# GENETIC DIVERSITY ANALYSIS OF *Artocarpus heterophyllus* Lam. USING SCoT MARKERS FOR ANTIOXIDANT AND ANTI–INFLAMMATORY PROPERTIES

**By**

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## THESIS

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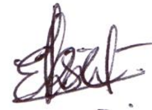
**KERALA, INDIA**

**2020**

## DECLARATION

I, hereby declare that the thesis entitled "**Genetic diversity analysis of A*rtocarpus heterophyllus* Lam. using SCoT markers for antioxidant and anti–inflammatory properties**" is a bonafide record of research done by me during the course of research and that the thesis has not previously formed the basis for the award of any degree, diploma, fellowship or other similar title, of any other University or Society



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# CERTIFICATE

This is to certify that this thesis entitled **"Genetic Diversity analysis of *Artocarpus heterophyllus* Lam. using SCoT markers for antioxidant and anti-inflammatory properties"** is a record of research work done by **Ms. Elsit Mariya C (2015-09-014)** under my guidance and supervision and that this has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to her.

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| α | Alpha |
| β | Beta |
| % | percentage |
| ° C | Degree Celsius |
| µg | microgram |
| ABTS | 2,2-Azinobis 3-ethylenzthiazoline-6-sulphonic acid |
| AChE | Acetylcholinesterase |
| ACE | Angiotensin Converting Enzyme |
| AEAC | Ascorbic acid Equivalent Antioxidant Capacity |
| AFB1 | Aflatoxin B1 |
| AFLP | Amplified Fragment Length Polymorphisms |
| approx. | approximately |
| bp | basepair |
| CDDP | Conserved DNA Derived Polymorphism |
| cDNA | Complementary DNA |
| CMV | cytomegalo virus |
| COA | College of Agriculture |
| CORAP | Conserved Region Amplification Polymorphism |
| COX-2 | Cyclooxygenase - 2 |

| | |
|---|---|
| CTAB | Cetyl Trimethyl Ammonium Bromide |
| DNA | Deoxyribo nucleicacid |
| DPPH | 2,2 diphenylpicrylhydrazyl |
| DW | Dry Weight |
| EDTA | Ethylene Diamine Tetra Acetic acid |
| EST | Expressed Sequence Tags |
| FCR | Folin–Ciocalteu Reagent |
| FRAP | Ferric Reducing Antioxidant Power Assay |
| g | gram |
| GAE | Gallic Acid Equivalent |
| GPS | Global Positioning System |
| HIV | Human Immuno Deficiency Virus |
| HRBC | Human Red Blood Cell membrane |
| HSV-2 | Herpes Simplex Virus type 2 |
| IFN-γ | Interferon-γ |
| iNOS | inducible Nitric Oxide Synthase |
| IPGRI | The International Plant Genetic Resources Institute |
| IRAP | Inter Retrotransposon Amplified Polymorphism |
| ISSR | Inter Simple Sequence Repeats |
| IU | International Units |

| | |
|---|---|
| JNTBGRI | Jawaharlal Nehru Tropical Botanic Garden and Research Institute |
| KAU | Kerala Agricultural University |
| Kg | Kilogram |
| Lam. | Lamark |
| LPS | Lipopolysaccharide |
| *M* | Molar |
| mg | Milligram |
| mL | Milliliter |
| mm | Millimetre |
| MPPCR | Microsatellite primed PCR |
| $Na_2CO_3$ | Sodium Carbonate |
| NBPGR | National Bureau of Plant Genetic Resources |
| NIH | National Institute Of Health |
| nm | Nanometer |
| OD | Optical Density |
| ORAC | Oxygen Radical Absorbance Capacity |
| ORF | Open Reading Frames |
| PAGE | Poly Acrylamide Gel Electrophoresis |
| PBA | P450-Based Analogues |
| PCR | Polymerase Chain Reaction |

| | |
|---|---|
| PGE2 | Prostaglandin $E_2$ |
| pH | Potential of Hydrogen |
| PSC | Peroxyl Scavenging Capacity |
| PVP | Polyvinylpyrrolidone |
| QE | Quercetin Equivalent |
| Q-PCR | Quantitative Polymerase Chain Reaction |
| RAPD | Random Amplification of Polymorphic DNA |
| RB | Round Bottom |
| RBIP | Retrotransposon-Based Insertion Polymorphism |
| REMAP | Retrotransposon Microsatellite Amplified polymorphism |
| RFLP | Restriction Fragment Length Polymorphism |
| RNA | Ribo Nucleic Acid |
| rpm | revolutions per minute |
| RSA | Radical Scavenging Activity |
| SCAR | Sequence Characterized Amplified Region |
| SCoT | Start Codon Targeted Marker |
| SNP | Single Nucleotide Polymorphisms |
| SRAP | Sequence Related Amplified Polymorphism |
| SSAP | Retrotransposon Based Sequence Specific Amplification Polymorphism |
| SSR | Simple Sequence Repeats |
| TAE | Tris Acetate EDTA |

| | |
|---|---|
| TE | Tris EDTA |
| TEAC | Trolox Equivalent Antioxidant Capacity |
| TFM | Targeting Fingerprinting Marker |
| TLC | Thin Layer Chromatography |
| TNF | Tumor Necrosis Factor |
| TOSC | Total Oxyradical Scavenging Capacity |
| TPC | Total Phenolic Content |
| TRAP | Targeted Region Amplified Polymorphism |
| UV | Ultra Violet |
| VNTR | Variable Number Tandem Repeat |

# 1. INTRODUCTION

*Artocarpus heterophyllus* Lam., commonly known as Jackfruit, belonging to the genus *Atrocarpus*, is one of the significant trees in tropical home gardens with widespread uses. It belongs to the Moraceae family along with other prominent members such as figs, mulberries and breadfruit. It is believed to be originated from Western Ghats of rainforest of India and Malaysia, commonly seen in south-eastern Asia, central and eastern Africa and also in the Caribbean islands, Florida, Brazil, Australia, Puerto Rico and many Pacific Islands (Rahman *et al*., 1999). In our country, jackfruit is most commonly seen in southern states like Kerala, Tamil Nadu, Karnataka, Goa, coastal Maharashtra and other states like, Assam, Bihar, Tripura, Uttar Pradesh and foothills of Himalayas. In March 2018 jackfruit was declared as the official fruit of Kerala.

The primary economic portion of jackfruit is the fruit which can be used both as raw and ripe, that significantly contributes to the nutrition for the people. The parts of jack tree such as leaves, wood, latex, ash of leaves, roots and bark have been reported to have medicinal properties from ancient times along with fruits. It has got protective activity against various parasitic infections and lifestyle disease. The plant is rich in phytocompounds like morin, artocarpin, dihydromorin, cynomacurin, flavonoids, stilbenoids, isoartocarpin, cycloartocarpin, artocarpesin, artocarpetin, artocarpanone, oxydihydro artocarpesin, norartocarpetin and cycloartinone (Wetprasit *et al*., 2000) making it potent antioxidant, anti-inflammatory, antibacterial, antifungal, anticariogenic, antineoplastic, cicatrizent and hypoglycemic (Yao *et al*., 2016).

The jackfruit is rich in useful antioxidant compounds with good carotenoids composition (Ko *et al*., 1998). Besides the total phenolic content in jackfruit, vitamin C (ascorbic acid), vitamin E (α-tocopherol), other non-enzymatic antioxidants like flavonoids and related polyphenol such as α-lipoic acid and glutathione are reported for the antioxidant activity (Swami *et al*., 2012).

The anti-inflammatory effects of the characterized phenolic compounds viz., artocarpesin ((5,7,2,4-tetrahydroxy-6-β-methylbut-3-enyl) flavone), norartocarpetin (5,7,2,4 tetrahydroxyflavone), oxyresveratrol (trans-2,4,3,5-tetrahydroxystilbene) (Venkataraman, 2001) and flavonoids like cycloartomunin, artonins A and B, artocarpanone, cyclomorusin, dihydrocycloartomunin, dihydroisocycloartomunin, cudraflavone A, artomunoxanthone and cycloheterohyllin, artocarpanone A and heteroflavanones A were reported in jackfruit (Wei *et al*., 2005).

Jackfruit being the largest edible fruit that attained acceptance in recent years, due to this reason more basic studies need to be conducted to find out the relationship between its medicinal properties and genetic variability. DNA-based molecular markers technique are important in plant genomeresearch with an applications in pharmacognostic identification and analysis

The degree of genetic diversity in jackfruit accessions from the National Bureau of Plant Genetic Resource (NBPGR) Thrissur and wild samples from forest areas were assessed using Start Codon Target (SCoT) markers (Collard *et al*., 2009). SCoT markers are based on a short conserved region in plant genes surrounding the ATG translation start (or initiation) codon that has been well characterized in previous studies (Joshi *et al*., 1997; Sawant *et al*., 1999) and are generally reproducible.

In this context, the study entitled "Genetic diversity analysis of *Artocarpus heterophyllus* Lam. using SCoT markers for antioxidant and anti-inflammatory properties" was undertaken to analyze the phytochemical components and molecular diversity in a set of twenty jackfruit genotypes with the following objectives.

1. To screen phytochemical constituents of edible fruit parts for antioxidant and anti-inflammatory activities.
2. To analyse the genetic diversity of *Artocarpus heterophyllus* Lam. samples collected from different areas of Kerala using SCoT markers.

## 2. REVIEW OF LITERATURE

The present study involved "Genetic diversity analysis of *Artocarpus heterophyllus* Lam. using SCoT markers for antioxidant and anti-inflammatory properties". A brief review of literature related to the study was collected, reviewed and arranged in following subtopics.

2.1 Introduction and Botanical Description of Jackfruit

2.2 Origin and distribution of the fruit

2.3 Importance of Jackfruit

2.4 Genetic Diversity studies in Jackfruit

2.5 Molecular Markers

2.6 Molecular Analysis

2.7 Phytochemical Composition

2.8 Pharmacological Aspects

2.9 Phytochemical assays

### 2.1INTRODUCTION TO JACKFRUIT

Jackfruit is a tropical climatric fruit from the family Moraceae, native to the rain forest of Western Ghats of India. It is the largest edible fruit that is a popular and inexpensive fruit in Southern Asia. It is known as poor man's food and is rich in moisture, nutrients, minerals and phytochemicals (Ranasinghe *et al*.,2019).

Jackfruit thrives in tropical, warm and humid, frost-free climatic area with well drained but moist soil, with a pH of 4.3 – 8.0 and with medium soil fertility. It must be irrigated in order to produce fruits in drought condition. Growth habits vary from tall and straight with a thin trunk to short with a thick trunk, also with soil type, environment and cultivar. The optimum temperature is 19 – 29˚C, altitude at approx. 1600 meters above sea level and the annual rain fall between 1000 and 2400 mm (Roy *et al*., 1996).

Jackfruit has got numerous nutritional and medicinal potential not only to the fruit but also to its leaves, stem, bark, root and latex (Prakash *et al.*, 2009). Fruits can be consumed as raw in salads and when ripened. It can also be preserved as jam, jelly, chips or can be cooked as a vegetable. Seeds can be boiled, roasted or baked and powdered to use as flour.

| | |
|---|---|
| **Kingdom** | : Plantae |
| **Subkingdom** | : Tracheobionta |
| **Superdivision** | : Spermatophyta |
| **Division** | : Magnoliophyta |
| **Class** | : Magnoliopsida |
| **Subclass** | : Hamamelididae |
| **Order** | : Urticales |
| **Family** | : Moraceae |
| **Genus** | : *Artocarpus* |
| **Species** | : *Artocarpus heterophyllus* |

**Figure 2.1** Jackfruit tree with fruit

**2.1.1 Botanical Description to Jackfruit**

Jackfruit is succulent, aromatic, flavorful, compound fruit with variable fruit characteristics (fruit flesh, size, shape, density of spines, colour, texture, odour, quality, and period of maturity as per (Jagadeesh *et al.*, 2007)) because of being widely propagated by seeds and cross pollinated by wind and insect (Boss *et al.*, 1985). Female spikes are seen after male spikes. It bears hundreds of small sessile flowers with fleshy receptacles and are characterised by a perianth, stigma and unilocular ovary. After pollination, fruit matures by three to seven months. Fruit consist of a fruit axis: a latex rich inedible fibrous core that held the syncrap fruit together, a perianth: the bulk fruit that extend from the lower fleshy true fruit commonly called bulb to exterior rind (green to yellow brown) of hexagonal, bluntly conical carpel apices that cover a thick, rubbery,

whitish to yellowish wall. True fruits are oblong cylindrical in shape and have a acid to sweetish (when ripe) banana flavoured flesh (aril) surrounds each seed. Seeds are light brown, rounded enclosed in a thin, whitish membrane.

## 2.2 ORIGIN AND DISTRIBUTION OF THE FRUIT

The jackfruit that is cultivated since prehistoric times throughout the tropical lowlands in South and Southeast Asia, parts of central and eastern Africa and Brazil. Bangladesh, India, Myanmar, Thailand, Vietnam, China, Philippines, Indonesia, Malaysia and Sri Lanka are the major jackfruit producers (APAARI, 2012).Since post European contact it has been introduced to many of Pacific islands like Fiji. It is also reported to have been introduced to Palau, Yap, Pohnpei, Nauru, Tabiteuea in Kiribati, Samoa and other islands (Fosberg *et al*., 1979).

Throughout in Bangladesh a wide and undocumented diversity of jackfruit is observed, but gave less importance in documentation and conservation of its genetic resources (Khan *et al*., 2010).

India is the leading producer of the jackfruit with wide distribution in Assam, Tripura, Bihar, Uttar Pradesh, the foothills of the Himalayas and South Indian States of Kerala, Tamil Nadu and Karnataka (Wangchu *et al*., 2013). In Indian agriculture and culture jackfruit has got a significant role. Major share of jackfruit production is by Assam and Tripura, the total annual production in Assam is estimated to be in the vicinity of 1,75,000 tons and its productivity has been improving even though the area and production has not shown any change (APAARI, 2012).

Jackfruit is known as the state's official fruit and is widely grown as an important tree in Kerala’s homesteads and also as a shade crop in coffee plantations.

## 2.3 IMPORTANCE OF JACKFRUIT

Jackfruit has got significant role in many areas like dietary practices, traditional folk medicinal practices, in agroforestery or environmental practices and for the biological activities of its phytocompounds and in the production of Value Added

Products that is it known for its multi-purpose utilities in providing food, timber, fuel, fodder, medicinal and industrial products.

The primary economic product of jackfruit is the fruit, used both when raw and ripe. The 100 g of ripe edible portion of jackfruit contains carbohydrate (18.9 g), protein (1.9 g), fat (0.1 g), moisture (77 %), fiber (1.1 g), total mineral matter (0.8 g), calcium (20 mg), phosphorus (30 mg), iron (500 mg), vitamin A (540 IU), thiamin (30 mg), having caloric value 84 (Boss *et al*., 1985). Jackfruit also contains fatty acids, ellagic acid, and amino acids like arginine, cystine, histidine, leucine, lysine, methionine, threonine, tryptophan and others. It serves as both vegetable and nutritious dishes during the season. It is considered as a poor man's food in South-East Asia (Bose *et al*., 1985; Singh *et al*., 1963).

The firm fleshed (crunchy crisp firm carpels at full ripeness and not that sweet as soft but high quality) and soft fleshed (soft and fleshy perianths on ripening with small, brous, soft fruit and spongy flakes with very sweet carpels) are the main two jackfruit types. Isopentyl isovalerate (28.4 %), butyl isovalerate (25.6 %) are the major aroma components in firm type. Isopentylisovalerate (18.3 %), butyl acetate (16.5 %), ethyl isovalerate (14.4 %), butyl isovalerate (12.9 %), 2 methylbutyl acetate (12.0 %) are that in soft type (Maia *et al*., 2004).

Jackfruit flesh is consumed as ripe fruits and fully ripened, it is also used as flavouring agent to ice cream and drinks. Baby food, cordials base, candies, fruit-rolls, marmalades, jackfruit leather are some pureed jackfruit products (Mushumbusi, 2015). Fermentation and distillation of ripened fruit is used in production of alcoholic liquor. The unripe stage (when the fruit is tender) is used to prepare pickles and also cooked, resembling meat (Tiwari and Vidyarthi, 2015). Young leaves and tender male flower were also eaten as vegetables. The seeds are also consumed as bolied or roasted chips or added to flour for baking and cooked in dishes (Shyamalamma *et al*., 2008). It is also used in preparation of nutritive drinks when mixed with milk.

Jackfruit has got a derivative of trimethylsilyl and methyl esters as free sugars and fatty acids were identified and quantified using gas liquid chromatography. The major sugars and fatty acids found to be the in all parts of jackfruit as fructose, glucose, sucrose and capric, myristic, lauric, palmitic, oleic, stearic, linoleic, arachidic acids respectively (Chowdhury *et al*., 1997).

In agroforestry or environmental practices, jackfruit tree can be planted on farms to control soil erosion, it's dense canopy provides shade to plantation crops like pepper, coffee, betel nut and cardamom. It is also used as an intercrop with coconut, durian, mango and citrus. It also helps in supporting pepper vine and yam. It serves as an excellent wind break and also as an ornamental to home yard with its glossy, medium to deep green foliage with its many products and bountiful production. Beside these it is an excellent silvopasture with its foliage and fallen fruit and rage "waste after removing pulp from fruit" to cattle, goats and pigs. The medium hardwood jackfruit wood is durable, termite and decay resistant with a resemblance to highly valued mahogany for building material, furniture, cabinet making. In the Philippines and Cebu it is popular for musical instrument (Guitar, ukuleles) and also used as fuel wood. Expensive art carvings and picture frames were designed using excavated roots of old jack trees. Latex obtained are used as glue in mending chinaware and as varnishes and sticky latex is used as bird or insect trap. The bark of the tree is boiled with alum, wood chips or sawdust to dye the characteristic color to robes of Buddhist monks (Elevitch *et al*., 2006).

The better options for popularizing jackfruit is the introduction of high yielding jackfruit varieties, proper harvesting and postharvest practices of the fruits such handling, transportation and storage. The development of novel processing technologies will minimize the postharvest and production losses . Waste management of processing industries that convert the jackfruit waste into value-added products are also better options for popularizing the jackfruit cultivation and consumption.

## 2.4. GENETIC DIVERSITY STUDIES IN JACKFRUIT

Genetic diversity is the total number of genetic characteristics that a species can have in their genetic makeup or it can be referred to as the variation at the level of individual genes. Cross pollination and seed propagation over a long period of time, increase in species diversity and genetic diversity within existing population of jackfruit was observed because of the evolutionary process of extinction, selection, gene drift, gene flow and mutation that results in wide variations in sweetness, flavour, taste, size, shape and bearing habit of fruit (Chandrasekhar *et al.,* 2018). Genetic diversity study is important to develop cultivars with increased yields, wider adaption, desirable qualities, pest and diseases resistance (Gadi *et al*., 2020).

Bangladesh is one of the major jackfruit producing countries of the world and it is considered a secondary centre of jackfruit diversity (Arora, 1998; Dhar, 1998; Hossain, 1996) and a lot of genetic variability studies were reported in jackfruit by researchers over there. Azad *et al*. (2007) surveyed five agro-ecosystem in Bangladesh and collected genetic variability information on the basis of the morphological and isozyme variations. Akter *et al*. (2018) evaluated and documented the qualitative fruit characters to determine the variation in jackfruit germplasm for the further variety development programme at Jamalpur location in Bangladesh. The hypothesis according to Khan *et al*. (2010) is that genetic erosion of valuable genetic diversity will occur due to the cultivation of jackfruit with desirable characteristics in the market exhibit a positive selection pressure over local or wild and therefore they must be conserved.

India is the largest producer of jackfruit with wide cultivation of jack principally in Karnataka, Assam, Tripura, Uttar Pradesh and other states like Andhra Pradesh and Tamil Nadu (Chandrasekhar *et al*., 2018) and seen as homesteads in other areas. Mitra and Maity, 2002 identified 35 superior clones from their collection and evaluation of over 1460 jackfruit trees in West Bengal. Wangchu *et al.* (2013) studied forty four superior jackfruit genotypes collected from three districts of West Bengal. In this study, genotypes were analyzed based on quantitative, qualitative and genetic

parameters and grouped into ten clusters for quantitative characters and eight for qualitative characters.

Reddy *et al*. (2004) characterized that jackfruit in south Karnataka with enormous variability in their qualitative and quantitative traits of fruit. Phaomei *et al*. (2018) studied diversity of twenty jackfruit in West Garo hills at Meghalaya using IPGRI descriptors and documented the trees with breeding and crop improvement characters. Muthulakshmi (2003) studied the genetic diversity of jackfruit in four topographical regions of Thrissur district in Kerala and she observed wide variation within tree, leaf, fruit characters and yield. Variability studies in fruit based on chemical composition, physical characters were also done by Jagadeesh *et al*. (2007a, 2007b).

One of the most key developments in the field of molecular genetics is the development and utilization of the molecular markers for the exploitation and identification of plant genetic diversity (Idrees *et al*., 2014). In Jackfruit, Schnell *et al*. (2001) analyzed that genetic diversity variation using AFLP markers in one interspecific hybrid champedak, twenty six jackfruit accession and one breadfruit accession. 49.2 % are polymorphic result was obtained based on 12 primer pairs.

The RAPD markers based morphological and molecular analysis of genetic diversity in twelve high yielding jackfruit accessions from different localities in south India was done by Simon *et al*. (2007) and observed low to moderate level genetic diversity by the calculated genetic dissimilarity matrix based on Squared Euclidian Distances. Krishnan *et al.* (2015) studied the genetic relationship among jackfruit selections during 2012–2014 from Kuttanad region, Kerala using RAPD technique. Ten primers produced maximum reproducible polymorphic bands of thirty primers used.

Shyamalamma *et al*. (2008) used AFLP markers to assess the diversity relations of 50 jackfruit accessions. 22 % were polymorphic based on eight primer pairs analysis. Li *et al*. (2010) also studied genetic diversity of 50 jackfruit accessions from three provinces in China based on AFLP marker analysis.

Kavya *et al*. (2017) made an attempt to identify the diversity in 20 jackfruit genotypes for selected pulp colour. Swarna, Lalbagh Madhura, Byrachandra, NSP, Ashoka Yellow were the best genotypes identified and NEL as best for trade purpose. Molecular diversity analysis was carried out using 22 SSR primers, among them six showed polymorphism among twenty genotypes. But no polymorphic primer was identified for distinct pulp colour.

Witherup *et al*. (2019) measured genetic diversity of 361 jackfruit from Bangladesh using 13 microsatellite loci and determined the changes over time in genetic diversity levels with the influence of planting material from nurseries.

DNA based molecular marker technologies that are easy, simple, abundant, independent from the environment, suitable for early and rapid evaluation, and having non-tissue specific characteristics, can be grouped as dominant and co-dominant, based on their different ability to show homozygosity (dominant marker) or heterozygosity (co-dominant marker) (Hartl, 1988).

## 2.5 MOLECULAR MARKERS

Molecular markers are the DNA sequences that are representative of the difference at the genomic level that can be readily detected and whose inheritance can be easily monitored (Agarwal *et al*., 2008; Kumar *et al*., 2009). They have been reported as the excellent agronomic sector tools for the analysis of genetic variation by linking phenotypic and genotypic variation (Grover and Sharma, 2016; Varshney *et al*. 2005). As described by Collard and Mackill (2008), the size and composition of a plant population and the number of genes segregating in a population are the characteristics that a good marker will depend upon.

The criteria for an ideal molecular markers are they must be simple, quick and inexpensive, with limited use of tissue and DNA samples, should be polymorphic with even distribution in genome, must have linkage to distinct phenotypes and require no prior information about the genome of an organism, that could give adequate resolution of genetic differences to create multiple, independent and reliable markers.

Non-PCR or hybridization based techniques (Lander and Botstein 1989), PCR-based techniques (O'Hanlon *et al*. 2000) and sequence-based marker techniques (Ganal *et al*., 2009) are the three major molecular marker techniques in popularity.

### 2.5.1 Hybridization Based Molecular Marker

#### *2.5.1.1 Restriction Fragment Length Polymorphism (RFLP)*

RFLP is a codominant, polymorphic marker with high genomic abundance and random distribution, first reported in for construction of genetic map by Botstein *et al*. (1980). Detection of polymorphism in RFLP is based on the DNA rearrangement in the recognition site of restriction enzyme due to the evolutionary process, mutations like insertion, deletion, single nucleotide polymorphism. It differentiates the individual by the size of fragmented DNA. The digested DNA were electrophoresed, blotted and hybridized with the labelled probe and visualized. The RFLP markers were employed for genetic diversity and population genetic study in varied plants like *Quercus phellos* (Coutinho *et al*., 2016), *Saccharum* spp. (Nerkar *et al*., 2015) and *Vigna radiata* (Islam *et al*., 2015).

### 2.5.2 PCR Based Molecular Markers

#### *2.5.2.1 Random Amplified Polymorphic DNA (RAPD)*

Random Amplified Polymorphic DNA (RAPD) is a quick, easy, dominant marker with high genomic abundance and random distribution throughout the genetic material, that require low quantities of template DNA for the assay. It was developed by Welsh and McClell (1990) and is a PCR based random genome profiling with use of single decamer arbitrary primer (William *et al*., 1990) and is efficient in screening DNA sequence based polymorphism at a very large number of loci without pre-sequencing of DNA . Vural *et al*. (2009) reported the crucial role of RAPD in assessment of genetic variability, relationships management of genetic resources and biodiversity, studies of phylogenetic relationships and in genome mapping and cultivar identification (DNA typing).

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

Amplified Fragment Length Polymorphism (AFLP) is a combination of both RAPD and RFLP. To overcome the limitation of reproducibility in RAPD, AFLP was developed. It works on the basis of selective amplification with adapter linked restriction enzyme digested DNA fragment (Lynch *et al*., 1998; Vos *et al*., 1995). The variations in the restriction sites result in AFLP banding profiles and are scored as dominant after PAGE (Kumar *et al*., 2009). It serves as an ideal marker with unique position on genome that generate fingerprints of any DNA regardless of its source can be generated, without any prior knowledge of DNA sequence.

***2.5.2.3 Simple Sequence Repeats (SSR)***

Simple Sequence Repeats (SSR) also known as Microsatellite or Variable Number Tandem Repeats (VNTR) are tandemly repeated short nucleotide motifs of one to six bases that are found throughout the genome. They are abundant, ubiquitous, easy to automate, codominant, universal, robust, reliable and reproducible markers belong to either coding or non coding region. With a pair of flanking locus-specific oligonucleotide primers, SSRs can be easily amplified by PCR (Litt and Luty, 1989; Weber and May, 1989) and resolved on a high resolution agarose gel.

***2.5.2.4 Inter Simple Sequence Repeats (ISSR)***

ISSR markers are simple, randomly distributed in the genome, exhibit mostly dominant inheritance patterns and require low quantities of DNA. They are used in gene mapping and clonal fidelity testing of *in vitro* derived plants species, plant varietal identification, taxonomic and genetic diversity studies (Nilkanta, 2017).

Zietkiewicz *et al*. (1994) first reported Inter Simple Sequence Repeat (ISSRs). It is also known as Microsatellite primed PCR (MPPCR) (Sharma *et al*., 1995) that combines the benefits of RAPD with reproducibility and specificity. It generates data based on information of DNA fragment which is intermediate to identical microsatellite repeat regions oriented in opposite directions.

### *2.5.2.5 Single Nucleotide Polymorphism (SNP)*

Single Nucleotide Polymorphisms (SNPs) are polymorphisms specific to differences at a single nucleotide (substitution, deletion or insertion) occur frequently. Syvanen *et al*. (2001) reported SNP as a novel type of DNA marker that gains popularity in genomic studies, cultivar discrimination in crop species where other marker types fails. Well established genomic library sequences and Expressed Sequence Tags (EST) available in public domains aids in identifying SNPs and flanking sequences to provide precise sequence information for designing allele specific PCR primers or oligonucleotide probes for genetic analysis (Pudake and Kumari, 2016).

### *2.5.2.6 Sequence Characterized Amplified Region (SCAR)*

SCAR is based on the specific amplification of a particular locus using sequenced RAPD marker termini based primers. The primers were designed as 22 – 24 bases long from the cloned RAPD fragments linked to a trait of interest. Paran and Michelmore (1991), reported Sequence Characterized Amplified Regions (SCARs) as derivative of RAPDs or AFLPs (Sharma *et al*., 2007) or any other multi-locus marker technique, overcoming the limitations of random profiling (Grover, 2016) such as dominance, low reproducibility and are quick and easy to use.

### *2.5.2.7 Sequence Related Amplified Polymorphism (SRAP)*

Li and Quiros, (2001) developed this simple, inexpensive, dominant marker technique for the amplification of coding regions or Open Reading Frames (ORFs) by using two primer amplification targeting GC rich exons and AT rich promoters, introns and spacers. The amplified products were resolved by agarose gel electrophoresis and scored based on the absence and presence of the bands. SRAPs have been used for the construction of linkage maps and in identification of quantitative trait loci, in gene tagging, evaluation of genetic variation at species level and in population genetic analysis of closely related hybrids(Gulsen *et al*., 2006; Uzun *et al.,* 2009).

#### *2.5.2.8 Conserved DNA Derived Polymorphism (CDDP)*

Collard and Mackill (2009) developed this technique fingerprint rice varieties. It is also employed in evaluating genetic diversity among crop plants. They generate polymorphism results by targeting the multiple copies of short conserved sequences found in the plant genome by specific primers designed to bind to these genes. Related abiotic and biotic stress genes are also targeted by these primers. Single long primer amplifications with a high annealing temperature improve the accuracy in CDDP. 200 – 1500 bp length DNA fragments produced were viewed using electrophoresis and autoradiography.Variation in CDDP is represented as length polymorphism within these regions as they share the same priming site but differ in their genomic distribution. .

#### *2.5.2.9 Start Codon Targeted Marker (SCoT)*

SCoT marker technique ia an ideal marker with al characteristics reported by Collard *et al.* (2008). They are designed based on the conserved region surrounding the translation initiation codon (ATG context), and therefore corresponds to functional genes and correlating characters (Xiong *et al*., 2011). Apart from other popular marker systems, SCoT is gene targeted marker with multi-locus nature. Studies in analyzing diversity and diagnostic fingerprinting has been established in Mango (Luo *et al*., 2012), Orchid (Bhattacharyya *et al*., 2015), Date palm (Al-Qurainy *et al*., 2015), Diospyros (Deng *et al*., 2015), *Elymus sibiricus* (Zhang *et al*., 2015), *Vigna unguiculata* (Igwe *et al*., 2017) and Taxus using SCoT marker were reported (Hao *et al*., 2018).

Targeting Fingerprinting Markers (TFMs) and mobile element-based molecular markers are some major advanced techniques of genetic diversity study. TFMs consist of following techniques: CDDP, PBA, IT, SCoT, SRAP, TRAP and CORAP. Mobile element-based molecular markers include: IRAP, REMAP and SSAP (Poczai *et al*., 2012) and RBIP (Ismail *et al*., 2016).

### 2.6 MOLECULAR ANALYSIS

Pure, high quality, intact DNA must be obtained for any further molecular studies in plants. But the presence of high levels of polysaccharides and secondary metabolites

in most of the plant species made that a tedious process. Many methods were developed by modifying Dellaporta *et al*. (1983) and Saghai-Maroof *et al*. (1984) to obtain good quality DNA from plants with rich polyphenolic and polysaccharide content. But they were typically time-consuming and no automation compatible. Nowadays a variety of commercial DNA isolation kits using membrane-based protocol are available to obtain DNA with all desired characteristics in less time and they are easy to perform.

The basic steps involved in the isolation of DNA from a plant cell are; degradation of the cell wall rich in polysaccharide and purification of the nucleic acid from the contaminants. The buffer with detergents like Cetyl Trimethyl Ammonium Bromide (CTAB) is often used for this. The presence of polysaccharides, polyphenols and other organic compounds may impede with the process of DNA isolation (Cota-Sánchez *et al*., 2006). Ethylene Diamine Tetra Aceticacid (EDTA), Sodium chloride and Tris Base are the other components of the lysis buffer.

The contaminants like polyphenolics, proteins, RNA need to be removed. Polyvinylpyrrolidone (PVP) helps in removing polyphenolics and enzymes like Proteinase K and RNase A were used for removing proteins and RNA. Proteins were further denatured by β-Mercaptoethanol and removed by the phenol/chloroform wash (Hillis *et al*., 1996). Impurities are withdrawn through extraction of the aqueous phase from organic mixture (Sambrook and Russel, 2001; Chomczynski and Sacchi, 2006). The DNA in the aqueous phase is then recovered at the end using ethanol and resuspended in TE buffer or sterile distilled water (Buckingham and Flaws, 2007). In membrane-based protocol DNA is bound to the silica membrane using binding buffers and further washed, recovered and stored.

### 2.6.1 Polymerase Chain Reaction (PCR)

PCR is a revolutionary method in molecular biology for *in vitro* amplification of specific sequences of nucleic acid. It was developed by the American biochemist, Kary Mullis in 1983 and his pioneering work was honored with Nobel prize in chemistry (1993). PCR is considered as a standard, essential molecular tool for functional analysis

of gene and protein, it permits acellular cloning and sequencing, diagnosis of infectious diseases, genetic diseases and genetic fingerprinting.

The basic steps of PCR are denaturation, annealing, extension and the basic ingredients for DNA amplification are DNA template, primers, nucleotides, DNA polymerase and buffer.

In the first step, the strands of template DNA are separated in the denaturation step by increasing the temperature to about 94 °C at which the hydrogen bonds cannot be maintained and the double-stranded DNA is denatured into single-stranded DNA (single-stranded DNA).

In second step annealing, temperature is reduced to 40 – 70 °C, ie. primer hybridization/annealing temperature. The primers, short single strand sequences complementary to template DNA to be copied, annealed. The higher the annealing temperature, the more selective the hybridization and more specific. Lowering the temperature will allow the rebuilding of hydrogen bonds and thus reassociation of the complementary strands.

In third step extension, primer extension is performed at a temperature of 72 °C, called extension temperature. The synthesis of the complementary strand with the help of heat stable enzyme from eubacterium *Thermus aquaticus*. With thermostable DNA polymerase and all the other components (like nucleotides- the building blocks, buffers that provide necessary ions for enzymes to work and maintain the right pH during the reaction cycles) the reaction is cycled through the different temperatures that allow amplification to occur. Each cycle theoretically doubles the amount of DNA present in the previous cycle and we get a bulk amount of amplified DNA in a very short time (Robert and Zhou *et al*., 2015).

Quantitative polymerase chain reaction (Q-PCR) is a method by which the amount of the PCR product can be determined, in real-time, and is very useful for investigating gene expression (Maddocks *et al*., 2017) and is a method of choice in molecular diagnostics laboratories. Q-PCR is also called as real time PCR. It takes advantage of

fluorescence chemistry to detect DNA amplification during each cycle of PCR and does not rely on conventional downstream analysis like electrophoresis.

Reverse Transcriptase PCR make use of reverse transcriptase enzyme to convert RNA molecules to cDNA and then amplify them. Those are the best means in detection of RNA viruses.

PCR brought revolutionary changes to the detection, identification and characterization of microbial contaminants (Settanni *et al*., 2005). It also help in detection of plant viruses, Cloning of genomic DNA or cDNA, Detection of mutations, Analysis of allelic sequence variations, Genetic fingerprinting of forensic samples, Nucleotide sequencing (Remmers *et al*., 2015).

## 2.7 PHYTOCHEMICAL COMPOSITION

*Artocarpus* species are known to contain many potential bioactive phytochemicals with biological properties. Studies have reported that jackfruit as rich source of many classes of phytocompounds such as carotenoids, flavanoids, phenolics, volatile acids sterols and tannins, and that their concentration changes with the variety (Arung *et al*., 2007; Chandrika *et al*., 2005; Ong *et al*., 2006; Venkataraman, 2001). Reports are available that the extracts and metabolites from *Artocarpus heterophyllus* leaves, bark, stem and fruit can be exploited in various biological activities including antibacterial, antiviral, anti tubercular, antifungal, antiplatelet, anti-arthritic, tyrosinase inhibitory and cytotoxicity and are cited below with several pharmacology studies of the natural products from *Artocarpus*.

According to Formica *et al*. (1995) polyphenolic compounds ubiquitous in plants show biological activities like antioxidant activity, anti inflammatory activity, inhibition of platelet aggregation, antimicrobial activities and antitumor activities . They are primarily classified into flavonoids namely flavones, flavonols, flavanones and anthocyanidins.

*Artocarpus* species contain phenolic compounds, including isoprenylated flavonoids, stilbenoids and 2-arylbenzofurans. 0.36 mg GAE/100 g DW [milligrams of Gallic acid equivalent per gram of dry weight] was the reported total phenolic content in jackfruit by Wongsa and Zamaluddien, 2005. Three phenolic compounds characterized for anti-inflammatory property were artocarpesin [(5,7,2,4 tetrahydroxy -6-β- methylbut-3-enyl) flavone], nor artocarpetin (5,7,2,4- tetrahydroxyflavone) and oxyresveratrol (trans-2,4,3,5-tetra hydroxy stilbene).

Flavonoids in *Artocarpus* species were classified according to their skeletons, namely chalcones, flavanones, flavan-3-ol, 3isoprenylflavones, flavones and of compounds devoid of a flavonoid skeleton. Other classes include modified flavonoids, with cyclised derivatives of 3-prenylflavones possessing a 2',4' dioxygenated or 2',4',5'-trioxygenated pattern of B-ring, namely oxepino flavones, piranoflavones, di hydrobenzoxanthones, furano dihydro benzoxanthones and pyrano di hydrobenzo xanthones. Another class of rearranged flavonoids, namely quinonobenzoxanthones, cyclopentenoxanthones, xanthonolides, dihydroxanthone, and cyclopentenochromone (Hakkim, 2006).

Compounds reported within the jackfruit tree bark includes a novel heterophylol skeleton, flavones pigments,triterpenic compounds,betullic acid and Cycloheterophyllin ($C_{30}H_{30}O_7$) (Barik *et al*., 1997; Lin and Lu, 1993; Prakash *et al*., 2009).

## 2.8 PHARMACOLOGICAL ASPECTS

### 2.8.1 Antioxidant Effect

Ko *et al*. (1998) reported the presence of many useful antioxidant compounds within jackfruit. There is always an increased demand for natural antioxidants in fruits and vegetables among food scientists, nutrition specialists, and consumers, as they are claimed to reduce the risk of chronic diseases and promote human health (Ribeiro *et al*., 2007). Antioxidant compounds present in jackfruit include water-soluble free radical scavenger Vitamin C (ascorbic acid) (Narasimham, 1990), chain-breaking antioxidant Vitamin E (α-tocopherol) and other non-enzymatic $O_2$ quenching antioxidants like

carotenoids, coloring pigments like beta-carotene, lycopene, lutein , flavonoids and α-lipoic acid and glutathione. (Cadenas and Packer 1996; Kagan *et al*., 2002; Sies 1996).

Antioxidant activity and phenolic content was dominant in seeds rather than the other edidle portions in jackfruit (Jagtap *et al*., 2010; Soong and Barlow, 2004). The ethanolic extracts of *Artocarpus heterophyllus* showed highest $IC_{50}$ value (410g/ml) in DPPH assay done by Soubir, 2007.

### 2.8.2 Anti-inflammatory Effect

Inflammation is a local defense reaction of our body in order to eliminate or limit the spread of injurious agent that are aimed at host defense and usually activated in most disease condition. The inflammatory response involves a complex array of enzyme activation, mediator release, fluid extravasations, cell migration, tissue breakdown and repair (Vane *et al*., 1995). Inflammation can also occur because of Trauma, Surgical procedures or in musculoskeletal muscle, oxidative stress-induced inflammation (Mitchell and Cotran, 2000). Anti-inflammatory agents are the drugs or medicines used to relieve swelling, pain and other symptoms of inflammation.

Fang *et al*. (2008) determined the inhibitory effects of artocarpesin, norartocarpetin and oxyresveratrol on proinflammatory mediators in lipopolysaccharide (LPS) activated RAW 264.7 murine macrophage cells. Artocarpesin suppressed the biochemical pathway behind NO and PGE 2 through inducible protein expressions and posses therapeutic potential against inflammation-associated disorders.

Inhibition of chemical mediators released from mast cells, neutrophils and macrophages during inflammation serves as a *in vitro* measure of flavonoid content in jackfruit. Cycloheterohyllin, Artonins B and Artocarpanone identified as inhibitors of superoxide anion formation in formyl-Met-Leu-Phe (fMLP) stimulated rat neutrophils. Artocarpanone was identified as inhibitor of the release of lysozyme from rat neutrophils stimulated with formyl-Met-Leu-Phe (fMLP), it also exhibit significant inhibitory effect on NO production and iNOS protein expression in RAW 264.7 cells (Wei *et al*., 2005).

Chanda *et al.* (2009) reported anti-inflammatory effects of protease fraction and the artocarpain-H extracted from the fruit stem latex of jackfruit by carrageenan induced rat paw oedema and Cotton pellet-induced granuloma model.

**2.8.3 Anti diabetic Effect**

Fernando *et al*. (1991), investigated the effects of hot water extracts of *Artocarpus heterophyllus* leaves on the glucose tolerance of normal human subjects and maturity-onset diabetic patients. When examined at oral doses equivalent to 20g/Kg of starting material significant improvement was observed in glucose tolerance in the normal subjects and the diabetic patients.

Kotowaroo *et al*. (2006) showed the property of inhibition of α-amylase activity of jackfruit leaf. The aqueous leaf extract with α-amylase and starch caused a prominant decrease in the enzyme activity in the assay. *In vitro* studies in rat plasma also showed the inhibitory activity of extract over the α-amylase activity. competitive inhibitor leaf extract act as a starch blocker of α-amylase and reduces the post- prandial glucose peaks.

**2.8.4 Anti Cancer Property**

Lignans, isoflavones and saponins in jackfruit prevent the formation of cancer cells in the body and fight against stomach ulcers (Baliga *et al*., 2011). Arung *et al*. (2010a) reported, norartocarpin, cudraflavone C, artocarpin, brosimone I, kuwanon C cudraflavone B and 6-prenylapigenin as more active than the clinical drugs (carmustine, vinblastine and 5-fluorouracil) used in the cytotoxic effects in B16 melanoma cells. Artocarpin was also reported with cytotoxic effects on *in vitro* cultured human T47D breast cancer cells (Arung *et al*., 2010b).

Chemoprotective compounds in jackfruit reduce the mutagenicity of aflatoxin B1 (AFB1), proliferation of cancer cells and are effective aid to prevent or treat lymphoma cancer (Ruiz Montanez *et al.,* 2014). Methanolic extract of *A. heterophyllus* seeds showed excellent toxicity on neoplastic cells and was nontoxic to normal cells, studies were conducted to evaluate its cytotoxic activity against A549, Hela and MCF-7 cell lines (Hari *et al*., 2014).

Jacalin has effect on cell proliferation or cytotoxicity on the A431 (human epidermoid carcinoma) and HT29 (human colorectal carcinoma) (Sahasrabuddhe *et al.*, 2006). The potent cytotoxic activity of many of the isoprenylated flavonoids was reported against various cell lines, including murine leukemia P388, KB, mouse L-1210 and colon 38 (Nomura *et al*., 1998).

### 2.8.5 Wound healing

Patil *et al*. (2005) studied the wound healing property of the ethanol extract of dried leaves and its various fractions on petroleum ether, butanol, butanone and methanol in rats. Best activity was shown by the methanol fraction of leaves.

Leaves were thought to possess wound healing effects, reduce pain, decrease abscesses and relieve ear problems (Gupta and Tandon, 2004; Morton, 1987).

## 2.9 PHYTOCHEMICAL ASSAYS

Phytochemicals or phytoconstituents are non-nutritional chemical compounds or bioactive compounds responsible for the protection of plant from biotic and abiotic stress. Phytochemical screening assays refer to the extraction, screening and identification of the medicinally active substances found in plants. Some of the bioactive substances that can be derived from plants are flavonoids, alkaloids, carotenoids, tannin, antioxidants and phenolic compounds.

Variety of solvents are used for extraction of bioactive compounds from the plants that vary in their polarity. The most commonly used one is methanol. Methanol followed by water, ethanol, ethyl acetate, choloroform are widely used for extraction. The active constituents are soluble in polar solvents so non polar solvents are used less.

The flavonoids and other related phytocompounds in *Artocarpus* are soluble in most organic solvents. Hakkim *et al.* (2006), reported complete extraction of the appropriate tissues of each of the *Artocarpus* species by cold percolation with methanol or ethanol, followed by solvent partition of the extract with benzene, chloroform, and finally with ethyl acetate as the method of choice in his experiment.

### 2.9.1 Total Phenolic Content Assay

Phenols contained by plants have good antioxidant, anti-mutagenic and anti-cancer properties and may contribute directly to antioxidative action. The total phenolic content was estimated colorimetrically using Folin-Ciocalteu reagent, as described by Singleton and Rossi, (1965). Modifications were reported by many researchers using readymade Folin-Ciocalteu and improved incubation periods (Bueno *et al*., 2012; Cicco *et al*., 2009; Gulcin *et al*., 2004; Verza *et al*., 2007).

Folin-Ciocalteu was 1$^{st}$ developed in 1927 for quantification of tyrosine ( Folin, 1927). It is a reagent mixture of sodium molybdate, sodium tungstate and other reagents. When it reacts with phenols, it gives a blue color due phosphotungstic phospho-molybdenum complex which can be quantified by visible-light spectrophotometry (Schofield *et al*., 2001). The maximum absorption of the chromophores depends on the alkaline solution and the concentration of phenolic compounds. It is necessary to use an enormous excess of the reagent to obtain a complete reaction since the reagent rapidly decomposes in alkaline solutions, but the excess reagent can cause precipitates and high turbidity, making spectrophotometric analysis impossible. To overcome this problem, lithium salts were included in the reagent, which prevented the turbidity (Folin, 1927).

### 2.9.2 Total Flavonoid Assay

One of the most commonly used procedures for total flavonoid determination is the spectrophotometric assay based on aluminium complex formation. This method was initially proposed by Christ and Müller (1960) for the analysis of herbal materials, was later modified several times. The aluminum chloride colorimetric method was modified from the procedure reported by Woisky and Salatino (1998). Quercetin was used to make the calibration curve.

### 2.9.3 Antioxidant determining assays

The studies on antioxidant activity or free radical scavenging activity of plants with biological active content are often done followed by measuring the total phenolics as well as total flavonoid content within them. Chemical antioxidant activity assays are also used

extensively to evaluate the potential bioactivity of phytochemical constituents in food items from plants. Some of the chemical antioxidant activity assays are oxygen radical absorbance capacity (ORAC) (Cao *et al*., 1993), Trolox equivalent antioxidant capacity (TEAC) (Miller *et al*., 1993), total radical-scavenging antioxidant parameter (TRAP) (Ghiselli *et al*., 1995; Wayner *et al*., 1985), 2,2 diphenylpicrylhydrazyl (DPPH) free radical (William *et al*., 1995), ferric reducing/antioxidant power (FRAP) (Benzie *et al*., 1996), total oxyradical scavenging capacity (TOSC) (Winston *et al*., 1998), 2,2-Azinobis 3-ethylbenzthiazoline-6-sulphonic acid radical ($ABTS^{\cdot+}$) scavenging assay (Miller *et al*., 1993) and peroxyl scavenging capacity (PSC) (Adom *et al*., 2005).

#### *2.9.3.1 DPPH Radical Scavenging Activity*

Lee *et al*. (2003) studied antioxidant activity of the Jackfruit pulp extracts using 1,1-diphenyl-2-picrylhydrazyl (DPPH) with some modifications. The antioxidant activity of a given compound or a complex matrix is evaluated by a stable radical, namely purple coloured 2,2-diphenyl-1-picrylhydrazyl (DPPH•), with an unpaired valence electron at one atom of its nitrogen bridge (Gomes *et al*., 2008), the decrease of the DPPH concentration (ie. formation of 1,1-diphenyl-2-picryl hydrazine, yellow colour) is measured from the decrease of absorbance at a characteristic wavelength 517 nm (Arteaga *et al*., 2008). Results were expressed as radical scavenging activity (% RSA).

$$\% \text{ RSA} = \left(\frac{\text{Acontrol} - \text{Asample}}{\text{Acontrol}}\right) * 100$$

### 2.9.4 Anti-inflammatory Assays

#### *2.9.4.1 In vitro anti-inflammatory activity assays*

Lysosomal enzymes released during inflammatory pathways cause tissue injury. Stability of lysosomal membrane is therefore considered as a limiting factor for inflammatory responses, due to the hindrance in releasing lysosomal constituents of activated neutrophil such as bactericidal enzymes and proteases. Chou (1997), mentioned HRBC or erythrocyte membrane as analogous to the lysosomal membrane.

Anti-inflammatory properties of *Centalla asiatica* (Chippada *et al*., 2011)*,* *Enicostemma axillare* (Leelaprakash *et al*., 2011), *Basella alba* linn. Var. alba (Kumar *et al*., 2011), *Symplocos cochinchinensis* (Lour) Moore ssp *laurina* (Vadivu *et al*., 2008)*,* *Rhizopora mucronata* (Kumari *et al*., 2015) and many other plants were determined using HRBC membrane stabilization assay.

Various other *in vitro* anti inflammatory studies are Inhibition of albumin denaturation, Protein inhibitory action, Anti-lipoxygenase activity, Xanthine oxidase assay, Acetylcholin esterase (AChE) inhibitory activity, Antimitotic activity.

***2.9.4.2 In vivo anti-inflammatory activity assays***

For the detection of orally active anti-inflammatory agents, carrageenan used induced inflammation is done. It is reported that edema formation due to carrageenan in the rat paw produce a biphasic event, with the release of histamine and serotonin in initial phase and prostaglandins, lysosomes, bradykinins and protease later. It also involves other inflammatory mediators (Yaksh *et al*., 2001) which are released as a result of tissue injury. Anti-inflammatory Activities of *Crotalaria pallid* Aiton leaves of Fabacae family were analysed using this method by Bulbul *et al*. (2017).

Kumar *et al.* (2014) studied antinociceptive and anti-inflammatory activities of Methanolic fruit extract of *Bridelia retusa* using various *in vivo* anti-inflammatory assays in experimental animals. Meera *et al*. (2017) identified some chemical constituents and evaluated the anti-inflammatory activity of crude ethanolic extracts of spine, skin and rind of jack fruit (*Artocarpus heterophyllus*) peel using *in vitro* and *in vivo* studies along with polyphenol and flavonoid contents quantification. In this study skin exhibited maximum anti-inflammatory activity and rind had preferential inhibition on Cyclooxygenase-2 and spine and skin inhibited both Cyclooxygenase-1 and 2 *in vitro.*

# 3. MATERIALS AND METHODS

The research work entitled "Genetic diversity analysis of *Artocarpus heterophyllus* Lam. using SCoT markers for antioxidant and anti-inflammatory properties" was carried out at Biotechnology and Bioinformatics division and Ethnomedicine and Ethnopharmacology division of Jawaharlal Nehru Tropical Botanic Garden and Research Institute (JNTBGRI), Palode during the period 2019-2020.

## 3.1 MATERIALS

### 3.1.1 Plant Material

*Artocarpus heterophyllus* Lam. leaves were used for the genetic diversity analysis and edible fruit and seed were used for the antioxidant potential screening. The one that showed the best antioxidant potential was tested for its anti-inflammatory properties. Eighteen different accessions were collected from NBPGR, Thrissur, and from the forest range of Bonacaud, Thiruvananthapuram (Table 3.1).

**Table 3.1** List of Jackfruit accessions used for the study

| Sl No. | Accession No. | Location | GPS |
|---|---|---|---|
| 1 | MGNL-IC 97625 | NBPGR,Thrissur | N10°31'0" E76°17'0" |
| 2 | NLTR-IC 97632 | | N10°31'0" E76°17'0" |
| 3 | MG91-IC9550 1B | | N10°31'0" E76°17'0" |
| 4 | MG91'-IC9550 1A | | N10°31'0" E76°17'0" |
| 5 | MG81 – IC 92254 | | N10°31'0" E76°17'0" |
| 6 | MV – IC 97633 | | N10°31'0" E76°17'0" |
| 7 | MG70 – IC 91665 | | N10°31'0" E76°17'0" |
| 8 | MGNL2-IC 97628 | | N10°31'0" E76°17'0" |
| 9 | MG 92- IC96148 | | N10°31'0" E76°17'0" |
| 10 | MGNL1-IC 97627 | | N10°31'0" E76°17'0" |
| 11 | WB1 | Bonacaud | N08°40'10.8" E77°C08'53" |
| 12 | WB2 | | N08040'10.8" E 770C08'53" |
| 13 | WB3 | | N 8°36'45.68" E 77°5'50.19" |
| 14 | WB4 | | N08041'30.5" E077011'42" |
| 15 | WB5 | | N8°40'58.716" E77°10'8.93" |
| 16 | WB6 | | N8°40'58.62" E77°10'8.808" |
| 17 | WB7 | | N8°40'55.756" E77°10'6.776" |
| 18 | WB8 | | N8°42'31.193" E77°10'21.001" |

### 3.1.2 Chemicals and Drugs

Methanol, DPPH (1,1-DiPhenyl-2-PicrylHydrazyl), Ascorbic Acid, Folin Ciocalteu reagent, Sodium Carbonate, Gallic Acid, Aluminium Chloride, Quercetin, Potassium Acetate, Carrageenan, (Formaldehyde) Formalin, Indomethacin, Acetyl salicylic acid (Aspirin), Alsever solution, Phosphate Buffer Saline, Hypo saline, Isosaline, Normal saline, Diclofenac sodium, Egg Albumin, Cetyl Trimethyl Ammonium Bromide (CTAB), Poly Vinyl Pyrrolidone (PVP), Mercaptoethanol, Proteinase K (Origin, Kerala), Ethylene Diamine Tetra Acetic acid (EDTA), Tris Base, Sodium Chloride, Chloroform, Phenol, Isoamyl Alcohol, Isopropanol, Absolute Ethanol, Sodium Acetate, RNase A(Origin,Kerala), PCR Reaction Mix (Takara Emerald).

### 3.1.3 Instruments

Soxhlet apparatus, Heating Mantle (Rivotek, Chennai), Rotary vacuum evaporator (Heidolph, Germany), Hot air oven (Universal, India), Grinder (Havells), Electronic balance (Mettler Toledo, India), Mini Spin Centrifuge (Eppendorf, India), pH Meter (Eutech, Singapore), Spectrophotometer (XP 3001 Xplorer, India), Water Bath (Lab Companion, China), Microwave oven (IFB, India), Gel Electrophoresis Apparatus (Bio Rad, California), Gel doc (UVP, U.K.), Nanophotometer (Implen, U.S.A.), PCR (Veriti-Applied Biosystems, U.S.A), Plethysmometer were used.

### 3.1.4 Experimental animals

Male Wistar rats (150 to 250 g) obtained from the Animal House of Jawaharlal Nehru Tropical Botanical Garden Research Institute, Palode. The experimental animals were divided into different groups and caged in polypropylene cages and maintained under standard environmental conditions with temperature 25 ± 2°C, the relative moisture of 60 ± 10%, room air changes 15 ± 3 times/h and 12-hour dark-light cycles. The animals were supplied with commercial rodent feed (Lipton India Ltd; Mumbai, India) and water. Animals were acclimatized for one week before the initiation of an experiment. All animal experiments were carried out based on the guidelines of the NIH and only after getting approval of the Institute Animal Ethics Committee.

## 3.2 METHODS

### 3.2.1 Molecular Analysis

#### *3.2.1.1 CTAB based DNA isolation protocol*

Genomic DNA extraction was done according to Cetyl Trimethyl Ammonium Bromide (CTAB) method (Murray and Thomson, 1980). On lowering salt concentration CTAB forms insoluble complex with nucleic acid. Rich nucleases activity in plants are reduced by quick freezing of the tissue using liquid nitrogen and thawing in extraction buffer that contains CTAB detergent and a high concentration of EDTA. The following procedure was followed to extract and quantify the DNA.

1) 150mg of *Artocarpus heterophyllus* Lam. leaf samples were cut into pieces using scissors and ground well to fine powder using liquid nitrogen in a mortar and pestle.

2) Added 2 % β-mercaptoethanol and polyvinylpyrrolidone to the extraction buffer and warmed at $65^0$C for 5 – 10 min.

3) The tissue powder was quickly transferred into a clear autoclaved 2ml eppendorf tube containing 1ml of prewarmed CTAB buffer and mixed well.

4) Added 4µl proteinase K and mixed again by inversion for one minute.

5) Incubated the tubes at $65^o$C for 20 min with occasional mixing and performed centrifugation at 13000 rpm for 5 min.

6) Transferred the supernatant to a fresh tube and added equal volume of phenol:chloroform:isoamylalcohol (25:24:1).Mixed by inversion and centrifuged at 10000 rpm for 5 min.

7) Supernatant was carefully transferred to a clean eppendorf tube and added equal volume of chloroform-isoamylalcohol (24:1). Mixed by inverting the tube to 20 – 25 times to form emulsion and was centrifuged at 13000 rpm for 10 min.

8) Supernatant was carefully decanted and transferred to a new tube and was precipitated by adding 1/10 of sodium acetate and equal volume of ice-cold isopropanol. Mixed by gentle inversion and incubated at $-20^o$C for 2 hours or overnight.

9) Centrifuge at 13000 rpm for 15 min. Discard the supernatant and wash the pellet twice using 100 µL 70% ethanol by centrifuging at 10,000 rpm for 5 min.

10) Decanted the supernatant and air dried DNA pellet at room temperature (15 – 25$^{o}$C) until the whitish pellet turned transparent.
11) Pellet was resuspended in 100μl TE buffer and stored at -20$^{o}$C for future work.

#### *3.2.1.2 RNase Treatment*

1μL RNase A (OrionX) was added to the eluted DNA and incubated at Room temperature for 30 minutes and stored.

#### *3.2.1.3 Agarose Gel Electrophoresis*

The quality of the isolated DNA samples was analyzed using Agarose Gel Electrophoresis. The DNA which was negatively charged moves towards cathode according to their size from the wells at the anode.

3μl of DNA aliquots from each sample was loaded in 0.8% agarose gel. The gel was run at 70V with a 1X tank TAE buffer in a horizontal gel electrophoresis unit for about 1 hour. The genomic DNA bands in the gel were visualized by Ethidium bromide staining under the EC3 Chemi HR 410 imaging system (UVP, U.K.).

#### *3.2.1.4 Nanophotometer analysis*

The quality and quantity of the DNA samples were analyzed by using Nanophotometer (Implen, USA). The instrument was calibrated with 1μl sterile water (elution buffer) as blank. Nanophotometer required a sample size of 1μl DNA for measuring the optical density and to identify the specified parameters. The instrument provided analytical data on,

- Concentration of DNA in the sample (μg/μl)
- Purity of DNA sample (260/ 280 and 260/230)

The concentration and purity of DNA is checked by measuring absorption at A260 and A 280 by using Nanophotometer. The A260/A280 ratio around 1.8 indicates a good quality of DNA. Ratio lesser than 1.8 indicates protein concentration and ratio of 2.0 or greater indicates RNA contamination. The visual assessment of DNA was done by using 0.8%

electrophoresis. As a result, the extracted, purified DNA was diluted to final concentration of 100µl and subjected to PCR amplification.

#### *3.2.1.5 PCR SCoT Amplification*

The DNA samples and primers were optimized for PCR parameters such as the dilution of stock DNA to be used as a template, annealing temperature. Most of the SCoT marker anneals at 50˚C. DNA samples were then subjected to PCR amplification with the randomly selected ten SCoT primers (Collard and Mackill, 2009; Luo *et al.,* 2010). The reaction mixture consisted of the following items:

**Table 3.2** PCR Reaction Mix

| **Reagents** | **Volume** |
|---|---|
| Master mix (2X) | 7.5 µl |
| Primer (0.8mM) | 1.2 µl |
| Template DNA(40ng) | 3 µl |
| Water | 3.3 µl |
| Total Volume | 15.0 µl |

**Table 3.3** List of SCoT Primers used

| **SCoT** | **Primer Sequence** | **%G-C** |
|---|---|---|
| 03 | CAACAATGGCTACCACCG | 56 |
| 10 | CAACAATGGCTACCAGCC | 56 |
| 11 | AAGCAATGGCTACCACCA | 50 |
| 15 | ACGACATGGCGACCGCGA | 67 |
| 16 | ACCATGGCTACCACCGAC | 56 |
| 21 | ACGACATGGCGACCCACA | 61 |
| 32 | CCATGGCTACCACCGCAC | 67 |
| 35 | CATGGCTACCACCGGCCC | 72 |
| 40 | CAATGGCTACCACTACAG | 50 |
| 45 | ACAATGGCTACCACTGAC | 50 |

Amplification was carried out on Veriti thermal cycler (Applied Biosystems, California) using the following temperature profile: Reactions began with a 5 min initial denaturation at 94˚C, followed by 35 cycles with 1 min at 94˚C, then 1 min at specific annealing temperature 50˚C and 2 min extension at 72 ˚C, a final extension step covering 10 min at 72 ˚C marked the end of the reaction. The amplified products were resolved in 1.5% agarose gel with ethidium bromide and were analyzed using a EC3 Chemi HR 410 imaging system (UVP, UK). A 100 bp DNA ladder (OrionX) was loaded along with the samples to compare the size of resultant bands.

#### *3.2.1.6 Data Analysis*

The band patterns obtained were scored as absent (0) or present (1). Using POPGENE version 1.32 number of alleles observed (na), effective number of alleles (ne), nei's genetic diversity index (h), shannon's information index (I) were calculated. NTSYS-pc version 2.02 software package were used to calculate Jaccard's similarity coefficient (Guha *et al*., 1999) and cluster analysis was performed using the UPGMA algorithm (Rohlf, 1993).

### 3.2.2 Phytochemical Analysis

#### *3.2.2.1 Sample Preparation for Soxhlet Extraction*

Mature cultivar jackfruits samples were collected from the NBPGR jackfruit conservatory, Thrissur and mature wild samples were collected from Bonacaud forest range, Trivandrum, during early 2020 fruiting season. The separated flakes and seeds were oven-dried at the Biotechnology and Bioinformatics Division, JNTBGRI. The dried flakes and seed samples were powdered using a grinder.

#### *3.2.2.2 Soxhlet Extraction*

Powdered samples (20g) were loaded in the soxhlet apparatus timble, made of blotting paper. Extraction was done after connecting sample loaded soxhlet with Round Bottom (RB) flask with solvent and chips/glass beads (to avoid bumping) and condenser with inlet and outlet water flow. The powdered sample was subjected to continuous extraction for about eight siphons in the soxhlet apparatus using methanol as solvent at

about 40°C. A fresh round bottom flask was taken and the extract solution was transferred to it after measuring its initial weight using electronic weighing balance. The solvents were distilled under reduced pressure using a rotary vacuum evaporator (Heidolph) at 40°C and 30 rpm. The final weight of round bottom flask with concentrated extract was taken and noted as final weight. The yield of the extract was measured by the equation

$$\text{Yield} = \left[\frac{FinalWeight\ of\ RB - InitialWeight\ of\ RB}{weightofsample}\right]*100$$

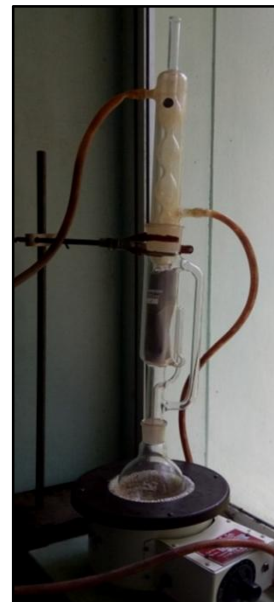

**Figure 3.1 Soxhlet Apparatus**

### *3.2.2.3 Total Phenolic Assay*

Folin–Ciocalteu colorimetric method described by Singleton *et al.* (1965) with some modifications were used to determine the total phenolic contents (TPC) in the fruits and seeds extracts.. Various concentrations of gallic acid (2-10 μg/mL) were used as standard and sample (200 and 500 μg/mL) were prepared using methanol. The whole reaction mix volume is 10 ml with 10% Folin–Ciocalteu reagent (5mL) and 7.5 % $Na_2CO_3$ (4mL). The tubes were shaken well and standed for for 30 minutes, the absorbance was measured then made at 760 nm against a blank. Triplicate values taken were used to generate the graph and determine the mg gallic acid equivalents (GAE) per gram of sample in dry weight (mg/g) *i.e.*, the total phenolic content in extract.

### *3.2.2.4 Total Flavonoid Assay*

Total flavonoids content of jackfruit flakes and seeds were determined by Chang *et al*. (2002) with minor modifications. The concentrations of samples (200 and 500 μg/mL) were prepared using distilled water. The total reaction of 4ml reaction mixture consist of 10% aluminum chloride (100 μL), 1M potassium acetate (100 μL) and 2.8mL distilled water. The mixture was mixed thoroughly and incubated for half an hour, absorbance was then taken at 415 nm. The total flavonoids content were expressed as mg of quercetin equivalents per gram extract.

#### *3.2.2.5 DPPH Radical Scavenging Activity*

The DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical scavenging capacity of the jackfruit flakes and seeds were measured according to the procedure described by Maksimovic *et al.* (2011) with slight modifications. The DPPH reagent solution was prepared by dissolving 3.9 mg of DPPH in 50 mL methanol (0.2mM). The different concentrations of extract (50, 100, 150, 200μg/mL) were mixed to react with 1ml DPPH solution. The complete 3mL reaction mixture was mixed thoroughly and incubated in dark for 30minutes at room temperature. The change in colour of the resulting solution was determined using spectrophotometer at 517 nm.

The DPPH radical scavenging capacity was calculated using the following equation and expressed as radical scavenging capacity (% RSC).

$$\% \text{ RSC} = \left[\frac{Acontrol - Asample}{Acontrol}\right] * 100$$

Control - mixture without extract

Concentrations (0.625,1.25,2.5,5,10 μg/mL) of ascorbic acid plotted against (%) of free radical scavenging activity gave standard curve for determing $IC_{50}$ value for the samples and standard.

### 3.2.3 *In vitro* Anti-inflammatory Analysis

#### *3.2.3.1 HRBC Membrane Stabilization Assay*

HRBC membrane stabilization assay is used to weigh up the anti inflammatory activity of the extract (Gandidasan, 1991). The blood sample was collected from healthy individuals and were blended with equivalent amount of sterilized Alsever solution. The blood was centrifuged at 3000 rpm and the packed cells were washed with 0.85 % isosaline (pH 7.4) and a 10% v/v suspension were made with isosaline. Various concentrations of extracts were prepared (100, 150, 200 and 250 μg/mL) using distilled water. The assay mixture comprises the drug, 1 ml of 0.15 M phosphate buffer (pH 7.4) and 2 ml of 0.36% hypo saline and 0.5 ml of the HRBC suspension. Diclofenac sodium was utilized as the reference standard and 2 ml of distilled water used as alternative to

hyposaline served as the control. All the assay mixtures were incubated at 37°C for 30 minutes and were centrifuged. The haemoglobin content in the supernatant solution were evaluated utilizing spectrophotometer at 560 nm. The percentage of hemolysis was computed by assuming the hemolysis produced in the presence of distilled water as 100%. The percentage of HRBC membrane stabilization or protection were calculated by using the following equation,

$$\% \text{ Inhibition} = 100 - \left[\frac{OD\ of\ test}{OD\ of\ control} * 100\right]$$

***3.2.3.2 Albumin Denaturation Assay***

Albumin Denaturation Assay according to Rahman *et al.*, (2015) with slight modifications was done to evaluate the percentage inhibition of protein denaturation by the flake extract of jackfruit. The reaction mixture (5 mL) consisted of 0.2 mL of egg albumin (from fresh hen's egg), 2.8 mL of phosphate-buffered saline (PBS, pH 6.4) and 2 mL of varying concentrations (100,150, 200,250 µg/mL) of extract. A similar volume of double-distilled water served as the control. Afterwards, the mixtures were incubated at 37 ± 2°C in a water bath for 15minutes and then heated at 55°C for five minutes. Then it is removed from water bath and left for cooling, after cooling, their absorbance was measured at 660 nm by using the vehicle as a blank. Diclofenac sodium in the concentrations of 2,4,6,8,10 µg /mL was used as the reference drug and treated similarly for the determination of absorbance. The percentage inhibition of protein denaturation was calculated by using the following formula:

$$\% \text{ inhibition} = 100 \times \left[ \frac{VC - Vt}{Vc} \right]$$

Where, Vt = absorbance of the test sample
Vc = absorbance of control.

Each experiment was done in triplicate and the average was taken. The extract concentration for 50 % inhibition ($IC_{50}$) was determined by the dose-response curve.

### *3.2.4.1 Carrageenan Induced Paw Study*

The anti-inflammatory activity of the methanolic extract of flakes of jack fruit MG 92 was studied by the method, Carrageenan-induced paw inflammation was produced by subplantar administration of 0.05 ml of freshly prepared carrageenan (1% w/v) to the left paw of each rat (Winter *et al*., 1962). Different groups of animals were pretreated with extract (200 and 250 mg/kg, p.o.) or with 5 ml/kg of distilled water (vehicle control) or 10 mg/kg reference drug (indomethacin) at 30 minutes before eliciting paw edema. The paw volume was measured by dipping the foot in the water bath of the plethysmometer up to the anatomical hairline on lateral malleolus and compared with control animals, which received only the vehicle. Measurement was done immediately before, first, second, third hour following carrageenan injection. The oedema inhibitory activity was calculated according to the following formula.

**Figure 3.2** Plethysmometer

$$\% \text{ inhibition} = \left[\frac{(control - test)}{control}\right] * 100$$

**Table 3.4** Drug Administration Pattern for Carrageenan Induced Paw Oedema**:**

| **Groups** | **Treatments** | **Volume (per orally) (mL)** | **No. of animals per group** |
|---|---|---|---|
| 1 | Distilled Water | 1 | 2 |
| 2 | 10 (mg/Kg) Indomethacin | 1 | 2 |
| 3 | J200(200 mg/kg) | 1 | 2 |
| 4 | J250(250 mg/Kg) | 1 | 2 |

#### *3.2.4.2 Formalin Induced Paw Oedema Study*

The anti-inflammatory activity of the methanolic extract of flakes of jackfruit MG92 was studied by the method, Formalin - induced paw oedema was produced by subplantar administration of 0.05 ml of freshly formalin (1% w/v) to the left paw of each rat. After 30 minutes of the oral administration of test materials different groups of animals were pretreated with extract (200 and 250 mg/kg, p.o.) or with 5 ml/kg of distilled water (vehicle control) or 10 mg/kg reference drug (Aspirin). The paw volume was measured using plethysmometer and compared with control animals, which received only the vehicle. Measurement was done immediately before, first, second, third hour following

The oedema inhibitory activity was calculated according to the following formula.

$$\% \text{ inhibition} = \left[\frac{(control - test)}{control}\right] * 100$$

**Table 3.5** Drug Administration Pattern for Formalin Induced Paw Oedema Study

| Groups | Treatments | Volume(per orally) (mL) | No. of animals per group |
|---|---|---|---|
| 1 | Distilled Water | 2 | 2 |
| 2 | 10 (mg/Kg) Aspirin | 2 | 2 |
| 3 | J200(200 mg/kg) | 2 | 2 |
| 4 | J250(250 mg/Kg) | 2 | 2 |

### 3.2.5 Statistical Analysis

The results obtained were expressed as mean ± standard deviation (SD) and presented as graphs and tables. Data were analyzed by using a statistical tool in Microsoft Excel 2007 with one way analysis of variance (ANOVA) followed by Duncan's test. ANOVA was performed to compare the significant differences between groups and Duncan's test was carried out for pairfed comparisons between groups. The level of significance was set at ***$p$<0.05.

# 4. RESULTS

This chapter includes the results of the research work entitled “Genetic diversity analysis of *Artocarpus heterophyllus* Lam. using SCoT markers for antioxidant and anti-inflammatory properties” carried out at the Biotechnology and Bioinformatics Division and Ethnomedicine and Ethnopharmacology division of Jawaharlal Nehru Tropical Botanic Garden and Research Institute (JNTBGRI), Palode during the period 2019-2020.

## 4.1 GENETIC DIVERSITY ANALYSIS

### 4.1.1 DNA Isolation

DNA samples of eighteen accessions isolated by CTAB method were used for the diversity analysis. Isolated samples were resolved in 0.8% agarose gel with ethidium bromide for quality analysis. The purity and quantity of the isolated materials estimated with Nanophotometer is represented in Table 4.1.

**Table 4.1** Quality and quantity of isolated DNA samples

| Sl. No. | Sample (accession no.) | Purity (260/280) | Concentration (ng/µl) |
|---|---|---|---|
| 1 | MG70 – IC 91665 | 1.8 | 17.9 |
| 2 | MG91-IC9550 1B | 1.9 | 32.5 |
| 3 | MG91’-IC9550 1A | 1.3 | 18.0 |
| 4 | MG 92- IC96148 | 2.0 | 19.5 |
| 5 | NLTR-IC 97632 | 1.1 | 20.6 |
| 6 | MGNL1-IC 97627 | 1.3 | 40.2 |
| 7 | MV – IC 97633 | 1.8 | 62.5 |
| 8 | MGNL2-IC 97628 | 1.2 | 40.3 |
| 9 | MG81 – IC 92254 | 1.9 | 38.6 |
| 10 | MGNL-IC 97625 | 1.6 | 53.4 |
| 11 | WB1 | 1.7 | 110.5 |
| 12 | WB2 | 1.7 | 14.2 |
| 13 | WB3 | 1.1 | 58.7 |
| 14 | WB4 | 1.6 | 108.3 |
| 15 | WB5 | 2.2 | 45.2 |
| 16 | WB6 | 1.9 | 158.6 |
| 17 | WB7 | 1.8 | 63.4 |
| 18 | WB8 | 0.5 | 16.5 |

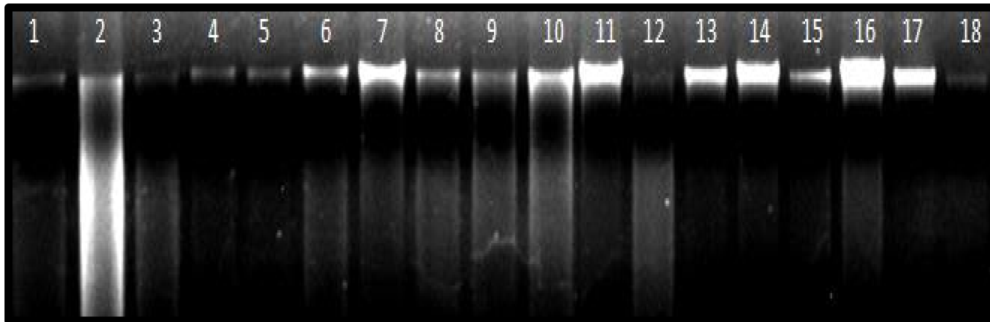


**Figure 4.1**: Genomic DNA isolated from 18 Jackfruit Samples. Lane 1– MG 70, Lane 2 – MG 91, Lane 3 – MG 91', Lane 4 – MG 92, Lane 5 – NLTR, Lane 6 – MG NL 1, Lane 7 – MV, Lane 8 – MG NL 2, Lane 9 – MG 81, Lane 10 – MG NL IC, Lane 11 – WB1, Lane 12 – WB2, Lane 13 – WB3, Lane 14 – WB4, Lane 15 – WB5, Lane 16 – WB6, Lane 17 – WB7, Lane 18 – WB8

### 4.1.2 SCoT Analysis

Initially SCoT marker screening was performed in the DNA samples of eighteen accessions. (Table 6). Among them, eleven accessions showed unambiguous and reproducible banding patterns, which were selected for the further genetic diversity analysis using SCoT markers. Out of the screened ten, eight primers amplified and showed polymorphism which were considered for diversity study and phylogenetic analysis .

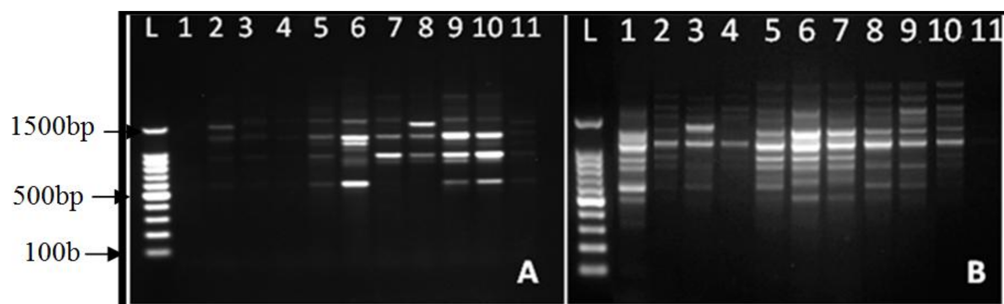


**Plate 4.1. Amplification products generated by SCoT primers with eleven DNA templates. 4.1.A** – SCoT 10, **4.1.B** – SCoT 11. Lane L – 100bp Ladder (Orion X), 1 – MG 91, 2 – MG NL 1, 3 – MV, 4 – MG NL2, 5 – MG 81, 6 – MG NL 1, 7 – WB1, 8 – WB3, 9 – WB4, 10 - WB6, 11 – WB7

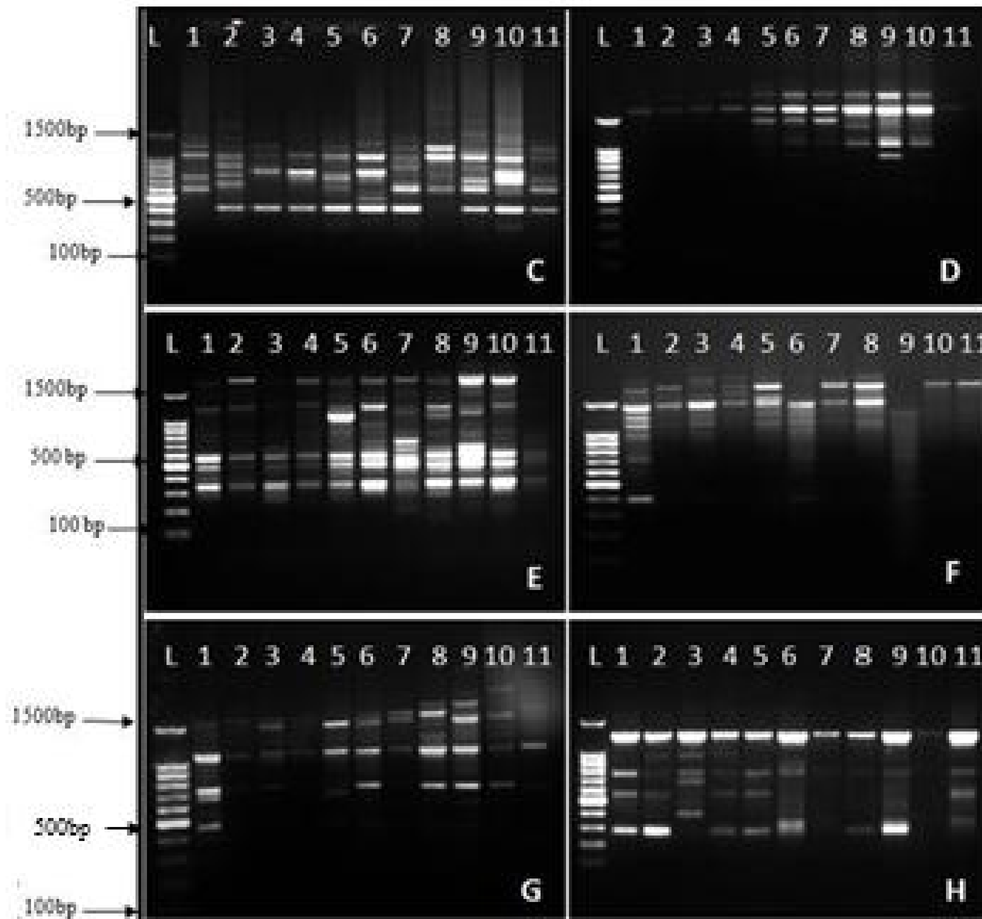


**Plate 4.2: Amplification products generated by SCoT primers with eleven DNA templates.** **4.2.C** – SCoT 15, **4.2.D** – SCoT 16, **4.2.E** – SCoT 21, **4.2.F** – SCoT 32, **4.2.G** – SCoT 35, **4.2.H** – SCoT 45. Lane L – 100bp Ladder (Orion X), 1 – MG 91, 2 – MG NL 1, 3 – MV, 4 – MG NL2, 5 – MG 81, 6 – MG NL 1, 7 – WB1, 8 – WB3, 9 – WB4, 10 - WB6, 11 – WB7

### 4.1.3 Genetic Diversity Revealed by SCoT markers

A total of 128 bands were produced by 8 SCoT primers among 11 *A. heterophyllus* accessions out of which 123 were polymorphic. The number of polymorphic bands ranged from 9 (SCoT 45) to 20 (SCoT 15) with an average of 15.37 bands per primers. Percentage polymorphism varied from 81.8% (SCoT 45) to 100% (SCoT 11, 21, 32) with an average polymorphism of 94.875% across all accessions (Table 4.2).

The observed number of alleles per locus (na), effective number of alleles (ne), Shannon's information index (I) and Nei's gene diversity were statistically generated using POPGENE (Yeh and Boyle, 1997) and shown in Table 4.4.

**Table 4.2 :** Number of Scorable polymorphic bands of each primer

| Primer ID | Total Number of Bands (TNB) | Number of Polymorphic Bands (NPB) | Number of Monomorphic Bands(NMB) | Polymorphic Ratio (%) |
|---|---|---|---|---|
| SCoT 10 | 13 | 12 | 1 | 92.3 |
| SCoT 11 | 15 | 15 | - | 100 |
| SCoT 15 | 21 | 20 | 1 | 95.2 |
| SCoT 16 | 19 | 18 | 1 | 94.7 |
| SCoT 21 | 15 | 15 | - | 100 |
| SCoT 32 | 15 | 15 | - | 100 |
| SCoT 35 | 20 | 19 | 1 | 95 |
| SCoT 45 | 11 | 9 | 2 | 81.8 |

Genetic similarity coefficients were generated based on Jaccards Coefficients. The similarity coefficients generated ranged from a minimum of 0.27 to a maximum of 0.57 with an average of 0.5. The highest genetic similarity (0.57) was found between MV and MGNL2 and the lowest genetic similarity (0.27) was found between MG NL 2 ,WB 1 and MG 91(Table 4.3).

The similarity coefficients generated from the SCoT data were used to construct the dendrogram based on the Jaccards coefficient. UPGMA clustering algorithm based on similarity matrix at similarity index of 0.47 grouped the accessions into 8 clusters (Figure 4.2) . Cluster I (MG 91) is the most distinctive among all. Cluster I (MG 91), II (MG NL 1), IV(WB 7), VI(WB 4), VII (IC) and VIII(WB3) consisted only single accession while cluster III got most similar two cultivar accessions (MV and MG NL2) and cluster V got one cultivar (MG 81) and two wild accessions(WB 3 and WB 4).

**Table 4.3:** Table showing Jaccard's Similarity Coefficient Values

| | MG 91 | MG NL1 | MV | MG NL2 | MG 81 | IC | WB1 | WB3 | WB4 | WB6 | WB7 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| MG 91 | 1.00 | | | | | | | | | | |
| MGNL1 | 0.44 | 1.00 | | | | | | | | | |
| MV | 0.37 | 0.47 | 1.00 | | | | | | | | |
| MG NL2 | 0.27 | 0.44 | 0.57 | 1.00 | | | | | | | |
| MG 81 | 0.39 | 0.48 | 0.45 | 0.44 | 1.00 | | | | | | |
| IC | 0.43 | 0.39 | 0.43 | 0.34 | 0.49 | 1.00 | | | | | |
| WB 1 | 0.27 | 0.37 | 0.36 | 0.33 | 0.51 | 0.36 | 1.00 | | | | |
| WB 3 | 0.40 | 0.38 | 0.45 | 0.36 | 0.42 | 0.41 | 0.44 | 1.00 | | | |
| WB 4 | 0.33 | 0.34 | 0.36 | 0.32 | 0.46 | 0.44 | 0.41 | 0.43 | 1.00 | | |
| WB 6 | 0.30 | 0.39 | 0.36 | 0.45 | 0.49 | 0.41 | 0.46 | 0.40 | 0.47 | 1.00 | |
| WB 7 | 0.40 | 0.37 | 0.40 | 0.43 | 0.40 | 0.34 | 0.28 | 0.35 | 0.36 | 0.36 | 1.00 |

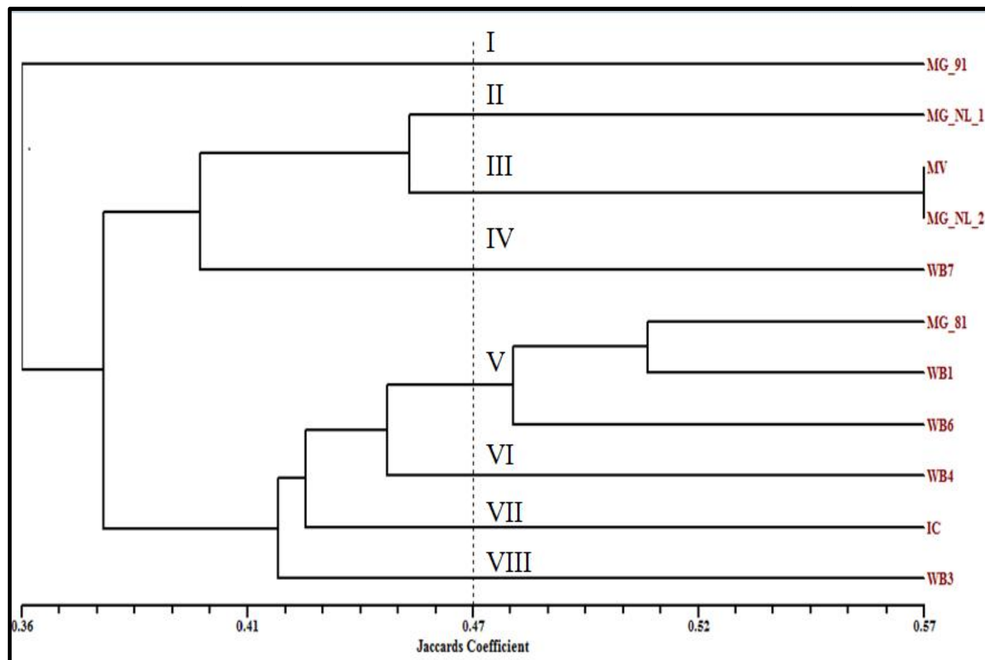


**Figure 4.2:** Dendrogram of the jackfruit accessions studied

**Table 4.4:** Table Showing summary of genetic variation statistics

| Locus | na | ne | h | I |
|---|---|---|---|---|
| ST10 | 2.0000 | 1.6544 | 0.3955 | 0.5847 |
| ST11 | 2.0000 | 1.6565 | 0.3963 | 0.5856 |
| ST15 | 2.0000 | 1.3810 | 0.2759 | 0.4483 |
| ST16 | 2.0000 | 1.6528 | 0.3950 | 0.5841 |
| ST21 | 2.0000 | 1.6777 | 0.4040 | 0.5938 |
| ST32 | 2.0000 | 1.4156 | 0.2936 | 0.4695 |
| ST35 | 2.0000 | 1.3379 | 0.2525 | 0.4197 |
| ST45 | 2.0000 | 1.7510 | 0.4289 | 0.6203 |
| **Mean** | 2.0000 | 1.5659 | 0.3552 | 0.5382 |
| **St. Dev** | 0.0000 | 0.1600 | 0.0690 | 0.0785 |

na = Observed number of alleles

ne = Effective number of alleles [Kimura and Crow (1964)]

h = Nei's (1973) gene diversity

I = Shannon's Information index [Lewontin (1972)]

The observed number of alleles per locus (na) is 2.0000, effective number of alleles (ne) ranged from 1.3379 to 1.7510, Shannon's information index (I) ranged from 0.4197 to 0.6203, Nei's gene diversity ranged from 0.2525 to 0.4289.

## 4.2 PHYTOCHEMICAL ANALYSIS

### 4.2.1 Extraction

The dried flakes and seeds were extracted by methanol using soxhlet apparatus for 4 hours. The percentage of yield, colour of extract and consistency were noted.

**Table 4.5** Yield of flakes extract

| Flakes | Extract Weight(g) | Yield (%) | Extract Color | Extract Consistency |
|---|---|---|---|---|
| MGNL 2 – IC 97628 | 1.5736 | 7.868 | Light Yellow | Thick |
| MGNL 1 – IC 97627 | 3.4965 | 17.4825 | Dark Brown | Sticky |
| MV – IC 97633 | 2.5051 | 12.5255 | Bright Yellow | Thick |
| MG 70 – IC 91665 | 1.2688 | 6.344 | Light Brown | Loose |
| MG 92- IC 96148 | 0.646 | 3.23 | Light Brown | Thick |
| MGNL - IC 97625 | 3.1751 | 15.8755 | Dark Brown | Sticky |
| MG 81 – IC 92254 | 6.44678 | 32.2339 | Light Brown | Thick |
| MG91' – IC 9550 1A | 3.6616 | 18.308 | Light Yellow | Sticky |
| NLTR – IC 97632 | 2.1108 | 10.554 | Dark Brown | Loose |
| MG 91 – IC 9550 1B | 1.6105 | 8.0525 | Light Yellow | Sticky |
| MGNL 2 – IC 97628 | 1.5736 | 7.868 | Light Yellow | Thick |
| MGNL 1 – IC 97627 | 3.4965 | 17.4825 | Dark Brown | Sticky |
| MV – IC 97633 | 2.5051 | 12.5255 | Bright Yellow | Thick |
| WB1 | 2.6416 | 13.208 | Light Yellow | Thick |
| WB2 | 2.8872 | 14.436 | Very Dark Brown | Sticky |
| WB3 | 12.6496 | 63.248 | Bright Yellow | Thick |
| WB4 | 2.5733 | 12.8665 | Light Yellow | Sticky |
| WB5 | 11.498 | 57.49 | Light Brown | Thick |
| WB6 | 11.382 | 56.91 | Dark Brown | Sticky |
| WB7 | 15.13 | 75.65 | Light Brown | Thick |
| WB8 | 7.8084 | 39.042 | Light Brown | Loose |

**Table 4.6** Yield of seed extract

| Seeds | Extract Weight (g) | Yield (%) | Extract Color | Extract Consistency |
|---|---|---|---|---|
| MGNL 2 – IC 97628 | 1.2061 | 6.0305 | Dark Brown | Thick |
| MGNL 1 – IC 97627 | 4.9272 | 24.636 | Dark Brown | Thick |
| MV – IC 97633 | 1.2748 | 6.374 | Dark Brown | Thick |
| MG 70 – IC 91665 | 1.1812 | 5.906 | Dark Brown | Thick |
| MG 92- IC 96148 | 0.7875 | 3.9375 | Dark Brown | Sticky |
| MGNL - IC 97625 | 1.505 | 7.525 | Light Brown | Thick |
| MG 81 – IC 92254 | 8.4686 | 42.343 | Dark Brown | Thick |
| MG91' – IC 9550 1A | 3.7335 | 18.6675 | Dark Brown | Sticky |
| NLTR – IC 97632 | 4.2618 | 21.309 | Light Brown | Thick |
| MG 91 – IC 9550 1B | 1.1923 | 5.9615 | Light Brown | Thick |
| WB1 | 0.4079 | 2.0395 | Very Dark Brown | Thick |
| WB2 | 5.7864 | 28.932 | Very Dark Brown | Solid |
| WB3 | 13.2304 | 66.152 | Dark Brown | Thick |
| WB4 | 0.5572 | 2.786 | Light Yellow | Sticky |
| WB5 | 10.88 | 54.4 | Light Brown | Thick |
| WB6 | 2.9092 | 14.546 | Dark Brown | Sticky |
| WB7 | 7.64 | 38.2 | Light Brown | Thick |
| WB8 | 10.696 | 53.48 | Light Brown | Thick |

### 4.2.2 Determination of Total Phenolic Content

The values of total phenolics in extracts were expressed as Gallic acid equivalents (GAE). The absorbance values of standard concentration were shown in Table 4.7. Concentration of phenolics in mg GAE/ g of extract were estimated for flakes and seeds of both cultivar and wild. The maximum phenolic content in cultivar varieties were determined in MG 92 flakes (25.375mg/g) and MG 91' seeds (91.930mg/g) and minimum phenolic content were in MG 91 flakes 2.89mg/g and MG 92 seeds 5.0833mg/g (Table 4.8). In wild varieties, the maximum amount of total phenolics were in WB2 flakes and seeds *ie.,* 219.536 mg/g and 233.344 mg GAE/g of extract and minimum of 3.15mg/g in WB 5 flakes and 14mg/g of WB 8 seeds of extract in wild variety (Table 4.9).

**Table 4.7** Absorbance Values of Standard Concentration(GA)

| Concentration (µg/ml) | Absorbance @ 760nm |
|---|---|
| 2 | 0.024±0.003 |
| 4 | 0.050±0.002 |
| 6 | 0.082±0.004 |
| 8 | 0.104±0.003 |
| 10 | 0.114±0.007 |
| 20 | 0.290±0.024 |
| 40 | 0.537±0.004 |
| 60 | 0.801±0.009 |
| 80 | 1.034±0.028 |
| 100 | 1.194±0.119 |

All the values are expressed as Mean± Standard Deviation, for n=3

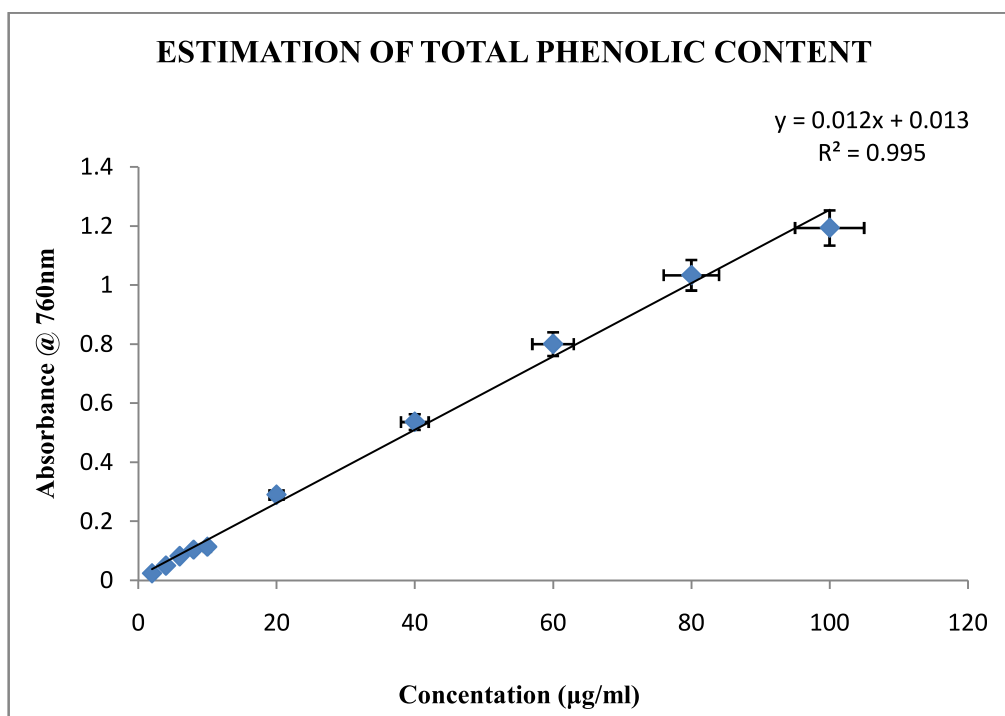


**Figure 4.3** Standard Curve for Gallic Acid

**Table 4.8** Absorbance Values of Cultivar Samples

| Sample | Concentration (µg/ml) | Flakes<br>Mean Absorbance | Seeds<br>Mean Absorbance |
|---|---|---|---|
| MG92 | 200<br>500 | 0.079±0.002<br>0.153±0.111 | 0.024±0.0005<br>0.047±0.004 |
| MG 91 | 200<br>500 | 0.014±0.008<br>0.044±0.002 | 0.034±0.002<br>0.105±0.001 |
| MG IC97625 | 200<br>500 | 0.048±0.002<br>0.084±0.001 | 0.196±0.002<br>0.518±0.004 |
| NLTR | 200<br>500 | 0.043±0.003<br>0.063±0.003 | 0.019±0.002<br>0.088±0.011 |
| MV | 200<br>500 | 0.032±0.003<br>0.200±0.004 | 0.015±0.001<br>0.134±0.004 |
| MGNL1 | 200<br>500 | 0.014±0.0005<br>0.058±0.004 | 0.150±0.007<br>0.331±0.030 |
| MG 70 | 200<br>500 | 0.021±0.004<br>0.042±0.007 | 0.096±0.001<br>0.161±0.004 |
| MG 81 | 200<br>500 | 0.031±0.034<br>0.047±0.004 | 0.035±0.001<br>0.114±0.002 |
| MGNL2 | 200<br>500 | 0.023±0.002<br>0.073±0.004 | 0.035±0.001<br>0.114±0.002 |
| MG 91' | 200<br>500 | 0.024±0.002<br>0.104±0.001 | 0.117±0.005<br>0.347±0.016 |

All the values are expressed as Mean ± Standard Deviation, for n=3

**Table 4.9** Absorbance Values of Wild Samples

| Sample | Concentration<br>(µg/ ml) | Flakes<br>Mean Absorbance | Seeds<br>Mean Absorbance |
|---|---|---|---|
| WB1 | 200<br>500 | 0.526±0.003<br>0.973±0.002 | 0.539±0.006<br>1.017±0.040 |
| WB2 | 200<br>500 | 0.670±0.005<br>1.006±0.012 | 0.728±0.007<br>1.027±0.050 |
| WB3 | 200<br>500 | 0.042±0.003<br>0.131±0.006 | 0.042±0.003<br>0.131±0.006 |
| WB4 | 200<br>500 | 0.350±0.006<br>0.927±0.008 | 0.536±0.002<br>0.959±0.005 |
| WB5 | 200<br>500 | 0.015±0.006<br>0.045±0.003 | 0.054±0.005<br>0.247±0.008 |
| WB6 | 200<br>500 | 0.076±0.002<br>0.186±0.003 | 0.096±0.002<br>0.293±0.005 |
| WB7 | 200<br>500 | 0.076±0.002<br>0.186±0.003 | 0.058±0.001<br>0.173±0.001 |
| WB8 | 200<br>500 | 0.035±0.002<br>0.047±0.001 | 0.044±0.003<br>0.104±0.003 |

All the values are expressed as Mean± Standard Deviation, for n=3

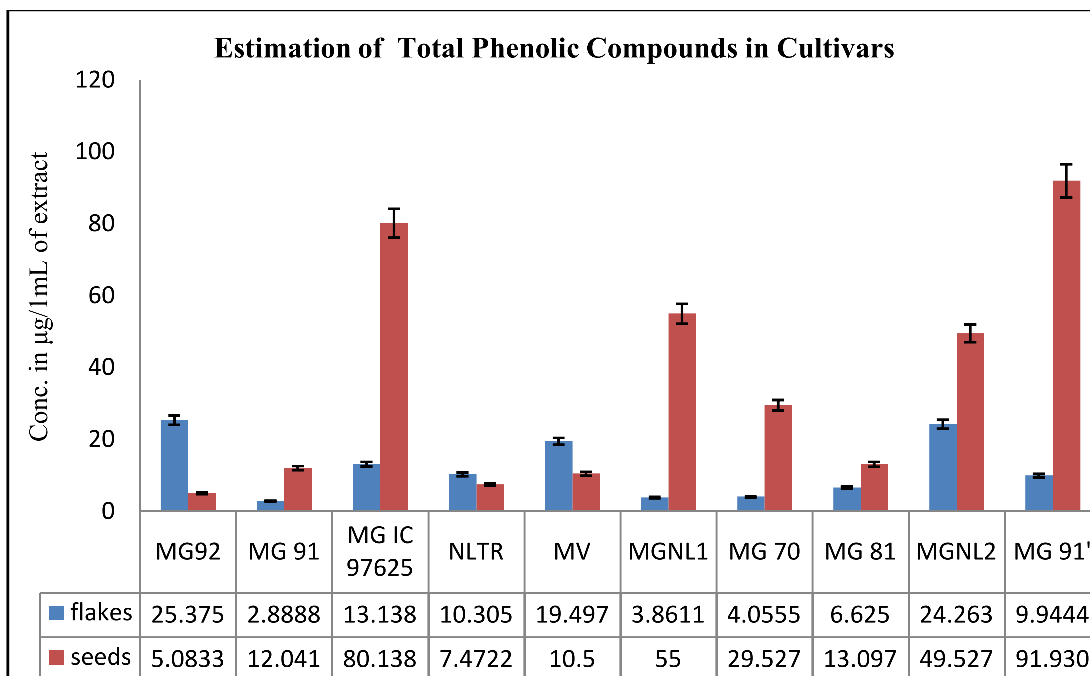


**Figure 4.4** Total phenolic content of methanolic extract of flakes and seeds of cultivar samples

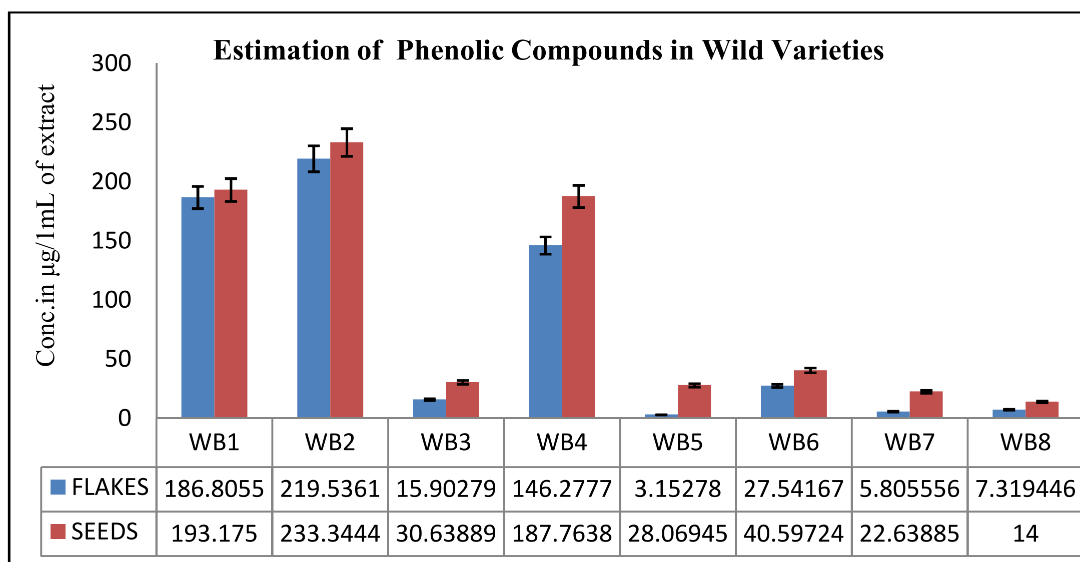


**Figure 4.5** Total phenolic content of methanolic extract of flakes and seeds of wild samples

### 4.2.3 Determination of Total Flavonoid Content

The values of total flavonoid in crude extracts were expressed as mg Quercetin equivalents (QE) per gram of extract. The absorbance values of standard and sample concentrations were shown in Table 4.10,4.11,4.12. The highest Flavonoid content in cultivar variety was determined in MV flakes; 673.7mg QE/g and MG NL 2 seeds; 183.3mg/g and minimum was in MG 70 flakes 74mg/g and MG81 seeds 20.62mg/g (Figure 4.7). In wild varieties WB1 flakes and seeds showed the highest amount of flavonoid content 69.5mg/g and 743.75mg/g respectively and the minimum was determined in flakes of WB7 26.875mg/g and seeds of WB6 26.25mg/g (Figure 4.8).

**Table 4.10** Absorbance Values of Standard Concentration (Q)

| Concentration (µg/ml) | Absorbance |
|---|---|
| 10 | 0.023±0.0020 |
| 20 | 0.059±0.0007 |
| 30 | 0.088±0.0056 |
| 40 | 0.114±0.0007 |
| 50 | 0.129±0.0156 |

All the values are expressed as Mean± Standard Deviation, for n=3

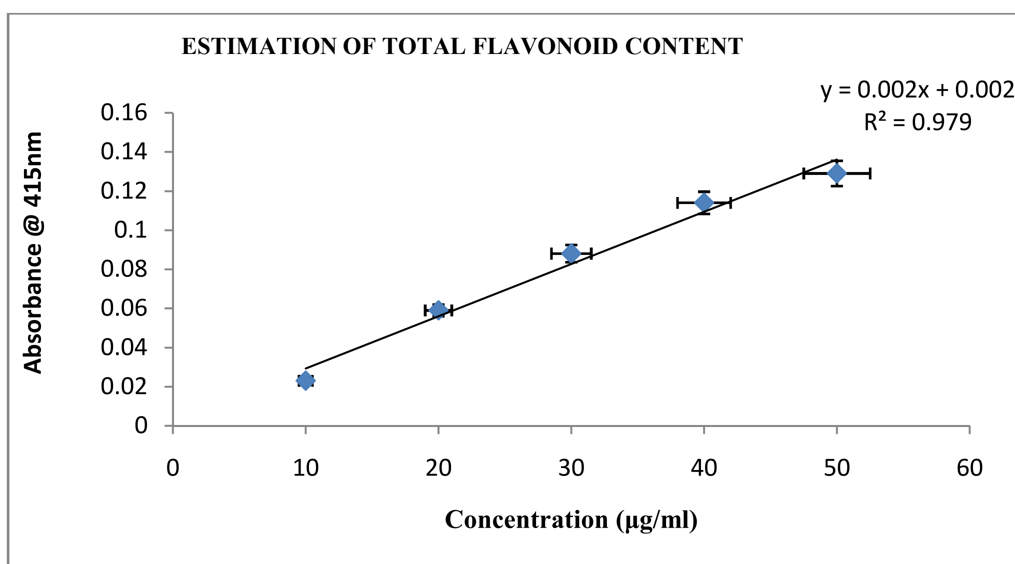


**Figure 4.6** Standard Curve of Quercetin

**Table 4.11** Absorbance Values of Cultivar Flakes

| Sample | Concentration (µg/ ml) | Flakes Mean Absorbance | Seeds Mean Absorbance |
|---|---|---|---|
| MG92 | 200<br>500 | 0.039±0.001<br>0.119±0.014 | 0.013±0.001<br>0.033±0.004 |
| MG 91 | 200<br>500 | 0.038±0.011<br>0.084±0.009 | 0.020±0.002<br>0.078±0.007 |
| MG IC97625 | 200<br>500 | 0.025±0.003<br>0.095±0.006 | 0.019±0.001<br>0.047±0.002 |
| NLTR | 200<br>500 | 0.019±0.004<br>0.042±0.004 | 0.019±0.001<br>0.070±0.015 |
| MV | 200<br>500 | 138.5±0.014<br>327.5±0.059 | 0.027±0.001<br>0.080±0.001 |
| MGNL1 | 200<br>500 | 0.044±0.002<br>0.163±0.021 | 0.105±0.012<br>0.338±0.040 |
| MG 70 | 200<br>500 | 0.030±0.002<br>0.077±0.019 | 0.018±0.001<br>0.069±0.001 |
| MG 81 | 200<br>500 | 0.037±0.001<br>0.107±0.024 | 0.007±0.002<br>0.029±0.001 |
| MGNL2 | 200<br>500 | 0.039±0.001<br>0.104±0.001 | 0.062±0.028<br>0.217±0.003 |
| MG 91' | 200<br>500 | 0.039±0.011<br>0.104±0.001 | 0.069±.0070<br>0.188±0.018 |

All the values are expressed as Mean± Standard Deviation, for n=3

**Table 4.12** Absorbance Values of Wild Samples

| Sample | Concentration (µg / ml) | Flakes Mean Absorbance | Seeds Mean Absorbance |
|---|---|---|---|
| WB1 | 200<br>500 | 0.124±0.003<br>0.437±0.002 | 0.198±0.002<br>0.520±0.018 |
| WB2 | 200<br>500 | 0.268±0.012<br>0.821±0.002 | 0.230±0.007<br>0.721±0.023 |
| WB3 | 200<br>500 | 0.009±0.020<br>0.016±0.004 | 0.012±0.002<br>0.021±0.006 |
| WB4 | 200<br>500 | 0.201±0.003<br>0.638±0.002 | 0.083±0.001<br>0.162±0.002 |
| WB5 | 200<br>500 | 0.035±0.016<br>0.037±0.006 | 0.015±0.014<br>0.014±0.003 |
| WB6 | 200<br>500 | 0.031±0.010<br>0.080±0.032 | 0.005±0.005<br>0.014±0.005 |
| WB7 | 200<br>500 | 0.005±0.016<br>0.015±0.006 | 0.026±0.034<br>0.031±0.006 |
| WB8 | 200<br>500 | 0.023±0.004<br>0.059±0.021 | 0.009±0.020<br>0.016±0.004 |

All the values are expressed as Mean± Standard Deviation, for n=3

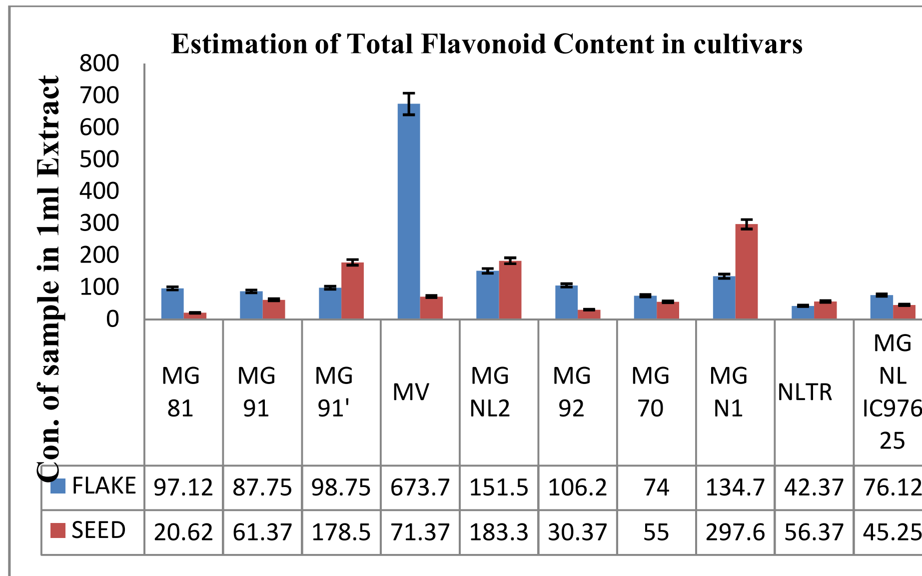


**Figure 4.7** Total flavonoid content of methanolic extract of flakes and seeds of cultivar samples

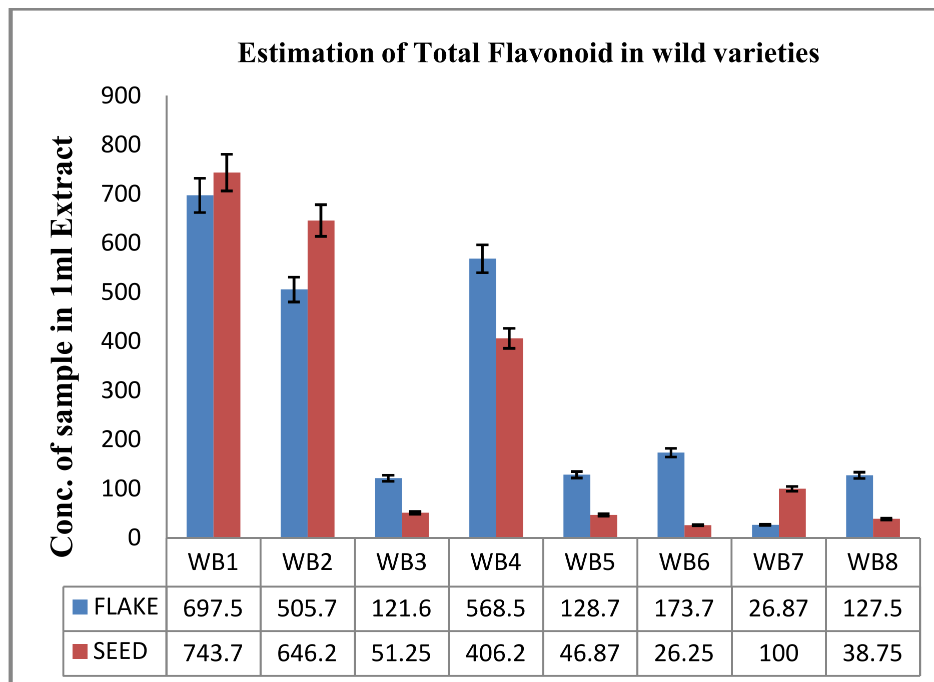


**Figure 4.8** Total flavonoid content of methanolic extract of flakes and seeds of wild samples

### 4.2.4 DPPH Radical Scavenging Assay

Antioxidant potential of methanolic extract was determined by using DPPH radical scavenging Assay. The $IC_{50}$ values were calculated using percentage of inhibition by radical scavenging of antioxidants in our extract. Percentage inhibition of samples were shown in Table 17.

**Table 4.13** Absorbance Values of Standard Concentration(Asc. Acid)

| Concentration (µg/ml) | Percentage of Inhibition |
|---|---|
| 0.625 | 9.868351 |
| 1.25 | 13.48689 |
| 2.5 | 25.22863 |
| 5 | 43.52364 |
| 10 | 72.42139 |

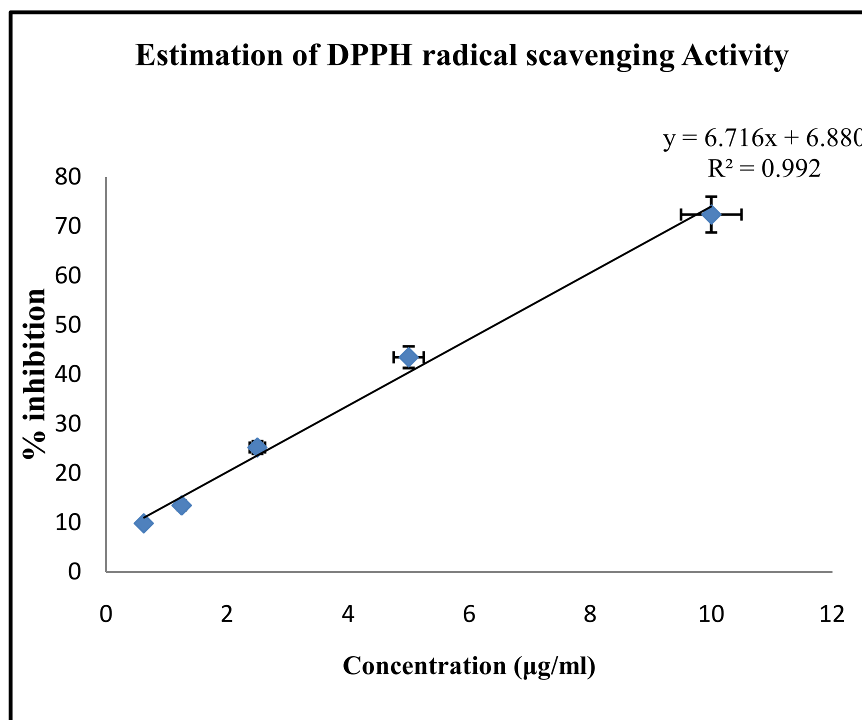


**Figure 4.9** Standard Curve for Ascorbic Acid (Asc. Acid)

**Table 4.14** Percentage of Inhibition of Various Cultivar Samples and its concentrations

| Con. (µg/ml) | MG 81 Flakes | MV Flakes | MG 91' Flakes | NLTR Flakes | MG91 Flakes | MG 70 Flakes | MG NL 1 Flakes | MG NL2 Flakes | MG NL IC Flakes |
|---|---|---|---|---|---|---|---|---|---|
| 200 | 29.3103 | 29.37 | 32.17 | 31 | 20.87 | 31.19 | 30.2 | 20.9 | 35.623 |
| 100 | 28.2489 | 26.56 | 16.81 | 28 | 18.81 | 27.6 | 21 | 18.8 | 28.36 |
| 50 | | | | | | | | 13.6 | 22.1406 |
| 25 | 23.2008 | 20.24 | 8.836 | 17 | 13.57 | 17.17 | 14.8 | 9.61 | 20.1764 |
| 12.5 | | | | | 9.609 | 17.45 | 4.95 | | 16.6182 |
| 6.25 | 23.0101 | 16.02 | 7.394 | 17 | | | | | 10.2051 |

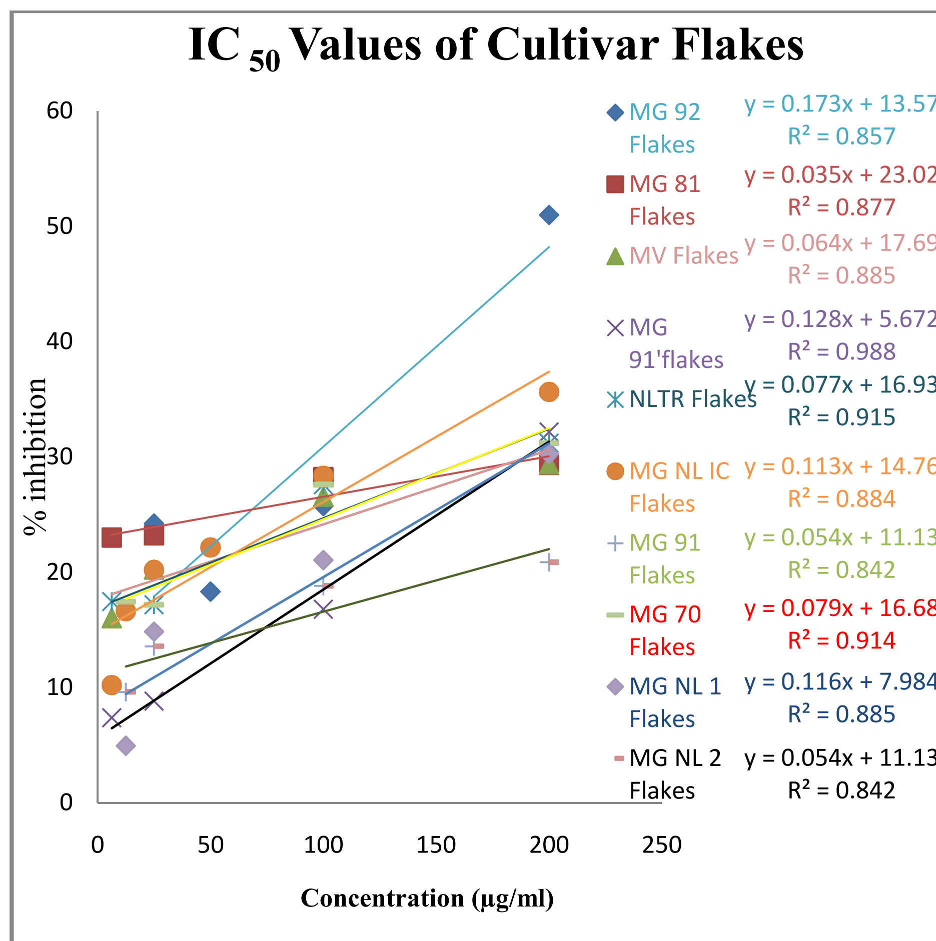


**Figure 4.10**. Graphical Representation for IC $_{50}$ of Cultivar flake

**Table 4.15** Percentage of Inhibition of Various Cultivar Seed Samples and its Concentrations

| Con. (µg/ml) | MG NL1 Seeds | MG 92 Seeds | MG NL IC Seeds | MG NL2 Seeds | MG 70 Seeds | MG 81 Seeds | MG 91' Seeds | MG 91 Seeds | MV Seeds | NLTR Seeds |
|---|---|---|---|---|---|---|---|---|---|---|
| 200 | 59.23 | 72.24 | 47.29 | 52.9 | 29.58 | 38.95 | 79.54 | 21.034 | 31.2 | 52.922 |
| 100 | 40.66 | 47.28 | 34.21 | 38.8 | 23.77 | 34.4 | 53.61 | 19.839 | 27.6 | 38.774 |
| 50 | 29.60 | 27.2 | 22.84 | | | | | | | |
| 25 | 26.65 | 24.54 | 23.24 | 23.2 | 18.3 | 26.94 | 36.98 | 14.572 | 23.6 | 23.173 |
| 12.5 | 21.06 | 18.31 | 16.37 | 13.2 | 17.8 | 24.2 | | | | |
| 6.25 | 23.15 | 22.85 | 21.98 | | | | 24.23 | 12.934 | 17.45 | 13.214 |

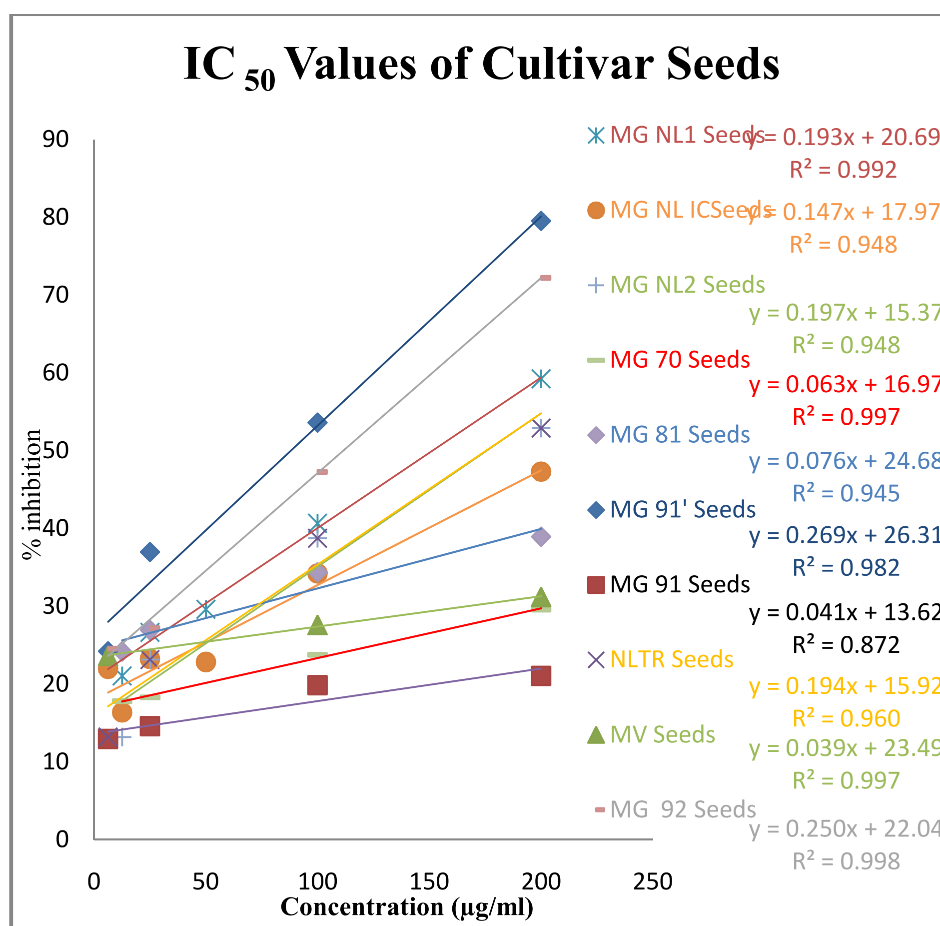


**Figure 4.11** Graphical Representation for IC $_{50}$ of Cultivar Seeds

**Table 4.16** $IC_{50}$ Values for the Cultivar Samples

| Flake Sample | $IC_{50}$ |
|---|---|
| MG 81 Flakes | 770.857 |
| MG 91 Flakes | 475.714 |
| MV Flakes | 682.413 |
| MG 91' Flakes | 335.036 |
| NLTR Flakes | 603 |
| MG NL2 Flakes | 682.413 |
| MG 92 Flakes | 210.578 |
| MG 70 Flakes | 665.172 |
| MG NL1 Flakes | 347.095 |
| MG NL IC Flakes | 665.172 |

| Seed Sample | $IC_{50}$ |
|---|---|
| MG 81 Seeds | 327.373 |
| MG 91 Seeds | 839.772 |
| MV Seeds | 419.876 |
| MG 91' Seeds | 94.125 |
| NLTR Seeds | 237.045 |
| MG NL2 Seeds | 175.829 |
| MG 92 Seeds | 419.876 |
| MG 70 Seeds | 510.606 |
| MG NL1 Seeds | 151.865 |
| MG NL IC Seeds | 217.891 |

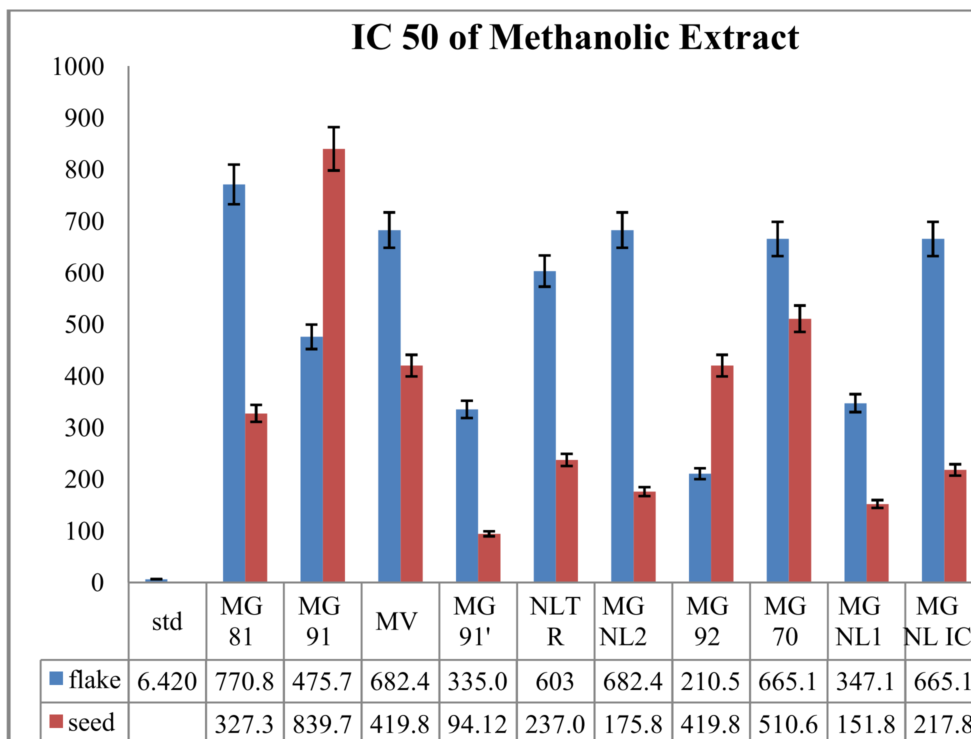


**Figure 4.12** $IC_{50}$ Values for Cultivar Samples

**Table 4.17** Percentage of Inhibition of Various Wild Flakes Samples and its Concentrations

| Conc. (µg/ml) | WB 1 F | WB 2F | WB 3F | WB 4F | WB 5F | WB 6F | WB 7F | WB 8F |
|---|---|---|---|---|---|---|---|---|
| 50 | 16.787 | 2.89 | 8.219 | 12.52 | 5.199 | 9.44 | 0.058 | 4.41 |
| 100 | 27.196 | 8.18 | 16.438 | 28.57 | 8.665 | 11.80 | 9.20 | 7.01 |
| 150 | 40.67 | 12.45 | 24.794 | 68.88 | 11.09 | 17.22 | | 9.92 |
| 200 | 46.99 | 15.46 | | 82.77 | 15.07 | 23.47 | 36.14 | |

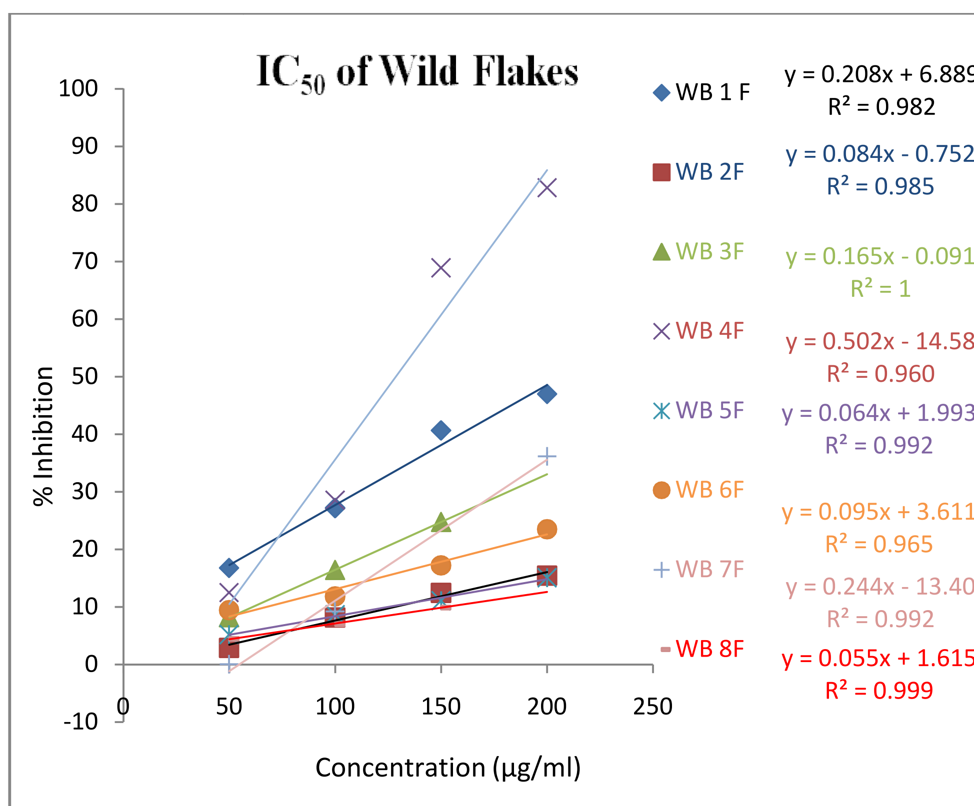


**Figure 4.13** Graphical Representation for IC $_{50}$ of Wild flakes

**Table 4.18** Percentage of Inhibition of Various Wild Seeds Samples and its Concentrations

| Conc. (µg/ml) | WB 1S | WB 2S | WB 3S | WB 4S | WB 5S | WB 6S | WB 7S | WB 8S |
|---|---|---|---|---|---|---|---|---|
| 50 | 10.23 | 19.79 | 0.66 | 17.661 | 9.272 | 19.58 | 13.537 | 5.199 |
| 100 | 15.52 | 37.609 | 0.658 | 39.618 | 17.93 | | 24.90 | 8.665 |
| 150 | 22.44 | 56.79 | 0.629 | 68.377 | | 60.69 | 37.48 | |
| 200 | 28.760 | 65.282 | 0.609 | 90.692 | 34.74 | 77.5 | 48.796 | 15.078 |

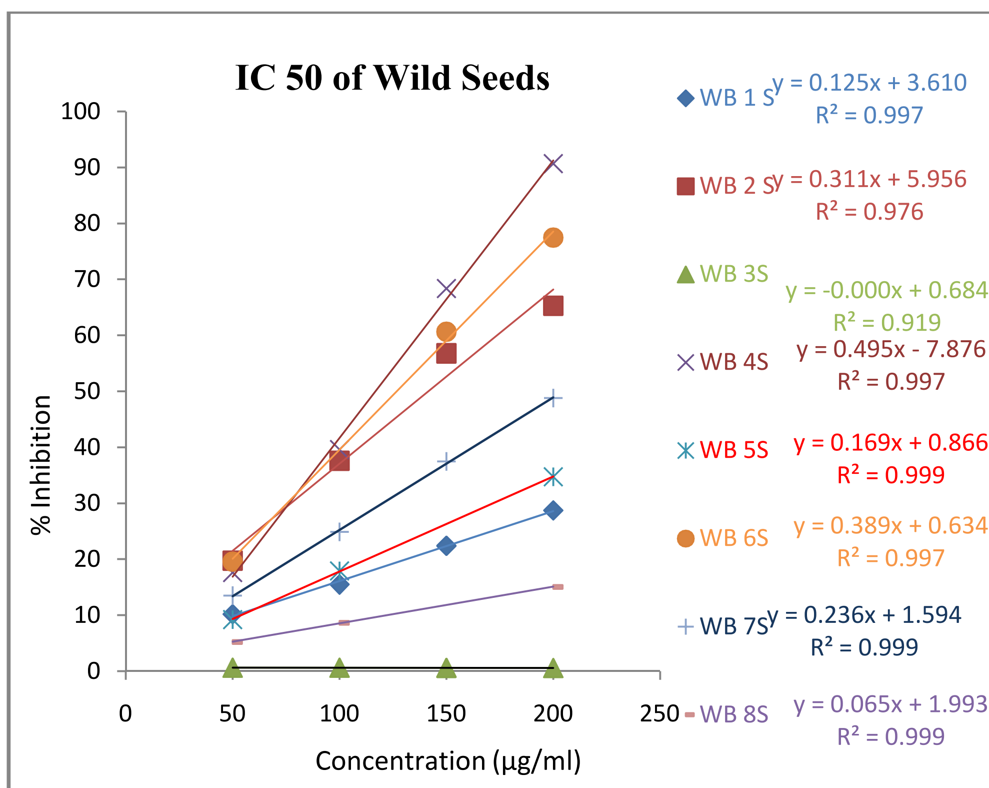


**Figure 4.14** Graphical Representation for $IC_{50}$ of Wild Seeds

**Table 4.19** IC $_{50}$ Values for the Wild Samples

| Flake Sample | IC $_{50}$ |
|---|---|
| WB1 | 207.264 |
| WB 2 | 596.133 |
| WB 3 | 303.581 |
| WB 4 | 257.290 |
| WB 5 | 750.109 |
| WB 6 | 488.305 |
| WB 7 | 488.305 |
| WB 8 | 879.73 |

| Seed Sample | IC $_{50}$ |
|---|---|
| WB1 | 371.12 |
| WB 2 | 141.620 |
| WB 3 | 705.69 |
| WB 4 | 234.3157 |
| WB 5 | 290.733 |
| WB 6 | 126.904 |
| WB 7 | 205.110 |
| WB 8 | 638.569 |

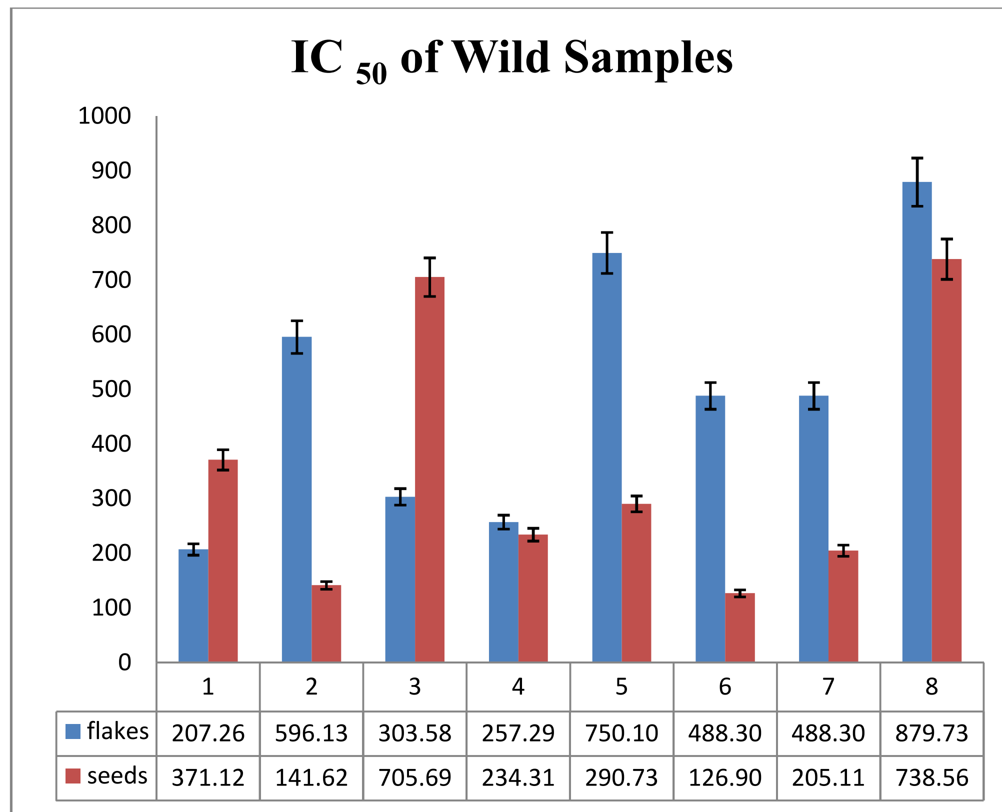


**Figure 4.15** IC $_{50}$ Values for Wild Samples

4.3 ANTI-INFLAMMATORY STUDIES

**4.3.1 *In vitro* Anti inflammatory Studies**

***4.3.1.1 HRBC Membrane Stabilization Assay***

The measure of anti inflammatory activity of the extract is measured on the basis of inhibition of HRBC membrane lysis due to the hypotonic treatments to the Red Blood Cells. Various concentrations of methanolic extract of Jackfruit sample is determined based on the standard diclofenac sodium. Extract showed maximum inhibition of 46.069 % at 200 µg/ml and IC $_{50}$ of 292.157. The results are shown in Table 4.20

**Table 4.20** % of protection HRBC Membrane Stabilization Assay

| Concentration (µg/ml) | % Protection sample | $IC_{50}$ sample | % Protection Standard diclofenac | $IC_{50}$ standard |
|---|---|---|---|---|
| 100 | 30.999±1.52 | 292.157 | 42.77963± 1.32 | 167.589 |
| 150 | 34.745±1.22 | | 47.44889±1.50 | |
| 200 | 40.704±2.36 | | 53.89615±1.56 | |
| 250 | 46.069±1.00 | | 59.3239 ±1.37 | |

Values are expressed as mean ± SD, for n=3

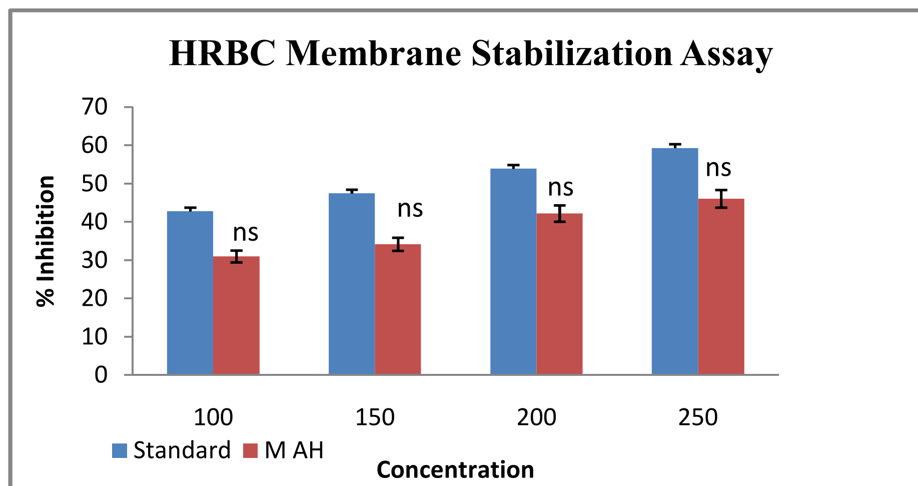


**Figure 4.16** HRBC Membrane Stabilization Assay

Values are expressed as mean ± SD, for n=3, one way ANOVA followed by Duncan's multiple comparison test, ns- no significant difference compared with standard.

#### *4.3.1.1 Albumin Denaturation Assay*

The measure of anti inflammatory activity of the extract is measured on the basis of inhibition of protein denaturation due to heat applied. Various concentrations of methanolic extract of jackfruit sample is determined based on the standard diclofenac sodium. Extract showed maximum inhibition of 35.42 % at 200 µg/ml and IC $_{50}$ of 445.34. The results are shown in Table 4.21.

**Table 4.21** % of protection in Albumin Denaturation Assay

| Concentration (µg/ml) | % Protection sample | $IC_{50}$ sample | % Protection Standard diclofenac | $IC_{50}$ standard |
|---|---|---|---|---|
| 100 | 23.76±0.0021% | 445.342 | 40.48405±0.015% | 178.7273 |
| 150 | 30.25±0.0028% | | 47.85479±0.005% | |
| 200 | 32.12±0.0021% | | 53.24532±0.005% | |
| 250 | 35.42±0.0035% | | 57.09571±0.005% | |

Values are expressed as mean ± SD, for n=3

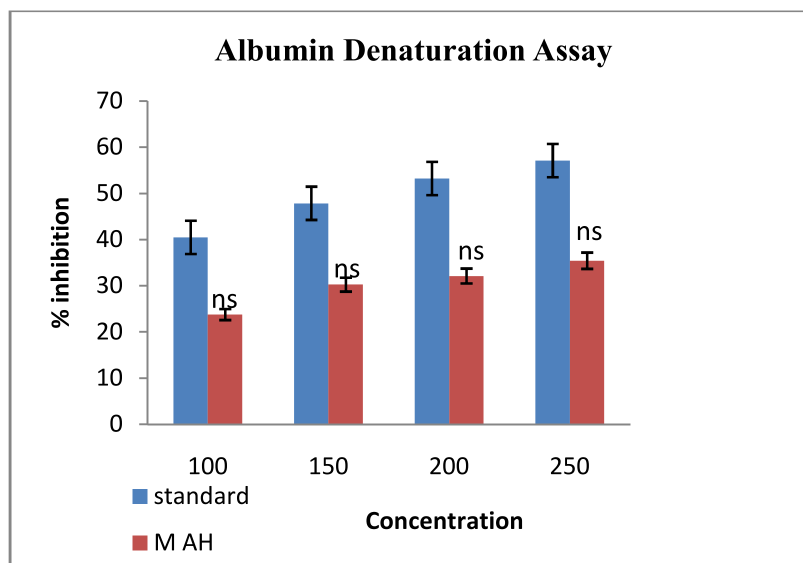


**Figure 4.17** Membrane Denaturation Assay

Values are expressed as mean ± SD, for n=3, one way ANOVA followed by Duncan's multiple comparison test, ns- no significant difference compared with standard.

### 4.3.2 *In vivo* Anti inflammatory Studies

#### *4.3.2.1 Carrageenan induced paw oedema in Wistar Rats*

The activity of methanolic extract of *A. heterophyllus* on carrageenan induced paw oedema was examined by the measuring of paw volume in adult Wistar rats. The percentage of inhibition is shown in Table 4.22 and the difference in paw volume is shown graphically in Figure 4.18. The maximum percentage of inhibition of paw oedema 37.5 % was exhibited by a dose of M AH (250mg/kg)

**Table 4. 22** Percentage of inhibition in Carrageenan induced paw oedema

| Sl.No. | Concentration (µg/ml) | Percentage Of Inhibition |
|---|---|---|
| 1 | Control | - |
| 2 | Indomethacin (10mg/kg) | 42.5±2.23%* |
| 3 | J200 (200mg/kg) | 33.3±3.28% |
| 4 | J250 (250mg/kg) | 37.5±3.32% |

Values are expressed as mean ± SD, for n=3 *P ≤ 0.05 compared with standard

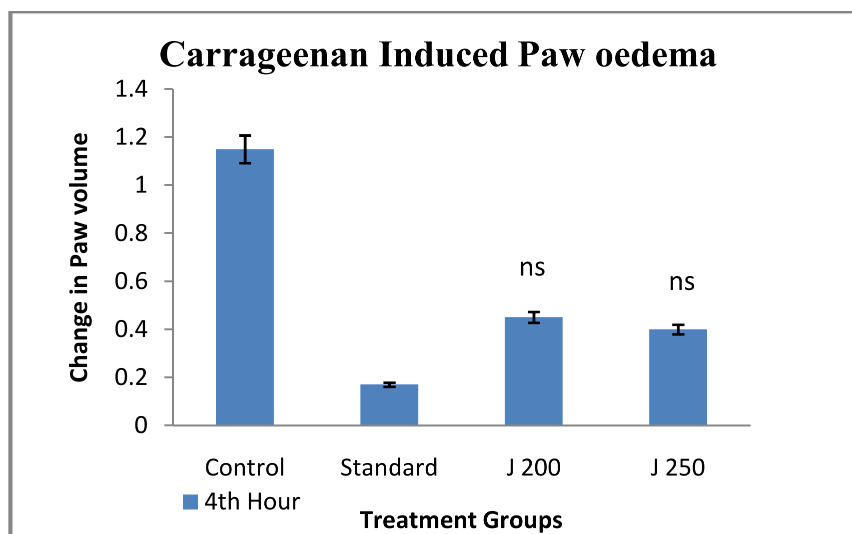


**Figure 4.18** Effect of methanolic extract of *A. heterophyllus* on Carrageenan induced paw oedema in rats

Values are expressed as mean ± SD, for n=3, one way ANOVA followed by Duncan's multiple comparison test, ***P≤0.05 and ns- no significant difference compared with the control group.

### *4.3.2.2 Formalin Induced paw oedema in Wistar Rats*

The activity of methanolic extract of *A. heterophyllus* on formalin induced paw oedema was examined by the measuring of paw oedema volume in adult Wistar rats. The percentage of inhibition is shown in table 4.23 and the difference in paw volume is shown graphically in Figure 4.19 . The maximum percentage of inhibition of paw oedema 32.5 %* was exhibited by a dose of M AH (250mg/kg).

**Table 4.23** Percentage of inhibition in Formalin induced paw oedema

| Sl. No. | Concentration (µg/ml) | Percentage Of Inhibition |
|---|---|---|
| 1 | Control | |
| 2 | Aspirin(10mg/kg) | 37.66±3.34%* |
| 3 | J200(200mg/kg) | 28.25±2.29% |
| 4 | J250(250mg/kg) | 32.73±2.15%* |

Values are expressed as mean ± SD, for n=3, *P ≤ 0.05 compared with standard

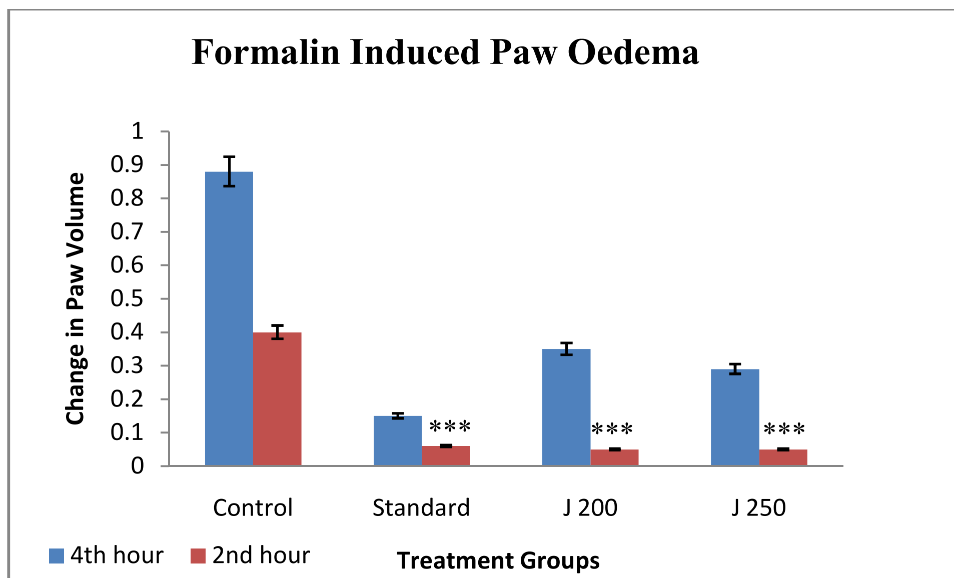


**Figure 4.19** Effect of methanolic extract of *A. heterophyllus* on Formalin induced paw oedema in rats

Values are expressed as mean ± SD, for n=3, one way ANOVA followed by Duncan's multiple comparison test, ****P* ≤ 0.05 and ns- no significant difference compared with control group.

# 5. DISCUSSION

*Artocarpus heterophyllus* Lam. is one of the important homeyard plants with diverse use for all its parts. It is reported with rich phytochemicals that possess great pharmacological potential (Wetprasit *et al*., 2000; Yao *et al*., 2016). They exhibit a wide range of variation in its fruit characteristics and morphology due to the open pollinated flowers and seed dispersal propagation. The main aim of the study included the identification of the accessions which exhibit the best antioxidant, anti-inflammatory properties among the collection (Table 3.1) and identification of the genetically variant ones within the collection using SCoT marker.

## 5.1 GENETIC DIVERSITY ANALYSIS

The present study reports the effective use of 8 SCoT primers for comparing genetic diversity among 11 accessions of *Artocarpus heterophyllus* Lam. SCoT analysis is highly reproducible, low cost and effective to use, with requirement of small amounts of DNA in addition to no prior information of DNA sequence. SCoT is gene targeted marker with multilocus nature unlike RAPD, AFLP and ISSR marker system, and it can generate more information correlated with biological traits and helpful in high genetic polymorphism (Xiong *et al*., 2011).

Quality of DNA is an essential prerequisite for molecular fingerprinting. In present study DNA with good quality produced amplicons of expected size in SCoT analysis. The non amplification of the remaining samples may be due to the hindrance by the polyphenols, protein and polysaccharides. The some of the most useful SCoT primers in present study were SCoT 15 and SCoT 35, generating 21 and 20 bands with polymorphic bands 20 and 19, respectively. Three primers (11,21,32) showed 100 % polymorphism. ScoT 21 and 45 primers showed high gene diversity (h = 0.4040, 0.4289) among the other locus analysed. Similarity values between all jackfruit accessions ranged from 0.27 (between MG NL2 TO MG 91) to 0.57 (between MG NL2 and MV).

High polymorphism (100%) is reported in the present study as in compliance that SCoT is better than other molecular markers in determining polymorphism with earlier

investigations in jackfruit by Schnell *et al.,* 2011 who reported 49.2 % polymorphism with AFLP markers. Simon *et al.* (2007) who observed low to moderate genetic diversity with RAPD markers then Kavya *et al*. (2017) who reported polymorphism in jackfruit using SSR primers.

The maximum similarity was observed between MV and MG NL 2 accessions. The lowest similarity was seen between MG NL2 & MG 91 and MG 91 & WB1. Genetic relationships amongst 11 jackfruit accessions were evaluated by the construction of a dendrogram based on scored data of 8 SCoT markers. UPGMA clustering algorithm based on similarity matrix at similarity index of 0.47 grouped the accessions into 8 clusters. Various clusters formed shows variations in genetic constitutions amongst them. Cluster I (MG 91) is the most distinctive among all which indicates that this group has less relation with other groups and is genetically variable. Cluster I (MG 91), II (MG NL 1), IV(WB 7), VI(WB 4), VII (IC) and VIII(WB3) consisted only single accession while cluster III got two most similar cultivar accessions (MV and MG NL2) and cluster V got one cultivar (MG 81) and two wild accessions (WB 3 and WB 4).

5.2 PHYTOCHEMICAL STUDIES

For the quantification of phytochemicals like phenolics and flavonoids, methanolic extract of samples were chosen. Since the active phytochemicals constituents are soluble in polar solvents (Chanda and Dave, 2009 ). Maximum extract yield percentage was obtained in wild variety WB 7, *i.e*., 75.65 % and minimum extract yield was in seeds of wild variety WB 4, 2.786 % (Table 4.5 & 4.6).

Quantification of Total phenolics and flavonoids in the samples were done. When quantified, jackfruit seeds were seen to possess more phenolics when compared with flavonoids (Figure 4.4,4.5,4.7,4.8). Soong *et al.*(2004) and Jagtap *et al.* (2010) reported a higher phenolic content in seeds of jackfruit compared to other edible portions in seeds. Based on the method of extraction and the solvent used, the amount of the phenolics got extracted also varies as reported by Zhang *et al.* (2017).

The total flavonoids were also higher in wild than the cultivar. On an overall viewpoint based on estimation of flavonoids, flakes showed more flavonoid content than the seeds (Figure 4.7 & 4.8). Among the cultivars, KAU identified Muttom Varikka (MV) variety with a bright yellow showed high flavonoid content that support the report by Erlund (2004), that the portion rich in flavonoid corresponds to their bright yellow colour. The higher content of phenolic and flavonoid in the wild than the cultivar may be due to the stressed environment in the wild that promote them for the active production of secondary metabolites.

The presence of phenolics and flavonoids like phytocompounds are responsible for antioxidant potential in jackfruit (Jagtap *et al*.,2010) with many other phytocompounds like vitamins, carotenoids etc. The ability to scavenge the radicals varies from one portion to another, from one fruit to another and also depends on the maturity patterns of the fruit. In the present study, antioxidant potential was determined by assessing the ability of scavenging the coloured, proton DPPH radical by the crude methanolic extract of our sample. It was observed the seeds possess more antioxidant potential than the flake sample (Figure 4.12 & 4.15). In reference with the polymorphism studies, some sort of genetic variation related to the phytoconstistuents.

## 5.3 ANTI-INFLAMMATORY STUDIES

Inflammation is a tightly regulated, principle response of the body invoked to deal with cellular or tissue injuries. It is characterized through mediator release, enzyme activation, fluid extravasations, cell migration, vasodilatation and neutrophil infiltration. The hormone mediators like histamine and bradykinin will cause the blood vessels to get widened allowing more blood to reach the inflamed tissue, turning them red and feel hot. They also cause irritations and generate pain signals to be sent to the brain. Natural foods rich in antioxidants are useful with phytochemicals belonging to phenolic and flavonoid classes in jackfruit, making it a rich antioxidant diet with anti-inflammatory properties.

In present study Anti inflammatory activity of flake extract was determined by both *in vitro* and *in vivo* assays like HRBC membrane stabilization assay, Albumin Denaturation Assay, Carrageenan and Formalin induced paw oedema assays. with high radical scavenging activity was selected so that they can be eaten as fresh without any loss of phytoconstituents during cooking.

HRBC membrane is similar to the lysosomal membrane that ruptures when a tissue gets inflamed. Its rupture will cause the release of lysosomal enzymes within, anti inflammatory drugs can inhibit the release of this lysosomal enzyme by stabilizing the lysosomal membrane (Mounnissamy *et al*.,2008). Similarly the hypotonicity induced RBC will undergo hemolysis and haemoglobin oxidation, treating it with the extract cause the inhibition of the hypotonicity induced damage and is considered as a measure of anti inflammatory activity (Ferrali *et al*., 1992).

The results demonstrated that MAH at the concentration of 100 µg/mL protects the hypotonicity induced haemolysis of RBC but is comparatively less. At the highest concentration (250 µg/mL), MAH showed maximum inhibition of 46.069%, as compared with the standard Diclofenac sodium which showed 59.32 % inhibition of RBC haemolysis. Dose-dependent inhibition of HRBC membrane stabilization was observed with all the extracts and diclofenac. In this study, the difference between diclofenac and the extracts was not found to be significant as in the study using *A. heterophyllus* peel skin and rind by Meera *et al*., (2017). The $IC_{50}$ value of extract was found to be 292.15 µg/mL as compared with the standard having $IC_{50}$ value of 167.58 µg/mL.

Heat mediated albumin denaturation assay is a method to determine the percent of inhibition of the plant material as a measure of protection of albumin from heat denaturation. Protein denaturation causes the production of auto antigens that leads to tissue damage and inflammation. Some of the anti inflammatory drugs thereby inhibit the protein denaturation and is considered as an anti inflammatory potential determining assay. The results also showed a dose dependent inhibition as in the case of HRBC

stabilization that increased concentration gives more protection from the heat denaturation (Grant *et al*., 1970). The $IC_{50}$ value of extract was found to be 445.34µg/mL as compared with the standard having $IC_{50}$ value of 178.72 µg/mL.

Carrageenan induced rat hind paw oedema is a common animal model assay to analyze orally active anti inflammatory drugs (Di Rosa *et al*., 1971;Saha *et al*., 2007). Carrageenan is a mucopolysaccharide phlogistic agent obtained from extract of *Chondrus* an Irish sea moss. Single dose of it produces acute, effective, non-toxic, non-immune, non-systemic effects with a high degree of reproducibility. Carrageenan induced oedema formation is associated with the release of histamine and serotonin that further leads to generation of prostaglandin, lysosomes, bradykinins and protease so it is referred to as a biphasic event. The MAH showed dose dependent response activity over a period of time and extract showed maximum inhibition of 37.5 % during induced paw edema. The extract with a dose of 250 mg/kg induced significant ($p<0.05$) anti-inflammatory activity at $4^{th}$ hour after carrageenan administration.

Formalin test is a valid and reliable model to assess the clinical pain and injury due to acute inflammation. It is a biphasic event with an early neurogenic component followed by tissue mediate response. One of the major biochemical events during formalin induction is the relative compositional variation of connective tissue constituents like glycoprotein, hexosamine, mucopolysaccharides, hydroxyproline and sialic acid. In the case of MAH administration, the maximum reduction in paw volume was observed at the dose 250 mg/kg after the 4th hour is 32.73%, which was not significantly comparable to that of the standard drug Aspirin (37.66%). The present study can further elaborated to phytocompound identification and purification.

# 6. SUMMARY

The research work entitled “Genetic diversity analysis of *Artocarpus heterophyllus* Lam. using SCoT markers for antioxidant and anti-inflammatory properties” was carried out at the Biotechnology and Bioinformatics Division and Ethnomedicine and Ethnopharmacology division of Jawaharlal Nehru Tropical Botanic Garden and Research Institute (JNTBGRI), Palode during the period 2019 – 2020.The objective of the study was to analyse the genetic diversity of *Artocarpus heterophyllus* Lam. samples collected from different areas of Kerala using SCoT markers and to screen phytochemical constituents of edible fruit part for antioxidant and anti-inflammatory activities.

Jackfruit is one among the most important trees in homestead gardens. It is believed to be indigenous to Western Ghats rainforest in India and Malaysia. It has got worldwide recognition for its rich nutrient content, versatile use in the food industry and also for its rich phytoconstituents with medicinal property. Phenolics and flavonoids are the major among the biologically active metabolites with the pharmacognic properties.

Eighteen accessions of jackfruit, both wild and cultivar, were collected for the phytochemical and genetic diversity analyses. Genomic DNA was isolated using modified CTAB method and resolved in 0.8% ethidium bromide agarose gel. Quantity and quality were assessed using Nanophotometer. The genetic polymorphism for the different accessions were done using ten SCoT markers resulting in variation in the banding pattern in resolving gel. Bands were then scored and analysed using POPGENEversion 1.32 and NTSYSpc version 2.02.

POPGENE analysis gave the observed number of alleles per locus (na) as 2.000. Effective number of alleles (ne) ranged from 1.3379 to 1.7510, Shannon’s information index (I) ranged from 0.4197 to 0.6203 and Nei’s gene diversity ranged from 0.2525 to 0.4289. Further genetic similarity coefficients were generated by NTSYS based on Jaccards Coefficients and ranged from 0.27 to 0.57 with an average of 0.5. The highest genetic similarity (0.57) was found between MV and MGNL2 and the lowest genetic similarity (0.27) was found

between MG NL 2, WB 1 and MG 91.

Dendrogram was constructed based on the Jaccards coefficient using UPGMA clustering algorithm based on similarity matrix. At similarity index of 0.47 the accessions were grouped into 8 clusters. Cluster I (MG 91) is the most distinctive among all and cluster III got most similar two cultivar accessions (MV and MGNL2).

Soxhlet extraction using methanol gave more solubility and more phytochemicals in extract. The yield of the extract showed no influence in the activity of phytochemicals within it. Antioxidant potential was determined using DPPH radical scavenging activity. Phytochemicals like phenolics and flavonoids were quantified by Folin Ciocalteu assay and Aluminium Chloride colorimetric assay respectively. The one with best antioxidant property was chosen for *in vitro* anti inflammatory assays like albumin denaturation assay, HRBC membrane stabilization assay and *in vivo* anti inflammatory like carrageenan induced paw oedema and formalin induced paw oedema studies .

High phenolic content was observed in seeds of WB2 (233.344 mg GAE/g)whereas WB1 flakes showed high flavonoids (697.5 mg GAE/g). MG 92 cultivar flakes showed the maximum radical scavenging activity among cultivar with $IC_{50}$ of 210.58, was used for the *in vivo* and *in vitro* anti-inflammatory studies. In anti-inflammatory studies, the percentage of inhibition shows a dose dependent relation, as the concentration of sample increases the activity of protection increases.

The variation in the phytoconstituents of jackfruit differs accordingly with the genetic conditions as well as environmental changes and also depends on the maturity patterns. It has been noted that phytoconstituents in the seed and flake samples showed great variation as in the PCR amplified products from corresponding leaf samples of the jackfruit accessions. Wild accessions studies are comparatively less but are a potent source for many phytoconstituents with medicinal properties that need to be investigated.

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# 8. APPENDICES

## APPENDIX I

**CTAB Buffer**

Tris HCl – 100mM (pH 8)

EDTA – 20mM (pH 8)

NaCl – 1.4M

CTAB – 2%

## APPENDIX II

**TE Buffer**

Tris - 10mM

EDTA – 1Mm

## APPENDIX III

**TAE Buffer**

Tris Base – 242g

Glacial Acetic Acid – 57.1 mL

0.5 M EDTA - 100ml

# APPENDIX IV

**Tracking Dye**

Bromophenol Blue – 0.25%

Xylene Cyanol – 0.25%

Glycerol - 30%

Water – 69.5%

# APPENDIX V

**Alsever Solution**

Sodium Citrate – 0.8%

Citric Acid – 0.05%

Dextrose – 2%

Sodium Chloride – 0.42%

# APPENDIX VI

**Phosphate Buffer Saline (pH 6.4) (1000mL)**

Sodium Chloride – 8g

Potassium Chloride – 0.2g

Di Sodium Hydrogen Phosphate – 1.44g

Di Hydrogen Sodium Phosphate – 0.12

**GENETIC DIVERSITY ANALYSIS OF *Artocarpus heterophyllus* Lam. USING SCoT MARKERS FOR ANTIOXIDANT AND ANTI–INFLAMMATORY PROPERTIES**

**ELSIT MARIYA C.**

**(2015-09-014)**

# Abstract of Thesis

**Submitted in partial fulfillment of the requirement for the degree of**

**B. Sc. – M. Sc. (INTEGRATED) BIOTECHNOLOGY**

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## ABSTRACT

The research work entitled "Genetic diversity analysis of *Artocarpus heterophyllus* Lam. using SCoT markers for antioxidant and anti-inflammatory properties" was carried out at the Biotechnology and Bioinformatics Division and Ethnomedicine and Ethnopharmacology division of Jawaharlal Nehru Tropical Botanic Garden and Research Institute (JNTBGRI), Palode during the period 2019 – 2020.

*Artocarpus heterophyllus* Lam., the official fruit of Kerala, is well known for its dietary uses from ancient times. Cultivar and wild accession comparative studies are not yet reported based on the genetic variation and phytoconstituents. Present study aimed to analyze the genetic variation and constituents of phytochemicals for anti-inflammatory property using Start Codon Targeted (SCoT) marker in both cultivar and wild accessions.

The variation in genetic diversity of both cultivar and wild accessions were analyzed using 10 SCoT markers. NTSYS and POPGENE were employed for the construction of dendrogram and estimation of genetic variation. MG 91 cultivar showed the highest variation with similarity coefficient 0.44 to MG NL1. The total phenolics and flavonoids of the flakes and seeds were estimated using phytochemical analysis.

High phenolic content was observed in seeds whereas flakes showed high flavonoids. Antioxidant potential was determined using DPPH radical scavenging activity. MG 92 cultivar flakes which showed the maximum radical scavenging activity with $IC_{50}$ of 210.58 was used for the *in vivo* and *in vitro* anti-inflammatory studies. In anti-inflammatory studies, the percentage of inhibition showed a dose dependent relation, as the concentration of sample increased the activity of protection also increased.

The variation in the phytoconstituents of jackfruit differs due to genetic as well as environmental conditions and also depends on the maturity patterns. It has been noted that phytoconstituents in the seed and flake samples showed great variation as in the PCR amplified products from corresponding leaf samples of the jackfruit accessions. Wild accessions studies are comparatively less but are a potent source for many phytoconstituents with medicinal properties that need to be investigated.