CAPITAL UNIVERSITY OF SCIENCE AND TECHNOLOGY, ISLAMABAD

Metabolic Engineering of *Artemisia carvifolia* Buch by *Agrobacterium* Mediated Genetic Transformation with *rol A* Gene for Enhancement of Flavonoids

by

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Dedicated to my father for his constant, unconditional love and support

&

To the memory of my mother, with love and eternal appreciation

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CERTIFICATE OF APPROVAL

This is to certify that the research work presented in the dissertation, entitled "**Metabolic Engineering of *Artemisia carvifolia* Buch by *Agrobacterium* Mediated Genetic Transformation with *rol* A Gene for Enhancement of Flavonoids**" was conducted under the supervision of **Dr. Erum Dilshad**. No part of this dissertation has been submitted anywhere else for any other degree. This dissertation is submitted to the **Department of Bioinformatics & Biosciences, Capital University of Science and Technology** in partial fulfillment of the requirements for the degree of Doctor in Philosophy in the field of **Biosciences**. The open defence of the dissertation was conducted on **July 12, 2024.**

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List of Publications

It is certified that following publication(s) have been made out of the research work that has been carried out for this dissertation:-

1. Khan, A. N., & Dilshad, E. "Enhanced Antioxidant and Anticancer Potential of Artemisia carvifolia Buch Transformed with rol A Gene". *Metabolites*, vol.13, no.3, pp.351, 2023.

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comfort for my studies. Their constant prayers, affection, and endorsement have empowered me to pursue and achieve my goals.

(Amna Naheed Khan)

Abstract

The plant's bioactive compounds known as secondary metabolites enjoy a range of biological activities. Flavonoids, owing to their ROS scavenging capacity, are well-known antioxidants. A good source of flavonoids is the *Artemisia* genus, but here in the plants of this genus, they are found in very low quantities. Thus, the present study was designed in which the medicinal plant *Artemisia carvifolia* Buch was selected to enhance the flavonoid content through genetic engineering with *rol A* gene.

For this purpose, first of all, the identification of *Artemisia carvifolia* Buch was conducted through DNA barcoding, involving the sequencing of the *psbA-trnH* region within the chloroplast DNA. Following DNA extraction, the amplification process utilized primers designed for the *psbA* and *trnH* genes. Successful PCR amplification resulted in a consistent 500 bp product across all DNA samples. The obtained sequence exhibited a 99.7% similarity to the *psbA-trnH* sequence of *A. carvifolia*, with only a two-base pair difference.

The flavonoids were detected in wild type *A. carvifolia* plants by HPLC and included vanillic acid (0.47 μg/mg DW), rutin (1.39 μg/mg DW), gallic acid (0.62 μg/mg DW), syringic acid (1.05 μg/mg DW), coumaric acid (1.65 μg/mg DW), caffeic acid (0.47 μg/mg DW), ferulic acid (1.39 μg/mg DW), and cinnamic acid (1.67 μg/mg DW). The genetic transformation of *A. carvifolia* was carried out with *Agrobacterium tumefaciens* strain GV3101 carrying *rol A* gene. The transformation of *rol A* gene was confirmed by PCR and the gene copy number was confirmed by Southern blot analysis. The successful and stable integration of the *rol A* gene was observed in all five transgenic lines of *A. carvifolia*. Transgenic lines T1, T2, and T4 displayed the presence of a single copy of the *rol A* gene, while T3 and T5 exhibited the integration of two copies of the gene.

In transformed plants, all detected flavonoids were increased up to several folds. The HPLC analysis revealed the presence of catechin (3.19 μg/mg DW) and geutisic acid (2.22 μg/mg DW) in transformed plants, which were absent in wild-type plants. By upregulating the expression levels of *PAL* and *CHS*, the *rol A* gene actively

contributed to the induction of flavonoid biosynthesis. This was confirmed by real-time qPCR which showed the higher expression levels of the genes for flavonoid biosynthesis enzymes phenylalanine ammonia-lyase *(PAL)* and chalcone synthase *(CHS)* in plants transformed with *rol A* genes, as the expression level was increased up to 9-20 folds and 2-6 folds, respectively. The *rol A* transgenic lines T3 and T5 carrying two copies of *rol A* gene, particularly showed higher expression of both *PAL* and *CHS* gene, with the highest expression in T3 line.

The pharmacological potential of wild type and *rol A* transformed plants was evaluated by determining their antioxidant and anticancer activity. Through assays, it was found that plants that were genetically modified with the *rol A* gene showed an increase in their antioxidant capacity up to several folds. The methanolic extracts of wild-type and transgenic plants showed antioxidant potential with transgenic lines demonstrating an average increase of 1.4-fold in the total phenolic content and 1-2-fold in the total flavonoid content as compared to wild-type plants. Similarly, total antioxidant capacity and total reducing power were increased up to an average of 1-2-fold and 1.5-2-fold respectively.

The evaluation of free radical scavenging activity in both the wild-type *A. carvifolia* and plants modified with the *rol A* gene was conducted through the DPPH scavenging assay. The transformed plant extracts exhibited the highest scavenging effect, as indicated by their lower IC_{50} values as compared to wild-type plant extracts. Notably, the radical-scavenging potential was prominently expressed in the *rol A* transgenic lines T3 and T5, with an IC_{50} value of 206.9 μg/mL and 225.65 μg/mL respectively. In contrast, the wild-type plants displayed a comparatively lower radical-scavenging efficiency, with an IC_{50} value of 627 μg/mL.

Furthermore, the methanolic extracts obtained from the plants under investigation were subjected to evaluation for their anticancer potential, targeting three distinct cancer cell lines comprising HeLa, HePG2, and MCF7. Mortality of the cancer cell lines was found to be increased after treatment with the methanolic extracts of plants. The results revealed that, when compared to the wild-type plant, all transgenic lines exhibited greater effectiveness against all cell lines. The mortality rate of HeLa, MCF7 and HePG2 cells after treatment with untransformed *A.*

carvifolia extract was 30%, 32%, and 36% respectively. After being treated with *rol A* transgenic extracts, the mortality rate in HeLa cells increased to 75%. Similarly, MCF7 and HePG2 mortality rates increased to 70% and 74%, respectively, upon exposure to transgenic cell lines.

Current findings indicate that the *rol A* gene significantly enhances the flavonoid content of *A. carvifolia*, thereby amplifying the plant's effectiveness as both an antioxidant and an anticancer agent. In conclusion, the observed results suggest that, *rol* genes play a pivotal role in the improvement of secondary metabolites, especially flavonoids, in *Artemisia carvifolia* Buch. Moreover, they have a positive impact on enhancing the plant's pharmacological potential when compared to its wild-type counterpart.

Keywords: *Artemisia carvifolia* Buch, *Agrobacterium tumefaciens*, *rol A* gene, flavonoids, phenylalanine ammonia-lyase, chalcone synthase, antioxidant assays, antiproliferative activity.

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Abbreviations

BAP	Benzyl amino purine
CaMV	Cauliflower mosaic virus
CHS	Chalcone synthase
CTAB	Cetyltrimethylammonium-bromide
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPPH	2,2-diphenyl-1-picryl-hydrazyl
EDTA	Ethylenediaminetetraacetic acid
EGCG	Epigallocatechin gallate
EMT	Epithelial-mesenchymal transition
HPLC	High-performance liquid chromatography
IAA	Indole acetic acid
IC_{50}	Inhibitory concentration at fifty percent
LB	Luria broth
LC/MS	Liquid chromatography/Mass spectrometry
matK	Maturase K
mRNA	Messenger ribonucleic acid
MS Medium	Murashige and Skoog medium
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NAA	Naphthalene acetic acid
nptII	Neomycin phosphotransferase gene
OD	Optical density
PAL	Phenylalanine ammonialyase
PCR	Polymerase chain reaction

PMs	Primary metabolites
psi	Pounds per square inch
rbcL	Ribulose bisphosphate carboxylase
Ri	Root inducing
RNA	Ribonucleic acid
rol	Root oncogenic loci
ROS	Reactive oxygen species
SDS	Sodium dodecyl sulfate
SMs	Secondary metabolites
Ti	Tumor inducing
TLR4	Toll-like receptor 4
TRAF6	TNF receptor-associated factor 6
trnH	Transfer RNA for histadine
UV	Ultraviolet
vir	Virulence
WHO	World Health Organization

Chapter 1

Introduction

1.1 Background of the Study

Medicinal plants constitute the oldest form of medical treatment, having been employed for numerous years in traditional medicine across various nations globally. The experiential understanding of their positive effects has been transmitted through generations within human communities [1]. Humans have historically depended on medicinal plants to combat illnesses and fulfil diverse health requirements. The World Health Organization (WHO) estimates that 80% of people on the planet rely on medicinal plants for their basic medical needs [2]. Natural substances play a crucial role as a reservoir of medicinal compounds, and presently, numerous contemporary drugs originating from conservational herbal medicine are employed in modern pharmacotherapy [3].

1.1.1 Traditional Medicines and Advancements

The official definition of traditional medicine can be characterized as "the collective information, skills, and practices rooted in the theories, opinions, and experiences native to diverse cultures, whether understandable or not. It is employed in maintaining health and in the prevention, identification, improvement, or cure of physical and mental illnesses" [4].

The extraction process stands as a pivotal stage in examining bioactive molecules derived from plant sources. Alongside more conventional methods, contemporary extraction procedures like ultrasound-assisted and supercritical fluid extraction methods are currently being employed [5]. Furthermore, the advancement of sophisticated tools for the qualitative and quantitative evaluation of phytochemicals, such as LC/MS and HPLC, has substantially enhanced phytochemical exploration [6].

Conversely, the biological characteristics of numerous plant species, conventionally used in conjunction with their bioactive constituents, have been elucidated thus far. Both the traditional bioassay-guided natural drug discovery method and contemporary approaches, such as high-throughput screening, and the innovative reverse pharmacognosy approach, have facilitated the identification of numerous bioactive phytochemicals. Nonetheless, medicinal plants continue to hold promise for the future, as the phytochemical composition and potential health benefits of numerous species remain unstudied or require more in-depth investigation [7].

1.1.2 Medicinal Plants: History, Distribution and Challenges

With the developments in the science of medicinal plants, it has become one of the oldest approaches for curing different ailments, especially in China, India, Egypt, and Greece. In ancient Persia, plants were frequently employed for sterilizing, medicinal, and aromatic purposes. Notably, the need for natural sources from the environment has escalated by 8% to 15% annually in Asia, Europe, and North America in past decades. More than 50,000 species (comprising over a tenth of total plant species) are used to make cosmetic and pharmaceutical goods. Medicinal plants do, however, vary in their geographic distribution, with wild populations providing the majority of medicinal herbs [8].

The term “medicinal plant” encompasses a diverse array of plants possessing therapeutic properties. These plants serve as abundant reservoirs of compounds

applicable to drug synthesis. Various plant parts may be used for medicinal purposes. Active compounds within these plant parts exert beneficial effects directly or indirectly and are employed as therapeutic agents. These active compounds, also known as substances, are produced and stored within the plant body, influencing physiological processes in living organisms [9].

Compounds derived from plants have the ability to considerably enhance the treatment of challenging illnesses, including cancer. Additionally, plant components are known for their capacity to inhibit the onset of specific diseases. The lethal and undesirable effects associated with conventional and allopathic medications have played a crucial role in the growing demand for herbal drugs and the rise in the number of herbal drug manufacturers. Simultaneously, there has been a decrease in the utilization of chemical drugs [10].

1.1.3 Genus *Artemisia*

The *Artemisia* genus stands as the biggest and extensively dispersed within the family Asteraceae (Compositae). Comprising over 500 diverse species, it predominantly originated in the temperate regions of Asia, Europe, and North America. *Artemisia*, a versatile genus, is recognized by various names such as 'Wormwood,' 'Sagebrush,' 'Mugwort,' or 'Tarragon'.

This genus is affiliated with the family Asteraceae, also known as the 'Compositae family,' 'thistle family,' 'sunflower family,' or 'daisy family.' The term '*Artemisia*' originates from the ancient Greek words 'Artemis' (The Goddess referring to the Greek Queen Artemisia). The term 'Wormwood' is linked to its historical use as a remedy for intestinal worms [11, 12].

Artemisia species, which are mostly perennial, biannual, or annual herbaceous plants or shrubs, exhibit different colors. They are categorized by a pungent smell and bitter taste accredited to the existence of terpenoids and sesquiterpene lactones.

Certain varieties are grown as cultivated crops, while others find application in the crafting of tea, tonics, alcoholic beverages, and medicinal products [13].

1.1.4 *Artemisia* species as Bioactive Wonders

In addition to non-volatile bioactive compounds, *Artemisia* species serve as a noteworthy reservoir of essential oils containing thujone, thujyl alcohol, cadinene, phellandrene, pinene, among others. These oils have been documented for their diverse biological activities, encompassing antibacterial [14], anti-malarial [15], anti-viral [16], anti-inflammatory [17], anti-oxidant [18], anti-helminthic [16], anti-diabetic [19, 20], hepatoprotective [21], anti-spasmodic [17], anti-cancer [22], anti-tumor [23], antifertility [20], anti-rheumatic [24], anti-pyretic [25], anti-hypertensive [26], anti-arthritis [27], and various other effects.

Artemisia species serve as abundant reservoirs of diverse biologically active compounds responsible for a multitude of pharmacological activities. Variations in the qualitative and quantitative makeup of bioactive compounds could be associated with factors such as environmental conditions, species diversity, geographic and climatic influences, genetic factors, plant age, soil characteristics, stage of vegetation, anatomical fragment of the plant, the season of harvesting, and the method employed for harvesting [28].

Artemisia species have been found to contain a variety of constituents, including both non-volatile and volatile compounds, flavonoids, phenolic acids, coumarin derivatives, monoterpene, and sesquiterpene. The *Artemisia* genus possesses a diverse array of antioxidant phenolic compounds, potentially contributing to its biotic effects, particularly in antioxidant, anti-malarial, and anticancer effects. Notably, it has been suggested that the antimalarial and anticancer effects of *A. annua* may stem not only from artemisinin but could also from synergistic interactions with flavonoids. Key antioxidant phenolic compounds found in *Artemisia* species are gallic acid, caffeic acid, epicatechin, ferulic acid, quercetol, kaempferol, sinapic acid, rutin, quercetin, chlorogenic acid, catechin, vanillic acid, luteolin, gentisic acid isoquercitrin, and apigenin [29]. Important phenolics and terpenoids of *Artemisia* species are given in Figure 1.1 [30].

A. annua has garnered notable attention for its efficacy against chloroquine and mefloquine resistant strains of *Plasmodium*. It has been reported that it

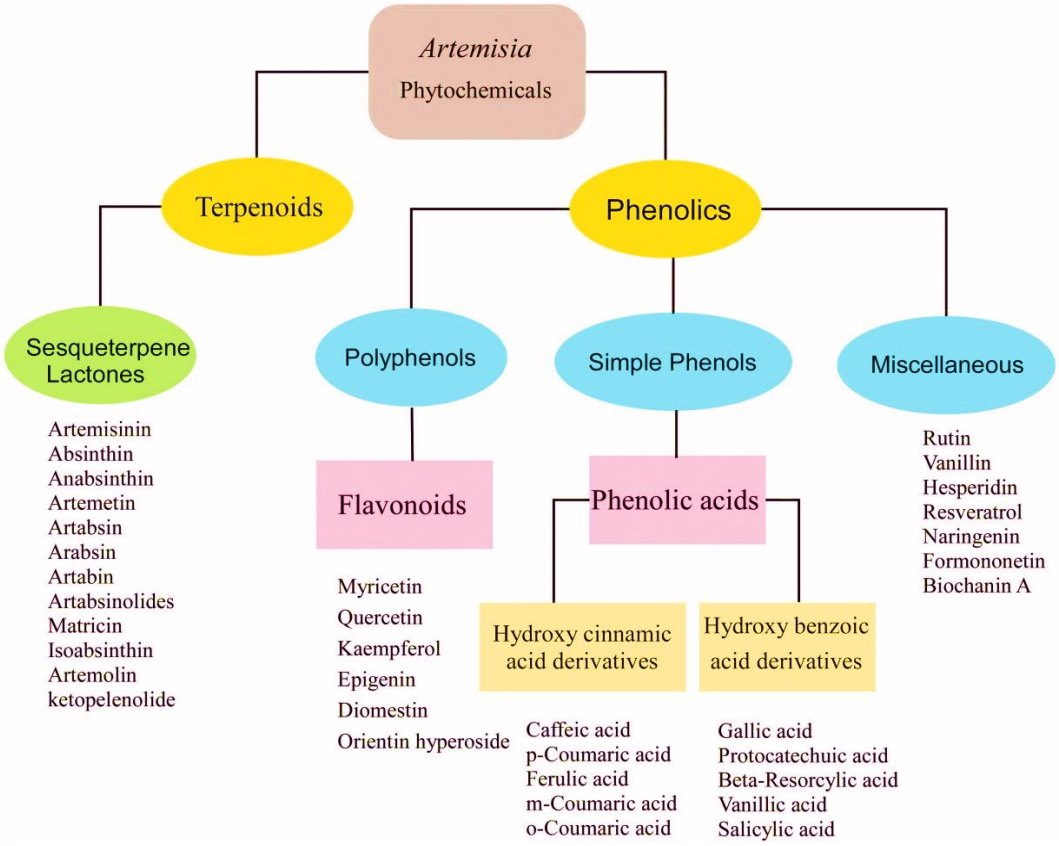


FIGURE 1.1: Phytochemicals of *Artemisia* genus [30].

eliminates the responsible parasites more rapidly than chloroquine, leading to a quicker symptomatic response [31]. The discovery of Artemisinin, a sesquiterpene lactone endoperoxide and the most crucial agile substance in *Artemisia annua* L. (*A. annua* L.), was credited to Professor Youyou Tu during the collaborative and classified initiative "Project 523". This project was initiated by China's former President Mao Zedong on May 23, 1967, with the objective of finding a novel medication to address intermittent fever, specifically malaria. Artemisinin, a semi-synthetic compound, is found in varying concentrations in all *Artemisia* plants [32].

Historically, *A. absinthium* has been employed for various purposes such as combating parasites, acting as an antiseptic, alleviating spasms, reducing fever, aiding digestion, stimulating the heart, restoring cognitive function, addressing liver inflammation, and enhancing memory. This plant is used by traditional Chinese Medicine to address conditions like acute bacillary dysentery, certain cancers, and neurodegenerative diseases [33].

Extracts from the entire herb of *A. asiatica* have traditionally been employed in oriental medicine to address issues related to inflammation, cancer, infections, and ulcerogenic diseases. *Artemisia vulgaris* (L.) has a history of use for its analgesic, anti-inflammatory, antispasmodic properties, and effectiveness in addressing liver diseases. Additionally, *Artemisia afra* (Jacq.) has been traditionally employed to treat a range of conditions including coughs, colds, colic, malaria, diabetes, headaches, dyspepsia bladder and kidney disorders, and is also utilized as a purgative [34].

Various organic fractions derived from *Artemisia nilagirica* leaf demonstrated antibacterial activity as opposed to both clinical and phytopathogenic bacteria. Moreover, the essential oil extracted from *A. scoparia*, which is rich in monoterpenoids and tested at concentrations ranging from 25 to 200 μg/mL, exhibited potent antioxidant properties and effectively scavenged radicals, particularly against hydroxyl ion and hydrogen peroxide. Chrysosplenetin and artemisetin, extracted from *A. sieversiana*, demonstrated remarkable antitumor efficacy specifically against melanoma B16. Additionally, the essential oil obtained from *A. sieversiana* displayed significant anti-inflammatory properties, attributed to the presence of azulenes in the essential oil [35]. The plant *Artemisia carvifolia* is gathered from its natural habitat for local applications in both medicinal and culinary contexts. Its medicinal uses are parallel to those of *Artemisia annua*, underscoring its significant role as an anti-malarial remedy [36]. Recognized as sweet wormwood in western regions, Qing Ho is a conventional Chinese herbal remedy. As a fragrant antibacterial plant, recent studies indicate its efficacy in eradicating malarial parasites, reducing fevers, and controlling bleeding. In tropical regions, it is frequently employed as a cost-effective and efficient anti-malarial solution. This plant comprises the essential oil artemisinin, a renowned component in Traditional Chinese drugs recognized for its anti-inflammatory, antipyretic, and hemostatic attributes [37].

1.1.5 *Artemisia's* Secondary Metabolites: Therapeutic Significance

In the natural environment, secondary metabolites and essential oils play a crucial role in safeguarding plants by serving as antibacterials, antifungals, antivirals,

and insecticides. They also contribute to plant defense against herbivores by diminishing their appetite for these plants. Additionally, they may attract specific insects, aiding in the dispersal of pollens and seeds, while simultaneously repelling undesirable insects. From a chemical standpoint, essential oils are intricate natural blends containing approximately 20 to 60 components, each present in varying concentrations. Typically, 2–3 major components make up a substantial portion (20–70%) of the oil, influencing its biological properties. These major components fall into two distinct groups with different biosynthetic origins: the primary group comprises terpenes, while the other consists of aliphatic and aromatic constituents. All these are characterized by their low molecular weight [38].

The intense and fragrant aroma exhibited by certain species within the *Artemisia* genus primarily arises from elevated levels of volatile terpenes (key constituents of their essential oils), particularly concentrated in foliage. The chemical makeup of essential oils derived from the *Artemisia* genus has undergone thorough investigation, with numerous studies conducted on various species across the globe. Numerous studies have indicated considerable intraspecific differences in the terpene contents of essential oils among *Artemisia* species [38].

In certain instances, the fluctuation in volatile components within these plants may be attributed to factors such as plant ontogeny or growth under varying altitudes. The quality and quantity of essential oils derived from *Artemisia* species are impacted by various factors, including the season of harvest, soil pH and fertilization, drying conditions and stage, geographic location, chemotype or subspecies, selection of plant part or genotype, as well as the method employed for extraction. Sesquiterpene lactones and flavonoids, identified among the secondary metabolites of the *Artemisia* genus, hold significant therapeutic importance. The limited production and low quantities of these crucial metabolites within plant cells have prompted a sustained focus on enhancing their in vitro production over the years. Enhancements in the levels of compounds possessing antioxidant properties, especially flavonoids, can be achieved through various means. This includes genetic transformation with specific genes directly linked to flavonoid biosynthesis, the application of elicitors, and or the introduction of wild strains of agrobacteria

[39, 40]. The latter involves the transfer of bacterial *rol* genes, exerting a nonspecific impact on the metabolism of plant cells.

DA-9601, a standardized extract derived from *A. asiatica*, has demonstrated chemopreventive effects in the context of mouse colon carcinogenesis initiated by azoxymethane and promoted by dextran sulfate sodium. The ethanolic extract obtained from *A. asiatica* has been documented to hinder the activation of inflammation in mouse microglial cells. This inhibition is evidenced by a reduction in nitric oxide production and a lowering of the levels of inducible nitric oxide synthase and pro-inflammatory cytokines. Additionally, the extract demonstrated protective effects on nerve growth factor-differentiated PC12 cells, shielding them from microglial cytotoxicity [41].

The sesquiterpene lactones artemisinin and arglabin, isolated from various *Artemisia* species, have gained approval as drugs for the cure of human malaria and cancer, respectively. Additionally, the terpenoid compound paclitaxel, known for its anticancer properties, is utilized against various types of cancer. Currently, clinical trials are underway to evaluate the potential of the flavone quercetin, a type of flavonoid, in the context of both prostate cancer treatment and primary prevention [42].

1.1.6 Flavonoids: A Natural Defense against Cancer

Flavonoids, a group of hydroxylated phenolic compounds found in plants, hold a distinctive position among secondary metabolites. They are categorized into various classes, with flavones, flavonols, flavanones, catechins, isoflavones, and anthocyanidins being the most prevalent. Much like terpenoids, flavonoids exhibit a diverse array of biological activities. These compounds have been shown to exert protective effects against numerous infectious and degenerative diseases, including cancer. Additionally, they demonstrate significant biological activities [43].

Flavonoids, as phytochemicals, contribute to the diverse colors observed in seeds, fruits, flowers, leaves, and stems. These compounds, recognized as a prominent class of natural aromatic chemicals, are reported to be the most prevalent phenolic

compounds in plants. Beyond their role in coloration, flavonoids serve as protective agents for plants, shielding them from various abiotic and biotic factors. This includes protection against insects, microbial contamination, extreme cold temperatures, and exposure to ultraviolet (UV) radiation to prevent serious damage [44]. Furthermore, flavonoids play a role in attracting pollinating insects, serving as chemical messengers within plants and contributing to the metabolism of auxin [45]. They are most prevalent in fruits, vegetables, teas, and cocoa in the human diet. They can chelate redox-active metals, reduce lipid peroxidation, and decrease other mechanisms that involve reactive oxygen species, which is what gives them their cardioprotective benefits [46].

It is widely recognized that flavonoids obtained from natural sources possess various beneficial properties, including anti-inflammatory, antioxidant, antiviral, and anti-tumor activities. Both in vivo and in vitro studies have demonstrated that flavonoids isolated from plants possess a diverse range of anticancer properties. Flavonoids, present in foods and beverages commonly consumed by humans, are associated with minimal risky side effects compared to manufactured anti-cancer drugs. Moreover, regular consumption of flavonoids, either through supplements or foods rich in these compounds, has been suggested to potentially reduce the risk of developing cancer [47].

Nevertheless, variations or fluctuations in the levels of flavonoids within plants have been observed across different stages of the plant's growth. For example, research has indicated that a specific cultivar of *A. annua* exhibited higher levels of compounds like casticin and chrysoplenetin in its leaves and flowers during the blossoming stage. Similarly, recent reports have highlighted differences among cultivars in terms of antioxidant capability, suggesting that antioxidant compounds, particularly flavonoids, may vary in their concentrations [48].

1.1.7 Metabolite Engineering with *Agrobacterium*: A Powerful Approach

A potent method for uplifting the synthesis of crucial secondary metabolites involves genetic transformation, particularly through the use of *Agrobacterium tumefaciens*

and *Agrobacterium rhizogenes*. Metabolite engineering involves various strategies, such as overexpressing and/or competing with the target pathway, alleviating rate-limiting steps, interrupting the catabolism pathway of the desired product, and obstructing alternative pathways [49]. *Agrobacterium tumefaciens*, in particular, serves as a robust tool for metabolic engineering in medicinal plants by enabling the overexpression and/or downregulation of specific genes and the utilization of transcriptional regulators. Several studies have shown how powerful *rol* genes are in inducing secondary metabolism in a wide range of plant groups. The genetic transformation of *A. carvifolia* using *A. tumefaciens* has been reported for *rol B* and *rol C* genes [50–52]. The plant oncogens *rol A*, *rol B, rol C* and *rol D* are found on the plasmids of *Agrobacterium rhizogenes*. These genes are introduced into the plant genome after agrobacterial infection and result in the development of tumors and hairy root disease [53]. The Rol A protein is a growth-stimulating DNA binding protein, encoded by *rol A* gene, whereas the Rol B protein encoded by *rol B* gene controls the auxin signaling pathway by functioning as a tyrosine phosphatase. The *rol C* genes' cytokinin glucosidase activity can initiate the production of a large variety of secondary chemicals in plants and cell cultures that have undergone transformation [54, 55].

1.2 Gap Analysis

1.2.1 Absence of Prior Studies on Genetic Transformation of *Artemisia carvifolia* Buch and Flavonoid Enhancement through *rol A* Gene

- **Gap:** The genetic transformation of *Artemisia carvifolia* with *rol A* gene and the function of the *rol A* gene in augmenting flavonoid synthesis in this plant has not yet been investigated.
- **Implication:** This gap underscores the need for novel research efforts focused specifically on the genetic transformation of *Artemisia carvifolia* plants with the *rol A* gene and to check the potential of the *rol A* gene to enhance

flavonoid content in *Artemisia* species. These efforts will also advance our understanding of metabolic engineering in this species.

1.2.2 Restricted Knowledge of the Role of *rol A* Gene in Cancer Prevention and Antioxidant Enhancement

- **Gap:** Cancer is one of the main causes of death worldwide. Research on the anticancer and antioxidant capabilities of *Artemisia carvifolia* plants through the transformation of *rol B* and *rol C* genes has been explored but the relevance of the *rol A* gene has not been investigated in this context.
- **Implication:** Our knowledge of how the *rol A* gene, in particular, enhances the antioxidant and anticancer properties of *Artemisia carvifolia* plants is lacking. This knowledge gap has to be filled with more research.

1.2.3 Uncertainty regarding Ideal Circumstances for *rol A* Gene Transformation

- **Gap:** The ideal circumstances for successfully transforming *Artemisia carvifolia* plants with the *rol A* gene of *Agrobacterium rhizogens* may not be well understood.
- **Implication:** In order to guarantee effective *rol A* gene integration and to subsequently increase flavonoid content, it is imperative to establish ideal transformation circumstances.

1.3 Problem Statement

One of the best sources of flavonoids is the genus *Artemisia*, which has more than 500 species. The level of these flavonoids in *Artemisia* plants is relatively low, and fluctuations in plants have also been noted during various stages of the plant's growth. There is a need to opt for strategies for the enhancement of these important metabolites.

1.4 Research Questions

This study will respond the given questions:

Research Question 1

What effects does the genetic transformation of *rol A* gene of *A. rhizogens* have on the *A. carvifolia* plants in terms of flavonoid content?

Research Question 2

What particular effect does the *rol A* gene have on the biosynthetic pathways leading to the manufacture of flavonoids in *A. carvifolia* plants?

Research Question 3

Which particular enzymes and pathways in *A. carvifolia* plants are affected by the *rol A* gene, which results in increased flavonoid production?

Research Question 4

Does this transformation affect the plants' ability to prevent cancer and function as antioxidants?

Research Question 5

What effects has the *rol A* gene introduction had on the general well-being, and morphology of *A. carvifolia* plants?

1.5 Research Objectives

The current research work was aimed to enhance the flavonoid content in the medicinal plant *Artemisia carvifolia* Buch through genetic engineering with *rol A* gene. The antioxidant and anticancer potential of transgenic plants were also assessed. The objectives were as follows:

Research Objective 1

To genetically transform and regenerate *Artemisia carvifolia* plants with *rol A* gene.

Research Objective 2

To determine the effect of *rol A* gene on the flavonoids biosynthetic pathway genes *PAL* and *CHS*.

Research Objective 3

To determine the effect of *rol A* gene on the flavonoid content as well as antioxidant and anticancer activity of plant.

Chapter 2

Literature Review

This chapter is concerned with the general background of the genus *Artemisia* and the phytochemistry of its species *Artemisia carvifolia*, role of flavonoids as antioxidant and anticancer agents, role of *rol* genes specifically *rol A* gene in the enhancement of the plant's secondary metabolism.

2.1 Medicinal Plants

A plant is classified as medicinal if it contains compounds suitable for therapeutic use or serves as a basis for the production of pharmaceuticals. Despite the advancement of various mainstream therapies, there is a growing trend towards herbal medicine driven by concerns about the escalating toxicities linked to conventional therapies [56]. Medicinal plants have long been employed in local communities worldwide as a source of relief. Even today, their significance remains paramount, serving as the primary or major healthcare system for approximately 85% of the world's population.

Besides this, these plants are also a source of drug development because 80% of all synthetic pharmaceuticals are made from them [57]. Various parts of medicinal plants such as seeds, stems, leaves, flowers, fruits, and roots are abundant sources of bioactive compounds [58].

2.1.1 History of the Medicinal Plants

Determining the precise historical moment of utilizing herbs for medicinal purposes is challenging. Evidence suggests that the cultivation of herbs for medicinal use dates back approximately 60,000 years. Written records about therapeutic plants can be traced back almost 5000 years ago in regions such as India, China, and Egypt, and at least 2500 years ago in Greece and Central Asia [59]. In olden times, individuals instinctively turned to nature to find remedies for their ailments, initially relying on both animals and plants. Given the limited understanding of the origin of diseases, the identification of useful plants for treatment, and the methods of their application during that era, reliance on empirical knowledge prevailed. As knowledge expanded, the reasons behind using specific medicinal plants for particular diseases became apparent. Subsequently, the utilization of medicinal plants transitioned from an empirical approach to one based on discovered facts. The initial documented evidence of preparing drugs from medicinal plants is found in writings on a Sumerian clay slab from Nagpur, dating back nearly 5000 years ago. Evidence from inscriptions indicates that Egyptians and Chinese, who utilized plants for medicinal purposes over 27 centuries BC, were among the earliest human societies to do so [59]. Ancient Greeks were also acquainted with the medicinal properties of certain plants, and both Hippocrates, the founder of Greek medicine, and Aristotle, a student of Hippocrates, employed medicinal plants in treating diseases. Following this era, Theophrastus, a Greek scientist, founded the School of Medicinal Plants. Subsequently, Pedanius Dioscorides, a surgeon and physician from 75-45 BC, living in the first century A.D., authored an encyclopedia titled "De Materia Medica". This comprehensive work comprised scientific studies on 600 therapeutic medicinal plants [60].

2.1.2 Medicinal Herbs and Traditional Medicine

Traditional herbal medicine has been employed for many years equally in developing and developed nations due to its natural origins and relatively fewer associated complications. The early history of medicine is closely intertwined with the history

of therapeutic plants, with some of the primary printed medical texts focusing on plants, as evidenced by the Ebers Papyrus from 1500 BC, which includes the names of numerous plants. Various forms of traditional remedies are extensively utilized in Asia, Africa, and Latin America to address fundamental health needs. This technique, sometimes known as supplementary or alternative medicine, is becoming more and more common in developed nations [61].

In the present day, as reported by the WHO, over 80% of the global inhabitants predominantly trust on traditional medications, primarily derived from plants, as a primary source of healthcare. This percentage encompasses not only a substantial portion of China, India, and all developing nations but also includes numerous advanced countries. While diseases are currently more frequently treated with synthetic drugs specifically developed in laboratories, and their proven efficacy in treating ailments has accounted to their widespread use, it is acknowledged that the use of certain medications can result in adverse effects on the body. As a result, there is a growing acknowledgement of the significance of medicinal plants and their derivatives, and public trust in their use continues to strengthen. At present, the basis for numerous historical medications, including Aspirin, Morphine, Digoxin (extracted from Foxglove), Quinine (derived from Cinchona bark), and Pilocarpine (from Maranham Jaborandi), rests on the pharmaceutical, clinical, and chemical research on traditional medicines primarily sourced from plants. Over half of all medications on the market today are thought to have their roots in medicinal plants. Phytotherapy is experiencing widespread adoption worldwide, marking continuous and substantial growth. Consequently, there is a global shift from synthetic compounds towards herbal drugs, characterized as a return to nature for disease prevention and pain management. Nature continues to serve as an abundant source of medicinal herbs [62].

2.1.3 Cancer Care: Plant-based Solutions

Cancer differs from infectious and environmental diseases brought on by antigens not found in our body system, as well as diseases linked to microbes and parasites.

Human malignancies can arise from a variety of causes, including genetic or epigenetic factors that cause normal cells to mutate [63]. The science of epigenetics examines how variations in heritable gene expression cause aberrant cells to proliferate. Aberrant gene function, changes in gene expression, apoptosis malfunctioning, angiogenesis initiating, loss of normal cell growth, development, and regulation, and metastasizing of these cells to other healthy tissue or organs are the causes of cancer. Metastasis is the term used to describe the expansion of cancer from its original site (cells or tissues) to a different healthy area of a tissue or organ. Early cancer diagnosis, prevention, and therapy are top priorities for a number of cancer research bodies [62].

Cancer ranks among the world's most serious public health issues in terms of both morbidity and mortality. Some of the most popular cancer treatment options include radiation, chemotherapy, and surgical tumor excision; however, these conventional methods have a number of detrimental side effects. Chemotherapy continues to be the most popular and successful cancer treatment strategy. It is intended to cause harm to cancer cells' genetic makeup. Damage triggers the activation of the DDR pathway, a system for signalling, detection, and repair. The DDR initiates events that result in the suppression of cell cycle progression and cell death by stimulating subsequent molecular pathways [64]. Cancer patients may have paresthesia, nausea and vomiting, exhaustion, persistent pain, and anorexia as side effects of their treatment. Additionally, the acute effect of oncopharmaceuticals can impair the kidneys, liver, lungs, and nerves, thus increases the likelihood of medication side effects associated with chemotherapy. Furthermore, long-term medication use can result in infertility and some metabolic syndromes, among other far-reaching effects on the patient's life and health [65].

Extensive research has tried the potential use of herbs in cancer treatment, and some chemicals derived from plants have demonstrated encouraging anticancer effects. Numerous investigations have confirmed the natural bioactive chemicals' anticancer effectiveness. The National Cancer Institute has examined around 36,000 plant species for their potential anticancer properties, with approximately 3,500 plant

species demonstrating consistent anticancer activity [66]. Antioxidants, which are abundant in many therapeutic plants, aid in the body's defense against dangerous free radicals. Additionally, there is a link between chronic inflammation and a higher risk of cancer. Certain medicinal plants have anti-inflammatory qualities that may aid in reducing inflammation and, thus, aid in the prevention and treatment of cancer. Some medicinal plants have substances that prevent angiogenesis, which limits blood flow to tumors and slows their growth. Additionally, certain medicinal plants may augment the effectiveness of conventional cancer treatments, such as chemotherapy or radiation therapy, while possibly mitigating their associated side effects. This holistic approach is commonly referred to as complementary and alternative medicine (CAM) [67].

The phytochemicals and byproducts of plants offer a possible substitute to boost treatment effectiveness and minimize side effects in cancer patients. Numerous phytochemicals under consideration are physiologically active, naturally occurring anticancer drugs. Testing natural extracts for possible anticancer biological activity is the initial step in creating an efficient and successful anticancer therapy with no side effects. Subsequently, active phytochemicals are purified via fractionation (bioassays-guided), and their effects are evaluated through both in vitro and in vivo [68].

Resveratrol, a natural organic molecule, a member of the polyphenols group, is produced by enzyme stilbene synthase and acts as an antioxidant. It has anti-inflammatory and anti-cancer effects in addition to its antioxidant qualities. Its pleiotropic action is achieved through regulating the activation of several signaling pathways, such as the DDR pathway. Shang et al. explored the anti-inflammatory and cardioprotective attributes of resveratrol in a rat model involving cardiomyopathy and sepsis. The study delved into the correlation between the PI3K/AKT/mTOR signaling pathway and NF-κB activity, while also examining the impact of resveratrol on both pathways. Rats administered resveratrol exhibited a suppression of myocardial tissue damage and a reduction in apoptotic cell count. Concurrently, there was an upregulation in the expression of PI3K, AKT, and mTOR proteins, coupled with a downregulation of factors linked to the

NF-κB pathway, such as TNF-α, IL-6, IL-1β, and toll-like receptor 4 (TLR4) [69]. Similarly, it was documented that resveratrol inhibited prostate cancer cells' survival, proliferation, and epithelial-mesenchymal transition (EMT) by reducing TNF receptor-associated factor 6 (TRAF6) levels. Elevated TRAF6 expression has been associated with heightened cell migration and the facilitation of gene transcription expressing proteins relevant to EMT [70].

A phenolic substance called gingerol is present in ginger rhizomes. The administration of gingerol demonstrated an augmentation of caspase-3 activation in a mouse model examined for metastasis of breast cancer. Furthermore, it led to a reduction in orthotopic tumor development and the migration of mammary tumor cells (4T1Br4) to brain, lungs, and bones. Similarly, in a nude mice (athymic BALB/c) that was injected with human lung adenocarcinoma, glycyrrhizin, the predominant bioactive compound in the roots of *Glycyrrhiza glabra* L, compacted thromboxane synthase activity and thus increased the nuclear antigen expression of cell [71].

Green tea, derived from *Camellia sinensis* of the family Theaceae, is known for its notable content of epigallocatechin gallate (EGCG). Animal models and cell lines have been used in various studies to evaluate the effectiveness of EGCG [72].

2.2 Genus *Artemisia*

The family Asteraceae, which has commercial and therapeutic significance, includes the genus *Artemisia* L. Some of this genus' species can be found in the southern hemisphere and are often located in the northern hemisphere's temperate zones. It is a widely distributed genus within the Anthemideae tribe and has over 500 species of plants and shrubs. Diana, the Greek goddess, is represented by the generic name "Artemisia", which is derived from "Artemis" [73].

The genus *Artemisia* is known for its alternate leaves, small capitulum, arranged in panicles, or shaped like a head, blossoming, rarely unaccompanied, involucral bracts that are arranged in a few rows, receptacles that shaped like semicircle, without

scales, and occasionally hirsute, and florets that are all tubular with achenes that are oblong and without pappus or sometimes a small scarious ring (Figure 2.1) [35].

This genus produces secondary metabolites and essential oils that are used to treat various ailments. These plants are mostly biannual, perennial, annual herbaceous ornamental, aromatic plants or shrubs, and medicinal plants. Numerous extracts derived from *Artemisia* species are used to treat conditions such as stress, anxiety, sleeplessness, irritability, and psychoneurosis. Numerous biological properties, including antimalarial, antiseptic, antibacterial, anticancer, hepatoprotective, and antirheumatic activities, are present in these species. The treatment of diseases like cancer, inflammation, hepatitis, malaria, and infections by fungi, bacteria, and viruses is routinely carried out with *Artemisia* [34]. The extensive biological diversity of Pakistan includes 42 species of the genus *Artemisia* (Asteraceae), which are distributed throughout the country's phytogeographical zones. The majority of these plants are located in the country's northern frontier regions, in the districts of Swat, Rawalpindi, Gilgit, Chitral, Skardu, Abbottabad, and Kashmir, as well as in the foothills and ranges of the Himalaya, Karakoram, and Hindu Kush mountains. Pakistan is home to a diverse range of organisms, including 38 species of genus *Artemisia* L. (Asteraceae) [74].

2.2.1 Ethnomedicinal Study of the *Artemisia* Genus

Genus*Artemisia* has 500 species spread across Asia, Europe, and North America. The vast steppe populations of Asia are dominated by these largely perennial herbs. The highest number of species are located in Asia, where China has 150 accessions, the former USSR has 174, Japan has roughly 50, and Iran has 35 species of the genus. Many disorders, including cancer, hepatitis, malaria, and infections caused by fungi, bacteria, and viruses are treated with *Artemisia* species on a regular basis. Certain *Artemisia* species are employed in the creation of fragrant wreaths and to produce the essential oils for the flavouring of vermouth [75].

In the past, *Artemisia absinthium* (L.) was employed as an anthelmintic, antispasmodic, stomachic, febrifuge, cardiac stimulant, and to restore deteriorating mental function and liver inflammation. It was also used to enhance memory [76]. In addition to being used as a purgative, *Artemisia afra* (Jacq.) has been used to treat a number of illnesses including diabetes, malaria, bladder and kidney diseases, migraines, dyspepsia, colic, and malaria. Traditional eastern medicine has utilized *Artemisia asiatica* (Nakai) to treat cancer, inflammation, infections, and ulcerogenic illnesses. The Egyptian medicinal plant utilizing *Artemisia judaica (L.)* as a remedy for digestive issues. Native Americans in the western United States have utilized *Artemisia tripartite* (Rydberg), or three-tip sagebrush, to treat headaches, wounds, colds, sore throats, and tonsillitis. The common Tibetan medicinal plant *Artemisia vestita* (Wall. ex Bess.) has been used extensively in China to treat a variety of inflammatory illnesses. *Artemisia vulgaris* (L.) has been utilized for liver problems, as well as an analgesic, anti-inflammatory, and antispasmodic. Traditional medicine has employed preparations of *Artemisia abrotanum* L. (also known as "southernwood") to treat various diseases, counting upper respiratory conditions. These days, the primary uses of this perennial plant are culinary and aesthetic [35].

2.2.2 Phytochemicals and Antioxidant Activity of *Artemisia* Species

According to the literature on phytochemical data for the genus *Artemisia*, the principal chemical components of the *Artemisia* species are terpenoids, caffeoylquinic acids, coumarins, flavonoids, acetylenes, and sterols. The *Artemisia* species *A. absinthium*, *A. annua, A. afra, A. maritima,* and *A. scoparia* (Waldst et Kit) are particularly abundant in terpenoids [35]. Many botanical extracts and phytochemicals found in plants have the potential to revolutionize the development of approaches for the cure of a wide range of illnesses. If the significance of their anti-oxidants and antimicrobial substances is fully acknowledged, that goal should be easily accomplished. Because flavonoids exhibit anti-inflammatory qualities and reduce pain sensitivity, they are vital. They are anti-inflammatory because

they prevent the production of prostaglandins by inhibiting the corresponding enzymes. Research conducted in vitro has demonstrated that plant-based polyphenols with antioxidant capacity are pharmacologically beneficial against neurological disorders. Because the aforementioned complexes can hunt down free radicals, they can additionally serve as anti-cancerous, anti-mutagenic, and cardio-protective compounds [77]. According to studies, polyphenols have the ability to mitigate cell damage and lower cholesterol, making them excellent chemopreventive agents [78]. Experts are searching for new, naturally occurring, safe antioxidants to substitute for synthetic antioxidants used in foods and medicines as a result of these phenomenal findings.

Many researchers have examined and validated the phytochemical and antioxidant activities of many *Artemisia* species, indicating that the species from the genus *Artemisia* are abundant in various phytochemical elements with more effective antioxidant potential. *Artemisia annua* is a highly studied plant that has prompted significant attention in the field of pharmacology. Several hundred secondary metabolites have been identified and confirmed thus far. The plant's phytochemical investigation confirms the existence of sesquiterpene lactones and sesquiterpenoid compounds, with a strong correlation between these components and artemisinin. Endoperoxide sesquiterpene lactone, a bioactive pharmacological component, can be found in this plant [79]. Studies verified that the ethanolic extract of *A. annua* leaves has a substantial concentration of flavonoids and phenolics [80]. In addition to *A. annua, Artemisia amygdalina* is also a significant plant with enormous medicinal and commercial importance. Flavonoids and alkaloids exist in its ethanolic, methanolic, chloroform, ethyl acetate, aqueous, and crude extracts; triterpenoids are only present in the ethanolic extract. Tannins are only found in the ethyl acetate extract; on the other hand, saponins are found in the ethanolic and aqueous extracts but not in the methanolic, chloroformic, or ethyl acetate extracts [81].

Possessing antibacterial, antitumor, antispasmodic, antiseptic, antimalarial, hepatoprotective, and antirheumatic properties, the methanolic extract of *Artemisia vulgaris* contains phenolics, flavonoids, and sesquiterpenoid type chemicals [82].

Additionally, it has been found that *Artemisia absinthium* possesses flavonoids and phenolic substances, both of which have antioxidant properties [83].

2.2.3 Anticancer Activity of *Artemisia* Species

Many natural medicines with higher efficacy for treating cancer can be found in medicinal plants. Plants produce several vital metabolites, such as polyphenol-containing flavonoids and lignin. The potential biological actions of these compounds, such as their anticancer activity, are assessed both in vitro and in vivo. Numerous researches demonstrated that *Artemisia* species are more effective cytotoxic and anti-cancerous options, as has been previously documented for other plants [84]. Additionally, it has been demonstrated that *Artemisia* species are toxic to cancer cells both in vivo [85] and in vitro [86]. One or more of the plant's important chemicals may be the cause of these behaviors. Of those substances, artemisinin is a highly potent constituent of numerous *Artemisia* species, most notably *Artemisia annua,* and it has superior cellular toxicity towards human lymphoid leukemia cells. Additionally, human colorectal and breast cancer cells can be inhibited in their proliferation by artemisinin and its related substances [87]. Additional substances derived from *Artemisia* species that are likewise significant anticancer components include terpenoids, sesquiterpene lactones, and flavonoids. The low molecular weight plants *Artemisia vulgaris* and *Artemisia absinthium* are also known to contain numerous useful chemicals. These substances include monoterpenes, lactones, lignans, flavonoids, and sesquiterpenes. These are thought to be these plants' primary potent anti-carcinogen chemicals [13].

Apoptosis, a planned cell death that is triggered by cell cycle arrest, has been shown in studies to be a critical effect of the active ingredients in *Artemisia* species. *Artemisia fukudo*'s efficacy as a preventative measure against cancer is supported by Kim et al. [88]. A study found that while *Artemisia capillaris's* smoke and aqueous extracts cause cellular death in the human myelogenous leukemia cell line (HL60), they had no effect on breast cancer (MCF-7) or other types of tumor cells. Yet, in human hepatoma cell lines, macromolecules found in *Artemisia capillaris* may promote apoptosis [89].

The water-soluble extracts of *Artemisia argyi* have significant activity against murine tumor cells but little activity against human tumor cell lines or breast cancer cell lines. A significant advancement in the therapy of breast cancer appears to be the production of apoptosis in human breast cancer MCF-7 cells through the mitochondrial pathway, as demonstrated by a study involving the use of smoke and water extracts of *Artemisia princeps*. Flavones are an essential chemical found in *Artemisia argyi* and *Artemisia Asiatic*. These compounds have the ability to inhibit some types of cancer by activating apoptosis, such as prostate cancer, stomach cancer, myeloid leukemia, human lung cancer, and melanoma. Likewise, *Artemisia turanica* Krash. n-hexane extracts have superior cytotoxic, antiproliferative, and anticancer properties against two leukemic cancer cell lines, primarily HL-60 and K562 [13].

Research has shown that important components such as flavonoids and phenolic acids are abundant in the ethanolic extracts of *Arnica montana* and *Artemisia absinthium*. These substances exhibit superior antioxidant activity and also exert cytoprotective effects on fibroblast cells that are susceptible to oxidative damage. *Arnica montana* and *Artemisia absinthium* are now both considered superior candidates for the treatment of skin conditions [83]. Human muscle cancer cells exposed to methanolic extracts of *Artemisia scoparia* have catastrophic effects on 88-93% of cancer cells, indicating the herb extract's anticancer potential [84].

2.3 *Artemisia Carvifolia* Buch

The family Asteraceae includes the species *Artemisia carvifolia* in the genus *Artemisia*. *Artemisia carvifolia* is commonly known as sweet wood and had already been described by Francis Buchanan-Hamilton, although William Roxburgh had actually first used the name [90].

The *Artemisia carvifolia* Buch taxonomic classification is as follows: [90].

- Class: Magnoliopsida

- Subclass: Asteridae
- Order: Asterales
- Family: Asteraceae
- Genus: *Artemisia*
- Species: *carvifolia*

2.3.1 Morphology of *A. Carvifolia* Buch

Artemisia carvifolia has light-yellow, multi-stellate blooms and has alternate leaves that resemble *Artemisia annua* in terms of morphology (Figure 2.1). They have simple broad leaves. It is a branched annual or biennial plant that grows 30 to 150 cm tall. The plant is cultivated in the wild for use as a medicine and food in the region. Moist river channels, outer forest margins, canyons, waysides, and coastal beaches are among its ecosystem, which ranges in elevation from low to 4,600 meters. Achenes are produced by perennials. India, the Himalaya, Assam, Burma, and China are among the countries where *Artemisia carvifolia* is native. The soil should be dry to moderately damp, and the perennials appreciate a sunny location [36].

2.4 Primary and Secondary Metabolites

The phrase "primary metabolites" describes all the substances required for life, including amino acids, carbohydrates, lipids, and nucleic acids. In addition to the intermediates and products of primary metabolism, vascular plants also have numerous chemical substances called secondary metabolites that are mostly involved in their defense systems. Despite not being necessary for the plant's survival, they do contribute to its health and sustenance. In addition to primary metabolites, the pathways leading to secondary plant products also include other pathways that have evolved specifically for a given plant family or genus. Lack of SMs (secondary

FIGURE 2.1: *Artemisia Carvifolia* Buch

metabolites), in contrast to PMs (primary metabolites), does not directly cause cell death, but it can have a negative long-term impact on the organism's ability to survive [91].

2.5 Plant Secondary Metabolites

Plant SM are diverse chemical compounds generated through metabolic pathways of plants descended from the basic primary metabolic pathways. These are chemical compounds, small in size, produced from primary metabolites during the growth period of plants, containing molecular weights of less than 3000 da. Various metabolites are found in many plant species with different chemical properties and compositions [92].

The collective term "plant SMs" refers to over 12,000 alkaloids, 40,000 terpenoids, and 8,000 phenyl propanoids that are only found in plants. SMs of plants, including terpenoids, phenolics, and nitrogen-containing compounds (alkaloids), are responsible for the color of vegetables, provide defense against microorganisms and herbivores, and function as signal molecules when plants are under stress [91].

Since ancient times, plant SMs and their derivatives have been used as therapeutic agents to treat various diseases. Numerous plant-based natural compounds have been used as the primary raw materials for a number of medications. Additionally, plant SMs have been employed as pharmacological probes, prototypes, and drug precursors.

Specifically, a significant amount of medications sold globally are derived from plants, and the pharmaceutical sector currently uses a number of bioactive substances [91].

Some specific plant SMs, like morphine, which was the first natural product (NP) to be extracted from the opium poppy (*Papaver somniferum*) in 1806 and employed as a drug precursor for a number of medications, have been used as drug precursors for several medicines [93]. Sesquiterpene lactone-containing artemisinin, which was derived from *Artemisia annua*, is used as an antimalarial medication to treat *Plasmodium falciparum*-caused malignant cerebral malaria. Paclitaxel is an antimitotic drug to treat breast and ovarian malignancies, although it prevents tubulin from polymerizing to create microtubules. It is extracted from *Taxus brevifolia*, a highly oxygenated tetracyclic diterpenoid [94].

2.5.1 Types of Secondary Metabolites

Primary metabolites are necessary for growth and development of plants and supplies for processes including photosynthesis, translocation, and respiration. Secondary metabolites that are derived from PM are generated by biosynthetic alterations such as methylation, hydroxylation, and glycosylation and are not involved directly in development or growth. Secondary metabolites obviously have more complex structural makeup than primary metabolites and include side chains [95]. Plant secondary metabolites have been classified into three main groups as depicted in Figure 2.2 [96].

(i) Nitrogen-containing compounds

(ii) Phenolic groups (built of simple sugars and benzene rings),

(iii) Terpenes and steroids (mostly made of carbon and hydrogen).

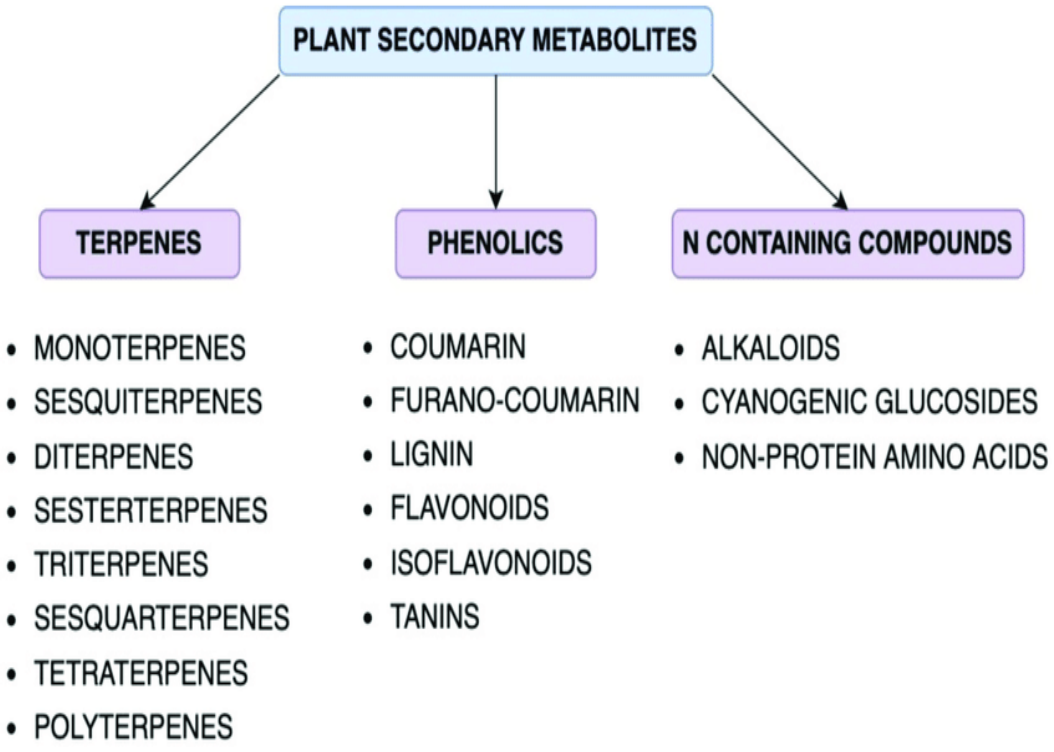


FIGURE 2.2: Major Types of Secondary Metabolites [96]

2.5.2 Terpenes

Terpenes are structurally diverse and substantial category of metabolites, with at least 35,000 distinct terpenes having been identified thus far. Terpenes are conveniently recognized as they consist of units of isoprene that can be altered by cyclisation processes. Depending on the number of isoprene molecules in their carbon skeleton, they are divided into various categories, as shown in Figure 2.2. Terpenoids contain active compounds that have antitubercular, anticancer, anxiolytic, and mutagenic properties [95].

2.5.3 Nitrogenous Compounds, the Alkaloids

Alkaloids are a diverse collection of secondary plant chemicals with a wide range of structural types (Figure 2.3), biosynthesis processes, and pharmacological potential.

In this family of chemicals, there are roughly 12,000 different types of molecules. Alkaloids are cyclic molecules with a restricted distribution in living things that include nitrogen in a negative state of oxidation. Either the heterocyclic ring itself contains nitrogen, or there may be an additional cyclic link. In addition to plants, insects, microbes, marine invertebrates, and animals are sources of alkaloids. Since many years ago, alkaloids have been utilized as medications, and they are still widely used today. Quinine and morphine are two examples of alkaloids that are found in nature [97].

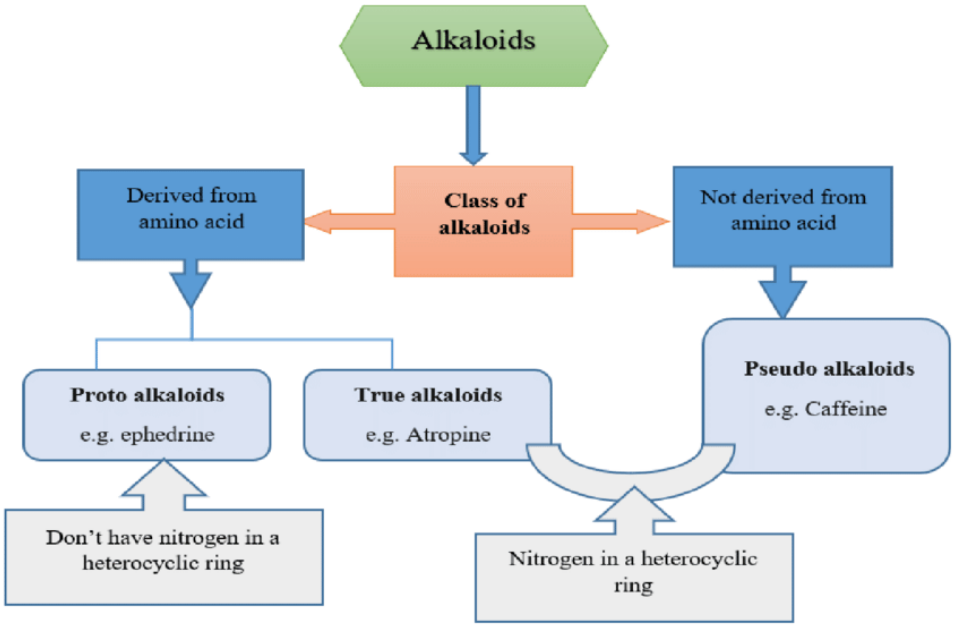


FIGURE 2.3: Structural Classification of Alkaloids [98]

2.5.4 Phenolic Compounds

Phenolics are one of the major types of SM and hold a special place among natural products due to their widespread dispersion throughout the plant kingdom. They are aromatic organic compounds containing at least one 7 hydroxyl group linked to the benzene ring. Phenolics are well-known for their effectiveness as therapeutic substances in curing various diseases, including diabetes, cardiovascular and neurological diseases, and cancer. Their ability to treat diabetes is mediated

through the adjustment of glucose metabolism [99]. Figure 2.4 provides a list of various phenolic classes [100].

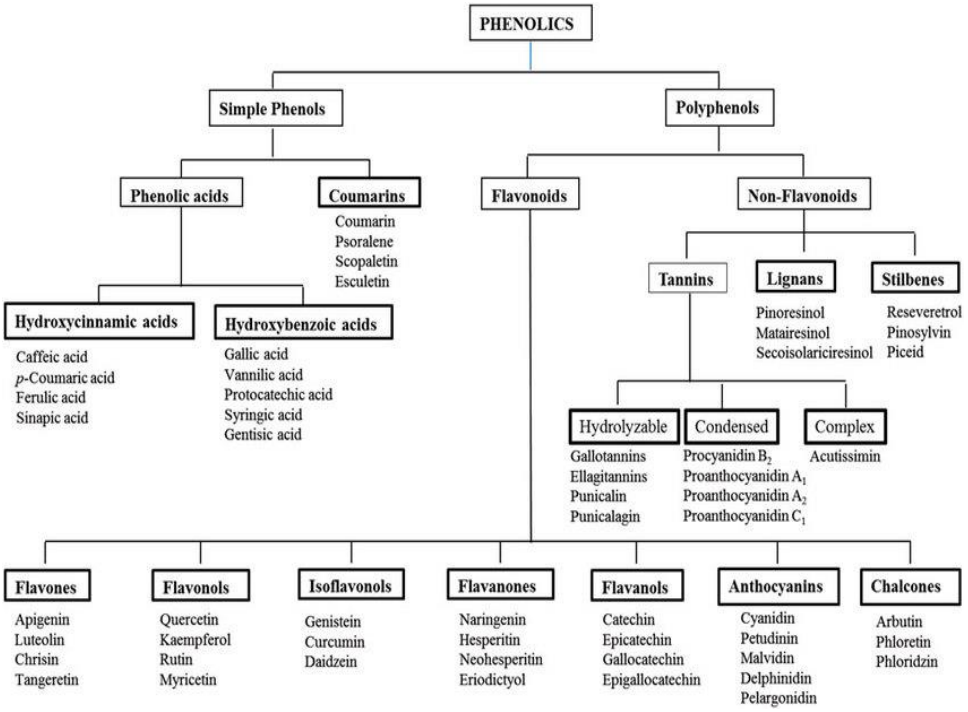


FIGURE 2.4: Schematic Diagram of Different Classes of Phenolic Compounds [100]

2.6 Flavonoids

More than 4000 distinct flavonoids with plant origins have been identified as of this point. These are most frequently observed in green plants, and are primarily found in the form of glycosides in different parts of plants. According to their chemical makeup, flavonoids have two benzene rings A and B connected by a heterocyclic pyrane ring C, thus make a fifteen-carbon skeleton as seen in Figure 2.5 [101].

Flavonoids are hydroxylated polyphenols with a common chemical structure of three rings (C6–C3–C6). Their quantity varies according to plant type, stage of developmental, and environmental factors for the growth of plant. It has long been believed that flavonoids, which give flowers their color and perfume, are generated in certain parts in plants. In order to promote spore germination, seedling growth, and development, they are utilized to draw pollinators. In

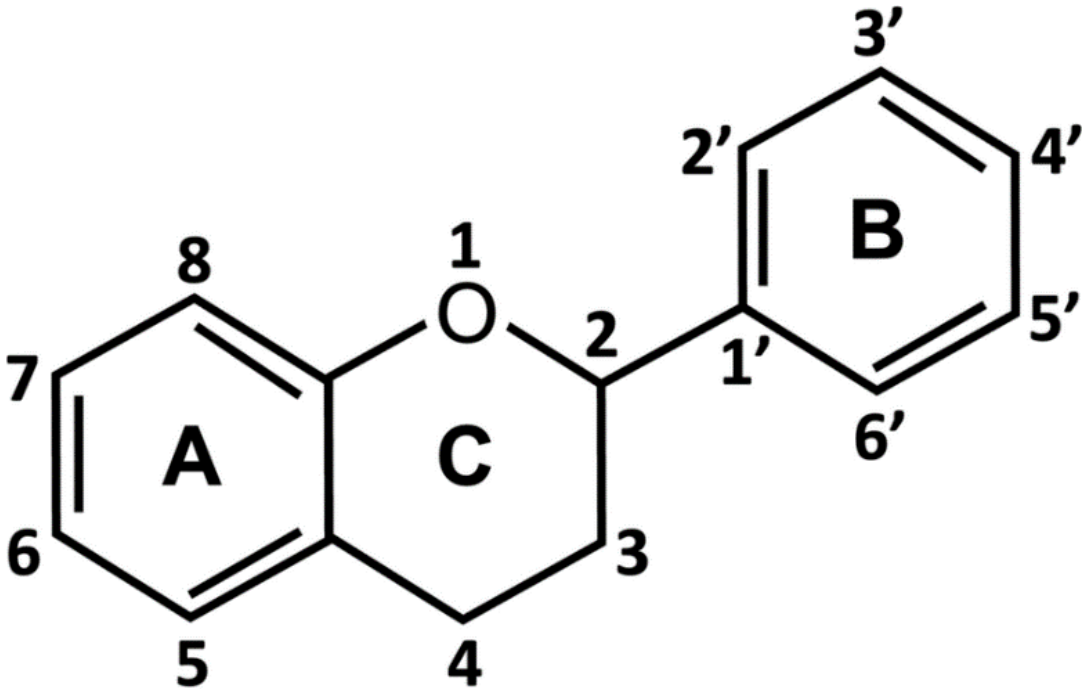


FIGURE 2.5: Basic Structure of Flavonoids [101]

addition to acting as ultraviolet filters, flavonoids help plants survive biotic and abiotic stress [102].

2.6.1 Classification of Flavonoids

They can be categorized into numerous classes. The classes include, flavones, flavanones, flavonols, flavanonols, isoflavones, catechins, chalcones, and anthocyanins. Figure 2.6 lists their general structures. The fundamental criteria that define flavonoids classes are differences in the degree of oxidation and substitution pattern of the C ring, while individual compounds within a class differ in the substitution pattern of the A and B rings [103].

2.6.2 Biosynthesis of Flavonoids

Phenylpropanoid pathway produce flavonoids from phenylalanine, generated through the shikimate pathway. The general phenylpropanoid pathway cover the first three steps in the phenylpropanoid pathway [105]. First, phenylalanine is transformed

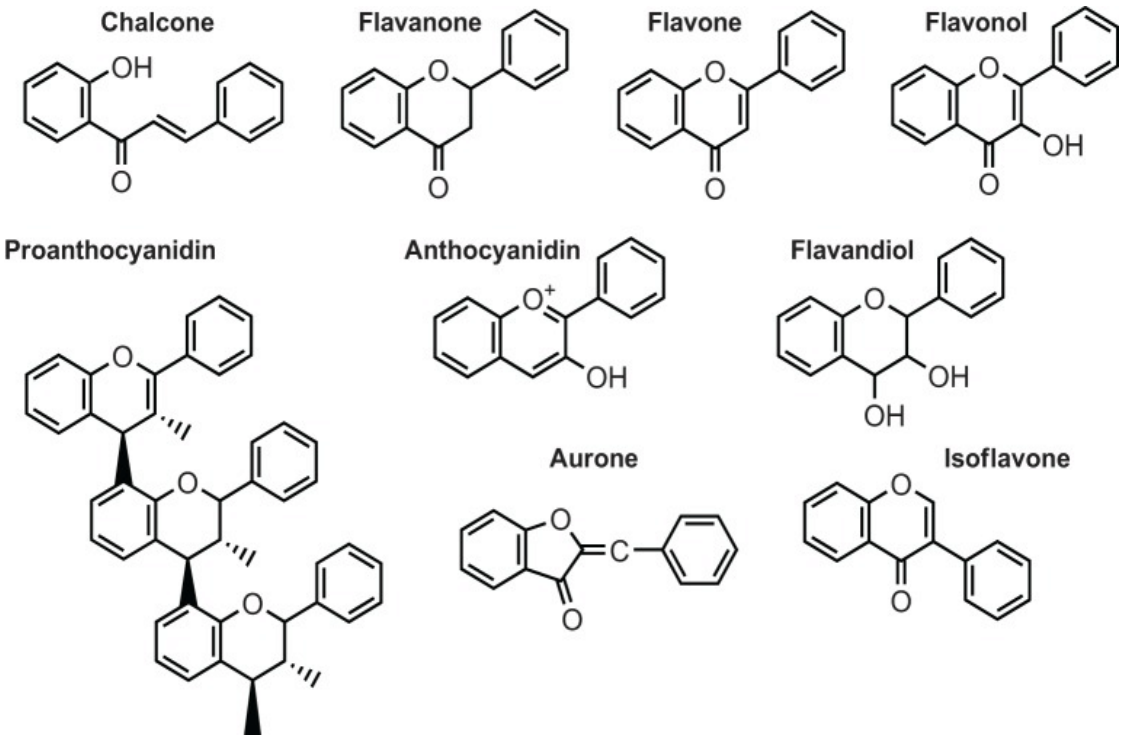


FIGURE 2.6: Structure of Different Classes of Flavonoids [104]

into p-coumaroyl-CoA by the activity of three enzymes: cinnamic acid 4-hydroxylase (C4H), 4-coumarate: CoA ligase (4CL), and phenylalanine ammonia lyase (PAL). The deamination of phenylalanine to trans-cinnamic acid, the first committed step in the overall phenylpropanoid pathway, is catalyzed by PAL. Furthermore, PAL is essential for controlling the carbon flux in plants from primary to secondary metabolism [106]. Plant cytochrome P450 monooxygenase, C4H, is engaged in the second phase. P-coumaric acid is created by the hydroxylation of trans-cinnamic acid, which is catalyzed by C4H. Additionally, this is the first oxidation step in the route that generates flavonoids. In the third step, the formation of *p*-coumaroyl-CoA occurs by enzyme 4CL by adding a co-enzyme A (CoA) unit to *p*-coumaric acid. The process of making particular flavonoids, which starts with chalcone synthesis, is initiated when *p*-coumaroyl-CoA enters the pathway for making flavonoids. The key and primary rate-limiting enzyme, specific for the flavonoid biosynthesis pathway is CHS, a polyketide synthase, and chalcone is the first key intermediate product produced by chalcone synthase enzyme, providing a basic structure for the subsequent synthesis of flavonoids in the flavonoid metabolic pathway. Even though the primary mechanism for flavonoid production is conserved in plants [107].

Numerous Fe2+/2-oxoglutarate-dependent dioxygenases, reductases, hydroxylases, and isomerases are among the enzymes that alter the basic flavonoid skeleton, depending on the species, giving rise to the many flavonoid subclasses as shown in Figure 2.7 [108]. Research on the *PAL* and *CHS* gene expression levels appears to be important for the synthesis of flavonoids.

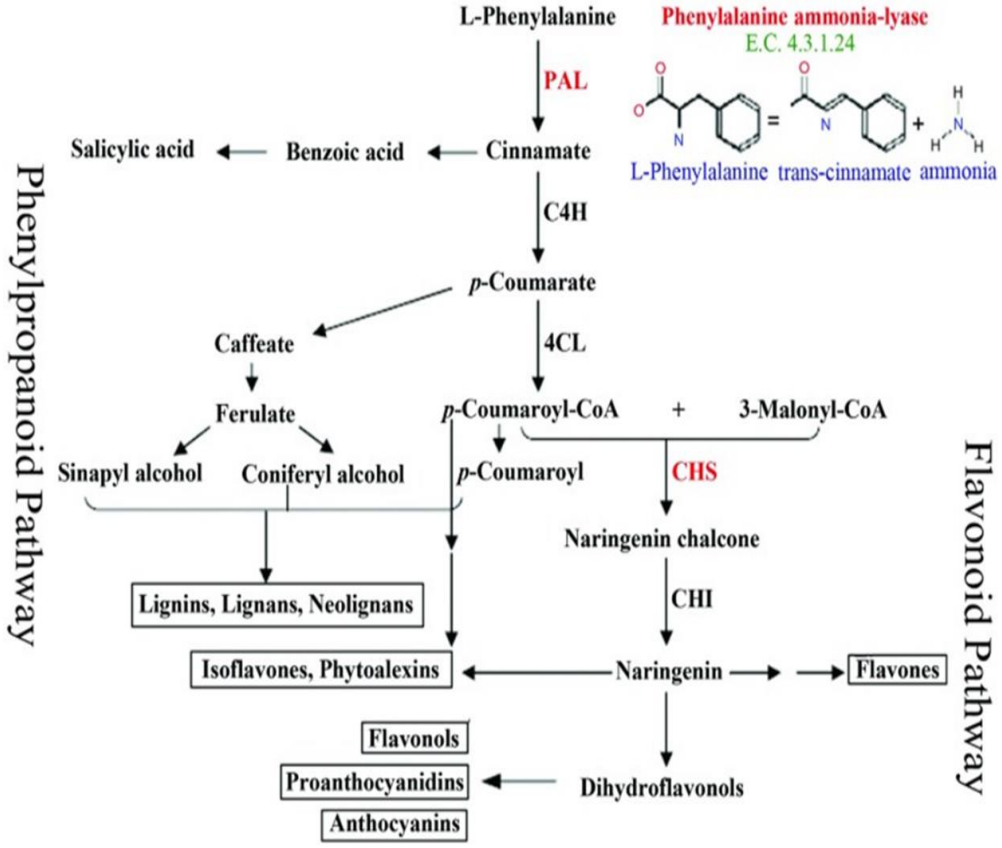


FIGURE 2.7: Flavonoids Production Pathways in Plants [108]

2.7 Pharmacological Activities of Flavonoids

2.7.1 Flavonoids as Antioxidant Agents

Reactive oxygen species (ROS) have the potential to oxidize cellular proteins, lipids and nucleic acids. Contributions of ROS to cellular aging, carcinogenesis, mutagenesis, and coronary heart ailments have been reported, possibly as a result of DNA damage, destabilization of membranes and low-density lipoprotein (LDL)

oxidation [109]. Flavonoids' antioxidant and chelating properties are thought to be responsible for the majority of their positive health effects. They have shown distinctive cardioprotective benefits due to their ability to inhibit LDL oxidation. It has been demonstrated that rat' mayocardial post-ischemic damage can be decreased by flavonoid-rich diets. According to studies, flavonoids have the ability to reduce alpha-tocopherol radicals, transfer electrons free radicals, chelate metal catalysts, antioxidant enzymes activation, and inhibit oxidases. Quercetin is one of the major dietary phytoconstituents among the variety of flavonoids that are widely distributed in plants, including seeds, fruits, vegetables, leaves, and so on. Scientists have been paying close attention to quercetin lately because of its antioxidant properties and medicinal properties, which include anti-inflammatory, anti-obesity, and anti-cancer effects [110].

2.7.2 Flavonoids as Anticancerous Agents

Uncontrolled cell proliferation and a malfunctioning cell cycle, which cause aberrant cells to invade and disseminate to other regions of the body, characterize the varied disease known as cancer. Increased stress, pollution, smoking, hazardous radiations, and ultraviolet radiation are the main external determinants of cancer, whereas hypoxia, oxidative stress, genetic alterations, and an apoptotic dysfunction are the key internal causes [111]. Increasing data explains that cancer is a metabolic disease caused by differing levels of mitochondrial dysfunction and metabolic changes [112]. Increased aerobic glycolysis, unbalanced pH, impaired lipid metabolism, elevated ROS production, and poor enzyme activities are the primary metabolic changes of the tumor cells [113–115].

These polyphenols' anticancer properties rely on a number of variables, including their concentration and chemical makeup. Different organs' malignant cells demonstrated varying degrees of sensitivity to flavonoids [116]. Flavonoids have been demonstrated in numerous studies to be able to control cellular metabolism, scavenge free radicals, and offer protection against disorders linked to oxidative stress. They exhibit a broad spectrum of anticancer actions, including the inhibition of ROS-scavenging activity of enzyme, involvement in cell cycle arrest,

induction of autophagy and cell death, and decrease of cancer cell multiplication and intrusiveness. While the underlying molecular pathways of several flavonoids' anticancer effects remain unclear, there is mounting evidence to support this claim. Flavonoids, which have been employed as chemopreventive agents for cancer, are extremely abundant in fruits and vegetables. For example, dietary fruits and vegetables, notably onion and apple, contain the flavonoid quercetin which is used to treat lung, prostate, stomach, and breast cancers. It was discovered through a meta-analysis of 12 researches including over 190,000 participants that eating a lot of flavonoids dramatically reduced the risk of getting breast cancer. High consumption of flavonols and flavones was found to lower the incidence of breast cancer [117]. Flavonoids have a variety of chemopreventive properties, including estrogenic/antiestrogenic activity, induction of detoxifying enzymes, antiproliferation or cell death, induction of cell cycle arrest, prevention of oxidation, control of the host immune system, anti-inflammatory activity, and modifications in cellular signaling [118]. Various mechanisms for anticancer activity of plant derived flavonoids are mentioned in Figure 2.8 [119].

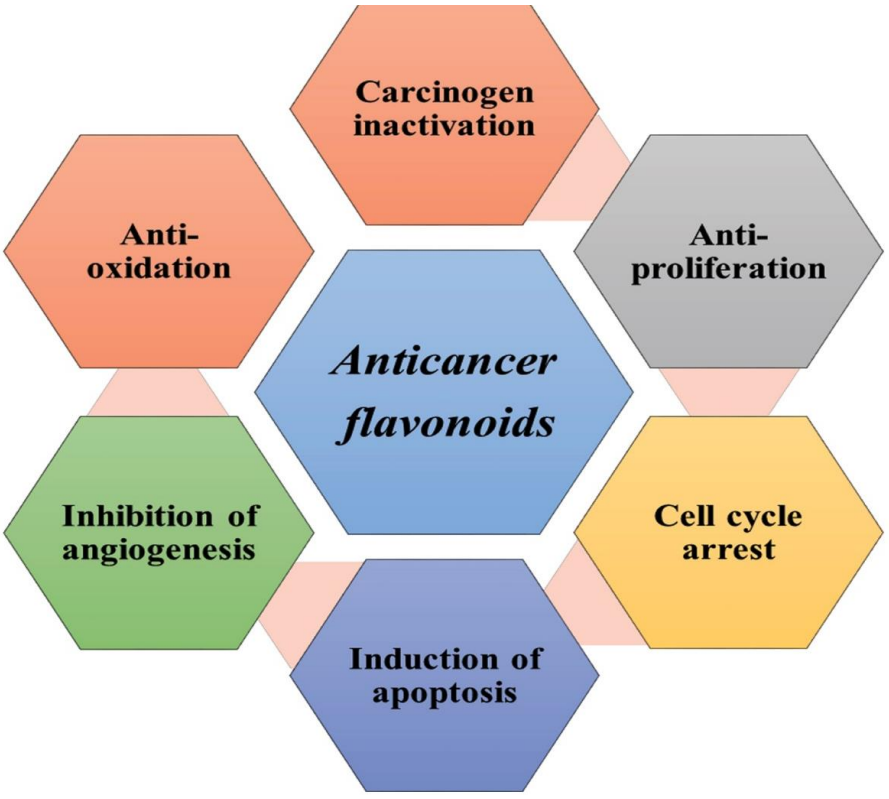


FIGURE 2.8: Various Mechanisms of Anticancer Activity of Flavonoids [119]

Flavonoids like apigenin, myricetin, luteolin, quercetin, and kaempferol have been shown in studies by Zutphen to reduce the mortality risk associated with respiratory tract tumors. Flavones have the ability to inhibit multiple protein kinases, including cyclin-dependent kinases (CDKs), dual-specificity tyrosine phosphorylation regulated kinase 1A (DYRK-1A), and glycogen synthase kinase-3 (GSK3). These kinases have all been connected to the emergence of cancer [116]. Individuals with a high flavonoid consumption have also been observed to have a decreased incidence of lung cancer [120]. It has also been noted that consumption of flavones in breast cancer tends to protect against mammary cancers [121].

Additionally, a low-fat diet that emphasizes eating lots of fruits, vegetables, fish, soybean curd, and other foods had a lower risk of developing breast cancer in Japanese women [122]. It has also recently been discovered that consuming tea and broccoli (which contain kaempferol) can prevent ovarian cancer. If more long-term research supports these findings, ovarian cancer prevention may make significant progress [123].

2.8 Flavonoids of *Artemisia* Genus

Plants are known to contain more than 4500 distinct flavonoids. One of the finest sources of flavonoids is the genus *Artemisia*, which has more than 500 species [124]. Around 50 flavonoids have been identified from the most well investigated species in this genus, *A. annua*, although flavonoids have also been found in *A. absinthium* L. [125], *A. asiatica* [126], and *A. herba-alba* [127].

Although diverse chemotypes demonstrate significant diversity, the level of these flavonoids in *Artemisia* plant is relatively lower, and morphological variations have also been noted in the same plant at various phases of growth, For instance, the elevated levels of casticin and chrysoplenetin were obtained from foliage of *A. annua* that was grown in the field during full bloom. Similar variations in antioxidant potential have been seen in several cultivars in another study, indicating heterogeneity in the concentration of antioxidants [48].

2.9 Strategies to Enhance Secondary Metabolites

The following strategies are utilized to boost secondary metabolite concentration because, as was previously said, they only account for less than 1% of all carbon in the plant.

2.9.1 Enhancement of the Secondary Metabolites through Elicitation

By subjecting undifferentiated cells to different elicitors like salicylic acid, methyljasmonate, chitosan, and heavy metals, the secondary metabolites content can be enhanced [128]. When pathogens or elicitors are introduced to plant cells, defense reactions begin to build up in whole plants or plant cell cultures and these defensive secondary plant products are produced. Even while secondary metabolite elicitation has been the subject of extensive research, the precise mechanism is still unknown. Numerous mechanisms, including messenger Ca^{2+}, elements affecting the integrity of cell membrane, blockage or stimulation of intracellular pathways, and changes in osmotic stress have been proposed in this area [129].

2.9.2 Metabolic Engineering for Enhancement of Secondary Metabolites

In order to achieve the required amount of a specific product or collection of products, a secondary metabolic pathway may be genetically modified by introducing or knocking out certain genes [130]. The last ten years have seen a lot of investigation on the biosynthetic processes that produce secondary plant chemicals. A brand-new area of study known as metabolic engineering also emerged at the beginning of the 1990s. Intentional alteration of cellular metabolism for the purpose of producing desired molecules is known as metabolic engineering. Recombinant DNA technology allows for the manipulation of many organisms' metabolic pathways [131].

Metabolic engineering, according to Bailey [132], is "the augmentation of cellular function through altering of cell's regulatory processes". After successful route elucidation and metabolite mapping, this mostly relies on finding restraining enzyme functions. With the proper use of genetic transformation, such restricting measures can be changed. The majority of methods used up to now have relied on the insertion of genes taken from more efficient organisms, usage of promoters that boost a target gene's expression, or antisense and co-suppression techniques to produce plants with knock-down versions of the desired features.

Incorporating additional genes to create novel phenotypes or enhancing the expression of already existing genes are both possible with transformation technology [133]. *Agrobacterium*-based transformation in plants is the most used type of transformation.

2.9.3 *Agrobacterium*-mediated Transformation

Agrobacterium tumefaciens, which is responsible for around 80% of the transgenic plants that have been created to date, has a significant contribution to the field of plant genetic engineering and fundamental molecular biology studies. Many "recalcitrant" species, including monocots and fungi, which are not part of *Agrobacterium's* natural host range, can now be changed, contrary to the previously held belief that just dicots plants and gymnosperms, or a few number of monocot species are capable of transformation by *Agrobacterium* [134]. In addition, extended DNA segments can be inserted into plants, and transformed cells often have one or a low number of copies of T-DNA incorporated into their genomes with less rearrangement [135].

2.9.4 Classification of *Agrobacterium*

A number of species are there for *Agrobacterium* but the two commonly utilized for genetic transformation purpose are *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes* [136].

Agrobacterium tumefaciens

In essence, *Agrobacterium tumefaciens* is a bacterium that inhabits soil and develops crown galls on dicotyledonous plant species and typically carries the Ti plasmid. In the vulnerable plant's nuclear genome, T-DNA from the Ti plasmid is transmitted [137]. Genes required for growth regulator autonomy and the synthesis of a wide range of opines, which are noble metabolites capable of being catabolized by the initiating *Agrobacterium*, are both found in T-DNA (Figure 2.9).

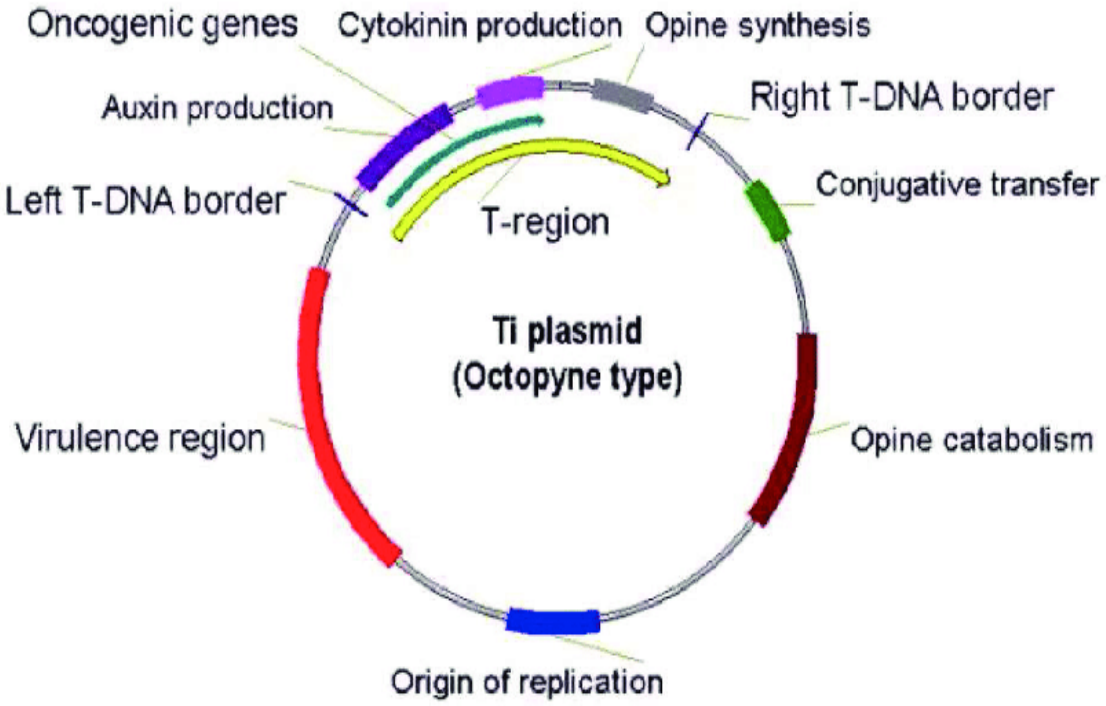


FIGURE 2.9: Ti-plasmid of *A. tumefaciens* [138]

Agrobacterium rhizogenes

Agrobacterium rhizogenes is a probable plant pathogen that produces hairy root development at the spot of illness. In a manner that is strikingly similar to *A. tumefaciens*, it causes a syndrome in dicotyledonous plants called hairy root disease. Additionally, it triggers the start of opine synthesis and pathogenicity [139]. The procedure of *Agrobacterium*-mediated plant transformation and its applications is the focus of extensive research in current era. It is remarkable that certain plant

components that were previously mysterious have been isolated and described, and many elements of the primary molecular processes underlying T-DNA transfer in the bacterial cells have been uncovered [140].

2.10 The *rol* Genes and Their Impact on Secondary Metabolites of Plant

The *rol* genes have a major impact on synthesis of secondary metabolite production. The *rol* genes are great activators of secondary metabolism in a variety of plant families, according to numerous studies [141]. The generation of secondary metabolites appears to be most effectively induced by the induction of three *rol* genes from T-DNA, *rol A, rol B*, and *rol C*, either singly or in combination (*rol ABC*) [142].

The Rol A protein known as growth-stimulating DNA binding protein, encoded by *rol A* gene, and *rol B's* tyrosine phosphatase activity controls the auxin signaling pathway [55]. In *Vitis amurensis* and *Rubia cardifolia*, *rol B* has been utilized to boost the manufacturing of resveratrol and anthraquinones, respectively. In transformed plants and cell cultures, the *rol C* gene's cytokinin glucosidase ability can promote the formation of numerous secondary chemicals, such as ginsenosides, indole alkaloids, tropane alkaloids, pyridine alkaloids, and anthraquinones [50, 143]. In contrast to wild-type plants, transformants carrying the *rol B* and *rol C* genes displayed a much higher artemisinin content. Semi-quantitative RT-PCR and southern blot analysis were used to examine transgenic *A. carvifolia* plants with elevated secondary metabolism and alteration in morphological features to see if there was a relationship with *rol* gene expression [50].

The *rol A* gene

The Rol A protein is most likely a DNA-binding protein, and has structural similarities to the E2 DNA-binding domain of the HPV [55]. It has been demonstrated

that the *rol A* gene stimulates the synthesis of nicotine. Anthraquinones (AQs) were synthesized at a 2.8-fold higher level in calli from *Rubia cordifolia* that expressed the *rol A* gene. An intriguing biotechnological feature of *rol A* is that it ensured exceptionally steady AQ levels in *R. cordifolia* calli while also creating the ideal environment for active callus formation. This impact remained constant over a seven years span of research investigation of the *rol A*-transformed callus line [142]. Transformants of the *rol A* gene have a very abnormal phenotype, includes dwarfism or semi-dwarfism, long internode lengths, wrinkled and dark-green leaves, and delayed aging. While comparing to the control plants, *Artemisia* transgenics of the *rol B* gene exhibit wider leaves and shoot apical dominance [144]. The *rol A* has a role in the metabolism of gibberellin as evidenced by the reduction in gibberellic acid content in tobacco plants transformed with the gene *rol A* [145].

Chapter 3

Research Methodology

3.1 Working Precautions

During the germination of seeds, tissue culture, and transformation of *Artemisia carvifolia* explants by *rol A* gene, sterilized conditions were kept in place for both human safety and to reduce the likelihood of contamination. The following safety precautions were implemented for this reason.

- Lab coats were utilized in the Laboratory during work.
- All apparatus used for growing microorganisms were sanitized by autoclaving for 20 minutes at 121^oC.
- Disinfectants such as spirit and 70% ethanol were used to clean benches and working area.
- The entire setup utilized in the experiment for surface sterilization was exposed to UV radiations in a Laminar flow hood for 15-20 minutes.
- Hands were washed properly by using a disinfectant soap before and after working.
- Waste materials were autoclaved before their disposal to reduce the chances of contamination.

3.2 Laboratory Glassware and Chemicals

Most of the plastic ware used in current research work was sterile. The glasses that were utilized were bought from Pyrex®. All of the glassware was thoroughly cleaned with detergent before being submerged in a 10% bleach solution for each experiment. After washing, the glassware was dipped in 10% bleach solution for 15 to 20 minutes and dried in an oven for 15 minutes. The glassware was then wrapped and placed in an autoclave set for 20 minutes at 120°C and 15 psi. Throughout the entire experiment, flasks were sealed with aluminium foil and firmly plugged with cotton. In the tissue culture study's tests, analytical and molecular biology-grade chemicals like sucrose, MS, agar, and phytoregulators were used. The reagents, chemicals and Murashige and Skoog (MS) medium were purchased from Sigma Chemical Co., USA. Agar was purchased from "DIFCO" laboratories, USA. Distilled water was used to prepare all solutions.

3.3 Inoculation Site and Instrumentation

The transfer room had a monthly cleaning and 95% ethyl alcohol treatment. Petri dishes, surgical instruments, and flasks holding distilled water were all sanitized in an autoclave for 20 minutes at 121^{o}C and 15 psi. Petri dishes and surgical instruments were autoclaved in a newspaper or aluminum foil.

3.4 Environment for Tissue Cultivation

Tissues were transferred aseptically using a Laminar Flow Cabinet in which HEPA filter was installed. The work surface of Laminar Flow Cabinet was cleaned with 95% ethyl alcohol before use. The cabinet was stocked with culture vessels, Petri dishes, distilled water, and surgical instruments. To guarantee sterility, the workplace and all equipment were then subjected to UV light for 20 minutes. About thirty minutes after turning off the UV light, tissue culture work began.

95% ethyl alcohol was used to dip surgical instruments. The equipment was once more immersed in ethyl alcohol, reflashed, and used after each manipulation.

3.5 Plant Identification through DNA Barcoding

3.5.1 Medium for Seed Germination

The present study employed the MS (Murashige and Skoog) medium for germination of seed. MS salts at half strength were prepared, augmented with 3% (w/v) sucrose, and then solidified with 0.5% (w/v) agar. Prior to adding agar, 1 N NaOH / HCl was used to bring the media's pH down to 5.8. For 20 minutes, the medium was autoclaved at 15 psi and 121^{o}C. The prepared media was used as seed germination media.

3.5.2 Collection of Plant Material

Seeds of *Artemisia carvifolia* were gathered from Astore, located in Pakistan's northern areas at an elevation of 8,500 feet and 35.3667° N and 74.8500° E. The plant material was then identified through DNA barcoding.

3.5.3 Seeds Sterilization and Germination

Following seed collection, they were surface sterilized for 30 seconds with 70% ethanol and then for 15 seconds using 0.1% (w/v) mercuric chloride ($HgCl_2$). The seeds were then dried on autoclaved filter paper after being rinsed with sterilized distilled water (three times) within a laminar flow hood. While maintaining the sterile conditions seeds were sown in petri plates on half strength MS medium. Subsequently, seeds were chilled at 4^{o}C for 2 days in dark. Subsequently, seeds in petri plates were subjected to incubation within growth chamber set at 25^{o}C with

16 h of photoperiod, light intensity maintained at 45 μE m^{-2} s^{-1} and 60% relative humidity under aseptic conditions. After the emergence of plantlets from seeds, further DNA extraction process was performed.

3.5.4 Genomic DNA Extraction from Plants

For extraction of genomic DNA from germinated plantlets, an efficient and simple plant genomic DNA extraction procedure (CTAB method) [146] was used with some changes. The composition of DNA extraction solutions is given in Table 3.1. The steps involved in the process of DNA extraction are given below:

- The plant material (200 mg) which was at a frozen state, underwent grinding into a finely powdered form using a pestle and mortar immersed in liquid nitrogen.
- When ground powder formed, 500 μL CTAB extraction buffer was introduced.
- The resulting mixture was then transferred to vial and SDS 20% (40 μL) was added to each vial.
- The vials are then vortexed for 30 sec.
- The vials were then positioned in a water bath for incubation at 65^{o}C for 35 minutes with frequent shaking.
- Phenol: chloroform was added at the ratio of 25:24 to each vial in quantity of 500 μL before placing on an inverted shaker for a duration of 20 minutes.
- To resolve phases, centrifugation was done out for 5 minutes at 14000 rpm (maximum speed).
- The upper aqueous phase (also called supernatant), which contains nucleic acids, was transferred to a new vial.
- Vials were shaken after being filled with chilled ethanol (100%) and then left at - 20^{o}C overnight to allow DNA to precipitate.

- The following day, vials were subjected to centrifugation at 14000 rpm for a duration of 15 minutes.
- The upper layer was drained away to obtain the pellet.
- Each vial received 500 μL of 70% ethanol, which was applied to wash the pellet three times.
- After draining the liquid and evaporating the ethanol, the pellet was then dissolved in 20-50 μL TE buffer containing RNases.
- The vials were kept at -20^{o}C in a refrigerator.
- By measuring the amount of light absorbed at wavelengths of 260 nm and 280 nm and determining the corresponding ratio, the DNA quality and quantity were evaluated.

Table 3.1: Composition of Plant DNA Extraction Solutions

Technique	**Chemicals Used**	**Composition**
Plant DNA Extraction	CTAB Buffer	1M Tris HCL (pH 8)
		0.5M EDTA (pH 8)
		5M NaCl
		3% CTAB
		3% PVP
		0.2% B-Mercaptoethanol
	70% Ethanol	70 mL ethanol in 30mL dH2O
	T.E Buffer	10 mM Tris (pH 8)
		1 mM EDTA

For CTAB Buffer, pH of EDTA and Tris HCL was adjusted to 8, autoclaved distilled water was used and chemicals were combined in a reaction bottle. The CTAB buffer was then stored at 4^{o}C.

3.5.5 Polymerase Chain Reaction

The PCR amplification was aimed to identify *Artemisia carvifolia* Buch by targeting the non-coding spacer region located between the *psbA* and *trnH* genes within the

chloroplast DNA. For this purpose, primers of "psbA: 5' -GTTATGCATGAACGTAATGCTC-3' and trnH: 5'- CGCGCATGGTGGATTCACAATC-3'" were used. The reaction of PCR took place in 25 μL total reaction mixture [147]. The composition of Master Mix and PCR program are given below in Table 3.2 and Table 3.3.

TABLE 3.2: Composition of Master Mix of PCR

Technique	**Reagent used**	**Concentration**
Polymerase chain reaction	dNTPs	0.2 mM
	DNA	50 ng
	F primer	0.25 μg
	R primer	0.25 μg
	Taq polymerase	0.2 U
	MgC12	2 mM
	PCR buffer	1X

TABLE 3.3: PCR Program for Amplification of Genes

Step	**Temperature**	**Duration**
Initial denaturation	94	3 mins
Cyclic denaturation	94	30 sec (35 cycles)
Cyclic annealing	54	30 sec
Elongation	73	45 sec
Final elongation	73	10 mins

3.5.6 Agarose Gel Electrophoresis

It was carried out to examine the PCR products. In order to prepare a 1.5% w/v agarose gel, 1.5 grams of agarose were melted in 100 milliliters of 1X TBE buffer in a microwave oven. To stain DNA, 2 μL of ethidium bromide solution was added. 0.2% bromophenol blue mixed with 40% sucrose was used as loading dye (3 μL) and was properly combined with DNA samples in the wells. A DNA sample is mixed with loading buffer to give it visible color and increase its viscosity, which causes the sample to sink into wells rather than float. The electrophoresis was carried out in a gel tank with 1X TBE buffer running for 60 minutes at 100 volts

and 60 mA. The presence of the product resulting from amplification was confirmed by exposing the agarose gel to a UV-trans illuminator following electrophoresis.

Ingredients of loading dye

Bromophenol blue (BPB)	0.25%
Sucrose solution	40%

Ingredients of 10 X TBE

Chemicals	Amount used	Final concentration
Boric acid	55 g	890 mM
Tris	108 g	890 mM
Na_2EDTA	9.3 g	20 mM (pH 8.3)

3.5.7 Purification and Sequencing of PCR-Derived Products

The Rapid PCR Purification System 9700 was used to purify the PCR product, and the ABI Prism 310 Automated DNA Sequencer was used to sequence the purified products using the dideoxy-chain termination method. Furthermore, by using the BioEdit software/alignment tool BioEdit (version 7.2.5.0), the sequences acquired were located and examined.

3.6 *Agrobacterium tumefaciens* mediated Genetic Transformation of *Artemisia carvifolia* Buch with *rol A* Gene

In the present study, the genetic transformation of *Artemisia carvifolia* Buch was carried out with *rol A* gene. For that experiment, *Agrobacterium tumefaciens*

strains GV3101 carrying the vector pPCV002-A containing the *rol A* gene was utilized. After transformation, the successful integration of gene, its expression and gene copy number were authenticated by molecular analysis. The procedure used is mentioned here.

3.6.1 Bacterial Strain and Plasmid Construction

For bacterial growth, Luria broth and nutrient agar were prepared in distilled water by maintaining pH 7. The *Agrobacterium tumefaciens* strain GV3101 carried the plasmid pPCV002-A (Figure 3.1), generously provided by Dr. Spena, Max-Planck-Institut fur Zuchtungsforschung, 5000 Koin 30, FRG [53], for the purpose of transformation. The plasmid's T-DNA region encompassed the coding region of the *rol A* gene with the CaM35S promoter regulating the *rol A* gene's expression. Additionally, the T-DNA region contained the *NPTII* gene (neomycin phosphotransferase gene) governed by the NOS promoter, along with NOS terminator sequences. The details of the construct are given in the Figure 3.1.

3.6.2 Plant Material for Transformation

The experiments of the present research were performed using *Artemisia carvifolia* seeds, procured from Astore, in Pakistan's northern regions. The seeds were preserved in airtight jars, sealed plastic envelopes, at a cool, dry place. After germination, the plantlets obtained were used for the transformation experiment.

3.6.3 Media Preparation for Enhanced Transformation and Shoot Development

Media employed for seed germination and shoot regeneration were prepared according to the reported protocol [50]. Table 3.4 shows the composition of seeds germination and regeneration media. To enhance the growth, plant growth hormones BAP and NAA at specific concentration were added in the media. The

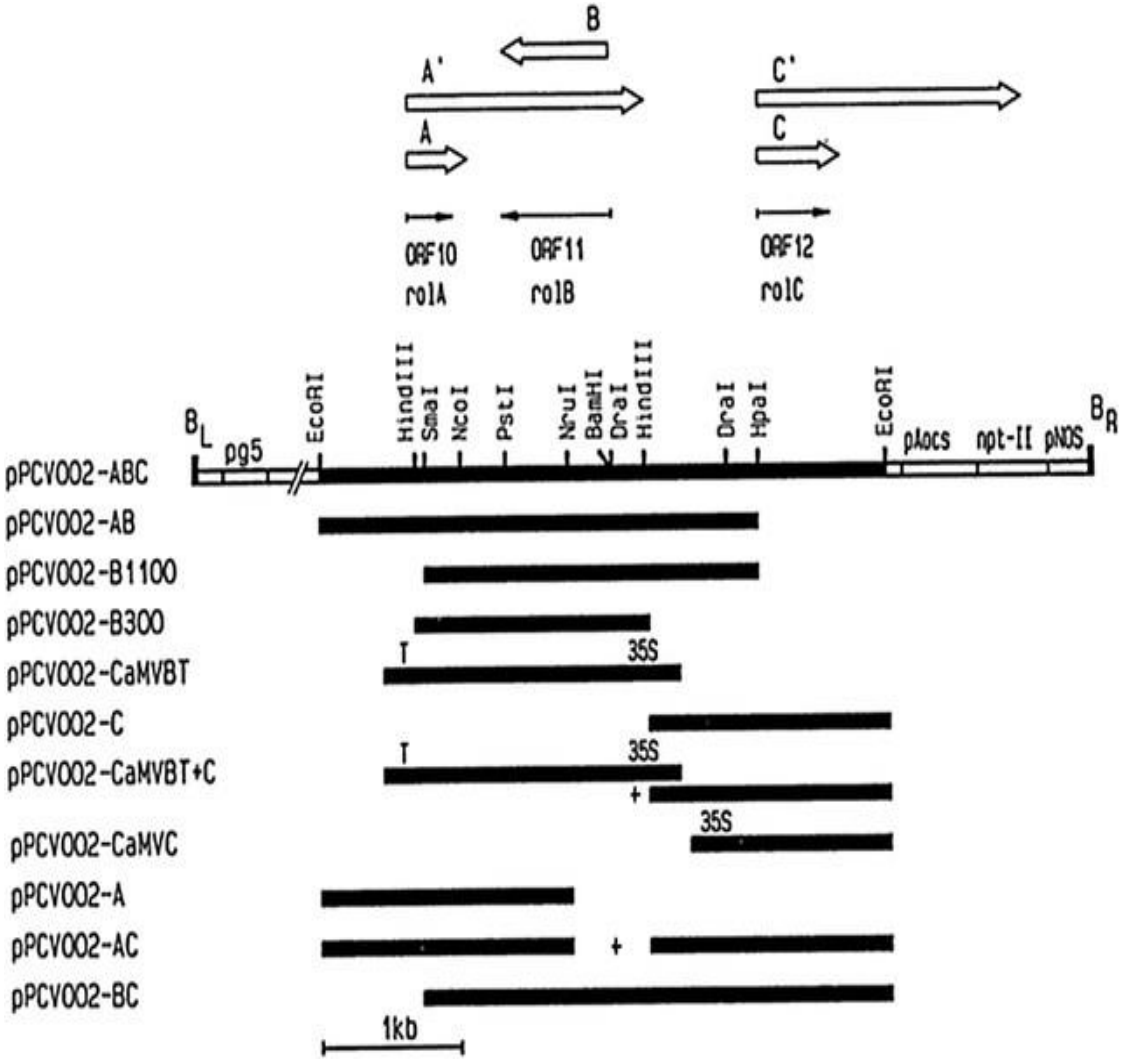


FIGURE 3.1: Map of Vector (pPCV002-A) Used for the Genetic Transformation of Artemisia carvifolia Buch [53]

pH was set to 5.8 and after autoclaving the media, BAP and NAA were added in the media in the combinations as given in Table 3.5 to make media for shoot organogenesis [50].

3.6.4 Sterilization of Seeds and Germination

Seeds were surface sterilized using 70% ethanol for different time durations like 30 sec, 1 min, 1.5 min, 2 min, followed by 0.1% (w/v) mercuric chloride ($HgCl_2$) for 15 sec. The seeds were then dried on autoclaved filter paper in a laminar flow hood, after being rinsed three times with autoclaved distilled water. To optimize

TABLE 3.4: MS Media for Germination of Seeds and Plant Regeneration

Experiment	**Medium**	**Components**	**Conc./L**
Seeds germination & Rooting media	$\frac{1}{2}$ MS Medium	MS basal salts	2.22 g
		Sucrose	20 g
		Agar	5 g
		Dist. H2O	1 L
Shoot regeneration	MS Medium	MS basal salts	4.4 g
		Sucrose	30 g
		Agar	5 g
		Dist. H2O	1 L

the media for seeds germination, seeds were placed in water, plain agar, $\frac{1}{2}$ MS and MS medium in small petri plates while maintaining the sterile conditions.

The best germination media found was then used in whole experiments. Under aseptic circumstances, these petri plates were then kept in a growth chamber with a temperature of 25^{o}C, a photoperiod of 16 hours, an illumination of 45 μE m^{-2} s^{-1}, and a relative humidity of 60%. The seedlings obtained from these germinated seeds were then used in further experiments.

3.6.5 Optimization of Explants and Media for Regeneration

Leaf, stem and stem with nodes were removed from one-month old plants of *A. carvifolia* and cut into 0.5-1 cm pieces under the laminar flow hood. These explants were then shifted to different regeneration media (Table 3.5).

Around 4-5 explants were cultured in one petri plate and total 15 petri plates were prepared for each media preparation. The experimentation was repeated many times. The best shooting media and explants with best regeneration was then used in transformation experiment.

TABLE 3.5: Compositions of Media for Shoot Regeneration and Rooting of Plants.

Medium	**Composition**
Shoot regeneration media 1	MS medium + BAP (0.1mg/L) + NAA (0.1mg/L)
Shoot regeneration media 2	MS medium + BAP (0.5mg/L) + NAA (0.1mg/L)
Shoot regeneration media 3	MS medium + BAP (1mg/L) + NAA (0.1mg/L)
Rooting media	MS+ NAA (0.1 mg/L)

3.6.6 Preparation of Explants for Transformation

Explants including leaf, stem and stem with nodes were excised from one month old in vitro grown *Artemisia carvifolia* seedlings, as mentioned in previous section, and precultured on shooting media (Table 3.6). All work was done in laminar flow hood to prevent contamination chances. In each petri plate, 5-6 explants were cultured [50].

3.6.7 Media Preparation for Transformation Experiment

For transformation procedure, co-cultivation and selection media were prepared as shown in Table 3.6. To prepare the medium, 500 mL flasks were used which were then sealed by using cotton and aluminum foil, and subjected to autoclaving for 20 minutes at 15 psi pressure and 121°C. Under a laminar flow hood, the autoclaved medium was poured into 75 mm diameter petri dishes, allocating 25 mL of medium to each dish.

Subsequently, the medium within the petri dishes solidified, benefitting from the laminar flow hood's sterile environment. The composition of co-cultivation media was same as preculturing media [50]. The composition of media used during transformation is given in Table 3.6.

TABLE 3.6: Components of Media Utilized in Transformation Protocol

Medium	**composition**
pre-culturing shooting media	MS medium+ BAP 0.5 mg/L + NAA 0.1 mg/L + 200 μM acetosyringone
co-cultivation media	MS medium+ BAP 0.5 mg/L + NAA 0.1 mg/L + 200 μM acetosyringone
selection media	MS medium+ BAP 0.5 mg/L+ NAA 0.1 mg/L+ kanamycin 50 mg/L + cefotaxime 300 mg/L
Rooting media	MS medium+ NAA 0.1 mg/L+ Kanamycin 30 mg/L

3.6.8 Transformation of *Artemisia carvifolia* with *Agrobacterium tumefaciens*

The *Artemisia carvifolia* genetic transformation was carried out employed the method previously outlined [50].

3.6.8.1 *Agrobacterium* Culture Preparation

Following the introduction of *Agrobacterium tumefaciens* (strain GV3101 harboring pPCV002-A) into a 100 mL flask that contained 50 mL liquid LB medium, augmented with antibiotic kanamycin (50 mg/L) under laminar flow hood, the bacterial cultures were left to incubate overnight in a shaker incubator at 120 rpm and 28°C. Once the cultures gotten 24 hours of growth and the optical density (OD) fell within the range of 0.2 to 0.8, the plants were transformed using the bacterial culture.

3.6.8.2 Transformation Protocol

1. The nodal explants that were excised from one-month old *Artemisia carvifolia* plants and precultured on preculturing media were removed and infected by *Agrobacterium* culture for various time spans (2.5, 5, 10, and 20 minutes).

2. Afterward, the explants were placed on autoclaved filter paper in large petri plate to get rid of extra bacterial culture and then co-cultivated in a medium

called co-cultivation medium containing 200 μM acetosyringone (previously called preculturing media) for different time durations i.e. 12, 24, and 48 hours. About eight to ten explants per petri plate were co-cultivated.

3. The growth chamber was set up with the plates containing the explants at 27^{o}C, 16 hours of photoperiod, 45 uEm^{-2} s^{-1} of illumination, and a relative humidity of 60%.

4. Following a two-day co-cultivation period, the explants underwent multiple washings with autoclaved distilled water. After utilizing diluted cefotaxime to eradicate bacteria, autoclaved distilled water was used for another wash.

5. After that, the explants were placed on large petri plate comprising autoclaved filter paper and moved to selection medium for regeneration of shoots (containing 50 mg/L kanamycin and 300 mg/L cefotaxime).

6. Following a two-week period, explants were moved to a fresh media for the initial 30 days. Subsequently, sub-culturing was carried out at three-week intervals thereafter.

7. The cefotaxime concentration was minimized to 150 mg/L after a span of two months and completely omitted after 3 months.

8. The 2-3 cm developed shoots were cut and put in rooting media (Table 3.6) for root development.

3.7 Transplantation to Pots and Acclimatization

Following the regeneration of modified plants, the wild type control plants and plants resistant to kanamycin were individually transplanted into small pots containing a soil mixture composed of equal parts of clay, sand, and peat. To facilitate proper drainage and prevent root damage, small pores were created at the bottom of the pots.

Transparent polythene bags were used to enclose the plants after transplantation to retain moisture. The acclimatization process took place over one month in a growth

chamber with conditions set at 25±2°C, a photoperiod of 16 hours, illumination of 45 μEm^{-2} s^{-1}, and a relative humidity of 60%. After the plants had undergone adequate hardening, they were transferred to a greenhouse, and diligent care was provided until reaching maturity. A thorough examination revealed several differences in the morphological characteristics between the control plants and the transgenic plants resistant to antibiotics. These variations in morphological parameters were carefully observed and documented.

3.8 Molecular Analysis of Transformed Plants

To ascertain the successful integration of *rol A* gene into the plant's genetic makeup following a transformation procedure, a comprehensive molecular examination was carried out, employing PCR and Southern blot analyses. The experimental design encompassed the extraction of genomic DNA for PCR analysis from transformed as well as untransformed plants, alongside the isolation of plasmid DNA from *Agrobacterium tumefaciens* strains harboring the pPCV002-A vector.

3.8.1 Genomic DNA Extraction from Plants

For extraction of genomic DNA from germinated plantlets, an efficient and simple plant genomic DNA extraction procedure i.e. CTAB method was used with some changes [146]. The composition of DNA extraction solutions is given in Table 3.1. The steps involved in the process of DNA extraction are given below:

- The plant material (200 mg) which was at a frozen state, underwent grinding into a finely powdered form using a pestle and mortar immersed in liquid nitrogen.
- When ground powder formed, 500 μL CTAB extraction buffer was introduced.
- The resulting mixture was then transferred to vial and SDS 20% (40 μL) was added to each vial.

- The vials were then vortexed for 30 sec.
- The vials were then positioned in a water bath for incubation at 65^{o}C for 35 minutes with frequent shaking.
- Phenol: chloroform was added at the ratio of 25:24 to each vial in quantity of 500 μL before placing on an inverted shaker for a duration of 20 minutes.
- To resolve phases, centrifugation was done out for 5 minutes at 14000 rpm (maximum speed).
- The upper aqueous phase (also called supernatant), which contains nucleic acids, was transferred to a new vial.
- Vials were shaken after being filled with chilled ethanol (100%) and then left at - 20°C overnight to allow DNA to precipitate.
- The following day, vials were subjected to centrifugation at 14000 rpm for a duration of 15 minutes.
- The upper layer was drained away to obtain the pellet.
- Each vial received 500 μL of 70% ethanol, which was applied to wash the pellet three times.
- After draining the liquid and evaporating the ethanol, the pellet was then dissolved in TE buffer containing RNases.
- The vials were kept at -20^{o}C in a refrigerator.
- By measuring the amount of light absorbed at wavelengths of 260 nm and 280 nm and determining the corresponding ratio, the DNA quality and quantity were evaluated.

3.8.2 Isolation of Plasmid DNA

To extract plasmid DNA, the standard protocol [148] was used with some changes. The alkaline lysis method was used in this regard, as described below. All solutions used for isolation of bacterial plasmid are mentioned in Table 3.7.

Bacterial culture *(Agrobacterium tumefaciens* strain GV3101 harboring plasmids pPCV002-A) that were grown previously was transferred to 15 mL tubes and centrifuged at 4000 rpm for 10 minutes at 4^oC to recover the cells. After decanting the tubes to remove the upper layer, the cells were permitted to air-dry.

- The bacterial pellets were collected and 200 μL of ice-cold alkaline lysis solution I was added, followed by thorough mixing via vertexing.
- Following this, the resultant suspension was carefully transferred into microfuge tube. Subsequently, 400 μL of freshly prepared alkaline lysis solution II was introduced to the microfuge tube. Ensuring thorough mixing, the contents were inverted rapidly 5 times before being chilled on ice for a duration of 5 minutes.
- After adding alkaline lysis solution III (300 μL), the contents underwent gentle mixing by rapid inversion numerous times, and subsequently left on ice for a duration of 3-5 minutes. This strategic utilization of low temperature and pH conditions serves to augment the renaturation of denatured DNA.
- The centrifugation at maximum speed was taking place for 15 minutes, leading to the formation of an upper layer carrying the renatured DNA, which was carefully shifted into new microfuge tube.
- An equal volume of phenol: chloroform at the ratio of 25:24 was then added for DNA purification. After vertexing the aqueous and organic phases together, the tubes underwent centrifugation for five minutes at maximum speed, and the upper layer was carefully moved to a fresh microfuge tube.
- The DNA was precipitated by introducing twice as much ethanol to this upper layer supernatant which was then kept at -20^oC for an hour.
- Next, the tubes were subjected to a 20-minute centrifugation at 14000 rpm in order to extract the precipitated DNA. After discarding the supernatant, the tubes were dried with paper towels.

- The pellet was cleaned two times with 70% ethanol to get rid of any leftover salt. The tubes were dried on paper towel to dry the pellet. Pellet was dried until smell of ethanol vanished.
- After dissolving the pellet in 60 μL of TE buffer, it was refrigerated at -20^{o}C to facilitate a subsequent PCR reaction.

TABLE 3.7: Solutions of Plasmid Extraction

Solutions	**Chemicals**	**Concentrations**
Solution 1	Tris	25 mM (pH 8.0)
	EDTA	10 mM (pH 8.0)
	Glucose	50 mM
Solution II	SDS	1%
	NaOH	0.2 N
Solution III	Sodium acetate	3 M (pH 4.8)
TE buffer	Tris	10 mM (pH 8)
	EDTA	1 mM

3.8.3 Polymerase chain reaction (PCR)

Polymerase chain reaction was conducted to check the presence of *rol A* gene. The PCR reaction was carried out in 25 μL final reaction mixture and the PCR conditions were followed according to reported method [149]. The composition of PCR mixture for detection of gene of interest, the primer sequences, PCR program for amplification of *rol A* gene are given below in Tables 3.8, 3.9 and 3.10 respectively. A programmed DNA thermal cycler was used to conduct the PCR study.

Table 3.8: Composition of Master Mix of PCR

Technique	**Reagent used**	**Concentration**
Polymerase chain reaction	dNTPs	0.2 mM
	DNA	50 ng
	F primer	0.25 μg
	R primer	0.25 μg
	Taq polymerase	0.2 U
	MgCl2	2 mM
	PCR buffer	5 μL

Table 3.9: Primer Sequences of Studied Genes

Gene	**Primer sequences**	**Length of amplified product**
Rol A	F: 5'-AGAATGGAATTAGCCGGACTA-3' R: 5'-GTATTAATCCCGTAGGTTTGTT-3'	308 bp
NPTII	F: 5'-AAGATGGATTGCACGCAGGTTC-3' R: 5'-GAAGAACTCGTCAAGAAGGCGA-3'	781 bp
β-Actin	F: 5'-ATCAGCAATACCAGGGAACATAGT-3' R: 5'-AGGTGCCCTGAGGTCTTGTTCC-3'	160 bp

Table 3.10: PCR Program for Amplification of Studied Genes

Step	**Temperature**	**Duration**
Initial denaturation	95	5 mins
Cyclic denaturation	95	30 sec (25 cycles)
Cyclic annealing	54	2 mins
Elongation	71	2 mins
Final elongation	71	12 mins

3.8.4 Agarose Gel Electrophoresis

This technique was conducted to check the PCR yields. In order to prepare a 1.5% w/v agarose gel, 1.5 grams of agarose were melted in 100 milliliters of 1X TBE

buffer in a microwave oven. To stain DNA, 2 μL of ethidium bromide solution was added. 0.2% bromophenol blue mixed with 40% sucrose was used as loading dye (3 μL) and was properly combined with DNA samples in the wells. The electrophoresis was carried out in a gel tank with 1X TBE buffer running for 60 minutes at 100 volts and 60 mA.

Ingredients of loading dye

Bromophenol blue (BPB)	0.25%
Sucrose solutio	40%

Ingredients of 10 X TBE

Chemicals	Amount used	Final concentration
Boric acid	55 g	890 mM
Tris	108 g	890 mM
Na_2EDTA	9.3 g	20 mM (pH 8.3)

3.8.5 Southern Blot Analysis

The DNA extracted from the transformed leaves through CTAB extraction process before, was used in the southern blot analysis of transformants. Subsequently, the fragments of DNA were then shifted to a nylon membrane that was positively charged, after the DNA had been digested and separated on agarose gel following the standard protocol [148]. The probe was made, and it was used to hybridize membrane-carrying plant DNA. Subsequently, the membrane was exposed by placing it over an x-ray film. All steps are explained below.

Subsequently, the membrane was exposed by placing it over an x-ray film. All steps are explained below.

Step 1: DNA Restriction

Digestion restriction was performed employing a restriction endonuclease in accordance with manufacturer guidelines (Fermentas). In 100 μL reaction, 3-5 μg of DNA extracted from transgenic plants underwent digestion with EcoRI and cleaved at a specific restriction site within the T-DNA area of the *rol A* construct. Following are the components used.

✓	DNA	5 μg
✓	EcoR1	5 μL
✓	PCR H_2O	up to 100 μL
✓	10X Buffer	10 μL

The digestion mixture was subjected to incubation in a reaction for 16 hours at 37^{o}C. Subsequently, the DNA was rinsed with 70% ethanol, precipitated using sodium acetate and ethanol, and reconstituted in TE buffer (15 μL).

Step 2: Agarose Gel Electrophoresis

Each sample received 5 μL DNA loading buffer and the digested DNA was separated on agarose gel (0.8% (w/v) TBE) with ethidium bromide (0.5 μg/mL (w/v)). A steady current of 40 mA was used during the 16 hours electrophoresis process.

Step 3: Membrane Transfer

When the digested DNA on the agarose gel had been completely separated, the gel was treated with distilled water and put in a clean dish containing 0.25 M

HCl solution to keep shaking for 20-25 minutes at normal room temperature. The gel was then immersed in the denaturation solution for 30 minutes while being continuously shaken at room temperature. After which it was neutralize in neutralization solution for half an hour at room temperature while being shaken. The gel was then rinsed with autoclaved distilled water. In another plate that contained 20X SSC, a sponge was partially immersed. In order to avoid air bubbles, Whatman filter paper the size of a sponge was placed on the sponge, followed by the gel moistened with 20X SSC. Three sheets of Whatman filter papers dipped in 20X SSC were placed on top of an equal-sized positively charged nylon membrane after it had been placed on the gel. A stack of 4 cm high tissue paper was placed on top of the setup, and then a glass plate with weight was placed on top of that. The apparatus was left in its current state before being dismantled the following day. The recovered nylon membrane was then cleaned with 2X SSC and left to dry. In order to fix the transferred DNA fragments, the nylon membrane was baked at 120^{o}C for half an hour. The components of solutions are given below in Table 3.11.

TABLE 3.11: Compositions of Solutions Used in Transfer of Restriction Fragments to Membrane

Solutions	**Components**	**Concentrations**
Denaturation solution	NaOH	0.5 M
	NaCl	1.5 M
Neutralization solution	Tris-HCL	0.5 M (pH 7.2)
	NaCl	1.5 M
20X SSC	NaCl	3 M
	Sodium citrate	0.3 M

Step 4: Procedure of DNA Labeling

The probe for the experiment consisted of PCR-amplified yields of *rol A* gene from plasmid. In order to label the probe, digoxigenin (DIG)-11-dUTP with DIG High Prime DNA Labeling reagents were used. In a nutshell, 4 μL probe (1μg DNA) was added in 12 μL autoclaved distilled water. The mixture was well mixed and then the DNA was denatured by heating the mixture for 10 minutes at 95°C

before being moved swiftly to ice for 3-5 minutes. Finally, 4 μL of DIG-High prime labeling mixture i.e. DIG-High prime, nucleotides, random primers, DIG-dUTP, klenow enzyme and buffer components were added. After mixing, it was incubated overnight at 37^{o}C. The following day, the mixture was heated to 65^{o}C for 10 minutes to halt the reaction before being kept at -20^{o}C until needed. Prior to use, the probe was immediately refrigerated on ice after being incubated at 95^{o}C for 10 minutes to cause denaturation.

Step 5: Hybridization Process

Without adding labeled DNA, the pre-hybridization process was carried out at 43^{o}C in 50-100 mL DIG Easy Hybridization solution. The hybridization solution was thrown away after 2 hours and replaced with the fresh (20-30 mL) solution. The hybridization was then carried out overnight at 43^{o}C after adding labeled DNA probe.

Step 6: Post Hybridization Process

For post hybridization washing process, first of all, the membrane was washed twice with solution (2X SSC, 0.1% SDS) at 20-25^{o}C for 30 minutes while being constantly stirred. After that, the membrane was washed twice with solution (0.5X SSC, 0.1% SDS) at 68^{o}C for 30 minutes while being constantly agitated.

Step 7: Immunological Detection

For immunological detection step, blocking and antibody solutions were prepared.

Blocking Solution

A 1X working solution was created by diluting the 10X blocking solution (kit component) at a 1:10 ratio with Maleic acid buffer.

Antibody Solution

The kit component, anti-digoxigenin-AP, was centrifuged for five minutes at 10,000 rpm. The necessary volume was then pipetted and added to the blocking solution at a ratio of 1:1000 (1μL / 10 mL).

According to the instructions provided in the DIG DNA labeling and detection kit (Roche) manual, immunological detection was performed on X-ray film using the CSPD (Chemiluminescent substrate for alkaline phosphatase detection) substrate. Following hybridization and stringency washes, the membrane was briefly rinsed in washing buffer for 5 minutes before being incubated for 30 min in 100 mL of blocking solution (kit component). The membrane was next incubated for 39 minutes in 20 mL antibody solution (40 mL / 50 cm^2) before being washed for 15 min in washing buffer solution. Following a 5-minutes equilibration in detection buffer, the membrane was exposed to CSPD with the DNA side facing up for 5 minutes at 20^{o}C in a plastic bag.

For the elimination of extra CSPD, the membrane was placed in two glass plates, covered with plastic sheets, and incubated at 37^{o}C for 10-12 minutes. After the development folder's edges were closed, the membrane was subjected to X-ray film (Kodak) overnight at -70^{o}C in order to improve signal intensity and then developed using X-ray developing solutions. The compositions of buffering reagents are given below (Table 3.12).

Table 3.12: Compositions of Buffering Solutions

Solutions	**Components**	**Concentrations**
Washing buffer (pH 7.5)	Maleic acid	0.1 M
	NaCl	0.15 M
	Tween 20	0.30%
Detection buffer (pH 9.5)	Tris-HCL	0.1 M
	NaCl	0.1 M
Maleic acid buffer (pH 7.5)	Maleic acid	0.1 M
	NaCl	0.15 M

3.9 Semi-quantitative RT-PCR

To verify the expression of *rol A* gene, semi quantitative RT PCR was used. The RNA was extracted from transformed and untransformed shoots of 4 months old plants by using the method given below in section 3.9.1, to check its purity or quantity by measuring the absorbance at wavelengths of 260 nm and 280 nm using a Nanodrop ND-2000 spectrophotometer (Thermo scientific) [50]. A 1.2% agarose gel was used to analyze RNA in order to determine its quality.

3.9.1 RNA Extraction

Young leaves were collected from four months old transformed and wild plants of *Artemisia carvifolia* and total RNA was taken out by using the Trizol reagent [50]. The following steps were taken place.

- With the aid of a cooled and clean, uncontaminated pestle and mortar, 100 mg of plant material was ground in liquid nitrogen and then transferred to a sterile vial (2 mL).
- The vial was filled with Trizol reagent (1mL/100mg of pulverized tissue), and it was vortexed for 15-20 seconds, afterwards, 3 metallic balls were added to the vial, homogenized in a tissue lyser and then placed at room temperature for 4-5 minutes.
- To the vial, chloroform (0.2mL/mL trizol) was added and the tubes were gently inverted to mix the mixture, followed by centrifugation at 4^{o}C at 13000 rpm for 15 minutes.
- The upper layer was moved to a fresh vial on ice. Afterwards, isopropanol (0.5mL/mL trizol) was added to it and the mixture was thoroughly blended by repeatedly inverting the vial, and then kept at room temperature for ten minutes.
- Afterwards, the vial was centrifuged at 13000 rpm for ten minutes at 4^{o}C to collect the pellet. The supernatant was removed and the pellet received 1 mL of 75% ethanol before being placed on ice for 30 minutes.

- Once more, centrifugation at 13000 rpm for ten minutes at 4^{o}C was done. The pellet was de-ethanolized and allowed to dry at room temperature.
- In order to prepare the pellet for usage, it was finally dissolved in 50 μL of DEPC treated water and kept at -70^{o}C.

3.9.2 cDNA Synthesis/ DNase Treatment

The RNA that was extracted from transformed and untransformed shoots of 4 months old plants was processed with Turbo DNAse (Ambion) to guarantee that all DNA had been completely removed from the RNA. For this purpose, following steps were applied.

Reverse transcription of 1 μg of RNA was conducted for 60 minutes at 42^{o}C in a 20 μL reaction volume with the following ingredients:

- 200 units of RevertAid M-MuLV reverse transcriptase (Fermentas),
- 20 units of RNase inhibitor,
- 1 mM dNTPs
- 1 X reverse transcriptase buffer,
- 5 μM Oligo (dT) 18 primer.

The reaction was finished by incubation at 70^{o}C for 5 minutes. For template, 1 μL of this cDNA reaction mixture was used and semi-quantitative reverse transcriptase-polymerase chain reaction was conducted with *rol A* gene primers as done previously to assess the expression of *rol A* gene, as primers of *rol A* gene used in PCR (Table 3.9) were also used in RT-PCR. The gel image of PCR products was scanned by using Kodak Molecular Imaging software and integrated density values were computed for each band. As an internal control, β-actin gene was used to ensure the reaction. For this, a control RT-PCR reaction was executed with β-actin gene primers (Table 3.9). PCR was conducted using a programmed DNA thermal cycler

(Biometra, USA). The reaction conditions for genes amplifications are mentioned in Table 3.13.

TABLE 3.13: PCR Program for Amplification of Genes

Step	**Temperature**	**Duration**
Initial denaturation	95	5 mins
Cyclic denaturation	95	30 sec (25 cycles)
Cyclic annealing	54 *(rol A)* 60 (β-actin)	1 min
Elongation	70	1 min
Final elongation	70	10 mins

3.10 Flavonoids Evaluation through HPLC-DAD System

Polyphenol levels in extracts obtained from both transformed and wild type *A. carvifolia* plants were quantified through HPLC-DAD analysis. The protocol adhered closely to the one previously reported [150] with minimum alterations.

3.10.1 Chemicals and Reagents

The chemicals or reagents like methanol, n-Hexane, acetonitrile, and ethyl acetate along with standards, vanillic acid, catechine, syringic acid, rutin, gallic acid, coumaric acid, caffeic acid, ferulic acid, geutisic acid, and cinnamic acid, were of HPLC rank.

3.10.2 Preparations of Standards

Methanol was used to prepare the standard stock solutions of flavonoids i.e. gallic acid, vanillic acid, rutin, catechine, syringic acid, coumaric acid, geutisic acid, caffeic acid, ferulic acid, and cinnamic acid at the concentration of 1000 μg/mL and stored at -20^{o}C for further usage. This freshly prepared stock solutions were

used to prepare different serial dilutions, 10, 20, 50, 100, 150, and 200 μg/mL in series, for the standard calibration curve (graph), from which the analysis of flavonoids (quantitative/qualitative) was performed.

3.10.3 Sample Preparation for Flavonoids Extraction

Young shoots were used for flavonoids extraction according to reported method. In a nutshell, flavonoids from 10 mg of dry powdered plant material were brought out in 300 μL of methanol at ambient temperature for 15 minutes using a sonicator bath. The extract was then centrifuged at 13000 rpm for 5 minutes, and the supernatant was transferred to a new vial. Supernatant was gathered after the process was carried out three times. The material was then dried in a vacuum cell at 50^{o}C and then placed in 200 μL of a methanol: water (2:1) solvent mixture, and then transferred to HPLC vials for additional analysis after being filtered.

3.10.4 Preparation of Mobile Phase for Experiment

The preparation of the mobile phase involved two components:

- ✓ Mobile phase A: Acetonitrile + 0.5% formic acid
- ✓ Mobile phase B: H_2O + 0.5% formic acid

3.10.5 Instrumentation and Conditions for HPLC

Flavonoid quantitative analysis was conducted using the HPLC–DAD system, coupled with a symmetry C-18 analytical column and an autosampler. The wavelength was set at 235-450 nm, and a pressure of 200 psi was applied. A mobile phase consisting of acetonitrile with 0.5% formic acid (A) and water with 0.5% formic acid (B), running at a flow rate of 1 mL/min was used to achieve separation. The injection volume was 10 μL, and the retention time was 27

min. Peak identification in the extracts was performed by contrasting retention indices with reference standards. Prior to sample and standard injection, the HPLC system underwent a pre-analysis phase where it pumped the mobile phase and equilibrated for 1 hour, ensuring the establishment of a stable baseline. For the proper maintenance of the HPLC system, a daily routine was implemented, involving running the mobile phase, followed by 50% and 100% methanol runs for 30 minutes each, after the last sample run. The mobile phase maintained a pH below 7.0 to avoid column dissolution. Detection of analytes occurred at specific wavelengths, along with their corresponding retention times (Table 3.14).

TABLE 3.14: Retention Time of Examined Flavonoids with Wavelength

S. No.	Standard flavonoids	Signal wavelength (nm)	Retention time (min)
1	Vanillic acid	257	9.116
2	Rutin	257	12.649
3	Gallic acid	279	3.679
4	Catechin	279	7.003
5	syringic acid	279	9.76
6	Coumaric acid	279	13.79
7	Geutisic acid	325	7.433
8	Caffeic acid	325	9.252
9	Ferulic acid	325	12.642
10	Cinnanic acid	325	13.79

3.10.6 Calibration Curve

The construction of the calibration curve involved plotting the analyte's chromatographic area (AU) against its concentration (mg/mL). A discernibly linear response was evident, as reflected by a correlation coefficient (R2) exceeding 0.99, indicating a high degree of linearity. The accuracy in determining standard concentrations fell within the range of 80-100%. This calibration process involved serial dilutions 10, 20, 50, 100, 150, and 200 μg/mL of all standard flavonoids. The resulting calibration curve, along with the derived linear regression equation were employed for quantifying flavonoids in the samples. That equation facilitates the conversion

of Peak Area values to concentrations, offering a robust method for quantifying flavonoids in the analyzed samples.

3.10.7 Peak Identification and Verification

The co-injection method was employed to unequivocally identify peaks corresponding to vanillic acid, catechine, syringic acid, rutin, gallic acid, coumaric acid, caffeic acid, cinnamic acid, geutisic acid, and ferulic acid. Each supposed peak for every standard was duly observed, and the corresponding areas were recorded from the initial sample that underwent the run.

3.10.8 Statistical Analysis

Statistical analysis was conducted on all experiments, with each sample subjected to triplicate measurements. The dataset underwent statistical evaluation utilizing t-test and Two-way ANOVA with Graphpad Prism software.

3.11 Evaluation of Genes Participating in the Flavonoid Biosynthetic Pathway via Real-Time qPCR

3.11.1 RNA Extraction

RNA extraction process is already explained in section 3.9.1

3.11.2 DNase Treatment

The RNA that was extracted from transformed and wild type shoots of four months old plants was processed with Turbo DNase (Ambion) to guarantee that all DNA

had been completely removed from the RNA, resulting in a purified RNA sample ready for subsequent analyses. For this purpose, following steps were applied.

- In the extracted RNA, DNase buffer was added at 0.1 volumes, resulting in a concentration of 5 μL for every 50 μL of RNA.
- Subsequently, 1 μL of DNase was carefully introduced to the RNA, and the mixture underwent thorough mixing before being subjected to incubation at 37^{o}C for 30 minutes. Afterwards, the DNase inactivation solution at 0.1 volumes was introduced.
- The resulting mixture was thoroughly mixed before being incubated for an additional 5 minutes at room temperature, after which, the mixture underwent centrifugation at 10,000 rpm for 5 minutes.
- Finally, the supernatant was carefully transferred to a fresh vial.

3.11.3 cDNA Synthesis

Following DNase treatment, extracted RNA was transformed into cDNA using an Invitrogen first strand cDNA synthesis kit in accordance with the manufacturer's instructions. In order to achieve that, 1 μg of RNA was transformed into cDNA by adding the following reagents to the reaction mixture until the final volume reached 12 μL [50].

Reagents	**Concentrations**	**Volume**
RNA	1 μg	X μL
dT15	50 μM	1 μL
dNTPs	10 mM	1 μL
DEPC H2O	-	X μL
Final volume	-	12 μL

After thoroughly combining all of the ingredients in a vial, the mixture was quickly centrifuged, allowed to incubate at 65^{o}C for five minutes, and then additional ingredients (listed below) were added.

Reagents	Volume
5xbuffer (kit)	4 μL
DTT (0.1 m)	2 μL
RNases out	1 μL

The mixture was mixed and briefly centrifuged before being incubated for two minutes at 37^{o}C. Then, after adding 1 μL of RT enzyme (kit), the mixture was subjected to incubation for 50 minutes at 37^{o}C and then for 15 minutes at 70^{o}C. At the end, by measuring absorbance at 260/280 nm using a Thermo Scientific Nanodrop ND-2000 spectrophotometer, cDNA synthesis was verified.

3.11.4 Real-time Quantitative PCR Analysis

Selected genes encoding phenylalanine ammonia-lyase (PAL) and chalcone synthase (CHS) were subjected to quantitative real time PCR in order to assess the potential effects of *rol A* gene on the expression of flavonoids biosynthesis genes by using the previously described method [50]. The reference gene was the β-actin. The gene specific primers (that target specifically) were used (Table 3.15). For experiment, a 1:4 dilution of cDNA was used. A 384-well platform was used for the qPCR, using iTAqTM Universal SYBR Green Supermix (BioRad, Hercules, CA, USA). The real time qPCR reaction conditions were as followed (Table 3.16).

TABLE 3.15: Genes Primer Sequences Examined by Real-time qPCR

Genes	Primer Sequences	Accession Number
CHS	F: AGGCTAACAGAGGAGGGTA	GQ468548.1
	R: CCAATTTACCGGCTTTCT	
PAL	F: ACACTCGGTTAGCTATTGCTGCAA	FJ481987.1
	R: CCATGGCGATTTCTGCACCT	
β-Actin	F: ATCAGCAATACCAGGGAACATAGT	EU531837
	R: AGGTGCCCTGAGGTCTTGTTCC	

TABLE 3.16: Real-time qPCR Reaction Conditions for Genes Amplifications

Step	**Temperature**	**Duration**
Initial denaturation	94	6 mins
Cyclic denaturation	94	10 sec (45 cycles)
Cyclic annealing	65	12 sec
Elongation	71	12 sec
Final elongation	71	10 mins

3.12 Pharmacological Evaluation of Transformed Plants

The antioxidant and anticancerous potential of *rol A* gene transformants were measured through in vitro antioxidant assays and MTT assay.

3.12.1 Antioxidant Potential Measurement through Bioassays

3.12.1.1 Material Preparation

Methanolic extracts of both wild type and transgenic plants of *A. carvifolia* were prepared with 1 g of dry powdered plant material [51]. The dried material was immersed in 3 mL of solvent mixture of methanol and chloroform (1:1). The mixture was then sonicated for 30 min and centrifuged at maximum speed for 5 min. the supernatant obtained was transferred to a fresh falcon tube. The remaining plant residue was subjected to all of the preceding stages three times, and the resultant supernatant was collected. This collected supernatant, containing the target components, was then subjected to drying in a vacucell for evaporation of the solvent. The final dried residue obtained was then dissolved completely in DMSO until it reached a final concentration of 100 mg/mL.

3.12.1.2 Determination of Total Phenolic Content

The determination of the total phenolic content in *A. carvifolia* transformed and wild type plant extracts was conducted utilizing a modified Folin-Ciocalteu method [151].

Assay Procedure

In order to determine the overall phenolic content, 98 μL of Folin-Ciocalteu reagent (1:10 distilled water) was combined with 4 μL of plant extract (100 mg/mL) and allowed to incubate for 5 minutes at room temperature. Next, the reaction mixture was mixed with 98 μL of 6% sodium carbonate. There was a 200 μL total reaction volume, and the positive and negative controls were DMSO and gallic acid, respectively. After thoroughly mixing of the solution, it was left in a dark, room temperature place for 90 minutes to develop color. Subsequently, a 96-well micro-plate reader (Biotek Elx 800) was used to determine the absorbance at 725 nm against MS as blank. The absorbance values obtained were then used in the following formula to determine the sample's total phenolic content:

$Y = 0.0732 \text{ x } X - 0.0205$

In this formula, 'Y' represents the sample's absorbance and "X" denotes the sample's total phenolic content, which needs to be calculated in μg/mL.

3.12.1.3 Determination of Total Flavonoid Content

The determination of the total flavonoid content in *A. carvifolia* transformed and wild type plant extracts was conducted utilizing aluminum chloride ($AlCl_3$) colorimetric method [151].

Assay Procedure

Total flavonoid content was determined using the aluminum chloride colorimetric method. In this assay, 10 μL of 10% aluminum chloride and 10 μL of 1 M potassium

acetate were combined separately with 4 μL of each sample (100 mg/mL) and positive control quercetin. The volume was then increased up to 200 μL with the help of distilled water. The reaction mixture's absorbance was measured at 405 nm after it was left at room temperature for thirty minutes. DMSO was used as negative control. The following formula was used to determine the sample's total flavonoid content:

Y = 0.0101X – 0.004

In this formula, 'Y' represents the sample's absorbance and "X" denotes the sample's total phenolic content, which needs to be calculated in μg/mL.

3.12.1.4 Determination of Total Antioxidant Capacity

The determination of the total antioxidant potential of *A. carvifolia* transformed and wild type plant extracts was conducted utilizing the methodology reported previously [151].

Assay procedure

The assay was run on a 96 well plate. Firstly, 4 μL of plant extract (100 mg/mL) was added to each well, followed by the addition of 196 μL total antioxidant capacity reagent. Following a 90-minute incubation period at 90^{o}C in a water bath, the mixture's color turned dark blue. It was then cooled, and absorbance was measured at 630 nm using a micro plate reader (Biotek, Elx 800). DMSO was utilized as the negative control and ascorbic acid as the positive control. The following formula was used to determine the sample's total antioxidant capacity:

Ascorbic Acid Equivalence = 100 / 2.651 x Absorbance of sample μg/mL

3.12.1.5 Determination of Total Reducing Power Assay

The determination of the total reducing power of *A. carvifolia* transformed and wild type plant extracts was conducted utilizing the reported method [151].

Assay Procedure

This experiment was run on a 96-well plate. First, 490 μL of 0.2 M phosphate buffer and 20 μL of plant extract (100 mg/mL) were added to the Eppendorf tubes. After that, 490 μL of potassium ferricyanide was added to the mixture and it was incubated for 20 minutes at 50^{o}C.

To the Eppendorf tubes, 500 μL of 10% trichloroacetic acid was additionally added. After that, the mixture was centrifuged for 10 minutes at 3000 rpm. 500 μL of the supernatant was separated and transferred into a fresh eppendorf tube. By adding 100 μL of ferric chloride to it, the ferric chloride's color changed to blue upon reduction.

This sample was taken and put into wells in an amount of 200 μL. After that, the samples' absorbance was measured on a microplate reader at 630 nm. As positive and negative controls, ascorbic acid and DMSO were employed respectively. The following formula was used to determine the sample's reducing power:

Ascorbic Acid Equivalence = 100 /2.7025 x Absorbance of sample μg/mL.

3.12.1.6 Evaluation of Antioxidant Activity via DPPH Free Radical Scavenging Assay

The DPPH radical scavenging assay was employed to evaluated the antioxidant activity of the plant extracts, following a reported method by Dilshad et al. [51, 151].

Step 1: DPPH Solution Preparation

3.9 mg of solid DPPH was dissolved in 100 mL of methanol to create 0.1 mM DPPH solution. DPPH solution was freshly prepared and used. It was shaken thoroughly for half an hour on a magnetic stirrer in the dark to make sure it was completely soluble.

Step 2: Positive Control Preparation

Methanol was used to dissolve ascorbic acid at concentrations of 40, 20, 10, and 5 ppm to serve as a positive control.

Step 3: Samples Preparation

The assay was performed by using four different concentrations of plant extracts. The concentrations used were 1000, 500, 250, and 125 ppm, prepared in DMSO at final volume of 1000 μL.

Step 4: Assay Procedure

The assay was run in a micro plate with 96 wells. 180 μL of freshly made 0.1 mM DPPH solution was combined with 20 μL of each plant extract, or DMSO in the case of a negative control. Triplicate assays were performed for each concentration. After that, the reaction mixture was incubated for half an hour at 37^{o}C. After incubation, the DPPH solution's color changed from deep violet hue to light yellow shade. This was checked by measuring the reaction mixtures' absorbance at 517 nm using a micro plate reader. A blank solution comprising methanol (180 μL) and DMSO (20 μL) was employed for spectrophotometric analyses. Using the following formula, the determination of the percentage of DPPH free radical scavenging was observed for each concentration of the test compound.

$$Percentagescavenging = \frac{absorbanceofcontrol - absorbanceoftestsamplex100}{Absorbanceofcontrol}$$

3.12.2 MTT (Methyl Thiazolyl Tetrazolium) Assay

The MTT assay was used to evaluate the antiproliferative activity of the plant extracts under investigation. In this assay, the tetrazolium salt MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) is changed by living cells' mitochondria into a formazan, a distinctive purple precipitate [152].

The assay is used to evaluate how pure substances, medications, plant extracts, or natural substances affect the ability of treated cells to proliferate. Three distinct cell lines MCF7 (derived from breast carcinoma), HePG2 (derived from hepatic carcinoma), and HeLa (derived from cervical cancer cells), were employed for that purpose [151, 153].

Sample Preparation via Methanol Extraction

Flavonoids exhibit solubility in methanol so methanol was used as solvent to prepare the samples. The methanolic extraction was made according to the reported procedure [151]. A total of 100 mg of dried powdered material obtained from *A. carvifolia* plants, both transformed and wild type, was subjected to extraction with 1mL of methanol using a Sonication bath for 1 hour at room temperature. The resulting extract underwent centrifugation, and the supernatant was dried off through a rota-evaporator set at 40^oC. Subsequently, these dehydrated samples were reconstituted in 100% (V/V) DMSO solution, achieving a final concentration of 40 mg/mL, which was then tested on all cancerous cell lines. All samples underwent filtration using a filter that is safe for DMSO.

Mammalian Cell Lines and Culture Conditions

The ATCC provided the HepG2, HeLa, and MCF-7 cell lines under the following ATCC numbers: HTB-22^{TM}, CCL-2^{TM}, and HB-8065^{TM} respectively. These cells were grown in DMEM medium lacking pyruvate (Gilbco, Ref. 42430-023), which was augmented with 10% of heat inactivated fetal bovine serum FBS (Gilbco, Ref. 10270) and 1% of penicillin/streptomycin (Gilbco, Ref. 15140-122). We obtained all of the components from Gibco (Invitrogen). At 37^oC, the cells were treated with 5% CO_2. Cultivated as monolayer cultures in T-75 flasks Costar, the cultures were sub-cultured twice a week at 37^oC in an atmosphere with 100 % relative humidity, 5% CO_2 in the air. The cells were kept at a low passage number (5 to 20) for optimal conditions.

A 12-well plate was seeded with cells from the cell lines under investigation, in order to perform the cytotoxicity assay [151]. A 200 ppm concentrations of plant extracts were applied to the medium in each well, following a 24-hour period of cell growth. Viability assessments were carried out 48 hours after treatment. Every experimental condition was run in triplicate. To ensure uniformity across all assays, controls comprised one where cells were treated only with the solvent (DMSO) and another where they were not given any treatment. MTT method was used to assess the variability of the cells or mortality of cancerous cells.

Preparation of Chemical Reagents used in MTT Assay

MTT solution at 0.25%

For this solution, 125 mg MTT was mixed with 50 mL PBS 1X. The solution was then filtered through a filter (0.22 μM) at the cabin. The solution was shielded with aluminium foil because it is sensitive to light and kept at 4^{o}C.

Succinic Acid Solution

295 mg succinic acid was mixed with 50 mL of PBS 1X. The solution was then filtered through a filter (0.22 μM) at the cabin and kept at 4^{o}C.

SDS/acetic acid/DMSO

This solution was made by mixing 4.5 g of SDS with 257.4 μL of acetic acid and 45 mL of DMSO. The solution was always prepared at the time of usage.

MTT Assay Procedure

The MTT assay was performed as reported earlier [151]. Following the removal of the growth medium, 1 mL of fresh culture medium was mixed with 0.63 mM of MTT and 18.4 mM of sodium succinate. The cells were then incubated at 37^{o}C

for a duration of 3 hours. After that, the liquid medium was discarded and the formazan was reconstituted in DMSO containing 0.57% CH_3COOH and 10% SDS. The absorbance was then assessed at 570 nm using UV2310 spectrophotometer.

3.13 Statistical Analysis

Statistical analysis was conducted on all experiments, with each sample subjected to triplicate measurements. The dataset underwent statistical evaluation utilizing t-test and Two-way ANOVA with Graphpad Prism software.

Chapter 4

Results

4.1 DNA Barcoding for Identification of Plant

4.1.1 Seeds Germination

Following 70% ethanol and 0.1% mercuric chloride surface sterilization, seeds were allowed to germinate on $\frac{1}{2}$ MS media without the addition of growth hormone.

After being chilled in the dark for two days at 4^{O}C, germination began within a week as shown in Figure 4.1. These plantlets were utilized to extract DNA for barcoding purposes after one month.

4.1.2 DNA Extraction and PCR

For PCR, the plant's genomic DNA was extracted using CTAB method from the one-month old germinated plants. By measuring the absorbance at 260 and 280 nm and determining the ratio, the purity and quantity of DNA were evaluated.

The chloroplast genomes psbA-trnH region, about 500 bp, was amplified successfully (Figure 4.2). The amplified yield was found by exposing the agarose gel to a UV-trans illuminator following electrophoresis.

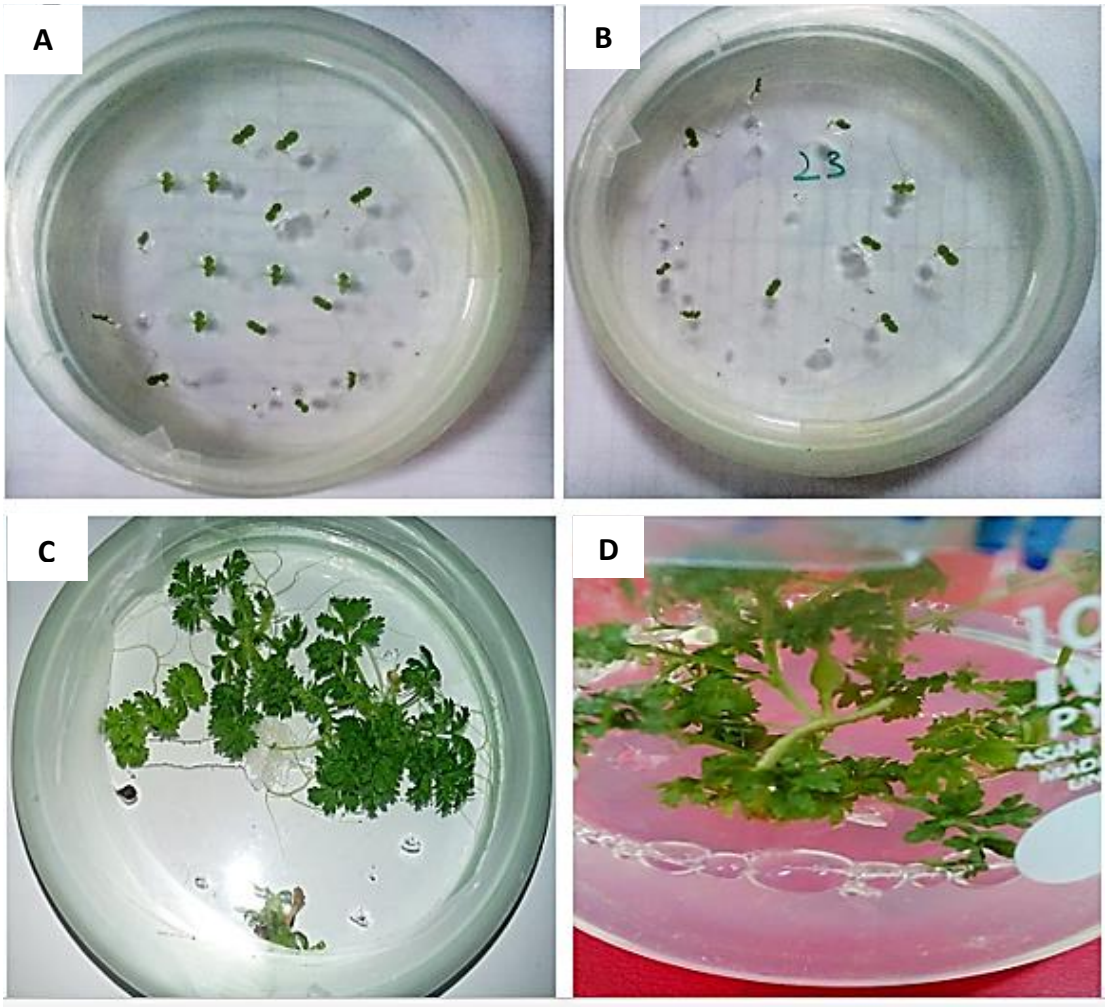


FIGURE 4.1: For DNA barcoding, germination of seeds after seven days (A & B), root-bearing plantlets after four weeks (C & D).

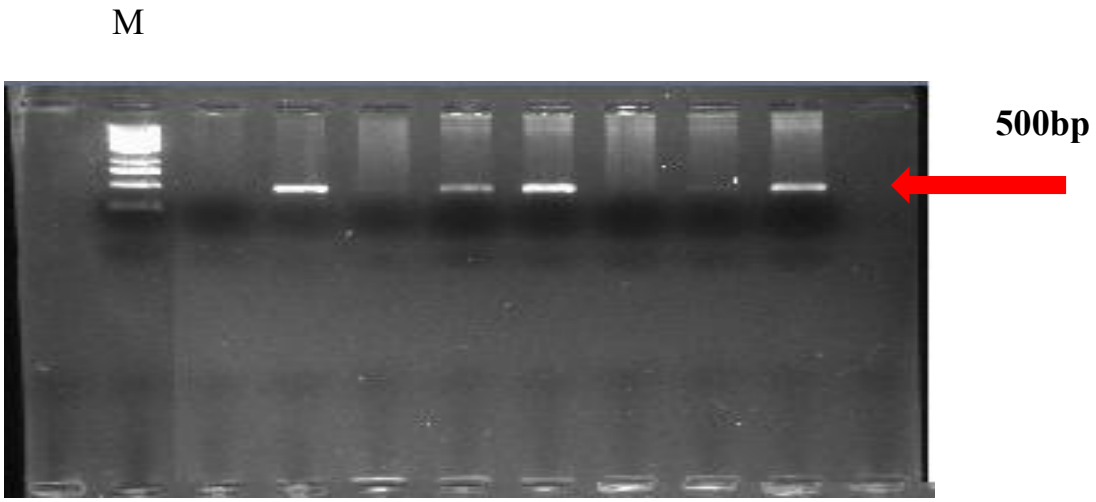


FIGURE 4.2: PCR amplified product (500bp) of chloroplast DNA region *psbA-trnH*. 'M' stands for 1kb of marker DNA

4.1.3 PCR Product Purification and Sequencing

The Rapid PCR Purification System 9700 (Marligen Biosciences, Ijamsville, MD, USA) was used to purify the PCR product. After that, the purified products were sequenced using the dideoxy-chain termination method in triplicate to check the reliability of nucleotides that were species-specific.

For the purpose of confirming the plant species, the GenBank Accession number [NCBI: FJ418751] was used as reference sequence Furthermore, by using the BioEdit software/alignment tool BioEdit (version 7.2.5.0), the sequences acquired were located and examined. The sequence was identified as *psbA-trnH* of *A. carvifolia* (99.7% similarity) with two base pairs difference after doing the CLUSTAL W in BioEdit programme and BLAST in NCBI. There was found cytosine instead of guanine at 233 bp and guanine instead of Thymine at 354 bp positions.

4.2 Transformation and Regeneration

The regeneration and transformation conditions were optimized for *A. carvifolia* by using different media conformations and *Agrobacterium tumefaciens* strain GV3101 carrying plasmid pPCV002-A.

4.2.1 Optimization Sterilization Conditions of Seeds

Seeds of *A. carvifolia* were surface sterilized using 70% ethanol for different time durations like 30 sec, 1 min, 1.5 min, and 2 min, then by 0.1% (w/v) mercuric chloride ($HgCl_2$) for 15 sec. Seeds were then rinsed three times with autoclaved distilled water. However, variations in germination efficiency were observed among the different treatments of 70% ethanol (Figure 4.3). Highest germination efficiency was observed at time duration of 1 minutes followed by 30 seconds. Germination efficiency was found lower at the time duration of 1.5 and 2 minutes (Figure 4.3). Similar findings were observed with reference to control of contamination. Best

sterilization was observed at 1 minutes with best germination efficiency as higher time duration resulted in lowering the germination efficiency.

4.2.2 Media Optimization for Seed Germination

Four types of media i.e. water, plain agar, $\frac{1}{2}$ MS and MS media were utilized for seed germination. The sown seeds were then kept in a growth chamber. The germination efficiency was higher on half MS medium, with a rate of 98%, compared to water, plain agar, and MS media where only 10%, 70%, and 75% of the seeds germinated respectively (Figure 4.4). After being chilled in the dark for two days at 4°C, germination began within a week. The seedlings obtained from these germinated seeds were then used in further experiments.

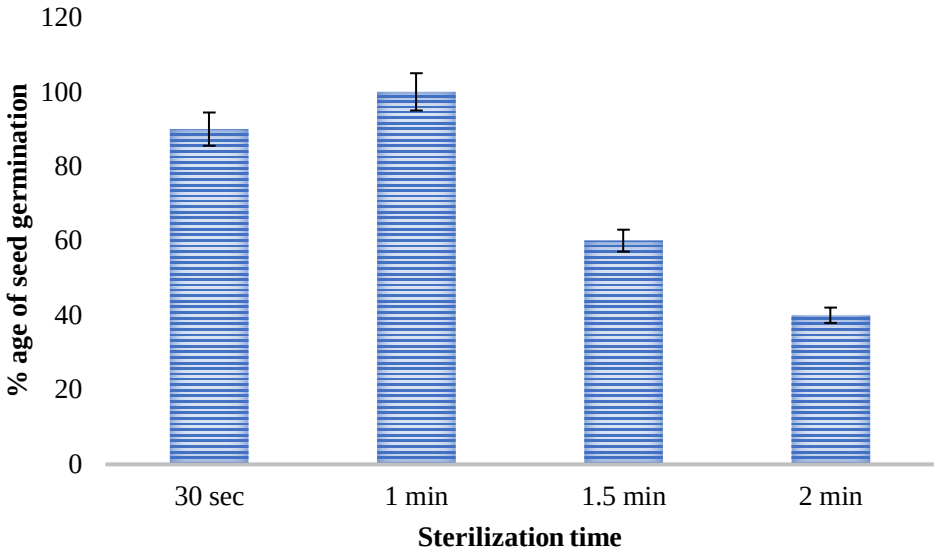


FIGURE 4.3: Percentage seeds germination with different duration of exposure of 70% ethanol.

4.2.3 Optimization of Media and Explants for Regeneration

Three different shoot regenerating media were used to pick one best regeneration media for the explants. Shoot regeneration medium 2 gave the best results followed

by shoot regeneration medium 3. Explants from leaf, stem, and stem with nodes (nodal explants) were put on media to check their transformation efficiency. Stem explants with nodal regions exhibited the highest regeneration efficiency on all media types, surpassing the regeneration observed with leaf explants (Figure 4.5). On shoot regeneration medium 2, nodal explants exhibited 55% regenerating efficiency. Statistical study of variance revealed a highly significant interaction ($P < 0.0001$) between the various types of media and the explants utilized for shoot organogenesis (Table 4.1).

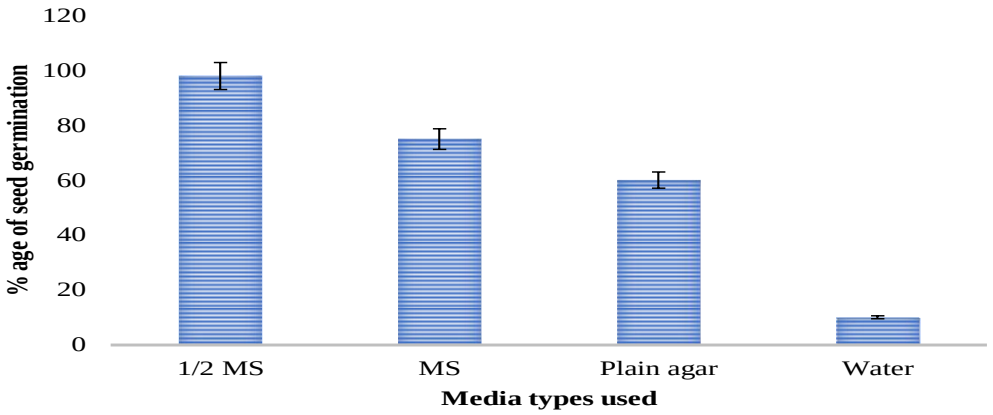


FIGURE 4.4: Impact of media on percentage of seeds germination

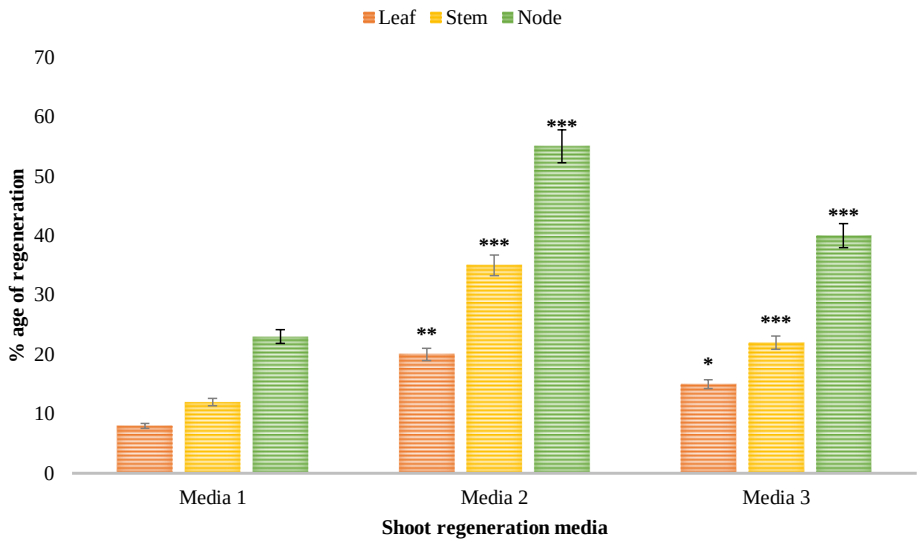


FIGURE 4.5: Impact of different shoot regeneration media and types of explants on plant regeneration efficiency. Asterisks show data groups with significant difference as compared to one with low regeneration efficiency (*$P < 0.05$, **$P < 0.01$ and ***$P<0.001$)

TABLE 4.1: Two way ANOVA for the Impact of Shoot Regeneration Media on Different Types of Explants

Source of Variation	**Degrees of Freedom**	**Sum of Squares**	**Mean Square**	**F-Value**	**Prob.**
Media	2	1165	582.3	145.6	0.0000
Explants	2	3505	1752	438.1	0.0000
Interaction	4	141.3	35.33	8.833	0.0000
Residual (error)	18	72	4		
Total	26	4883			

Based on the aforementioned results, shoot regeneration media 2 was chosen as best regeneration media for evaluating the transformation efficiency of construct containing *rol A* gene. Stem explants containing nodes (nodal explants) were employed as the plant material for the transformation experiments.

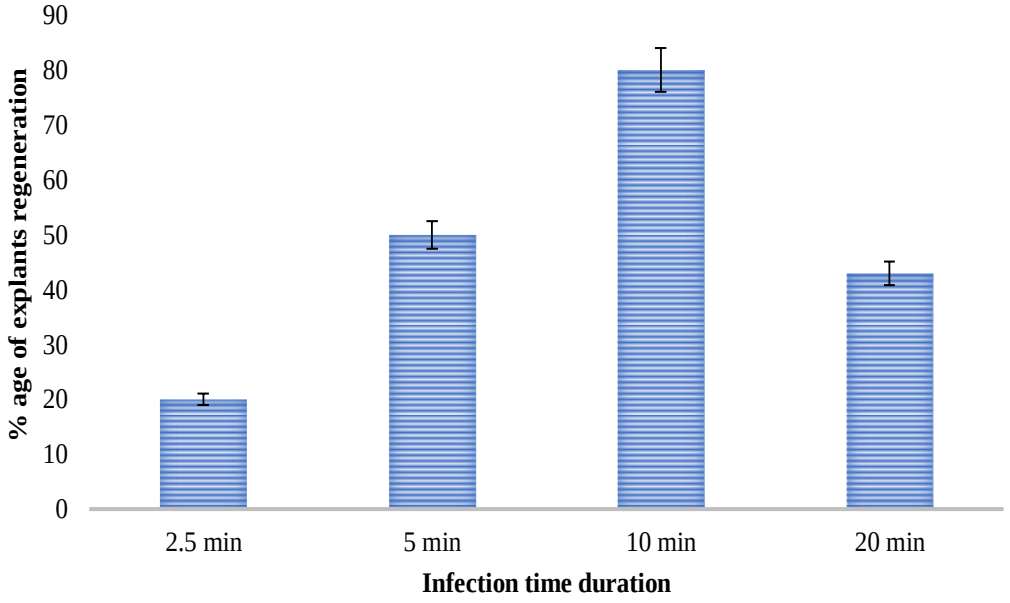
4.2.4 Genetic Transformation

4.2.4.1 Impact of Infection Time Duration and Co-cultivation Time Period on Transformation Efficiency

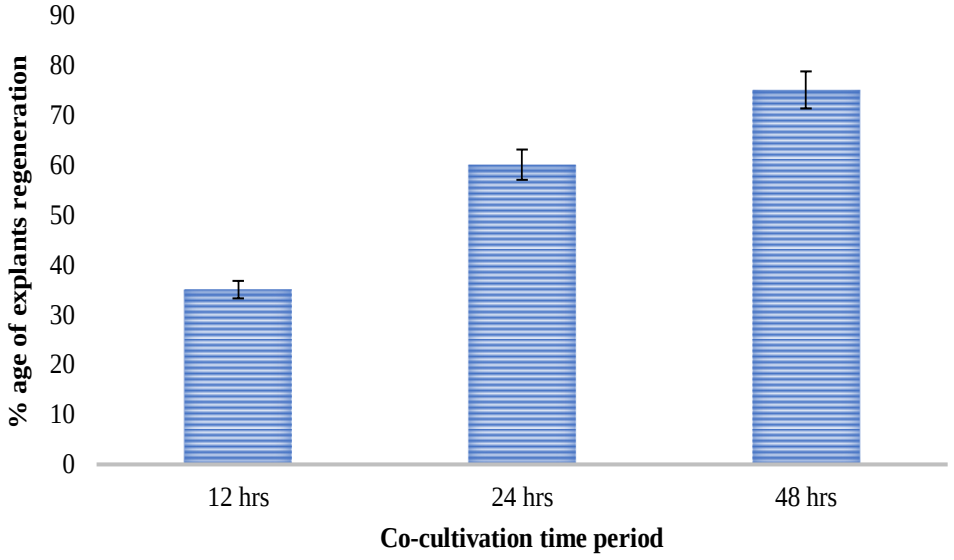
The nodal explants excised from one-month old *Artemisia carvifolia* plants were precultured on preculturing media and then infected by *Agrobacterium* culture for varying time durations such as 2.5 min, 5 min, 10 min and 20 min. All infection times gave different responses. Explants subjected to a 10 minutes infection time demonstrated the highest transformation efficiency (Figure 4.6 A). Prolonging the infection time was suboptimal, as it resulted in the death of the explants. After which they were co-cultivated for 12 hrs, 24 hrs, and 48 hrs (Figure 4.6 B). A co-cultivation period of 48 hours proved to be optimal for achieving the maximum transformation efficiency. Extending the co-cultivation duration led to the overgrowth of bacteria, negatively impacting the transformation process.

4.2.4.2 Selection of Transformed Explants

After co-cultivation, the explants underwent multiple washings with autoclaved distilled water and diluted cefotaxime to eradicate bacteria. The explants were then transferred to selection media for shoot regeneration (Figure 4.7) containing 50 mg/L kanamycin and 300 mg/L cefotaxime.



(A)



(B)

FIGURE 4.6: Impact on the explant regeneration of infection duration (A) and co-cultivation time period (B)

FIGURE 4.7: Ex-plants on co-cultivation (A) and selection media (B).

4.2.4.3 Transgenic Shoots and Roots Regeneration

In the transformation experiment, a total of 500 explants were employed and a 45% transformation efficacy was observed. Based on the number of regenerated shoots relative to the total number of explants that were infected, transformation efficiency was computed. Despite encountering various contaminations, only five *rol A* transformants successfully reached maturity on the selection media. Selected shooting media (MS medium+ BAP 0.5 mg/L+ NAA 0.1 mg/L+ kanamycin 50mg/L + cefotaxime 300mg/L) showed best shoot regeneration response (Figure 4.8). Furthermore, after achieving a significant number of shoots that are of a reasonable length, they were subjected to rooting on rooting media. Rooting media containing 0.1 mg/L NAA with 30 mg/L kanamycin was used for rooting response. Positive response on rooting was observed with 70% efficiency (Figure 4.9). Plants successfully reached maturity after going through co-cultivation and passing through different rounds of selection on shooting and rooting media (Figure 4.10).

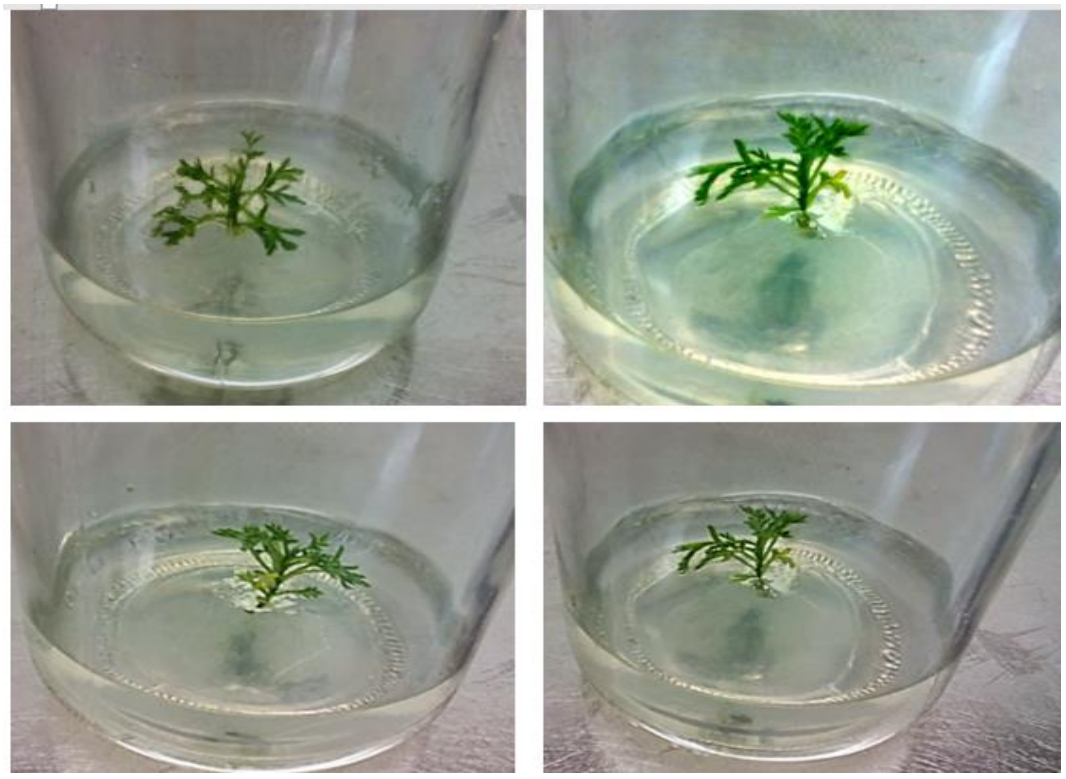


FIGURE 4.8: Shoot regeneration of *rol A* transformed explants on selection media containing 50 mg/L kanamycin and 300 mg/L cefotaxime.

FIGURE 4.9: Rooting response of *rol A* transformed explants on selection media containing 30 mg/L kanamycin.

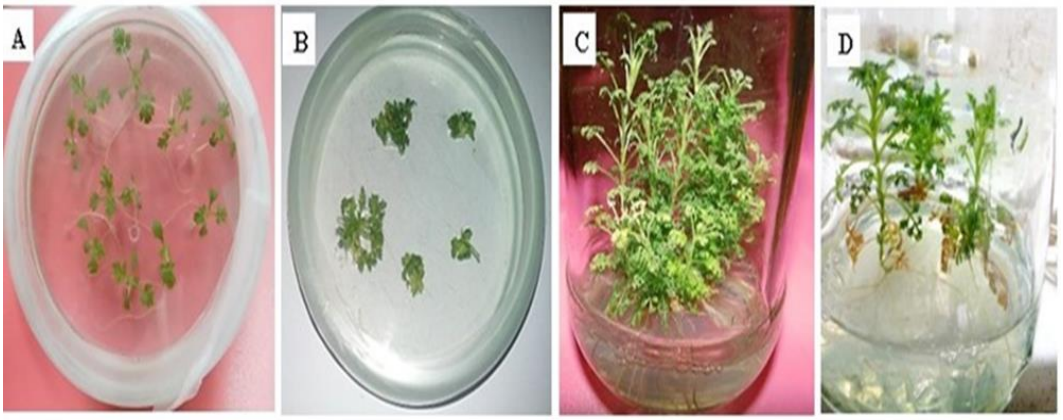


FIGURE 4.10: All steps of genetic transformation of *Artemisia carvifolia*. (A) Seeds germination, (B) co-cultivation, (C) regenerated wild type plant, (D) regenerated *rol A* transgenic plant

4.2.4.4 Morphological Assessment of *rol A* Transformed Plants

Morphological distinctions were illustrated in Figure 4.11, between transgenic plants carrying *rol A* gene and wild type plants. Notably, the transformed plants exhibited dwarfness and a harder stem texture compared to the wild type plants. Additionally, *rol A* transgenic plants showcased shorter, narrower, and dark-colored leaves. Furthermore, these transgenic plants demonstrated an accelerated growth rate when cultivated on the selection media in comparison to the wild type plants.

FIGURE 4.11: Morphology of *rol A* transformed plants (A), and wild type plants (B) of *Artemisia carvifolia* Buch

4.2.4.5 Transitioning to Pots for Plant Acclimatization

The plants exhibited survival reactions upon being moved to the pots; however, the primary factor impeding their survival was fungal infection. The plants required a long time to acclimatize because they were so delicate. It took them over a month to become used to the growth room's conditions. After a few months, they had matured enough and were gradually hardened enough to be moved into the greenhouse (Figure 4.12).

FIGURE 4.12: Acclimatization of wild type (A) and *rol A* transgenic plants (B) in pots.

4.3 Molecular Analysis

A. carvifolia was successfully transformed with *A. tumefaciens* strain GV3101 carrying the *rol A* gene. For the *A. carvifolia rol* gene transformants, PCR results revealed 308 bp for *rol A* gene and 781 bp for the *nptII* gene amplified products

(Figure 4.13). The plasmid DNA of GV3101-PCV002-A yielded similar amplified products. Besides this, wild-type plants did not exhibit that these genes were present in their genome.

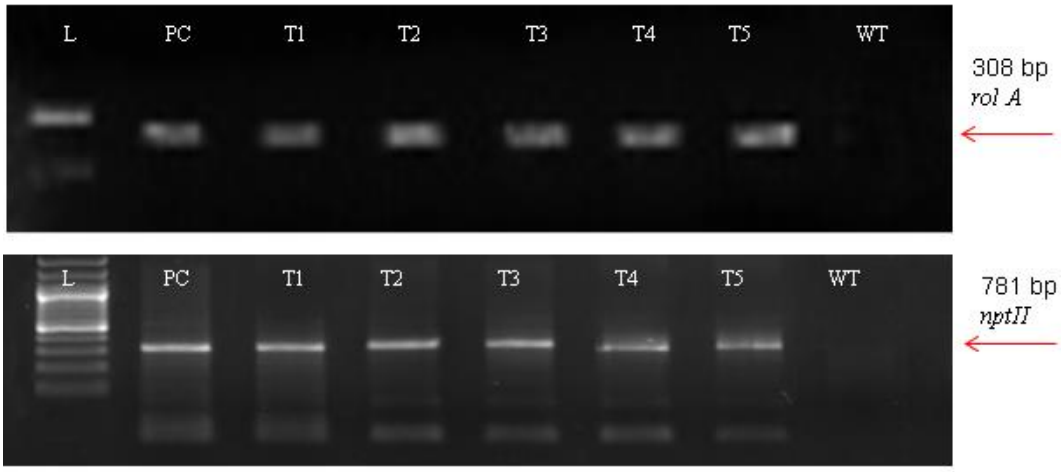


FIGURE 4.13: PCR result of transgenic *Artemisia carvifolia* harboring *rol A* genes showing 308 bp for *rol A* and 781 bp for the *nptII* gene. T1-T5 represents *rol A* transgenic lines. "L" represents the ladder. "WT" represents wild type plants and "PC" represents plasmid DNA as possitive control.

4.4 Evaluation of Copy Number and Expression Level of Transgenic Plants through Southern Blot Analysis and Semi- quantitative RT-PCR

The incorporation of *rol A* gene into the plant genome was confirmed by Southern blot analysis, which also provided information on the number of copies of gene present in various transgenic lines. All five transgenic lines of *A. carvifolia* showed the successful stable integration of *rol A* gene. One copy of *rol A* gene was seen in transgenic lines T1, T2 and T4. Whereas T3 and T5 showed two copies of the integrated gene as shown in Figure 4.14. Although there were differences in the expression level of these genes among studied lines, RT-PCR validated gene expression in all transformed lines. The *rol A* transgenic lines T3 and T5 in Figure

4.15 displayed the highest levels of expression due to double copy numbers as visible in southern blot analysis results (Figure 4.14). β-actin was chosen as the internal control (housekeeping gene) which showed similar expression in wild type plants and all transgenic lines.

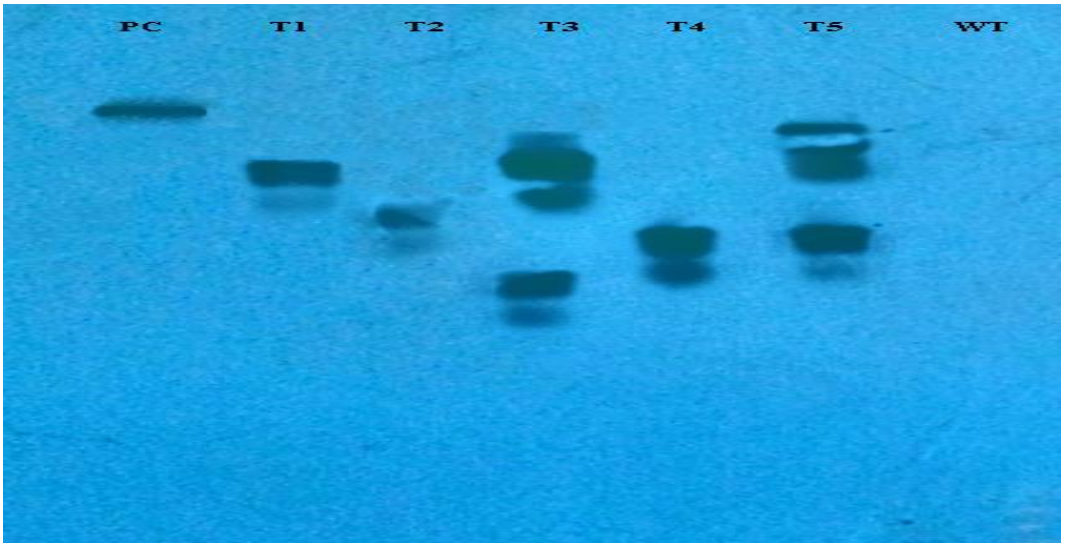


FIGURE 4.14: Southern blot analysis of PCR-positive plants demonstrating the incorporation of *rol A* gene into the *Artemisia carvifolia* genome. T1-T5 represents *rol A* transgenics. "PC" indicates plasmid DNA as possitive control. "WT" represents wild type plants with no *rol A* gene. Transgenic lines T3 and T4 showed two copies of integrated gene.

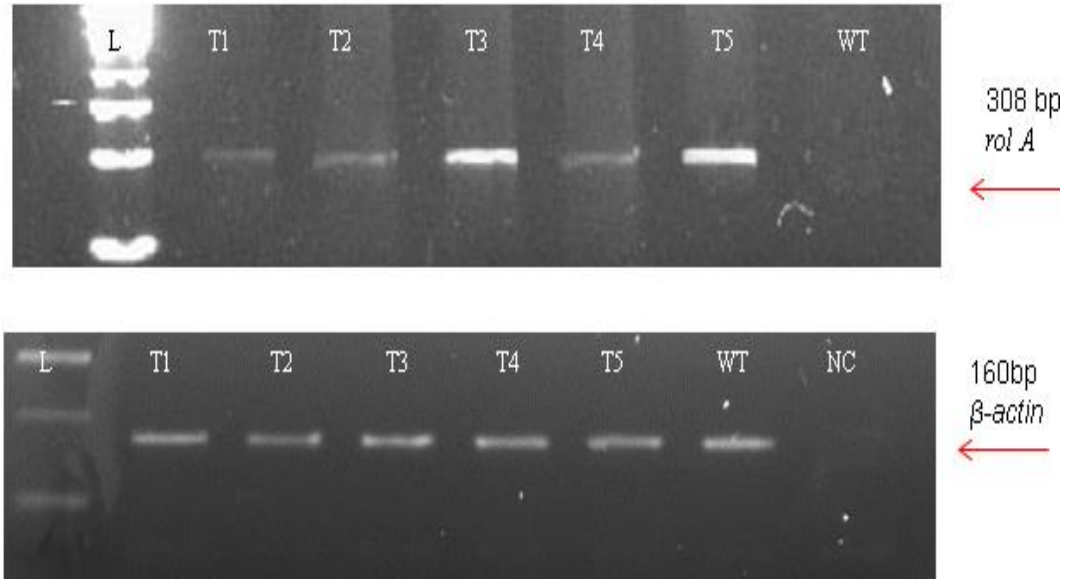


FIGURE 4.15: Semi quantitative RT PCR using the β-actin (160 bp) amplification as an internal control. T1-T5 represents *rol A* transgenics. "L" represents the ladder. "WT" represents wild type plants with no *rol A* gene. "NC" indicates the negative control.

4.5 HPLC-DAD-Based Analysis of Flavonoids

Methanolic extracts of shoots of both transgenic and wild type *A. carvifolia* plants were prepared and then an HPLC-DAD system was used for the detection and quantification of flavonoids. The HPLC profile obtained was evaluated against the absorption spectra and retention duration of ten standard compounds or flavonoid markers including vanillic acid, rutin, catechine, gallic acid, syringic acid, caffeic acid, coumaric acid, geutisic acid, ferulic acid and cinnamic acid. As illustrated in Figure 4.16, unlike the wild type plants, the flavonoids catechin and geutisic acid were discovered in the transformed plants only. Figure 4.17 shows the HPLC chromatogram of standard flavonoids. Vanillic acid, syringic acid, gallic acid, coumaric acid, ferulic acid, caffeic acid and cinnamic acid were present in both wild type plants and transformed plants but the concentration of these phenolic compounds was enhanced in *rol A* gene transformants (Figure 4.16).

The vanillic acid content in wild type plants was 0.47 μg/mg DW but in the transformed plants it reached the highest 1.82 μg/mg DW in T3, showing a 4-fold increase. Rutin levels in wild type plants were 1.39 μg/mg DW, highest increase was observed in T5, showing a 2.7-fold increase of rutin. The gallic acid concentration was 0.62 μg/mg DW in wild type plants, increasing to 3.89 μg/mg DW with up to 6.2-fold in *rol A* transformants. Syringic acid wild type content was 1.05 μg/mg DW, increasing up to 3.7-fold to (3.90 μg/mg DW) highest in T5 transformed plants. The concentration of coumaric acid in wild type plants was 1.65 μg/mg DW but in transformed plants, it reached 2.85 μg/mg DW, showing the 1.7-fold increase in transformed plants. The concentrations of caffeic acid, ferulic acid, cinnamic acid in wild type plants were 0.47 μg/mg DW, 1.39 μg/mg DW, 1.67 μg/mg DW respectively and in transformed plants, these reached 2.60 μg/mg DW, and 5 μg/mg DW, 4.23 μg/mg DW respectively showing a 5.5-fold increase of caffeic acid, a 3.6-fold increase of ferulic acid, a 2.56-fold increase of cinnamic acid in transformed plants. The results of HPLC showed that all flavonoids were increased up to the highest level in T3 and T5 transformed plants due to the integration of a double copy number of *rol A* gene found in southern blot analysis (Figure 4.14). Catechin and geutisic acid were absent in wild type plants and the concentration

of catechin in transformed plants was 3.19 μg/mg DW and the amount of geutisic acid was 2.22 μg/mg DW in transformed plants. The chromatograms of standards and all samples of wild type plant and transgenic lines are shown in Figure 4.17 to Figure 4.23. Statistical analysis was also conducted, where the production levels of investigated phenolic compounds in *rol A* transgenic plants exhibited an extremely significant difference (P<0.0001) when compared to the wild type *A. carvifolia* plants (Table 4.2).

TABLE 4.2: Two-way ANOVA for HPLC Analysis

Source of Variation	**Degrees of Freedom**	**Sum of Squares**	**Mean Square**	**F-Value**	**Prob.**
Polyphenols	9	117.4	13.04	54.64	0.0000
Transgenic lines	5	120.2	24.04	1592	0.0000
Interaction	45	20.15	0.4478	2933	0.0000
Residual (error)	120	0.9835	0.008196		
Total	179	258.7			

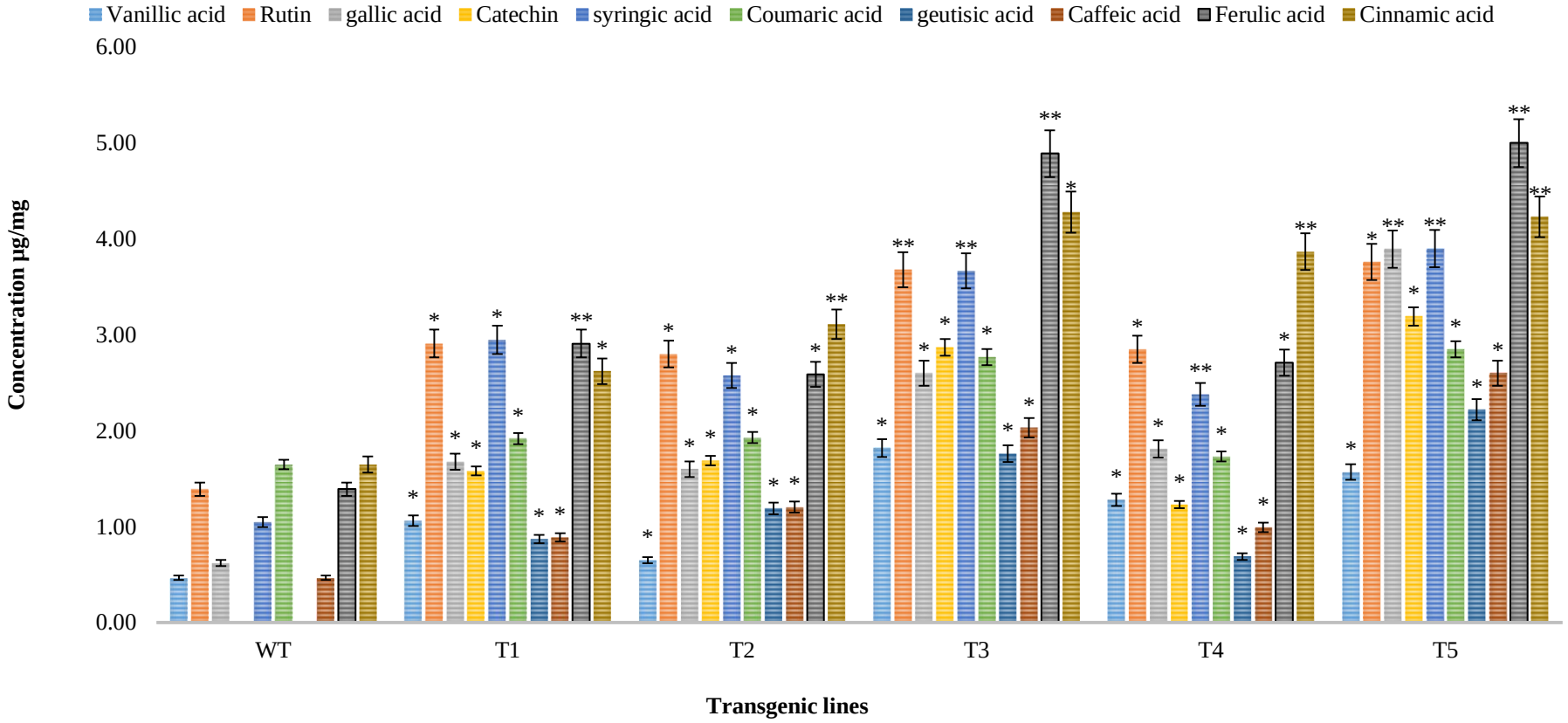


FIGURE 4.16: Quantitative analysis of flavonoids by HPLC in *A. carvifolia rol A* transgenic lines and wild type plants. T1-T5 shows transgenic lines. "WT" indicates wild type *A. carvifolia* plant. *Asterisk* depicts the statistically significant difference in data compared with control plants at *P $<$ 0.05 and **P $<$ 0.01.

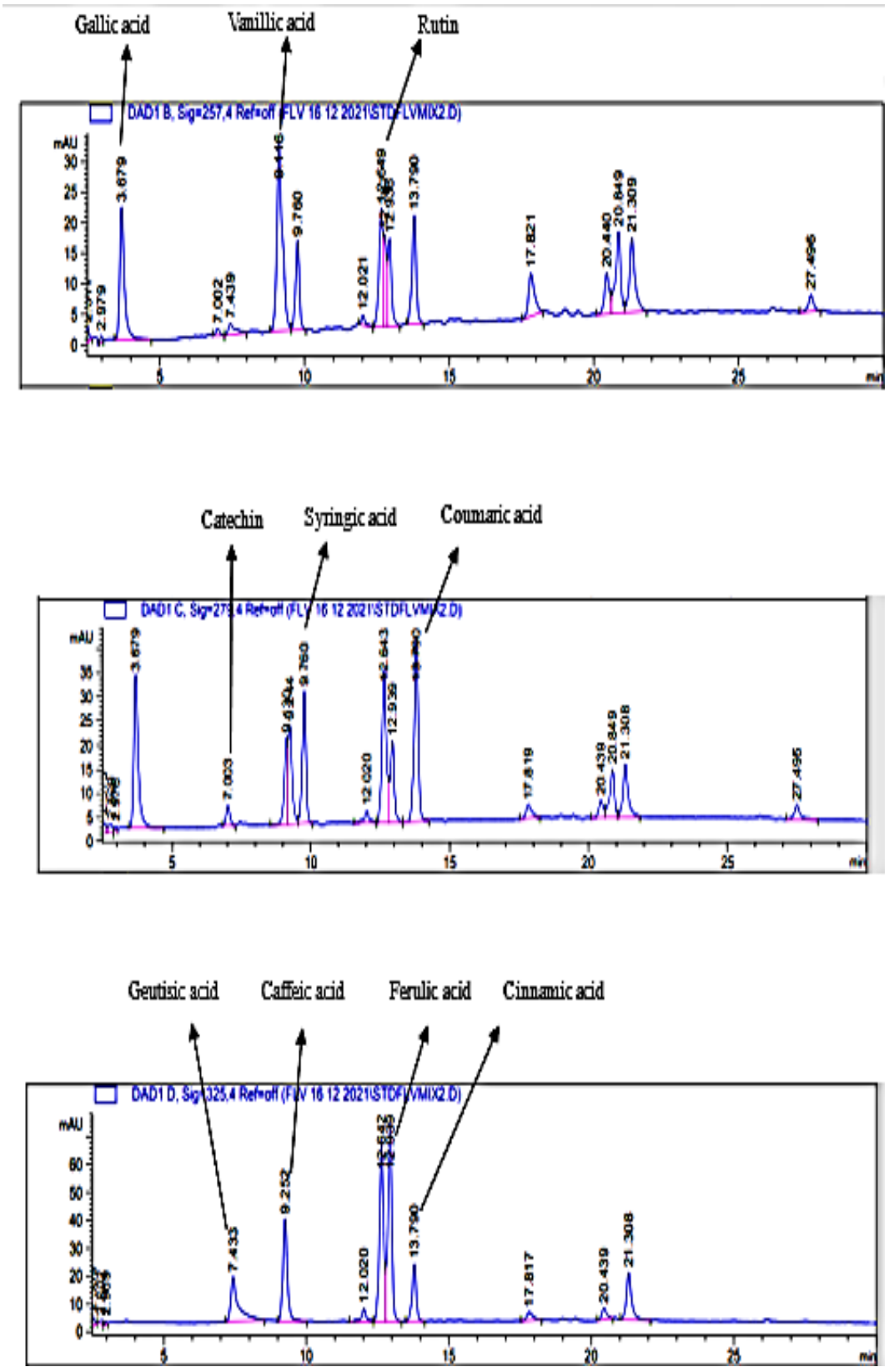


FIGURE 4.17: HPLC Chromatogram of standard flavonoids showing peaks of vanillic acid, rutin, catechine, gallic acid, syringic acid, caffeic acid, coumaric acid, geutisic acid, ferulic acid and cinnamic acid.

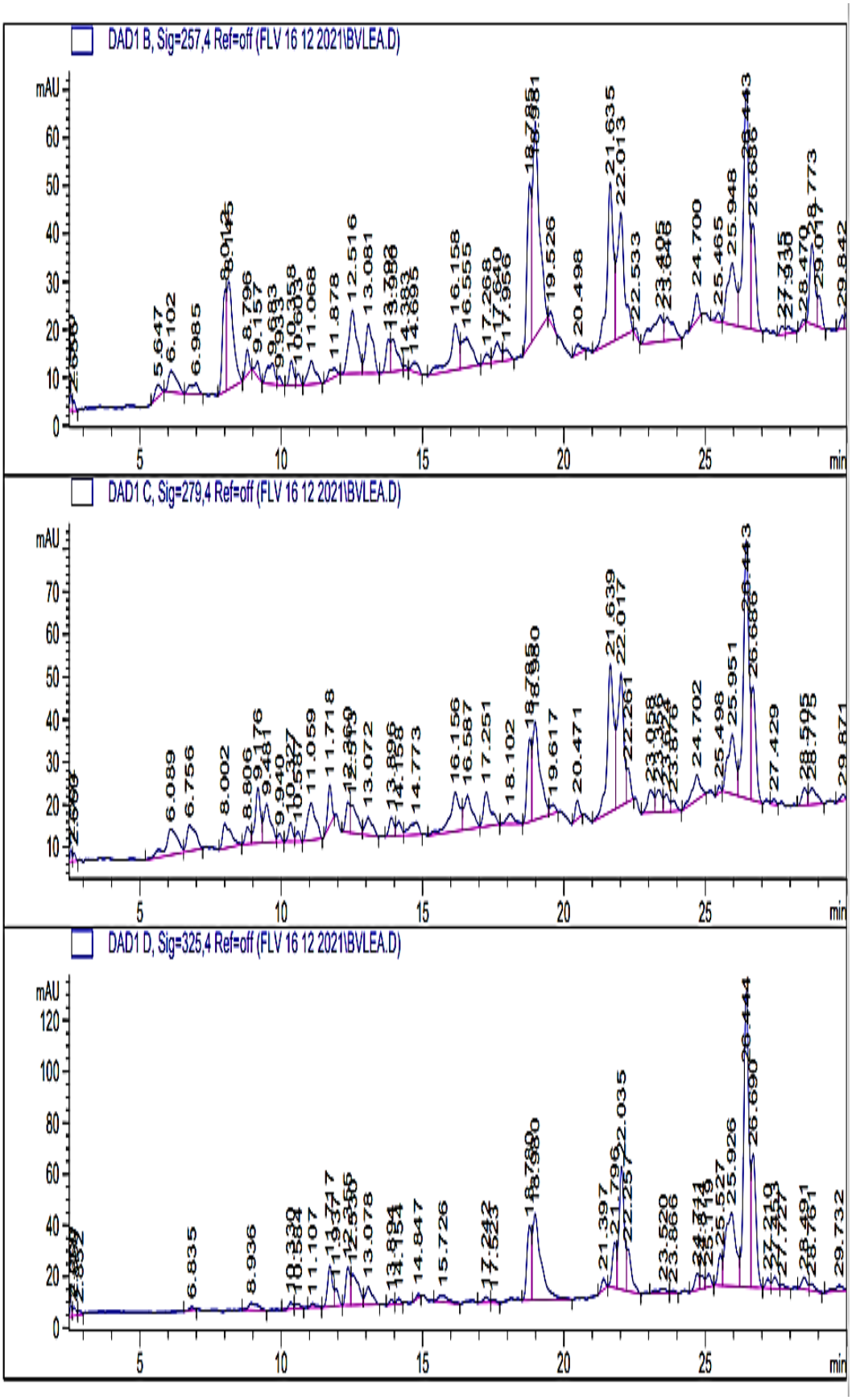


FIGURE 4.18: HPLC chromatogram of wild type *Artemisia carvifolia* plants. The graphs show the absence of catechin and geutisic acid.

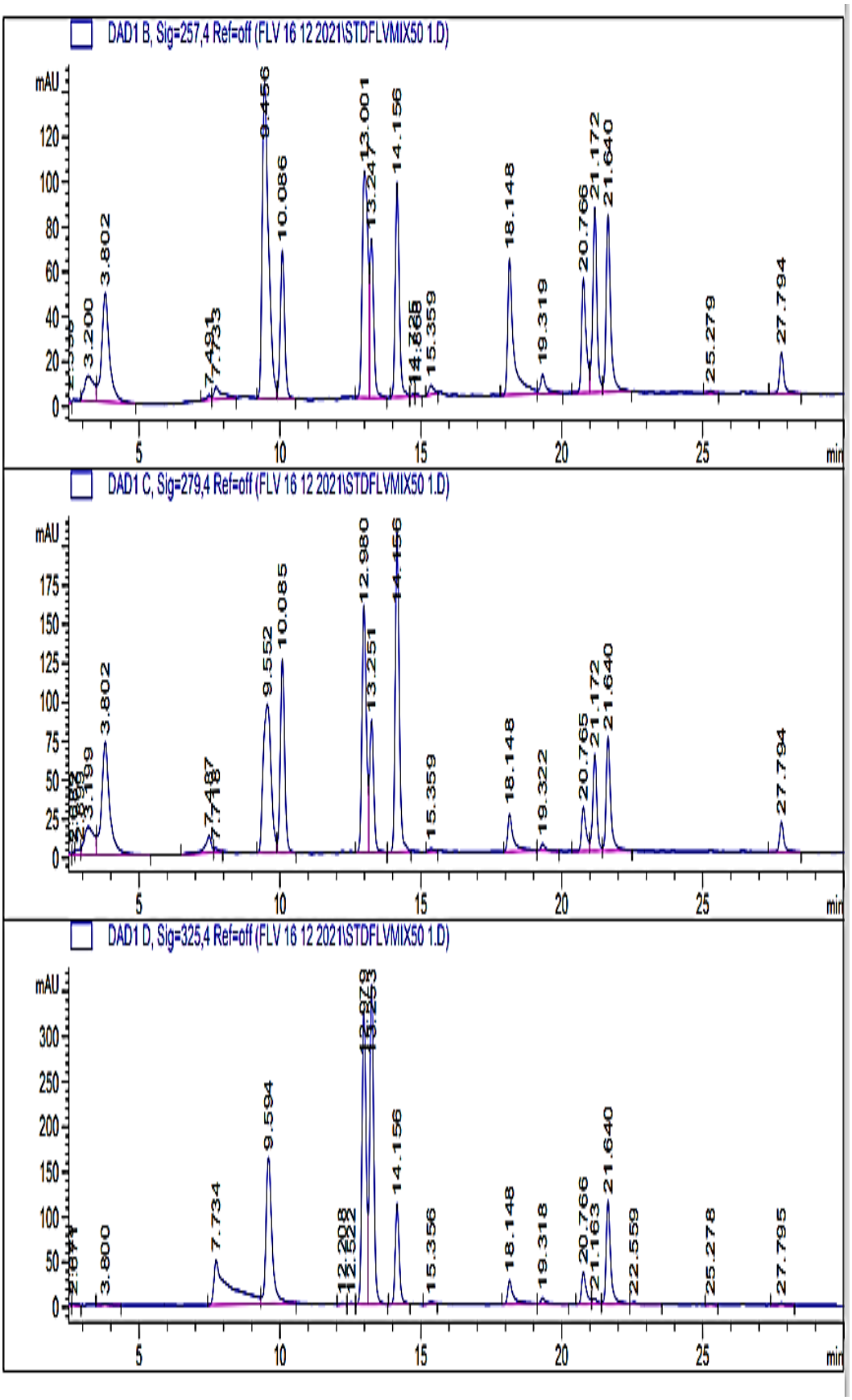


FIGURE 4.19: HPLC chromatogram of transgenic line T1

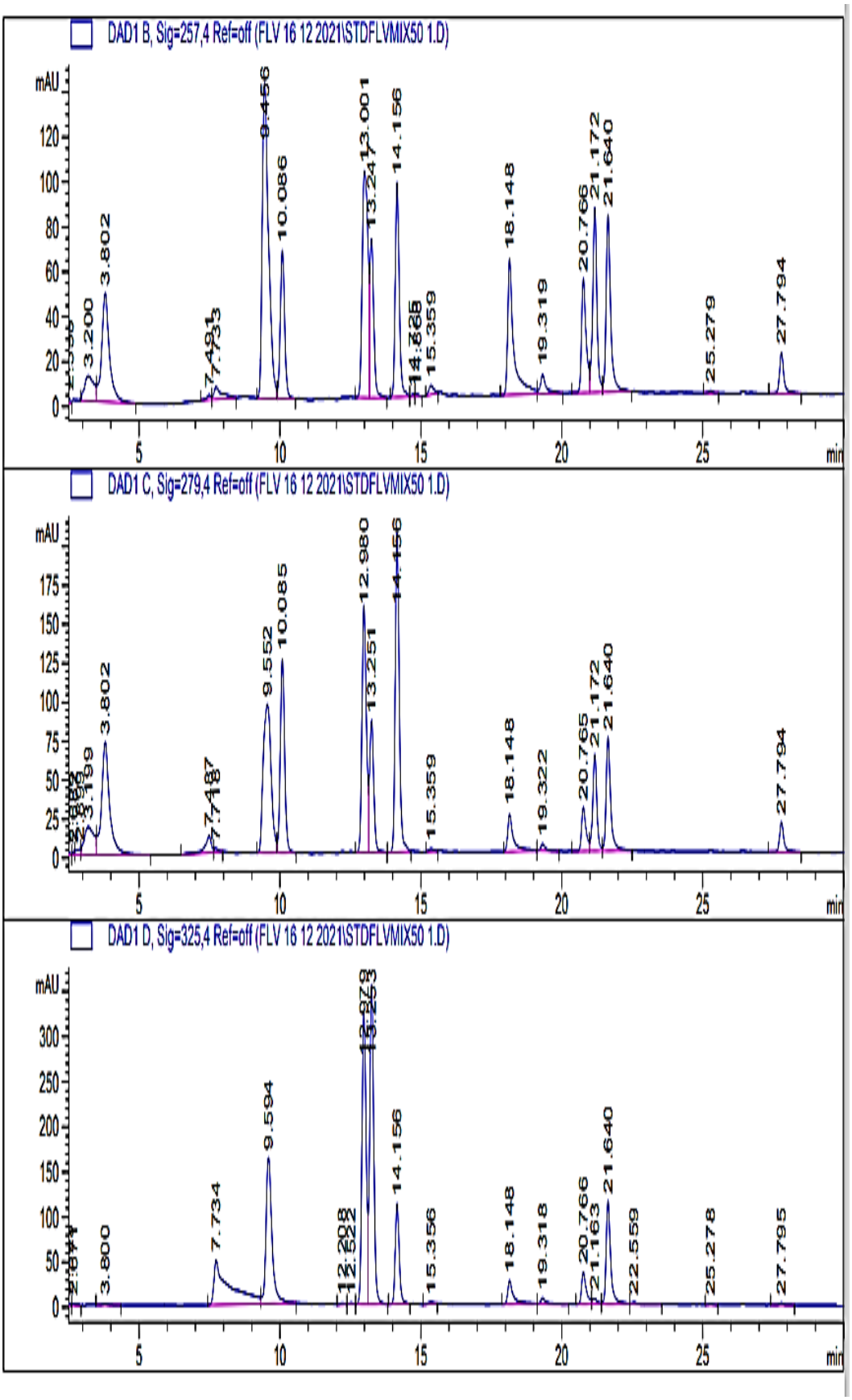


FIGURE 4.20: HPLC chromatogram of Transgenic line T2

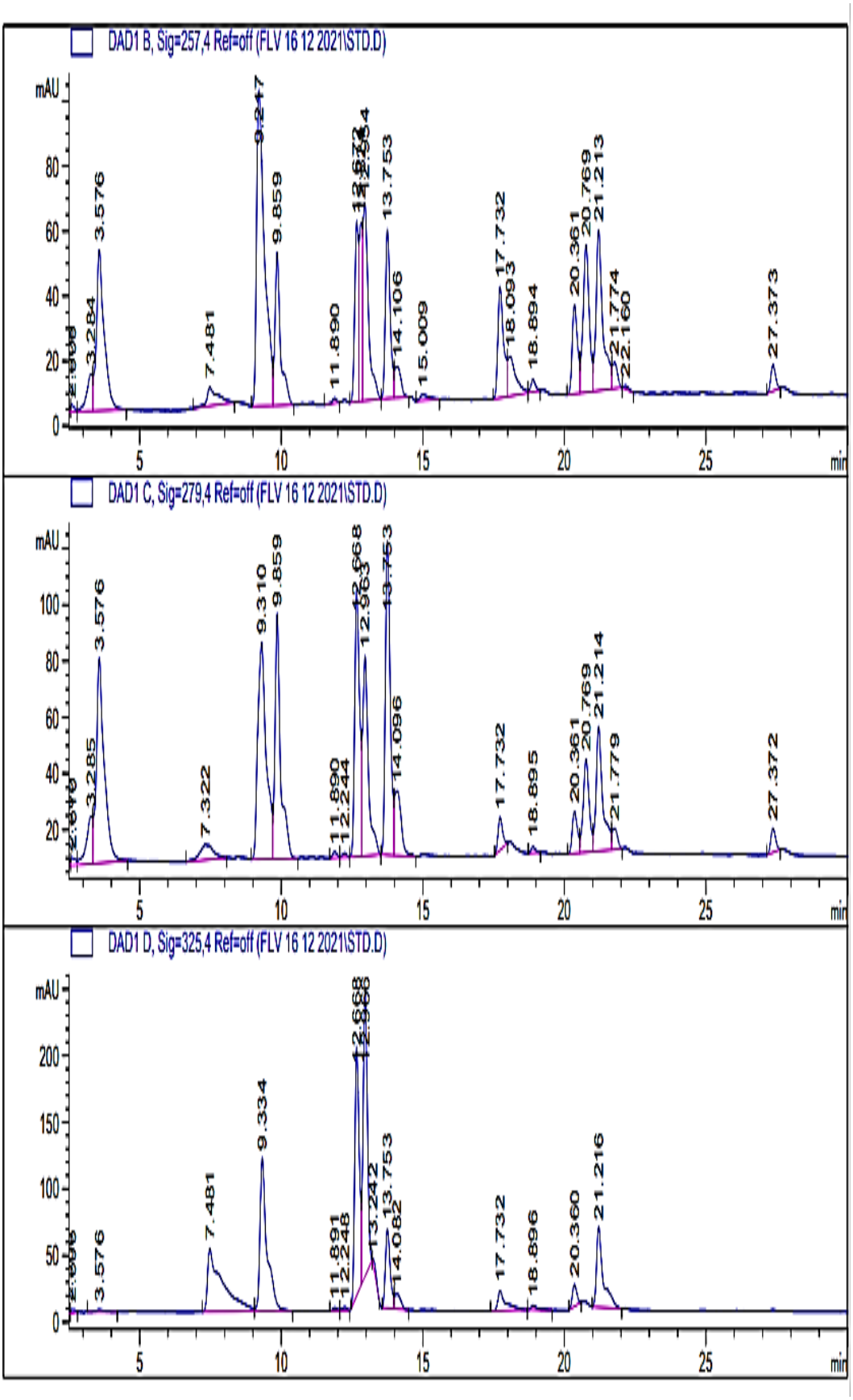


FIGURE 4.21: HPLC chromatogram of transgenic line T3

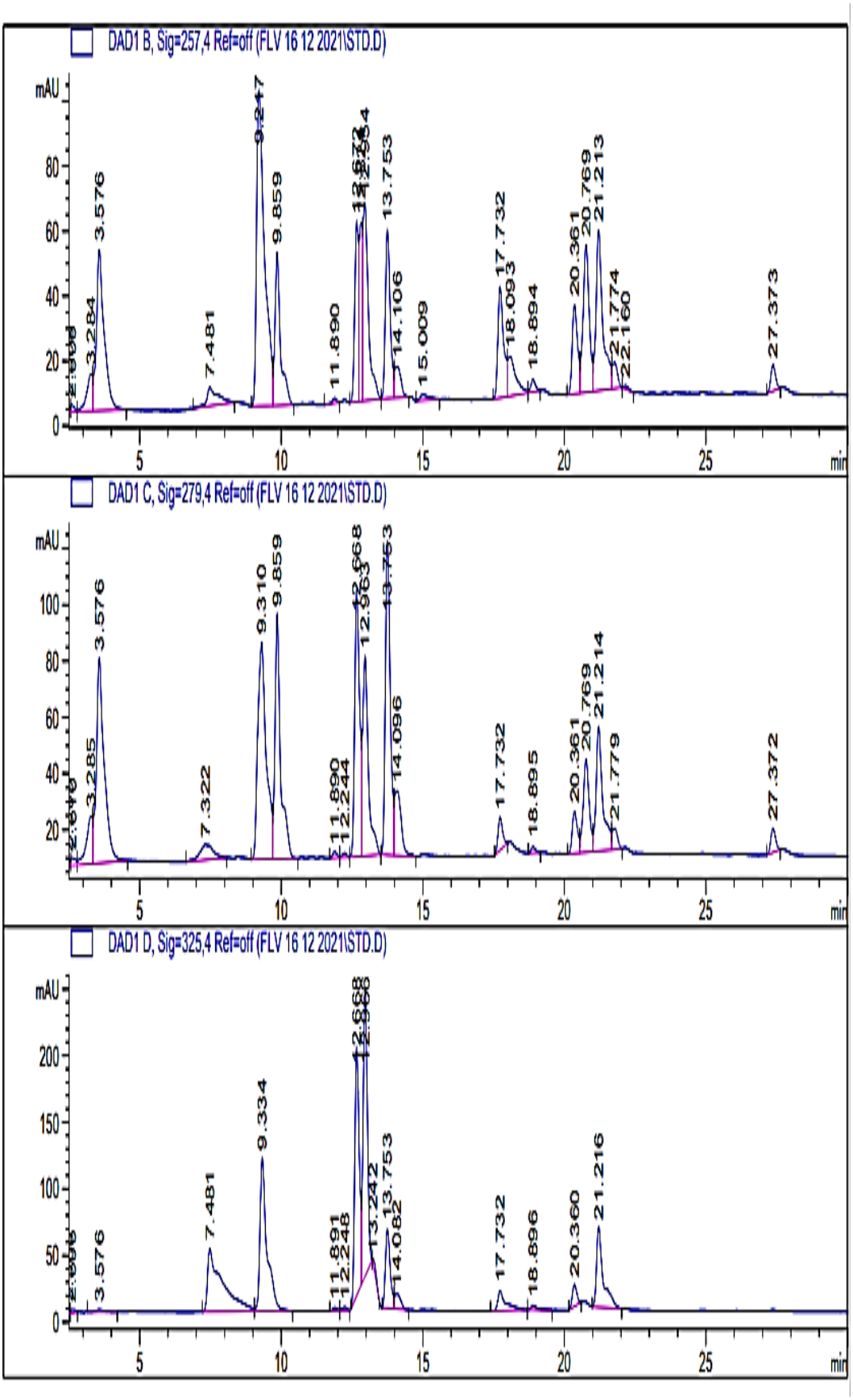


FIGURE 4.22: HPLC chromatogram of transgenic line T4

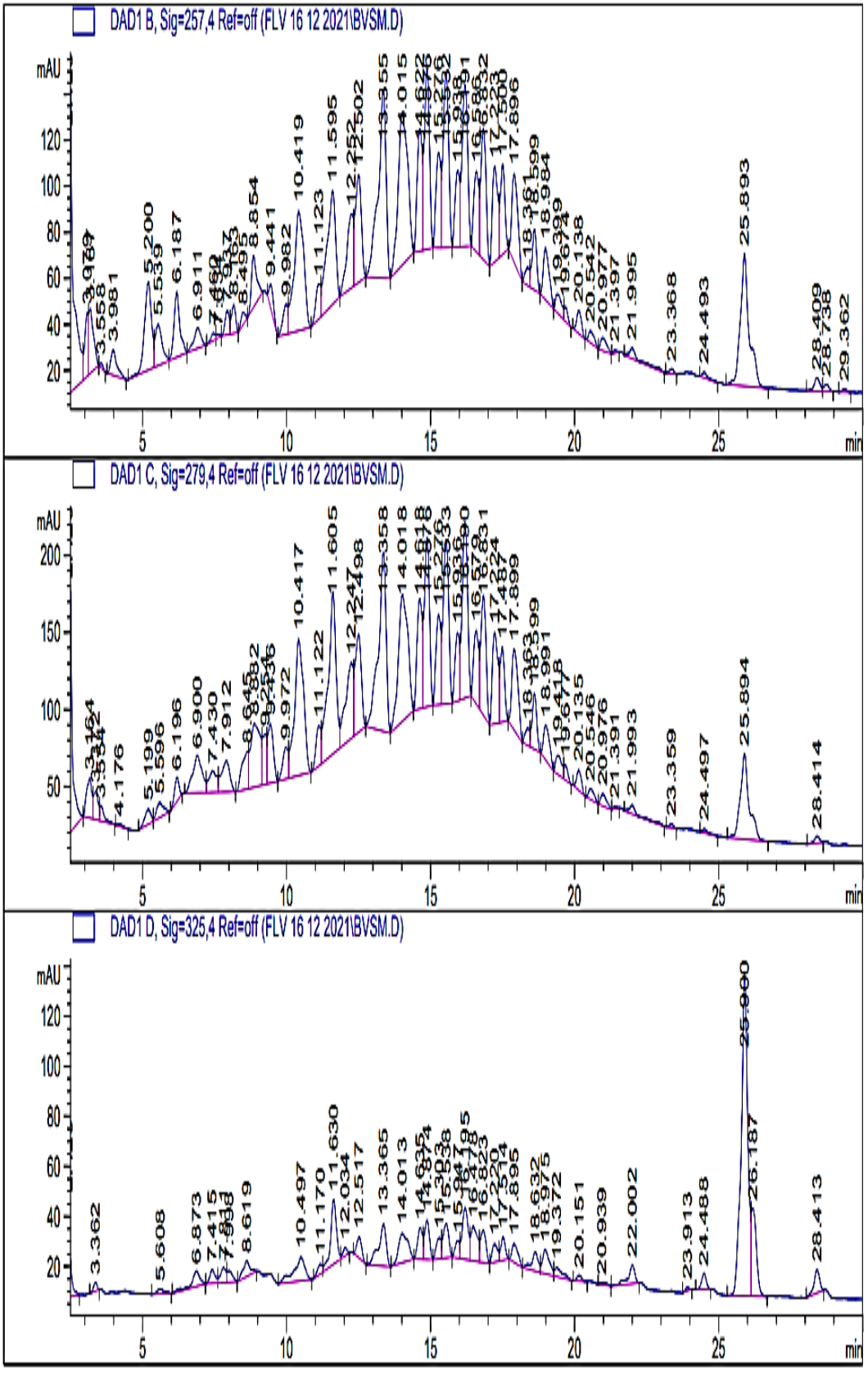


FIGURE 4.23: HPLC chromatogram of transgenic line T5

4.6 Expression Analysis of Flavonoid Biosynthetic Pathway Genes through Real-Time qPCR

To evaluate the expression of two flavonoid biosynthetic genes (*PAL* and *CHS*), RT-qPCR was performed. In RT-qPCR, significant changes and higher expression degrees of the investigated genes of flavonoids biosynthetic pathway in plants transformed with *rol A* gene were observed. The expression of *PAL* and *CHS* genes was much low in wild type plants as shown in Figure 4.24.

The *PAL* gene was highly expressed in the transformed plants as compared to *CHS* gene expression in untransformed plants as an expression of *PAL* gene was 9-20 folds higher and expression of *CHS* gene was 2-6 folds higher in transformed plants.

The *rol A* transgenic lines T3 and T5, which both carried two copies of *rol A* gene, particularly showed higher expression of both *PAL* and *CHS* gene, with the highest expression in T3 followed by T5 transgenic line plants.

These results showed that *rol A* gene plays role in inducing flavonoid biosynthesis by enhancing the expression of their biosynthetic genes i.e., *PAL* and *CHS*. The *rol A* gene showed extremely significant effect ($P<0.0001$) on the production of flavonoids in *A. carvifolia* plants (Table 4.3).

Table 4.3: Two-way ANOVA for Flavonoid Biosynthetic Genes

Source of Variation	**Degrees of Freedom**	**Sum of Squares**	**Mean Square**	**F-Value**	**Prob.**
Genes of metabolic pathway	1	513.2	513.2	504.1	0.0000
Transgenic lines	5	812.8	162.6	159.7	0.0000
Interaction	5	209.6	41.91	41.17	0.0000
Residual (error)	24	24.43	1.018		
Total	35	1560			

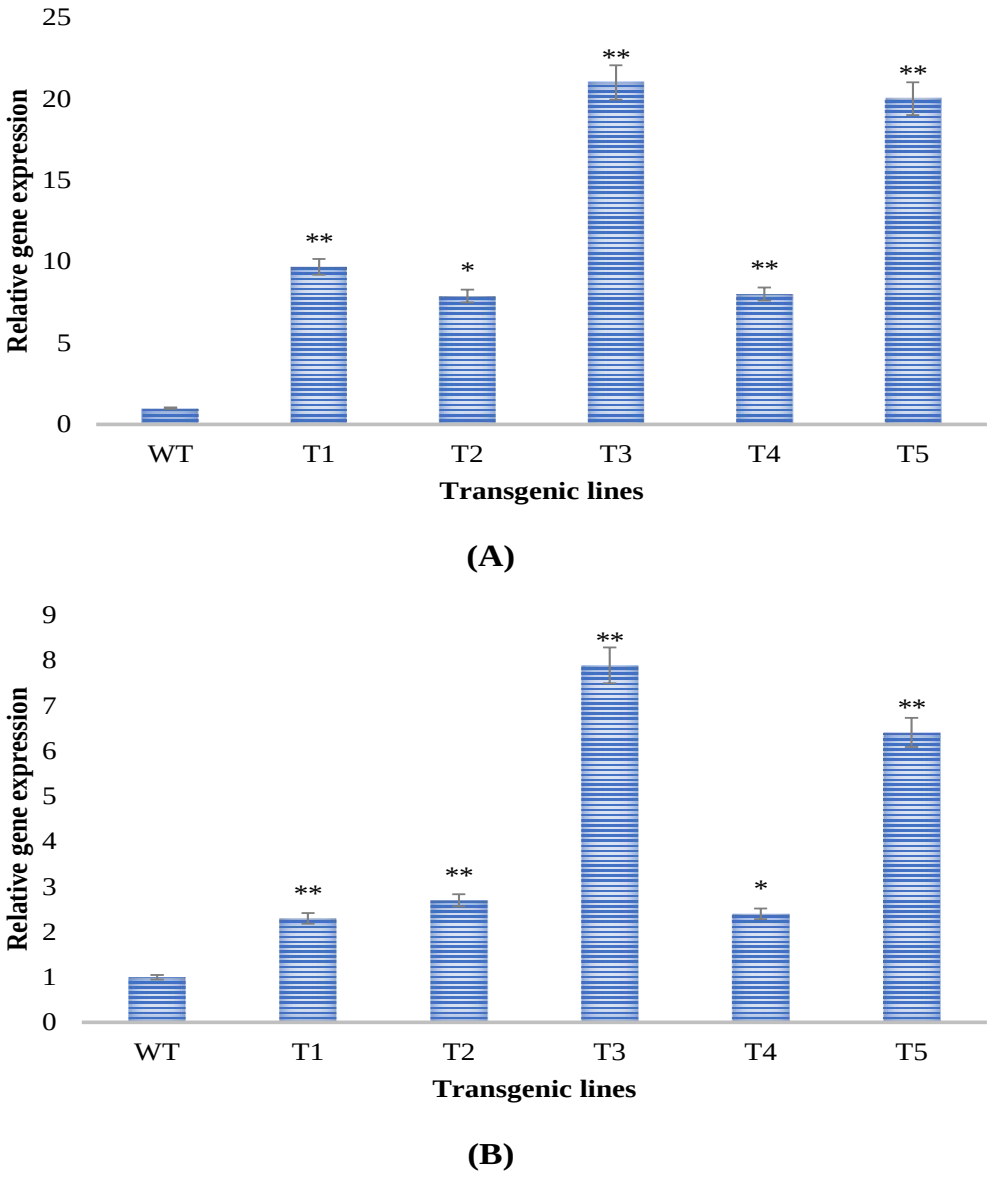


FIGURE 4.24: Quantitative real-time PCR analysis of flavonoid biosynthetic pathway genes. *PAL* (A) and *CHS* (B) in wild type and *rol A* transgenic of *A. carvifolia* plants. *Asterisk* depicts the statistically significant difference in data compared with control plants at *P $<$ 0.05 and **P $<$ 0.01.

4.7 Analyzation of the Antioxidant Potential of *rol A* Gene Transformed and Untransformed *A. Carvifolia*

The antioxidant potential of *Artemisia carvifolia rol A* transgenic plants and wild type plants was assessed by using antioxidant assays.

4.7.1 Total Phenolic Content

To analyze the total phenolic capacity of samples of wild type and *rol A* transformed *A. carvifolia* plants, Folin-Ciocalteu reagent was used. The total phenolic content was calculated as the equivalent of gallic acid (mg/g of DW) at 725 nm. TPC in wild type plants was 50 mg/g whereas *rol A* transgenic lines demonstrated an average increase of 1.4-fold in the phenolic content and the level of phenolic content was highest in line T3 (84 mg/g) followed by T5 (80 mg/g) as shown in Figure 4.25 A.

4.7.2 Total Flavonoid Content

The total phenolic content was calculated as the equivalent of quercetin (mg/g of DW) at 405 nm with $AlCl_3$ colorimetric method. TFC in wild type was 20 mg/g compared to 1-2-fold increase in *rol A* transgenic lines. In transgenic lines T1, T2, T3, T4 and T5, the TFC was measured as 27 mg/g, 30 mg/g. 32.9 mg/g. 27 mg/g and 31 mg/g respectively (Figure 4.25 B).

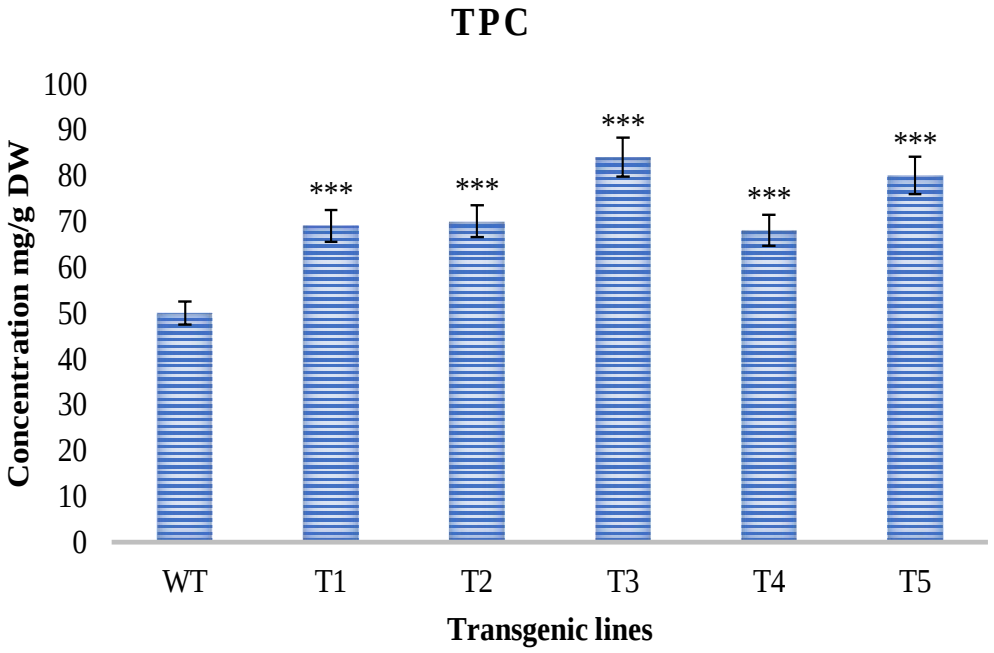
4.7.3 Total Antioxidant Capacity

Total antioxidant capacity was determined in terms of ascorbic acid equivalence (mg/g of the DW) at 630 nm. TAC was increased up to an average 1-2-fold, as in wild type TAC was measured 40 mg/g but in transformed plants it was enhanced in all transgenic lines with highest level in T3 (60.7 mg/g) and then in T5 (50.9 mg/g) as shown in Figure 4.25 C.

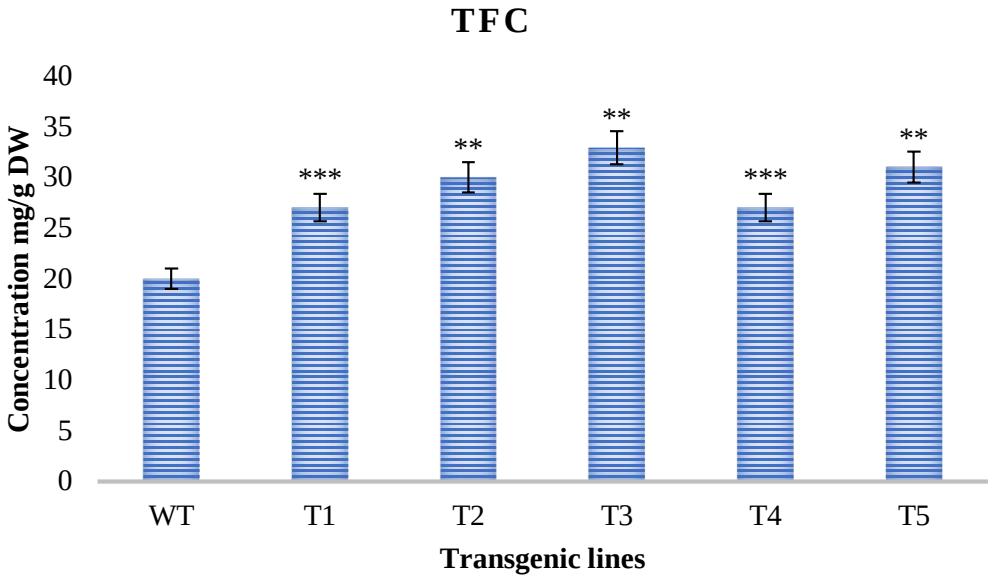
4.7.4 Total Reducing Power

Similarly, total reducing power was also significantly increased in *rol A* transformed plants. In wild type plants, total reducing power was 70 mg/g. In transgenic line T1, T2, T3, T4, T5 the TRP was measured as 99.4 mg/g, 100 mg/g, 130 mg/g, 97mg/g, and 125 mg/g respectively, showing an average increase of 1.5-2-fold in

total reducing power (Figure 4.25 D). The *rol A* gene showed extremely significant effect (P<0.0001) on the antioxidant capacity of the *A. carvifolia* plants (Table 4.4). All these results indicated that the transgenic plant's antioxidant capacity was increased by the incorporation of the *rol A* gene.



(A)



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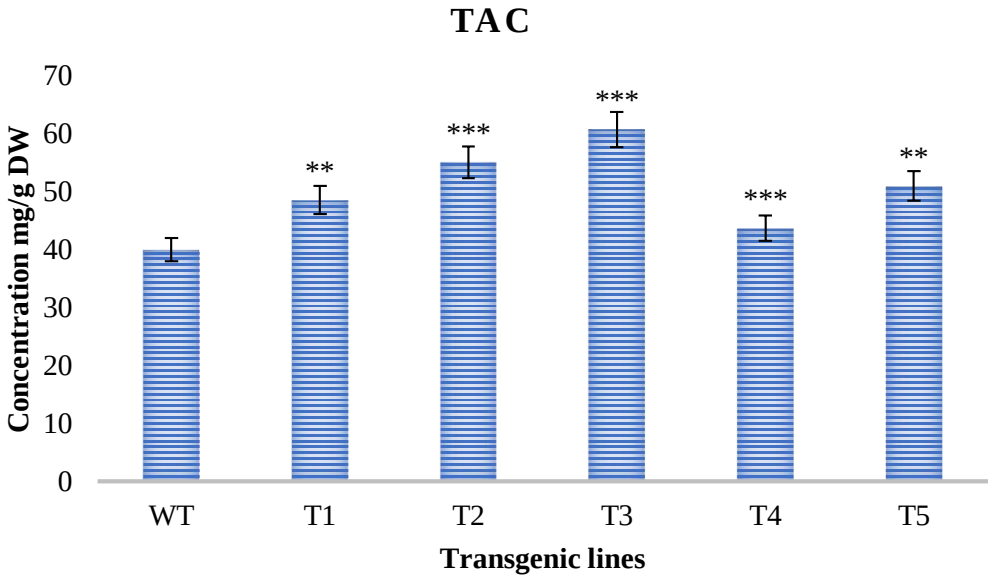
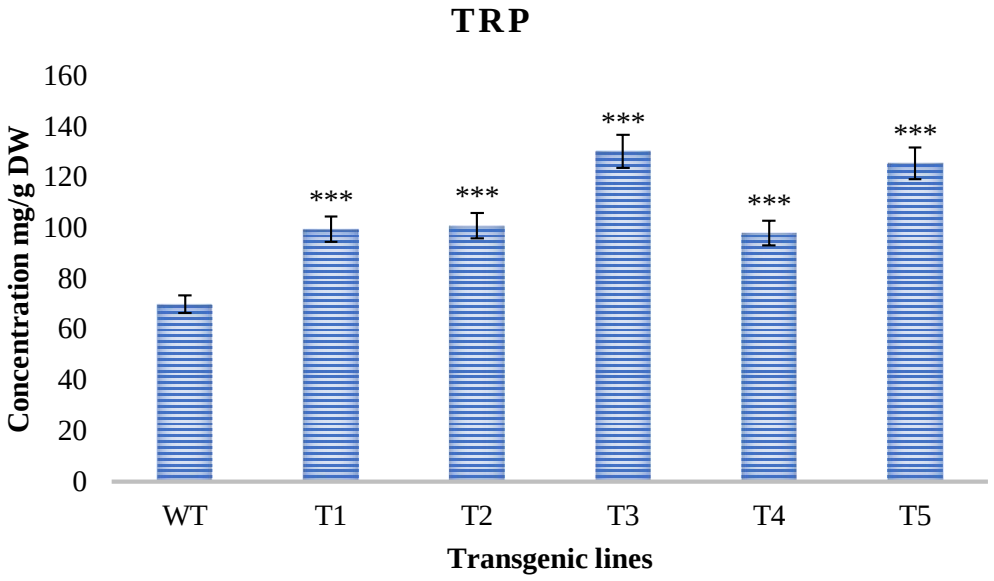


(C)



(D)

FIGURE 4.25: Evaluation of antioxidant potential through different antioxidant assays. TPC (total phenolic content) (A), TFC (total flavonoid content) (B), TAC (total antioxidant capacity) (C) and TRP (total reducing power) (D). "WT" represents wild type plants. T1-T5 indicates *rol A* gene transgenic lines. Error bars indicate the S.E of three means. *Asterisk* shows the significant variance in results in comparison with wild type plants at *P $<$ 0.05, **P $<$ 0.01 and ***P$<$0.001.

TABLE 4.4: Two-way ANOVA for Antioxidant Assays

Source of Variation	**Degrees of Freedom**	**Sum of Squares**	**Mean Square**	**F-Value**	**Prob.**
Interaction	15	3330	222.0	68.68	0.0000
Transgenic lines	5	7898	1580	488.8	0.0000
Antioxidant assays	3	56270	18760	5804	0.0000
Residual (error)	48	155.1	3.232		
Total	71	67660			

4.8 DPPH Free Radical Scavenging Assay

DPPH free radical scavenging is an efficient and widespread technique for determining the antioxidant activity of plant extracts. In this study, the DPPH assay was used to test the ability of transformed and untransformed plants to scavenge free radicals. According to results obtained, the *rol A* gene transformed plants showed significant increase in antioxidant potential as compared to untransformed wild type plants as shown in Figure 4.26. The extract of *rol A* transgenic line T3 displayed the maximum radical scavenging ability with an IC_{50} value of 206.9 μg/mL compared to the wild type plants having IC_{50} of 627 μg/mL. Similarly, the extracts of T1, T2, T4, and T5 exhibited more efficacy with less IC_{50} values (379, 450, 365.7 and 225.65 μg/mL respectively) as compared to wild type plants. The *rol A* gene showed extremely significant effect ($P<0.0001$) on the free radical scavenging ability of transformed plants of *A. carvifolia* as shown in Table 4.5.

TABLE 4.5: Two-way ANOVA for DPPH Free Radical Scavenging Assay

Source of Variation	**Degrees of Freedom**	**Sum of Squares**	**Mean Square**	**F-Value**	**Prob.**
Interaction	15	472.6	31.51	17.32	0.0000
Transgenic lines	5	3281	656.2	360.7	0.0000
Concentrations	3	22130	7376	4055	0.0000
Residual (error)	48	87.33	1.819		
Total	71	25970			

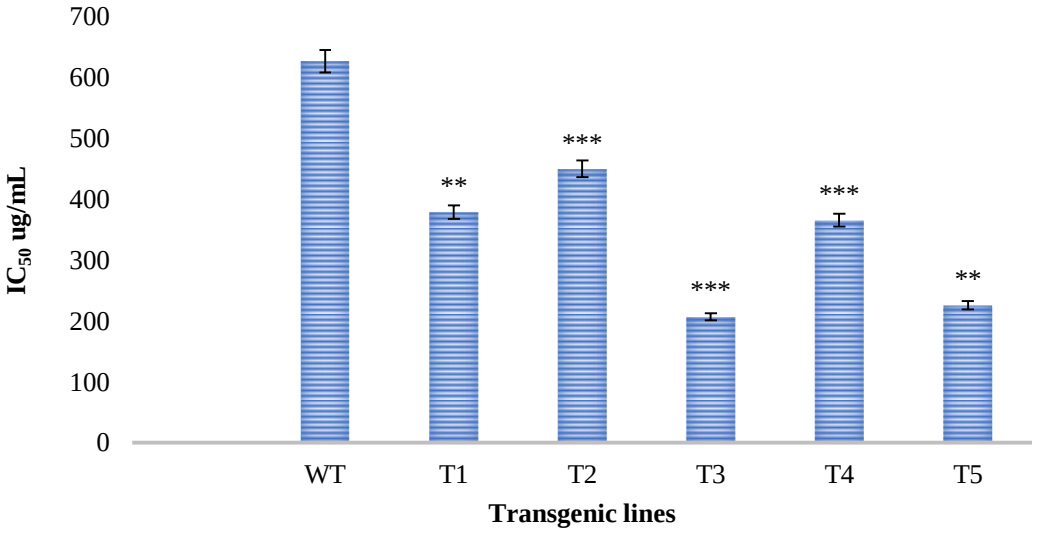


FIGURE 4.26: Results of the DPPH assay of extract of five *rol A* integrated transgenic lines and wild type plant extract (WT) of *Artemisia carvifolia*. *Asterisk* shows the significant variance in results in comparison with wild type plants at **P < 0.01 and ***P < 0.001.

4.9 Measurement of Anticancerous Activity against Cancer Cell Lines through MTT Assay

The antiproliferative activity of untransformed and transgenic plants of *rol A* gene were checked by treating three different cell lines namely HeLa, MCF7, and HePG2 with the methanolic extracts of plants and thus the cell viability of all cell lines was evaluated. The results obtained showed that all transgenic lines were more effective against all cell lines as compared to wild type plant as shown in Figure 4.27 - Figure 4.29. Mortality rate of HeLa cells after treatment with untransformed *A. carvifolia* extract was 30% but after treating with *rol A* transgenic extracts, it increases upto 75%. Similarly, the mortality rate of MCF7 and HePG2 cells after treating with wild type plant extract was 32% and 36% respectively. After treatment with transgenic cell lines, the mortality rate of MCF7 and HePG2 was increased upto 70% and 74% respectively. Transgenic line T3 and T5 shows maximum effect on the viability of cancer cells. Thus, *rol A* gene showed extremely significant effect (P<0.0001) on the enhancement of anticancerous properties of the plants under study (Table 4.6).

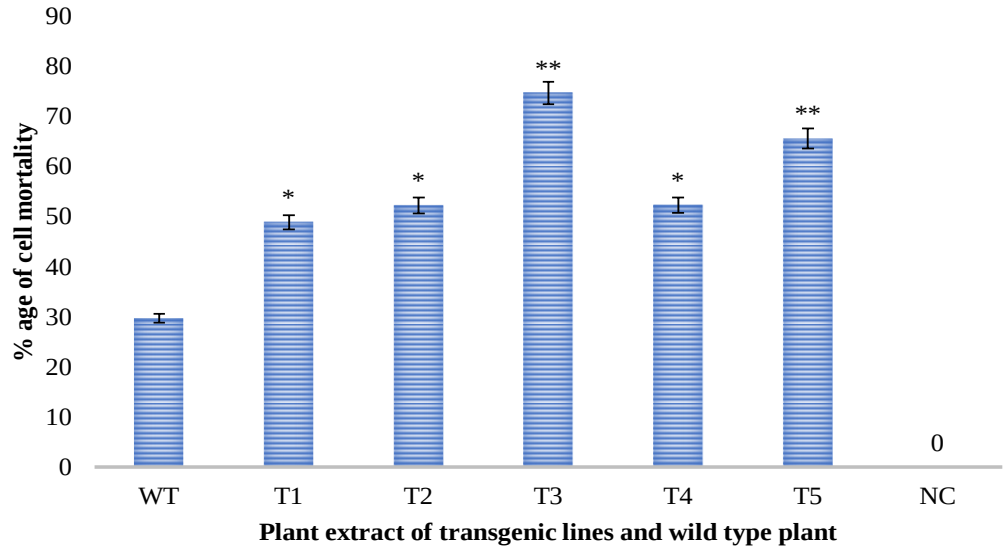


FIGURE 4.27: MTT assay for determination of cytotoxic activity of plant extracts (40 mg/mL) against HeLa cancer cell lines. "NC" indicates negative control. *Asterisk* shows the significant variance in results in comparison with wild type plants at *P $<$ 0.05 and **P $<$ 0.01.

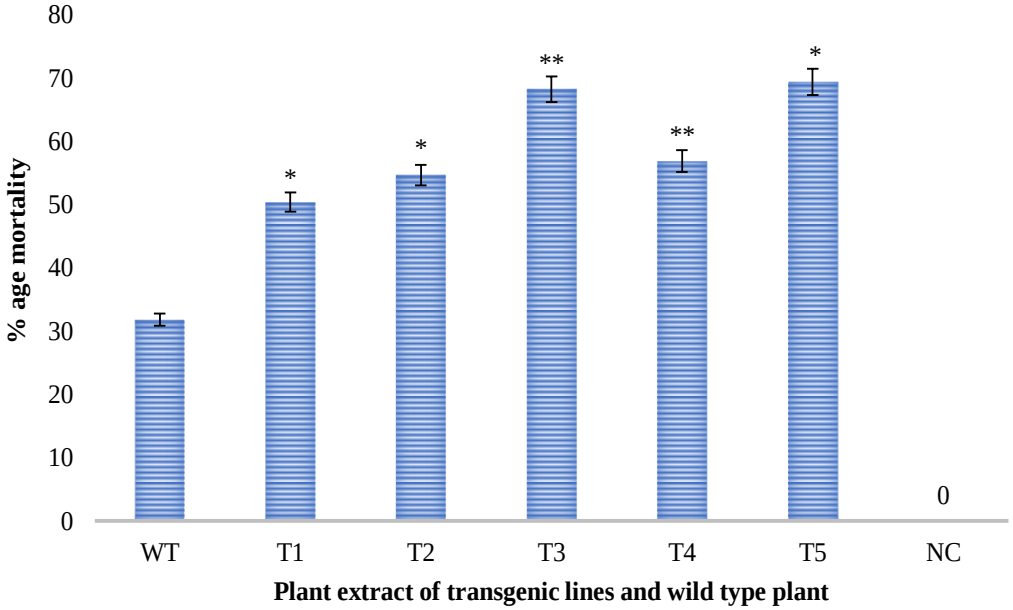


FIGURE 4.28: MTT assay for determination of cytotoxic activity of plant extracts (40 mg/mL) against MCF7 cancer cell lines. "NC" indicates negative control. *Asterisk* shows the significant variance in results in comparison with wild type plants at *P $<$ 0.05 and **P $<$ 0.01.

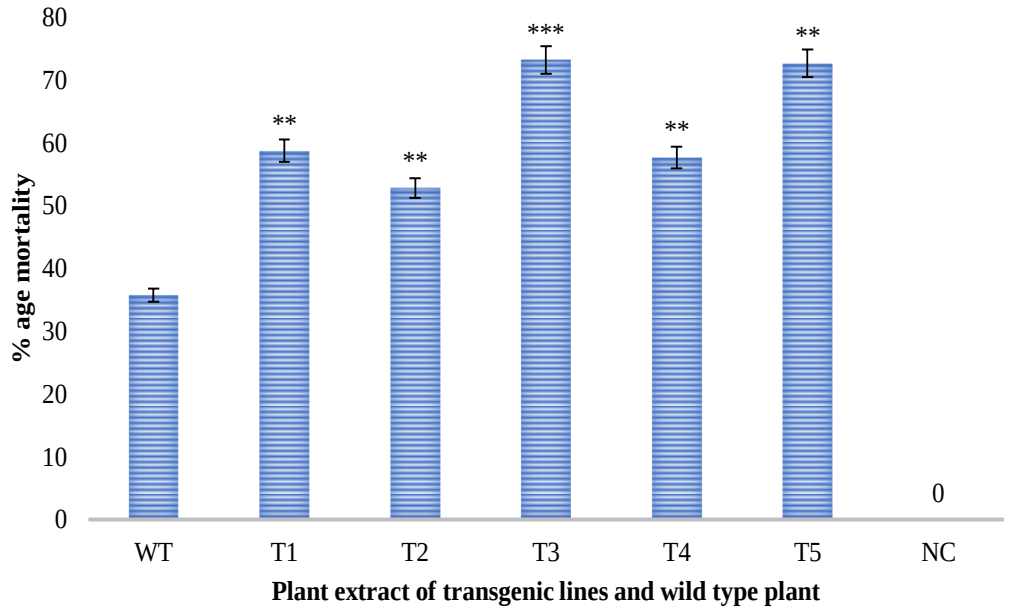


FIGURE 4.29: MTT assay for determination of cytotoxic activity of plant extracts (40 mg/mL) against HePG2 cancer cell lines. "NC" indicates negative control. *Asterisk* shows the significant variance in results in comparison with wild type plants at *P $<$ 0.05, **P $<$ 0.01 and ***P$<$0.001.

TABLE 4.6: Two-way ANOVA for MTT Assay

Source of Variation	**Degrees of Freedom**	**Sum of Squares**	**Mean Square**	**F-Value**	**Prob.**
Cancer cell lines	2	202.2	101.1	118.2	0.0000
Transgenic lines	6	34110	5684	6645	0.0000
Interaction	12	291.4	24.28	28.38	0.0000
Residual (error)	42	35.93	0.8554		
Total	62	34640			

Chapter 5

Discussion

The following research was conducted to increase the secondary metabolite specially flavonoids in *Artemisia carvifolia* plants by transformation with *rol A* gene. Therefore, after identification through DNA barcoding, in the current study *A. tumefaciens* (strain GV3101) harboring *rol A* gene mediated genetic transformation of *Artemisia carvifolia* Buch to enhance secondary metabolites was successfully achieved. Following DNA extraction, shoots of transgenic plants that were growing on media for selection underwent PCR confirmation. Southern blotting was used to assess the transgene copy number after PCR confirmation of the transgenic lines. The *rol A* gene expression was confirmed by the semi-quantitative RT- PCR. The increase in flavonoid content was evaluated through HPLC. It was further investigated that *rol A* gene expression was linked with the increased level of important genes of flavonoid biosynthetic pathway. Furthermore, different antioxidant assays confirmed the enhancement of secondary metabolite content and plant's antioxidant and anticancerous capacity.

5.1 DNA Barcoding for *Artemisia carvifolia* Identification

Following surface sterilization with a solution of ethanol (70%) and mercuric chloride (0.1%), seed germination was initiated on $\frac{1}{2}$ MS media devoid of any growth promoting hormone. The germination process commenced in a week,

facilitated by a two-day cold treatment in the absence of light at 4^{o}C. Following one month, the resulting young plants were utilized to extract DNA, specifically for barcoding purposes. CTAB method was followed for DNA extraction purpose. Upon assessment using a spectrophotometer at 260/280nm, the extracted DNA demonstrated satisfactory quality and quantity. Furthermore, to verify the quality of the DNA, electrophoresis (0.8% agarose gel) was carried out.

DNA barcoding involves identifying species by analyzing the nucleotide diversity in short DNA segments. This method is widely employed as an efficient tool for the quick and accurate identification of plant species. Utilized in the identification of biological specimens, DNA barcoding relies on DNA sequences from either the organelle or nuclear genome [154]. In the realm of traditional medicine, medicinal plants assume a crucial and dispensable role in treating various ailments. However, the herbal industry faces challenges such as the substitution and contamination of therapeutic plants with thoroughly resembling species. The effectiveness of a medicine diminishes when it is contaminated, and in certain instances, substitution with toxic adulterants can have lethal consequences. Therefore, the accuracy of formulation is crucial for ensuring the effectiveness of medicinal herbs.

Traditional techniques for identifying medicinal plants encompass organoleptic methods (relying on the senses: taste, sight, smell, touch), macroscopic and microscopic methods (relying on characteristics like color, shape, and appearance), and chemical profiling (utilizing techniques such as TLC, HPLC-UV, HPLC-MS). Nevertheless, neither of these methods is adept at easily identifying similar species in processed items. The former approach necessitates trained personnel for macroscopic and microscopic analysis, while the latter approach can be influenced by physiological and storage conditions, potentially impacting chemical profiles or markers. Validation at the DNA level offers greater reliability due to the stability of DNA as a macromolecule, unlike RNA. DNA remains unaffected by exterior influences and is present in altogether tissues, adding to its robust nature. Consequently, the creation of DNA-based markers is crucial for the verification of therapeutic herbs. The potential of DNA barcoding lies in its ability to serve as a practical and standardized tool for species-level identification. This technique

holds promise for various applications, including forensic analysis and determining animal diets when the food lacks identifiable morphological criteria [154].

The concept of using 'DNA barcode' as identifiers for taxonomic classification was initially introduced in 2003 [155]. In the examined poisonous plants, the non-coding *trnH-psbA* intergenic spacer emerges as the most promising candidate. Moreover, the chloroplast genes of the *rbcL* and *matK* have also demonstrated suitability as DNA barcodes in the examined loci. They exhibit high universality, sequence quality, coverage, and discriminatory capability. Combining these two regions can enhance species discrimination to achieve optimal results [156].

In the realm of plant species identification, four established barcodes (*matK, trnH–psbA*, *rbcL,* and *ITS*) are primarily regarded for their significance in biodiversity research and preservation efforts [157]. The chloroplast (cp) serves as a vital organelle, playing a pivotal role in plant photosynthesis and various other essential biochemical processes. Chloroplast DNA proves to be a fitting choice for examining phylogenetic relationships among species. In contrast to traditional DNA fragments, the chloroplast (cp) genome displays a relatively conserved structure with subtle variations. This distinctive attribute has been harnessed across a multitude of research areas, including but not limited to species identification and the formulation of molecular markers. The conserved yet subtly variable features of the cp genome contribute to its utility in advancing scientific investigations and applications [158]. This stability is particularly advantageous in fields like species identification, where the conserved regions provide a reliable foundation for distinguishing between different species. Sequences exhibiting lower mutation rates are essential for conducting phylogenetic analyses at higher taxonomic levels, whereas higher mutation rates become crucial for distinguishing closely related species [159].

In the present investigation, the process employed for the identification of *A. carvifolia* involved the initial step of surface sterilizing the seeds, followed by germination on a specific medium reported previously [50]. The choice of a specific germination medium is crucial for promoting optimal growth conditions. Subsequently, DNA extraction was conducted from the shoots of the germinated

plantlets, employing a previously established protocol [146]. The utilization of a reported protocol ensures the reliability and consistency of the DNA extraction process, contributing to the accuracy of the overall identification methodology.

Ensuring the reproducibility of Polymerase Chain Reaction (PCR) results over an extended period is contingent upon the paramount importance of both the quality and quantity of DNA [146]. The presence of secondary compounds in plant tissues adds an extra layer of complexity to the requirement for obtaining high-quality genomic DNA. This often compels researchers to experiment with various extraction methods customized for their specific plant species of interest, aiming to yield substantial amounts of DNA while maintaining its quality. The challenges are further heightened with the advent of high-throughput, next-generation sequencing, especially when simultaneous DNA extraction is needed for multiple species [160]. Various methods have been explored to address the difficulties associated with acquiring suitable DNA from plants. In addressing these challenges, the DNA extraction protocol implemented in this study emerged as particularly effective. This protocol not only demonstrated superiority in preserving the quality of DNA but also proved adept at yielding substantial quantities, contributing to the sustainability and accuracy of molecular investigations in medicinal plant research.

Earlier studies have indicated the efficacy of the place located between the *trnH* and *psbA* genes (non-coding spacer region) of chloroplast DNA as a dependable means for identifying *A. carvifolia*. This particular region has been previously utilized for the discrimination of *A. carvifolia* from other plant species [50]. In the specific case of identifying *A. carvifolia*, the *psbA-trnH* genes region of cp DNA was subjected to amplification through PCR, employing primers designed for the *psbA* and *trnH* regions. This targeted amplification approach, using the mentioned chloroplast DNA region and PCR primers, served as a valuable tool in the precise identification of *A. carvifolia* Buch.

Phylogenetic analysis was undertaken on the chloroplast DNA (cpDNA) *psbA–trnH* sequences derived from a sample pool comprising seven haplotypes sourced from 243 individuals across ten populations of *A. halodendron* [161]. In a study [162] the *psbA-trnH* region was employed for the identification of fourteen medicinal

plant species in Pakistan. This region stands out as the second most frequently utilized barcode for identifying medicinal plant species. Notably, it demonstrated the highest amplification rate at 100% and a maximal discriminatory rate of 83% when compared to various other loci tested, as reported [163]. This underscores the effectiveness of the *psbA-trnH* region as a valuable tool in the identification of medicinal plant species, emphasizing its widespread adoption in botanical research and contributing to the advancement of species discrimination methodologies.

In our study, the DNA, once extracted, underwent amplification using primers specific to the *psbA* and *trnH* genes. The reaction mixture was prepared and PCR was performed following the established protocol. Successful amplification was achieved, producing an amplified product (500 bp) from all DNA samples. As it is advised that an ideal barcode should be of a shorter length to facilitate easy DNA extraction, amplification, and subsequent sequencing.

To further validate the identity, the amplified products were sequenced using the dideoxy chain termination method. Subsequently, the resulting sequences were then matched to the *A. carvifolia* standard *psbA-trnH* sequence that was available on the NCBI web platform. This comparative analysis serves as a crucial step in confirming the accuracy and reliability of the identified DNA, aligning with established standards for molecular identification in botanical research.

The *trnH-psbA* DNA barcode has shown promise as a candidate gene for the effective identification of both bitter and sweet almond varieties, along with their associated species. The findings revealed that among the tested 14 specimens, the *trnH-psbA* plastid genes (1058 bp), were identified as the most variable region within the coding genes. This suggests a higher level of nucleotide diversity in this specific area [164].

To verify and validate the sequences acquired from the studied species of plant, sequencing was done in triplicate through ABI Prism 310 Automated DNA Sequencer, yielding consistent outcomes and thus confirming the existence of nucleotides specific to the species. The CLUSTAL W (function in BioEdit software) was used for identification purpose and comparison of sequences. Mini-barcodes are appealing

due to their enhanced performance, particularly in instances involving degraded DNA extracted from herbal materials [165].

5.2 *Agrobacterium tumefaciens* Mediated Genetic Transformation of *A. carvifolia* Buch

In the present study, seeds of *A. carvifolia* were surface sterilized by using 70% ethanol for different time durations like 30 sec, 60 sec, 80 sec, and 120 sec. The best results were obtained when seeds were sterilized for only 1 min with ethanol. The media was optimized for seeds germination. For that purpose, seeds were placed in water, plain agar, MS and $\frac{1}{2}$ MS containing media. Among these all, half MS was found as the best suitable medium with high seed germination efficiency (98%) for transformation experiments. Furthermore, medium with different combination named SRM 1, SRM 2, and SRM 3 were used for shoot regeneration to figure out the superlative medium for shooting. As shoot regeneration medium 2 (0.5 mg/L BAP + 0.1 mg/L NAA) showed highest regeration efficiency so it was used in transformation experiments. BAP and NAA were supplemented in MS medium to enhance the growth. The transformation protocol was initially established by Vergauwe et al. in 1996, marking the first successful procedure for the rapid regeneration of *Artemisia* plants. Prior to this, there had been no developed method to achieve efficient regeneration in a short time frame. In 1998, Vergauwe further delved into the study of various critical parameters influencing the efficiency of *A. annua* transformation [166, 167]. Previously, in vitro shooting and rooting was achieved successfully in transformed *Artemisia aucheri* Boiss. Explants from leaves were cultured in a media formulated with MS components [168]. To enhance the efficiency of the transformation system for *A. annua* using *A. tumefaciens*-mediated methods, an investigation was conducted into various influential factors. These factors encompassed the *A. tumefaciens* strain selection, identification of the plant genotype, determination of the preculture period, assessment of the composition of the infection bacterium suspension, examination of co-cultivation methods, and establishment of the co-cultivation period [169]. The insights derived from this

research, along with findings from other relevant reports, were subsequently utilized to generate *rol* gene transformants of *A. carvifolia*.

Explants from leaf, stem and stem with nodes (nodal explants) were checked on above mentioned different shoot regeneration media to found their regeneration efficiency. Nodal explants exhibited the highest regeneration efficiency on all media types but on shoot regeneration media 2, it exhibited 55% regeneration efficiency and interaction was found statistically significant ($P < 0.0001$) among the media and explants utilized. Thus, nodal explants were then used in further experiments. Nodal explants were cut from one-month old seedlings of *A. carvifolia* and cultured on shooting media before and after transformation with supplements of BAP and NAA.

From literature review, it was found out that nodal regions of stems cultured on MS basal medium showed highest transformation efficiency. Stem explants with nodal regions demonstrated the highest transformation efficiency (50%) across all types of media, surpassing the performance of leaf explants (25%). Also the media when added with 0.1 mg/L NAA and 0.5 mg/L BAP showed the best results [170]. Similar outcomes were attained when nodal explants of *A. japonica* were cultivated on auxins (NAA) and BAP containing MS medium. Multiple shooting was observed in the explants cultured on BAP containing medium [171].

The time frame of infection and co-cultivation has a major impact on transformation efficiency. Because T-DNA is integrated into plant genomic DNA during co-cultivation, it is a crucial component of plant genetic transformation. It was followed according to reported protocol [50]. In present study, the impact on transformation efficiency of infection time duration and time period of co-cultivation was evaluated. The nodal explants (removed from one-month old seedlings) were infected by *Agrobacterium* culture for different time durations such as 2.5 min, 5 min, 10 min and 20 min. Among all timings, 10 minutes infection time was found as the best optimal duration as it demonstrated the highest transformation efficiency and prolonging the time of infection resulted in the demise of the explants. Different co-cultivation time frames like 12 hrs, 24 hrs and 48 hrs were experimented and time duration of 48 hours was proved to be optimal for achieving the maximum

transformation efficiency. By prolonging the co-cultivation duration, the chances of contamination were increased.

According to a previous report [170], achieving optimal regeneration responses on selection media, an infection time of 10-15 minutes is enough. Prolonged infection periods, however, lead to the demise of the explants. Similarly, co-cultivation period of 48 hours is best according to reported research [170]. In *A. annua,* co-cultivation for 48 to 60 hours was demonstrated to be beneficial for improved transformation, however, co-cultivation for longer periods of time did not improve transformation efficiency [167]. Maximum transient transformation efficiency was achieved by co-cultivating for two days as opposed to one or four days [171]. Other plants in the Asteraceae (Compositae) family also depend strongly on the duration of infection. For instance, during the transformation of an *Agrobacterium* medium, the transient activity and transformation frequency decreased as the bacterial load and infection period increased [172]. In a study, *W. coagulans* was genetically transformed with *Agrobacterium* containing *rol A* genes successfully. The explants used for transformation were exposed to bacteria for five to ten minutes. A longer incubation period resulted in explant's contamination with the bacteria, whereas a shorter period of incubation led to low transformation efficiency. Although the length of co-cultivation varied, optimal results were achieved through a 48 hours co-cultivation period. Following co-cultivation, the plants were transferred to the regeneration medium. The transformed cells were screened using media containing 50 mg/L of kanamycin [173].

In the current study, the selection media contained 300 mg/L of cefotaxime and 50 mg/L of kanamycin for the purpose of selecting transformed explants. Certain monocots and some dicot plants that are unable to secrete acetosyringone can benefit from the addition of acetosyringone, which increases transformation efficiency [169]. When 200 μM acetosyringone was added, efficiency rose to 83% [174]. To boost bacterial pathogenicity, acetosyringone and growth hormones are added to the cocultivation media. The selection media contained 300 mg/L of cefotaxime and 50 mg/L of kanamycin, which were used to choose the transformed explants [175]. According to a number of studies, 50 mg/L of kanamycin is an

adequate concentration for the selection of transgenic *Artemisia* genus plants [176]. *Agrobacterium* growth has been demonstrated to be controlled at 300 mg/L cefotaxime by some reports [177]. A study used the disc-diffusion assay to test the effects of various cefotaxime concentrations on all four bacterial strains. The outcome demonstrated that cefotaxime (>200 mg/L) was more successful in getting rid of every bacterial strain [174].

Rooting was successfully achieved on a medium comprising half-strength MS, enriched with NAA (0.1 mg/L). Positive response on rooting was observed with 70% efficiency. Literature involving *Artemisia annua* genetic transformation by *A. tumefaciens* supports the use of NAA. Researchers found that rooting *Artemisia annua* on 0.05-2.0 mg/L and 0.1 mg/L NAA produced the best results [169]. Improved rooting was observed in the plants transformed with the *rol A* gene when grown on the media supplemented with NAA (0.5 mg/L) and BAP (0.1 mg/L) similar to previously acclimatized transgenic plants (*rol ABC*) [178].

The plants that which survived were moved to the pots. Following the introduction of *rol A* gene through transformation, noticeable morphological differences were observed between transgenic plants carrying *rol A* gene and wild type plants. The *rol A* transgenic plants exhibited characteristics such as shorter, narrower, and darker-colored leaves and they demonstrated a more rapid growth rate on the selection media. Comparable leaf morphological changes were reported in the case of *Ajuga bracteosa* plant transformed with *rol A* gene, where the leaves also displayed varying degrees of (moderate to severe) wrinkling coupled with epinasty [179]. Likewise, alterations or modifications in the appearance and morphology of leaves attributed to the *rol A* gene were documented in tobacco plant [180]. The plants that underwent transformation displayed a stunted growth compared to the wild type plants, aligning with earlier findings reported by researchers that *rol A* gene induces dwarfness in transgenics of *Ajuga bracteosa*, tuber, tomato, tobacco and soybean [145, 179–182]. When *Withania coagulans* was transformed with *rol A* gene, explicit morphological variations were observed. The colour of transformed plants was found to be deeply green, whereas in case of control plants it was not that deep [173]. All *rol A* soybean transformants exhibited notable differences in plant phenotype,

particularly in terms of plant height, overall appearance, plant character, and leaf structure. Furthermore, a distinct alteration in leaf morphology was observed, with the soybean plants that underwent *rol A* transformation displaying leaves of an elliptical shape [183]. Comparable observations were documented in *Artemisia annua*, where plants subjected to transformation with *rol B* and *C* genes displayed stunted growth, and thus exhibit shorter stature compared to the control group. The contrast in leaf morphology was evident, with control plants showcasing broad leaves, while the transformed counterparts presented extremely short and narrow leaves. The stems of transgenic plants exhibited a firmer texture in comparison to the delicate stems of control plants. Although transformed plants exhibited increased branching, this did not have a discernible impact on their overall growth rate [150].

Significant disparities in both growth and morphology were evident in this study. While genetic transformation of *A. carvifolia* using *A. tumefaciens* has been reported for *rol B* and *rol C* genes, there is currently no documented instance of the application of the *rol A* gene in this process. Previously, a research group generated *rol B* and *rol C* genes transgenics of *A. carvifolia*, showing distinct morphological variations as compared to wild-type plants. The transformed plants with *rol B* exhibited broader leaves and increased inflorescence, showcasing accelerated development on the selection media. In contrast, *rol C*-transformed plants demonstrated resistance to regeneration, characterized by a narrower leaf blade and shorter internodes. The findings underscore the morphological heterogeneity induced by the different *rol* genes in comparison to the control or untransformed plants [50]. In an alternative research study, lettuce underwent genetic transformation through the incorporation of the *rol C* gene via *A. tumefaciens* (GV3101 strain). The transformation process involved around 300 explants, with an estimated transformation efficiency ranging between 65–75%. Interestingly, only three transgenic lines successfully matured. The noteworthy outcome of this genetic modification was observed in all altered plants, showcasing distinct phenotypic changes in contrast to the control group. These changes included reductions in leaf area, internodal lengths, stem heights, and alterations in inflorescence patterns [184]. Furthermore, *A. annua* and *A. dubia* plants upon genetic transformation

with the combined *rol ABC* genes exhibited heightened stature and broader leaves [185]. This phenomenon is likely attributed to the collaborative impact of *rol* genes. The observed morphological changes could be a consequence of hormonal imbalances induced by *rol* genes, known to disrupt the auxin-to-cytokinin ratio in favor of cytokinin [186].

Both PCR and southern blotting verified the *Artemisia carvifolia* transgenic plants. PCR results from every transgenic plant were positive. The Southern blot experiment revealed that transgenic lines had both single as well as double copies of gene. The outcomes resembled those of a study where single and double copy numbers were observed in *rol A* gene transformants [179].

According to some reports, *Agrobacterium* transformation systems in *Artemisia annua* have been optimized. Southern blotting has verified the integration of the gene of interest, and 90% of the plants have a double number [187]. About 90% of the regenerated plantlets that were potted in the greenhouse survived. That is comparable with the findings of Fazal et al. According to them, following their acclimatization, 95% of the young plants of *Artemisia absinthium* were effectively transplanted into the soil and kept growing in the field [188].

5.3 Analysis of Flavonoids through HPLC

Numerous chromatographic methods i.e. gas chromatography (GC), paper chromatography (PC), capillary electrophoresis (CE), thin-layer chromatography (TLC), and high-performance liquid chromatography (HPLC), have been used to determine flavonoids. The two most popular techniques for characterizing and quantifying known flavonoids are mass spectrometry (MS) and high performance liquid chromatography (HPLC) combined with DAD/PDA detectors [189].

The identification and separation of nonvolatile substances can be accomplished with high selectivity and sensitivity using the simple HPLC method, which eliminates the need for additional derivatization steps. This is the most desirable method for identifying specific flavonoids in all their matrices, including foods, medicines,

biological samples, and beverages, due to its availability, flexibility, and variety of separation columns and coupling detectors [190].

Typically, heated reflux extraction techniques are used to extract flavonoids from a variety of solvents, including water, acetone, methanol, ethanol, or combination of these solvents. In order to extract polyphenols from plant samples, they must be freeze dried, air dried, or oven dried. In a study, plant extracts of *Dryopteris erythrosora* that were first dried in the air before being dried in an oven showed the highest level of total flavonoid content when compared to plant extracts that were dried in an oven directly [191].

In this current investigation, the examination of flavonoids in both *Artemisia carvifolia* wild type and plants with the incorporated *rol A* gene was conducted, encompassing both qualitative and quantitative assessments. Plant material that had been dried and powdered was extracted using methanol. Water and acetonitrile were employed as the mobile phase. According to HPLC analysis, a substantial increase in all flavonoids up to several times was noticed, and certain flavonoids, like catechin and geutisic acid, were present in transgenic plants only but absent in control plants. Prior research [151] examining the *A. annua* transgenic plants (containing *rol B* and *rol C* genes) revealed similar effects of the *rol* genes. A discernible elevation in the quantity of identified flavonoids was observed. In the *rol B* transgenic plants, the levels of quercetin, isoquercetin, and caffeic acid rose up to four times, six times, and three times, respectively. In contrast, *rol C* gene containing transformed plants displayed three-fold increases in rutin and quercetin, a five-fold increase in isoquercetin, and a 2.6-fold increase in caffeic acid [151]. The HPLC analysis of *A. carvifolia*, encompassing both control and transgenic of *rol B* and *rol C*, unveiled the presence of catechin and apigenin exclusively in the transgenic plants. Notably, the concentration of caffeic acid exhibited a 2.4-fold increase in *rol B* transformants and a 2-fold increase in *rol C* transformants in comparison to the wild type. Additionally, the concentration of quercetin escalated by 6-fold in *rol B* transgenics and 4-fold in *rol C* transgenics. In the control plants, the initial content of isoquercetin was 400 μg/g DW, demonstrating a 1.9-fold increase (770 μg/g DW) in *rol B* transgenics and a 1.6-fold (660 μg/g DW) in *rol*

C gene transgenics. Moreover, the content of rutin in wild-type plants, initially at 300 μg/g DW, experienced a 2.4-fold increase (720 μg/g DW) in *rol B* transgenics and a 1.6-fold increase (570 μg/g DW) in *rol C* transformants [51]. As stated in the introduction, polyphenols had been found in various *Artemisia* plants before, albeit in much smaller amounts. It is well known that *rol ABC* genes are dependable enhancers of the synthesis of secondary metabolites. Plant secondary metabolism has been reported to be strongly stimulated by *rol* genes. Increased flavonoid and phenolic content were noted by Kiani et al. in *rol ABC* transgenics of *A. dubia* [192].

It has been demonstrated that the *rol A* gene stimulates the production of nicotine [193]. *Rubia cordifolia* calli expressing *rol A* generated 2.8 times more anthraquinones (AQs) than control calli [142]. One intriguing biotechnological feature of *rol A* is that its integration in *R. cordifolia* calli generated conditions for vigorous callus growth while also guaranteeing remarkably stable levels of AQs. During a seven-year period of *rol A*-transformed callus line observation, this effect remained constant [142].

Earlier research has identified flavonoids or polyphenols in various *Artemisia* species such as *A. absanthium* [194], *A. abrotanum* [195], *A. annua* [151], *A. asiatica* [196], *A. capillaris* [196], *A. afra* [197]. When *A. dubia* dried plant material of both control and transformed were subjected to phytochemical analysis, the results showed that the transformed plant material produced more flavonoids and phenolic compounds than the untransformed shoots and roots [192].

5.4 Analysis of Expression of Flavonoid Biosynthetic Genes

Metabolic engineering is one rapidly expanding area of plant biotechnology that has increased bioactive compounds production in plant cultures in vitro by interfering with biosynthetic pathways in recent decades [198]. According to a number of reports, the accumulation of flavonoids in plant tissue is directly correlated

with the expression of genes *PAL* and *CHS*. The PAL enzyme is essential to the biosynthesis of flavonoids because it catalyzes the flow of primary metabolites into the phenylpropanoid pathway, which is the route by which flavonoids are biosynthesized. The enzymatic activity responsible for catalyzing the condensation of 4-coumaroyl CoA into the initial flavonoid, namely naringenin chalcone, is facilitated by chalcone synthase (CHS) which has its role as an acyltransferase. CHS stands as the inaugural enzyme within the flavonoid pathway. It is noteworthy that this particular biochemical reaction is regarded as a rate-limiting step crucial to the biosynthesis of flavonoids across diverse plant species [107].

By raising the expression levels of *PAL* and *CHS*, the *rol A* gene is known to play a role in inducing flavonoid biosynthesis. This was confirmed by real-time qPCR. The outcomes are consistent with previous reports that showed both of these genes' expression levels to be higher in *rol* genes transformed *Brassica rapa* [199], *Artemisia carvifolia* [51], and *Lactuca serriola* [200] plants contrast to normal plants. Noticeable alterations in the expression of genes associated with the flavonoid biosynthetic pathway (*PAL* and *CHS*), were evident in *rol* gene transgenics of *A. carvifolia* when compared to non-transformed plants. The qPCR analysis unambiguously demonstrated a substantial upregulation of both target genes in the transformed plants, with *PAL* exhibiting particularly heightened expression.

In *rol B* transformants, the increase in *PAL* gene expression ranged from 8 to 21-fold, contrasting with a 3 to 6-fold increase for *CHS*. Similarly, in *rol C* transformants, *PAL* expression showed a 10 to 19-fold elevation, while *CHS* displayed a 3 to 5.8-fold increase [51].

5.5 Evaluation of Antioxidant Capacity of Wild Type and Transgenic plants of *A. carvifolia*

Antioxidants work by reducing or eliminating free radicals because they are chemical compounds that donate electrons to free radicals, combining to form inactive

compounds that cannot harm an organism's body [201]. One of the primary substances that functions as a powerful antioxidant against free radicals is the phenolic group [202]. The associations between phenolic compounds, flavonoids, and the antioxidant potential of medicinal plants have been extensively documented and studied.

The results of this study show that transformed *A. carvifolia* methanolic extracts have significantly higher antioxidant qualities. Several assays were used to assess the antioxidant capacity of both transformed and untransformed *A. carvifolia* plants. Plants that were genetically modified with the *rol A* gene showed increase in their antioxidant capacity up to several folds. TPC in *rol A* transgenic lines demonstrated an average increase of 1.4-fold in the phenolic content as compared to wild type plants. Evaluation of TFC showed 1-2-fold increase in *rol A* transgenic lines. TAC was increased up to an average 1-2-fold in transformed plants. Similarly, total reducing power was also significantly increased in *rol A* transformed plants. Comparable results were also observed in other published studies on *Artemisia carvifolia* [51], *Artemisia annua* [151], *Lactuca serriola* L. [200], and *Lactuca sativa* L. [184] in which *rol* genes transformation boost the antioxidant potential in transformed plants. The transformed *Artemisia carvifolia* plants' increased antioxidant capacity as a result of the *rol* genes transformation is consistent with the reported findings [51]. The transformation of *Artemisia dubia* with *rol* genes resulted in a notable increase in pharmacological activities, including antioxidant, antitumor, and cytotoxic activities. This suggests that *rol A* gene transformation is a useful tool for inducing and enhancing antioxidant potential in medicinally important species [203]. Compared to transformed roots and untransformed controls, transformed shoots displayed higher levels of antioxidant activity. The shoot's high antioxidant activity was predicted given that it had a higher flavonoid and phenolic content than its roots [192].

The scavenging activity of free radicals in *Artemisia carvifolia* wild type and plants altered with the *rol A* gene was assessed using the DPPH scavenging assay. Scavenging free radicals is an essential part of treating chronic illnesses like cancer and heart disease. The transformed plant extracts exhibited the highest scavenging

effect, as indicated by their lower IC_{50} values, which indicate a greater capacity for protection. The ability to scavenge radicals was increased in the transgenic lines carrying the *rol A* gene. With an IC_{50} value of 206.9 μg/mL, the *rol A* transgenic line T3 demonstrated the highest capacity to scavenge radicals, while the wild type plants showed an IC_{50} value of 627 μg/mL. The outcomes matched the discovery that lower IC_{50} values were observed in *rol* genes transformed *Artemisia* plants for DPPH free radical scavenging activity [51, 151]. The methanolic extracts derived from *Artemisia carvolia rol B* and *rol C* transgenics exhibited elevated antioxidant capacity, greater reducing power, and enhanced protection against DNA damage induced by free radicals. Notably, within the transgenic plants, those carrying the *rol B* gene demonstrated slightly higher activity compared to the *rol C*-transformants [51]. Similarly, an analysis of the free radical scavenging activity revealed that the extracts from the hairy roots of the *Artemisia absinthium* L. transformed with *rol B* and *rol C* genes had the highest antioxidant activity [204]. Research has been conducted to investigate the connection between *R. cardifolia* transformed with *rol B* and *rol C*'s secondary metabolism stimulation and the generation of ROS (reactive oxygen species). They found that the transformed *R. cardifolia* cells had a significant decrease in the amount of intracellular ROS, demonstrating the potential ROS suppressor role of the *rol B* and *rol C* genes. The genes encoding the ROS detoxifying enzymes were also more highly expressed in conjunction with this reduction in ROS [205].

5.6 Evaluation of Cytotoxicity of Wild Type and Transformed Plants on Different Cell Lines

Globally, cancer ranks second in terms of both mortality and morbidity, after cardiovascular disease. According to estimates from the World Health Organization (WHO), 7 million people die from cancer each year and about 11 million people receive a diagnosis of the disease [206]. The most recent study states that there are 5.2 million cancer-related deaths and 8.2 million new cases in Asia. Lung cancer (8%), colorectal cancer (6.2%), and breast cancer (13.5%) afflict the majority of

people [207]. While several therapeutic modalities, including radiation, hormone therapy, immunotherapy, chemotherapy, and targeted therapy, are available to treat cancer, they come with major drawbacks and potential side effects.

Natural products derived from plants have drawn a lot of interest lately because of their varied pharmacological characteristics, which include cytotoxic and anticancer effects. In addition to their many anti-cancer qualities, these plants have the capacity to either enhance the cytotoxicity of cytostatic medications when used in combination with other medications or lessen some of their negative side effects [208]. The MTT cytotoxicity assay is a colorimetric method frequently used to evaluate the cytotoxic properties of various medications, chemicals, environmental contaminants, and plant extracts [209].

Through a variety of pathways, natural flavonoids exhibit anti-inflammatory, anti-tumor, and anti-oxidant properties. By inhibiting the NF-kB pathway in various cancers, these natural flavonoids suppress cell proliferation and cell cycle arrest, triggers apoptosis in colorectal, breast, and prostate cancers, and reduce the activity of nucleoside diphosphate kinase-B in bladder, lung, and colon cancers [210]. The phenolic compounds have antimutagenic, anticarcinogenic, and gene-expression-modifying properties in addition to being antioxidants. Because they can halt DNA mutations in key genes like tumor suppressor or oncogene genes, flavonoids are well known among secondary metabolites for preventing or postponing cancer [211, 212].

In this work, three distinct cell lines HeLa, MCF7, and HePG2 were treated with plant methanolic extracts to test the antiproliferative activity of untransformed and transgenic plants containing the *rol A* gene. The obtained results demonstrated that, in comparison to the wild type plant, all transgenic lines were more effective against all cell lines. After being treated with *rol A* transgenic extracts, the mortality rate in HeLa cells rose to 75%. Similarly, MCF7 and HePG2 mortality rates increased to 70% and 74%, respectively, following treatment with transgenic cell lines. The outcomes matched the previously published research, which showed that HeLa and MCF7 had 60% viability when treated with wild type *A. annua* extract and up to 40% and 35%, respectively, when treated with *rol B* transgenics.

Furthermore, it was discovered that when *Artemisia annua* was transformed with the *rol B* and *rol C* genes, its cytotoxicity against the MCF7, HepG2, and Hela cell lines was significantly higher than it was in the wild type plant [151]. The methanolic extract of the plant reduced the viability of cancer cells to 80%, while the n-hexane extract lowered it to 70%. The cytotoxic effect was significantly amplified when both extracts were applied in combination, revealing a synergistic interaction between flavonoids and artemisinin [52]. It has been documented that extracts derived from different species of *Artemisia* have cytotoxic and anticancer properties against a number of cancer cells [213, 214]. Additionally, *A. ciniformis'*s organic and aqueous fractions have demonstrated an inhibitory effect against H_2O_2-induced cytotoxicity in H9c2 cells [215]. *A. ciniformis*'s dichloromethane fraction significantly cytotoxicated a gastric cancer cell line [216]. Concentration-dependent cytotoxicity of *A. indica* essential oils against four cancer cell lines has been demonstrated [217]. Similar to this, *A. capillaris* and *A. herba alba* essential oils effectively suppressed the growth of oral cancer cells of human and cell lines of severe lymphoblastic leukemia respectively [218, 219].

Comparably, some *Artemisia* species have been shown to be effective against cancer cell lines such as HeLa, P388 murine leukemia, and molt-4 human leukemia in previously published research articles [220–222]. The impact of transformation (using *rol ABC* genes) on *A. dubia*'s cytotoxic and antitumor potentials was reported. The findings indicated that *A. dubia*'s cytotoxicity and antitumor potential have increased as a result of transformation with *rol ABC* genes. The findings revealed that the introduction of *rol ABC* genes has augmented the cytotoxic effects and antitumor capabilities of *A. dubia* [192].

Chapter 6

Conclusion and Future Prospects

The purpose of the current research work was to genetically transform *Artemisia carvifolia* Buch plants with *rol A* gene to check the possible effects of this gene on the plant's biosynthetic pathways of secondary metabolites and thus any enhancement in the potential of the plant as an antioxidant and anticancer.

The *psbA-trnH* sequence found in the chloroplast DNA of *Artemisia carvofolia* plants was used for identification purpose. This sequence served as a valuable tool for the successful identification of this particular plant. Moreover, this genetic marker can be used effectively in identifying different plant species of genus *Artemisia*. The specificity and uniqueness of the *psbA-trnH* gene sequence make it a reliable molecular marker, aiding researchers in distinguishing between different species, and thereby contributing to the accuracy and precision of plant species identification.

Nodal explants from one-month-old *Artemisia carvifolia* plants were precultured before being infected with *Agrobacterium* culture and cocultivated. The selection media for shoot regeneration contained kanamycin and cefotaxime, and NAA with kanamycin was used for rooting. PCR confirmed the successful transformation of *rol A* gene. Despite encountering contaminations, five *rol A* transformants out of 500 explants reached maturity, resulting in a 45% transformation efficiency. Rooting was successful in 70% of cases using 0.1 mg/L NAA and 30 mg/L kanamycin.

Southern blot analysis confirmed the stable integration of *rol A* genes into the genome of *A. carvifolia* across all five transgenic lines. Copy number variation was observed, with T1, T2, and T4 having one copy of the integrated gene, while T3 and T5 displayed two copies. Despite differences in gene expression levels among the lines, RT-PCR validated the expression in all transformed lines. Notably, transgenic lines T3 and T5, with double copy numbers according to southern blot results, exhibited the highest levels of gene expression.

By using the methanolic extracts of both transgenic and wild-type *A. carvifolia* plants, detailed analysis of flavonoids was effectively conducted. For this analysis, HPLC with a DAD detector was used and identified as an optimal and reliable method for detecting and quantifying flavonoids. The results revealed a significant increase, up to several times, in the levels of all flavonoids in the *rol A* transformed plants compared to the wild type, thus indicating the effectiveness of *rol A* gene in enhancing secondary metabolites. The flavonoids, such as catechin and geutisic acid, were exclusively present in the transformed plants and absent in the wild-type plants.

Metabolic pathways of flavonoid production in *A. carvifolia* plants and the impact of *rol A* gene on the genes of these pathways were analyzed through real-time qPCR. Transformed plants with the *rol A* gene exhibited significant changes and higher expression levels of genes (*PAL* & *CHS*) associated with the flavonoid biosynthetic pathway. Notably, the expression of investigated genes was markedly lower in wild-type plants. The *PAL* gene expression was much higher, up to several folds as compared to *CHS* gene expression. All these changes in gene expression were considered to be due to *rol A* gene introduction and which thus proved to play a role in inducing flavonoid biosynthesis.

The wild-type *A. carvifolia* plants' methanolic extracts, upon evaluation for pharmacological potential, displayed antioxidant and anticancer properties, which were notably enhanced in plants transformed with the *rol A* gene. The heightened antioxidant, and anticancer activities observed may be attributed to the increased accumulation of polyphenols (flavonoids). The transformed plants demonstrated enhanced antioxidant potential as evidenced by lower IC_{50} values. The impact of

the *rol A* gene on enhancing the anticancer properties was extremely significant ($P<0.0001$). When treated with *rol A* transgenic extracts, the mortality rate in Hela cells, MCF7, and HePG2 mortality rates increased significantly.

Future Prospects

- The combined effect of *rol ABC* genes construct in *Artemisia carvifolia* should be performed to determine their impact on the synthesis of flavonoids and their comparison with individual *rol A*, *rol B*, and *rol C* could be studied.
- Additional investigations could explore gene expression patterns in T1 and T2 generations using real-time qPCR and Northern blot analysis.
- The genetic transformation of a maximum number of *Artemisia* species with the *rol A* gene could be extended to establish an augmented, stable, and enduring source of vital metabolites
- To enhance the flavonoid content, additional genes involved in their biosynthesis could be introduced into *Artemisia carvifolia* through genetic transformation.
- Conducting additional in-vivo and in-vitro assays is recommended to assess various medicinal properties of flavonoids across different species of *Artemisia*.
- The entire genome of *Artemisia* can be sequenced that could prove valuable in forecasting the genes 'molecular functions across various species of *Artemisia*.
- The enhancement of flavonoid (polyphenol) production in this species has reshaped our understanding of where and how these compounds are synthesized in the plants. These discoveries will contribute to the development of more effective strategies for increasing the synthesis of this valued beneficial medication. This, in turn, will expand its potential applications in the chemotherapy of diverse diseases.

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Appendix-A

Electronic Databases

https://www.ncbi.nlm.nih.gov/

https://www.genome.jp/tools-bin/clustalw

https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome

https://bioedit.software.informer.com/