammed and Prakash, 2007)

- **Powerful Antioxidant**

Antioxidants help to prevent all kinds of diseases and also slow down the aging process. A study of more than 120 plant foods, published in the Journal of Nutrition reported ginger was ranked number one among the five richest food sources of antioxidants, including berries, walnuts, sunflower seeds, and pomegranates. Test-tube and animal research has shown that ginger inhibits the production of free radicals. Ginger also enhances the body's internal production of antioxidants (Rehman et al, 2011)

- **Antiemetic**

Ginger has been shown to exert potent antiemetic properties and has been compared to standard drugs used in combating nausea and vomiting (Abdel-Aziz et al, 2006)

- **Morning Sickness**

A Double-blind randomised placebo-controlled trial of 120 women participants less than 20 weeks pregnant was conducted in March 1999–November 1999. The participants had experienced morning sickness daily for at least a week and had had no relief of symptoms through dietary changes. Random allocation of 125 mg ginger extract
(EV.EXT35; equivalent to 1.5 g of dried ginger) or placebo was given four times per day for 4 days. Nausea, vomiting and retching was measured by the Rhodes Index of Nausea, Vomiting and Retching. The nausea experience score was significantly less for the ginger extract group relative to the placebo group after the first day of treatment and this difference was present for each treatment day. Retching was also reduced by the ginger extract although to a lesser extent. No significant effect was observed on vomiting. Follow-up of the pregnancies revealed normal ranges of birth weight, gestational age, Apgar scores and frequencies of congenital abnormalities when the study group infants were compared to the general population of infants born at the Royal Hospital for Women for the year 1999–2000 (Willetts et al, 2003)

• **Motion Sickness**

One of the most famous reports on the effects of ginger on motion sickness was reported in the British medical journal The Lancet (Mowrey and Clayson, 1982). Motion sickness was induced by being subjected to a rotating, tilted chair while blindfolded under controlled conditions. It was found that ginger was significantly more effective in reducing motion sickness than the antihistamine dimenhydrinate and a placebo.

• **Relieves Chemotherapy related nausea**

A large study found that doses of half a gram to a gram of ginger daily could help relieve nausea in patients going through chemotherapy. The study showed that all doses of ginger relieved nausea significantly better than the placebo (Ryan et al, 2009)
- **Prevention of Nausea after gynecological laparoscopy**

Ginger has been used by Chinese people for gastrointestinal symptoms including nausea and vomiting. It has been reported to be as effective as metoclopramide and better than placebo for the prevention of postoperative nausea and vomiting after major gynecological surgery and day case gynecological laparoscopy (Visalyaputra et al 1998).

- **Anti-inflammatory**

The anti-inflammatory properties of ginger have been known and valued for centuries. During the past 25 years, many laboratories have provided scientific support for the long-held belief that ginger contains constituents with antiinflammatory properties. The original discovery of ginger's inhibitory effects on prostaglandin biosynthesis in the early 1970s has been repeatedly confirmed. This discovery identified ginger as an herbal medicinal product that shares pharmacological properties with non-steroidal anti-inflammatory drugs (Grzanna et al, 2005).

- **Rheumatism and Musculoskeletal disorders**

Ginger is described in Ayurvedic and Tibb systems of medicine to be useful in inflammation and rheumatism. It is suggested that at least one of the mechanisms by which ginger shows its ameliorative effects could be related to inhibition of prostaglandin and leukotriene biosynthesis, i.e. it works as a dual inhibitor of eicosanoid biosynthesis (Srivastava and Mustafa 1992).
• Manages the effects of Diabetic complications

A recent study into the hypoglycemic potentials of ginger was studied in rats. At a dose of 500 mg/kg, raw ginger was significantly effective in lowering serum glucose, cholesterol and triacylglycerol levels in the ginger-treated diabetic rats compared with the control diabetic rats. The ginger treatment also resulted in a significant reduction in urine protein levels. In addition, the ginger-treated diabetic rats sustained their initial weights during the treatment period. Moreover, ginger decreased both water intake and urine output in the STZ-induced diabetic rats. The present results indicate that raw ginger possesses hypoglycemic, hypocholesterolaemic and hypolipidaemic potential. Additionally, raw ginger is effective in reversing the diabetic proteinuria observed in the diabetic rats. Thus, ginger may be of great value in managing the effects of diabetic complications in human subjects (Al-Amin et al, 2006).

• Improves Circulation

Ginger has been found to be beneficial in reducing platelet aggregation which leads to coronary artery disease, while having no effect on blood lipids or blood sugar. Healthy people, patients with C.A.D.(coronary artery disease) and non-insulin dependent diabetes sufferers were all the subjects of an Indian study which found that a 10g single dose of powdered ginger, "significantly reduced platelet aggregation" in C.A.D. patients (Bordla et al, 1997)
• Colon Cancer prevention

A recent study investigated the effect of ginger on the initiation and post-initiation stages of 1,2-dimethylhydrazine (DMH)-induced colon carcinogenesis in male Wistar rats. In the presence of a known colon carcinogen, DMH, plasma lipid peroxidation (TBARS, lipid hydroperoxides and conjugated dienes) and cancer incidence were significantly increased whereas enzymic (GPx, GST, GR, SOD and CAT) and non-enzymic antioxidant concentrations (GSH, vitamins C, E, and A) were decreased as compared to control rats. The number of tumors as well as the incidence of cancer was significantly decreased on treatment with ginger. In addition, ginger supplementation at the initiation stage and also at the post-initiation stages of carcinogenesis significantly reduced circulating lipid peroxidation and significantly enhanced the enzymic and non-enzymic antioxidants as compared to unsupplemented DMH-treated rats. Ginger supplementation suppresses colon carcinogenesis in the presence of the procarcinogen DMH (Manju and Nalini 2005)

• Helps Manage Prostate Cancer

A recent study evaluated the effect of whole ginger extract in mice given human prostate cancer xenografts. It was found that whole ginger extract exerts significant growth-inhibitory and death-inductory effects in a spectrum of prostate cancer cells. (Kama et al, 2011).
Morphological Characteristics and medicinal importance of three studied species of Zingiber genus:

Zingiber officinale Roscoe:

- Morphology: Height up to 1.3 m. leaves sessile, up to c. 15 x 2(3) cm, linear-lanceolate, glabrous. Inflorescence on an up to 25 cm erects peduncle. Bracts green with a paler margin. Flowers are yellow with a purple, yellow-spotted labellum; anther dark purple. Rhizomes branched, yellowish inside, thickened, fleshy, and strongly aromatic. Pseudostems 50--100 cm. Leaves sessile; ligule slightly 2-lobed, 2–4 mm, membranous; leaf blade lanceolate or linear-lanceolate, 15–30 x 2–2.5 cm, glabrescent. Inflorescences arising from rhizomes, ovoid, 4–5 x ca. 1.5 cm; peduncle to 25 cm; bracts pale green, sometimes yellowish at margin, ovate, ca. 2.5 cm, apex mucronate; bracteoles equaling bracts. Calyx ca. 1 cm. Corolla yellowish green; tube 2–2.5 cm; lobes lanceolate, ca. 1.8 cm. Central lobe of labellum with purple stripe and cream blotches, oblong-ovate, shorter than corolla lobes; lateral lobes ovate, ca. 6 mm, free nearly to base.
Figure 1.3- Morphological features and rhizomes of *Zingiber officinale* Roscoe

- **Medicinal Importance:** *Zingiber officinale* Roscoe. is medicinally most important species of ginger and most of the therapeutic properties of ginger is due to presence of 6-gingerol which is only found in *Zingiber officinale*. The medicinal properties of *Z. officinale* are attributed to its spicy, pungent constituents, mainly gingerols, which stimulate the thermoregulatory receptors (Jancso-Gabor, 1980). Gingerols and shoagols possess a wide range of pharmacological and physiological effects, which include cardiovascular, gastro-intestinal (antiemetic, antinausea, antiulcer), antioxidant, anti-inflammatory, antimicrobial (analgesic, sedative, antipyretic, antibacterial), as well as thermogenic activities (Bone and Gupta, 1997 and Kikuzaki, 2000). Ginger has been shown to be effective against tumour growth, rheumatism and migraine and is active as an antioxidant (Schulick, 1993), hence ginger is an ingredient in various commercial natural products in emerging nutraceuticals and functional foods.
Z. officinale has been increasingly used recently because of its low toxicity and its broad spectrum of biological and pharmacological applications (Kadota et al., 2003; Kim et al., 2005; Kim et al., 2007; Kuhad et al., 2006; Lam et al., 2007; Lee et al., 2005; Sekiwa et al., 2000; Shukla and Singh, 2007; Surh, 2002; Wei et al., 2005; Yokosuka et al., 2002 and Young et al., 2005).

The anticancer effects of Z. officinale are thought to be attributed to various constituents including (6)-gingerol, (6)-paradol, shogaols, zingerone, and Galanals A and B (Suekawa et al. 1984). Ingenol and [6]-shogaol, isolated from ginger rhizome, demonstrated antiviral activity. (10)-gingerol has been reported as active inhibitor of M. avium and M. tuberculosis in vitro. Gingerol and related compounds have also been investigated for antimicrobial activities. [6]-gingerol and [12]-gingerol, isolated from ginger rhizome, demonstrated antibacterial activity against periodontal bacteria (Miri et al. 2008). In vitro research indicates that gingerols and related shogaols exhibit cardio depressant activity at low doses and cardiotonic properties at higher doses (Wang et al. 2003). Both (6)-shogaol and (6)-gingerol, are reportedly potent enzymatic inhibitors of prostaglandin, thromboxane, and leukotriene biosynthesis.

The antioxidant properties of Z. officinale have been shown in various investigations. The antioxidant activity of Z. officinale was shown to be as effective as vitamin C in lowering lipid peroxidation by influencing the enzymatic blood level of superoxide dismutase, catalase, and glutathione peroxidase (Ahmed et al. 2000). The lipid peroxidation lowering associated with ginger consumption was also demonstrated in apolipoprotein-E deficient mice (i.e., mice that are prone to develop atherosclerosis). A number of animal studies have shown that ginger lowers cholesterol levels.
Experimental animal data suggest a strong positive effect of *Z. officinale* on plasma lipid composition that may be important for the prevention of atherosclerotic events. The antioxidant action of *Zingiber officinale* has been proposed as one of the major possible mechanisms for the protective actions of the plant against toxicity and lethality of radiation (Jagetia *et al.*, 2003; Haksar *et al.*, 2006) and a number of toxic agents such as carbon tetrachloride and cisplatin (Amin and Hamza, 2006; Yemitan and Izegbu, 2006), and as an anti-ulcer drug (Siddaraju and Dharmesh, 2007). It has been shown that [6]-gingerol is endowed with strong anti-oxidant action both in vivo and in vitro, in addition to strong anti-inflammatory and anti-apoptotic actions (Kim *et al.*, 2007). This makes it a very effective agent for prevention of ultra violet B (UVB)-induced reactive oxygen species production and COX-2 expression, and a possible therapeutic agent against UVB-induced skin disorders.

❖ *Zingiber montanum* (J. Koeing) Link ex A. Dietr:

- **Morphology:** Rhizome thick, 1-2 cm, perennial, fleshy, horizontal, aromatic, light yellow inside. Leafy shoots 1 – 1.5 m high. Leaves subsessile; ligule very short, c. 2 mm long, pubescent; lamina 20 – 40 x 3 – 4 cm, linear-lanceolate, apex acute, base slightly rounded, upper surface glabrous, lower pubescent. Inflorescence borne separately from leafy shoot; peduncle 10-25 cm long, clothed with pubescent sheaths; spike 6 – 8 cm long, red. Bracts 3 – 3.5 x 3 – 3.6 cm, broadly ovate, subacute with narrow membranous margins, purplish brown, pubescent. Bracteoles 2 – 2.5 x 1.2 – 1.5 cm, obtuse, 3-toothed. Calyx c. 1.5 cm long, truncate, white,
membranous, unilaterally split, glabrous. Corolla 2.3 – 2.5 cm long, pale yellow; lobes lanceolate, pale yellow, dorsal lobe c. 3.2 x 1.5 cm, cymbiform; lateral lobes c. 2.5 x 1 cm, linear-lanceolate, reflexed. Labellum 3-lobed, ca. 2.5 cm wide, yellowish-white, suborbicular, apex emarginated with crisped margins. Lateral lobes c. 8 x 5 mm, obliquely obovate, erect. Filament short, anther-thecae c. 1 cm, crest longer than thecae. Style long, filiform; stigma obconical, margins ciliate. Epigynous glands linear, free from each other. Ovary 3 – 4 mm long, pubescent. Capsule c. 1.5 cm in dia., ovoid. Seeds very small, purple.

Figure 1.4- Morphological features and Rhizomes of *Zingiber montanum* (J. Koeing) Link ex A. Dietr
• **Medicinal Importance:** *Zingiber montanum* has been proven to be extremely useful for human health and thus developed into creams and massage oils for relieving muscle pain. A number of pure compounds isolated from the plants have been shown to possess antimicrobial (Wasuwat *et al.*, 1989), topical and oral anti-inflammatory (Kuroyanagi *et al.*, 1980; Tuntiwachwuttikul *et al.*, 1980, 1981; Panthong *et al.*, 1990; Ozaki *et al.*, 1991; Pongprayoon *et al.*, 1995, 1996; Panthong *et al.*, 1997; Jeenapongsan *et al.*, 2003), and antioxidative activity (Kuroyanagi *et al.*, 1980; Tuntiwachwuttikul *et al.*, 1980, 1981; Kanjanapothi *et al.*, 1987; Ozaki *et al.*, 1991; Pongprayoon *et al.*, 1995), as well as activity as a smooth-muscle relaxant (Kanjanapothi *et al.*, 1987). Terpinen-4-ol (24–32%) and sabinene (34–44%) were found as the major constituents of the rhizome oil of *Z. cassumunar* Roxb. and their antimicrobial activities were reported in comparison with the commercial terpinen-4-ol (Wasuwat *et al.*, 1989; Giwanon *et al.*, 2000). The rhizome oil of *Z. cassumunar* Roxb. from Malaysia was found to exhibit high activity against yeasts (Bin *et al.*, 2003).

**Zingiber zerumbet** (L.) Roscoe ex Sm:

• **Morphology:** *Zingiber zerumbet* Sm also known as the Shampoo Ginger is a vigorous ginger with leafy stems growing to about 1.2 m tall. It is a perennial, so from autumn until spring it goes dormant above ground as the leafy stems shrivel and die away, leaving the pale brown, creeping stems (rhizomes) at ground level. In the spring the plant springs up anew. The 10-12 blade-shaped leaves 15–20 cm long grow in an alternate arrangement on thin. Among the leafy stems the
conical or club-shaped flower heads burst forth on separate and shorter stalks. These appear in the summer, after the leafy stems have been growing for awhile. The flower heads are reddish-green 3–10 cm long with overlapping scales, enclosing small yellowish-white flowers that poke out a few at a time. As the flower heads mature, they gradually fill with an aromatic, slimy liquid and turn a brighter red color. The flower stalks usually remain hidden beneath the leaf talks.

Figure 1.5- Morphological features and Rhizomes of *Zingiber zerumbet* (L.) Roscoe ex Sm

- **Medicinal importance:** *Zingiber zerumbet* (L.) Roscoe ex Sm, also known as the “shampoo-ginger”, is used in toothache. The cooked and softened rhizome is pressed into the hollow and left for as long as needed. To ease a stomach ache, the ground and strained rhizome material is mixed with water and drunk. Similarly, Awapuhi Pake is widely cultivated and eaten, or made into a tea for indigestion as
well as increased circulation of the blood and to increase sense of well-being. Flavonoids and aromatic compounds from the rhizomes of *Zingiber zerumbet* have been isolated which are of potent medicinal use (Dae *et al.*, 2004). An extract from *Z. zerumbet*, "Zerumbone", has been found to induce apoptosis, or programmed cell death, in human liver cancer cells (Huang *et al.*, 2005). Anti-inflammatory and antinociceptive activities of *Zingiber zerumbet* methanol extract is already proven (Zakaira *et al.*, 2010, Sulaiman *et al.*, 2010, Chien *et al.*, 2008). Antifungal property of *Zingiber zerumbet* is also been reported (Jantan 2003).
Literature review on molecular documentation study of ginger:

Because of immense medicinal value of ginger (Z. officinale), much of the available documented information is on its biochemical aspects (Singh et al., 2000, Wohlmuth et al., 2006). Being a poorly studied crop, literature on molecular characterization of ginger (Z. officinale) is limited. Little work has been done on the molecular characterization of North-East (NE) Indian germplasm. Genetic diversity analysis based on morphological characters is not always reliable. Disagreement in germplasm classification between morphological characters and molecular data is reported in literature (Wahyuni et al., 2003). Since their discovery, molecular markers have played a leading role in delineating the diversity in most living organisms. Each marker type has its own advantages and disadvantages but their purpose oriented selection and stringent experimentation has always produced useful information. RAPD markers are markers of choice because of their simplicity in use and low cost. RAPD markers have been used to evaluate the genetic diversity of other species of Zingiber genus (Saowaluck and Yingyong, 2010-Zingiber montanum and Mohanty et al., 2011 -Zingiber rubens). For genetic diversity analysis using RAPD and ISSR method 46 Zingiber officinale accessions from different countries including different places of India has been reported by Kizhalkayil and Sasikumar (2010). In another study of RAPD analysis of Zingiber officinale by Rout et al. (1998), RAPD markers were used to evaluate the genetic stability of micropropagated plants. Fifteen primers were used to amplify DNA from in vivo and in vitro plant material to assess the genetic fidelity. All RAPD profiles from micropropagated plants were monomorphic and similar to those of field grown control plants i.e no variation was detected within the micropropagated plants. Using
phylogenetic analysis and metabolic profiling, Jiang et al. (2006) studied diversity within and among ginger species and found that *Z. officinale* from different geographical origins were indistinguishable. In another study, 16 elite cultivars (clones used for commercial cultivation) of ginger (*Z. officinale*) were characterized using cytological and RAPD markers, and significant variations among cultivars were detected (Nayak et al., 2003). Palai and Rout (2007) also reported RAPD analysis using twelve primers for identification and analysis of genetic variation within eight high yielding (cultivated) varieties of ginger for evaluating genetic relationship among them. In India, cultivation of traditional as well as improved clones is common. Majority of commercial clones was developed through clonal selection from traditional types. Kizhakkayil and Sasikumar (2010) in their study of Indian ginger (*Z. officinale*) reported geographic bias and high genetic similarity among the clones. They included one clone from NE India (from Meghalaya) which grouped distinctly apart from all other clones and showed highest genetic variability. These studies were, however, not enough to explain the status of genetic diversity in *Z. officinale*. Although the diversity studies conducted so far included germplasm from India, China and few other countries surrounding it, no such effort has been made to study the diversity of ginger (*Z. officinale*) from NE India, which is considered as one of the centers of diversity of many species, including ginger. It is pertinent to mention here that while several methods of DNA fingerprinting are reported in the literature, the AFLP method has seldom been used for analysis of *Zingiber officinale* genome. The only available report is that of Wayhuni et al (2003) using a manual DNA sequencer. Such study however could not identify any unique molecular marker related with the traits of interest focusing on size and color of ginger.
MATERIAL AND METHODS
Materials:

For species specific marker development in three species of Zingiber genus (Zingiber officinale, Zingiber montanum and Zingiber zerumbet), the plant materials were collected from different parts of eastern and northeastern India, viz., West Bengal, Sikkim, Assam, and Meghalaya (Table-1.1). The samples consisted of six collections of Z. officinale, five collections of Z. montanum and five collections of Z. zerumbet.

Due to being medicinally most important ginger species, further study concentrates only on wild landraces of Zingiber officinale Roscoe. to develop trait (medicinal) related DNA marker. Total fifteen landraces (Rhizome) of Zingiber officinale Roscoe germplasm were collected from Uttar Banga Krishi Vishwavidyalaya (UBKV) Coochbehar, West Bengal, India (Figure-1.6). UVKV collected these landraces from different northern districts of West Bengal, viz. Jalpaiguri, Coochbehar, Darjeeling, North Dinajpur (Table-1.2). Voucher specimens of all these studied samples are preserved at the Central National Herbarium (CAL), Botanical Survey of India, Howrah, for future reference.
<table>
<thead>
<tr>
<th>SI No</th>
<th>Name of the plant</th>
<th>Common name</th>
<th>Family</th>
<th>Latitude, Longitude &amp; Elevation (m)</th>
<th>Place of Collection</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Zingiber officinale Roscoe.</td>
<td>Garden Ginger</td>
<td>Zingiberaceae</td>
<td>26° 22' N 89° 29' E 45 m</td>
<td>Kamat Abutara Coochbehar West Bengal</td>
</tr>
<tr>
<td>2</td>
<td>Zingiber officinale Roscoe.</td>
<td>Garden Ginger</td>
<td>Zingiberaceae</td>
<td>26° 32' N 88° 46' E 75 m</td>
<td>Uttar Balaguri Dist-Jalpaiguri West Bengal</td>
</tr>
<tr>
<td>3</td>
<td>Zingiber officinale Roscoe.</td>
<td>Garden Ginger</td>
<td>Zingiberaceae</td>
<td>26° 22' N 89° 29' E 45 m</td>
<td>Tufanganj Bazar, Coochbehr West Bengal Source-Bhutan</td>
</tr>
<tr>
<td>4</td>
<td>Zingiber officinale Roscoe.</td>
<td>Garden Ginger</td>
<td>Zingiberaceae</td>
<td>26° 32' N 88° 46' E 75 m</td>
<td>Birpara market Dist-Jalpaiguri West Bengal</td>
</tr>
<tr>
<td>5</td>
<td>Zingiber officinale Roscoe.</td>
<td>Garden Ginger</td>
<td>Zingiberaceae</td>
<td>26°49’ N 87°49’ E 53 m</td>
<td>North Dinajpur Dist, Old Alluvial zone, West Bengal</td>
</tr>
<tr>
<td>6</td>
<td>Zingiber officinale Roscoe.</td>
<td>Garden Ginger</td>
<td>Zingiberaceae</td>
<td>23°080’N 93°030’E</td>
<td>Chajing, 10 Km from Imphal, Manipur</td>
</tr>
<tr>
<td></td>
<td>Species</td>
<td>Habitat Description</td>
<td>Latitude</td>
<td>Longitude</td>
<td>Altitude</td>
</tr>
<tr>
<td>---</td>
<td>--------------------</td>
<td>---------------------------------------------</td>
<td>-----------</td>
<td>-----------</td>
<td>----------</td>
</tr>
<tr>
<td>7</td>
<td><em>Zingiber montanum</em> (J.Koeing) Link ex A.Dietr</td>
<td>Cassumunar Zingiberaceae Ginger</td>
<td>27°15' N</td>
<td>88°35' E</td>
<td>922m</td>
</tr>
<tr>
<td>8</td>
<td><em>Zingiber montanum</em> (J.Koeing) Link ex A.Dietr</td>
<td>Cassumunar Zingiberaceae Ginger</td>
<td>27°15' N</td>
<td>88°35' E</td>
<td>922m</td>
</tr>
<tr>
<td>9</td>
<td><em>Zingiber montanum</em> (J.Koeing) Link ex A.Dietr</td>
<td>Cassumunar Zingiberaceae Ginger</td>
<td>27°15' N</td>
<td>88°35' E</td>
<td>922m</td>
</tr>
<tr>
<td>10</td>
<td><em>Zingiber montanum</em> (J.Koeing) Link ex A.Dietr</td>
<td>Cassumunar Zingiberaceae Ginger</td>
<td>27°15' N</td>
<td>88°35' E</td>
<td>922m</td>
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<tr>
<td>12</td>
<td><em>Zingiber zerumbet</em> (L.) Roscoe ex Sm</td>
<td>Shampoo Zingiberaceae Ginger</td>
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<td>88°36' E</td>
<td>512m</td>
</tr>
<tr>
<td>13</td>
<td><em>Zingiber zerumbet</em> (L.) Roscoe ex Sm</td>
<td>Shampoo Zingiberaceae Ginger</td>
<td>250 40'N</td>
<td>91054' E</td>
<td>973 m</td>
</tr>
<tr>
<td>14</td>
<td><em>Zingiber zerumbet</em> (L.) Roscoe ex Sm</td>
<td>Shampoo Zingiberaceae Ginger</td>
<td>250 40'N</td>
<td>91054' E</td>
<td>969 m</td>
</tr>
<tr>
<td>Accession No.</td>
<td>Scientific name</td>
<td>Family</td>
<td>Latitude, Longitude &amp; Elevation</td>
<td>Place of Collection</td>
<td></td>
</tr>
<tr>
<td>---------------</td>
<td>-----------------</td>
<td>--------</td>
<td>--------------------------------</td>
<td>---------------------</td>
<td></td>
</tr>
<tr>
<td>G-04-07</td>
<td><em>Zingiber officinale</em></td>
<td>Zingiberaceae</td>
<td>26° 22' N 89° 29' E 45 m</td>
<td>Kamat Abutara, Coochbehar, West Bengal</td>
<td></td>
</tr>
<tr>
<td>G-23-05</td>
<td><em>Zingiber officinale</em></td>
<td>Zingiberaceae</td>
<td>26° 32' N 88° 46' E 75 m</td>
<td>Uttar Balaguri, Dist-Jalpaiguri, West Bengal</td>
<td></td>
</tr>
<tr>
<td>GCP-08</td>
<td><em>Zingiber officinale</em></td>
<td>Zingiberaceae</td>
<td>26° 22' N 89° 29' E 45 m</td>
<td>Tufanganj Bazar, Coochbehr, West Bengal</td>
<td></td>
</tr>
</tbody>
</table>

Table 1.1- Name (Scientific & Common), Family, GPS Data, place of collection of three species of *Zingiber* genus
<table>
<thead>
<tr>
<th>Code</th>
<th>Species</th>
<th>Family</th>
<th>Latitude</th>
<th>Longitude</th>
<th>Height</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>PGS-08</td>
<td><em>Zingiber officinale</em></td>
<td>Zingiberaceae</td>
<td>26° 32' N</td>
<td>88° 46' E</td>
<td>75 m</td>
<td>Birpara market, Dist-Jalpaiguri, West Bengal</td>
</tr>
<tr>
<td>G-01-07</td>
<td><em>Zingiber officinale</em></td>
<td>Zingiberaceae</td>
<td>25° 56' N</td>
<td>87° 58' E</td>
<td>53 m</td>
<td>North Dinajpur, Dist, Old Alluvial zone, West Bengal</td>
</tr>
<tr>
<td>GCP-05</td>
<td><em>Zingiber officinale</em></td>
<td>Zingiberaceae</td>
<td>26° 32' N</td>
<td>88° 46' E</td>
<td>75 m</td>
<td>Jaigaon, Dist-Jalpaiguri, West Bengal</td>
</tr>
<tr>
<td>G-05-07</td>
<td><em>Zingiber officinale</em></td>
<td>Zingiberaceae</td>
<td>26° 32' N</td>
<td>88° 46' E</td>
<td>75 m</td>
<td>Madarihat locality (Before Birpara), Dist-Jalpaiguri, West Bengal</td>
</tr>
<tr>
<td>GCP-22</td>
<td><em>Zingiber officinale</em></td>
<td>Zingiberaceae</td>
<td>18°56'N</td>
<td>82°96'E</td>
<td>3031 m</td>
<td>Pattangi, Orissa</td>
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<tr>
<td>GCP-29</td>
<td><em>Zingiber officinale</em></td>
<td>Zingiberaceae</td>
<td>27°03'N</td>
<td>88°18'E</td>
<td>2250 m</td>
<td>Gorubathan, Dist-Darjeeling</td>
</tr>
<tr>
<td>GCP-27</td>
<td><em>Zingiber officinale</em></td>
<td>Zingiberaceae</td>
<td>26° 22' N</td>
<td>89° 29' E</td>
<td>45 m</td>
<td>Jambari south, Gosanimari, Coochbehar, West Bengal</td>
</tr>
<tr>
<td></td>
<td>Scientific name</td>
<td>Family</td>
<td>GPS Data</td>
<td>Place &amp; Date of collection</td>
<td></td>
<td></td>
</tr>
<tr>
<td>----</td>
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<td>----------</td>
<td>---------------------------</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G-03-05</td>
<td><em>Zingiber officinale</em></td>
<td>Zingiberaceae</td>
<td>26° 32' N 88° 46' E 75 m</td>
<td>Odlabari Dist-Jalpaiguri, West Bengal</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IG-1</td>
<td><em>Zingiber officinale</em></td>
<td>Zingiberaceae</td>
<td>26° 32' N 88° 46' E 75 m</td>
<td>Manglabari, Jaigaon Dist-Jalpaiguri, West Bengal</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GCP-18</td>
<td><em>Zingiber officinale</em></td>
<td>Zingiberaceae</td>
<td>26° 32' N 88° 46' E 75 m</td>
<td>Uttar Madarihat Dist-Jalpaiguri, West Bengal</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GCP-44</td>
<td><em>Zingiber officinale</em></td>
<td>Zingiberaceae</td>
<td>26° 22' N 89° 29' E 45 m</td>
<td>IISR. Callicut, but grown in UBKV, Cooch behar, West Bengal</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GCP-16</td>
<td><em>Zingiber officinale</em></td>
<td>Zingiberaceae</td>
<td>26° 22' N 89° 29' E 45 m</td>
<td>Chawarah Hathkhola Dinhata Coochbehar West Bengal</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 1.2- Scientific name, Family, GPS Data, Place & Date of collection of the wild *Zingiber officinale* Roscoe germplasm.
Figure 1.6- Photograph of fifteen wild landraces of *Zingiber officinale* Roscoe. Landraces used in the study
Methods:

Extraction of Nucleic acids:

- DNA extraction from fresh leaves of Ginger:

DNA was extracted from fresh leaves of 3 months old plants by the 2% CTAB method (Bousquet et al., 1990).

Required solutions:

- Extraction buffer
  - 2% CTAB Cetyl Trimethyl Ammonium Bromide
  - 100mM Tris HCl
  - 20mM EDTA
  - 1.4 M NaCl
  - 1% PVP (Polyvinylpyrrolidone)
  - 0.2% β-mercaptoethanol
- Absolute Ethanol (Chilled)
- Chloroform : Isoamyl Alcohol (24:1)
- Phenol (1M tris equilibrated, pH-8.0)
- 3M Sodium acetate
- Isopropanol
- RNaseA (10mg/ml)
- Tris-EDTA Buffer : 10mM Tris-Cl, 1mM EDTA
Protocol:

- 500 mg leaves were taken
- Leaves were cut into small pieces
- Pieces of leaves were crushed with liquid nitrogen in mortar and pestle in 4°C (cold room).
- After grinding, the tissue was mixed with 2 ml CTAB mixture buffer (2x).
- It was mixed properly i.e. in such a way that CTAB (2x) reach everywhere.
- Then the samples were floated in Centrifuge tube in hot water bath at 65°C for 45 mins.
- At interval of every 5-10 mins the microfuge tubes (CTAB + sample) were gently inverted for remixing.
- Then equal volume of chloroform: Isoamyl alcohol (24:1) was mixed gently.
- Then it was centrifuged at 12000 rpm for 10 min in RT.
- The upper aqueous layer was separated in a fresh centrifuge tube.
- Equal volume of Chloroform: Isoamyl alcohol (24:1) was added then.
- Then it was centrifuged at 10,000 rpm for 10 min in 24°C.
- The upper aqueous layer was transferred in a fresh tube.
- One volume of Isopropanol was added and mixed gently.
- It was left overnight at -20°C.
- Then it was centrifuged for 10 mins at 10,000 rpm in 4°C.
- Pellet was washed with 1ml of 70% ethanol.
• Pellet was dried and was dissolved in TE buffer 500 μl)
• Samples were then transferred into eppendorf tubes

PURIFICATION

• 10 μl RNase A (10mg/ml) was added and incubated in 37°C for 1/2 hour
• Then equal volume i.e., 500 μl of saturated phenol (pH 8.0) was added in each tube.
• Centrifuge at 10,000 rpm for 10 min
• The aqueous layer was separated.
• Equal volume of chloroform- isoamyl alcohol (1:1) was mixed.
• Then it was centrifuged at 10,000 rpm for 10 min
• The aqueous layer was separated again.
• Then 1/10 volume of 3M NaOAc and 2 volume chilled alcohol was added.
• It was left for overnight at -20°C.
• Next day the tubes were centrifuged at 10,000 rpm for 10 minute
• The pellet was washed in 70% alcohol.
• It was then dried in vacuum drier until the smell of ethanol eliminated
• Finally DNA was dissolved in minimum amount (20-40 μl of TE buffer )
DNA extraction from dried rhizome of Ginger:

DNA was also extracted from dried rhizome powder of plants by the 4% CTAB method (Syamkumar et al., 2003).

Required solutions:

- Extraction buffer
  - 4% CTAB (Cetyl Trimethyl Ammonium Bromide)
  - 100 mM Tris HCl
  - 20 mM EDTA (Ethylene Diamine Tetra Acetic acid)
  - 2.0 M NaCl (Sodium Chloride)
  - 1% PVP (Polyvinylpyrrolidone)
  - 0.3 % β-mercaptoethanol
- Absolute Ethanol (Chilled)
- Chloroform : Isoamyl Alcohol (24:1)
- Phenol (1M tris equilibrated, pH-8.0)
- 3M Sodium acetate
- Isopropanol
- RNaseA (10mg/ml)
- Tris-EDTA Buffer : 10mM Tris-Cl, 1mM EDTA

Protocol:

- 500mg of dried rhizome powder was taken.
- Freshly prepared (pre heated at 60°C) extraction buffer (100mM Tris-Cl, pH-8, 20mM EDTA, 2M NaCl, 4% CTAB, 0.3% β-mercaptoethanol, 1% PVP) was
added to each sample and was incubated at 60°C in water bath with occasional mixing in 5 min interval.

- Then equal volume of chloroform: isoamyl alcohol (24:1) and was mixed by inversion for 15 mins.
- Then the tubes were centrifuged at 10,000 rpm at 24°C for 10 mins and carefully transfer the upper aqueous layer to another tube.
- Then 0.6 volume of isopropanol added to each tube and kept in -20°C for overnight.
- Next day, the tubes were centrifuged at 10,000 rpm at 24°C for 10 mins.
- The supernatant was discarded and pellet was washed (8000 rpm for 5 mins 24°C) with 80% ethanol.
- The pellet was dried in vacuum concentrator and was dissolved in 1 ml TE buffer.
- Then the whole solution was transferred to 2 ml microcentrifuge tube and was labeled properly.
- 1ml Phenol (1m tris equilibrated, pH-8.0) was added, after proper mixing, the microcentrifuge tubes were centrifuged at 12,000 rpm at 4°C for 10 mins.
- After centrifugation, the upper aqueous layer was transferred to a fresh tube and equal volume of chloroform:isoamyl alcohol (24:1) was added to each tube. Then centrifugation was done at 12,000 rpm at 4°C for 10 mins.
- This step was repeated once.
- The upper aqueous layer was transferred to a fresh tube and equal volume of ice cold ethanol was added to precipitate the DNA.
- Then centrifugation was done at 10,000 rpm at 4°C for 10 mins.
• The pellet was dried in vacuum concentrator and was dissolved in appropriate amount of TE buffer (20-40 µl).

• **RNA extraction from fresh leaves of Ginger:**

RNA was extracted from fresh leaves by Trizol lysis method

**Required solutions:**

• TRIZOL as lysis buffer
• Chloroform
• Isopropanol
• 75% ethanol

All the solutions were made with 0.1% DEPC treated dH₂O.

**Protocol:**

- 500 mg leaves were taken
- leaves were cut into small pieces
- Pieces of leaves were crushed with liquid nitrogen in mortar and pestle in 4°C (cold room).
- 1ml TRizol reagent was added to the sample and transferred to the eppendorf tubes.
- Vortexing was done for proper mixing.
- Centrifugation was done for 5 min at 12,000 rpm at 4°C to remove chunks of tissue.
- Supernatant was transferred to new tube and left at RT for 5 min.
- 200 µl Chloroform (no isoamylalcohol) was added to each tube and was mixed well.
- After keeping at RT for 5 min, centrifugation was done at 12,000 rpm for 15 min at 4°C. The top aqueous phase contains the RNA. The interphase contains DNA, and the organic phase DNA and protein.
- Supernatant was taken and 500 µl of Isopropanol was added to the tubes.
- The tubes were kept overnight at -20°C.
- Centrifugation was done for 15 min at 12,000 rpm at 4°C to form a white pellet at bottom of tube.
- The pellet was washed with 75% ethanol (8000 rpm at 4°C for 5 min) and was allowed to dry in vacuum.
- The pellet was resuspended in 50µl DEPC dH₂O

❖ Qualitative assessment of extracted DNA and RNA by gel electrophoresis

The standard method used to separate, identify and purify nucleic acid (DNA and RNA) is electrophoresis through agarose gels. The technique is simple, rapid to perform and capable of resolving mixtures of nucleic acid fragment that cannot be separated adequately by other sizing procedures, such as density gradient centrifugation. The migration rate of DNA /RNA through agarose gels is additionally dependent upon the molecular size of the DNA / RNA, the agarose concentration, the conformation of the DNA and the applied current. A DNA fragment of a given size migrates at different rates
through gels containing different concentrations of agarose. Thus, by using gels of different concentrations, it is possible to resolve a wide size-range of DNA fragments.

**Used buffers**

- 50X Tris-acetate (TAE) - 242g Tris base, 57.1ml glacial acetic acid, 100 ml 0.5M EDTA (pH-8)
- 5X Tris-borate (TBE) – 54 g Tris base, 27.5 g boric acid, 20 ml 0.5 M EDTA (pH 8.0)

**Preparation of the agarose gel**

- Required amount of powdered agarose (0.8%) was measured and poured into electrophoresis buffer (TAE)
- The slurry was heated in a microwave oven until the agarose is completely dissolved.
- The solution was cooled to 50°C and 7 μl of ethidium bromide was added for every 100 ml of agarose slurry (from a stock of 10mg/ml).
- Gel mold, sealer and combs were cleaned with ethanol.
- Setup the mold on a equal plane so that the agarose will be distributed uniformly throughout the mold.
- Agarose gel solution was poured into the mold slowly without the formation of any air bubbles.
- Mold was left undisturbed until the agarose solidified completely.
The electrophoresis buffer was prepared in 1xTAE buffer and gel tank was filled up by the buffer.

The sealers were removed carefully and mold was placed in the gel tank having the electrophoresis buffer.

Pre-run was carried out for 30 minutes for removing any impurities in the gel.

**Loading the samples in the agarose gel**

DNA / RNA samples were mixed with loading buffer and were loaded in the slots of the submerged gel.

**Gel-loading buffer:**

**6x Gel Loading Buffer**

a) 0.25% bromophenol blue

b) 0.25% xylene cyanol

c) 40% (w/v) sucrose in H₂O (DEPC dH₂O in case of RNA)

**Procedure of running the gel:**

1. 3 μl of 6X gel loading dye was added to 3-4 μl of each DNA/RNA sample before loading the wells of the gel.

2. 1 well contained good quality λ DNA or RNA marker as molecular weight standards
3. Then submarine electrophoretic gel was run at 80 V till the loading dye has migrated 1/3 of distance in the gel.

Documentation of gel:

After the run was complete the gel was seen under UV trans-illuminator and the picture was documented using gel documentation system.

❖ Conversion of mRNA to cDNA:

After total RNA isolation mRNA fraction within total RNA population is converted to cDNA using Universal RiboClone cDNA Synthesis System (Promega, USA) following manufacturer’s instructions. After conversion cDNA was stored in -20°C.
**DNA Fingerprinting Techniques**

**Random Amplified Polymorphic DNA (RAPD) analysis:**

**Principle:**

The standard RAPD technology utilizes short synthetic oligonucleotides (10 bases long) of random sequences as primers to amplify nanogram amounts of total genomic DNA under low annealing temperatures by PCR (Williams et al., 1990). Amplification products are generally separated on agarose gels and stained with ethidium bromide. At an appropriate annealing temperature during the thermal cycle, oligonucleotide primers of random sequence bind several priming sites on the complementary sequences in the template genomic DNA and produce discrete DNA products if these priming sites are within an amplifiable distance of each other. The profile of amplified DNA primarily depends on nucleotide sequence homology between the template DNA and oligonucleotide primer at the end of each amplified product. Nucleotide variation between different sets of template DNAs will result in the presence or absence of bands because of changes in the priming sites.
Basic steps of RAPD fingerprinting method:

- **Isolation of Genomic DNA**: Total genomic DNA was extracted from the sample.

- **PCR Reaction**: PCR reaction was carried out random primer. Here only one primer acted both as forward and reverse primer. Annealing temperature was kept low as compared to normal PCR (36°C). Total number of cycles should be 35 to 45.

- **Separating gel fragments by Gel electrophoresis**: After PCR reaction the fragments were separated in 1.5% agarose gel in 1X TBE buffer.

- **Documentation of gel**: After the run was complete the gel was seen under UV trans-illuminator and the picture was documented using gel documentation syste
Protocol:

- Setting up the reaction for RAPD assay

1. The template DNA was taken out from 4°C and were kept on ice
2. All the reagents necessary for RAPD analysis were taken out from stocks (-20°C) and were kept them in ice and were allowed to thaw.
3. Then the reagents were mixed in the proportion specified (shown below) against each one for a total 25 μl reaction volume.

<table>
<thead>
<tr>
<th>Components</th>
<th>Volume in μl</th>
<th>Concentration of stock</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primers (both forward and reverse)</td>
<td>1.0</td>
<td>100μm</td>
</tr>
<tr>
<td>dNTPs</td>
<td>2.5</td>
<td>1mM</td>
</tr>
<tr>
<td>PCR buffer</td>
<td>5 / 2.5</td>
<td>5X / 10X</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>1.0</td>
<td>15mM</td>
</tr>
<tr>
<td>Taq Polymerase</td>
<td>0.50</td>
<td>5-10 units / μl</td>
</tr>
<tr>
<td>Sterilized water + DNA (30-40 ng)</td>
<td>17.5</td>
<td>–</td>
</tr>
</tbody>
</table>

4. Taq Polymerase was added at the end while making the cocktail
5. The cocktail was distributed in each 0.2 ml microfuge tube

6. 30-40 ng of template DNA was pipette out and was added to each tube. The DNA template and the reaction mix was mixed properly by pipetting in and pipetting out.

7. Proper labeling of samples with marker is done.

8. All the tubes were placed in thermocycler and run the programme.

Running the reaction in thermocycler

The temperature profile of RAPD reaction is given below:

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature</th>
<th>Time</th>
<th>No of cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>94°C</td>
<td>2 min</td>
<td>1</td>
</tr>
<tr>
<td>Denaturation</td>
<td>94°C</td>
<td>1 min</td>
<td></td>
</tr>
<tr>
<td>Annealing</td>
<td>36°C</td>
<td>1 min</td>
<td>45</td>
</tr>
<tr>
<td>Extension</td>
<td>72°C</td>
<td>2 min</td>
<td></td>
</tr>
<tr>
<td>Extended Extension</td>
<td>72°C</td>
<td>10 min</td>
<td>1</td>
</tr>
</tbody>
</table>
• **Gel Electrophoresis:**

1. The amplified PCR products were resolved by running a 1.5 % agarose gel.
2. Electrophoresis is done either with 1x TBE, because better resolutions can be seen with 1x TBE.
3. The samples were taken out from the thermocycler and 5 μl of 10x loading buffer were added to each sample.
4. Each sample was loaded in the well of the gel and run the gel at 80 volts for 3 to 4 hours.
5. When the loading dye reach 10-11 cm towards the bottom edge of the gel we get better resolution of amplified products.

• **Documentation of gel:**

After the completion of the run, the gel was seen under UV trans-illuminator and the picture was documented using gel documentation system.

❖ **Amplified Fragment Length Polymorphism (AFLP)**

**Principle:**

Amplified Fragment Length Polymorphism is a DNA fingerprinting technique developed by Zabeau and Vos and subsequently developed by Vos et al (1995). This method is based on PCR amplification of selected restriction fragments of a total digested genomic DNA. After labeling, amplified products are separated by electrophoresis. AFLP technology combines the power of restriction fragment length polymorphism
Basic Steps of AFLP fingerprinting:

Figure 1.8- Schematic diagram showing basic steps of AFLP fingerprinting
• **Restriction Digestion:**

Restriction fragments of the genomic DNA are produced by using two different restriction enzymes: a frequent cutter (the four-base restriction enzyme Mse-I) and a rare cutter (the six-base restriction enzyme EcoRI). The frequent cutter serves to generate small fragments, which amplify well and which have the optimal size range for separation on a sequence gel, whereas rare cutters limits the number of fragments to be amplified.

**Ligation of oligonucleotide adapters:**

Double-stranded adaptors consist of a core sequence and enzyme-specific sequence. Therefore, adapters are specific for either for EcoRI site or the Mse-I site. Usually restriction and ligation takes place in a single reaction. Ligation of adapters to the restricted DNA alters the restriction site in order to prevent a second restriction from taking place after ligation has occurred. The core sequence of the adapters consists of a known DNA sequence of 20 nucleotides, which will be used latter as primer in the PCR.

• **Pre-Amplification:**

This step is a normal PCR where the adapters are used as primers. This first PCR, called Pre-amplification, allows a first selection of fragments by only amplifying the DNA restriction fragments that have ligated an adapter to both extremities. Additionally to the adapter sequences, the primers for the pre-selective amplification have a supplementary base. This extra base enables another first selection by amplifying \( \frac{1}{4} \) of
the fragments that have ligated an adapter to both extremities. These first three steps can be run and visualized on a 1.5% agarose gel.

- **Selective Amplification:**

  The aim of this step is to restrict the level of polymorphism and to label the DNA. For this second amplification, three more nucleotides are added at the 3' end of the primer used for the pre amplification (adapters sequence +3 nucleotide). These two additional nucleotides make the amplification more selective and will decrease the number of restriction fragments amplified (polymorphism). Moreover one of the primers (usually the EcoRI primer) is labeled with a fluorescent dye, and will allow the visualization of DNA during migration.

- **Electrophoresis:**

  Previously the PCR products were denatured and run on acrylamide gel (DNA sequencer). In our study, samples were run on an ABI Prism 310. A thin capillary containing a polymer replaces the usual acrylamide gel. The electrophoresis conditions we used for fragment analysis can resolve DNA fragments differing just by one base pair. Samples are loaded in a track, and run one after the other through the capillary. All fragments are separated with regard to length, smaller fragments running first. Once passing the laser, a dye attached to the primer is excited and emits a florescent signal that is then collected by a computer. The results of fluorescence are visualized on the computer as peaks, called Electropherogramms. Each peak corresponds to a band on a normal acrylamide gel.
AFLP Plant Mapping Protocol

I) Annealing Adaptor Pairs: Annealing of the adaptor pairs for the restriction-ligation involves the following steps

a. From the AFLP Ligation and Preselective Amplification Module, the tubes labeled Msel Adaptor Pair and EcoRI Adaptor Pair were taken out.

b. Then tubes were heated in a water bath at 95 °C for 5 minutes.

c. After that the tubes were allowed to be cool to room temperature.

d. Spin in a microcentrifuge for 10 seconds at 1400 × g (maximum)

II) Preparing enzyme master mix:

Enzyme Master Mix was prepared to perform the restriction-ligation reactions for all DNA samples. This involves following steps:
The following reagents were combined in a sterile 0.5 mL microcentrifuge tube for one DNA sample:

a. 0.1 µL 10X T4 DNA ligase buffer with ATP

1X T4 DNA Ligase Buffer with ATP: 50 mM Tris-HCl (pH 7.8), 10 mM MgCl2, 10 mM dithiothreitol, 1 mM ATP, 25 µg/ml bovine serum albumin.

b. 0.1 µL 0.5 M NaCl

c. 0.05 µL 1 mg/mL BSA (diluted from 10 mg/mL stock)

d. 1 Units Msel

e. 5 Units EcoRI

f. 1 Weiss Units T4 DNA Ligase (or 67 cohesive end ligation units)

Sterile distilled water was added to bring the total volume to 1 µL.

After proper mixing it was spin down in a microcentrifuge for 10 seconds.

It was stored in ice until ready to aliquot into individual reaction tubes.
III) Preparing Restriction-Ligation Reactions:

The following reagents were combined in a sterile 0.5 mL microcentrifuge tube

a. 1.0 μL 10X T4 DNA ligase buffer that includes ATP

b. 1.0 μL 0.5M NaCl

c. 0.5 μL 1.0 mg/mL BSA (dilute from 10 mg/mL if necessary)

d. 1.0 μL Msel adaptor

e. 1.0 μL EcoRI adaptor

f. 1.0 μL Enzyme Master Mix

0.5 μg (500ng) genomic DNA was added in 5.5 μL sterile distilled water.

Then it was mixed thoroughly and was placed in a microcentrifuge for 10 seconds.

Then the thebes were incubated at 37 °C for 2 hours.

IV) Diluting Restriction-Ligation reactions:

After 2 hours of incubation, the restriction-ligation samples were diluted to give the appropriate concentration for subsequent PCR.

Add 189 μL of TE0.1 buffer was added to each restriction-ligation reaction and then mixed gently.
**V) Preselective amplification:**

The following reagents were combined in a sterile 0.2 mL microcentrifuge tube

a. 4.0 μL diluted DNA prepared by restriction-ligation

b. 1.0 μL AFLP pre-selective primer pairs

c. 15.0 μL AFLP Core Mix

Then the samples were placed in a thermal cycler.

**Thermal cycler parameters for pre-selective amplification**

<table>
<thead>
<tr>
<th>HOLD</th>
<th>CYCLE</th>
<th>HOLD</th>
<th>HOLD</th>
</tr>
</thead>
<tbody>
<tr>
<td>72 °C</td>
<td>94 °C</td>
<td>56 °C</td>
<td>72 °C</td>
</tr>
<tr>
<td>2 min.</td>
<td>20 sec.</td>
<td>30 sec.</td>
<td>2 min.</td>
</tr>
</tbody>
</table>

**VI) Verifying Successful Amplification:**

After Pre-amplification 10 μL of each reaction mixture was loaded in a 1.5% agarose gel in 1X TBE buffer at 4V / cm for 3–4 hours.

Then the gel was stained with ethidium bromide and the gel was viewed on a UV transilluminator. A smear of product from 100–1500 bp was clearly visible
VII) Diluting Pre amplified reactions mixture:

The pre-selective amplification products was diluted for next step in the following combination in a 0.2 ml microcentrifuge tube

a. 10.0 μL pre-selective amplification reaction product

b. 190.0 μL TE0.1 buffer

Then mixing was done properly
VIII) Selective Amplification:

The following reagents were combined in a sterile 0.2 mL microcentrifuge tube:

a. 3.0 μL diluted preselective amplification reaction product

b. 1.0 μL Msel Primer at 5 μM

c. 1.0 μL EcoRI primer at 1 μM (tagged with fluorescent dye)

d. 15.0 μL AFLP Core Mix

Then the samples were put in thermal cycler using the parameters shown below:

<table>
<thead>
<tr>
<th>HOLD</th>
<th>CYCLE</th>
<th>Number of Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>94 °C</td>
<td>94 °C</td>
<td>66 °C</td>
</tr>
<tr>
<td>2 min.</td>
<td>20 sec.</td>
<td>30 sec.</td>
</tr>
<tr>
<td></td>
<td>94 °C</td>
<td>65 °C</td>
</tr>
<tr>
<td></td>
<td>20 sec.</td>
<td>30 sec.</td>
</tr>
<tr>
<td></td>
<td>94 °C</td>
<td>64 °C</td>
</tr>
<tr>
<td></td>
<td>20 sec.</td>
<td>30 sec.</td>
</tr>
<tr>
<td></td>
<td>94 °C</td>
<td>63 °C</td>
</tr>
<tr>
<td></td>
<td>20 sec.</td>
<td>30 sec.</td>
</tr>
</tbody>
</table>
 IX) Preparing the Loading Buffer for the ABI PRISM 310

A loading buffer mix was prepared consisting of the following reagents in the proportions shown below for each sample:

a. 12.0 µL deionized formamide

b. 0.5 µL of GeneScan-500 [ROX] size standard

<table>
<thead>
<tr>
<th>Temp (°C)</th>
<th>62 °C</th>
<th>72 °C</th>
<th>Temp (°C)</th>
<th>61 °C</th>
<th>72 °C</th>
<th>Temp (°C)</th>
<th>60 °C</th>
<th>72 °C</th>
<th>Temp (°C)</th>
<th>59 °C</th>
<th>72 °C</th>
<th>Temp (°C)</th>
<th>58 °C</th>
<th>72 °C</th>
<th>Temp (°C)</th>
<th>57 °C30 sec.</th>
<th>72 °C</th>
<th>2 min.</th>
<th>Temp (°C)</th>
<th>56 °C30 sec.</th>
<th>72 °C</th>
<th>2 min.</th>
</tr>
</thead>
<tbody>
<tr>
<td>94 °C</td>
<td>62 °C</td>
<td>72 °C</td>
<td>94 °C</td>
<td>61 °C</td>
<td>72 °C</td>
<td>94 °C</td>
<td>60 °C</td>
<td>72 °C</td>
<td>94 °C</td>
<td>59 °C</td>
<td>72 °C</td>
<td>94 °C</td>
<td>58 °C</td>
<td>72 °C</td>
<td>94 °C</td>
<td>20 sec.</td>
<td>57 °C</td>
<td>30 sec.</td>
<td>72 °C</td>
<td>20 sec.</td>
<td>56 °C</td>
<td>30 sec.</td>
</tr>
<tr>
<td>20 sec.</td>
<td>30 sec.</td>
<td>2 min.</td>
<td>20 sec.</td>
<td>30 sec.</td>
<td>2 min.</td>
<td>20 sec.</td>
<td>30 sec.</td>
<td>2 min.</td>
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<td>2 min.</td>
<td>20 sec.</td>
<td>30 sec.</td>
<td>2 min.</td>
<td>2 min.</td>
</tr>
</tbody>
</table>

60°C 30 min.  

4°C forever

Thermal cycler parameters for selective amplification
X) Loading and Electrophoresis on the ABI PRISM 310:

Add 12.5 μL of the loading buffer was added to a sample tube with 0.5 μL of the selective amplification product.

Then the tubes were heated to 95 °C for 3–5 minutes and then quick-chilling was done in ice.

Then Samples were loaded in the 96-well sample tray and electrophoresis was performed in automated DNA sequencer.
• Fingerprinting data analysis and study of phylogenetic relationship:

Molecular phylogenetics is the use of the structure of molecules such as DNA or protein to gain information about an organism's evolutionary relationships. The result of a molecular phylogenetic analysis is expressed in a phylogenetic tree. A phylogenetic tree or evolutionary tree is a branching diagram or 'tree' showing the inferred evolutionary relationships among various biological species based on similarities and differences in their genetic component. The taxa joined together in the tree are implied to have descended from a common ancestor. In a rooted phylogenetic tree, each node with descendants represents the inferred most recent common ancestor of the descendants and the edge lengths in some trees may be interpreted as time estimates. Each node is called a taxonomic unit (HTUs) as they cannot be directly observed. The common methods used to infer phylogenetic trees are neighbor joining, maximum parsimony, maximum likelihood and Bayesian inference.

Amplified DNA alleles are scored as present or absent in each plant. Electrophoretic DNA bands / peaks of low visual intensity that could not be readily distinguished as present or absent are considered ambiguous markers and were not scored. For diversity analysis, bands / peaks are scored as present (1) or absent (0) to form a raw data matrix. Genetic distance is calculated by Jaccard's coefficient (Jaccard 1908), which is as follows: \( S_{ij} = \frac{N_{ij}}{(N_i + N_j + N_{ij})} \) where \( S_{ij} \) is the similarity index between the ith and jth genotype, \( N_{ij} \) is the number of bands present in both genotype, \( N_i \) is the number of bands present in the ith genotype but absent in the jth genotype, and \( N_j \) is
the number of bands absent in the ith genotype and present in the jth genotype. The average similarity matrix is used to generate a tree for cluster analysis by UPGMA (Unweighted Pair Group Method with Arithmetic mean) method. The average similarity matrix was used to generate a tree for cluster analysis by using software NTSys v 2.1.
c) Medicinal compounds assay and correlative studies with DNA Fingerprinting data

❖ Antioxidant assay by DPPH method:

Principle
DPPH (1, 1-Diphenyl-2-picrylhydrazyl) is a stable free radical with red color (absorbed at 517nm). DPPH has an unpaired valence electron at one atom of nitrogen bridge (Eklund et al., 2005). If free radicals have been scavenged, DPPH will generated it's color to yellow. Scavenging of DPPH radical is the basis of the popular DPPH antioxidant assay (Alma et al., 2003; Karioti, et al., 2004;Kordali, et al., 2005). This assay uses this character to show herbs free radical scavenging activity. The inhibitory percentage of DPPH was calculated according to the following equation:

\[
\% \text{ inhibition} = \left( \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \right) \times 100\%
\]

Procedure
1. 100 mg ginger powder was weighted.
2. Then 5 ml of 90% methanol was added to each tube.
3. The tubes were kept in room temperature for 1 hr with intermittent shaking.
4. Then 1 ml (0.004%) DPPH was added.
5. It was kept again in room temperature for 30 min.
6. The absorbance was taken at 517 nm in a UV-VIS Spectrophotometer.
7. The O.D. value of the sample to be tested is put in the above formula and the percent of inhibition is determined then.

❖ HPLC analysis of 6-Gingerol:

Principle:
The contents of 6-gingerol in landraces of Zingiber officinale are analyzed by High Performance Liquid Chromatography (HPLC) equipped with a reversed phase C18 column. The compound was identified and quantified based on retention time using 6-gingerol as HPLC external standard.

Procedure:

1. Sample preparation:
Fresh mature ginger rhizomes were graded, washed through with tap water, peeled and cut into cross-sections of 2 ± 1 mm thickness. Cut ginger samples were dried in an air dryer at 55 ± 2°C to achieve 10-12% moisture content.

2. Analysis of 6-gingerol content:
The contents of 6-gingerol were analyzed by high performance liquid chromatography (HPLC 1100, Agilent, Germany) equipped with a reversed phase column C18 (Hypersil ODS 250mm x 4.0mm i.d., 5 μm). Elution was isocratic using a mixture of HPLC grade
acetonitrile and water (55:45 v/v) flow rate 1.0ml/min, temperature 30°C. A Variable Wavelength Detector (VWD) set at 282nm was used.

The compounds were identified and quantified based on retention time using 6-gingerol as HPLC external standard. 10g of cut ginger were blended with 50 ml methanol (HPLC grade) by electrical blender for 1 min and centrifuged at 5,000 rpm for 5 min. The supernatant was subsequently filtered through a 0.20 μm nylon membrane filter (Whatman, England). A 20 μl ginger extract was then subjected to HPLC for the 6-gingerol analysis. The compound was identified and quantified based on retention time using 6-gingerol as HPLC external standard.
Karl Pearson Correlation Coefficient and Multiple Regression Analysis:

Correlations between different morphological and biochemical parameters were examined using Karl Pearson correlation. The association between AFLP markers and the quantitative traits was estimated through stepwise Multiple Regression Analysis (MRA), where each quantitative trait was treated as dependent variable, while the AFLP markers treated as independent variables. The analysis was based on the model: \( Y = a + b_1m_1 + b_2m_2 + \ldots + b_jm_j + \ldots + b_nm_n + d + e \) which related the variation in the dependent variable (\( Y \) = accession means for a quantitative trait) to a linear function of the set of independent variables \( m_j \), representing AFLP markers. The \( b_j \) terms are the partial regression coefficients that specify the empirical relationships between \( y \) and \( m_j \), \( d \) represents between accession residuals, which is left after regression, and \( e \) is the random error of \( Y \) that includes environmental variation (Virk et al., 1996, Kar et al., 2008). To select independent variables for the regression equation, F values with 0.045 and 0.099 probabilities were used to enter and remove, respectively (Affifi and Clark, 1984). All analysis was performed using the same SPSS software package. \( R^2 \) denotes the square of \( r \), the correlation coefficient. Selected markers were further tested with linear curve fitting, using linear models for confirming the significance of \( \beta \)-statistics for each band identified by MRA. Beta can be defined as standardized regression coefficient = \( BS_x/BS_y \), where \( B \) is the regression coefficient or slope and \( S_x/S_y \) are the standard deviations of independent (x) and dependent (y) variables (Affifi and Clark, 1984; Kar et al., 2008; Ruan et al., 2009).
Cloning and sequencing of Chalcone synthase cDNA:

After synthesis of double stranded cDNA from total mRNA, Chalcone Synthase gene cDNA was amplified by PCR reaction using gene specific primers designed based on the sequence information obtained from published chalcone synthase cDNA sequence (GenBank accession no- DQ486012). The sequences of these two primers are:

**CHS-F: 5’-ATCGCCTGCAGGCTACCCAT-3’**

**CHS-R: 5’-GCAGTCTACCAATACCGTAG -3’**.

PCR reaction was set up using following reagents in given amount:

<table>
<thead>
<tr>
<th>Reagents</th>
<th>AMOUNT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Template DNA</td>
<td>40ng</td>
</tr>
<tr>
<td>Forward Primer</td>
<td>0.5 µL</td>
</tr>
<tr>
<td>Reverse Primer</td>
<td>0.5 µL</td>
</tr>
<tr>
<td>dNTP (1mM)</td>
<td>2.5 µL</td>
</tr>
<tr>
<td>Buffer (10X)</td>
<td>2.5 µL</td>
</tr>
<tr>
<td>Distilled H₂O</td>
<td>Amount required for volume makeup</td>
</tr>
<tr>
<td>Taq polymerase</td>
<td>0.5 µL</td>
</tr>
</tbody>
</table>
The temperature profile of PCR reaction is given below:

Then the PCR product was run in 1.5 % agarose gel. A sharp DNA band at around 1500 bp was observed.

This fragment was gel extracted using Gel extraction kit (Qiagen, USA)

Requirement:

1. Agarose Gel containing DNA fragments.
2. QG Buffer.
3. PE Buffer.
4. EB Buffer.
5. 1.5 ml Microcentrifuge tube.
7. Isopropanol
Procedure:

1) The gel portion containing the DNA fragment was excised out by a sterile knife and taken into a clean 1.5ml microcentrifuge tube.

2) The weight of the excised fragment was measured.

3) 3 volume of QG buffer was added to 1 volume of gel.

4) The tube was then incubated at 50 °C for 10 min to dissolve the gel completely. The tube was vortexed every 2 to 3 min during incubation.

5) After the gel slice had dissolved completely 1 gel volume of isopropanol was added to the sample & mixed.

6) A QIA quick spin column was placed in a collection tube & the sample was applied to the column for binding of the desired DNA fragment to the column & centrifuged at 13000 rpm for 1 min. The flow through was discarded & the column was placed back to the same collection tube.

7) 500 ul of QG buffer was added to the column & again centrifuged at 13000 rpm for 1 min.

8) 750 ul of PE buffer (ethanol+ sodium acetate) was added to the column & centrifuged at 13000 rpm for 1 min.

9) The flow through was discarded & the column was recentrifuged for additional 1 min.
10) The column was then placed in a clean 1.5 ml microcentrifuge tube to elute the DNA. 50 ul of EB buffer was added to the column & kept for 5 min for the proper elution of DNA which was bound to the column membrane.

11) 7 ul of the eluted DNA mixture was run on a 1% agarose gel to observe the proper elution of desired DNA fragments which was expected to give a band of 1.8 kb.

The size of Chalcone Synthase complete cDNA is 1173 bp NCBI accession No.-.
Cloning in pGEM-T easy vector:

- 'TA' Cloning:

TA cloning is a subcloning technique that doesn't use restriction enzymes and is easier and quicker than traditional subcloning. The technique relies on the ability of adenine (A) and thymine (T) (complementary basepairs) on different DNA fragments to hybridize and, in the presence of ligase, become ligated together (Figure-1.10). PCR products are usually amplified using Taq DNA polymerase which preferentially adds an adenine to the 3' end of the product. Such PCR amplified inserts are cloned into linearized vectors that have complementary 3' thymine overhangs. Commercialized kits with pre-prepared vectors and PCR reagents are currently sold, greatly speeding up the process.

Figure 1.10- Basic principle of 'TA' cloning
The pGEM-T Easy Vectors are linearized vectors with a single 3'-terminal thymidine at both ends. The T-overhangs at the insertion site greatly improve the efficiency of ligation of PCR products by preventing recircularization of the vector and providing a compatible overhang for PCR products generated by certain thermostable polymerases. These Vectors are high-copy-number vectors containing T7 and SP6 RNA polymerase promoters flanking a multiple cloning region within the α-peptide coding region of the enzyme β-galactosidase. Insertional inactivation of the α-peptide allows identification of recombinants by blue/white screening on indicator plates.

Figure 1.11- Genetic map of pGEM-T easy Vector (Promega, USA)

- T7 RNA polymerase transcription initiation site 1
- multiple cloning region 10–128
- SP6 RNA polymerase promoter (−17 to +3) 139–158
Preparation of LB/Ampicillin/ IPTG/X-GAL Plates:

Reagents required:

a) IPTG stock solution (0.1M)

b) X-Gal (50mg/ml) dissolved in N,N'-dimethyl formamide

c) LB Medium (pH-7.0)

d) Ampicillin (100μg/ml)

Preparation of plate:

15g agar was added to 1 litre of LB medium. Then the medium was autoclaved and then allowed to cool to 50°C. 30-35ml medium was poured in each plate. After solidification is complete, 100μl of 100mM IPTG and 20 μl of 50mg/ml X-Gal was added and allowed to absorb for 30 minutes at 37°C prior to use.
• **Ligation of cDNA fragment into Vector:**

  a) pGEM-T Easy vector tube (Promega, USA) and control insert DNA tubes were briefly centrifuged for collecting the contents at the bottom.

  b) Then the ligation reactions were set up as described below: 2X Rapid ligation buffer was vigorously shake before use.

    | Reagents                        | Standard reaction | Control      |
    |--------------------------------|-------------------|--------------|
    | 2X Rapid ligation buffer       | 5 µl              | 5 µl         |
    | pGEM-T Easy vector             | 1 µl              | 1 µl         |
    | PCR product                    | (70ng)            | –            |
    | Control inserts DNA            | –                 | 2 µl         |
    | T4 DNA ligase (3 Weiss unit/ µl)| 1 µl              | 1 µl         |

    De-ionized water to make the final volume 10 µl

    This reaction mixture were mixed well by pipetting and then incubated overnight at 4°C for getting maximum number of ligated vectors.

• **Transformation into Host cell:**

  a) 20 µl of Host cell (JM109) was taken and mixed with 10ml LB medium.

    Then incubated overnight at 37°C in a shaker incubator.

  b) Next day 150 µl from this overnight broth was taken and added to 10ml of fresh LB medium and was incubated at 37°C for 2.5 hrs in a incubated shaker.

  c) After 2.5 hrs the culture was centrifuged at 6000rpm for 5min at 4°C.
d) Pellet was taken. 1 ml chilled CaCl$_2$•2H$_2$O (50mM) was added. Then the mixture was centrifuged at 6000rpm for 5min at 4°C.

e) Soup was discarded. 200µl CaCl$_2$•2H$_2$O (50mM) was added and was vortexed.

f) Then the tubes were kept in ice for 2 hr.

g) Then 10µl of ligation mixture was added to it. Then it was again kept in ice for 20 min.

h) Heat shock was given to the cells at 42°C for 90 sec in a water bath.

i) Then the tubes were immediately inserted into ice and kept there for 15-20 min.

j) Then 800µl fresh LB medium was added and tubes were incubated for 1.5 hrs at 37°C.

k) Then the mixture was centrifuged at 6000rpm for 5 min at 4°C.

l) The pellet obtained was dissolved in 200µl LB medium.

m) Then plating was done in LB / Ampicillin / IPTG / X-Gal plate.

n) Then the plates were incubated overnight at 37°C in a shaker incubator.
Figure 1.12- Transformed colonies (Blue) with ligated product in 100 µl of cells in LB agar with Ampicillin (50µg/ml)

o) Next day white colonies were selected from the plates and these white colonies were dissolved in 10ml LB (Amp+) tubes and were incubated at 37°C for overnight.

p) Next day the culture was centrifuged at 6000 rpm at 37°C for obtaining the pellet. Then Plasmid isolation was carried out from this pellet.
• Plasmid DNA Isolation by Alkaline Lysis Method:

Principle:

Plasmids are double stranded, circular, self-replicating extra chromosomal DNA molecule. Alkaline lysis method for rapid purification of plasmids exploits the topological difference between circular plasmids and linear chromosomal fragments. Cells are first treated with solution I which contains glucose to maintain the osmolarity, Tris EDTA buffer is used as EDTA being a chelating agent chelates the magnesium ions thus rendering DNAase enzyme inactive. Cells are lysed by treating with alkali (NaOH) and a detergent, Sodium dodecyl sulphate (SDS) (Solution II). SDS denatures bacterial proteins and NaOH denatures the plasmid and chromosomal DNA. The incubation time for SDS treatment is very crucial. However, in the case of plasmids the strands remain closely circularized since they are linked by the interwined backbones of double helix. In contrast, strands of linear/ nicked DNA are free to separate completely. When this mixture of denatured plasmid and chromosomal DNA is neutralized by the addition of sodium acetate or potassium acetate (Solution III), renaturation occurs. Renaturation of plasmid is rapid and accurate since the strands are already in close physical proximity. Linear molecules generated by random shearing of chromosomal DNA renature less accurately forming networks of DNA that can be removed from lysate by centrifugation together with denatured protein and RNA. Plasmid DNA remains in solution and can be precipitated using ethanol/ isopropanol. Agarose gel electrophoresis separates plasmid DNA by its size and shape. A preparation of plasmid DNA has predominantly supercoiled
form that runs faster by virtue of its compact structure. Introduction of single or
double stranded breaks lead to presence of relaxed and linear forms
respectively. These forms run more slowly since their open structures experience
more resistance passing through the gel matrix. These are seen as bands above
the gel matrix. Vector or a plasmid carrying an insert runs slower than the
plasmid alone.

Reagents required:

1) Alkaline Lysis Solution I (GTE) (Plasmid Preparation)
   
<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>50Mm</td>
</tr>
<tr>
<td>Tris-HCl (pH 8)</td>
<td>25mM</td>
</tr>
<tr>
<td>EDTA (pH 8)</td>
<td>10Mm</td>
</tr>
</tbody>
</table>
   
   Prepare Solution I from standard stocks in batches of 100ml, autoclave for 15
   minutes at 15 psi (1.05 Kg/cm²) on liquid cycle, and store at 4°C.

2) Alkaline Lysis Solution II (Plasmid Preparation)

   NaOH—0.2(N) (freshly diluted from a 10N stock)
   SDS—1% (w/v)

   Prepare Solution II fresh and use at room temperature.

3) Alkaline Lysis Solution III (Plasmid Preparation)

   5M potassium acetate  60 ml
   Glacial acetic acid   11.5 ml
   H₂O                   28.5 ml
The resulting solution is 3M with respect to potassium and 5M with respect to acetate. Store the solution at 4°C and transfer it to an ice bucket just before use.

4) Gel running buffer TAE buffer Stock Solution/L 50X
5) EDTA (0.5 M, pH 8.0)
6) 10X Tris EDTA (TE)
7) Tris-Cl (1M) pH 8
8) Isopropanol
9) 6X Gel loading Buffers: 0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol, 30% (v/v) glycerol in H2O.
10) Ethidium Bromide (10 mg/ml)

Protocol:

a) 1.5 ml of overnight culture of *E. coli* XL1Blue cells grown in LB-Amp broth was taken in an eppendorf.

b) It was centrifuged at 10000 rpm for 10 mins at 4°C.

c) The supernatant was discarded and to the pellet 100μl of solution I was added and mixed by vortexing.

d) To it 200μl of solution II was added and mixed by inverting the vial.

e) Immediately 150μl solution III was added and the vial was placed in ice for 30 min.

f) It was centrifuged at 15000 rpm for 20 mins at 4°C.
g) The pellet was discarded and the supernatant was collected in a fresh vial.

h) To it 0.6 volume of isopropanol was added and the vial was kept at -20°C for 30 min.

i) The vial was centrifuged at 15000 rpm for 20 mins at 4°C.

j) The supernatant was discarded and the pellet was washed with 70% ethanol and kept for air drying.

k) The pellet was dissolved in Tris EDTA buffer (pH 8).

l) Required amount of loading dye was added to the sample and the sample was loaded in 1% agarose gel (prepared in 1X TAE buffer) and was electrophoresed.

![Figure 1.13- Isolated Plasmids as seen in 1.5% agarose gel](image)

**Figure 1.13- Isolated Plasmids as seen in 1.5% agarose gel**

*Lane-1, 3 pGEMT vector without insert (3000bp)*

*Lane-2, 4 pGEMT vector with insert (4173bp)*
• Sequencing in Automated DNA Sequencer:

Sequencing was done in 3130XL Genetic Analyzer (Applied Biosystems, USA).

• Reagents required:

1. Ready Reaction mix
2. Big Dye terminator sequencing buffer.

The Sanger PCR reaction was carried out by adding reagents as described below:

• Ready Reaction mix-0.4μl/reaction
• 5X buffer- 2 μl
• DNA (Plasmid)- 150-200ng
• T7,SP6 Primer [T7-Forward primer,SP6-Reverse primer]-100ng/μl
• Deionized water to make final volume 10 μl.

The temperature profile of PCR reaction is given below:

![Temperature profile of PCR reaction](image-url)
• **Purification of Extension product:**
  a) 10 μl of deionized water was added to each PCR tube.
  b) Total 20 μl was taken into an microcentrifuge tube.
  c) 2 μl 125mM EDTA was added.
  d) 2 μl of 3M Sodium Acetate was added (pH-5.2)
  e) 50 μl 100% ethanol was added.
  f) Mixed well and was kept at room temperature for 20min.
  g) Then the tubes were centrifuged at 13,000 rpm for 20 min.
  h) Then the washing was done by adding 200 μl 70% Ethanol.
  i) Then the tubes were centrifuged at 12,000 rpm for 20 min.
  j) The samples were air dried.

• **Loading and Electrophoresis on the ABI PRISM 310:**
  a) 12 μl High Dye Formamide was added to each tube and kept in dark for 20min.
  b) Then the tubes were heated at 95°C for 5 min.
  c) Then the tubes were transferred to ice immediately.
  d) Then samples were loaded (10 μl) in micro-titter plate and sequencing was done.

❖ **BLASTN analysis:**

BLASTN analysis was performed with obtained sequence of Chalcone Synthase cDNA (of both high and low antioxidant / 6-gingerol containing landraces) with submitted database in NCBI.
Results and Discussion
Species specific AFLP marker development in three species of *Zingiber* genus:

Seven primer pairs were used in AFLP fingerprinting for all the landraces of three species of *Zingiber* genus viz- *Zingiber officinale*, *Zingiber montanum* and *Zingiber zerumbet*. The AFLP alleles which are unique in *Zingiber officinale* landraces, using seven primer pairs and their respective size of alleles in bp is shown in Table-1.3. Similarly alleles, specific for *Zingiber montanum* and *Zingiber zerumbet* landraces with their respective size (in bp) is shown in Table-1.4 and Table-1.5 respectively. Such data on DNA characterization reveals that landraces of each species being similar regardless of their place of collection. The study identified species specific AFLP markers for the three *Zingiber* species. This suggests that DNA fingerprinting (viz. AFLP) may be used as a dependable identifying parameter for species identification even if collected from different locations. The fact that in this study DNA was extracted from leaves also establishes that in species identification and documentation by DNA fingerprinting methods flowers are not essential as is needed for classical taxonomic studies. The infraspecies level variation among landraces of each of the three species is represented by alleles which show variation within landraces of a particular species. AFLP patterns of representative land race of each of the three species using six pairs of primers given in Figure-1.14a and Figure 1.14b.

The dendogram obtained after analysis using NTSys v 2.1 Software indicated three major clusters, each representing one of the three different species of *Zingiber* genus used in the study (Figure-1.15). In the first cluster, six landraces of *Zingiber officinale* clustered together, showing 92.5% similarity among them. The second group
consisted of five landraces of the *Zingiber montanum* grouping together with 95% similarity within the cluster. Similarly, in the third group, five landraces of *Zingiber zerumbet* are clustered together with 95.5% similarity within them. From this dendogram it appears that *Zingiber montanum* and *Zingiber zerumbet* are phylogenitically more closely linked to each other than to *Zingiber officinale*.

The DNA markers represented by unique peaks for each of the three species of the *Zingiber* genus generated in the present study suggests these as a useful reference tool for species identification that circumvents problems associated with morphological or biochemical markers. The frequency of occurrence of unique species specific peaks in AFLP analysis of DNA isolated from crude drug (plant) preparation (Misra et al., 2007) could be used in assay for the presence of a specific species population. AFLP, in particular, has been the method of choice for discriminating between closely related species and authentication of herbs (Passinho-Soares et al., 2006). The significance of this study stems from the fact that it provides an authentication tool to detect adulterants in the crude drug preparations and to maintain the quality standards in the herbal drug industry.
<table>
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Table 1.3- Species specific alleles No and their respective size (in bp) of Zingiber officinale
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Table 1.4- Species specific alleles No and their respective size (in bp) of *Zingiber montanum*
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Table 1.5- Species specific alleles no and their respective size (in bp) of Zingiber zerumbet
Zingiber officinale  
Zingiber montanum  
Zingiber zerumbet  

Primer pair used: EcoRI-ACG + Msel-CAG

Zingiber officinale  
Zingiber montanum  
Zingiber zerumbet  

Primer pair used: EcoRI-AAG + Msel-CTT

Zingiber officinale  
Zingiber montanum  
Zingiber zerumbet  

Primer pair used: EcoRI-ACA + Msel-CTG

Figure 1.14a- Representative species specific AFLP pattern of three species of Zingiber genus using three pairs of primers
Figure 1.14b- Representative species specific AFLP pattern of three species of *Zingiber* genus using three pairs of primer
Figure 1.15- Cluster diagram showing phylogenetic relationship among landraces of three *Zingiber* species and species specific clustering.
Molecular documentation of some wild landraces of *Zingiber officinale*, collected from different areas of North Bengal

**Random Amplified Polymorphic DNA (RAPD) analysis:**

Sixteen decamer primers were used for the PCR reactions and all the sixteen primers could detect polymorphism significantly in the genomic DNA through RAPD analysis. Representative RAPD fingerprinting pattern of studied fifteen landraces of *Zingiber officinale* using three random decamer primers are shown in Figure-1.16. The primer names and the number of polymorphic bands obtained are given (Table-1.6). Out of total 204 bands scored from the sixteen primers, 176 bands were found to be polymorphic and only 28 bands were found to be monomorphic. The binary data scored from all the sixteen primers were used for the calculation of Jaccard’s similarity index. This similarity index helped us to evaluate the genetic relatedness and also the genetic variation of the different landraces of *Zingiber officinale*. This data allowed the development (through NTSYS software) of a dendogram revealing the genetic distances between these genotypes. In the dendogram (Figure-1.17) three clusters were observed. Cluster I contains five genotypes (GCP-18, G-05-07, G-03-05, G-23-05, PGS-08), cluster II contains six genotypes (GCP-16, G-04-07, GCP-08, GCP-27, GCP-05, IG-1) and cluster III contain four (GCP-29, GCP-22, G-01-07, GCP-44) genotypes. The extent of genetic relatedness among *Zingiber officinale* Roscoe genotypes varied considerably (0.703 to 0.912). Highest similarity (0.989) was observed in G-04-07 and GCP-27. Least genetic similarity (0.703) was observed between GCP-16 and G-01-07.
<table>
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<th>Primer Name</th>
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<th>No. of Total bands</th>
<th>No. of polymorphic bands</th>
<th>No. of monomorphic bands</th>
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<td>5'GGAACCCACA3'</td>
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<td>OPAB-06</td>
<td>5'GTGGCTTGGGA3'</td>
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<td>5'AGCAGCGAGG3'</td>
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Table 1.6- Primers used for the RAPD analysis (Name and Sequence). Their number and type of bands of fifteen landraces of *Zingiber officinale* landraces.
Primer used- OPAL-13

Primer used- OPAA-16

Primer used- OPBH-01

Figure 1.16- Representative RAPD picture of fifteen wild landraces of *Zingiber officinale* collected from different areas of North Bengal. M1-1 Kb ladder, M2-100bp ladder
Figure 1.17- RAPD based phylogenetic tree of fifteen wild landraces of *Zingiber officinale* showing phylogenetic relationship among them.
Amplified fragment Length Polymorphism (AFLP) analysis:

Fifteen wild landraces of *Zingiber officinale* have been used for AFLP analysis using six pairs of primer combinations; Representative AFLP fingerprinting pattern of all fifteen landraces using primer pair EcoRl-ACG / Msel-CAG which shows maximum (98.27%) variation among landraces and using primer pair EcoRl-AAC / Msel-CTG which showed least (88.52%) variation are shown in Figure-1.18 and Figure-1.19 respectively. Analysis of data of all the genotypes using seven primer pairs showed that out of total 837 alleles, 781 alleles were polymorphic and 56 alleles were monomorphic (Table-1.7). The similarity matrix, developed from AFLP binary data, was used to determine phylogenetic relationships between these landraces. The extent of this relatedness among these *Zingiber officinale* genotypes was found to vary considerably (0.671-0.890). Highest similarity (0.890) was observed between G-04-07 and GCP-27; few more pairs viz PGS-08 and G-23-05, G-03-05 and GCP-18 landraces also showed significant similarity (0.843 & 0.831 respectively). Least genetic similarity (0.671) was found between GCP-16 and G-01-07. UPGMA cluster analysis was used to determine genetic diversity and phylogenetic relationship among the landraces; this is represented in the dendogram (Figure-1.20). In the dendogram three clusters were observed. Cluster I contains five genotypes (GCP-18, G-05-07, G-03-05, G-23-05, PGS-08), cluster II contains six genotypes (GCP-16,G-04-07,GCP-08, GCP-27,GCP-05, IG-1) and cluster III contain four (GCP-29, GCP-22,G-01-07,GCP-44) genotypes.
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Table 1.7- Primers used for the AFLP analysis, type of bands (Total and Polymorphic) and percent of Polymorphism of fifteen landraces of *Zingiber officinale* landraces (*-Fluorescent dye tagged*)
Figure 1.18- AFLP Fingerprinting pattern of fifteen landraces of *Zingiber officinale* landraces, collected from different areas of North Bengal.

Primer pair used: EcoRI-ACG + Msel- CAG which shows highest (98.27%) polymorphism.
Figure 1.19- AFLP fingerprinting pattern of fifteen landraces of *Zingiber officinale* landraces, collected from different areas of North Bengal.

Primer pair used: EcoRI-AAC+ MseI-CTG which showed lowest polymorphism (88.52%)
Figure 1.20- AFLP based phylogenetic tree of studied wild landraces of *Zingiber officinale* showing phylogenetic relationship among the landraces
Medicinally important compounds analysis of *Zingiber officinale* landraces correlated with DNA profiling data:

- **Total Antioxidant potential determination:**

  Total antioxidant potential of the fifteen landraces of *Zingiber officinale* was evaluated by DPPH colorimetric assay method. All samples were analyzed in triplicates. The results obtained from the analysis of total antioxidant activity of *Zingiber officinale* landraces by DPPH method are shown in Table-1.8 and Figure-1.21. According to the data obtained significant variation were observed among different landraces for antioxidant potential. Some landraces (viz. GCP-18, G-05-07, G-03-05, G-23-05, PGS-08) have high antioxidant potential, some landraces (GCP-29, GCP-22, G-01-07, GCP-44,) have low antioxidant potential in comparison, and some landraces (GCP-16, G-04-07, GCP-08, IG-1, GCP-27, GCP-05) have intermediate value. The average value of antioxidant potential of all these landraces is 85%. Nan-Chen *et al.* (2008) evaluated antioxidant potential of some species of Zingiber genus viz, *Zingiber kawagoii* and *Zingiber oligophyllum* in Taiwan where values are found to be as low as 42% and 32% respectively. Stoiilova *et al.* (2007) have used air-dried roots of *Zingiber officinale* from Vietnam. In their study the *Zingiber officinale* varieties have showed upto 91% antioxidant activity. Maizura *et al.* (2011) reported antioxidant value of *Zingiber officinale* in Malaysia to be 79%. In another study on cultivated varieties of *Zingiber officinale* of India by Shirin and Jamuna (2010), antioxidant potential was shown to be 80%.
<table>
<thead>
<tr>
<th>Sample name</th>
<th>1st Value</th>
<th>2nd Value</th>
<th>3rd Value</th>
<th>Mean Value of triplicates (% of DPPH reduced)</th>
<th>Standard Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>GCP-18</td>
<td>89.47</td>
<td>89.23</td>
<td>89.27</td>
<td>89.32</td>
<td>0.074237</td>
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<tr>
<td>G-05-07</td>
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<td>88.56</td>
<td>89.09</td>
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<td>0.250488</td>
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<tr>
<td>G-03-05</td>
<td>88.32</td>
<td>88.53</td>
<td>87.46</td>
<td>88.10</td>
<td>0.327329</td>
</tr>
<tr>
<td>PGS-8</td>
<td>89.53</td>
<td>89.63</td>
<td>89.84</td>
<td>89.66</td>
<td>0.091348</td>
</tr>
<tr>
<td>G-23-05</td>
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<td>88.39</td>
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<td>GCP-27</td>
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<td>85.68</td>
<td>85.84</td>
<td>85.73</td>
<td>0.051747</td>
</tr>
<tr>
<td>GCP-05</td>
<td>84.34</td>
<td>85.43</td>
<td>85.97</td>
<td>85.58</td>
<td>0.479386</td>
</tr>
<tr>
<td>GCP-8</td>
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<td>85.06</td>
<td>85.47</td>
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<td>0.145717</td>
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<tr>
<td>G-04-07</td>
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<td>85.17</td>
<td>85.21</td>
<td>85.13</td>
<td>0.057831</td>
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<tr>
<td>GCP-16</td>
<td>85.02</td>
<td>85.13</td>
<td>84.84</td>
<td>84.99</td>
<td>0.084525</td>
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<tr>
<td>GCP-22</td>
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<td>82.42</td>
<td>82.58</td>
<td>82.43</td>
<td>0.078387</td>
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<tr>
<td>GCP-29</td>
<td>81.72</td>
<td>81.71</td>
<td>82.25</td>
<td>81.89</td>
<td>0.178357</td>
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Table 1.8- Total Antioxidant potential with Standard Error of fifteen landraces of *Zingiber officinale*

<table>
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<tr>
<th></th>
<th>6-Gingerol</th>
<th>81.09</th>
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<th>81.19</th>
<th>0.141892</th>
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<td>81.01</td>
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<td></td>
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<td>0.141892</td>
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<td>GCP-44</td>
<td>81.31</td>
<td>81.43</td>
<td>81.60</td>
<td>81.44</td>
<td>0.08413</td>
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<tr>
<td>IG-1</td>
<td>85.74</td>
<td>85.66</td>
<td>85.88</td>
<td>85.74</td>
<td>0.21682</td>
</tr>
</tbody>
</table>

- **Estimation of active principle (6-Gingerol) content in *Zingiber officinale* landraces**

The contents of 6-gingerol in the wild land races of were assayed by high performance liquid chromatography (HPLC). The compound was identified and quantified based on retention time using 6-gingerol as HPLC external standard. HPLC chromatogram of two representative landraces (one is of high 6-gingerol content and other is of low 6-gingerol content) and also of the standard (Sigma-Aldrich) is shown in Figure-1.22. While the land races showed variation in 6-gingerol content, it was interesting to note a grouping into high, medium and low levels of 6-gingerol among the different landraces (Table-1.9 and Figure-1.23). A comparative study showed high content of 6-gingerol in GCP-18,G-05-07, G-03-05,G-23-05,PGS-08 landraces, medium content in GCP-16,G-04-07,GCP-08, GCP-27,GCP-05, IG-1 landraces and low 6-Gingerol content in GCP-29,GCP-22,G-01-07,GCP-44 landraces of studied *Zingiber officinale*.
Different methods of chromatography are reported in the literature for assessment of 6-gingerol in *Zingiber officinale*. Gas Chromatographic-Mass-Spectrometric (GC-MS) methods (Harvey, 1981; Chen *et al*., 1986) have been used for analysis of 6-gingerol and its major natural degradation product 6-shogaol. This method however does not appear to be best suited for chemical assay of 6-gingerol; the high temperature associated with GC-MS analysis has been shown to cause conversion of the thermally labile 6-gingerol to 6-shogaol. High-Performance Thin Layer Chromatography (HPTLC) method has also been developed to determine the quantity of 6-gingerol in rhizomes of *Zingiber officinale* (Rai *et al*., 2006). Several High Performance Liquid Chromatographic (HPLC) analysis for assessment of 6-gingerol have been reported (Yoshikawa *et al*., 1993; Nakazawa and Ohsawa, 2002; He *et al*., 1998; Bhattarai *et al*., 2001) till date as this appears to be the preferred method for assessment the content of 6-gingerol.
<table>
<thead>
<tr>
<th>Sample name</th>
<th>1st value (mg/g of fresh tissue)</th>
<th>2nd value (mg/g of fresh tissue)</th>
<th>Mean value (mg/g of fresh tissue)</th>
<th>Standard Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>GCP-18</td>
<td>98.6</td>
<td>97.43</td>
<td>98.01</td>
<td>0.585</td>
</tr>
<tr>
<td>G-05-07</td>
<td>102.7</td>
<td>100.23</td>
<td>101.46</td>
<td>1.235</td>
</tr>
<tr>
<td>G-03-05</td>
<td>96.5</td>
<td>95.32</td>
<td>95.91</td>
<td>0.59</td>
</tr>
<tr>
<td>PGS-08</td>
<td>110.6</td>
<td>110.1</td>
<td>110.35</td>
<td>0.25</td>
</tr>
<tr>
<td>G-23-05</td>
<td>110.7</td>
<td>110.2</td>
<td>110.45</td>
<td>0.25</td>
</tr>
<tr>
<td>IG-1</td>
<td>69.40</td>
<td>69.80</td>
<td>69.60</td>
<td>0.45</td>
</tr>
<tr>
<td>GCP-27</td>
<td>66.36</td>
<td>65.76</td>
<td>66.06</td>
<td>0.3</td>
</tr>
<tr>
<td>GCP-05</td>
<td>61.11</td>
<td>60.89</td>
<td>61.00</td>
<td>0.11</td>
</tr>
<tr>
<td>GCP-08</td>
<td>64.81</td>
<td>63.76</td>
<td>64.28</td>
<td>0.525</td>
</tr>
<tr>
<td>G-04-07</td>
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<td>65.31</td>
<td>65.86</td>
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<tr>
<td>GCP-16</td>
<td>67.42</td>
<td>67.32</td>
<td>67.37</td>
<td>0.05</td>
</tr>
<tr>
<td>G-01-07</td>
<td>36.34</td>
<td>35.67</td>
<td>36.00</td>
<td>0.335</td>
</tr>
<tr>
<td>GCP-22</td>
<td>34.24</td>
<td>34.12</td>
<td>34.18</td>
<td>0.06</td>
</tr>
<tr>
<td>GCP-29</td>
<td>31.62</td>
<td>30.98</td>
<td>31.3</td>
<td>0.32</td>
</tr>
<tr>
<td>GCP-44</td>
<td>37.41</td>
<td>36.67</td>
<td>37.04</td>
<td>0.37</td>
</tr>
</tbody>
</table>

Table 1.9- 6-gingerol content (mg/g of fresh tissue) of fifteen landraces of *Zingiber officinale* landraces with standard error
Figure 1.21- Bar diagram showing total antioxidant potential in studied landraces of *Zingiber officinale*

X-axis: Sample accession number,

Y-axis: Total antioxidant potential (% of DPPH reduced)
Figure 1.22- Representative HPLC Chromatogram of 6-gingerol content in *Zingiber officinale* landraces [X-axis: Time Y-axis: mAU]

(A) 6-Gingerol standard from SIGMA

(B) Representative chromatogram of landrace (GCP-18) possessing high 6-gingerol content (Peak area: 68, 72312)

(C) Representative chromatogram of landrace (GCP-22) possessing 6-gingerol content (Peak area: 20, 32168)
Figure 1.23- Bar diagram showing 6-gingerol content in studied landraces of *Zingiber officinale*.

**X-axis:** Sample accession number,

**Y-axis:** Content of 6-gingerol (mg/g of fresh tissue)
• Correlative studies of RAPD analysis and total antioxidant content in *Zingiber officinale* landraces:

The similarity matrix scored from the RAPD analysis based binary matrix led to the development of a dendogram using NTSYS software (Figure-1.17). The three clusters observed in the dendogram showed close genetic relationship among landraces within each cluster, while each cluster being distantly related to each other. Interestingly, the three clusters were found to be correlated with total antioxidant potential of the landraces (Figure-1.24); the most distantly related clusters (viz. Cluster-I and Cluster III) contain landraces possessing highest (89.32% - 87.30%) and lowest (82.43% - 81.44%) antioxidant potential. The data showed that the intermediate cluster viz. Cluster-II contains landraces which possess intermediate level of antioxidant (85.74 % - 84.99 %).

• Correlative studies of AFLP analysis and 6-Gingerol content in *Zingiber officinale* landraces for development of Trait (medicinal) related DNA Marker:

The similarity matrix scored from the AFLP analysis based binary matrix led to the development of a dendogram using NTSYS software (Figure-1.20). The three clusters observed in the dendogram showed close genetic relationship among landraces within each cluster, while each cluster being distantly related to each other. Interestingly, the three clusters were found to be correlated with 6-gingerol content of the landraces (Figure-1.25); the most distantly related clusters (viz. Cluster-I and Cluster III) contain landraces possessing highest (110.70 % - 96.5 %) and lowest (47.41 % - 31.62 %).
amount of 6-gingerol. The data showed that the intermediate cluster viz. Cluster-II contains landraces which possess intermediate level of 6-gingerol (77.42 % – 64.81 %).

Correlations between morphological and biochemical (viz. antioxidant, 6-gingerol content) parameters (quantitative traits) of the studied landraces were examined using Karl Pearson correlation test. The Karl Pearson correlation coefficients for different morphological and biochemical parameters are shown in Table-1.10. A strong positive correlation was observed between total antioxidant potential and 6-Gingerol content (r = +0.902 which was significant at p<0.01). The association between AFLP markers and the quantitative traits was estimated through stepwise Multiple Regression Analysis (MRA), where each quantitative trait was treated as dependent variable, while the AFLP markers treated as independent variables. After Multiple Regression analysis, total four alleles (generated by primers EcoRI-AAG / Msel-CTC, EcoRI-ACC / Msel-CTG, EcoRI-AAC / Msel-CTG, and EcoRI-ACG / Msel-CAG) showed positive correlation with 6-Gingerol content. Out of them, the 149 base pair allele generated by EcoRI-AAG / Msel-CTG showed maximum ($R^2$=0.985) correlation with 6-Gingerol content and highly significant (p<0.01, t=13.243). The regression coefficient ($\beta$) was also very high (0.941). Another marker is of 73 base pair generated by EcoRI-AGC / Msel-CTG shows moderate correlation with 6-Gingerol content ($R^2$=0.930, t=7.543, $\beta$= 0.613) shown in Table-1.11.
### Table 1.10 - Karl Pearson correlations for various morphological and biochemical parameters

<table>
<thead>
<tr>
<th>Pearson Correlation Coefficient</th>
<th>VAR-01</th>
<th>VAR-02</th>
<th>VAR-03</th>
<th>VAR-04</th>
<th>VAR-05</th>
<th>VAR-06</th>
</tr>
</thead>
<tbody>
<tr>
<td>VAR-01</td>
<td>1.000</td>
<td>0.902**</td>
<td>0.013</td>
<td>-0.243</td>
<td>-0.189</td>
<td>-0.253</td>
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<tr>
<td>VAR-02</td>
<td>0.902**</td>
<td>1.000</td>
<td>0.002</td>
<td>-0.276</td>
<td>-0.158</td>
<td>-0.346</td>
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<tr>
<td>VAR-03</td>
<td>0.013</td>
<td>-0.002</td>
<td>1.000</td>
<td>0.733**</td>
<td>0.555*</td>
<td>0.249</td>
</tr>
<tr>
<td>VAR-04</td>
<td>-0.243</td>
<td>-0.276</td>
<td>0.733**</td>
<td>1.000</td>
<td>0.449</td>
<td>0.524*</td>
</tr>
<tr>
<td>VAR-05</td>
<td>-0.189</td>
<td>-0.158</td>
<td>0.555*</td>
<td>0.449</td>
<td>1.000</td>
<td>0.443</td>
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<tr>
<td>VAR-06</td>
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<td>0.249</td>
<td>0.524*</td>
<td>0.443</td>
<td>1.000</td>
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</table>

** Correlation is significant at the 0.01 level (2-tailed).  
* Correlation is significant at the 0.05 level (2-tailed).
<table>
<thead>
<tr>
<th>Biochemical parameter</th>
<th>Primer pair used</th>
<th>AFLP marker</th>
<th>r</th>
<th>$R^2$</th>
<th>Standardised coefficient $\beta$</th>
<th>t value</th>
<th>Standard Error (SE)</th>
<th>P Value</th>
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</thead>
<tbody>
<tr>
<td>6-gingerol content</td>
<td>EcoRI-AAG + Msel-CTG</td>
<td>149 bp</td>
<td>0.992</td>
<td>0.985</td>
<td>0.941</td>
<td>13.243</td>
<td>6.0001</td>
<td>0.001</td>
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<tr>
<td></td>
<td>EcoRI-AGC + Msel-CTG</td>
<td>73 bp</td>
<td>0.970</td>
<td>0.930</td>
<td>0.613</td>
<td>7.543</td>
<td>4.3268</td>
<td>0.001</td>
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</table>

Table 1.11- Coefficients for dependent variable in the stepwise MRA for association of AFLP markers with 6-gingerol content in *Zingiber officinale* landraces
Figure 1.24 - RAPD based clustering in the phylogenetic tree of fifteen landraces of *Zingiber officinale* correlated with total antioxidant potential
Figure 1.25- AFLP based clustering in the phylogenetic tree of fifteen landraces of *Zingiber officinale* correlated with 6-gingerol content.

<table>
<thead>
<tr>
<th>Sample name</th>
<th>6-Gingerol content (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GCP-18</td>
<td>98.01±0.032</td>
</tr>
<tr>
<td>G-05-07</td>
<td>101.46±0.043</td>
</tr>
<tr>
<td>G-03-05</td>
<td>95.91±0.456</td>
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<td>PGS-8</td>
<td>110.35±0.564</td>
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<tr>
<td>G-23-05</td>
<td>110.45±0.234</td>
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<tr>
<td>IG-1</td>
<td>69.60±0.343</td>
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<tr>
<td>GCP-27</td>
<td>66.06±0.454</td>
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<tr>
<td>GCP-05</td>
<td>61.00±0.123</td>
</tr>
<tr>
<td>GCP-8</td>
<td>64.28±0.343</td>
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<tr>
<td>G-04-07</td>
<td>65.86±0.544</td>
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<td>GCP-16</td>
<td>67.37±0.565</td>
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<tr>
<td>G-01-07</td>
<td>36.00±0.345</td>
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<tr>
<td>GCP-22</td>
<td>34.18±0.782</td>
</tr>
<tr>
<td>GCP-29</td>
<td>31.30±0.658</td>
</tr>
<tr>
<td>GCP-44</td>
<td>37.04±0.111</td>
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Single Nucleotide Polymorphism analysis of Chalcone Synthase gene of *Zingiber officinale*, correlated with medicinal potential

Chalcone Synthase is the rate limiting enzyme of Phynyl Propanoid Pathway which is responsible for production of secondary metabolites including antioxidants and 6-Gingerol in ginger. In an attempt to understand the molecular mechanism for variation in 6-gingerol content among the landraces, SNP analysis of Chalcone Synthase gene (cDNA) of high and low antioxidant as well as 6-gingerol containing wild landraces (four from each group) of *Zingiber officinale* was performed. The sequence of Chalcone Synthase cDNA (1173bp) of high and low 6-gingerol containing landraces (representative) obtained after cloning in pGEM-T easy vector (Promega, USA) and subsequently sequencing in automated DNA sequencer with converted amino acid sequences are shown in Figure-1.26 and Figure-1.27. BLASTN analysis of obtained sequence of Chalcone Synthase cDNA was performed and it was observed that this sequence showed 100% homology and similarity with submitted (in NCBI sequence id-DQ486012.1) complete cDNA sequence of Chalcone Synthase gene (Figure-1.28a and Figure 1.28b) of *Zingiber officinale*. Single Nucleotide Polymorphism was detected between high and low 6-gingerol containing landraces with respect to single base i.e. seven SNPs were detected between high and low antioxidant / 6-Gingerol containing landraces. It is interesting to note that all the landraces of high antioxidant potential have similar SNPs whereas all landraces of low antioxidant potential contain specifically different SNPs that are consistent among the low antioxidant / 6-gingerol containing landraces. On conversion of the nucleotide sequence to amino acid sequence in six reading frames, it was observed that two SNPs caused change in amino acid sequence.
whereas rest five SNPs do not show any change in corresponding amino acid presumably due to degeneracy of codon. At position 142 and 291 of chalcone synthase polypeptide, Serine (S) in high antioxidant / 6-gingerol containing landraces has been changed to Leucine (L) and Asparagine (N) respectively in low antioxidant / 6-gingerol containing landraces (Figure-1.29 a,b & Figure-1.30 a,b). SNP markers associated with economic traits in plants are developed in many species. In rice SNP in Wx gene associated with amylase content have been developed (Ayres et al. 2000). In wheat, SNPs in Pinb gene (Huang and Coder, 2005) associated with grain hardness and Rht gene (Ellis et al. 2005) associated with dwarfism have been developed. SNP markers also developed in Onion (Acala et al, 1997), Barley (Chiapparino et al, 2003), Soybean (Jang and Lee, 2003), Oilseed mustard (Gupta et al, 2004) associated with trait of interest. These markers have the potential to be used in MAS and may enable extrapolation into transgenes. In this study SNP markers have been established in Zingiber officinale Roscoe, which are correlated with antioxidant potential / 6-gingerol content.
Figure 1.26 - cDNA sequence (with converted amino acid sequence) of Chalcone Synthase gene of representative (GCP-18) Zingiber officinale landraces containing high medicinal potential
Figure 1.27- cDNA sequence (with converted amino acid sequence) of Chalcone Synthase gene of representative (G-01-07) *Zingiber officinale* landraces containing low medicinal potential
Nucleotide Sequence (1173 letters)

<table>
<thead>
<tr>
<th>Query ID</th>
<th>Description</th>
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Database Name: nr
Description: Nucleotide collection (nt)
Program: BLASTN 2.2.28+

Graphic Summary

Distribution of 103 Blast Hits on the Query Sequence

Color key for alignment scores

Figure 1.28a- Graphic summary of BLASTN analysis of Chalcone synthase cDNA sequence in NCBI
Figure 1.28b- BLASTN analysis of chalcone synthase cDNA sequence (obtained in this study) which shows 100% similarity with submitted (Accession no-DQ486012.1) sequence in NCBI
Figure 1.29a- Portion of sequenced Chalcone Synthase cDNA of *Zingiber officinale* showing four Single Nucleotide Polymorphisms (Red), among them three causes no change in amino acid but one change (S to L) is observed (Green).

(A)-representing landrace (GCP-18) of High medicinal potential.

(B)- representing landrace (G-01-07) of Low medicinal potential.

![Figure 1.29a](image-url)

Figure 1.29b- Portion of the sequence showing C→T polymorphism in Chalcone Synthase cDNA at position 149 causing change in amino acid (Serine → Leucine).
Figure 1.30a- Portion of sequenced Chalcone Synthase cDNA of *Zingiber officinale* showing three Single Nucleotide Polymorphisms (Red), among them two causes no change in amino acid but one change (S to N) is observed (Green).

(A)- representing landrace (GCP-18) of High medicinal potential.

(B)- representing landrace (G-01-07) of Low medicinal potential.

Figure 1.30b- Portion of the sequence showing C→T polymorphism in Chalcone Synthase cDNA at position 281 causing change in amino acid (Serine → Asparagine).