A DISSERTATION ON

Assessment of Genetic Diversity in *Tagetes minuta* germplasm

SUBMITTED TO THE

DEPARTMENT OF BIOSCIENCES

INTEGRAL UNIVERSITY, LUCKNOW

IN PARTIAL FULFILLMENT

FOR THE

DEGREE OF MASTER OF SCIENCE

IN BIOTECHNOLOGY

BY

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M.Sc. Biotechnology (IV semester)

Department of Biosciences

Integral university, Lucknow

UNDER THE SUPERVISION OF

DR. Tripta Jhang

(Principal Scientist)

CENTRAL INSTITUTE OF MEDICINAL & AROMATIC PLANTS

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(वैज्ञानिक तथा औद्योगिक अनुसंधान परिषद)
पोस्ट आफिस - सीमैप, लखनऊ-226015, भारत

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TO WHOM IT MAY CONCERN

This is to certify that the dissertation entitled, "Assessment of Genetic Diversity in *Tagetes minuta*" is an original research work carried out by Ms. Mariyam Malik, (Enroll.no: 2000102400), under the guidance of Dr Tripta Jhang, Principal Scientist, Division of Plant Breeding and Genetic Resource Conservation, CSIR-CIMAP, Lucknow during 1st February, 2022-31st May, 2022, for the partial fulfillment of the requirements for the award of degree of Master of Science in Biotechnology of the Integral University, Lucknow and that no part of this work has been presented earlier for any degree or diploma in this or any of the universities.

Tripta Jhang
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This is to certify that the study conducted by Ms. **Mariyam Malik** during the months Feb-May, 2022 reported in the present thesis was under my Co-supervision. The results reported by her are genuine and script of the thesis has been written by the candidate herself. The thesis entitled is "**Assessment of Genetic Diversity in Tagetes minuta germplasm"** is therefore, being forwarded for the acceptance in partial fulfillment of the requirements for the award of the degree of M. Sc Biotechnology, Department of Biosciences, Integral University, Lucknow, (U.P).

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TO WHOM IT MAY CONCERN

This is to certify that **Ms Mariyam Malik** a student of M.Sc. Biotechnology (II Year, IV semester), Integral University has completed her four months dissertation work entitled "**Assessment of Genetic Diversity in Tagetes minuta germplasm"** successfully. She has completed this work from Department of Biosciences, Integral University, under the guidance of **Dr. Tripta Jhang,** Principal Scientist, Plant Breeding and Genetic Resource Conservation Division at CSIR- Central Institute of Medicinal and Aromatic Plants, Lucknow. The dissertation was a compulsory part of her M.Sc. degree. I wish her good luck and bright future.

(**Dr. Snober S. Mir**)
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DECLARATION

I declare that this thesis entitled, " Assessment **of Genetic Diversity in *Tagetes minuta* germplasm**" submitted to INTEGRAL UNIVERSITY for the partial fulfilment of the Master degree in Biotechnology is a presentation of my original research work and has been composed solely by myself. The work was done under the guidance of **Dr. Tripta Jhang,** Principal Scientist, Plant Breeding and Genetic Resource Conservation Division at CSIR-Central Institute of Medicinal and Aromatic Plants, Lucknow. It has not been submitted, in whole or in part, in any previous application for a degree. Wherever contributions of others are involved, every effort is made to indicate this clearly, with due reference to the literature, and acknowledgement of elaborative research and discussions.

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M.Sc Biotechnology

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Mariyam malik

Abstract

Tagetes minuta, also known as wild marigold, is a plant in the Asteraceae family. The plant's essential oil is prized for its presence in practically every part of the plant, with a small percentage in the stem and roots. Z—ocimene, limonene (hydrocarbons), and acyclic unsaturated monoterpenes ketones, dihydrotagetone, tagetones (E, Z), and ocimenones are the primary ingredients (90-95%) of Tagetes minuta essential oil. The plant has pharmacological, phytotoxic, antibacterial, antifungal, insecticidal, nematicidal, antiviral, antimicrobial, and antiviral activities, and is used in the perfume and flavor industries. Because there is a high demand for its oil, this species has a lot of potential for large-scale cultivation. In the present study the highest were Limonene (9.551), (*Z*)-Tagetone (13.983), *(E)*-Tagetenone (17.160)

CONTENTS

List of Abbreviations

BPB	Bromo phenol blue
Conc.	Concentration
M	Molar
mM	Millimolar
CTAB	Cetyl tri ammonium bromide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
EtBr	Ethidium Bromide
EDTA	Ethylene diamine tetra acetic acid
Mg	Milligram
MI	millilitre
ng	Nanogram
PCR	Polymerase Chain Reaction
U	Unit
µg	Microgram
µl	Microliter
%	Percentage
TBE	Tris Borate EDTA
PVP	Poly vinyl pyrrolidone
rpm	Revolution per minute

Kb	Killobase pair
V	Volt
UV	Ultraviolet
EO	Essential oil
TE	Tris EDTA

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Chapter-1

INTRODUCTION

Tagetes minuta L. is a natural aromatic plant from South America that is commonly referred as "Gainda" in India. It grows organically from spring till the beginning of winter, when it has completed its life cycle. This essential oil has wide applications as flavoring and in perfume. Besides, it is well known for its biocide properties. Various studies on *T. minuta* reported that there are variations in the essential oil composition according to the harvesting location as described by (Zygadlo et al. [Keitas y col), the growth stage by (Daghero et al*),* the different parts of the plant by Weaver et al. and that there are different chemotypes within the species as described by (Gil et al.) All of these factors necessitate a regional investigation of the native *T. minuta* essential oil. The genus is found in the southern United States, Argentina, and Mexico. Most species of this genus are branching, annual or perennial herbs or shrubs used for horticulture and essential oil production. Analgesics, antiseptics, carminatives, diuretics, antispasmodics, anthelmintics, stimulants, vermin repellents, and therapy of stomach and intestinal ailments have all been used by members of the genus *Tagetes. Tagetes minuta*, sometimes known as African marigold, is a very scented annual perennial herb that grows as a weed and/or is cultivated for trade '*Tagetes* oil'. Because of its antimicrobial, anti-inflammatory, antifungal, insecticidal, and acaricidal properties, *T. minuta* is employed as a natural source of raw material in indigenous remedies. *T. minuta* essential oil is also widely used in the culinary, flavoring, pharmaceutical, perfumery, and cosmetic industries. Tagetes oil is mostly produced for commercial use in Argentina, Australia, Brazil, France, Spain, Venezuela, Iran, and other nations. Antibacterial, anti-inflammatory, hypotensive, larvicidal, insecticidal, and aphicidal properties have been described for Tagetes oil and its terpene components. (Zygadlo et al., Vasudevan et al., and Hethelyi, et al.) describe in the current work a research of the chemical composition of essential oil from *Tagetes minuta* L. leaves and flowers as an effective biocide. The chemical makeup of *T. minuta* essential oil has been researched earlier in several countries. Dihydrotagetone, (Z)-ocimene, (Z)-tagetone, (E)-tagetone, (Z)-ocimenone, (E)-ocimenone, terpineol, and

limonene were the most common monoterpenoids found in essential oils of various plants. Essential oil content and components, on the other hand, have been documented to vary greatly depending on climatic and agricultural circumstances, growing season, growth/ harvesting stage, plant sections, cultivation tactics, and origin. Considering the huge potential of *Tagetes* oil and the conducive agroclimatic conditions for its cultivation in foot hills of north India, the present study was carried out to investigate variations in essential oil yield and composition of *T minuta* crop in different growth stages, viz. flowering initiation, full flowering, and maturity**.**

Fig1: field of *Tagetes minuta* (vanphool) CSIR-CIMAP

Fig2(a): Plant of *Tagetes minuta* **Fig2(b): flower of *Tagetes minuta***

Although several technologies for extracting aroma principles from natural resources have been developed, hydro-distillation remains the most extensively utilized process for distilling essential oils on a commercial basis because to its economic viability. Depending on how the plant materials are distilled, hydro-distillation can be split into three types: (a) water distillation; (b) water-steam distillation; and (c) steam distillation. Hydro-distillation is a traditional method for the extraction of bioactive compounds from plants. Plant materials are packed in a still compartment, then an appropriate amount of water is added and the mixture is brought to a boil. Direct steam can also be injected directly into the plant sample. By chilling the vapor mixture of water and oil with water, the vapor mixture is condensed. The condensed mixture flows from the condenser to a separator, which separates the oil and bioactive chemicals from the water automatically (Azmir et al., 2013). Although it has various advantages, such as the absence of organic solvents in the process, no requirement for dehydration of the plant materials, and faster extraction durations, it is limited by high temperature applications for heat-sensitive phenolic compounds (Azmir et al., 2013)**.**

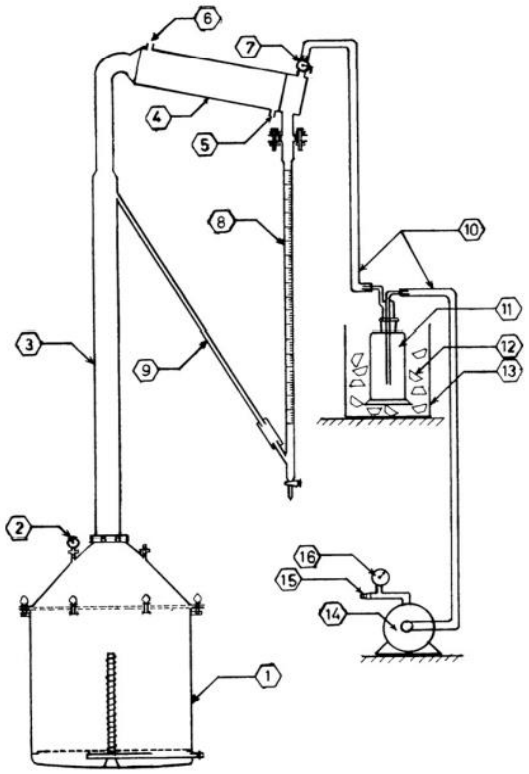


Fig 3. Hydro distillation - Mini distillation apparatus (MDA) for vacuum distillation of essential oils. (1) Distillation vessel, (2) temperature gauge, (3) cohobation head column, (4) condenser, (5) coolant inlet, (6) coolant outlet, (7) air vent/vacuum inlet, (8) receiver-cum separator, (9) recycling pipe, (10) heavy duty vacuum tubing, (11) cold trap, (12) ice cubes, (13) beaker, (14) vacuum pump, (15) vacuum regulator and (16) vacuum gauge

1.1Taxanomy of *Tagetes minuta*

Domain : Eukaryotes
Kingdom : Plantae
Phylum : Spermatophyta
Subphylum: Angiospermae
Class : Dicotyledonae
Order : Asterales
Family : Asteraceae
Genus : *Tagetes*

Species : *minuta*

Species of *Tagetes*

- *Tagetes apetala*
- *Tagetes arenicola*
- *Tagetes argentina*
- *Tagetes biflora*
- *Tagetes campanulata*
- *Tagetes daucoides*
- *Tagetes elliptica*
- *Tagetes elongata*
- *Tagetes epapposa*
- *Tagetes erecta*
- *Tagetes filifolia*
- *Tagetes foeniculacea*
- *Tagetes foetidissima*
- *Tagetes hartwegii*
- *Tagetes iltisiana*
- *Tagetes inclusa*
- *Tagetes lacera*
- *Tagetes laxa*
- *Tagetes lemmonii*
- *Tagetes linifolia*
- *Tagetes lucida*
- *Tagetes mandonii*
- *Tagetes mendocina*
- *Tagetes micrantha*
- *Tagetes microglossa*
- *Tagetes minima*
- *Tagetes minuta*

- *Tagetes moorei*
- *Tagetes mulleri*
- *Tagetes multiflora*
- *Tagetes nelsonii*
- *Tagetes oaxacana*
- *Tagetes osteni*
- *Tagetes palmeri*
- *Tagetes parryi*
- *Tagetes patula*

Table 1.1 Common Names The various common as well as local names of *Tagetes minuta* are as below:

English	Wild Marigold, Mexican Marigold
Hindi	Jungli Gainda
Spanish	huacatay, chinchilla, enana
Angola:	Ekaibulo
Argentina	Chil Chil
Brazil	chinchilla, coora, cravo de mato
Germany	Wilde Sammetblume
Madagascar	Mavoadala

1.2 Morphology and habit of *Tagetes minuta*:

Tagetes minuta is a strongly scented annual herb that grows to heights of 50-150 cm and has erect and sometimes highly branched stems. The 3-30 cm long and 0.7-8 cm wide leaves are mostly opposite but often alternate in the upper parts of the plant. The leaves are dark green to slightly glossy green, pungent, glabrous, and compound with 9-17 leaflets. The leaf margins are acute and serrate, while the leaflets have linear-lanceolate margins that are finely serrulate and 2-4 cm long, with orange translussscent glands. The undersurface of the leaves has small, multicellular, punctate glands that are orangish in

color and produce a liquorice-like aroma when punctured. The glands can also be found on the stem and involucre bracts. The heads are numerous and are usually supported by flat-topped cymes that are 10 to 15 mm long, 2-3 mm wide, and surrounded by four or five fused involucre bracts. Per capitula, there are usually 3 to 5 lemon-colored ray florets and 10 to 15 yellow-orange disc florets (Soule, 1996). The achenes are cylindrical in shape, dark brown to black in colour, and 10 to 12 mm long, with a pappus of 1-4 tiny, unequal Like scales 2-3 mm long and 3-5 ovate to lanceolate scales 0.5-1 mm long.

1.3 Ecology and habitat

The plant thrives in moist and dry environments in the tropics and subtropics, from sea level to respectable heights (3000 m), with soil pH ranging from 4.3 to 6.6 (Holm et al. 1997). Wild marigold is a weed that can invade waste ground, roadsides, gardens, orchards, and vine yards, making it a concern for pastures and a variety of crops (Hulina, 2008). Because of its preference for disturbed environments, the species has been able to colonise a wide range of locations around the globe (Meshkatalsadat et al. 2010**).** The plant thrives in regions with a lot of loose soil. For excellent development, a minimum of 50 cm of rainfall spread across the season or its equivalent in irrigation water is required (Singh et al, 2003). (Campos, 1997) associate species *of Tagetes minuta* are usually ruderal broad-leaved species like Bidens pilosa L. and Bidens sub-alternans DC. In a temperate area, where the night temperature drops, the plant grows best and produces the highest quality oil. During the reproductive season of its plants, temperatures between 12 and 30 degrees Celsius are ideal for producing high-quality oil. The crop prefers soils that are mostly acidic. Once in contact, the seeds stick to the hair of animals and human clothing, and are thus transmitted by humans, domesticated by wild animals (Singh et al. 2003).

1.4 Phenology

Flowering occurs in September. When the crop is fully bloomed, it is harvested in October-November, and the seeds are gathered at the end of November. In mid-August, removing the apical meristem is thought to be advantageous for controlling upright growth and lateral branching (Singh et al. 2003).

1.5 Agro-Practices

The plant is propagated by seed, either by sowing or transferring nursery-grown plants into the main field. Kumar and Singh (2008) did research in Palampur, H.P., and discovered that the 30th and 31st MSW (Meteorological Standard Week), which corresponds to the 23rd to 5th of August, is the best time for transplanting in the main field since it yields the most leaf and blossom biomass. Kumar and Singh (2008) also believe that seedlings that are 45 days old are suitable for transplantation. The crop lasts 4 to 6 months, depending on when it is sown, the crop technique used, and the weather conditions. For preserving ideal crop geometry, nursery growing is preferable than direct sowing. For the best herb and oil yield, use a medium light to medium heavy soil that is free of weeds. In addition to 20-30 tones' ha-1 farm yard manure used during soil preparation, the crop requires 120 kg N, 60 kg P, and 40 kg K per hectare. For optimal crop growth and output, enough soil moisture is essential throughout the growing season. From mid-June to mid-November is the best time to plant crops in the western Himalayas (Singh et al. 2003). Various spacings of 30x30 cm (Rao et al. 2000), 45x45 cm (Kumar and Singh, 2008), and 30x45 cm (Singh et al. 2003) have been investigated, with 30x45 cm being the most ideal. Plant growth, essential oil composition, and phenolic content of this species were all promoted by soil inoculation by plant growth boosting rhizobacteria such *Pseudomonas fluorescens* and *Azospirillum brasilense*, according to Cappellari et al. (2013). Singh et al. (2008) studied the effects of row spacing and nitrogen levels on *Tagetes minuta* herb, essential oil production, and oil quality.

1.6 Elite varieties

The Central Institute of Medicinal and Aromatic Plants (CIMAP) has published "Vanphool," an enhanced variety derived from an open population taken from the Almorah hills that has a high oil yield (61 kg ha-1), dihydrotagetone (32 percent), and (Z)-tagetone (16.7 percent) (Kumar et al. 1999). Institute of Himalayan Bioresource Technology (IHBT), Palampur (Anonymous, 2001) has created an enhanced cultivar called "Him gold."

1.7 Secretory glands

Simon et al. (2002) investigated the presence, structure, and distribution of secretory structures in *Tagetes minuta's* several organs. During leaf senescence, the secretory structures of essential oils collapse (Sharma et al. 2003). Lopez et al. (2009) investigated the secretory cavities in the leaf and reproductive structure of *Tagetes minuta* and discovered that those in the leaf blade half were significantly larger (150-200m) than those at the midvein (70m).

1.7 Molecular studies

Shahzadi et al. (2010) used a modified cetyltrimethylammonium bromide buffer procedure to recover high-quality DNA from fresh leaf tissues. The sun- and shade-dried tissues yielded a significant amount of DNA, as well. PCR was used to successfully amplify the DNA taken from fresh leaves using arbitrary RAPD primers**.**

1.8 Molecular markers

Molecular marker are identifiable DNA sequences, found at specific location on the chromosome, transmitted by the laws of inheritance from one generation to next. Molecular markers can be useful tools to both facilitate characterization *Tagetes* breeding program (Marker Assisted Selection, MAS) or to aid in characterization of *Tagetes* species (varieties). Different kind of molecular markers are in use, such as AFLPS, RAPDs, RFLPs, microsatellites and SNPs. They may differ in variety of ways such as their technical requirements, the amount of time, money and labor needed, the number of genetic markers that can be detected throughout the genome; and the amount of genetic variation found at each marker in a given population. The information provided by the markers for the breeder will vary depending on the type of marker system used. Each one has its own advantages and disadvantages. Microsatellite also known as Simple Sequence Repeats (SSRs) are iterations of 1-6 bp nucleotide motifs, dispersed random and ubiquitously throughout the genome. They are most polymorphic and reliable markers till date. They are designed from the conserved sequence regions flanking the microsatellite motifs. These markers amplify microsatellite loci across genotypes that typically generate size polymorphic fragments because of the difference in the number of repeats motifs at the locus.

ISSR (Inter simple sequence repeats) markers is sophisticated variant of Microsatellite primed PCR technique. In this technique anchored di-or tri nucleotide repeats serve as single primer for the region flanking the SSR motif and the amplified product are resolved on polyacrylamide gel and banding patterns are revealed either by autoradiography or by Ethidium Bromide staining (Zietkiewiez, Rafalski, and Labuda, 1994). In this technique PCR, complex fingerprinting like patterns were obtained that revealed inter as well as intraspecific polymorphism from wide variety of eukaryotic taxa (Zietkiewicz, Rafalski, and Labuda,1994; Kosia 1995; Wolf, Zietkiewiez, and Hofstra, 1995; Huttel, 1996(Weising 1994).

Objective:

1. Explore natural variation in *Tagetes minuta* population
2. To assess the genetic diversity in *Tagetes minuta* accessions

Chapter-2

REVIEW OF LITERATURE

Plants produce a diverse range of secondary metabolites as they grow and develop. Essential oils, also known as ethereal or volatile oils, are among the most important compounds of aromatic plant secondary metabolism. The term 'essential' denotes that the oil contains the plant's distinct scent or essence. In general, essential oils are volatiles, which are complex mixtures of natural, aromatic oily liquid blends with a strong fragrance. They are usually liquid at room temperature, but some are solid or resinous, and their colors range from pale yellow to emerald green and from blue to dark brownish, depending on the plant species that produces them. The biosynthesis and storage of essential oils are not limited to specialized plant parts, but occur in a variety of plant parts including leaves, flowers, seeds, fruits, roots, and barks. Steam distillation, hydro distillation, cold pressing, and solvent extraction are all methods used to extract essential oils. They are thought to protect against pathogens and pests by acting as antifeedants, anti-bacterial, antivirals, antifungals, and insecticides. Essential oils in a number of plants suppress the growth of neighboring plants through allelopathic effects, giving the producing plant a competitive advantage. Chemically, essential oils (EOs) fall into two biosynthetically related groups: terpenes and aromatic compounds with low molecular weight, the majority of which are monoterpenes. A single plant species' essential oil may contain 20 to 60 components or more in varying concentrations. They are distinguished by two or three major constituents present in high concentrations (20-90 percent) and various minor constituents present in trace amounts. While it has been proposed that the major components of EOs are the primary determinants of their biological properties, there is also evidence that Minor/trace components of EOs play an important role in determining biological properties such as antimicrobial activity via synergism. The biosynthesis of essential oils is limited to approximately 3000 plant species from more than 60 families, with approximately 300 plants serving as the foundation for the production of the majority of the world's economically important essential oils. However, most of these plant species are underutilized at the subsistence and commercial levels, despite their importance to humanity.

Wild marigold secondary metabolites include monoterpenes, sesquiterpenes, phenols, flavonoids, and thiophenes. Primary metabolite (carbohydrates, amino acids, lipids, and nucleotides) serve as a precursor for secondary metabolites, which do not contribute to plant growth or development. Secondary metabolites, on the other hand, play an important role in plant defense against abiotic stresses (Mahajan et al., 2020). The aroma of the wild marigold plant is caused by the presence of "terpenoids," which are produced in plants via the mevalonic acid (MVA) pathway in the cytoplasm and the 2-methylerythritol 4-phosphate (MEP) pathway in the chloroplast or plastid.

2.1 DISTRIBUTION AND NATURAL HISTORY

T. minuta is native to most of the world's temperate forests and mountain regions. It originated in South America and has spread as a weed throughout the world*. T. minuta* is native to most of the world's temperate forests and mountain regions. It originated in South America and has spread as a weed throughout the world. South Africa, Australia, Nigeria, India, Uruguay, East Africa (Kenya), Brazil, France, Chile, Bolivia, and the Chaco region of Paraguay have all reported it (Gardner et al. 1991; Espinar 1967; Craveiro et al. Chagonda and Makanda 1999; Chalchat et al. 1995; Rao et al. 1999). France, Kenya, Argentina, and Australia are the world's top producers of *"Tagetes* oil." It can be found in India at altitudes ranging from 1000 to 2500 meters. Thappa and colleagues (1993). The main growing regions where it occurs in natural habitat are Himachal Pradesh, J&K, and the hills of Uttar Pradesh.The wild growth of *T. minuta* in these areas is India's most important source of "*Tagetes* oil." Handa et al. (1963), Singh et al. (1995), and Bansal et al. (1999).

2.2 CLIMATE AND SOIL

Tagetes minuta grows in areas with loose soil and blooms in October and November. The highest quality essential oil is produced in moist temperate regions where nights are relatively cool during the plant's growth and flowering season. During the reproductive phase of the crop, an average temperature of 12176 is desired for the production of the highest grade of oil. This crop prefers soil that is slightly acidic (pH - 5.5 to 7.0). A well-drained sandy loam to clay loam soil with adequate humus promotes plant growth. A mini

mum of 50 cm of rainfall spread over the season, or its equivalent in irrigation water, is required to successfully raise the crop.

2.3 FOLK MEDICINE

Since ancient times*, Tagetes minuta* has been used in medicinal teas and condiments. Rees (1817) The plant's decoction is made by extracting the entire herb with hot water and is used as a beverage as well as a remedy for common colds, digestive system complaints, stomach upsets, diarrhea, and liver ailments. The decoction is served warm and can be sweetened to taste. Neher, 1968.

2.4 BIOLOGICAL PROPERTIES

Tagetes minuta oil has broncho dilatory, sedative, hypotensive, spasmolytic, and anti-inflammatory properties (Razdan et al. 1986). Dysdercus koenigi is biologically effective against juvenile hormones (Saxena and Srivastava 1973). The plant exhibits photosensitization and antiviral activity due to thiophenes (Chan et al. 1975), as well as antimicrobial activity due to the acyclic mono terpene ketones that are the primary constituents of its oil (Danes et al. 1988). The oil has a 100% inhibitory effect on gramme +bacteria, a 95% inhibitory effect on gramme - bacteria, and a 100% inhibitory effect on fungi (Hethelyi et al. 1986; Lima et al. 1993). One of the constituents, E-ocimenone, is responsible for the oil's larvicidal activity against *Aedes aegypti* mosquito larvae (Maradufu et al. 1978). Nematodes are parasitic on Tagetes roots, according to Steiner (1941). Well et al. (1992) report that Tagetes has insecticidal properties.

2.5 Harvesting and Distillation

The essential oil is found in the crop's leaf and flower parts, but not in the stem. As a result, the crop is harvested above ground at the point where the green leaves attach to the stem. A higher leaf and inflorescence to stem ratio is preferable for increased oil recovery. The crop is harvested when it is fully bloomed to maximize herb yield and oil quality (Singh et al. 1995). The biomass is chopped with a chaff cutter, and the thick stem is removed. This aids in filling the distillation tank and lowers distillation costs. Steam distillation is used to extract the essential oil from the crop. Herb material harvested at full

bloom is distilled when it is fresh, or within two to three days of harvesting. Before distillation, green foliage should be spread on the floor in the shade rather than piled in heaps. Experiments have shown that distilling fresh herbs yields a higher quality oil than sun-dried or stored material. Maximum oil content is obtained from fully matured plants, but at the expense of lower herb yield due to leaf shedding. At any stage of operation, stored biomass or essential oil should not be exposed to sunlight, moisture, or high temperatures. These elements degrade oil quality.

2.5 Storage of Oil

The oil of wild marigold is a pale yellow to dark yellow liquid. It is immediately dried after distillation. To prevent auto-oxidation, the oil is stored in stainless steel amber-colored glass, or aluminum containers that are filled to the brim. It is recommended that the oil be kept cool and away from light and humidity.

2.6 Application of *T.minuta* derived essential oil

T. minuta has as a long history of human intrigue and is utilized as beverage, condiment, ornamental, medicinal decoction, and in ritual/religious practices. In southern South America where the plant is native, its leaves, stems, and flowers are used as a culinary herb in Peru, Ecuador, and parts of Chile and Bolivia, where it is called by the Incan term, huacatay. The huacatay paste is used to make the popular potato dish called ocopa. In Peru, since the ancient Inca civilization, *T. minuta* is made into puree and seasoning that give local dishes a unique herbal flavor which is thought to taste like a mixture of mint, coriander, and basil oils. The plant is also popular for making ethnomedicinal teas in some areas. Tagetes has been utilized as a rich source of essential oil. Orange-yellow carotenoid is found abundantly in powders and concentrates of *T. minuta* florets. This carotenoid has been recognized, isolated, and avowed by the European Union (INS-Number E161b) for use in coloring food, and flavoring various food products, at a usage level in condiments and relishes such as pasta, vegetable oil, margarine, mayonnaise, salad dressing, baked goods, confectionery, dairy products, ice cream, yogurt, citrus juice and mustard. Tagetes oil has potential to protect food crops on the farm and in storage,

thereby increasing food security, particularly in undernourished communities of the world. The oil also provides an opportunity for developing an environmentally friendly and a nontoxic acaricide to enhance the production of milk, beef, and hides/skin in the livestock industry. Stimulant action through negative regulation on GABAergic function results in sedating, hypotensive, antidepressant, broncho dilatory, anti-inflammatory, spasmolytic and antifeedant bio-activities. It also has few health advantages like treatment for colds, respiratory problems, stomach disorders, antispasmodic, insecticide, antiseptic, anti-parasitic, and sedative. It has immense use in aromatherapy as well.

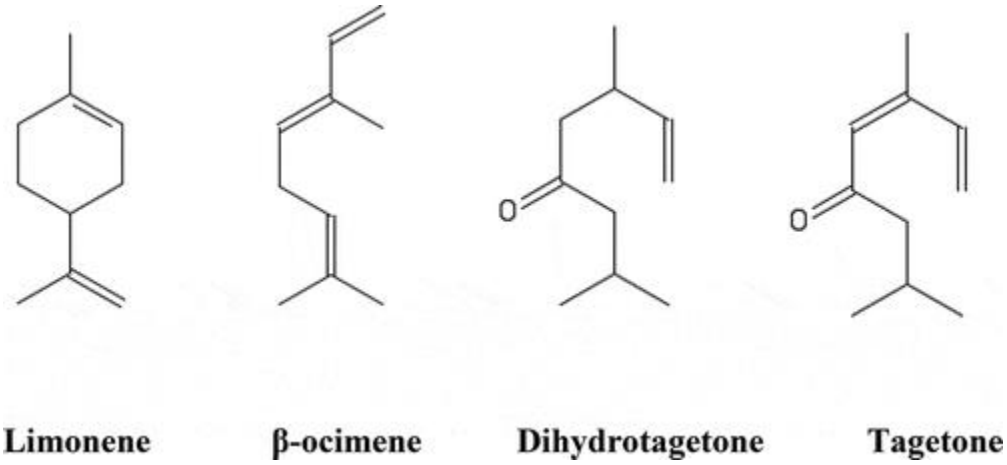
2.7 CHEMICAL ASPECTS

Essential oil composition

The importance of *T. minuta* from the presence of essential oil in nearly every part of its plants, excluding the stem (Singh et al., 2003). *T. minuta* oil contains a variety of secondary metabolites, including acyclic monoterpenes, sesquiterpenes, flavonoids, and thiopenes. Its essential oil contains significant amounts of (Z)-ocimene, (Z)- and (E)-tagetone, dihydrotagetone, and (Z)- and (E)-tagetenone**.** The chemical composition of essential oils from *T. minuta* differs greatly due to anthropological and environmental factors. These factors include harvesting method, geographical location, harvesting stage, parts used, and the climatic conditions under which the plant develops. The main compound in wild marigold essential oil varies according to geographical location. The constituents of *T. minuta* essential oil (Z)—ocimene and dihydrotagetone were significantly lower in Madagascar than in Rwanda, Turkey, India, and France. *T. minuta* essential oil from South Africa contained (E)-tagetone, dihydrotagetone, and (Z)-tagetone for chemotype 1, and (Z)—ocimene, (E)-ocimenone, and (Z)-ocimenone for chemotype. The essential oil composition of *T. minuta* revealed a higher content of tagetones in the UK sample, while an elevated proportion of ocimenes and ocimenones was reported in the Egypt and South Africa samples. (Senatore, F. Napolitano et al, Antibacterial activity of essential oils from *Tagetes minuta* L. (Asteraceae) with varying chemical compositions. Essential oils of *T. minuta* from Iran contained higher levels of -terpineol, (Z)-ocimene, (E)-ocimenone, dihydrotagetone, (Z)-tagetone, and (Z)-ocimenone. The main components of Himachal Pradesh essential oil were (Z)-ocimene and Dihydrotagetone.

Aside from a wealth of knowledge about the species' morphological, chemical, and genetic diversity, little is known about the environmental factors that influence this plant's qualitative and quantitative essential oil profile. Variation in the percentage of essential oil compounds is an important concept, particularly in commercially important crops, because chemistry has a direct impact on consumer product performance. Some authors have reported that the composition of Tagetes oil secondary metabolites varies with environmental factors such as soil, nitrogen levels, sunlight exposure, elevation, and temperature fluctuations (Moghaddam and Omidbiagi, 2007).

Chemical structure of major oil compounds of *T. minuta*.



Limonene **β-ocimene** **Dihydrotagetone** **Tagetone**

Table 2.1 Effect of different locations on essential oil components of *T.minuta*

Country	Major oil component
Madagascar	limonene > (Z)- beta -ocimene > (E)-ocimene > p-cymene > caryophyllene > (Z)- tagetenone
Egypt	Cis-beta- ocimene > (E)-ocimenone > (Z)-ocimenon > cis-tagetone
South Africa	cis-beta- ocimene > dihydrotagetone >cis-tagetone

Uk	Dihydrotagetone >cis-tagetone > trans-tagetone
Iran	Dihydrotagetone >cis-tagetone > trans-tagetone
Brazil, France, and Hungary	(Z)-beta-ocimene > (Z)- and (E)-tagetenone > (Z)-and (E)-tagetone > dihydrotagetone
North American	(Z)- beta -ocimene > (Z)- and (E)-tagetenone > dihydrotagetone > (Z)- and (E)-tagetone
Rwandan	(Z)- beta -ocimene > (Z)- and (E)- tagetone > (Z)- and (E)-tagetenones > dihydrotagetone
India (Kashmiri)	(Z)- beta -ocimene > (Z)- and (E)- tagetenone > dihydrotagetone > (Z)- and (E)-tagetone
India (Himachal Pradesh)	(Z)- beta -ocimene > dihydrotagetone > (Z)- and (E)-tagetone > (Z)- and (E)- tagetenone
India (Lucknow)	(Z)-tagetone > (Z)-tagetenone > dihydrotagetone, (E)-tagetone and (Z)- beta -ocimene > (E)- tagetone
India (Hyderabad	Dihydrotagetone > (Z)- beta -ocimene and (Z)-tagetone
Zambia	Dihydrotagetone > (Z)- beta -ocimene > (Z)- and (E)-tagetone > (Z)- and (E)- tagetenone
Turkey	Dihydrotagetone > (Z)- beta -ocimene > (Z)- and (E)-tagetenone> (Z)- and (E)-tagetone

Biosynthesis of the compounds of T. minuta oils

T. minuta essential oils are primarily terpenoid compounds, primarily monoterpenes and a few sesquiterpenes, and are not direct products of secondary metabolism. Three types of acetyl coenzyme A plant molecule that biosynthesizes mevalonic acid (MA), the source of terpenoid compounds. Before MA is converted to terpenoid, it is first converted into isopentenyl pyrophosphate (IPP). Two phosphorylation of MA with adenyl triphosphate (ATP) are required to produce the intermediate phospho-3-pyrophospho-5-mevolonate. The following steps are decarboxylation, hydrolysis, and dehydration, which result in the formation of IPP, which is later isomerized to give dimethyl allyl pyrophosphate (DMAPP). The reaction of IPP with DMAPP produces geranyl pyrophosphate (GPP), a monoterpenoid compound. GPP is then used as the primary precursor in the synthesis of a variety of monoterpenoid and sesquiterpenoid compounds. In tagetes oil, there are both acyclic and cyclic monoterpenes. Simple reactions like hydrolysis, dehydration, oxidation, and reduction result in the chemical modification of GPP or neryl pyrophosphate (NPP), which leads to the formation of acyclic monoterpenes. (Z)- and (E)-ocimene, (Z)- and (E)-tagetone, and (Z)- and (E)-tagetenone are examples of acyclic monoterpenes (also called ocimenones). Nonetheless, cyclic monoterpenes are formed by the cyclization of NPP, which is catalyzed by cyclases enzymes. The cyclic monoterpenes found in tagetes oils include limonene, piperitone, piperitenone, and others.

2.8 Gas chromatography mass spectrometry (GC-MS)

Mass spectrometry via gas chromatography (GC-MS) The essential oil was analyzed using a Zebron-5MS column (ZB-5MS 30 m 0.25 mm 0.25 um) (5 percent -phenylmethylpolysiloxane) on an Agilent 5977A MSD and 7890B GC system, Chemetrix Ltd.; Agilent Technologies, DE (Germany). Mass spectrometry via gas chromatography (GC-MS) The essential oil was analyzed using a Zebron-5MS column (ZB-5MS 30 m 0.25 mm 0.25 um) (5 percent -phenylmethylpolysiloxane) on an Agilent 5977A MSD and 7890B GC system, Chemetrix Ltd.; Agilent Technologies, DE (Germany) The column and temperature conditions were as follows: GC grade helium at a flow rate of 2 mL/min and splitless 1 mL injections. Temperatures for the injector, source, and oven were set to

280°C, 280°C, and 70°C, respectively. 15 °C/min to 120 °C, then 10 °C/min to 180 °C, then 20 °C/min to 270 °C and held for 3 min were the ramp settings.

2.9 Chemical reagents

The chemicals used were acetic acid, thiobarbituric acid (TBA), trychloroacetic acid, sodium dodecyl sulphate (SDS), 2, 2-diphenyl-1-picrylhydrazyl (DPPH), 2, 2′-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS), ethanol, methanol, ethyl acetate, n-hexane, Iron (II) sulphate (FeSO4), sodium hydroxide (NaOH), ascorbic acid and butanol. All chemicals reagents were of analytical quality and were bought from reliable commercial source.

Determination of antioxidant property

Antioxidant property of the extracted essential oil of *T. minuta* flower was evaluated in vitro by spectrophotometric method against DPPH, ABTS and lipid peroxyl radicals.

2.10 Pharmaceutical importance:

Essential oils of *Tagetes minuta*

T. minuta essential oils have been extensively studied in vivo and in vitro for various bioactivity properties, and have been reported to have antimicrobial, insecticidal, nematicide, acaricidal, and repellent action against a variety of human, livestock, and plant pests and pathogens. The chemical composition of essential oils from *T. minuta* collected from around the world has been studied and compared. These factors include the harvesting method, the geographical location of the target plant, the growth stage at harvest, the plant parts used, and the climatic conditions in which the plant grows. Variations in the chemical composition of EOs from the same plant species result in chemotypes, which are the result of biological variations caused by, among other things, the effects of different soils, temperature, weather conditions, and light. This implies that the chemical composition of plants that are botanically identical can differ. A comparison of the chemical composition of *T. minuta* EOs from Madagascar, for example, revealed that the constituents (Z)—ocimene and dihydrotagetone were significantly lower than those of *T. minuta* EOs from India, Turkey, Rwanda, and France. Several studies have

found chemotype variations in *T. minuta* essential oils. Untargeted GC-MS data analysis and hierarchical clustering analysis (HCA) of *T. minuta* EOs from South Africa, for example, revealed two major chemotypes. OPLS-DA revealed that (E)-tagetone, dihydrotagetone, and (Z)-tagetone were the characteristic marker constituents for chemotype 1, while (Z)—ocimene, (E)-ocimenone, and (Z)-ocimenone were the characteristic marker constituents for chemotype 2. constituents for chemotype (Wanzala et.al,2016) The study identified two chemotypes: one with a higher proportion of tagetones in the UK sample and another with a higher proportion of ocimenes and ocimenones in the Egypt and South Africa samples.

Antibacterial activity of *Tagetes minuta* essential oil

Antibacterial activity is one of the most widely studied areas in essential oil research. Antibacterial activity of *T. minuta* EOs against a range of human, plant and animal pathogenic bacteria has been studied. A study was conducted to assess the antimicrobial and antioxidative activities of essential oils of three South African plants including *T.minuta* Agar diffusion assays were used to determine the antimicrobial activities of EOs against two Gram-positive bacteria - Bacillus subtilis and Staphylococcus aureus - and two Gram-negative bacteria - Escherichia coli and Pseudomonas aeruginosa. *Tagetes minuta* EOs whose major components were (Z)-β ocimene and dihydrotagetone exhibited a concentration dependent antibacterial activity against the test bacteria, where the activity of the EOs increased with concentrations.

The antimicrobial effect of the essential oils was evaluated against two Gram-negative bacteria Salmonella typhi and E. coli and two Gram-positive bacteria S. aureus and B. subtilis. In addition, microdilution method was used to assess the minimum inhibition concentrations (MICs) of the EOs on the test pathogens. There was a concentration dependent antibacterial activity of *T. minuta* EOs whose main components were dihydrotagetone, (E)-β-ocimene, tagetone, (Z)-β-ocimene, limonene and epoxy ocimene.

Antifungal activity of *Tagetes minuta* essential oils

Antibacterial activity is one of the most widely studied areas in essential oil research. Antibacterial activity of *T. minuta* EOs against a range of human, plant and animal pathogenic bacteria has been studied. A study was conducted to assess the antimicrobial

and antioxidative activities of essential oils of three South African plants including *T. minuta*. Agar diffusion assays were used to determine the antimicrobial activities of EOs against two Gram-positive bacteria - *Bacillus subtilis* and *Staphylococcus aureus* - and two Gram-negative bacteria - *Escherichia coli* and *Pseudomonas aeruginosa. Tagetes minuta* EOs whose major components were (Z)-β ocimene and dihydrotagetone exhibited a concentration dependent antibacterial activity against the test bacteria, where the activity of the EOs increased with concentration. However, Gram-positive bacteria were more sensitive to the EOs than Gram-negative bacteria, a finding that concurred with previous studies, which reported that Gram-negative bacteria are generally more resistant to the effects of EOs than Gram positive bacteria. In another study, the chemical composition, antioxidant, antimicrobial and cytotoxic activity of *T. minuta* EOs were evaluated. The antimicrobial effect of the essential oils was evaluated against two Gram-negative bacteria *Salmonella typhi* and *E. coli* and two Gram-positive bacteria *S. aureus* and *B. subtilis*. In addition, microdilution method was used to assess the minimum inhibition concentrations (MICs) of the EOs on the test pathogens. There was a concentration dependent antibacterial activity of *T. minuta* EOs whose main components were dihydrotagetone, (E)-β-ocimene, tagetone, (Z)-β-ocimene, limonene and epoxy ocimene. The MICs for *S. typhi, E. coli, S. aureus* and *B. subtilis* were 150 ± 8, 165 ± 9, 67±8, and 75±7 µg/mL of *T. minuta* EOs, respectively. Similar to the previous findings, the EOs had a significantly stronger antibacterial effect on the Gram-positive bacteria than on Gram negative bacteria. Anti-bacterial activity of limonene enantiomers ((+), (−) and the racemate) singularly and in combination (1:1) with 1,8-cineole has been reported against S. aureus and P. aeruginosa. Anti-bacterial activity of *T. minuta* EOs obtained from different geographical regions and having different chemical profiles has also been studied. In one such study, the antibacterial activity of EOs extracted from aerial parts of *T. minuta* from Egypt, South Africa and the United Kingdom and having different percentage chemical compositions were tested against eight bacterial pathogens by determining the minimum inhibitory concentrations using broth dilution method. The EOs had great inhibitory activity against Gram-positive bacteria (*B. cereus*, *B. subtilis*, *S. aureus* and *S. faecalis*) than Gram-negative ones (*Proteus mirabilis*, *Pseudomonas aeruginosa*, *E. coli,* and *S. typhi)*. The study further showed variation in bioactivity of

essential oils from the three geographical locations which was attributed to differences in the percentage compositions of the major and minor essential oil constituents with the EOs from the United Kingdom exhibiting a higher antibacterial activity compared to EOs from Egypt and South Africa. Essential oils from a single plant may comprise of hundreds of major and minor components resulting in a myriad of possible biological interactions and anticipated effects. It has been reported in literature that generally, essential oils that contain phenolic structures such as carvacrol, eugenol and thymol produce the highest antimicrobial activity. However, more often than not, the level of antibacterial activity of EOs is usually as a result of unique complex synergistic and antagonistic biological interactions among different constituents of the EOs. Systematic and comprehensive investigations such as subtraction bioassays should therefore be conducted to accurately determine the levels of biological interaction, mechanisms and combinations that are responsible for the antibacterial activity of active components of the EOs, which have been isolated by bioassay guided fractionations. As previously stated, many studies have established that EOs are more efficacious towards Gram-positive than Gram-negative bacteria. The variation in sensitivity of Gram-positive and Gram-negative bacteria to EOs can be attributed to structural differences in cell walls of the two microbe groups. Roughly, 90% of the cell wall of Gram-positive bacterial is made up of peptidoglycan (murein), a mesh-like polymer of sugar and amino acids coupled with other molecules such as teichoic acid and proteins. This configuration of Gram-positive bacteria cell wall easily allows hydrophobic compounds to penetrate the cell and thus act on either the cell wall within the cytoplasm or both, thus making the Gram-positive bacteria more susceptible to antibacterial effects of EOs and other similar natural compounds. On the contrary, Gram-negative bacteria possess a more complex cell wall that has a much thinner peptidoglycan surrounded by an outer membrane consisting of proteins and lipopolysaccharide (LPS). It has been suggested that the presence of LPS and more specifically its O-side chain is responsible for greater resistance of Gram-negative bacteria to hydrophobic EOs by restricting their diffusion into the cytoplasm.

Antifungal activity of *Tagetes minuta* essential oils

Essential oils and plant extracts in general have been reported to have antifungal activity against a broad range of pathogenic fungi. High levels of activity against *Botrytis cinerea*

were reported in 13 out of 345 plant extracts. In the study, among the 49 essential oils analyzed, the most commonly occurring components in oils exhibiting high levels of antifungal activity were: limonene, 1,8-cineole, α-pinene, β-pinene, β-myrcene and camphor, which have been identified as some of the common components of *T. minuta* essential oils. Limonene, 1,8-cineole, α-pinene and camphor have been shown to have antifungal activity against a wide range of fungi such as *Rhizoctonia solani, Fusarium oxysporum, Penicillium digitatum, Aspergillus niger, Verticillium fungicola* and *Trichoderma harzianum*. *Tagetes minuta* essential oils have been evaluated for their fungicidal effects against a broad range of fungal pathogens. Essential oils extracted from leaves of *T. minuta* had higher activity than oils extracted from flowers of the same plant against eight phytopathogenic fungi namely: *Rhizoctonia solani, Fusarium solani, Sclerotinia sclerotiorum, Fusarium oxysporum pisi, Sclerotium rolfsii, Pyricularia grisea, Fusarium oxysporum lentis* and *Alternaria solani.* The percent inhibition of EOs from flowers at 1000 µg mL-1 were in the range of 8.9 to 35.1% with the highest activity reported *against F. oxysporum pisi* and the lowest against *P. grisea*. Leaf EOs on the other hand had the highest and least activity against *S. rolfsii* and *F. oxysporum lentis,* respectively. Concentration-dependent antifungal activity of EOs of *T minuta* extracted from aerial parts. *Aspergillus niger and Candida albicans* with corresponding MIC values of 135±15 and 115±8 µg/mL, respectively. Similarly, a concentration-dependent antifungal activity of *T minuta* EOs against *the fungus A. niger and yeast C. albicans* was reported with the activity increasing with increase in the concentration of essential oil. However, in some studies, diluted oils have been shown to result in larger zones of inhibition than those of undiluted oils. These cases of deviation from the expected results may be explained by the hypothesis that diluted oils diffuse more easily in the media compared to undiluted oil.

Insecticidal activity of *Tagetes minuta* essential oils

Chemical control of pests using insecticides remains one of the most effective strategies in pest management. Indeed, synthetic insecticides have played a major role in the control and management of many economically important pests thus boosting crop yields and animal productivity and enhancing global food security .Defense mechanism against pests are usually good sources of new insecticidal substances and comprise the main

focus by scientific community however, increased public awareness of the negative effects associated with synthetic insecticides such as high persistence of residues, toxicity to non-target organisms, increased cases of development of resistance in some pests, health risk to humans and general damage to environment has generated interest in biopesticides with botanical-based ones experiencing the greatest revival. Most plant-insect interactions are chemically mediated by secondary metabolites. Generally, plants that show well-developed seeking to develop plant-based insecticides. Essential oils as candidate for novel insecticides have numerous advantages; they are easy to extract, are biodegradable and hence ecofriendly, have low persistence in soil and water, and require less stringent approval and regulatory mechanisms due to their long usage history. Insecticidal activity of *T. minuta* essential oil against head lice, *Pediculus humanus capitis*, a human *ectoparasi*te that causes *Pediculosis capitis* has been studied. In the study, a linear regression model reported a Lethal Time (LT50) value of 16.4 ± 1.62 min with histopathological analysis of the treated adults showing pronounced disassembly of actin and myosin filaments. In another study, *T. minuta* EOs whose main constituents were limonene (13.0%), piperitenone (12.2%), and α-terpineol (11%) produced a dose-dependent toxicity against the cabbage aphid *Brevicoryne brassicae* with high dose (125.8 µlL-1 air) resulting in >90% insect mortality. *Tagetes minuta* essential oils from fresh and dried plant materials have also been reported to be highly effective against the larvae of *Anopheles stephensi* with the LC50 values of 1.0532 and 1.0315 mg/L, respectively. In another study, significant insecticidal activity of the EOs from aerial parts of *T. minuta* was reported against *Aedes aegypti* larvae. Essential oils of Tagetes minuta were also found to have repellent and antifeedant activity against diamond back moth*, Plutella xylostella (L.) (Lepidoptera Yponomeutidae)*. Essential oils from three genotypes of *T. minuta* were screened for their potential toxicity against three stored product beetle species: *Callosobruchus maculatus (Fabricius), Sitophilus oryzae (Linnaeus) and Tribolium castaneum (Herbst)*. The EOs from genotype 1 of *T. minuta* (TM-1) induced 100% adult mortality in the three beetle species at dosages of 50,000 ppm and 500 µg/insect in fumigant and contact toxicity tests, respectively. Furthermore, essential oils from TM-1 deterred oviposition and suppressed egg hatchability by 81% and 91%, respectively in *T. castaneum* at a dosage of 70, 000 ppm.

Acaricidal activity of *Tagetes minuta* essential oils

Plant essential oils have been studied extensively for their biocidal effect against a broad range of economically important arachnids especially ticks and mites revealing promising results. Control of tick species that infest livestock is especially of great importance in the livestock industry as it boosts productivity. Ticks cause extensive socio-economic losses to livestock industry by weakening the affected and infested animals through blood loss, reduction in the quality of the hides and more importantly, the ticks acting as vectors and reservoirs of a broad range of viral, rickettsial, bacterial and protozoan pathogens which are responsible for great economic losses globally. A study to evaluate the acaricidal activity of essential oils from leaves and stems of *T. minuta* against several tick species demonstrated that EOs of *T. minuta* had over 90% efficacy against four tick species at a concentration of 20%, an efficacy comparable to a number of referenced conventional acaricides. In another study, EOs of *T. minuta* whose major constituents were dihydrotagetone (54.10%) limonene (6.96%), tagetone (6.73%) and (E)-β-ocimene (5.11%) were shown to have significant activity against larvae, nymph and adult R. microplus. Essential oils of *T. minuta* from different plant parts in varied growth stages were investigated for their biocidal activity against Varroa destructor, an ectoparasitic mite of the *Apis mellífera.* The study findings revealed that the medium percentages of dead mites after six hours of treatment were 97.7, 98.3 and 100 for EOs from leaves of bloomed plants, leaves of non-bloomed plants and flowers, respectively. In a closely related study, *T. minuta* essential oils whose major components were (E)-β-ocimene (62.8%), (Z)-ocimenone (10.2%), (E)- ocimenone (6.6%), limonene (5.8%) and dihydrotagetone (4.2%) were evaluated for their biological activity against V. destructor. The percentage values of mite mortality sprayed with the EOs concentrations of 3, 4 and 5% were 64, 72 and 72%, respectively. In addition, significant acaricidal efficacy of *T. minuta* essential oil against V. destructor adult mite when administered through pulverization has been reported.

Repellent activity of *Tagetes minuta* essential oils

Repellents have been defined as substances that acting locally or at a distance, deter an arthropod from flying to, landing on or biting host human and/or animal skin or a surface

in general. Currently, considerable research efforts have been directed towards the development of alternative repellents to synthetic chemicals for control of insects and arthropods most of which are vectors to important diseases affecting humans and livestock. This is especially important because overreliance on synthetic chemicals is not without serious environmental and health concerns. Among the potential candidates in this regard are essential oils. Indeed, essential oils belonging to plants from numerous species have been studied extensively to assess their repellent properties with a number of them being the basis of some commercial repellent formulations. *Tagetes minuta* essential oils have been studied for their repellent activities against a number of human and animal parasites such as ticks and mosquitoes.

Repellency against ticks

The essential oils of *T. minuta* were investigated for their effect on the climbing response of adult R. appendiculate using a dual–and no–choice assay arrangement. Both assay methods used in this investigation independently showed a significant repellent effect of *T. minuta* essential oils against the test. The repellent effect was however less in no-choice assay compared to the dual-choice assay, especially for lower doses. Moreover, the study confirmed that *T. minuta* essential oils had effect on the adult *R. appendiculas'* both in the presence and absence of host-derived stimuli. In a related study, the repellency of essential oils of *T. minuta* obtained from three agro-ecological zones in Kenya was evaluated against *R. appendiculatus* using a dual-choice tick climbing assay apparatus. The study established a significant difference in chemical composition and dose-dependent repellency of *T. minuta* essential oils from the three agroecological zones. The higher activity of essential oil from one of the three regions was associated with the presence of relatively high amount of guaiacol, compared to oils from the other two regions, with synthetic blend assays showing that guaiacol and guaiacol-alloocimene blend had the highest repellency. In addition to *R. appendiculatus*, the activity of essential oils of *T. minuta* has been studied against other types of ticks. Anti-tick properties of the essential oils of *T. minuta* obtained by hydro-distillation from a combination of flowers, leaves and soft stems were evaluated for anti-tick properties against Hyalomma rufipes. *Tagetes minuta* EOs whose major components were (Z)-β-ocimene (28.5%), (E)-β-ocimene (16.83%) and 3-methyl2-(2-methyl-2-butenyl)-furan (11.94%) had a dose-

dependent repellent activity against H. rufipes. Furthermore, probit analysis revealed a repellent EC50 of *T. minuta* EO to be 0.07 ml/mL and 0.07 ml/mL for male and female ticks, respectively. Additionally, the essential oils were found to induce delayed molting in 60% (s. e±4.7) of nymphs after 25 days in comparison.

Repellency against mosquitoes

The repellent activity of *T. minuta* essential oils against some mosquito species has been investigated. The chemical composition and repellent effect of *T. minuta* essential oil on the host-seeking female *Anopheles arabiensis* mosquitoes was investigated using the human-bait technique. This study which was designed to simulate field situation was based on the proportion of host-seeking female *A. arabiensis* mosquitoes landing on the bait (human forearm) and biting it following its treatment with either the essential oils of *T. minuta* or Vaseline (a non-perfumed petroleum jelly), which was used both as a diluent as well as a control. There was a significant dose response effect of repellency with more test mosquitoes significantly landing and biting the control arm treated with pure petroleum jelly than the arm treated with the essential oil of *T. minuta* whose main constituents included (Z)-β-ocimene, (E)-βocimene, (Z)-tagetone, (E)-tagetone, dihydrotagetone, and piperitenone. In another study, the repellency effect of forty-one essential oils that included those of *T. minuta* against three mosquito species namely: *Aedes aegypti, Culex quinquefasciatus and Anopheles stephensi* was investigated. The repellent test employed the human-bait technique with the testing period lasting up to 8 hours. Furthermore, since the test mosquitoes included both day and night biters, different testing periods were used i.e 0800 to 1600 h for A. aegypti and 1600 and 2400 h *for A. stephensi* and *C. quinquefasciatus*. The protection time was calculated as the duration between the application of a repellent and the first two bites or two bites in successive observations. *Tagetes minuta* essential oils offered a protection of 60 minutes against *A. aegypti* and 480 minutes (the maximum protection time) for both *A. stephensi and C. quinquefasciatus*. In a closely related study, promising repellent activity of *T.minuta* essential oil against the three aforementioned mosquito species was obtained with the test oils providing 86.4, 84.2 and 75% protection against *A. stephensi, C. quinquefasciatus, and A. aegypti*, respectively after a study period of 6 hours. In addition to mosquitoes, *T. minuta* EOs have been investigated for their repellent properties against

other insects. *Tagetes minuta* EOs whose main constituents were (Z)-tagetone, (E)-β-ocimene and dihydrotagetone were found to have good repellent activity against Triatoma infestans (the insect vector of Chagas disease). At a concentration of 0.5% (w/v), the essential oils of *T. minuta* produced an average repellency of 94.7%.

Nematicidal activity of *Tagetes minuta* essential oils

Plant-parasitic nematodes are important parasites that cause great damage to many agricultural crops resulting in losses estimated in the order of billions each year. Botanicals have received much attention in the management of nematodes where they are used in four main ways: 1) used as part of the plant directly, 2) using extracts of parts of the botanicals, 3) using compounds of botanicals possessing nematicidal activity and 4) using plants as oil seed cakes, mature crop residue and organic amendments. The toxicity of two compounds; (Z)-β-ocimene and dihydrotagetone isolated from the EOs of *T. minuta* was investigated against the eggs and juveniles of Meloidogyne incognita, a plant-parasitic nematode. Tagetes minuta essential oils at a concentration of 4%, 3%, 2% and 1% were strongly toxic to both the eggs and juveniles of the test nematode. Moreover, the egg-hatch inhibitions by the above oil concentrations were in the range of 72 to 79% in 14 days with the inhibitory activity being concentration-dependent. The study additionally showed that of the two compounds, dihydrotagetone was more toxic to the eggs of *M. incognita* and the compound killed the juveniles much faster (in 72 hours) compared to (Z)-β-ocimene (in 96 hours). In addition to plant-parasitic nematodes, *T. minuta* essential oils have been studied for their nematicidal activity against animal gastrointestinal nematodes such as Haemonchus contortus, an important pathogenic nematode of ruminant. In the study, two bioassays: an egg hatch test (EHT) and larval development test (LDT) were used. *Tagetes minuta* essential oils displayed a dose-dependent effect in the EHT, inhibiting 98.1% of H. contortus larvae hatching at a concentration of 2.5 mg mL-1. Furthermore, the effective EOs concentration that suppressed 50% (EC50) of egg hatching was 0.53 mg mL-1. With regard to the larval development test, *T. minuta* EOs at a concentration of 10 mg mL-1 inhibited 99.5% of H. contortus larval development with an EC50 value of 1.67 mg mL-1.

Allelopathic effect of *Tagetes minuta* essential oils

Allelopathy, which is a biochemical interaction among higher plants in which certain plants release metabolites that have either stimulatory or inhibitory effects on the growth of the receptor plants has been recognized and studied for man year. Allelopathy is generally recognized as an important ecological factor that determines the structure and composition of plant communities. This phenomenon of certain plant species possessing the capability to affect surrounding plants has been widely documented and is currently being explored as a potential method of weed control and management. *Tagetes minuta* essential oils and their principal components in the vapor phase were studied for their allelopathic activity on roots of Maize (*Zea mays L.).* The volatile oils of *T. minuta* whose main components were limonene, β-pinene and α-pinene strong inhibitory and oxidant effect on the root of Z. mays seedlings. The phytotoxic action of *T. minuta* EOs against Z. mays was attributed to increased lipid peroxidation rates indicating an induction of ROS-generated oxidative stress. With regard to the terpenes studied, ocimenone had the highest inhibitory effect on the root growth and the highest oxidative value. Thus, the high phytotoxicity action of *T. minuta* EOs was attributed to its high content of ocimenone. In another study, the allelopathic potential of EOs of *T. minuta* against three invasive weeds: *Chenopodium murale L., Phalaris minor Retz.,* and *Amaranthus viridis* L. was investigated. The results of the study established a concentration-dependent response in which volatile oils of *T. minuta* reduced germination in the three test weeds with maximum reduction observed in *C. murale* followed by P. minor and least in A. viridis. Moreover, LC50 values for *C. murale* and P. minor were lower (0.761 and 0.822 mg/petri dish, respectively) compared to those of *A. viridis* (2.745 mg/petri dish). Similarly, seedling length also followed the same pattern with maximum reduction observed in C. murale. Two compounds that have been commonly isolated from *T. minuta* EOs i.e α-pine and its isomer β-pinene have been shown to have strong allelopathic effects, and their ecological role on the allelopathic interactions among plants is well documented. The phytotoxic effect of aqueous extracts and essential oils of *T. minuta* on the callus growth and induction on four plant species; *Oryza sativa (Dongjinbyeo), Brassica campestris subsp. Napus var. pekinensis, Raphanus sativus var. acanthiformis and Sesamum indicum (Ansanggae)* have been studied. Species specific inhibitory trends from the

essential oils and the aqueous extracts were observed. The percentage of total callus induction on *O. sativa* though slightly decreased in proportion to the concentration of the EOs was not significantly different to the control. For *B. campestris var. pekinensis,* however, the percentage of total callus induction was significantly inhibited by 20 µl essential oil. With regard to the callus growth, when tested in 20 µl essential oil, callus growth of the four receptor species decreased significantly compared to that of the control. The callus relative growth rate (RGR) in 20 µl essential oil was repressed in the following order: *S. indicum, B. campestris, O. sativa and R. sativus.*

Antioxidant activity of *Tagetes minuta* essential oils

An antioxidant is defined as any substance, which when present at low concentrations in combination with an oxidisable substrate, significantly delays or prevents oxidation of the substrate. Oxygen though a vital component for living system, is a highly reactive atom that is capable of forming damaging molecule (free radicals). Free radical-mediated oxidation of various biological substances has been associated with numerous diseases such as cardiovascular disease, Alzheimer's disease, arthritis, inflammation, diabetes, Parkinson's disease and several types of cancers. To prevent oxidative damage, many diseases have for a long time been treated using antioxidants. The use of synthetic antioxidants has raised health concerns especially after some such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) were reported to be toxic and potentially carcinogenic, by inducing DNA damage. As a result, there is increased need to find alternative naturally-based antioxidants especially from plants and plant metabolites. Indeed, numerous studies have been conducted on essential oils as potential sources of natural antioxidants. The antioxidant activity of essential oils isolated from *T. minuta* flowers was studied with the hydrogen atom-or-electron donating ability of the Eos assessed using the 2, 2- diphenylpicrylhydrazyl (DPPH) radical scavenging assay. The DPPH scavenging activity of the EOs was compared with those of the standard antioxidant, ascorbic acid. The DPPH free radical scavenging activity of the essential oil was lower (73.4%) at a concentration of 200 µg/mL, in comparison to that of ascorbic acid (94.1%) at similar concentration. *Tagetes minuta* EOs at a 200 µg/mL concentration reduced the concentration of DPPH free radical with an efficacy of about 75 µg/mL concentration of the standard antioxidant. Moreover, *T. minuta* EOs achieved

50% reduction of the DPPH radical with EC50 value of 86.35 µg/mL. A study that similarly used the DPPH radical scavenging assay to evaluate the antioxidant activity of *T. minuta* essential oil whose main constituents included, trans-ocimenone (51.7%), cis-tagetone (17.7%) and cis-ocimenone (7.7%), reported an EC50 value of 0.8mg/mL. Another study similarly employed the DPPH radical scavenging assay to evaluate the antioxidant properties of both the essential oils and solvent extracts of *T. minuta.* In the study, the active constituents were isolated by thin layer chromatography (TLC) and high-performance liquid chromatography (HPLC). The active fractions were later detected using gas chromatography/mass spectrometry (GC-MS) and nuclear magnetic resonance (NMR), with the active components tested quantitatively for their radical scavenging activity by Ultraviolet-visible spectroscopy (UV/Vis). *Tagetes minuta* essential oils whose main chemical components included (E)-β ocimene (15.90%), L-verbenone (15%) and limonene (8.02%) were reported to have strong antioxidant activity with an LC50 of 1.49 g/l-1 after 30 minutes of evaluation.

Anticancer activity of *Tagetes minuta* essential oils

Cancer is a leading cause of death worldwide, accounting for more than eight million deaths in 2012. It is estimated that roughly 60% of drugs currently used for cancer treatment have been isolated from natural products with plants being the most important sources. Systematic screening of plants especially those that are prominently used in various types of ethnic medicine has led to the discovery of important drugs such as taxol, camptothecin and camptothecin. Essential oils from a wide range of plants have been extensively studied for their anticancer activities including melanoma, leukemia, glioblastoma, and oral cancers, besides breast, cervix, colon, liver, pancreas, prostate and uterus cancers. Anticancer activity of and EOs was conducted on two human promyelocytic leukemia cell lines (HL-60 and NB4) and an experimental animal model cancer cell line, Ehrlish ascites carcinoma cells (EACC). For in vivo studies, three treatments: initiation, pre-initiation and post initiation were used to study the chemopreventive and/or chemotherapeutic efficacies of the two essential oils based on the survival of tumor (EACC) transplanted in female mice. In the case of the in vitro tests, *T. minuta* essential oils showed a higher anticancer activity against NB4 and EACC cell lines compared to O. basilicum EOs, while O. basilicum essential oils exhibited higher

bioactivity than *T. minuta* EOs against HL-60 cell line. For in vivo studies, the pre-initiation treatments were more effective for both essential oils compared to initiation and post-initiation treatments on the tumor (EACC) transplanted female mice. In another closely related study, chemical composition, antioxidant, antimicrobial and cytotoxic bioactivities of the two aforementioned EOs was studied. In the study however, the activity of the EOs against two human tumor cell lines namely, nasopharyngeal cancer cell line (KB) liver hepatocellular carcinoma cell line (HepG2) was studied using a modified MTT assay. At lower concentrations, (200µg/mL. Additionally, IC50 values for KB and HepG2 were 75±5 and 70±4 µg/mL of *T. minuta* essential oils, respectively. In another study, *T. minuta* EOs whose main constituents were (Z)- ocimenone (15.9%), (E)-ocimenone (34.8%) and (Z)-β-ocimene (8.3%) showed potent cytotoxic activity against MCF-7 breast tumor cells, with an IC50 of 54.7±6.2 µg/mL. Furthermore, 1,8-cineole, a common occurring constituent in *T. minuta* EOs has been shown to induce apoptosis of human colon cancer cells.

Other activities of *Tagetes minuta* essential oils

There have been numerous reports of bioactivities of *Tagetes minuta* essential oils and their components including: antidepressant activity via negative modulation on GABAergic function, tranquilizing, hypotensive, bronchodilatory, spasmolytic and anti-inflammatory bioactivities and antifeedant activities. Essential oils have been considered relatively non-toxic to mammals and hence completely safe to use because they are ‘natural’. However, most EOs can be toxic at high concentrations, especially when taken orally. Some EOs can be toxic when applied topically or orally even at very low concentration due to the presence of toxic components. In vitro and in vivo studies have shown that *T. minuta* EOs are phototoxic. It is for this reason that the Scientific Committee on Consumer Products (SCCS) has recommended a maximum level of 0.01% *T. minuta* EOs in leave-on products (except sunscreen cosmetic products), provided that the terthiophene content in the EOs does not exceed 0.35%. Additionally, SCCS recommends that *T. minuta* extracts and oils should not be used as ingredients of sunscreen products. With the exception of aromatherapy, there is little information in literature on safety considerations in using *T. minuta* EOs. Thus, comprehensive toxicity tests are required in order to ascertain the safety of using the EOs of *T. minuta* in

pharmaceuticals, agrochemicals, food and beverages and in sociocultural ethnic practices, among other applications.

Table 2.3 Pharmaceutical activities of *T. minuta*

Antibacterial	Target species/cells	Oil/Solvent with Maximum inhibition	Reference
	Xanthomonas axonopodis	Essential oil	Gakuubi, et al., (2016)
	Escherichia coli	Staphylococcus aureus	Kwamboka, et al., (2016).
	"Cryptococcus spp"	Flower Essential oil	Ismail, et al., (2013).
Antiviral	Ranikhet disease virus	Ethanol extract	Bhakuni, et al., (1969)
	"Carnation ring spot virus	carnation vein mottle viruses"	Singh et al., (2002).
	"Herpes simplex virus	Ethanol extract Pharmacology	Ghaemi, et al., (2004)
Insecticidal	Plutella xylostella	Essential oil	Reddy, et al., (2015).
	"Pediculus humanus capitis	Sitophilus oryzae	Reddy, et al., (2015)

	Zabrotes subfasciatus	Essential oil	Weaver, et al., (1994)
	Brevicoryne brassicae	Floral extract	Motazedian, et al., (2014).
	Rhipicephalus appendiculatus"	Essential oil	Wanzala, et al., (2014).
	Anopheles arabiensis; A. stephensi	Essential oil	Amer et al., (2006).
Anti-cancerous	Human promyelocytic leukemia cell lines	Essential oil	Mahmoud, et al., (2014).
	"Nasopharyngeal and liver hepatocellular cancer cell lines"	Essential oil	Ali et al., (2014).
	Breast tumor cells	Essential oil	Murata et al., (2013).
	"Human colon cancer cells"	Essential oil	Murata et al., (2013).
Acaricidal	Rhipicephalus. Microplus "Varroa destructor"	Essential oil R. sanguineus	Andreotti, et al., (2013).
	"R. microplus	Essential oil	Andreotti, et al., (2013).
Nematicidal	Meloidogyne incognita	Essential oil	O.K. et al., (2007).

Antifungal	Rhizoctonia solani, Sclerotinia sclerotiorum, S. rolfsii	Leaf essential oil	Ismail, et al., (2013).
	"Botrytis cinerea	Leaf essential oil	Saha et al., (2012).
	"R. solani	Fusarium oxysporum	S. Walia, et al., (2012).
	""A. niger	Candida albicans"	G.K. et al., (2012).
	" Cryptococcus spp"	Flower Essential oil	O.N. et al., (2004)

Trade and market demand

According to Singh et al. (2006), about 3t of high-quality oil is produced annually in Himachal Pradesh (India), with prices ranging from Rs.1935-2160 per kg, compared to Rs. 1170-1260 per kg in other parts of North India (Jhunjhunwalla, 2004) Because of the rising demand for its oil, this species has a lot of potential for large-scale cultivation. According to Cravero et al. (1988), Brazil is a major producer of "*Tagete*s Oil." In 1984 (Lawrence, 1985) [35], global oil production was roughly 1.5 tones. *Tagetes minuta* oil is most commonly produced in South Africa, India, Zimbabwe, Egypt, France, and Argentina. The production of Tagetes oil in South Africa was predicted to be 6.5 tonnes in 2003. Zimbabwe and India are predicted to produce 2 and 4 tonnes per year, respectively. However, Anonymous (2014) estimates that global demand for *Tagetes minuta* oil for all purposes will exceed 12 tonnes. *Tagetes minuta* oil is a particularly expensive product because the yield of extraction is just 0.1-0.4 percent. Around 25 tonnes of raw plant material and 12.5 to 17.5 kg of *Tagetes minuta* oil are produced per acre. Due to decreasing availability, FOB prices for high-quality oil have recently surged to roughly $ 190-250 kg-1. Low-quality Tagetes oil can be purchased for as little as $ 90

kg-1 (about Rs. 6120) (Anonymous, 2014). The cost of a litre of *Tagetes minuta* oil is $177.78 in the United States (approximately Rs.12, 119.26) 2016 (Anonymous).

Imports of *Tagetes* essential oil India brought in USD 10,028 worth of "*Tagetes* oil" in a total quantity of 42 litres. Switzerland is the greatest importer of "*Tagetes* oil," with USD 9,037 in imports, followed by France and the United States, with USD 677 and USD 147 in exports, respectively.Bombay Air Cargo handled 98 percent of all imports, followed by Nhava Sheva Sea, which handled 2% of all imports. The average price of "*Tagetes* oil" per unit in the International Journal of Chemical Studies is USD 241.21, and the average value per shipment is USD 346 (Anonymous, 2016b).

Exports of *Tagetes* essential oil

The oil is labelled with the HS Code 3301. India exported a total of 475 litres of *"Tagetes* oil" for USD 54,572. Germany is the greatest consumer of "*Tagetes* oil," accounting for USD 49,580 in exports, followed by Taiwan and the United States, with USD 2,160 and USD 997 in imports, respectively. Delhi Air Cargo was responsible for 100% of exports, while Chennai Air Cargo was responsible for 0% of exports. The average price per unit of "*Tagetes oil*" is USD 114.96, with an average cargo value of USD 1,605. (Anonymous, 2016b)

Fig:4 Products of *Tagetes minuta* oil

Molecular Markers

Molecular markers, useful for plant genome analysis, have now become an important tool in this revolution. Molecular markers are routinely employed in various aspects of plant genome analysis such as taxonomy, phylogeny, ecology, genetics and plant breeding. Molecular markers include biochemical constituents (e.g secondary metabolites in plants) and macro molecules for, example proteins and deoxyribonucleic acids (DNA). Analysis of secondary metabolites is, however, restricted to those plants that produce a suitable range of metabolites which can be easily analyzed and which can distinguish between varieties. These metabolites which are being used as markers should be ideally neutral to environmental effects or man agement practices. Hence, amongst the molecular markers used, DNA markers are more suit able and ubiquitous to most of the living organisms. Due to complex relationships between *Tagetes* minuta species and huge intraspecific variability in morphological and biochemical traits, the morphological differentiation of these species is very difficult. So, a combination of morphological, karyotypic, chemical, and molecular markers for an unambiguous conclusion about cultivated basils is required (Wetzel et al. 2008). Molecular diversity in *Tagetes minuta* has been studied by several workers employing random amplified polymorphic DNA (RAPD),inter-simple sequence repeat (ISSR), amplified fragment length polymorphism (AFLP), sequence-related amplified polymorphism (SRAP), simple sequence repeat (SSR), internal transcribed spacer (ITS) (Labra et al. 2004; De Masi et al. 2006; Carović-Stanko et al. 2010, 2011; Shinde et al. 2010; Moghaddam et al. 2011; Aghaei et al. 2012; Chen et al. 2013; Mahajan et al. 2015; Malav et al. 2015; Chowdhury et al. 2017). These have been studied along with the morpho-chemical traits of the *Tagetes minuta* accessions.

DNA Based Markers

- Codominant inheritance
- Frequent occurrence in genome
- Selective neutral behaviour
- Easy access (availability)
- Easy and fast assay

- High reproducibility
- Easy exchange of data between laboratories.

Molecular markers are utilized to evaluate DNA polymorphism and are generally classified as hybridization-based markers and polymerase chain reaction (PCR)-based markers. In the former, DNA profiles are visualized by hybridizing the restriction enzyme-digested DNA, to a labelled probe, which is a DNA fragment of known origin or sequence. PCR-based markers involve in vitro amplification of particular DNA sequences or loci, with the help of specifically or arbitrarily chosen oligonucleotide sequences (primers) and a thermostable DNA polymerase enzyme. The amplified fragments are separated electrophoretically and banding patterns are detected by different methods such as staining and autoradiography. PCR is a versatile technique invented during the mid-1980s Ever since thermostable DNA was introduced in 1988. The primer sequences are chosen to allow base specific binding to the template in reverse orientation. PCR is extremely sensitive and operates at a very high speed. Its application for diverse purposes has opened up a multitude of new possibilities in the field of molecular biology.

Microsatellite and Minisatellite

The term microsatellite was coined by Litt and Lutty, while the term minisatellites was introduced by Jeffrey. Both are multi locus probes creating complex banding patterns and are usually non-species specific occurring ubiquitously. They essentially belong to the repetitive DNA family. Fingerprints generated by these probes are also known as oligonucleotide fingerprints. The methodology has been derived from RFLP and specific fragments are visualized by hybridization with a labelled micro- or minisatellite probe. Minisatellite are tandem repeats with a monomer repeat length of about 11-60 bp, while microsatellites or short tandem repeats/simple sequence repeats (STRs/SSRs) consist of 1 to 6 bp long monomer sequence that is repeated several times. These loci contain tandem repeats that vary in the number of repeat units between genotypes and are referred to as variable number of tandem repeats (VNTRS) (i.e a single locus that contains variable number of tandem repeats between individuals) or hypervariable regions (HVRS) (i.e. numerous loci containing tandem repeats within a genome

generating high levels of polymorphism between individuals). Microsatellites and minisatellites thus form an ideal marker system creating complex banding patterns by simultaneously detecting multiple DNA loci. Some of the prominent features of these markers are that they are dominant fingerprinting markers and codominant STMS (sequence tagged microsatellites) markers. Many alleles exist in a population, the level of heterozygosity is high and they follow Mendelian inheritance. Microsatellites have become markers of choice because they are locus specific, often co-dominant, multi-allelic and transferable across species and even genera (Gupta et al., 1996). Microsatellites are arrays of nucleotides from 1 to 6 bases in length and are common components of genes and whole genomes (Tautz and Schotterer 1994). SSR markers have been developed for a range of applications in scientific research including measuring genetic diversity and genetic mapping (Varshney et al., 2005).

ISSR MARKER

In this technique, reported by Zietkiewiez et al., primers based on microsatellites are utilized to amplify inter-SSR DNA sequences. Here, various microsatellites anchored at the 3e end are used for amplifying genomic DNA which increases their specificity. Inter simple sequence repeat (ISSR) technique is a PCR based method, which involves amplification of DNA segment present at an amplifiable distance in between two identical microsatellite repeat regions oriented in opposite direction. The technique uses microsatellites, usually 16–25 bp long, as primers in a single primer PCR reaction targeting multiple genomic loci to amplify mainly the inter- SSR sequences of different sizes. The microsatellite repeats used as primers can be di-nucleotide, tri-nucleotide, tetranucleotide or penta-nucleotide. The primers used can be either unanchored (Gupta et al., 1994; Meyer et al., 1993; Wu et al., 1994) or more usually anchored at 3’ or 5’ end with 1 to 4 degenerate bases extended into the flanking sequences (Zietkiewicz et al., 1994). The technique combines Most of the benefit AFLP and microsatellite analysis with the universality of RAPD. ISSRs have high reproducibility possibly due to the use of longer primers (16–25mers) as compared to RAPD primers (10-mers) which permits the subsequent use of high annealing temperature (45– 60 ◦C) leading to higher stringency. The studies on reproducibility show that it is only the faintest bands that are not

reproducible. About 92–95% of the scored fragments could be repeated across DNA samples of the same cultivar and across separate PCR runs when detected using polyacrylamide (Fang & Roose, 1997; Moreno et al., 1998). 10 ng template DNA yielded the same amplification products as did 25or 50 ng per 20µl PCR reaction. The annealing temperature depends on the GC content of the primer used and usually ranges from 45 to 65 ◦C. ISSRs segregate mostly as dominant markers following simple Mendelian inheritance (Gupta et al., 1994; Tsumura et al., 1996; Ratnaparkhe et al., 1998; Wang et al., 1998). However, they have also been shown to segregate as co-dominant markers in some cases thus enabling distinction between homozygotes and heterozygotes (Wu et al., 1994; Akagi et al., 1996; Wang et al., 1998; Sankar & Moore, 2001).

Chapter-3

MATERIAL AND METHODS

The experimental material comprised of 35 genotypes of *Tagetes minuta* obtained from Research Farm of CSIR –central Institute of Medicinal and Aromatic Plants, Lucknow. Plants were harvested at flowering stage. Essential oil was done in fresh herbs by hydro-distillation using Clevenger types apparatus.

Table 3.1 Sample isolated from field:

1	Tm1	*T. minuta* accession
2	Tm2	*T. minuta* accession
3	Tm3	*T. minuta* accession
4	Tm4	*T. minuta* accession
5	Tm5	*T. minuta* accession
6	Tm6	*T. minuta* accession
7	Tm7	*T. minuta* accession
8	Tm8	*T. minuta* accession
9	Tm9	*T. minuta* accession
10	Tm10	T. minuta accession
11	Tm11	*T. minuta* accession
12	Tm12	*T. minuta* accession
13	Tm13	*T. minuta* accession
14	Tm14	*T. minuta* accession
15	Tm15	*T. minuta* accession
16	A1-1-6	Vanphool
17	A1-4-5	Vanphool
18	A1-4-7	Vanphool
19	A3-2-7	Vanphool
20	A3-4-3	Vanphool

21	A3-6-3	Vanphool
22	A-4-9-8	Vanphool
23	A5-2-2	Vanphool
24	A5-4-5	Vanphool
25	A6-3-3	Vanphool
26	A8-7-5	Vanphool
27	A8-7-8	Vanphool
28	A8-9-16	Vanphool
29	A8-11-4	Vanphool
30	B1-1-8	Vanphool
31	B1-5-4	Vanphool
32	B1-5-6	Vanphool
33	B1-5-11	Vanphool
34	B1-6-14	Vanphool
35	B2-7-8	Vanphool
36	B2-3-11	Vanphool
37	B5-2-2	Vanphool
38	B6-1-4	Vanphool

3.2 Preparation of buffers and Reagents

S.N.	Buffer/ solution	Composition/ preparation
1	1M Tris-Cl (Ph8.0;1L)	121.1 g of Tris base was mixed with 600ml of dd H2O by stirring and the pH was adjusted to 8 by adding HCl. THE Final volume was made up to 1L, autoclaved and stored at room temperature.
2	CTAB 20% (100 ml)	20g of CTAB was added to 100 ml of dd H2O and heated in water bath to dissolve completely
	0.5 M EDTA (Ph 8.0; 500 ml)	93.5 g of EDTA was dissolve in 200 ml of dd H20 and pH was adjusted to 8 by adding NaOH pellets. The final volume was

		made up to 500 ml, autoclaved and stored at room temperature
4	5 M NaCl (500ml)	146.1g of NaCl was mixed with 500 dd H2O by stirring, autoclaved and stored at room temperature.
5	High salt T.E. Buffer	20ml NaCl (15M), 1 ml Tris Cl (1M) & 0.2ml EDTA (0.5M) are mixed and the final volume was made up to 100 ml, autoclaved and stored at room temperature
6	10x TBE Buffer(1L)	108 g Tris base and 55 g Boric acid was dissolved in 800 ml dd H2Oand 40 ml of 0.5M EDTA was added to it. Now the final volume was made up to 1 L, autoclaved and stored at room temperature. The buffer was diluted to 0.5X and used accordingly when needed
7	Chloroform: isoamylalcohol (24:1)	96 ml of chloroform was added with 4 ml of isoamylalcohol
8	2.1 70% Ethanol	70ml of Absolute ethanol was added with 30ml distilled water.

3.2 Methodology

Phenotype data mining: The following 15 genetically and agronomically important traits were investigated. these listed phenotypic characters were scored as per standard descriptors for *Tagetes minuta*.

Table 3.4 Phenotypic characteristic No. of primary branch, Total branch, Spikelet length, Spikelet width, Spikelet width, No. of invocules / primary branch, No. of spikelet/per invocules, Phyllary no. of flower, Phyllary no. of leaf , Plant height, Vertical canopy, Biomass, Horizontal canopy, Root weight.

3.3 GAS CHROMATOGRAPHY

Sample preparation

The fresh aerial part s of different species of *Tagetes minuta* were harvested in the Central Institute of Medicinal s Aromatic Plants. The fresh aerial parts of the *Tagetes* species (100 g) were immersed in 1.0 L of water in a round bottom flask and subjected to hydro-distillation for 2 h using a Clevenger apparatus. The essential oils were recovered, the water was completely removed with anhydrous sodium sulfate, and the samples were stored at 4°C prior to their analysis. The essential oil samples were obtained in triplicate. Hydro distillation is the most common method used for essential oil analysis, whose main advantages are the high yield, short processing, and use of non-toxic solvents.

3.4 GENOMIC DNA ISOLATION

Genomic DNA was isolated from the leaves of 35 genotype under study by using a protocol developed by Khanuja et al., (1999) specifically for the medicinal and aromatic plant as follows. Healthy and young leaves were taken and blot dried using sterile filter papers. Fresh plant tissue was placed into a mortar and pestle and the tissue was ground finely using liquid nitrogen (3g fresh tissue or 0.5g dry tissue). Freeze-dried plants can be ground at room temperature. In either case, a fine powder is best for extracting DNA. The material was transferred in polypropylene tube and 3 ml of freshly prepared extraction buffer was added and mixed by inversion to the slurry. The material with extraction buffer was incubated at 60ºC in a shaking water bath for 1-2 hr. 3ml of Chloroform: Isoamyl alcohol (24:1) was added and mixed by slurry. This was centrifuged at 10,000rpm for 10-15 min. The upper aqueous layer was carefully transferred to another 10 ml polypropylene tube 0.7 volume of chilled isopropanol was added and the mixture was incubated at -20ºC for 15 minutes to precipitate the DNA. After 1 h, the mixture was mixed slowly and carefully producing fibrous nucleic acid which was scooped and transferred to a 1.5 ml microfuge tube and centrifuged at 10,000rpm for 10 min. Decant the supernatant without disturbing the pellet and subsequently wash twice with 500µl ice-cold 70% ethanol. Decant the ethanol remove the residual ethanol by drying in a Speed Vac. Dry the pellet long enough to remove alcohol, but without completely drying the DNA. Dissolve the DNA pellet in 20µl TE buffer (10mM Tris, pH 8, 1 mM EDTA). The pellet may need to be warmed, in order to dissolve in it.

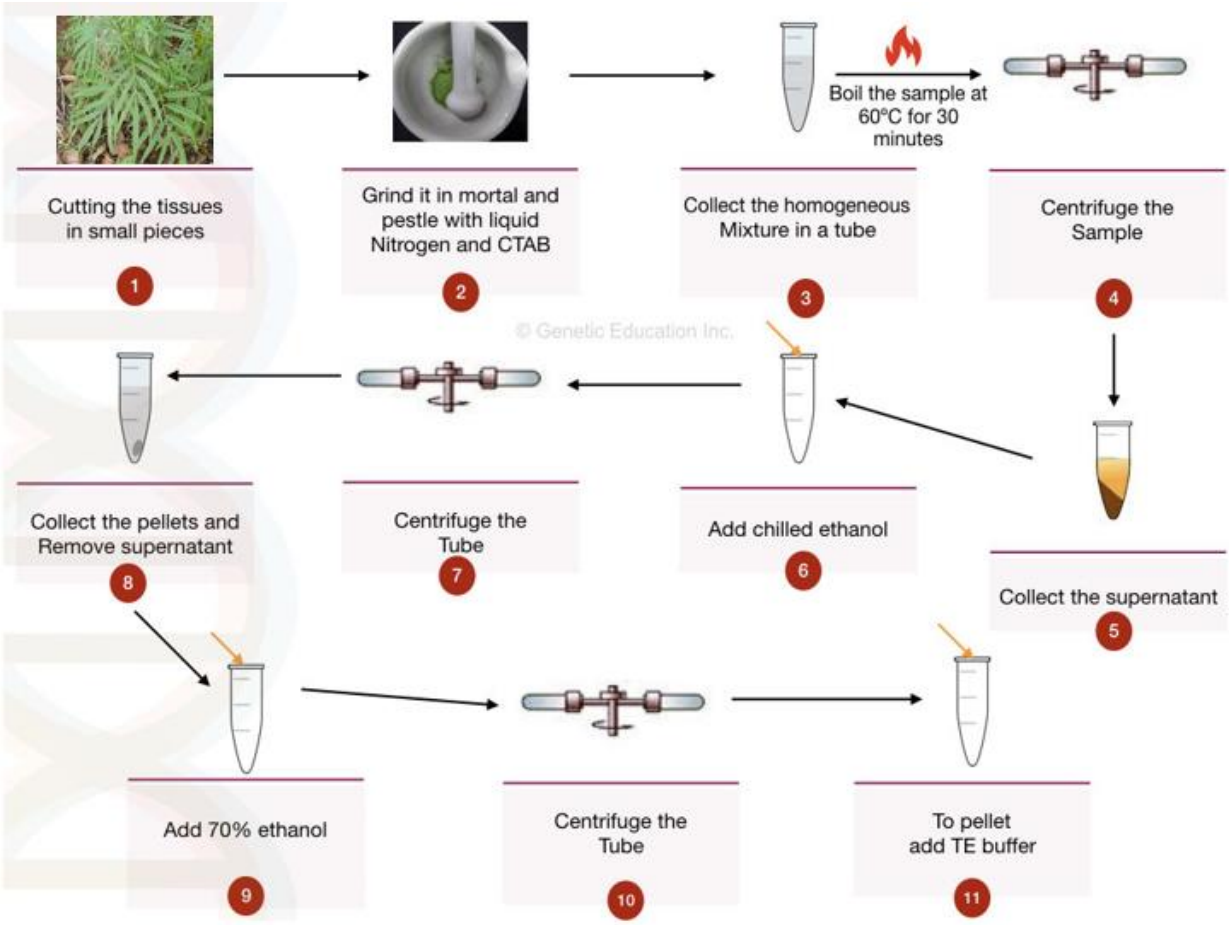


Fig4: DNA extraction by CTAB method

3.5 QUANTIFICATION OF DNA

Using a Nanodrop spectrophotometer, the concentration and purity of extracted genomic DNA were determined (ND-1000 Spectrophotometer). Light with a wavelength of 260 nm is absorbed by nucleic acid. The A ratio of 1.8 is widely regarded for DNA. An optical density of 1 at 260 nm corresponds to a DNA concentration of 50ng/l for double-stranded DNA The absorbance at 260 nm is used to calculate the concentration of nucleic acid for quantification. The purified DNA was used after being diluted in 50µl MQ water.

3.6 GEL ELECTROPHORESIS

The purity of purified DNA was determined by running 2µl of each DNA sample through a 0.8 % agarose gel. 2.4g of agarose was weighed and dissolved in 300ml of 0.5X TBE

Buffer by boiling until a clear solution was obtained. After the gel had cooled, Ethidium Bromide (3g/ml) was added to it. After placing the combs, the gel was poured into the casting tray. After solidification of gel, the combs were removed very carefully without breaking the wells formed. The gel tray was placed in the electrophoretic tank containing 0.5X TBE buffer. Aliquots (2µl) of DNA from each sample were loaded with 1x loading dye (5µl). Electrophoresis was performed at 5V/cm for 2 hours, and the results were photographed and analyzed using a gel documentation system (Syn gene GBox).

3.7 PCR AMPLIFICATION

Polymerase chain reactions (PCR) for amplification of DNA preparation were performed in a10µl volume. Making a solution containing Taq DNA polymerase, PCR buffer, dNTPs, gel loading dyes, and $MgCl_2$ and MQ water, fluorescence dye, Primer 1µl and add 1µl (25ng) of template DNA in 10µl of reaction volume. The DNA amplification was carried in a using the Bio-Rad C1000 Touch Thermal Cycler using the following thermal profile: Initial denaturation at 94ºC for 4 minutes; followed by 32 cycles of denaturation at 94ºC for 1 minute; followed by annealing at respective temperature for 1 minute; elongation at 72ºC for 1:30 minutes and final extension at 72ºC for 7 minutes.

Screening of PCR products

For the detection of PCR amplicons, they were resolved on 3% agarose SFR gel (Amresco Inc., Solon, OH, USA), (9 g of agarose was weighed and dissolved in 300 ml of 0.5X TBE Buffer by boiling, till the transparent solution was obtained) containing Ethidium bromide (2.5µg/ml). After pre-run of the gel for half an hour, the PCR products were loaded on the gel by mixing 3x of loading dye along with a 50 bp DNA ladder (MBI Ferment as) at 120V for3h in 0.5X TBE Buffer. The resolved amplicons were observed and photographed under gel documentation.

Steps	Temperature	Time duration	Denaturation
Step1	95	4min	

Step2	94ºC 50ºC- 55ºC 72ºC	1 minute 1 minute 1:30 minutes	 Annealing
Step3			Extension

Chapter-4

RESULTS AND DISCUSSION

DNA Quantification

Genomic DNA of plant samples was isolated using CTAB method (Khanju et al 1999). The quality was determined by running it on 0.8% agarose gel and while the quantity was determined with the help of gel documentation system and the quantity

Table 4.1 DNA quantification of *Tagetes* species plant leaf samples.

S. No	Genotype	Yield (µl)
1	Tm1	300(µl)
2	Tm2	250(µl)
3	Tm3	300(µl)
4	Tm4	150(µl)
5	Tm5	200(µl)
6	Tm6	150(µl)
7	Tm7	100(µl)
8	Tm8	200(µl)
9	Tm9	250(µl)
10	Tm10	250(µl)
11	Tm11	300(µl)
12	Tm12	350(µl)
13	Tm13	350(µl)
14	Tm14	350(µl)
15	A1-1-6	250(µl)

16	A1-4-5	200(μl)
17	A1-4-7	50(μl)
18	A3-2-7	50(μl)
19	A3-4-3	100(μl)
20	A3-6-3	100(μl)
21	A-4-9-8	50(μl)
22	A5-2-2	100(μl)
23	A5-4-5	100(μl)
24	A6-3-3	50(μl)
25	A8-7-5	50(μl)
26	A8-7-8	50(μl)
27	A8-9-16	150(μl)
28	A8-11-4	50(μl)
29	B1-1-8	50(μl)
30	B1-5-4	25(μl)
31	B1-5-6	100(μl)
32	B1-5-11	100(μl)
33	B1-6-14	150(μl)
34	B2-7-8	100(μl)
35	B2-3-11	100(μl)
36	B5-2-2	250(μl)
37	B6-1-4	350(μl)

Appreciable DNA yield was obtained in all species ranging up to as high as 350ng in the genotype tm7 to as low as 100ng. The low yield of was due to availability of less leaf tissue, due to poor growth of the plant. The samples which yield more than 50kb were further diluted. These were diluted to 25-50ng for making working stock. The quality and quantity of purified DNA was determined by using 0.8% agrose gel.

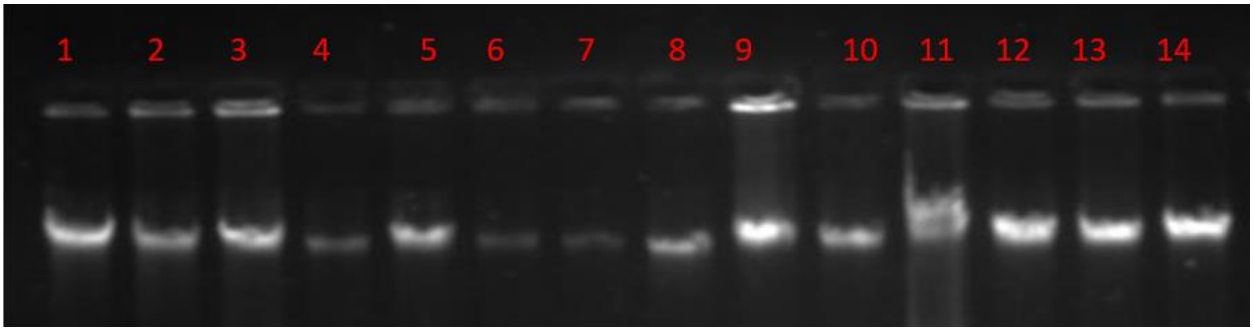


Figure 5: Quality and quantity of crude DNA (Tagetes minuta accession) as observed on 0.8% agrose gel.

Appreciable DNA yield was obtained in all species ranging up to as high as 350ng in the genotype to low as 50ng. The low yield of was due to availability of less leaf tissue, due to poor growth of the plant. The samples which yield more than 50kb were further diluted. These were diluted to 25-50ng for making working stock. The quality and quantity of purified DNA was determined by using 0.8% agrose gel.

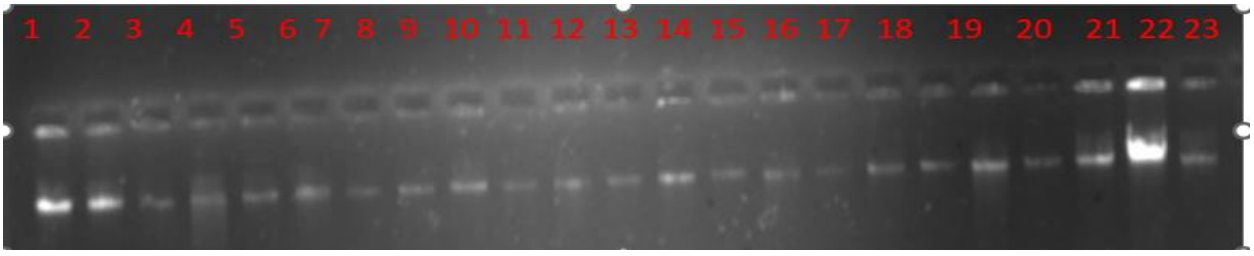


Figure 6: Quality and quantity of crude DNA (vanphool) as observed on 0.8% agrose gel

The hydro distillation of tagetes minuta (flower) produced a yellow liquid essential oil with a nice odour that is characteristic of the plant (table 3-1). The yield of essential oil from fresh flowers was shown in the table below.

Table 4.2 Physical properties of *T. minuta* essential oil

S.No	Essential oil	Colors	odor
1	A2-9-10	Light yellow	Intense aromatic
2	A3-4-3	Light yellow	Intense aromatic
3	A3-4-11	Light yellow	Intense aromatic
4	A3-6-3	Light yellow	Intense aromatic
5	A3-7-7	Light yellow	Intense aromatic
6	A8-7-5	Light yellow	Intense aromatic
7	A8-7-8	Light yellow	Intense aromatic
8	A8-7-13	Light yellow	Intense aromatic
9	A8-9-14	Light yellow	Intense aromatic

10	A8-10-1	Light yellow	Intense aromatic
11	B2-1-12	Light yellow	Intense aromatic
12	B2-6-1	Light yellow	Intense aromatic
13	B3-4-1	Light yellow	Intense aromatic
14	B3-5-7	Light yellow	Intense aromatic
15	B3-7-5	Light yellow	Intense aromatic
16	B8-2-7	Light yellow	Intense aromatic

Table 4.3 Percentage yield of essential oil extracted from Tagetes minuta essential oils

S.No	Essential oil	Flower weight(gm)	Oil yield (µl)
1	A2-9-10	68	400
2	A3-4-3	40	300
3	A3-4-11	72	400
4	A3-6-3	56	430

5	A3-7-7	66	505
6	A8-7-5	68	540
7	A8-7-8	44	300
8	A8-7-13	70	500
9	A8-9-14	42	300
10	A8-10-1	68	500
11	B2-1-12	68	400
12	B2-6-1	60	400
13	B3-4-1	54	300
14	B3-5-7	32	200
15	B3-7-5	32	180
16	B8-2-7	74	500

Analytical Analysis (GC/MS):

GC analyses of the essential oil samples was carried out using Agilent gas chromatography model 7890A equipped with flame ionization detector (FID) and a HP-5MS capillary column (30 m length x 0.25 mm internal diameter; 0.25 um film coating). Helium was used as a carrier gas at a flow rate of 1.0 ml/min. Temperature programming was done from 60 to 280 °C at 4 °C /min with initial and final hold time of 10 min. Injector and detector temperatures were 220 and 250 "C, respectively. Injection size was 0.1µl neat and split ratio was 1:100. The percentages of the individual constituents were calculated by electronic integration of the FID peak areas without response factor correction.

Table 3.5 Chromatographic Parameters for *Tagetes* Essential Oil Analysis by GC

Parameters	GC/MS *Tagetes* essential oil
Oven initial temperature (°C)	60
Ramp rate 1 (°C min^{-1})	3
Oven final temperature 1 (°C)	240◦C
Ramp rate 2 (°C min^{-1})	5
Oven final temperature 2 (°C)	290◦C
Final hold	240°C for 2 min
Injector (°C)	290◦C
Detector (°C)	290◦C

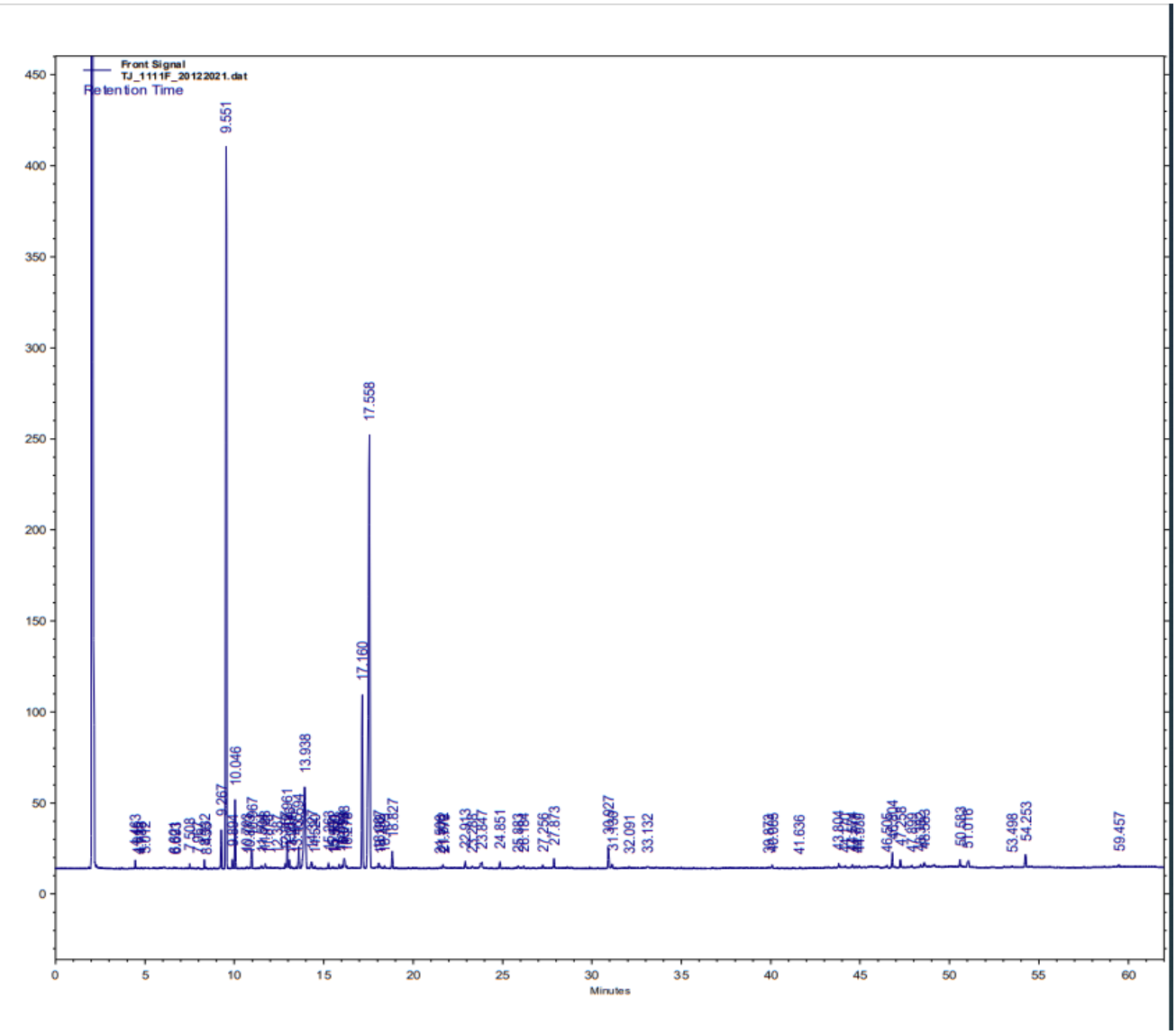


Fig7: GC spectra of the major compound present in essential oil.

GC Chromatogram of flower: The chemical compositions of the essential oil of *T. minuta* flower are shown in fig 7 and the GC-MS analysis of the essential oil of *T. minuta* flower from this study revealed 98 compounds present in the essential oil. The Major components were Limonene (9.551), (*Z*)-Tagetone (13.983), *(E)*-Tagetenone (17.160)

CONCLUSION

The findings of this study demonstrate that there is significant variability in *Tagetus minuta* genotypes and which can be harnessed in increasing the essential oil content. The essential oil of the minuta plant has a wide range of bioactive components and might be used to develop new antibacterial and antioxidant agents. The GC-MS analysis of the essential oil of *T. minuta* flower from this study revealed 98 compounds present in the essential oil. The major components were Limonene (9.551), (*Z*)-Tagetone (13.983), *(E)*-Tagetenone (17.160).

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APPENDIX-I

Sr. No.	Name
1.	Autoclave
2.	Centrifuge
3.	DNA Electrophoresis Unit
4.	Freezer (-20
5.	Microwave
6.	Ice Flake Machine
7.	Gel Documentation System
8.	PCR Machine
9.	pH Meter
10.	Spin win (Micro centrifuge)
11.	Vortex
12.	Water bath
13.	Weighing Machine
14.	Nano drop (1000)
15.	Water Purification System
16.	Sonicator
17.	Buchi Rotavapour R-300

2. Plastic and Glassware used in this study

1.	Beaker
2.	Tips (1ml, 200µl, 10 l)
3.	Conical Flask
4.	Measuring Cylinder
5.	Micro centrifuge Tube
6.	PCR Tubes

7.	Pipettes
8.	Mortar and Pestle
9.	Polypropylene Oakridge tube
10.	Graduated Bottle
11.	Round bottom flask
12.	Muslin cloth
13.	Crucible
14.	Conical bottom centrifuge tube
15.	Mini centrifuge tubes

Reagents for Agrose gel electrophoresis

1. **6x loading dye**

Reagents	Final conc.	Volume (for150ml)
Bromophenol blue	0.25	10mg
Xylene cyanol	0.25	10mg
Glycerol	30	1.2ml

2. **0.8% Agrose gel**

Reagent	Stock	Volume for (300 ml)
TBE	10x	15ml
		2.4ml
		270ml

3. **3% Agrose gel**

Reagent	Stock	Volume for (300 ml)
TBE	10x	15ml
Agrose		2.4ml
Double distilled water		270ml