Effect of Ascorbic Acid and Activated Charcoal on the Secondary Metabolite Enhancement of Callus Culture of *Ocimum Basilicum*

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DECLARATION

I, Maryam Talib D/O Talib Hussain, Registration no 02272011-015, MPhil Biotechnology scholar, Department of Biotechnology, Faculty of Biological Sciences, Quaid-i-Azam University Islamabad, Pakistan, hereby declare that the quoted data in the thesis entitled "Effect of Ascorbic Acid and Activated Charcoal on the Secondary Metabolite Enhancement on the Callus Culture of *Ocimum Basilicum*" is based on genuine work carried under the supervision of Dr. Bilal Haider Abbasi and has not been submitted or published somewhere else.

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Dedication

I dedicate this thesis to my Father Talib Hussain without whom I am nothing!

(Maryam Talib)

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List of Abbreviations

ABTS	(2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)
CAA	Cellular Antioxidant Assay
DPPH	2, 2-diphenyl-1-picrylhydrazyl
DW	Dry weight
FW	Fresh weight
FAO	Food and Agriculture Organization
FRAP	Ferric reducing antioxidant power
HPLC	High Performance Liquid Chromatography
NAA	1-Naphthaleneacetic acid
Mm	Millimeter
RA	Rosmarinic acid
TFP	Total flavonoid production
TPC	Total phenolic content
WHO	World health organization
µl	micro-liter
Rpm	revolution per minute
FC	Folin- Ciocalteu
mM	milli-molar

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(Maryam Talib)

ABSTRACT

Ocimum basilicum (Sweet basil) belongs to family *Lamiaceae* is a medicinal plant with different anti-microbial, analgesic and anti-oxidant properties. Sweet basil is very useful to cure a variety of diseases which includes breathing, gastrointestinal and heart problems. In this study we devised a methodology to uplift biomass and important phytochemical synthesis in callus cultures of *O. basilicum*. Among various elicitation approaches, abiotic elicitation with ascorbic acid and activated charcoal has proven to be efficient. In this study, Ascorbic acid and activated charcoal were used as abiotic elicitors, with concentration of ascorbic acid (A.A 10 and 20 mg/L). Ascorbic acid produced high levels of total flavonoid content 20 mg/L (10.3 mg/L DW) with respect to control. Total phenolic content A.A 1mg/L (11.61 mg/L DW) followed by A.A 5mg/L (11.4 mg/L DW). Activated charcoal produced maximum levels of Total phenolic and Total flavonoid content at control (11.9 mg/L DW) and (8.71mg/L DW). Antioxidant activities (DPPH, ABTS and FRAP) done at callus cultures of *O. basilicum*, when ascorbic acid as an elicitor was applied to callus, large amounts of caffeic acid rosmarinic acid and chicoric acid were detected through HPLC analysis. This study will contribute a better knowledge of the important effects of ascorbic acid and activated charcoal on biochemical parameters in *Ocimum basilicum* callus cultures.

INTRODUCTION

Plants are the beneficial source of cures for wide range of human illnesses. According to WHO (World Health Organization) report, 80 percent of people living in developing countries utilize herbal medicine to treat their different health problems. Plants are the important source for the discovery of novel and secure therapeutic medicines, herb screening for pharmacological activities and phytochemical constituents is one of the most vital field of research (Ahmed and Aujla 2012). Many of the medicines used today are derived from plants (Muralidharan and Dhananjayan 2004).

Ocimum basilicum, which is also known as Sweet basil belongs to the family *Lamiaceae*. It is one of the most commonly grown commercial herb. It is a local herb of Asia, Africa and India, and grown in temperate climates all over the world (Paton et al. 1999; Pripdeevech et al. 2010; Tchoumbougnang et al. 2006). This herb has been used for centuries to treat a variety of diseases including cardiac arrest, gastrointestinal and breathing problems (Makri and Kintzios 2008; Pripdeevech et al. 2010). Furthermore, it has analgesic, antioxidant and antimicrobial properties. The presence of essential oils, phenols and flavonoids which are the physiological and biological active components is credited with this favorable health prospect (Prakash and Gupta 2005). Caffeic acid, chichoric acid, rosmarinic acid and eugenol are the most important phenolic which are found in basil, have broad range of economic and health applications (Jayasinghe et al. 2003; Lee and Scagel 2009). Basil has square, branching stems with opposite leaves, bilabiate calyx and corolla with brown and black seeds (Kaya et al., 2008).

Purple basils, cinnamon basil, fine leaf basil, lemon basils, sweet basils, bush basil and anise basil are the most common types of basils. White fly, aphids and leafhoppers are the most common pests seen on basil plant. Leaf spot, Fusarium and Botrytis are the most common basil illnesses. Basil is drought sensitive, with previous research reporting significant reductions in essential oil content, dry and fresh matter production, chemical constituents like carbohydrate content, protein and proline content (Asadollahi et al. 2013; Ekren et al. 2012). The origin of word basil is the subject of debate, Basil is said to be an abbreviated form of Greek: Basilikon phuton which means imperial herb. Others claim that

the term *Ocimum Basilicum* comes from the Greek words okimon, which means smell and basilikon means regal (Selvakkumar et al. 2007). Sweet basil is an annual and perpetual herbaceous plant that is autogamous and aromatic. Throughout the summer basil produced huge green leaves that are about 2 inches long (Blank et al. 2012; Ch et al. 2015). The usage of sweet basil in the treatment of various diseases drew a lot of attention in Indian medicine's ancient systems (Unani and Ayurvedic). Basil is aromatic herb and have popular source of flavoring principles that is utilized in herbal remedies (Akbari et al. ; Marwat et al. 2011).

Leaves and flowering portions of Sweet basil are used as digestive, cordial agent, stomachic, anti-inflammatory agent, aromatic and flatus relieving. They have been used to cure odor loss, snake bites, skin ailments, insect stings and skin inflammation (Grieve 2013). *O. Basilicum* essential oil has been utilized in traditional medicine to treat variety of ailments. The aerial component of the plant has long been employed as an anti-inflammatory agent, fragrant, digestive, stomachic and cordial agent (Chang et al. 2009).

It has been used to treat various kind of ailments like anxiety, stomach pain, inflammation, headache, wheeze, contraction and arthropod bites (Murugan et al. 2007; Twilley et al. 2018). It also has anti-diabetic, anti-inflammatory characteristics as well as lower the blood glucose level and also possess antioxidant, antibacterial and antifungal capabilities which have been demonstrated in previous studies (Ahmad et al. 2016; Ahmed et al. 2019; Mousavi et al. 2018; Stanojevic et al. 2017). Traditional usage of this plant include use as a flavoring agent in the culinary industry as well as use in oral and dental treatments (da Silva Gündel et al. 2018). It is abundantly grown in Iran and people have utilized it as a vegetable and a medicinal herb (Akbari et al. 2018). The seeds of sweet basil were used as a nutritional fiber in Asian liquor and pudding (Hajmohammadi et al. 2016). Wheeze, headache, dysentery and skin infections are all treated with it (Labra et al. 2004).

In-vitro methods for the industrial production of different plant metabolites promised to solve the problem of varying product standard in nature which is effected by a variety of environmental conditions such as pests, climate and diseases. Selection of high yield producer lines can force the biosynthesis of interested chemicals in plant cell cultures (Shibli et al. 1997; Zenk and MH 1978). Biotechnology allows scientists to grow tissues, cells and

organ or complete organism in-vitro and genetically alter them to produce desired substances. World's population is growing continuously, there is a tremendous pressure on accessible land to provide food and meet demand. As a result, the available land should be utilized efficiently for other purposes such as the manufacture of medications and chemicals from plants (Tatli 2012). Plant dependent in-vitro systems for example organ cultures, callus cultures and cell suspension and genetic alteration to promote the development of interested plants and plant products are both interesting potential in biotechnology (Badea and Basu 2010).

In response to stresses like injury or pathogen attack, plants produce disordered mass of cells which is known as callus (Neely 1979). In medicine, the term callus refers to the hardening of dermal tissue, it comes from the Latin word callum, which means thick. The term callus was first used in plant biology to describe the rapid proliferation of cells and deposition of cellulose that occurs when plant is injured. Unorganized cell masses are collectively known as callus. A single transformed cell can produce callus, and a lot of callus cells are totipotent meaning they can revive the entire plant body (Nagata and Takebe 1971; Steward et al. 1958). PGR's (Plant growth hormones or phytohormones) are actually plant hormones that regulate a variety of morphological and physiological benefits in plants (Bhojwani and Razdan 1986; Srivastava 2002). Plant growth regulators are produced by plants, many plant species can thrive without the use of exogenous media supplementation (Baksha et al. 2005; Hussey 1982; Wala and Jasrai 2003).

1.1.AIMS AND OBJECTIVES

- To determine the effect of ascorbic acid and activated charcoal as an elicitor in the secondary metabolite enhancement of callus culture of *Ocimum Basilicum.*
- To determine Total Flavonoid Content (TFC), Total Phenolic Content (TPC) and free radical scavenging activity (DPPH).
- To identify the secondary metabolites through HPLC.

LITERATURE REVIEW

2.1. Characteristics of *Ocimum Basilicum*

O. Bassilicum or sweet basil is one of the Lamiaceae family's most economically valuable fragrant herb (Purushothaman et al. 2018). It's a fragrant yearly herb. It can grow to a maximum height of 60 cm and germinates 14 to 21 days after sowing. It has round, pointed and complementary leaves. Basil blossoms are fragrant, small and come in variety of colors including violent and red. It bears black seeds that are small. Its growing span is between 170 and 180 days, depending on the environmental circumstances. During the germinating season it can be picked two to three times (Asadollahi et al. 2013; Bączek et al. 2019; Makri and Kintzios 2008). According to certain research, a large amount of phenolic compounds (Caffeic acid and rosmarinic acid) which have strong antioxidant properties are found in sweet basil (Lee and Scagel 2009; Surveswaran et al. 2007).

Fig 2.1 *Ocimum Basilicum* (Sweet Basil)

https://www.gardenia.net/plant/ocimum-basilicum

2.2. Taxonomy

Kingdom	Plantae
Phylum	Magnoliophyta
Class	Magnoliopsida
Order	Lamiales
Family	Lamiacaea
Genus	Ocimum
Specie	Basilicum

2.3. Morphology

Ocimum has around 30 different shrubs and herbs species. It had a wide range of morphology, bloom color, growth habits, stem and leaves and chemical content (Calín-Sánchez et al. 2012; Filip 2017; Jirovetz et al. 2003). Color of seed is black while the shape of seed is oval, color of leaf is green and leaf margin is somewhat undulate. Flowering season is from October to December. Parts of Sweet basil which are commonly used are blooming top and leaves. Sweet basil is a moderate sized herb with a powerful smell with even or velvety touch. The herb's leaves are full length, uncomplicated, oval and antipodal in shape. They have 3-5 cm long serrated petiole. Flowers of Sweet basil are 8 to 10 mm long and are ordered in bunch of 6 to 10 flowers in circle. The petals might be purple, white or pinkish in color. On both sides of the herb's leaves there are glandular and non-glandular hairs (Ahmed and Aujla 2012).

2.4. Geographical Distribution

O. Basilicum is a fragrant, herbaceous plant that is grown in different parts of the world (Acharya et al. 2020). Sweet basil, which is thought to have evolved in the warmer

Indo-Malayan areas, is plentiful throughout the Pak-Indo subcontinent's humid and warmer areas. It thrives in a variety of settings including high grounds and wilds. Pollination is carried out with the help of insects (Ahmed and Aujla 2012). *Ocimum Basilicum* is native to high grounds of India, Sindh and Persia but it is also grown in a number of Mediterranean countries which includes Turkey (Nadkarni 2010). The ideal temperature for vegetation is between 21 and 30 degree Celsius, basil thrives in warm climates grows best over long days in the sun (Putievsky 1983).

2.5. Medicinal Uses

2.5.1. Medicinal Uses in Ancient Times

Sweet basil is familiar for its traditional therapeutic properties and is formally recognized in a number of nations (Lawrence 1985). *O. Basilicum* leaves and blossoms are used as a stimulant and anthelmintic in traditional medicine, and basil tea can help with diarrhea and vomiting. Plant oil is used to cure contractions, mental weariness, rose cold, sting of bee and snake bites as a first aid therapy (Demir and Özcan 2001). Sweet basil can be used to treat wheeze, stomach disorder, gouty arthritis and fever and it can also be used inside the body to cure inflammation of the urinary bladder and ureters, Bright's disease and haemorrhoid piles (Nadkarni 2010). Sweet basil essential oil has been used in traditional medicine to treat a variety of ailments. The areal component of the plant for example has long been used as fragrant, anti-inflammatory, flatus relieving, ingestion, stimulant and stomach problem (Marwat et al. 2011). Folk Chinese medicine has employed basil polysaccharides to cure cancer (Zhan et al. 2020).

2.5.2. Medicinal Uses of Sweet Basil in Modern Era

Sweet basil essential oil is economically important because of its broad use in makeup, nourishment and drug industries (Al-Maskri et al. 2011). In diabetic rats, a tincture ethanol of basil leaves can lower blood sugar and advanced glycation finished product (Widjaja and Rusdiana 2019). Saponins, alkaloids, flavonoids and tannins are some of the important essential oil molecules found in *O. Basilicum* leaves (Bansod and Rai 2008;

Dharmagadda et al. 2005; Kadian and Parle 2012). Anti-inflammatory, anti-convulsant, stomach pain relieving and diuretic characteristics are all found in *O.Basilicum* leaves (Choi and Hu 2008; Gahruie et al. 2017). Polysaccharides of basil leaves has anti-aging properties, immune-modulatory, anti-inflammatory as well as anti-bacterial, immunity boating properties and are useful in the treatment of diabetes mellitus (Feng et al. 2019).Basil is used in cosmetics such as oils, fragrances, moisturizers, shampoos and detergents. Basil tea helps with ingestion, gas elimination, contractions, nausea and loose motions. It is also used to cure mental exhaustion, wheezing and nervous problems (Grieve 2013).

2.6. Chemical Constituents and Nutritional Composition

Aldehydes, terpnes, alcohols and phenylpropanoids have been identified as major essential oil components which are found in sweet basil (ANTIĆ et al. 2019). The principle phenolic components that are found in *O. Basilicum* are flavanol-glycosides and phenolic acids (Javanmardi et al. 2002). Arachidonic acid, Stearic acid, Mystiric acid, Palmitic acid, Linoleic acid, Lauric acid, Oleic acid and Capric acid are the primary fatty acids that are found in basil species. Anti-oxidant capability is affected by increased temperature and light circumstances (Castronuovo et al. 2019). Eugenol and n-Cinnamate, Methyl chavicol, Ocimene, Geraneol, 1, 8 cineole, Borneol and B-Caryphyllone are some of the essential oils that are found in basil (Taie and RADWAN 2010). Chavicolandterpenoids and Eugenol are the vital essential oils which are present in basil (Gang et al. 2001; Grayer et al. 1996; Nacar and Tansi 2000).

2.7. Pharmacological Properties

Plant derived compounds have been employed in medicine, either in its natural state or after chemical alteration (Ramawat and Mérillon 2008). Sweet basil has a wide range of curative uses. Candida albicans yeast, bacterial strains of E.coli, Staph. Aureus, P.aeruginosa and E. faecalis were all tested using the essential oil of sweet basil. The oil of Ocimum basilicum has the highest MIC (Minimum Inhibitory Concentration) against Candida albicans (Ahmed and Aujla 2012; Kashyap et al. 2011).Sweet basil is used to treat different disorders like muscle pain, inhaling problems, fungicides and diabetes. It works as an anti-

inflammatory substance, wheeze, faintness, stomachache, vomiting, headache, painful or swollen testicles, Diarrhea with belly cramps, febrile condition, insensibility, immobility and worried temperament (Sarfraz et al. 2011; Sekarl et al. 2009).

2.7.1. Antioxidant Activity

Five distinct extracts of Ocimum basilicum. L and Origanum vulgare L. were examined for their antioxidant activities. Their antioxidant activity differed, which could be explained in part by the amounts of flavonoids and phenolics in the O. vulgare and O. basilicum extracts studied (Miraj and Kiani 2016). Nitric oxide radical scavenging assays, DPPH, hydroxyl and reducing potential assays were used to access the antioxidant activity of carbon tetrachloride, ethanolic and choloform extracts of O.basilicum in vitro. When compared to typical antioxidants, the ethanolic extract of sweet basil manifest higher antioxidant activity (Issazadeh et al. 2012). To evaluate the antioxidant impact of a preparation, a formulation containing 3 percent thick basil extract was studied. The preparation had significant impacts on skin bumpiness, skin moisture content, skin aging, skin dryness as well as increase in energy. The findings revealed that topical use of preparation comprising basil extract has anti-aging properties (Rasul and Akhtar 2011). A similar experiment was carried out on ethanolic extract of sweet basil leaves, which showed considerable superoxide radical scavenging and nitric oxide activity, showing their robust antioxidant properties (Meera et al. 2009).

2.7.2. Antimicrobial Activities

Essential oils from Ocimum species, particularly sweet basil, have been studied for anti-oxidant, anti-fungal and anti-bacterial properties. Mycotoxins can be produced by food-borne fungus, which can induce food and cereal deterioration. Basil oil has been found to be one of the most beneficial natural compounds in experiments conducted on various chemicals. *E. chevalieri, Alternaria. Sp, Cladosporium herbarum, Aspergillus flavus, Eurotium chevalieri, Botrytis cinerea* and *Eurotium amstelodami* were the seven fungi isolated from plants. *E. chevalieri* was the most delicate to basil oil while *A. flavus* was the most withstanding to basil oil (Jakowienko et al. 2011). Sweet basil oil was effective against

the fungus *Penicillium brevicompactum, Penicillium aurantiogriseum, Penicillium chrysogenum* and *Penicillium galbarum* (Kocić-Tanackov et al., 2012). *P. chrysogenum* has the highest reactivity, with 1.5 percent basil extract that is totally inhabiting the growth (Piyo, A et al., 2009). The poisonous fungus *F. moniliforme, A. parasiticus, A. flavus* and *A. ochraceus* were completely restricted by basil oil at 3000 ppm (Soliman and Badeaa, 2002). When conventional antibiotics and *Ocimum basilicum* essential oil were mixed, the medicinal effects on the bacterial strains *P. aeruginosa* and *Staph. aureus* were examined. *O. basilicum* essential oil were mixed with existing conventional antibiotics, their antibacterial action may be enhanced that results in symbiotic activity against clinically important bacterial strains. Linalool maybe responsible for sweet basil essential oil's antimicrobial properties (Araujo Silva V et al., 2016).

2.8. Plant Tissue Culture

Sterile culture of tissues, cells, organs or complete plants under synchronized environmental and nourishing conditions is known as tissue culture (Thorpe, 2007). Plants have been our sole source of fats, carbohydrates and protein for nourishment and shelter. Plants also produce a variety of secondary metabolites that are employed as medications, perfumes, as preservative medium for food, dye, seasonings, agro-chemicals and bio-pesticides (Phillipson, 1990; Balandrin and Klocke, 1988; Fowler and Scragg, 1988). Chemistry of the bulk of the plant species has yet to be defined, plants will pursue to produce unique products in addition chemical models for new treatments in the future generations (Cox and Balick, 1994). Plant cells have ability to give rise to any cell type, which implies each cell in a culture keeps its inherited information and thus synthesize the same chemicals as the mother plant. The following are some of the benefits of this technology over traditional agriculture fabrication:

- It is unaffected by periodic and geographical changes, as well as provisional conditions.
- It has a well-defined manufacturing system that confirms consistent product yield, standard and supply.
- It is unaffected by political influences

- It is convincible to make unique compounds that aren't found in mother plant.
- Downstream reconstruction and product are both well-organized.
- Plant cells can also undertake regiospecific and stereo bio-transformations to create new chemicals from inexpensive ingredients.
- Production speed is quick (Ravishankar and Venkartaraman, 1993).

2.8.1. Micro-propagation

The process of vegetative development and proliferation from plant seeds or tissues is known as micro-propagation. It is done on growing media in sterile and suitable circumstances, employing a variety of plant tissue culture methods (Zhou and J.Y, 2006; Leifert et al., 1989; Bhojwani et al., 1996). Tissue culture is founded on the notion of totipotency, which refers to a plant's potential to develop into a new plant from its tissues and cells (Fowler et al., 1993). Many plants under traditional cultivation do not vegetate, blossom and give rise to seed under particular climatic circumstances, and they develop and multiply over lengthy periods of time. Micro-propagation ensures a steady supply of therapeutic plants while using the least amount of time and area possible (Prakash and J. Van, 2007).

The following are some of the benefits of in-vitro micro-propagation of therapeutic plants:

1. Finding and producing clones with appropriate traits.
2. It is possible to create novel and improved genetically altered plants.
3. A faster multiplication rate.
4. Cryopreservation of inheritable material.
5. Secondary metabolite production.
6. Maintenance of plant species that are at verge of extinction.
7. The environment can be modified or managed to fit the plant's specific requirements.
8. Year round- availability of the plant (Gamborg et al., 1976).

2.8.2. Callus Cultures

Plant tissue culture is a crucial technology in both basic research and commercial applications. Un-differentiated callus cells restore injured tissue in all important terrestrial

plant families. For Biotechnological administrations, these callus cells can be cultured in-vitro. Callus cell cultures can be made from any section of the plant. If explants from plant tissues are acquired under sterile circumstances to avoid to avoid microbial contamination and cultivated on solid gel media that is supplied with growth hormones like cytokinin or auxin they progressively expand in-vitro a mass of cells that span from colorless and structure-less to pale brown. Callus cultures can be kept in- vitro forever by moving the cells on a regular basis. If maintained in adequate growth media that differs from typical culture media, callus cultures can be re-differentiate into whole plants. While certain callus cultures require dark circumstance to grow, others thrive in specific day to night conditions (Bonner, 1936).

MATERIALS AND METHODS

All of the research work was done under the supervision of Dr. Bilal Haider Abbasi at the Plant Cell Culture Laboratory, Department of Biotechnology, Quaid-i-Azam University Islamabad.

3.1. Apparatus

Spirit lamp, autoclave (KP- 30L, ALP Tokyo Japan), forceps, spatula, blades, pH meter (Jenway 3305), Laminar flow transfer cabinet, electrical balance and Glassware like petri dish, measuring cylinders, Erlenmeyer flask were used in this study.

3.2. Callus Culture Establishment

Explants of 2-3 mm leaves were aseptically removed from a 20-days old plantlet and placed on Murashige and Skoog basil medium that is supplemented by plant growth regulator 2.5 NAA. Callus were sub-cultured on MS media with same Plant growth regulator composition and a light intensity of 40 µmol/m^2/s under a 16/8 h (dark/light) photoperiod. MS medium without any plant growth regulator was used as a control. Callus cultures were collected on 28th day and dried in the oven.

3.3. Analytical Methods

Ahmad at al. (2010) provided a methodology for extraction from dried samples. Fresh culture was dehydrated at 45 degree cellsius overnight in incubator (Memmert- INB 200, 230V, Germany). 50 mg of dried callus was mixed in 500 µl of MeOH (Sigma Aldrich), then sonicated for almost 60 minutes at 25 degree cellcius at a 45 KHz ultrasonic frequency. Vortexing for 5 minutes and centrifugation at 10,000 rpm for 15 minutes (Spectrafuge^{TM} 24D micro centrifuge, Labnet internation) were used to collect the extract. The supernatant was removed from the settled powder and maintained at 4 degree Celsius for further analysis such

as determination of free radical scavenging activity DPPH, total phenolic content (TPC) and total flavonoid content (TFC).

3.4. Determination of Total Phenolic Content

The total phenolic content was determined using Singleton et al., (1965). 20 micro-liters of extract were combined 90µl of Folin- Ciocalteu reagent and the incubated for almost 5 minutes at room temperature. After incubation, the mixture was diluted with 90µl of sodium carbonate and with the help of micro-plate reader (Thermo Scientific Multiskan GO) readings were collected at 630nm. The calibration curve was plotted using gallic acid as the standard and the results were expressed as gallic acid equivalents (GAE)/g of DW.

Total phenolic content (mg/l) = DW (g/l) × TPC (mg/g)

This formula is used to calculate total phenolic production and is shown in mg/l

3.5. Total Flavonoid Content

Total flavonoid content was determined by using Fazal et al, (2016) methodology with minimal changes. 20 micro liter of plant extract was combined with 10µl of aluminum chloride $AlCl_3$ and potassium acetate. Each sample was then diluted water and then incubated for almost 30 minutes. Using a micro plate reader, readings were taken at 415nm under UV-visible spectrophotometer. Total Flavonoid content was calculated using quercetin standard calibration curve, with data expressed as quercetin equivalents (QE)/g of DW.

Total flavonoid production (mg/l) = DW (g/l) × TFC (mg/g)

To calculate Total flavonoid content this formula was used and shown in mg/l.

3.6. Antioxidant Activities

These three assays i.e. DPPH, FRAP and ABTS were performed to determine antioxidant potential of callus treated with ascorbic acid and activated charcoal.

3.6.1. DPPH (Free Radical Scavenging Assay)

The scavenging potential of free radicals was determined using the method of Fazal et al., (1965). 20 micro liters of extract was mixed with 180µl of DPPH solution at room temperature and then incubated for one hour in dark. Using a micro reader plate readings were taken at 517nm. In each case ascorbic acid was employed as a control. The following equation was used to calculate free radical scavenging activity:

% scavenging DPPH free radical = 100 × 1 --AE/AD

Where AE represents Absorbance of Dilution and AD represents the absorbance of pure DPPH as standard.

3.6.2. FRAP (Ferric Reducing Antioxidant Power) Assay

FRAP potential of callus culture treated with ascorbic acid and activated charcoal was done via given protocol of (Benzie and Strain 1996) by adding FRAP solution composed of 300mM of (acetate buffer), containing 20mM of hydrated iron chloride (FeCl3.6H2O) and 10 mM of (TPTZ). 190µl of FRAP solution was mixed with 10µl of callus extract and final volume as kept 200 microliters and reaction mixture was incubated at room temperature for fifteen minutes and then readind of absobtaion was taken at wavelength of 630nm through Micro-plate reader. All the procedure was repeated 3 times and antioxidant potential of all the samples were shown in the form of TEAC which is abbrivated for (Trolox C equivalent antioxidant capacity).

3.6.3. Antioxidant ABTS Assay

Protocol of (Tagliazucchi et al., 2010) is used to determine ABTS assay in which ABTS solution was applied to determine the antioxidant ABTS assay of all callus cultures. For this purpose, ABTS solution was made by pouring potassium persulphate (2.45mM) to ABTS salt (7 mM) in equal proportion with incubation in dark for up to for 16 hours. Initially the absorbance of the solution was fixed to 0.7 at a wavelength of 734 nm. After that callus

extract was readily mixed with the solution and remained incubated at room temperature for 15 min in dark. Absorbance of this mixture was analyzed by (ELX800, BioTek Instruments) Microplate reader at wavelength of 734 nm. This protocol was repeated thrice, and antioxidant potential of these samples were shown in form of TAEC.

3.7. HPLC Analysis

Following steps were used for the preparation of extract: 100 mg of dried material was homogenized in 500 micro liter HPLC grade methanol for 1 minute at 19,000 rpm using a blender (Ultraturrax, T25 basic) and then extraction was carried out in an ultrasonic bath USC1200TH (Prolabo; inner dimension 300 mm into 240 mm into 200 mm, provided with an electrical supply of 400W, supreme heating power of 400W and fluctuating frequencies, provided with a computerized timer, temperature regulator and frequency) during 1 hour at 30 kHz frequency and 25 plus minus 2 degree Celsius. Each sample was then centrifuged at 10,000 rpm for 5 minutes after extraction. The supernatant was removed and vaporized at 40 degree Celsius, then re-suspended in 0.1M (1 ml) 4.8 PH buffer (citrate phosphate) carrying 5 units per ml beta-glucosidase from almonds (Sigma Adrich) and incubated for almost 4 hours at 37 degree Celsius to clarify chromatograms and liberate glycine. Following that, each sample was centrifuged at 10,000 rpm for 5 minutes. And then the supernatant was sieved using a (Millipore) 0.45 micro-molar syringe filter before HPLC exploration. A Varian liquid chromatographic set-up consisted of Varian prostar 335 photodiode array detector (PAD), Varian Prostar 230 pump, Varian Prostar 410 auto sampler and Metachem Degasit was used for photochemical analysis, which was managed by Galaxie version 1.9.3.2 software. Separation was carried out at 35 degree Celsius using a Purospher RP-18 (Merck) column (250 into 4.0 mm, i.d. 5 micro-meter). The mobile phase consists of solvent A with 0.2 percent acetic acid and solvent B with methanol and a non-linear gradient of A-B: 90:10 (v/v) to 30:70 (v/v), from 0 to 40 minutes, A-B: 30:70 (v/v) to 0:100 (v/v) from 41 to 50 minutes and A-B: 0:100 (v/v) from 51 to 60 minutes. The chemicals were identified by comparing their retention periods and UV spectra at wavelength of 280nm. Each standard was quantified 5 point calibration curves with correlation value of 0.999.

3.8. Statistical Analysis

Experimental treatments were carried out in triplicate in the same laboratory under the same conditions. For the development of all graphs, the mean value of different treatments cited to analysis using the Graph Pad prism (Windows, v8.0)

RESULTS AND DISCUSSION

4.1. Biomass Accumulation

After 30 days of inoculation of plantlets were formed. These Plantlets were used for culturing on previously optimized protocol. Leaf explants up to 0.5 cm^2 were inoculated on MS media enriched with plant growth hormone in combination BAP with NAA (5 mg/L and 1 mg/L, respectively (Abbasi et al. 2010). Various concentrations of ascorbic acid and activated charcoal were prepared and applied to the media. A substantial difference occurred in biomass accumulation under varied ascorbic acid and activated charcoal concentrations.

Maximum biomass accumulation occurred at ascorbic acid treated culture A.A 10 mg/L (233.14 g/L) followed by A.A 20 mg/L (216.28). While minimum biomass accumulation was noted at control (143.71) as shown in figure (4.1).these results shows that an increase in the biomass accumulation of *Ocimum basilicum* occur with increase in concentration of ascorbic acid. The color of callus treated with ascorbic was noted to be light green and callus was compact as shown in figure (4.2).

Activated charcoal has antagonistic effect on the biomass accumulation of *Ocimum basilicum*. Decrease in the biomass accumulation occur with increase in concentration of activated charcoal. Maximum biomass accumulation was noted for control (157.28 g/L) as shown in figure (4.3). Minimum biomass accumulation was noted at the highest concentration of activated charcoal treated callus A.C 500 mg/L (71.42 g/L). There is a browning effect of activated charcoal on the color of callus which increase with increase in concentration of A.C as shown in figure (4.4). Similarly for dry biomass maximum (9.71 g/L) was noted on control and minimum on the high concentration of activated charcoal A.C (4.28 g/L).

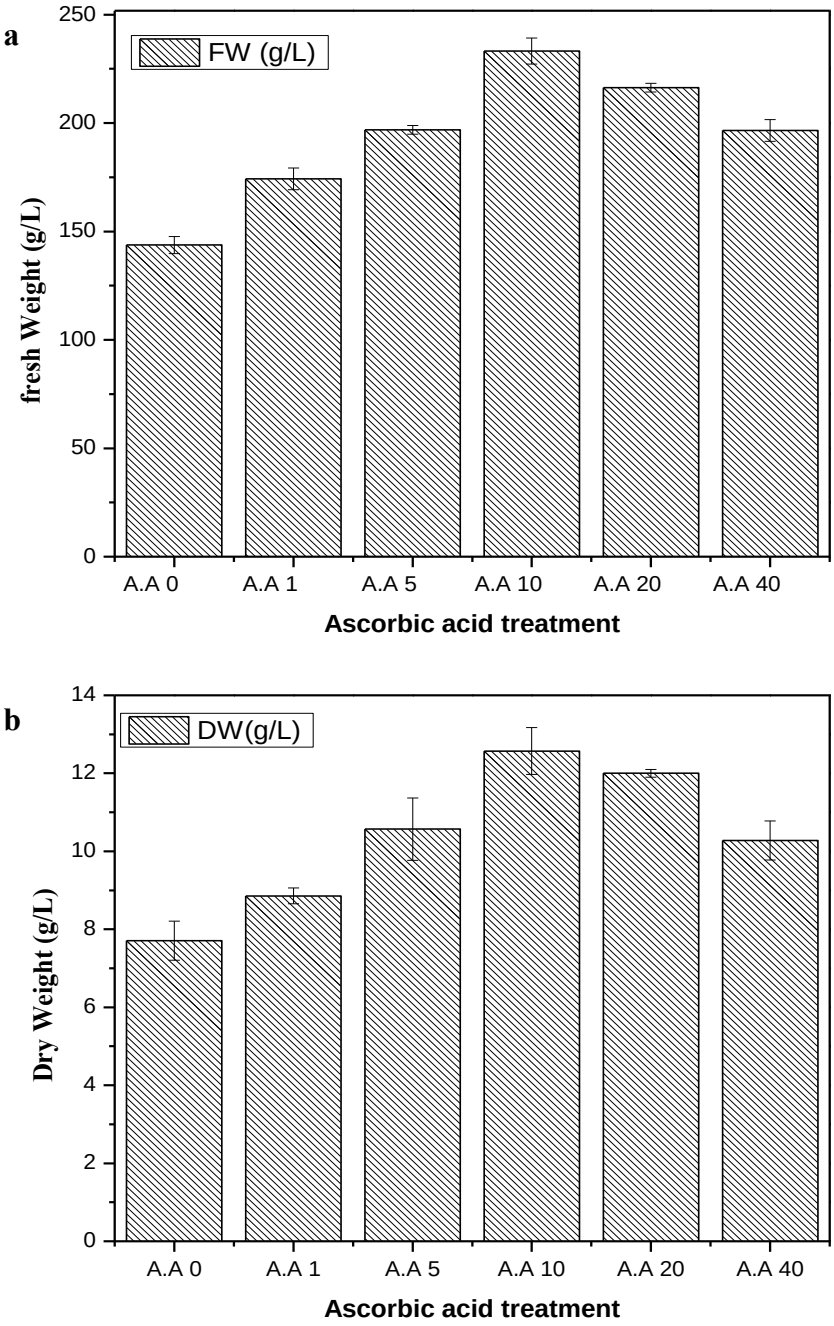

Figure 4.1: Biomass accumulation in callus culture of *Ocimum basilicum* grown on culture media treated with ascorbic acid. (a) shows fresh biomass while (b) shows dry biomass accumulation.

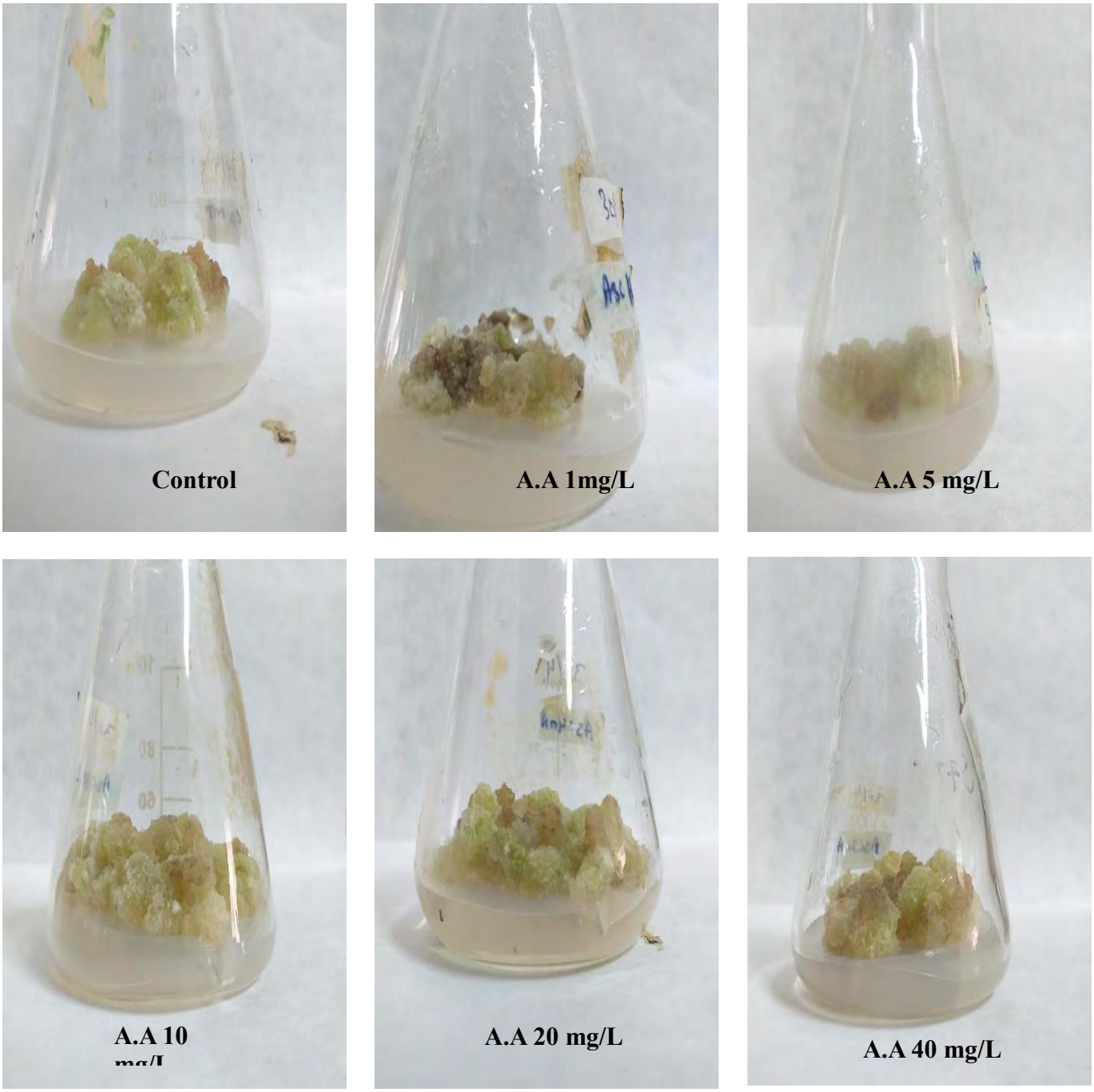

Figure 4.2: Effect of ascorbic acid on biomass and morphology of *Ocimum basilicum*. Maximum biomass was noted for A.A 20 mg/L while minimum was noted on control.

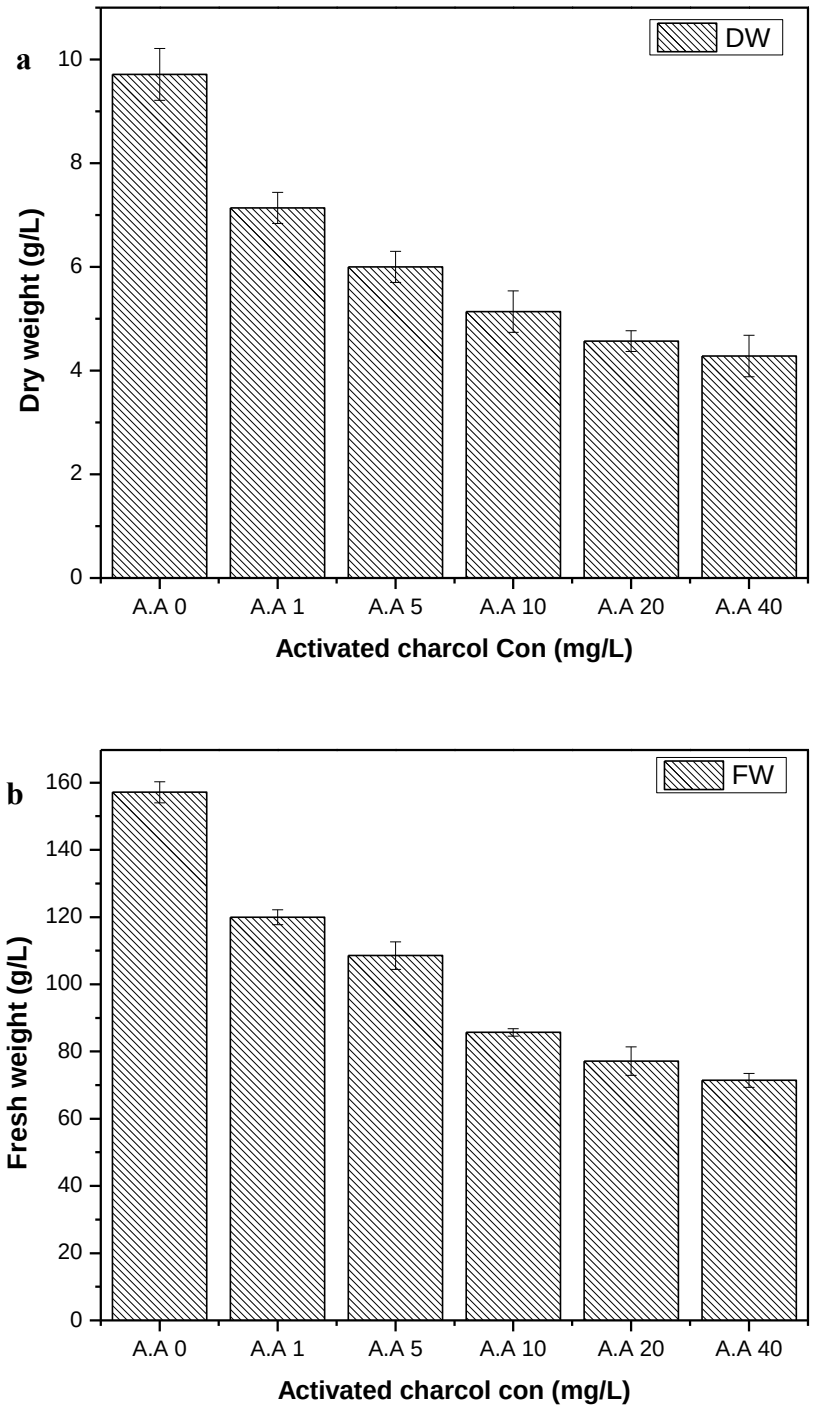

Figure 4.3: Biomass accumulation in callus culture of *Ocimum basilicum* grown on culture media treated with activated charcol. (a) shows dry biomass while (b) shows fresh biomass accumulation.

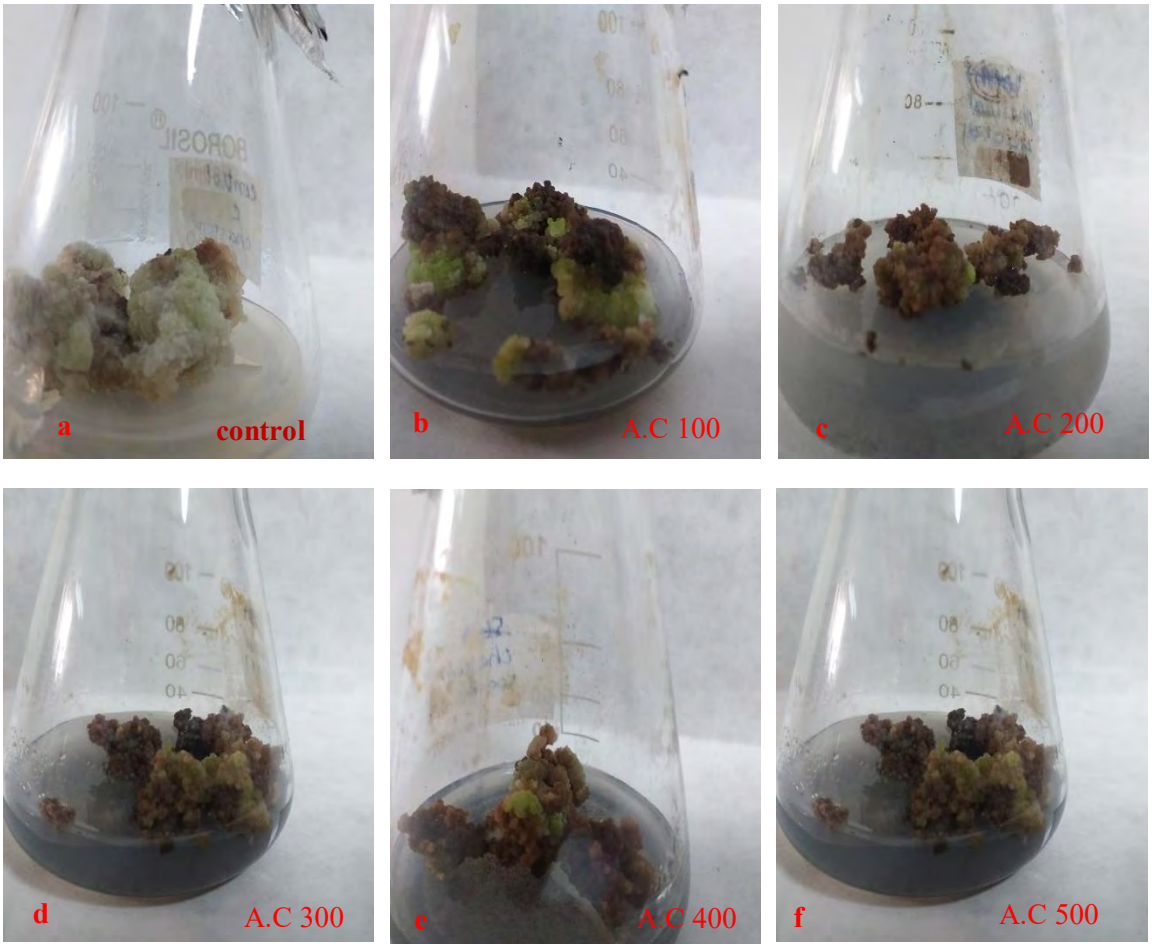

Figure 4.4: Effect of Activated charcoal on biomass and morphology of *Ocimum basilicum.*

4.2. Total Phenolic Content and Total Flavonoid Content

Various concentration of ascorbic ascid (AA) and activated charcol (AC) were used as a stimuli to enhance the phenolic and flavnoid contents in callus culture of *O. basilicum*. Highest amount of totatl phenolic content were noted on the minimum ascorbic acid concentreation A.A 1 mg/L (11.61 mg/L DW) followed by A.A 5 mg/L (11.4 mg/L DW), while minimum phenolic content was noted for highest concentration A.A 40 mg/L (7.43 mg/L). Similarly, maximum flavonoid accumulation occur in callus treated with A.A 20

mg/L (10.3 mg/L DW). Minimum flavonoid content were observed to be in the control (6.71 mg/L) figure 4.5.

In activated charcoal treated callus culture maximum phenolic and flavonid content accumulation occur in control culture (11.9 mg/L DW) and (8.71 mg/L DW) respectively. Minimum accumulation of both phenolic and flavonoid was noted in high conecntration treated activated charcoal culture i.e A.C 500 mg/L (TC = 5.23 mg/L DW) and (TFC = 8.35 mg/L DW). There is corelation between total phenol and flavonoid content accumulation in activated charcol treated callus figure 4.6.

Table 4.1: Total phenolic and flavonoid content in callus culture of *O.Basilicum*

Elicitor 1 Ascorbic acid (con mg/L)	**TPC (mg/L DW)**	**TFC (mg/L DW)**	**Elicitor 2 Activated charcol (con mg/L)**	**TPC (mg/L DW)**	**TFC (mg/L DW)**
A.A 0 (control)	8.49	6.71	A.C 0 (control)	11.9	8.71
A.A 1	11.61	7.1	A.C 100	10.73	6.06
A.A 5	11.4	7.25	A.C 200	8.64	3.37
A.A 10	8.85	9.1	A.C 300	7.87	3.25
A.A 20	8.94	10.37	A.C 400	7.72	3.02
A.A 40	7.43	8.35	A.C 500	5.23	2.95

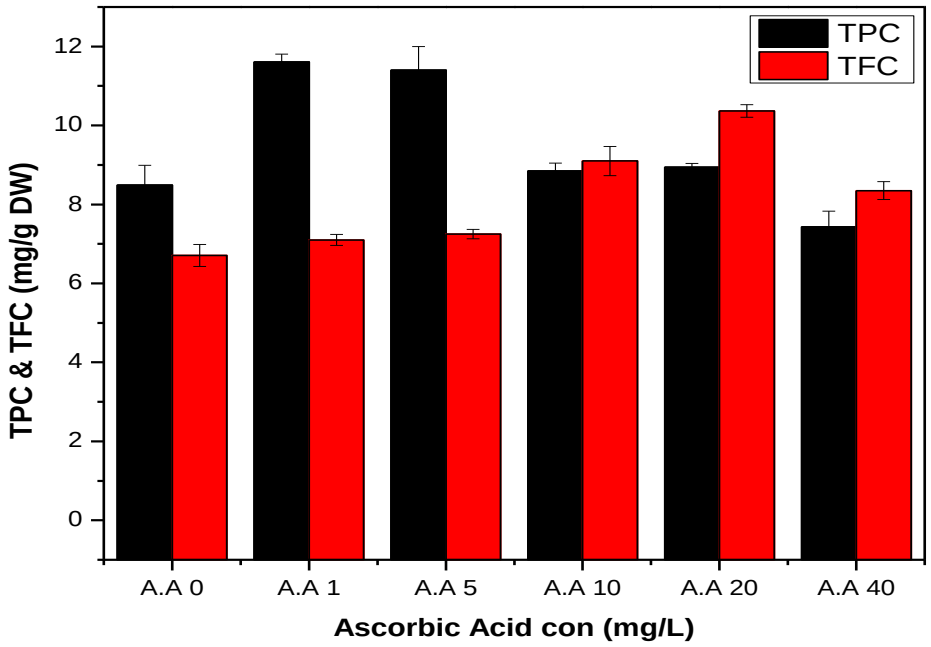

Figure 4.5: Total phenolic and flavonoid contents of *O.Basilicum* callus culture treated with ascorbic acid

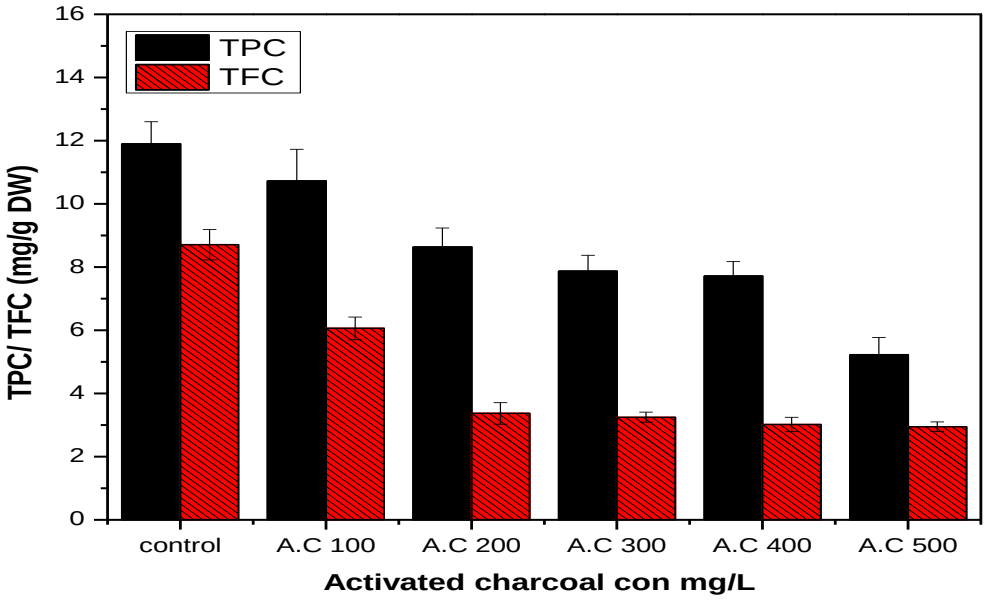

Figure 4.6: Totatl phenolic and flavonoid content in callus culture of *O.Basilicum* treated with different concentrations of activated charcol.

4.3. Antioxidant Activities

4.3.1. DPPH

DPPH assay was conducted to examine the antioxidant potential of the callus cultures of *0. Basilicum*. It was noted that highest antioxidant potential (93.2%) was recorded at A.A 20mg/l ascorbic acid concentration as compared to control cultures.

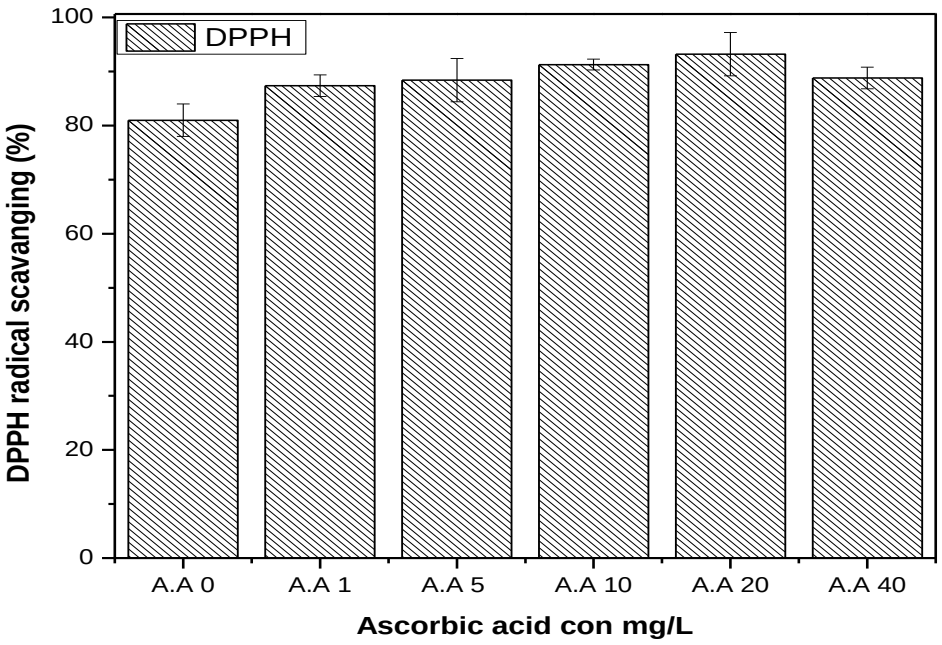

Figure 4.7: DPPH free radical scavenging Activity (%) in response to Ascorbic acid.

There was not much change in the DPPH assay for callus culture treated with activated charcoal. However, maximum DPPH value (91.6 %) was noted at A.C 300mg/L while minimum (88.2 %) was noted for control.

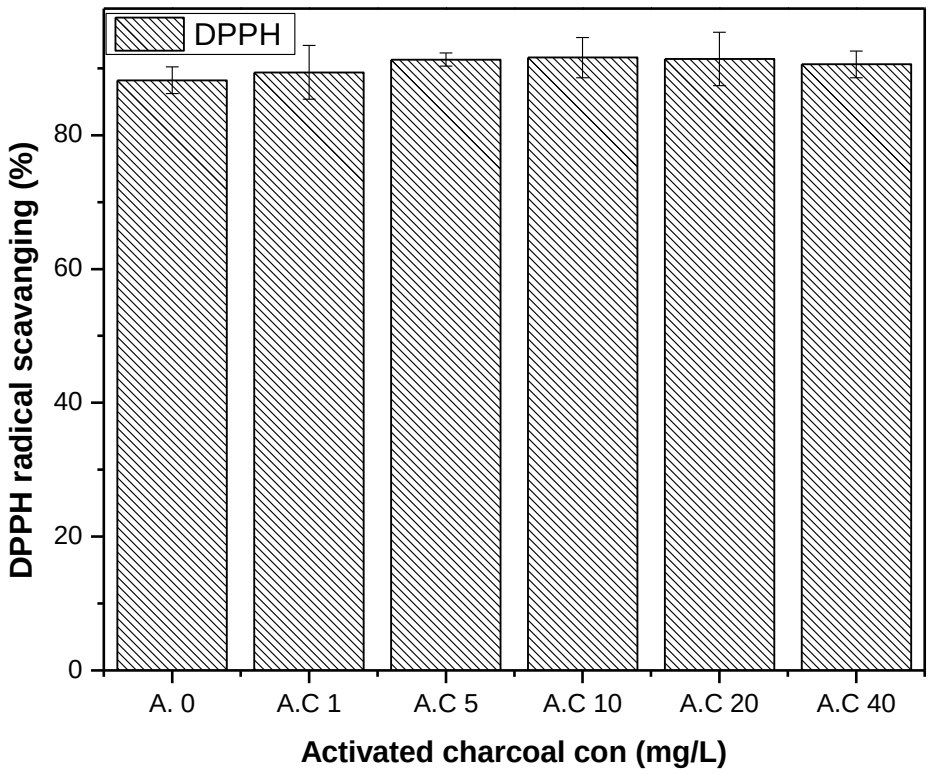

Figure 4.8: DPPH free radical scavenging Activity (%) in response to activated charcoal.

ABTS was found greater in A.A 100 20 mg/L than all concentrations i.e. (1060.46 TEAC μM). An increase of 3.3 fold occur in the ABTS antioxidant activity as compared to the control. Minimum ABTS activity was noted for control (316.2 TEAC μM). This shows that ascorbic acid has a positive response on the ABTS antioxidant activity. Similarly, high FRAP antioxidant activity was measured for A.A 5 mg/L treated callus culture (661.3 TEAC μM) followed by A.A 20 mg/L treated culture (618.8 TEAC μM). Minimum FRAP antioxidant activity was observed foe control without any elicitor culture.

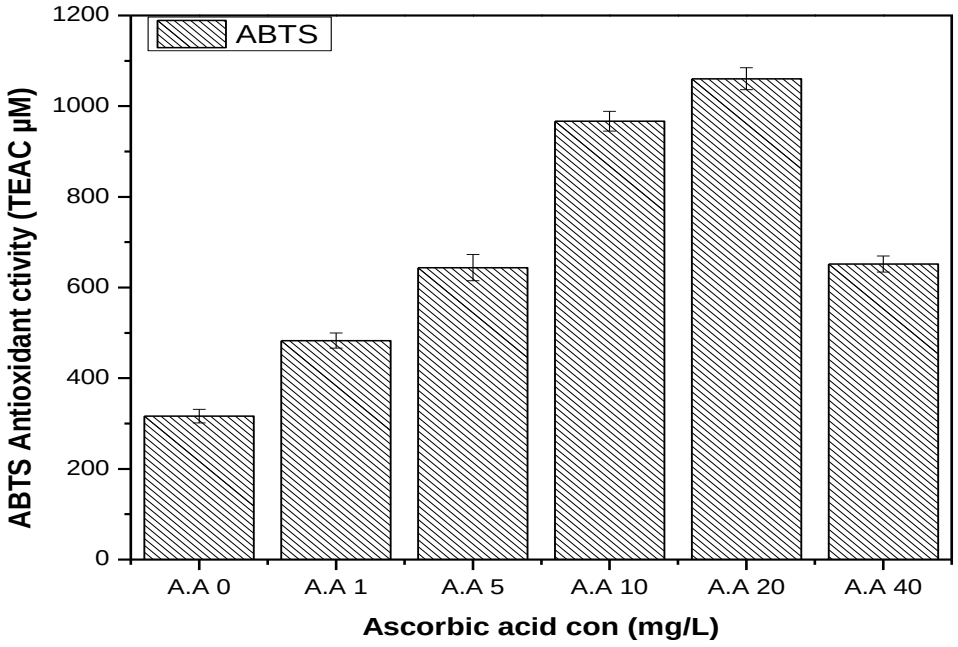

Figure 4.9: In vitro ABTS antioxidant activity of ascorbic acid treated cultures (TEAC: Trolox C equivalent antioxidant activity, expressed in µM).

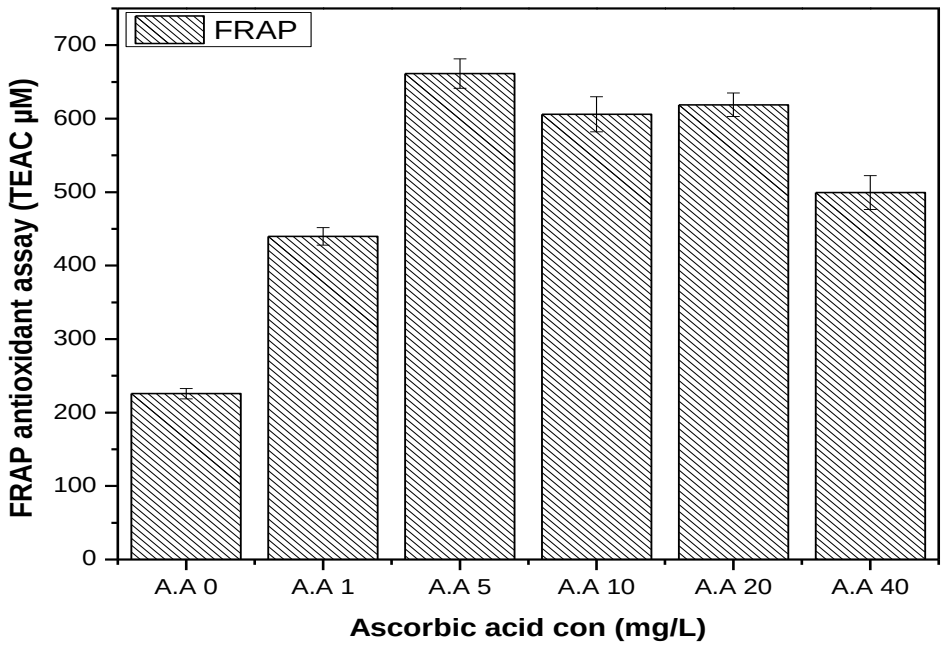

Figure 4.10: Ferric Reducing Antioxidant Power Assay of ascorbic acid treated callus cultures of *O. Basilicum* (TEAC: Trolox C equivalent antioxidant activity, expressed in µM).

Cellular antioxidant assay was also performed for all the treated callus culture of O. *Basilicum*. It was observed that A.A 10 mg/L have maximum value of CAA, while all other have smaller value than that. A.A 10 (85.6 %) followed by A.A 20 mg/L (82%) were maximum noted CAA values.

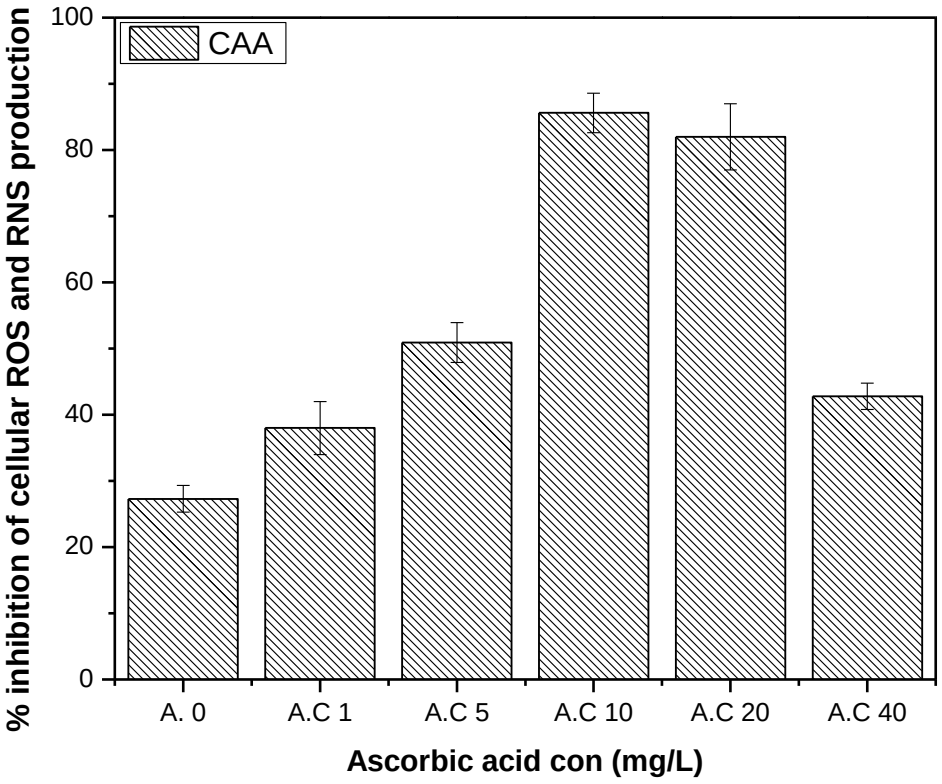

Figure 4.11: Control shows lower value in comparison to other values.

4.4. HPLC based Analysis

In this study accumulation of caffeic acid, chicoric acid and rosmarinic acid in callus cultures of *Ocimum Basilicum* were quantified using RP-HPLC method. HPLC results revealed that eminent accumulation of these hydroxycinnamic acid was recorded for ascorbic acid grown callus cultures. Upon evaluation of growth dynamics it was noted that the maximum

accumulation of phytochemicals occurred at elicited culture as compared to the control. Elicitor can be applied according to their nature and requirements. Ascorbic acid were applied and then analyzed three basic plant metabolites were analyzed which are hydroxycinnamic derivatives (caffeic acid, chicoric acid and rosmarinic acid). Metabolites were analyzed on the bases of comparison of retention times and UV spectra with standards used through HPLC in treated cultures. According to the results major phytochemical quantified was chicoric acid. There is almost 3 fold increase in the chicoric acid accumulation (79.73 ± 1.87 mg/g DW) in elicited culture A.A 10 mg/L as compared to control (25.07 ± 0.85 mg/g DW). There is an increase in chicoric acid accumulation in all elicited callus culture. The second major quantified phytochemical was rosmarinic acid. Enhanced accumulation of rosmarinic acid occur in a correlation with chicoric acid at moderate concentration of A.A 10 mg/L. An increase of almost 3.5 fold (41.04 ± 1.54 mg/g DW) was noted in the callus culture treated with elicitor as compared to control (12.00 ± 0.81 mg/g DW). Caffeic acid quantification shoed enhanced accumulation in the elicited callus culture as compared to the normal control culture. Maximum caffeic acid (119.72 ± 3.56 µg/g DW) was quantified at A.A 10 mg/L, while minimum accumulation was observed for control (55.62 ± 2.01 µg/g DW).from the table 4.2 it is cleared that there is a correlation between the phytochemicals in all elicited callus culture of O. Basilicum treated with ascorbic acid.

Table 4.2: Determination and quantification of medicinally important phytochemicals in ascorbic acid treated callus cultures of *O. Basilicum.*

Concentrations (mg/L)	**caffeic acid (µg/g DW)**	**Chicoric Acid (mg/g DW)**	**Rosmarinic Acid (mg/g DW)**
Control	55.62 ± 2.01	25.07 ± 0.85	12.00 ± 0.81
A.A 1	59.09 ± 3.02	27.98 ± 1.12	13.57 ± 0.93
A.A 5	81.62 ± 4.04	47.09 ± 1.85	23.77 ± 0.51

A.A 10	119.72 ± 3.56	79.73 ± 1.87	41.04 ± 1.54
A.A 20	84.79 ± 4.04	77.75 ± 1.84	27.28 ± 2.48
A.A 40	62.75 ± 3.02	29.96 ± 0.88	15.14 ± 1.45

4.5. Discussion

Plants have been widely investigated for their necessary ingredients and nutritional properties for decades. Higher plants can produce a variety of low molecular weight compounds in addition to the main primary metabolites, which are referred to as secondary metabolites (Wink 1988). Plant secondary metabolites are a collection of organic compounds produced by plants in response to biotic and abiotic stressors, as well as other disturbing circumstances (Verpoorte et al. 2002).

The use of biotechnological treatments to produce secondary metabolites in various plant culture techniques is an appealing alternative to isolation from entire plant material (Skrzypczak-Pietraszek et al. 2014). Using plant cell cultures as a biological platform for the synthesis of secondary metabolites, in combination with elicitation strategies such as the introduction of physical and chemical entities to the cell culture or growth conditions, is a promising approach for sustainable production in the modern medicinal and aromatic industry (Murthy et al. 2014).

Ascorbic acid is a naturally occurring antioxidant molecule that is essential for plant cell response to salt stress. Increased levels of ascorbic acid had a growth-promoting impact, as the callus development rate was even faster than that of non-elicited calli (control) (Rehman et al. 2014). When calli were cultivated in the presence of 20 mg/L AA, the FW increased to 233.14 when compared to control. Plant growth and development, as well as antioxidant capacity, are dependent on these non-enzymatic antioxidants (Barth et al. 2006; Pavet et al. 2005) According to a study, additional ascorbic acid (4.0 mM) strengthened the

stem and roots of salt-stressed chickpea plants and enhanced fresh and dry biomass (Beltagi 2008).

The production of non-enzymatic antioxidants (phenolics and flavonoids) as part of the anti-oxidant defence mechanism is regarded as a reference standard. Their accumulation differs according on the environment, and it can be aided by including elicitors in the growing media. Polyphenols and flavonoids abound in *Ocimum Basilicum* (Kaya et al. 2008). ROS generation by activated charcoal and ascorbic acid, which starts complicated reactions and influences cell metabolic processes are the cause of increased phenolics and flavonoids production. Plant defence mechanisms produce phenolics and flavonoids, which act as natural antioxidants in such situations (Choi and Hu 2008; Chung et al. 2018).

Overall, the TPC and TFC of the control were the highest, and they had an effect on the other concentrations. Previously, similar findings were published (Ali and Abbasi 2013). The suspension culture results of Artemisia absinthium L. are consistent with our findings (Ali et al. 2015). Our findings are similarly consistent with earlier research, with Chitosan and other elicitor concentrations (Petriccione et al. 2015; Treutter 2006; Vosoughi et al. 2018).

The DPPH assay is commonly used to assess the antioxidant capacity of plant cell-derived metabolites. The procedure is efficient, cost-effective, and accurate (Ali and Abbasi 2013; Anjum and Abbasi 2016). (Ali and Abbasi 2013; Anjum and Abbasi 2016). In the ABTS experiment, the antioxidant activities revealed diverse findings as the concentration of ascorbic acid increased. CAA and FRAP ascorbic acid had better results in DPPH than the others and in comparison to our findings (Abbasi et al. 2010). Applied light on *Ocimum basilicum* yielded higher DPPH and FRAP results, although our study's results demonstrate a larger value of ABTS than light reactions.(site basil light) (omar) Our findings are backed up by previous research that found that ascorbic acid improved antioxidant activity in *Caralluma tuberculata* callus culture (Rehman et al. 2014). Antioxidant activity studies indicated a notable correlation with secondary metabolites. Ascorbic acid significantly boosted phytochemical levels in Ocimum Basilicum callus culture, resulting in higher antioxidant generation capability. There was also a substantial correlation between phenolic profile and

antioxidant ability, which is consistent with earlier research (Kim et al. 2003; Moran et al. 1994)

When a plant is under stress or is exposed to unfavorable conditions, a variety of bioactive constituents are released. To protect the plant's life, these substances include low molecular weight phytochemicals such as phenolics and alkaloids (Khan et al. 2019). The polyphenol profile of an Ocimum basilicum callus culture changed significantly after exposure to *ascorbic acid*. Almost a 3 fold increase in the accumulation of metabolites was observed after treatment with the ascorbic acid as compared to control. There was a positive correlation in all the identified phytochemicals with increase in concentration. Treatment of A.A 20 mg/L was the most optimized concentration for enhanced phytochemicals in callus culture of *Ocimum Basilicum.*

Although the mechanism behind the synthesis of excess metabolites is unknown, ascorbic acid has been identified as a promising abiotic elicitor in in vitro cells for the development of essential bioactive compounds in several investigation (Khan et al. 2019; Rehman et al. 2014).

Conclusion

The current study used an elicitation technique to boost biomass and important phytochemical synthesis in *Ocimum Basilicum* callus cultures. Abiotic elicitation with ascorbic acid and activated charcoal has proven to be effective among other elicitation procedures. In this work, ascorbic acid (A.A) and activated charcoal (A.C) were used as abiotic elicitors, and moderate dosages of ascorbic acid (A.A 10 & 20 mg/L) were found to be effective. Overall, it was discovered that ascorbic acid produced the highest levels of biomass, total phenolic content, and total flavonoids. In vitro antioxidant activities (DPPH, ABTS, and FRAP) as well as cellular antioxidant activities were reported to be highest in cultures grown at A.A 20 mg/L when compared to control cultures. Higher concentrations of both types of elicitors, on the other hand, were found to have inhibitory effects. Furthermore, when ascorbic acid was applied to the callus, higher quantities of rosmarinic acid, chicoric acid, and caffeic acid were found in comparison to the control. In comparison to activated charcoal, ascorbic acid was found to be a stronger elicitor for important metabolite accumulation in Ocimum basilicum callus culture. This study will help significant contribution in understanding the crucial effects of ascorbic acid and activated charcoal on biochemical parameters in Ocimum Basilicum cultures, as well as the large-scale production of useful metabolites.

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