Journal of Food Quality

Increasing Nutritional Content through Physiological Interventions in Plant Commodities

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Review Article

Potential Probiotics Role in Excluding Antibiotic Resistance

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Background. Antibiotic supplementation in feed has been continued for the previous 60 years as therapeutic use. They can improve the growth performance and feed efficiency in the chicken flock. A favorable production scenario could favor intestinal microbiota interacting with antibiotic growth promoters and alter the gut bacterial composition. Antibiotic growth promoters did not show any beneficial effect on intestinal microbes. *Scope and Approach*. Suitable and direct influence of growth promoters are owed to antimicrobial activities that reduce the conflict between host and intestinal microbes. Unnecessary use of antibiotics leads to resistance in microbes, and moreover, the genes can relocate to microbes including *Campylobacter* and *Salmonella*, resulting in a great risk of food poisoning. *Key Findings and Conclusions*. This is a reason to find alternative dietary supplements that can facilitate production, growth performance, favorable pH, and modulate gut microbial function. Therefore, this review focus on different nutritional components and immune genes used in the poultry industry to replace antibiotics, their influence on the intestinal microbiota, and how to facilitate intestinal immunity to overcome antibiotic resistance in chicken.

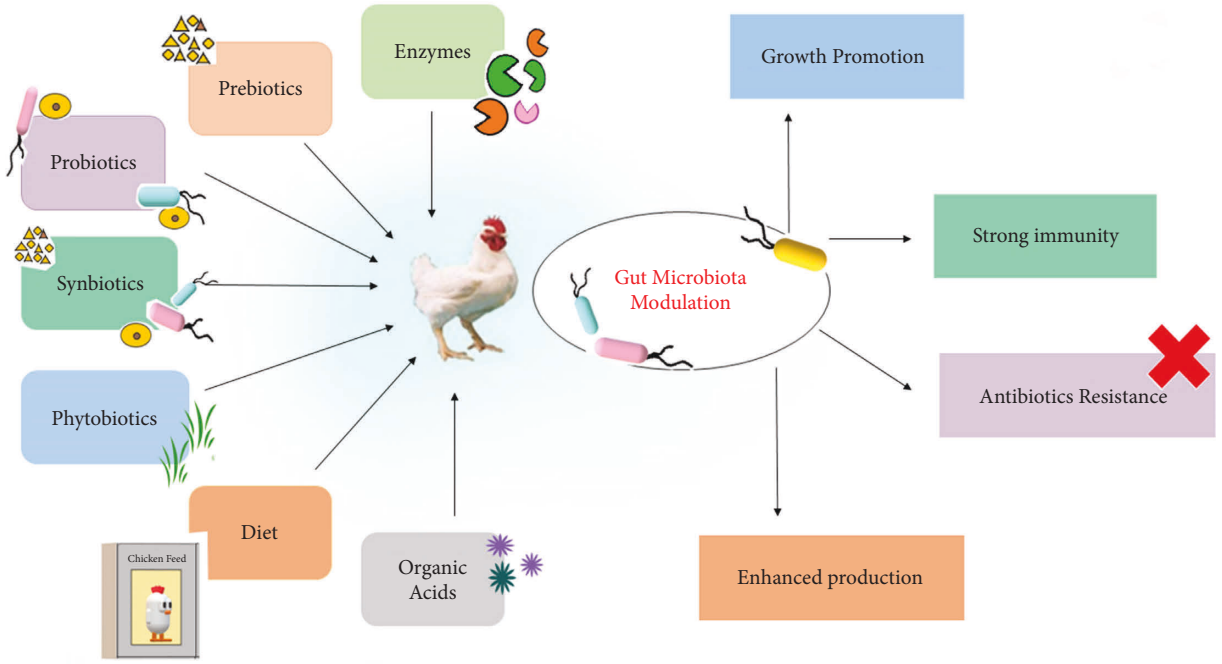


Figure 1: A schematic diagram of gut microbiota and introduction of different regulators involved in different functions.

1. Introduction

Bacterial resistance is a serious problem all over the world, especially in medical and agriculture fields. Bacteria displayed resistance to antibiotics and resulted in enhancing threats to human and animal health. Identifying mechanisms of resistance and investigating all the identified antimicrobial agents for clinical use are important. Curative and subtherapeutic uses of antimicrobials for animals are of increasing interest regarding the disclosure and distribution of resistant zoonotic bacterial pathogens [1]. A serious threat emerges due to antibiotic resistance with global deaths estimated by 2050 to reach 10 million people every year, but it is challenging to quantify the associated excess morbidity and mortality [2]. Due to disease problems and social pressure, there is a need to make important regulations on how to use particular antibiotics in livestock and poultry production. Potential alternatives need to implement to control different diseases and improve the quality of food through animal production and meat quality.

Supplementation of antibiotics is useful for stabilizing gut health, increasing growth performance, and preventing intestinal pathogens. Due to antibiotic resistance, the European Commission has banned the production and supplementation of antibiotics as growth promoters in the feed [3]. Different alternatives of antibiotics have been introduced including enzymes, organic acids, prebiotics, probiotics, and herbs to control pathogens by stimulating intestinal microflora in poultry production. The purpose of antibiotics alternatives is for feed preservation and antimicrobial activity [4–6]. The interaction of intestinal microbiota and the immune system through the use of antibiotic alternatives will be discussed in more detail in this review. Gut microflora plays a significant role in the chicken's physiological health, immunity, and nutrition. Schematic Figure 1 displayed different functions related to the use of gut microbiota. Different changes arise in gut microflora that can influence the feed efficiency accompanying bird status during health and disease. There are two subclasses of gut microbiota including the luminal microbiota and mucosa-attached microbiota. These could be affected by the nutrient availability, effects of antimicrobial substances, and the passage rate of diet [7, 8].

2. Morphological and Functional Development of the Small Intestine

Hatching results in maximum morphological changes in the small intestine. The intestine attains more weight as compared to whole body weight gain. The absence and presence of the feed are most important for small intestine development, but maximum and relative growth is less in the absence of feed. After 2–3 days posthatch, the crypts begin to form and reach a plateau. During the first 2 days, villi increase in length rapidly; a plateau reaches at first 10 days posthatch in the jejunum [9, 10]. The width of the jejunal villi increases marginally, and the optimum width was reached at 7 days posthatch. The density of jejunal villi reaches a constant level at 9 days posthatch [11]. The cell death or apoptosis occurs at the villi tips, which correspond to the physiological turnover. Dead epithelial cells and macrophages peel from villi into the lumen. Aberrant cell proliferation and maximum apoptosis occur due to contortion in the lumen of the intestine. More apoptosis is observed in the villus tip as compared to the villi tips of healthy chickens during the malabsorption syndrome, whereas acute inflammation arises due to infiltration of intestinal tissue/villi

by heterophils, which provoke the production of cytokines in the affected villi epithelium [12, 13].

The capacity of the birds to absorb carbohydrates is detectable during the 18^{th} embryonic day. A moderate level of absorbable capacity is in the hatch birds and then becomes maximum after a few days. With the increase of intestinal surface area, the absorptive capability also increases that occurs during morphological development. That is why enhancing the absorption surface results in the high uptake of nutrients that is significant for the synthesis and growth of tissues and organs. The regional activity of mucosal enzymes is linked with the digestive capability in particular intestinal regions. Mucin protein that is acidic in nature expressed from 17 days of eggs incubation to 3 days posthatch [14–16]. The production of neutral mucin that is linked with the mucus layer coordinates with the colonization of the intestine by microbes. *In vitro* studies on chickens and rats have proved that bacteria, for example, *Lactobacillus* strains, attach to the intestinal mucin and conflict for adhering to the epithelial or mucin layer happened between commensal bacteria and pathogens [17, 18].

3. Intestinal Microbiota

Colonization or aggregations of bacteria, viruses, and fungi in the skin, gut, genital, and respiratory tracts are described as microbiota. The microbiota has an important role in the suitable functioning of various physiological processes including host tissue development, nutrient absorption, and metabolism including immune system development [19]. Gut microbiota are closely associated with the lives of livestock, poultry, and of course human being due to their importance in overall health, well-being, and productivity. The gut environment's effects on the growth of normal intestinal bacteria have increasing commensal components that are accompanied by food-producing animals [20]. Intestinal epithelial cells, the immune system, and a microbial bunch are three important parts of the gastrointestinal tract (GIT) ecosystem [21]. Microbiota and host interlinking is very crucial for regular immune functioning. Microbiota regulates the growth of immune cells, the production of different molecules that facilitate the immune system including antibodies, host defense peptides (HDPs), and intestinal villi length and width [22]. Microbiota in GIT not only affects the host as a source of providing digestive enzymes but also increases nutrient absorption, defense, and destruction of pathogens and facilitates the growth of a healthy immune system. Irregular maturation of microbiota can result in the form of alternating of intestinal microbial colonization correlated with sensitivity, diabetes, obesity, diabetes, and abnormal immune defense system or responses [23]. The newly hatched chicks have differences in intestinal microbiota development; that is why there will be different responses to antibiotic treatment and diseases. Moreover, in animal kingdom, the growth and development of healthy gut microbiota is a very important stage in the beginning days after hatch that affects future growth and fitness [24, 25]. Initial days of chicks after hatching are very important for developing the normal microbial community. It shows that before going out of hatchery, young chicks have the most advanced stable microbiota [26]. The development of GIT is much faster than the development of other organs during the first week after hatching, and it is crucial for chicks to achieve genetic potential [27].

The primary and foremost assignment of the gut is the absorption of nutrients from feed and the expulsion of feces and urine. Moreover, chicks have a distinctive microbiota community that could be modulated by host secretions, dietary nutrients, and the host systemic responses [28]. Microbes regulate the various host physiological metabolisms in the gut and interact with each other and also with the host. Different genera that are associated with effective performance are *Lactobacillus*, *Clostridium*, and *Ruminococcus* [26]. There are two clusters of *Clostridium* species including IV and XIV, which are prominent in the microbiota of avian cecal, which is important butyric acid producers regarding growth booster function. Butyrate for epithelial cells is a crucial energy source in ceca and prohibits the inflammatory responses by a substitute on proinflammatory cytokines [29, 30]. There are more than 200 nonstarch polysaccharides enzymes (NSPs) and various pathways linked with the production of short-chain fatty acids (SCFAs) identified in a metagenomics analysis of cecal microbiota. These SCFAs provide energy to the chickens and decrease the cecal pH that inhibits pathogen growth and increases mineral absorption and ultimately growth performance [31].

4. Negative Impact of Antibiotics on Intestinal Microbiota

The basic purpose of antibiotics usage as therapeutics and growth promoters in animals and humans since the 1940s is to save lives and eradicate the uncountable microbes that cause diseases [32, 33]. It was reported that the United States utilizes an estimated 24.6 million dollar antibiotics annually as growth promoters. Antibiotics are obtained from either natural resources or synthetic drugs that play a critical role in the gut. Antibiotics have been widely associated with the poultry industry for decades, but there is the reduction in gut microbes and their toxic metabolites due to antibiotics [34]. Concurrently, overuse and irregular antibiotics supplementation have been declared to be notable bacterial resistance development. There is a threat to animal and human treatments due to bacterial resistance as they spread to genes for antibiotic resistance or may also interchange plasmid with intra- or interspecie [35, 36]. Antibiotic prophylactic usage in animal feed has been banned in the European Union (EC Regulation, No. 1831/2003). There has been a great challenge to nutritionist and poultry farmers due to this prohibition. There is an example of necrotic enteritis in poultry that is controlled with antibiotic growth promoters (AGPs) added in feed. Due to the prohibition of AGPs, there has been a great incidence of necrotic enteritis cases in poultry. Therefore, there is prompt demand for discovering antibiotics alternatives to regulate and maintenance of gut ecosystem balance and improve the overall performance of the birds [37].

Therapeutic and nontherapeutic usage of antibiotics causes the selection pressure for potential exits for *Salmonella* to obtain antimicrobial resistance genes from resident poultry microbiota. Previously, the abundance and diversity of antibiotic resistance genes (ARGs) were underestimated based on bacterial culture and ARGs identification that were intensified by the increase of sequence-novel ARGs [38, 39]. Recently, different approaches including metagenomics have been utilized for bacterial communities analysis and ARGs in bird diet [40]. The ARGs-harboring bacterial hosts were significantly influenced by bacterial colonization alteration due to antibiotics [41, 42]. Interaction between factors affecting the gut microbiota is shown in Figure 1.

5. Immune System

Biological structures, metabolism, and hemostasis that can protect the birds from different harmful organisms including bacteria, viruses, and protozoa are the immune system. The innate and adaptive are two types of the immune system. The innate immune system contains physical and chemical barriers including blood proteins, phagocytic cells, and blood complement serum proteins that function with antibodies to help the destruction of target cells. Whenever the innate immune system fails to invade pathogenic organisms, the adaptive immune system responds to counteract by recognizing the specific molecular functions on the outer surface of the pathogens. This system includes B and T cells and humoral immunity [43]. The immune system and physiology in birds also seem to parallel that of mammals due to the origin of the common reptilian ancestor and the lymphomyeloid tissues that are full of hematopoietic cells that evolved from epithelial or mesenchymal enlarge [44]. In the avian immune system, immune organs including the bursa of Fabricius, thymus, spleen, and lymphoid organs are fully developed when hematopoietic stem cells enter the bursal or thymic analogs and become efficient B and T cells [45]. The subtherapeutic doses of antibiotics were used since the 1950s in feed to improve growth performance in broiler chickens [46]. The increased knowledge of this concern with the antibiotic resistance development and the prevalence of its transfer to human pathogens has led to a European ban on the utilization of antibiotics in animal feed as growth promoters. Alternative ways are required to control microbial outgrowth and to prevent microflora imbalances in poultry. An alternative strategy is to modulate the expression of antimicrobial proteins (AMPs) such as β-defensin gallinacin-6 on the surfaces of the mucosa of chicken GIT [47]. Currently, several chicken antimicrobial peptides, belonging to the cathelicidin, liver-expressed antimicrobial peptide (LEAP), and β-defensin families, have been discovered. These are synthesized, are available after detecting the invading microbes, and rapidly neutralize a large range of microbes. AMPs have similarities among themselves regarding biophysical properties due to different species but their sequence is rarely similar. But some particular degree of identity is present either in the sequence of amino acids or the pre-region such as in cathelicidins. The AMPs possess a net positive charge that can attach to the negatively charged phospholipids groups on the bacterial membrane through electric interactions [48].

Recent studies have reported that beta-defensin family is involved in a crucial function in avian immunity, defending as the first line of defense against pathogens [49]. In the avian genome, only the beta-defensin family is present, also known as gallinacin or avian β-defensins (AvBDs) [50]. Avian β-defensins attach to a huge number of microbes including Gram positive, negative bacteria, yeast, and fungi [51]. Due to the response of multiple factors, beta-defensin is expressed and upregulated in the dendritic cells, keratinocytes, peripheral blood cells, epithelial cells lining the respiratory, gastrointestinal, and urogenital tracts including cytokines (interleukin) IL-1α, IL-1β, tumor necrosis factor-alpha (TNF-α), interferon-γ (IFN-γ), insulin-like growth factor1 [52], bacteria, lipopolysaccharides [53], yeast [54], and other stimulants such as PMA, isoleucine, and 1,25-dihydroxy vitamin D3 [55].

6. Regulation of Beta-Defensin in GIT

Microbial colonization of the avian gut possesses coincidence with gene expression of defensin, which plays a role as peptides defending against a huge number of microbes [56]. The responses of the gut defensin seem less predictable to the acute microbial challenges in down- and upregulation of the avian genes. Moreover, there are multiple factors challenged by microbes that could have an effect on GIT gene expression as well as the breed and age of the birds. The gene expression of avian β-defensin-1 (AVBD-1) and 4 in the duodenum were recorded elevated in the hatch and 7-day-old birds kept the low hygienic environment as compared to birds kept in the high hygienic environment due to potent gut antimicrobial activity, while AVBD-10 was found to maintain in all ages and environments [57, 58]. Figure 2 shows the antibacterial activity of sAVBD-6 against *Clostridium perfringens*.

AVBD 12 possesses a unique feature as a chemoattractant for avain immune cells and dendritic cells that can be related to the AvBDs application as a chemotherapeutic agent in the mammalian host. This is the reason that analogs of AVBD 12 were used to investigate the chemotherapeutic feature [59]. It has been proven that the antibacterial activity of avian β-defensin 7 plays an effective role to control the multidrug-resistant *Salmonella* strain after incubation with infected macrophages in the mouse. There was a significant reduction in the liver bacterial load that causes a significant increase in survival affected with a systemic lethal *Salmonella* infection. This can indicate that AVBD-7 could be used as a candidate of interest alternative to conventional antibiotics against bacterial infections [60].

7. Regulation of Cathelicidins in GIT

Four cathelicidins have been reported in the chickens until now including cathelicidin-1 (CATH-1), CATH-2, CATH-3, and CATH-B1 [61]. Cathelicidins possess a strong antimicrobial activity against various types of microbes including enveloped viruses, bacteria, and fungi at low

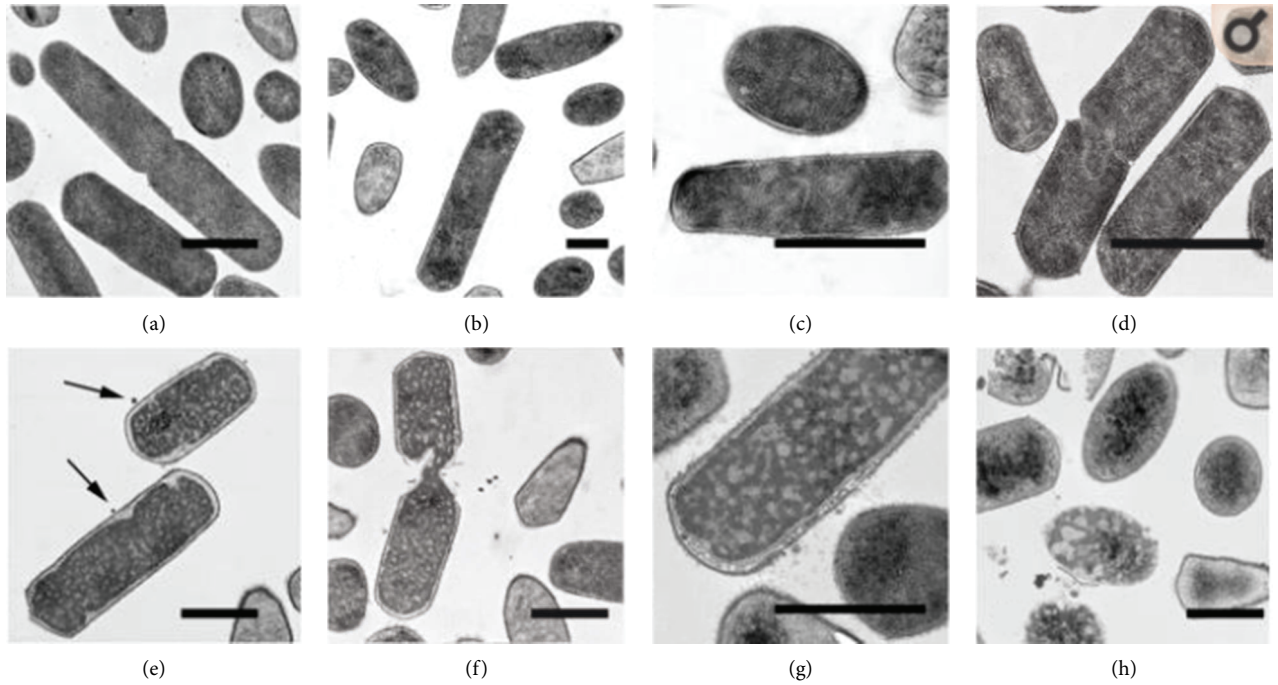


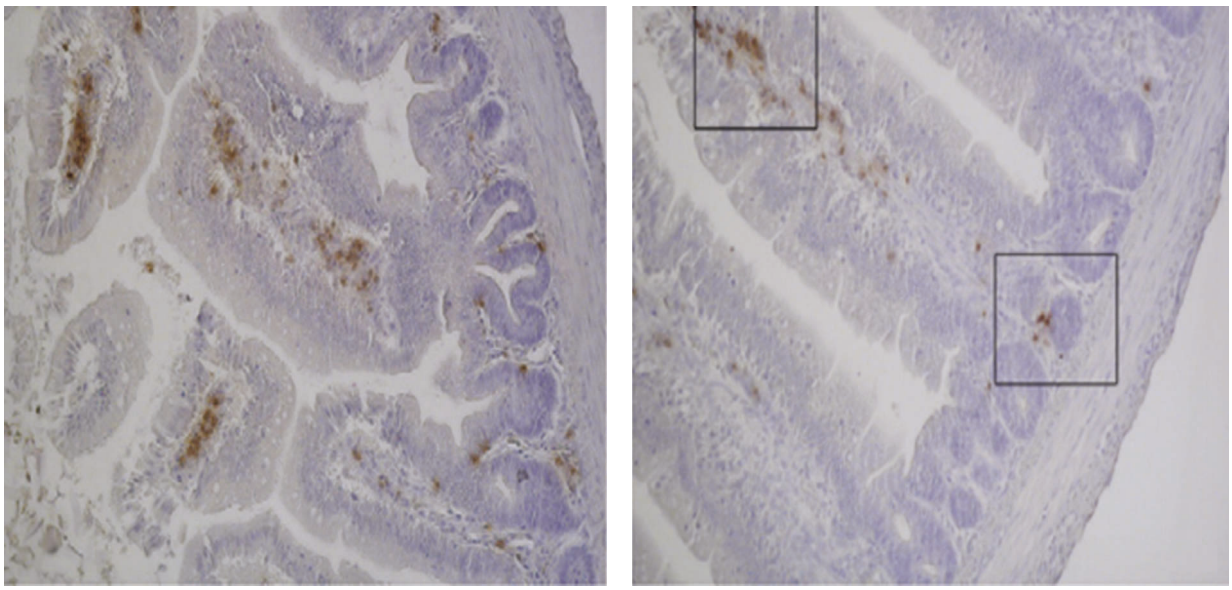
Figure 2: Transmission electron microscopy of *C. perfringens* cells incubated with synthetic AvBD-6. Bacteria incubated in a minimual medium for 30 min were undamaged. In contrast, bacteria incubated for 30 min with an increasing concentration of sAvBD-6 exhibited dose-dependent changes in the ultrastructure (a). Granulation of the intracellular material was already observed at 1.56 μm/ml (b). Irregular septum formation in dividing cells was observed at 1.56 μm/ml (c) and 6.25 μm/ml (d). At 12.5 and 25 μm/ml, cells exhibited retracting cytoplasm (e), lysis at the septa of dividing cells (f), cytoplasmic membrane degradation (g), and complete cell lysis (h) [47].

concentrations. Due to the cationic property in CATH molecular structure, it binds with the bacterial or fungal membranes containing negatively changed components. That is why the hydrophobic side chains are a lipid bilayer and disturbance resulting in pore formation. There are different models including carpet, barrel stave, and aggregate channel models of pore formation. Microbial exposure to the low concentration of peptides causes membrane permeability and proton motive force losses during complete lysis at high concentrations. There is another possibility that the negatively charge nature of the DNA, RNA, or proteins could lead to the prevention of DNA replication, protein synthesis, and function [62]. There was local infiltration of mature CATH-2 that was shown from heterophils after 8 and 48 h stimulation of *Salmonella enteritidis* in jejunum villus lamina propria of broilers of 4 days of age as shown in Figure 3 after immunohistochemistry. Moreover, CATH-2 could not express in intestinal epithelial cells from control or *Salmonella*-challenged broilers. CATH-2 exhibited dominant fungicidal and bactericidal activity against many microbes including specific chicken *Salmonella* isolates. CATH-1-3 has been reported to stop the LPS-induced cytokines to release from mouse macrophage cell line. Unlike CATH-1 and CATH-3, CATH-2 possesses a single proline residue at its center that can destabilize helical conformation and might be important for its interaction with biological membranes [63]. Enormous infiltration of CATH-2 positive cells eventuated in jejunal villi lamina propria of infected chickens at 8 h (a) and lesser extent at 48 h (b) as shown in Figure 3.

A moderate level of CATH-1 was expressed in the gizzard, small and large intestine, while CATH-2 expression was reported moderate in the cecal tonsil tissues and there was a low expression level throughout the intestinal tract [64]. Chicken CATH-B1 was expressed in the bursa of Fabricius and restricted to secretary epithelial cells due to the close proximity of M cells [65]. The highest expression level of CATH-1, CATH-2, and CATH-3 was found in the large intestine of the Baladi (local) breed, while only CATH-2 showed moderate expression levels in the duodenum [66].

8. Probiotics

Microbes fed directly or probiotics have been previously defined as "live microbial feed supplement with beneficial effect to host animal by improving its intestinal balance" [67]. Currently, bacterial species that are used as lactic acid producing (*Lactobacillus bulgaricus*, *Lactococcus lactis*, *Lactobacillus acidophilus*, *Lacticaseibacillus casei*, *Lactiplantibacillus plantarum*, and *Ligilactobacillus salivarius*), *Streptococcus thermophiles*, *Enterococcus faecium*, *Enterococcus facecalis*, *Bifidobacterium* sp., fungi (*Aspergillus oryzae*), and yeast (*Saccharomyces cerevisiae*) are used as probiotics [68, 69]. It is suggested that probiotics, when fed early in life, can influence the intestinal environment and



(a) (b)

Figure 3: Localization of CATH-2 in jejunal tissues of *S. enteritidis*-infected chicken. Chickens were infected with 1×104 CFU nalidixic-resistant *S. enteritidis* PT4. Jejunum tissue sections from infected and control chickens were taken 8 and 48 h after infection and were applied with Giemsa stained followed by immunostaining with anti-CATH-2 antibody. Massive infiltration of CATH-2 positive cells occurred in jejunal villi lamina propria of infected chickens at 8 h (a) and lesser extent at 48 h (b) [63].

favor the establishment of beneficial bacteria, thus reducing the likelihood of pathogenic colonization [70, 71]. The proposed mechanism of probiotics include: (a) competitive exclusion and antagonism of pathogens through the maintenance of beneficial commensals, (b) altering metabolism decreasing bacterial ammonia production and enzyme activity while increasing digestive enzyme activity, (c) boosting feed intake and digestion, (d) neutralizing enterotoxins, and (e) stimulating immune system [72]. There are some possible mechanisms that may be responsible for the competitive exclusion of pathogens. These include competition for binding sites of the mucosa, nutrients, or inhibitory substances production like volatile fatty acids or bacteriocin, which are antibacterial for pathogenic bacteria. Preparations are normally fed orally to newly hatched chicks in order to prevent colonization by pathogens in the rearing environment [73, 74]. Interestingly, some studies reported that undefined preparations have a beneficial influence on the necrotic enteritis prevalence including reduced mortality and cecal colonization, for example, demonstrated the lowering in the colonization of *C. perfringens* and subsequent reduction in the incidence of necrotic enteritis [75]. Another field study found that the use of undefined microflora preparations delayed the intestinal proliferation of *C. perfringens* and the presence of necrotic lesions [76], while the performance of probiotics depends on the blocking receptor sites of pathogen adhesion, production of antimicrobial peptides, transfers in the intestinal microbial structure, and immunomodulation in chickens [77].

There are many probiotics that are available with various commercial names in the market. But *Lactobacillus acidosis* is an important bacterial organism that provides the best acidic environment (pH 5–6.5) for the growth of villi in the intestinal wall to increase the surface for nutrient absorption [78]. Additionally, five effective strains *Pediococcus acidilactici* (*P. acidilactici*), *Enterococcus faecium* (*E. faecium*), *Bifidobacterium animalis*, *Lactobacillus reuteri*, and *L. salivarius* were investigated that these strains can inhibit a range of common pathogens in *in vitro* condition [79]. Lactic-acid-producing bacteria produce bacteriocins, lactic acid, peroxides, and antibiotics. These factors play an important role in the colonization of intestinal mucosa by probiotic bacteria by preventing the binding of pathogens and hence competition for attachment sites. Different beneficial bacteria produce different antibodies. The bacteriocin is produced from the bacterial genus *Enterococcus*, which possesses an inhibitory influence on pathogens *Clostridium* and *Listeria* spp. in broilers [80]. Acidophilin, lactocidin, and acidolin are produced from *Lactobacillus acidophilus*, while lactolin is produced from *L. plantarum*. Additionally, the lantibiotic nisin is produced by different *Lactococcus lactis* spp. Bacteriocin-like inhibitory substances are produced from *Bacillus cereus*, which has an inhibitory effect on *Staphylococcus aureus* and *Micrococcus luteus* with activity in the range of pH 2–9 [81]. Acidophilin, acidolin, lactobacilli, and blasticidin show *in vitro* inhibitory activity against *Klebsiella, Proteus*, *Salmonella*, *Shigella*, *Vibrio*, *Staphylococcus*, *Pseudomonas*, and *Escherichia coli*. Due to the supplementation of probiotics, there are a large number of goblet cells in the avian intestinal villi that suggested the substances produced during bacterial fermentation may take part in the development and maturation of goblet cells. The second mechanism is known as a tight junction a unique structure that establishes the epithelial barrier integrity,

Table 1: Probiotics and beneficial effects.

Probiotics	Biological functions	Reference(s)
E. faecium NCIMB 10415	Supplementation increases chicken body weight and FCR	[82]
Probiotic-FMB11(*Lactobacillus*)	Increase body weight and reduce cost of production	[83]
Lactobacillus (2 strains), *Bifidobacterium*, *Enterococcus*, *Pediococcus*	Increase more growth and no residual effect as compared to avilamycin-containing product	[84]
CE and MCE cultures	Feed CE and lowering colonization of *S. typhimurium* and *Campylobacter* as compared to the MCE	[85]
Probiotic Bio Plus 2B (*B. licheniformis*, *B. subtilis*)	Enhance egg production, reduce the ratio of damaged eggs, and reduce serum and egg yolk cholesterol and triglyceride levels, effective on FCR	[86]
Saccharomyces cerevisiae	Regulate intestinal microflora balance and humoral immune responses and also upregulate the expression of IL-1β and downregulate the TLR-4	[82, 87]

which inhibits the entrance of pathogenic bacteria and macromolecules. These are dynamic protein structures that can regulate their function [81]. Different probiotic strains and beneficial effects are shown in Table 1.

9. Prebiotics

Yeast cell walls (YCW) consist of mannoproteins, β-1,3-glucan, β-1,6-glucans, chitin glucans, and glucophospholipid surface proteins that are related to the plasma membrane. YCW is well-known possessing prebiotic properties with efficacy for regulating the immune system and intestinal microbiome [88]. Prebiotics with the proinflammatory response were investigated to inhibit the disease, as inflammation stimulates the host immunity against the disease [89]. The utilization of prebiotics, such as *Saccharomyces cerevisiae,* mannan-oligosaccharides (MOS), fructooligosaccharides (FOS), and beta-glucan has been applied in many experiments in chickens [90–92]. The Actigen™ (prebiotic) or MOS is second-generation yeast developed by using a technology called nutrigenomics that deals with changes in the gene expression of intestinal cells. Basically, Actigen™ is mannan-oligosaccharides a specific product that has been acquired from the outer cell of yeast (*Saccharomyces cerevisiae* var.),which improves growth performance [93]. The uses of MOS improve and maintain intestine health, hence leading to efficient absorption and conversion of nutrients into body weight [54]. The β-glucan is a long-chain polysaccharide and prebiotic that is extracted from yeast or fungal cell wall. Receptors of β-glucan recognition are present on sentinel cells, stimulating the production of cytokines and expansion of lymphocytes [94]. There are three major types of lymphocytes including NK cells, T cells, and B cells that play an important role in innate immunity, regulation of adaptive immunity, and production of antibodies against antigens [53]. Chitosan oligosaccharides that consist of 1–4 β-linkage with 2–10 sugar units of glucosamine 2–10 sugar, extracted from chitin, reported that supplementation in a broiler diet could regulate the immune system and increase nutrient availability, digestibility, and feed conversion ratio [54]. There is an increase in body weight gain of broilers 34 days after hatching due to the *in ovo* injection and also affected the intestinal microbiota [95]. But *in ovo* supplementation of GOS could replace prolonged water supplementation [96]. There are some novel extracted prebiotics that are acquired after processing of the softwood trees including galactoglucomannan oligosaccharides-arabinoxylans (GGMO-AX) and galactoglucomannan oligosaccharides (GGMO). Moreover, these contain glucose, galactose monomers, and mannose [97]. *In vitro* conditions investigated that *Lactobacillus* could grow faster on GGMO than MOS. It is reported that *Lactobacillus* could grow faster on GGMO than MOS. It has been described that colonization of *Salmonella typhimurium* in the liver, ceca, and ileum when supplemented with 0.2% GGMO in a broiler diet and enhances the growth performance and healthy intestinal morphology by clearing *S. typhimurium* as compared to the control treatment [98].

Xylan is the main part of cereal fiber such as corn cobs, hulls, straws, bran, and raw source of xylo-oligosaccharides (XOS). By the degradation of xylan from xylanase of fungi, steam or mineral acids diluted solutions can produce the XOS [99]. XOS might enhance growth performance, intestinal villus height, the proportion of *lactobacillus*, and levels of organic acids including butyrate, acetate, and lactate in the ceca of chickens. There is an increase in antibody titer against influenza H5N1 and thus improve humoral immunity in chickens by XOS supplementation [100, 101]. The supplementation of autolyzed yeast in the broiler diet would help provide cellular components and cell wall carbohydrates. *Saccharomyces cerevisiae* (an autolyzed yeast) contains 29–64% β-glucans, 13% protein, 9% lipids, and 31% mannan-oligosaccharides. Supplementation of yeast in ruminant feed depends on the enhanced rumen cellulolytic bacteria, energy delivered from diet, and finally the performance of the animals [102]. Fructooligosaccharides (FOS) and mannan-oligosaccharides (MOS) are two important beneficial bacterial groups that can cause the proliferation of *Lactobacillus* and *Bifidobacterium* and limit the number of *Salmonella* and *E. coli* (Table 2). These bacteria bind with MOS through the fimbriae, not with epithelial cells, which cause the bacteria to expel out with the feces [91]. FOS decreased the *S. enteritidis* in the excreta and colonization in the ovaries of layers. However, FOS upregulated the toll-like receptor-4 (TLR-4) and enhanced IgA-positive cells in the ileal mucosa. YCW exhibited strong anti-inflammatory effects than antibodies or a control diet, which causes lowering the liver relative weight because of systemic inflammation [103]. It was reported that the heterophil: lymphocyte ratio (H:L ratio) and basophil counts were

Table 2: Prebiotics and their biological functions.

Prebiotics	Biological functions	Reference(s)
FOS (fructooligosaccharide) or fructans	Create positive effect on the growth of *Bifidobacterium* and *Lactobacillus* bacteria and reduce pH that results in inhibition of *E. coli*.	[91]
Chitosan oligosaccharides (COS), extracted from chitin	Increase the weight of the bursa, thymus, IgG, IgA, and IgM in the serum and antibody titers against NDV and also, improve ileal digestibility.	[54, 106]
IMO	Increase the *Bifidobacterium* count in the gut and decrease the *S. typhimurium* count.	[107]
Mannan-oligosaccharide (MOS)	Inhibit the adhesion of bacteria with gut epithelial cells and improve intestinal immunity and microflora.	[82]
Yeast β-D-glucan	Trigger macrophage proliferation, production of inducible nitric oxide synthase causing nitric acid production that can kill *Salmonella enterica*, and regulate macrophage gene expression of interleukin-1(IL-1), IL-18, and TNF-α (tumor necrosis factor-α).	[54]
Galacto-oligosaccharides (GOS)	Inhibit the *Lactobacillus intestinalis* and *Faecalibacterium prausnitzii* in the broilers ceca and also enhance the concentration of *bifidobacteria* and lactobacillus in feces.	[96]
Xylo-oligosaccharides (XOS)	Lactate produced from *L. Crispatus* that could be used by butyric acid-producing bacteria. In response to this, butyrate can trigger MUC-2 gene expression, exert anti-inflammatory effects, and prevent necrotic enteritis.	[99]

higher in birds fed antibiotic-free control and 0.5% FOS diets than in birds fed antibiotics or other prebiotics-added diets [104]. Results from pathogen-challenged animal models in evaluating the effect of FOS supplementation on pathogen colonization suggested a reduced susceptibility to either *Salmonella* spp. or *E. coli* infection in broiler chickens [105]. These results suggest that the FOS supplementation in broiler diets may reduce the susceptibility to *Salmonella* colonization.

Supplementation of yeast β-d-glucan and *S. enteritidis* have interaction effects on AvBD-1 mRNA expression (at 15 day postinoculation (DPI), $P = 0.004$), AvBD-10 (at 7 DPI) and liver-expressed antimicrobial peptide-2 (LEAP-2; at 15 DPI $P < 0.001$) in jejunum. It was found that LEAP-2 showed higher expression in the SE-infected group as compared to the other groups at 15 days postinoculation (DPI) while in early infection found lower expression levels in the spleen. AvBD-1 exhibited the highest expression level in the glucan-supplemented and SE-infected birds. AvBD-10 gene showed higher mRNA expression in the jejunum at 7 DPI in birds infected with S*almonella* with no beta-d-glucan supplementation as compared to the control birds. AvBD-10 mRNA gene expression in the jejunum at 7 DPI was found to be lower in the birds given glucan and *Salmonella* infected as compared to the glucan-treated and uninfected birds. By the use of yeast β-D-glucan, the overall growth performance of broilers was affected but has a strong response of protective way against *Salmonella* infection (Table 2). *Salmonella* infection causes decreasing growth performance in birds due to the disruption of the intestinal mucosa and strong inflammatory responses [53]. *S. enteritidis* colonization in the intestine could be inhibited by the supplementation of yeast β-D-glucans causes the production of β-defensin in intestine mucosa. Moreover, there may be two reasons in the spleen: (1) during the early stage of infection with local infection of *S. enteritidis* in the intestine, there might be not enough stimulants for the origination of immune response in the spleen. The cells are stimulated due to infection started circulating in the body and enter into spleen and (2) the higher *Salmonella* load in the spleen in early infection period causes lower AvBDs gene expression which could be compromised to the production of defensin through immune evasion mechanism[53].

10. Synbiotics

When probiotics are combined with prebiotics, then they form synbiotics. As mentioned earlier that probiotics and prebiotics have been described to provide a positive influence on GIT of the birds [37]. The development of gut morphology and nutrient absorptions are contributed to enhancing the growth performance of chickens due to feeding synbiotics [108, 109]. A probiotic and FOS when used singly reduce the colonization of *S. enteritidis* in the intestine but show more effective utilization when used in combination [110]. In contrast, using in combination of multiple strain probiotics (containing 11 *Lactobacillus* strains) or prebiotics such as isomalto-oligosaccharide (IMO) alone for the purpose of cecal bacterial microflora and the concentration of ceca volatile fatty acids (VFAs) and non-VFA of the chickens, synbiotics does not exhibit 2-fold synergic effects [108]. Synbiotics have the great potential to be utilized as antibiotics alternatives for improving overall growth and decreasing pathogenic load in the chickens [111, 112].

There were histomorphological changes that occurred in the small intestine of chickens when synbiotics were used in the ovo stimulation. On day 1, both *L. salivarius* and *L. plantarum* enhanced the villi height, width, and surface of the duodenum of the chickens. Moreover, Brudnicki et al. showed that RFO prebiotics with ovo stimulation of broiler chickens enhanced the absorption rate of yolk sacs in the day-old chicks (Table 3) [96]. The retention of yolk sac in the population at the end of 14 days of posthatching was 0% in

Table 3: Synbiotics and their biological functions.

Synbiotics	Biological functions	Reference
Lactobacillus spp., lactose	Improved FCR and body weight	[114]
B. subtilis, FOS	Reduced incidence of diarrhea and mortality	[68]
A prebiotic fructooligosaccharide and four probiotic bacterial strains (*Lactobacillus reuteri, Enterococcus faecium, Bifidobacterium animalis, and Pediococcus acidilactici*)	More hen day egg production in supplemented hens than in nonsupplemented	[115]

the *in ovo* stimulated group as compared to 30% in the control group. From this, it is concluded that the major source of immunoglobulins contribution to the passive immunity in newly hatched chicks and initiation of early growth posthatching is the yolk sac, and faster yolk sac resorption results in the greater shifting of maternal antibodies into chicken's bloodstream [113]. For early colonization of the embryonic gut with benefit microbes, *in ovo* stimulation is a powerful and effective tool that can result in improved health, performance, and welfare of the chickens.

11. Organic Acids

Organic acids are carboxylic and fatty acids that possess a chemical structure R–COOH. Acetic acids, formic acid, propionic acid, butyric acid, lactic acid, malic acid, fumaric acid, and citric acid have been used in the poultry industry due to the importance of their physiochemical properties (Table 4). The utilization of an organic acid mixture in poultry feed not only improves the growth performance but also better carcasses characteristics [116]. It has been described that (formic, phosphoric, formic, tartaric, malic acid citric, and lactic acids (an acidifier mixture) were added to the chicken feed at the rate of 0.15%, and body weight gain was achieved. This improved performance may be due to the reduction of pH values in the gut, decrease in the number of pathogens that are tactful to lowering pH, or increase in the number of acid-loving *Lactobacillus* and exert direct antimicrobial effects [117]. The fundamental interest in organic acids usage instead of the use of antibiotics is that there are no residues in the meat or environment and any microbial resistance [118]. Many research work have described that organic acids in the diet have affected the height and area in the duodenum, jejunum, and ileum of chickens significantly [119]. There has been an increase in villi height, crypt depth, and surface area in the colon and jejunum of rats by supplementation of butyrate [120]. Broilers fed a diet having formic acid have the longest villi (1,273 μm) as compared to control (1,088 μm), whereas birds fed the organic acids possess deeper crypts in jejunum as compared to antibiotic-fed birds (266 vs. 186 μm) [121]. It has been described that to boost the normal crypt cell proliferation. There will be an increase in fast-growing tissues and maintenance. Butyrate concentrations (0.2%, 0.4%, or 0.6%) in broiler feed had improved the villi length and crypt depth in the duodenum and might be highly beneficial to young birds in intestinal development [122]. Supplementation of 3% butyric acid and fumaric acid and 2% formic acid mixed in the bird feed was experienced the highest duodenal, jejunum, and ileal villus height, respectively. The development in villi height of different parts of the small intestine might be attributed to the contribution of the intestinal epithelium as a natural barrier against pathogenic bacteria and toxic substances. These pathogenic substances cause a disturbance in the normal microflora or may change the permeability of intestinal epithelium and facilitate the takeover of the pathogen resulting in alteration of the ability to digest and absorb nutrients that leads to chronic inflammatory processes in the intestinal mucosa [81]. Due to the property of low pH organic acids, it may be helpful in preventing the transfer of bacteria from the diet or environment [123]. However, the reduction in the *Coliform* or *E. coli* count was more enormous than those of lactic acid-producing bacteria or *Lactobacilli* count in the ileum or the cecum. Less susceptibility to pH changes may be the reason for higher *Lactobacilli* that confirms that *Lactobacilli* in the gut are less sensitive to pH changes or reduction [122]. The *Lactobacilli* growth will be a boost in response to acidic pH and early growth of chickens.

12. Enzymes

Corn starch comprises amylose and amylopectin. Most starch sources are composed of 70–80% of amylopectin. Amylopectin contains α-1,4 and α-1,6 glucosidic bonds. α-amylase can degrade α-1,4 glucosidic bonds, but amylopectase is needed to degrade amylopectin [127]. Moreover, high concentrations of insoluble nonstarch polysaccharides (NSPs) are present in the corn including xylan and cellulose that have the ability to decrease digestive enzymes activity [128]. There is a large amount of native trypsin inhibitors in the corn, ranging from 0.56 to 1.87 mg/g dry matter, which inflict restriction on the enzyme to access the substrate associated with high digesta viscosity in chickens. The growth of *C. perfringens* has a much friendlier environment in the upper gut of chickens due to slow digestion passage rate, impaired nutrient digestion, and increased water intake that affect negatively on gut health [129]. The new season grains diets starch could be hydrolyzed by the supplementation of NSP-degrading enzymes that can reduce digesta viscosity and increase nutrient digestibility of the chickens fed a corn-based diet [130].

The supplementation of multienzyme (xylanase, amylase, protease, and phytase) enhances the optimum utilization of fibers and increases intestinal microbiota leading to the availability of important minerals and better growth performance of broiler chickens [128]. For degradation of NSPs in barley based diet, exogenous enzymes are used that can cause significant variation between gut microbial communities except between duodenum and jejunum. *Salmonella* that can be transmittable horizontally can be

Table 4: Functions of different organic acids in intestinal microbiota.

Organic acids	Biological functions	Reference
Butyric acid, acetic acid	Acting as the fuel of intestine, energy generation enhances development of host epithelial cells and villi height and is in the duodenum.	[8]
Citric acid	Lower the pH and cause increasing acid-loving lactobacillus	[117]
Fumaric acid	Increase Jejunum villus height	[124]
Formic acid	Decrease *Clostridium* count in the ileum	
Propionic acid	Directly act on the cell wall of Gram negative and result in lowering the pH in the GIT	[126]

controlled by the application of exogenous enzymes. The effectiveness of exogenous enzymes depends on different factors including animal strain, digesta viscosity rate, sex, diet composition, and type of supplemented enzyme [131, 132]. Yadav and Jha demonstrated the linking of growth-promoting effects of enzymes with mucosal morphology of the small intestine [133]. Moreover, the increase in the membrane enzyme activity and role in the last step of digestion cause the reduction in crypt depth of jejunum, ultimately improving growth performance in chickens by xylanase supplementation in diet [134]. Exogenous protease plays an important role by reducing the undigested protein from diet or caudal gut inflammation reduction and maintaining tight junction integrity [135].

The use of exogenous enzymes (xylanase, β-glucan, amylase, protease, phytase, lipase, and α-galactosidase) is important in poultry diets, which is composed of corn and soybean meal because these contain various anti-nutritional factors including NSPs and protein inhibitors that can disturb the normal digestion and nutrients absorption in the gut [136, 137]. Phytic acid is a crucial anti-nutritional factor due to the property of bonding with proteins, minerals, and starches prohibiting them to dissolve in GIT and thus not being available for chickens [138]. In chickens, the activity of phytase at the brush border of GIT is very low; this is the reason for supplementation of phytase in the feed for maximizing phytase activity for the availability of phosphorus and energy contents [139]. Dersjant-Li et al. reported that crop is the primary site for the bacterial phytase [140]. Maximum phytase utilization in chicken GIT will ensure the reduction of phytate phosphorus pollution in the environment when manure mix with the land and chicken will not face phosphorous deficiency problems. The reduction of digesta viscosity and FCR of chickens provided with different varieties is caused due to the proper use of exogenous microbial xylanase [141]. A most important factor in exogenous enzyme supplementation in the wheat-added feed is a significant level of arabinoxylans [142]. The most positive effects of xylanase supplementation on the growth performance of broilers in this research seemed to be related to improved nutrient digestibility, decreased viscosity of digesta, longer villi, as well as increased villus length-to-crypt depth ratios [143]. Supplementation of exogenous xylanase led to increasing numbers of *Lactobacilli*, which was confirmed by Nian et al., leading to the reduction of *Coliform* in the ileal contents, but *Salmonella* was not detected, while in cecal content, *Coliform* and *Salmonella* were increased simultaneously [144].

13. Herbal Extracts or Phytobiotics

Plant-derived compounds added into the diet to improve livestock productivity by melioration of feed properties, improvement of nutrient digestibility, absorption, and elimination of pathogens in the gut are phytogenic feed additives. According to their origin and treatment, a variety of plant derivatives used as nonwoody, herb flowering, spices (herbs with concentrated smell or taste commonly added to human food), like cinnamon, corridor, pepper, chili, oregano, and garlic (Table 5). Some are extracted from the fruits such as flavonoids that are water-soluble used in poultry feed as additives [145]. Phytobiotics possess many properties in poultry feed including palatability and quality (taste), growth promotion, gut function (improve health and absorption), carcass meat safety, and reduced microbial loads [84]. Different phytobiotics perform different functions including triggering the favorable bacterial growth including *Lactobacilli* and *Bifidobacteria*, acting as immune-stimulatory substances, and acting as protective shield against microbial attack in intestinal tissues, by decreasing virulence properties by enhancing microbial species hydrophobicity [146].

Essential oils from anise, citrus peels, and oregano along with antibiotic growth promoter reduced microbial activity in the cecum, colon, and terminal ileum, decreased chyme contents of volatile fatty acids and reduced bacterial colony count as well as biogenic amines. Relief from antimicrobial activity and its related product in small intestine results in volatile fatty acids counteracts intestinal pH stabilization and helpful for digestive enzyme activity. The formation of biological amines is causing toxicity by decarboxylation of limiting essential amino acids such as cadaverine from lysine and skatole from tryptophan [147, 148]. Using these feed additives can alter morphological changes in intestinal tissues and benefits the digestive tract by increasing villi length and reducing crypt depth in the jejunum and colon in broilers [149]. Hydrophilic extract of liquid fresh green tea at the level of 0.1 or 0.2 g/kg in a broiler diet can increase body weight gain, carcass weight, feed efficiency, and dressed weight, reducing the cholesterol content in serum and yolk [147]. The inclusion of ginger powder (0.5, 1, 1.5%), in a broiler diet, showed increased breast and thigh muscle yield and reduced abdominal fat content at a 1.5% ginger powder inclusion level due to the anti-cholesterimic effect. Thyme and cinnamon at 0.5 and 1% inclusion rate favorably changed antimicrobial balance (reduced total bacterial count and *E. coli* form group in jejunum and large intestine) in broiler's gastrointestinal tract [150].

14. Feed and Nutritional Management

The fibrousness, hardness, and coarseness of feed particles are referred to as the diet texture. The presence of these particles in the diet contributes to benefits to the digestive system of birds. In a broiler diet, lack of structure or texture affects the bird growth performance in modern commercial poultry production [160, 161]. Feed intake can be affected due to feed particle size and grain type being used and vary with the age of birds. Beak pasting from the fine grinding of wheat is an important reason results due to wheat gluten and enhances digesta viscosity with associated depression in feed intake [162]. It was reported that whatever the method of grinding (hammer or roller mill) of sorghum, broiler consumed feed according to the coarseness of feed and surface of ground grain is inversely related to the feed intake [163]. Pelleting feed positively affects feed intake and improves feed consumption due to the complete balance of nutrients available to chickens [164]. The high feed consumption has

Table 5: Effects of different phytobiotics on intestinal microbiota.

Phytobiotics	Biological functions	Reference
Chinese herbal polysaccharides (astragalin and achyranthan)	Enhance hemagglutination inhibition antibody titers, bursa of Fabricius index, and splenocyte proliferation	[151]
Essential oil of *Oreganum aetheroleum*	Increase humoral immune responses against *E. coli*	[137]
Garlic (*Allium sativum*)	Lower the lipid content and cholesterol in plasma, broad-spectrum antibacterial properties acting against Gram positive and Gram negative	[152]
Turmeric (*Curcuma longa*)	Enhance levels of serum antibodies to an Eimeria microneme protein, MIC2, and enhanced cellular immunity as measured by concanavalin A-induced spleen cell proliferation	[153]
Black cumin (*Nigella sativa* L. powder)	Enhance immune cells and intestinal health against Newcastle disease and significant decreased total counts of *Coliform* bacterial in the jejunum	[137]
Moringa oleifera	Reduce the activity of pathogenic bacteria and molds and improves the digestibility of other foods, helping chickens express their natural genetic potential	[154]
Ginger	Increase the absorptive surface area of the intestine and thus increase the absorptive capacity, resulting in higher body weight gain and lower FCR	[155]
Euphorbia hirta	Improve the microflora balance, decrease *E. coli* and *Salmonella* population, and stimulate the *Lactobacillus* spp. proliferation anti-dengue activity	[156]
Thyme (*Thymus vulgaris*)	Improve endogenous digestive enzyme secretion and activate immune response and antibacterial, antiviral, and antioxidant actions	[157]
Capsicum and *Curcuma longa* oleoresins	Reduce gut lesion scores in necrotic enteritis-afflicted birds, increase numbers of macrophages in the intestine, and regulate expression of genes associated with immunology	[158]
Cinnamaldehyde, a constituent of cinnamon (*Cinnamomum cassia*)	Increase 17 and 42% body weight gains following *Eimeria acervulina* and *E. max*ima infections and 2.2-fold higher *E. tenella*-stimulated parasite antibody responses, compared with the control	[159]

been observed in the pellet-fed birds due to an increase in the bulk density of pelleted feeds, which facilitates easy hold and an increase in feed intake (FI) was observed to vary from 2.8% to 64% resulting in increased growth performance and decreases the proportion of maintenance energy [165]. The application of whole grains to chickens has been widely used to lower feed handling and processing costs; improve foregut development, gut microflora, and prevention of coccidiosis; decrease ascites-related mortality; and enhance digestive enzymes secretion [166].

Mash pre-starter feed significantly affected the small intestine length. Digestion is associated with related enzyme proportion secreted from the pancreas and intestine that regulate digestion. The increase in weight of pancreas, protease, and amylase activity significantly ($P < 0.05$) was described in the response to feeding crumble pre-starter diet (CPD), but the activity of lipase was not affected. Birds fed with CPD exhibited greater body weight gain (BWG) than birds fed mash pre-starter diet (MDP) at 10 days of age [167]. In contrast, the activity of pancreatic enzymes was described as decreasing the pelleting of broiler feed. The amylase activity was decreased in crumble-fed chicks than mash feed fed chickens. Moreover, the increased villi height of chicks pelleted diet fed noticed enhanced growth performance and also increased the area of intestine for absorption [168]. Mash farm feed decreases the number of *Coliform* and *Enterococcus* while enhancing *C. perfringens* and *Lactobacillus* in the chicken's ileum as compared to pelleted feed [169]. The corn supports a low percentage of *Clostridia*, *Enterococci*, and *Lactobacilli* while wheat favors a high percentage of *Bifidobacteria* [170]. The low numbers of *Firmicutes* and *Bacteroidetes* from day one hatch to day 42 as birds are transferred from starter to finisher diet and for fermenting starch to sugars [171]. Gut microbiota are very important components in the gut for intestinal ecology that is why the gut is considered a forgotten organ. The composition of gut ecology, the effect of feed supplements on the gut microbiota modulation, and finally the harmful and beneficial effects of microbiota are all dependent on a better understanding and interactions of gut microbiota with other organisms. However, the most advanced technique is the only evidence available on how gut microbiota are affected by specific dietary components in the main parts of the gut including the small intestine, crop, and ceca. The role of microbiota cannot be negotiated in the different physiological, nutritional, immunological, and developmental processes in the chickens [133].

15. Age and Sex

The important factor that affects the gut cell density, bacterial composition, and metabolic function is the age of birds. With the advances in bird's age, there are sequential modifications in the composition of gut microbiota, due to the substitution and set up of more stable bacterial taxa [54]. Chickens are highly susceptible to pathogens during the neonatal period and relatively face problems after the rest of life. It was reported that *L. delbrueckii, C. perfringens*, and *Campylobacter coli* chicks at the age of 3rd day and *L. acidophilus, Enterococcus*, and *Streptococcus* chicks from 7

to 21 d of age, while *L. Crispatus* chicks at 28 and 49 days of age in the gut, different composition at different periods of age [172]. The main gizzard contains *Lactobacillus, Enterococci*, lactose-negative *Enterobacteria*, and *Coliform* [28]. The lowest bacteria density was found in the duodenum due to a dilution of the digest by bile secretion, containing *Clostridia*, *Streptococci*, *Enterobacteria*, and *Lactobacilli* and a short passage of time interval [173]. Ileal bacteria community was examined to 16S rRNA gene sequences, and *lactobacillus* (70%) as the major group, *Clostridiaceae* (11%), *Streptococcus* (6.5%), and *Enterococcus* (6.5%) were found [171]. The cecum as compared to the ileum possesses a wide range, rich, and steady microbiome community including anaerobes. There were significant changes observed at 6 weeks from day-old in the cecal microbiota community [174].

Male chickens exhibited a faster growth rate as compared to female chickens due to sexual differences in growth and development. This difference in growth rate may be associated with the difference in gut microbiota between sexes that can affect significantly nutrient digestion, absorption, and metabolism, which are associated with the immune and health status of birds. Alternation of the gut microbiome is directly related to the body weight of animals including pigs, chickens, and humans [21]. Lee et al. investigated that female broiler chickens harbor a number of *Bacteroidetes*, *Firmicutes*, and *Proteobacteria*. There are *Shigella* and *Moraxellaceae* associated with *Proteobacteria* causing relative abundance in female gut microflora, while male broiler chickens are associated with the enriched relative abundance of *Bacteroidetes* and *Firmicutes*, but the major difference between male and female growth in harboring microbiota are two genera *Bacteroides* and *Blautia* [7, 174]. It is concluded that biological processes such as sex hormones secretions differences cause the differences in microbiota in the ceca of male and female chickens [174].

16. Bacteriophages

Bacteria-eating viruses called bacteriophages are reported as an alternative to antibiotics in the resistance to bacterial diseases. Bacteriophages are particularly host-specific in nature, targeting a specific bacterial group, and did not affect the immune system of humans or animals, and normal gut microflora. These viruses increase in number inside the infected host cell or bacterial cell so-called lytic infection cycle and, by bacteriolysis, come out from the cells. Bacteriophages inject their DNA into the host cytoplasm and replicated utilizing the metabolic components of the infected host cell and encoding genes [175, 176].

It is investigated that bacteriophages were isolated and used in different experiments to decrease the colonization of *S. Typhimurium* and *S. enteritidis* in the cecum [177]. There is a decrease in the colonization of positive control groups. From the 7 DPI beginning of the experiment to the end at 15 DPI, all chicks exhibited no colonization of *Salmonella* in the cecum, which concluded that bacteriophage treatment is effective for *Salmonella* treatment. The use of antibiotics against the *salmonella* resistance strains results in high economic losses in the poultry industry. Uses of antibiotics kill the pathogenic bacteria and impact normal microflora and secondary infections. Bacteriophage supplementation has potential beneficial effects as compared to antibiotics supplementation due to the specific nature of bacteriophages. There will be a reduction of bacterial load in the intestine of newborn chicks if it could be possible to administer five succeeding dosages of bacteriophages orally [177]. By the combination of bacteriophage P22 and antibiotics inhibited the growth of *S. Typhimurium*. This combination of two factors reduces the development of antibiotic resistance in *S. Typhimurium*. There was a reduction in relative expression levels of genes regulating efflux pump (acrA, acrB, and tolC) and outer membrane (ompC, ompD, and ompF) [178]. Huang and Nitin grow bacteriophages that is based on edible antimicrobial coatings T7 phages (#BAA-1025-B2) on fish feed, a fish pathogen *Vibrio*, and a bacterium *E. coli* for treatment of human and fish pathogens, especially in a hydroponic system. This edible whey protein isolate coating was found to be beneficial in increasing the load of phages on fish feed pellets and decreasing the loss of phage activity during feed storage. This coating facilitates increased durability of phages in the stimulated gastric environment, and there is a significant reduction of bacteria in stimulated intestinal digestion [179].

17. Conclusions and Future Perspectives

The potentiality of these components as nutritional sources for the overall performance and prevention of enteric infections can improve the gut microflora and immune system in chickens. There are many natural sources that have been used as an alternative therapy against depression, osteoporosis, diabetes, and cancer. Rather than an antibiotic, there will be just another option to find new alternative sources from plants, animals, and other origins so that can be rich in nutrients and minerals to provide the nutrients to the broilers. In addition, the studies on the agonistic and synergetic effect of different feed additive sources are important to know so that the gap between information on their combined effects may be filled. The beneficial use of natural resource products in regulating the gut microflora population and immune system should be used in poultry against enteric infections to overcome antibiotic resistance. The nutrients may also encourage using these natural resources in the feed to improve the growth performance of poultry and alternately consumer, and human health.

Data Availability

No data were used to support this study.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Irfan Ahmed, Zhengtian Li, and Sharoon Shahzad contributed equally to this work.

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Research Article

Foliar Application of Copper Oxide Nanoparticles Increases the Photosynthetic Efficiency and Antioxidant Activity in *Brassica juncea*

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In the last few decades, use of copper oxide nanoparticles (CuO NPs) has been increased significantly that eventually included as a growth stimulator. This makes it essential to examine their impact on several plants. In the study detailed here, we investigated the effects of CuO NPs on the growth, physiological efficiency, biochemical assays, and antioxidant system in the mustard plant. Varying concentrations (0, 2, 4, 8, and 16 mg/L) of CuO NPs were applied at 25 days after sowing (DAS), and sampling took place at 30 and 45 DAS. The results indicate that CuO NPs-treated plants registered an increase in the growth and biomass over their respective control. Among different concentrations of CuO NPs (0, 2, 4, 8, and 16 mg/L), 8 mg/L proved to be the optimum foliar spray treatment and increase the chlorophyll content, net photosynthetic rate, leaf proline content, and antioxidant enzymes activity. We concluded that CuO NPs interact with meristematic cells triggering biochemical pathways conductive to an enhancement of the growth attributes. Further studies are needed to investigate the mechanisms of CuO NPs in mustard.

1. Introduction

Nanotechnology is one of the most fascinating and emerging fields to explore new things. The role of NPs has been increased for making commercial products, and they are also heavily used in industrial applications, causing health concerns by entering into the food chain. In literature, both effects (positive and negative) of NPs have been reported in plants as well as in animals. The most commonly used nanoparticles in agriculture fields are Ag, Cu, ZnO, Al, Si, Ce, Ti, and Au [1]. A number of research investigations have been conducted to evaluate the impact of NPs in agriculture. Increasing demand for NPs in various fields raises the concern about their impact on the environment and food chain. Phytotoxicity of ZnO NPs had been reported in diverse crop plants such as *Brassica napus*, *Raphanus sativus*, and *Lolium perenne* by Lin and Xing [2]. The positive result of metal oxide NPs has been reported in wheat seeds, which show evidence of enhanced nutrient use efficiency, photosynthetic activity, grain quality, and increased yield [3]. Application of SiO_2 NPs and TiO_2 NPs in soybean crop increased the germination, improved growth, and nitrate reductase activity [4]. The positive impact of NPs in plant growth raises hope for farmers as they can be used as an alternative for harmful chemical fertilizers, but still, extensive research needed in this field.

Among different nanoparticles, CuO NPs are extensively used in various fields, like in superconductors, batteries, gas sensors, etc. [5]. In agriculture, CuO NPs have been used as pesticides, herbicides, fertilizers, additives for soil remediation, and growth regulators [6]. Both positive and negative impacts of CuO NPs have been reported in plants. Shende et al. reported the positive effect of biogenic CuO NPs on the growth of pigeon pea [7]. A positive effect of CuO NPs also

reported in *Vigna radiata* [8] and *Cajanus cajan* [9]. In contrast to this, the toxic effect of CuO NPs at higher concentrations has also been reported. Reduced photosynthetic rate, transpiration rate, and photosynthetic pigment have been observed by 1000 mg/L of CuO NPs in rice [10]. Moreover, inhibitory effects of CuO NPs in a dose-dependent manner are also found in mustard [11]. Similarly, the toxic effect of CuO NPs is also reported in pea [12] and *Hordeum vulgare* [13]. In comparison to other NPs, further investigation needed to find out the impact of CuO NPs in plants at the physiological and biochemical levels.

Mustard (*Brassica juncea*) is cultivated all over the world as an oilseed crop. In India, mustard is the second most important crop for oil production. Across the world, India covered 13% of area for the mustard cultivation. India is the third largest producer of the oilseed crops [14].

From the published data, it was hypothesized that nanoparticles can also be used as micronutrients to increase plant growth performances and their yields. Cu works as a micronutrient in plants, so it was assumed that nano form of Cu could also be beneficial in the same way. Following all these data, a study was designed to investigate and explore the impact of CuO NPs on the improvement and overall physiology of mustard plants. Foliar sprays of CuO NPs with concentrations (0, 2, 4, 8, or 16 mg/L) were given on mustard plants. Characterization of nanoparticles was performed using a scanning electron microscope. To our knowledge, very meagre literature is available for CuO NPs, which mediates the positive response in plants. In our study, CuO NPs show a positive effect on *B. juncea* at a lower concentration in comparison to control.

2. Material and Experimental Methods

2.1. Plant Materials. The seeds of mustard (*B. juncea*) were procured from New Delhi (Indian Agriculture Research Institute), India. The seeds selected for sowing were healthy and uniform in size. Surface sterilization of the seeds was performed using 1% sodium hypochlorite solution for the duration of 10 min than repeatedly washed with double-distilled water.

2.2. Nanoparticles Sources. Nanoparticles were purchased from Sigma-Aldrich Division Pvt. Limited. Stock solutions of 16 mg/L of CuO NPs were prepared by dissolving the CuO NPs in double-distilled water, and the final volume was made up to 500 mL in a volumetric flask. After then, required quantities, i.e., 2, 4, 8, or 16 mg/L of CuO NPs, were prepared from the initial stock solution. A surfactant, Tween-20 (purchased from Sigma-Aldrich), was added to the CuO NPs solution before spraying to get a maximum attachment of NPs on foliage.

2.3. Experimental Design and Pattern of Treatment. An experiment was conducted in earthen pots with a randomly set design. Surface sterilized seeds were sown in pots. The soil used in this experiment was sandy loam and equally mixed with green manure in the ratio of 6 : 1. Seeds were germinated in natural circumstances in the net house of the Department of Botany, Aligarh Muslim University, Aligarh, India. Total twenty-five pots were required for this experiment. These twenty-five pots were differentiated into five sets, and every set consists of five pots. Each of the 5 sets was representing one treatment with five replicates. When plants were 25 days old, spraying of CuO NPs water (control), 2, 4, 8, or 16 mg/L was given. After 30 and 45 days of sowing (DAS), plants were collected to assess the various parameters.

2.4. Microscopic Observations of the Nanoparticles. Microscopic studies had been completed using the scanning electron microscope. For this, the leaf samples were fixed in glutaraldehyde (2.5%) and in 0.05 M potassium phosphate buffer at pH 7.1 for 8 h. After this. sample was dehydrated in an ethanol series. CuO NPs were characterized by scanning electron microscopy (SEM) on JEOL JSM–6360 at 15 kV.

2.5. Measurement of Growth Characteristics and Leaf Area. Growth characteristics such as root length and shoot length, fresh and dry mass of root and shoot were measured at 30 and 45 DAS. The method followed by Khan et al. [15] taken into account to measure the growth parameters of plants. For measuring the area of leaves, a portable instrument was used know as a leaf area meter. In our experiment, ADC Bioscientific (UK) leaf area meter had been used.

2.6. Measurement of Chlorophyll Content (SPAD Value). Chlorophyll content was measured in the intact leaves of plants with the help of a SPAD chlorophyll meter (SPAD-502; Konica, Minolta sensing, Inc., Japan).

2.7. Leaf Gas Exchange Parameter. Photosynthetic characters, viz., rate of net photosynthesis (P_N), stomatal conductance (g_s), transpiration rate (E), and internal carbon dioxide concentration (C_i) of leaves were measured as performed by Khan et al. [15], when leaves were entirely stretched during 11 : 00 and 12 : 00 h by using portable instruments known as infrared gas analyzer (IRGA). The model of IRGA used in our study was LI-COR 6400 (Lincoln, NE, USA). The instrument was stabilized at 25°C air temperature, 85% relative humidity, 600 μmol mol^{-1} CO_2 concentration, and PPFD 800 μmol mol^{-2} s^{-1}.

2.8. Analysis of Biochemical Parameter

2.8.1. Enzyme Assay. To measure the carbonic anhydrase (CA) activity method given by Dwivedi and Randhawa was used [16]. The activity of CA was measured in the fresh leaves of plants. Leaves were chopped into minute pieces and transferred into the solution of cysteine-HCl. This sample was then incubated at 4°C for the time of 20 min. After 20 min, these small pieces of leaves were blotted and transferred to the test tube containing phosphate of pH 6.8. To the test tube, 0.002% solutions of bromothymol blue indicator and bicarbonate (HCO_3) were added followed by

incubation of the test tube at 4°C for twenty minutes. In last, this reaction mixture was titrated against 0.5 N hydrochloric acid solution (HCl). In this solution, 0.2 mL of methyl red was added as an indicator.

Protocol given by Jaworski was applied to determine nitrate reductase (NR) activity [17]. For NR, the fresh leaves were taken and cut into small pieces. Pieces of the leaves were transferred to vials made up of plastic, which had 1.25 mL phosphate buffer with a pH of 7.5. In the plastic vials, potassium nitrate solution and isopropanol solution were added. This reaction mixture was incubated for 2 h at 30°C. Then, 0.02% N-1 naphthyl ethylenediamine dihydrochloride and sulphanilamide were added to the mixture. The absorbance of this mixture was measured with the help of a spectrophotometer (Elico model No. SL 171) at 540 nm wavelength.

To measure the activity and levels of diverse antioxidant enzymes like catalase (CAT), peroxidase (POX), and superoxide dismutase (SOD), the method used by Khan et al. was followed [15].

2.8.2. Endogenous Proline Level. Levels of proline was measured in the fresh leaves of the plants, and for this, the method given by Bates et al. was used [18]. For proline estimation, extraction of the leaf samples was obtained using a sulfosalicylic acid solution. In this extract, the same amount of glacial acetic acid and solution of ninhydrin was added. The final solution was heated at 100°C for the duration of 1 hour, and then, the reaction was terminated by placing the sample into using an ice bath and then to this terminated sample, 5 mL of toluene was added vigorously. Two separate layers were formed by the addition of toluene. The absorbance of the upper layer was taken on a spectrophotometer (Elico model No. SL 171) at 520 nm wavelength.

2.9. Statistical Analysis of Data. Experimental data of our studies were calculated by using analysis of variance (ANOVA) with the help of the SPSS software (SPSS, Chicago, USA). To differentiate between the mean values of treatment, least significant difference (LSD) was calculated at a significance level of $P \leq 0.05$.

3. Results

3.1. Phenotypic Character. Plants grown with CuO NPs showed a positive increase in growth biomarkers (length, fresh mass, and dry mass of shoot and root) in comparison with control plants at both the stages of plant growth (30 and 45 DAS) (Figures 1(a)–1(f)). Moreover, the maximum increase of growth parameters was reported in the plants exposed to 8 mg/L of CuO NPs at 30 and 45 DAS. However, increased with 16 mg/L of CuO NPs were statistically significant to the values of control while at par with that of 2 mg/L of CuO NPs.

3.2. Leaf Area, SPAD Value, and Photosynthetic Traits. Exogenously applied CuO NPs significantly increased leaf area and SPAD chlorophyll levels over the control (Figures 2(a) and 2(b)). However, the maximum leaf area and SPAD values were recorded, when the plants were subjected with 8 mg/L of CuO NPs, and were about 23.15% and 29.73% (leaf area), 23% and 37.5% (SPAD value) at 30 and 45 DAS, respectively.

The photosynthetic parameters (P_N, C*i*, E, and g_s) were increased as the growth progressed from 30 to 45 DAS irrespective of the treatment (Figure 2). However, concentrations (4 and 8 mg/L) increased it further at both stages. Out of the various concentrations, 8 mg/L was more effective and increased photosynthetic parameters at 30 and 45 DAS over their control. However, at higher concentrations, photosynthetic parameters were significantly decreased.

3.3. Activity of NR and CA. It was evident from Figures 3(a) and 3(b) that leaf CA and NR activity were increased with the advancement of the age of the plant. It further increased with the concentration (8 mg/L) of CuO NPs at both stages of growth. Maximum CA and NR activity were noted in the plants at 8 mg/L of CuO NPs which were 34.32% and 29.55% higher as compared to the control, respectively, at 45 DAS.

3.4. Antioxidant Enzymes. Improvement in the activity of the antioxidant enzyme (POX, SOD, and CAT) was noteworthy in every concentration (2, 4, 8 or 16 mg/L) of CuO NPs at both stages of growth (30 and 45 DAS). The minimum activity of the enzyme reported in control plants which were sprayed with distilled water only while CuO NPs (8 mg/L) showed the maximum increased in antioxidant level (Figures 3(c)–3(e)). The percent increase by 8 mg/L of CuO NPs in POX was 21% (30 DAS) and 53.4% (45 DAS), CAT was 23% (30 DAS) and 56% (45 DAS), and SOD was 23% (30 DAS) and 54.79% (45 DAS).

3.5. Proline Content. Leaf proline content was also increased with the advancement of the age of the plant (Figure 3(f)). It was further increased by the concentrations (4 and 8 mg/L) of CuO NPs, whereas maximum proline was recorded in the leaf of the plant grown in the presence of 8 mg/L of CuO NPs at both the stages of growth (30 and 45 DAS). The higher concentration was proved to be inhibitory, and it decreased the level of proline.

4. Discussion

Copper was considered an essential element for plant growth and metabolism. It plays a significant role in the electron transport chain of photosynthesis, mitochondrial respiration, responses in oxidative stress, hormonal signalling, etc. Copper also acts as a cofactor in many enzymes such as cytochrome c oxidase, amino acid oxidase in superoxidase dismutase as reported by Mazhoudi et al. in [19]. Scientists are working on nanoparticles of metal to find out its feasibility in improving the growth and productivity of the crop. Various studies have been performed, but no clear-cut picture of its role in plants has been ascertained. Some of them show positive while others show negative impact. It

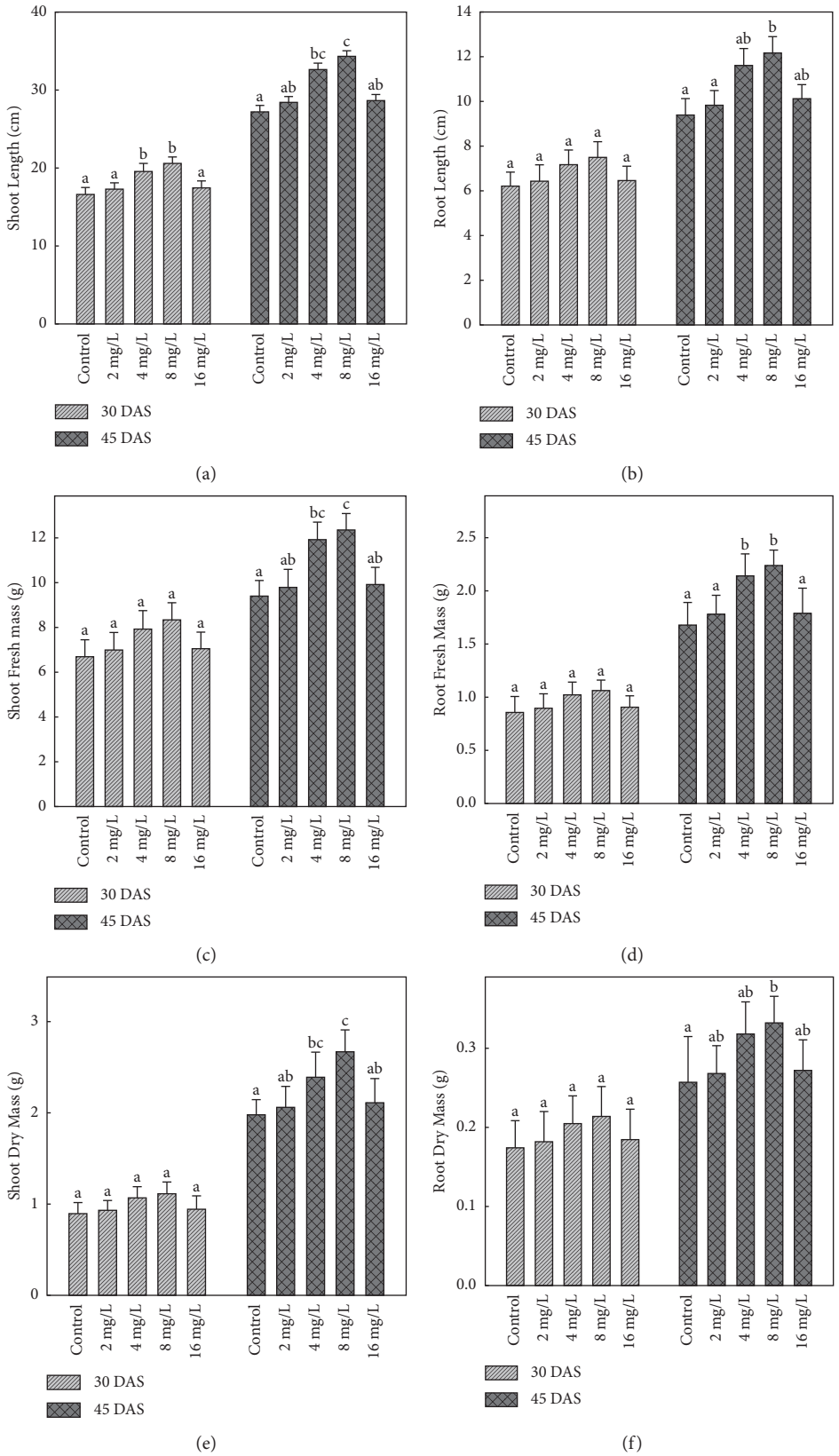


Figure 1: Effect of nanoparticles (NPs) on the length of shoot (a), root length (b), fresh mass of shoot (c), root (d), dry mass of shoot (e), and root (f) of mustard at 30 and 45 DAS. All the data are the mean of five replicates ($n = 5$), and vertical bars shows standard errors (±SE). (Different alphabet represents the significant difference ($p < 0.05$) of treatments compared to the control group).

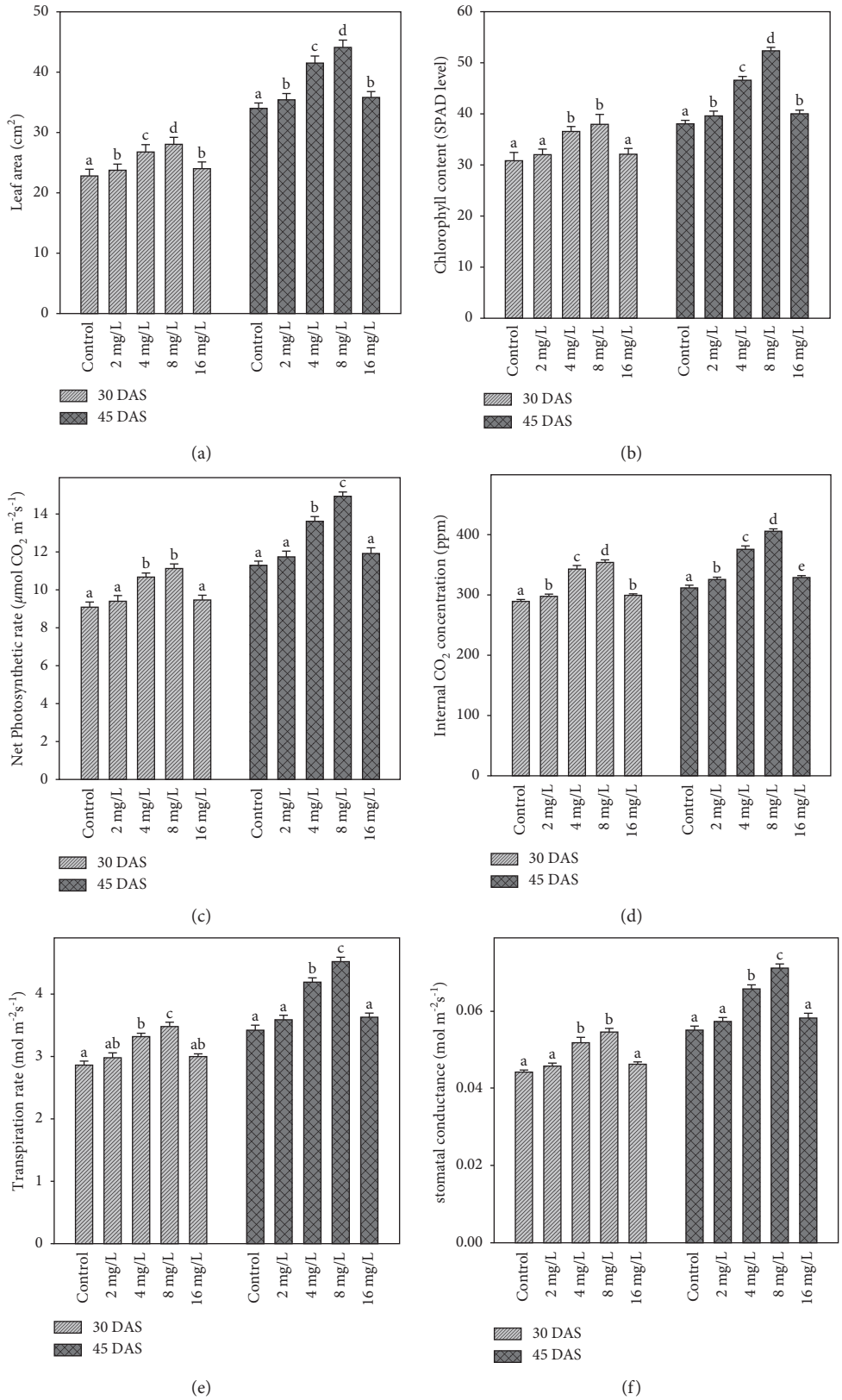


Figure 2: Effect of nanoparticles (NPs) on the (a) leaf area, (b) chlorophyll content, (c) net photosynthetic rate, (d) internal CO_2 concentration, (e) transpiration rate, and (f) stomatal conductance of mustard at 30 and 45 DAS. All the data are the mean of five replicates ($n = 5$), and vertical bars shows standard errors (±SE). (Different alphabet represents the significant difference ($p < 0.05$) of treatments compared to the control group).

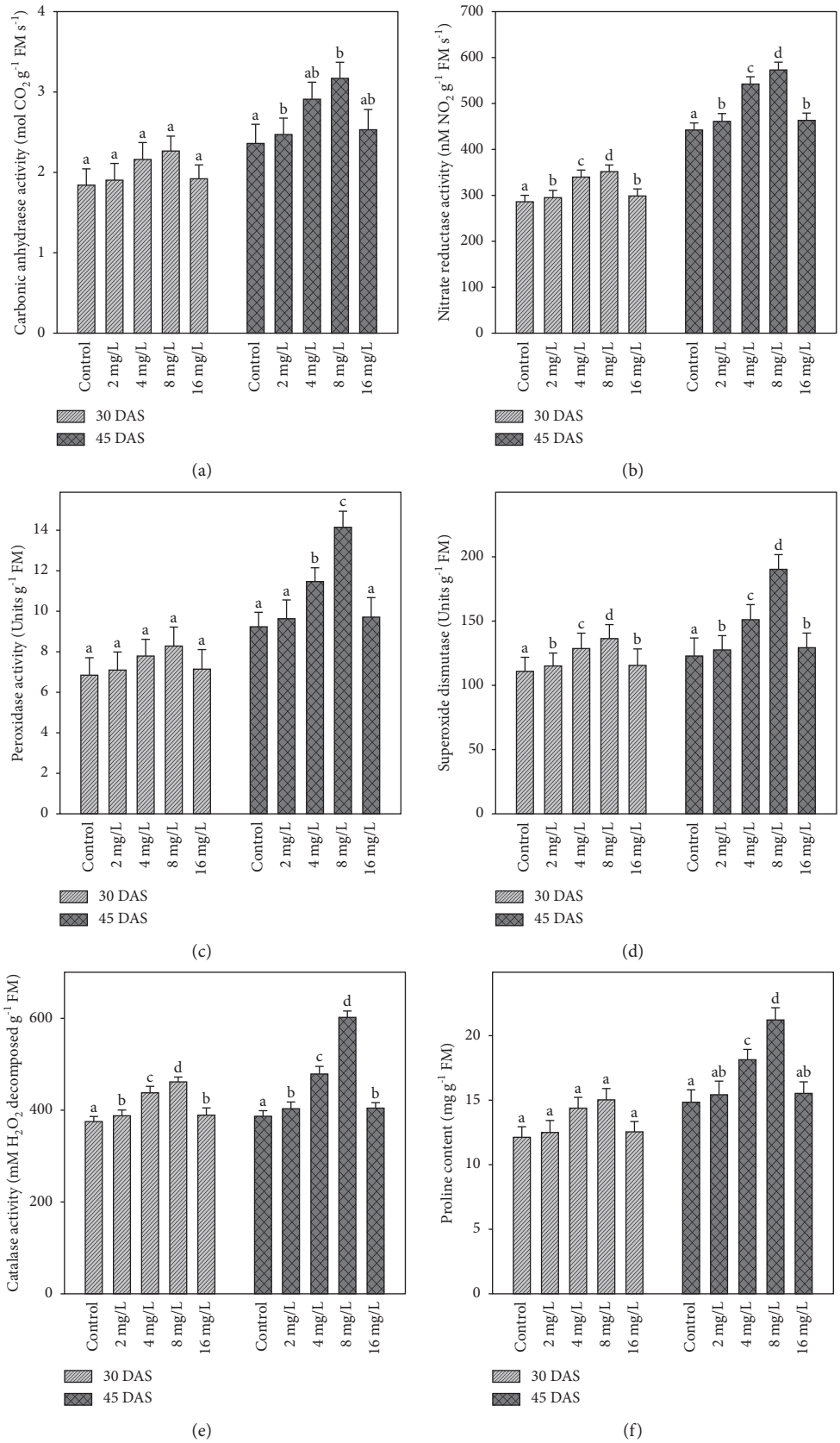


Figure 3: Effect of nanoparticles (NPs) on the (a) carbonic anhydrase activity, (b) nitrate reductase activity, (c) peroxidase activity, (d) superoxide dismutase, (e) catalase activity, and (f) proline of mustard at 30 and 45 DAS. All the data are the mean of five replicates ($n = 5$), and vertical bars shows standard errors (±SE). (Different alphabet represents the significant difference ($p < 0.05$) of treatments compared to the control group).

depends on the type of nanoparticle size and also on the concentration of nanoparticles. The result of nanoparticles also varies from one plant species to another species. In the present observation, it was noted that spraying of CuO NPs on the leaves of mustard seedling leads to a significant change (Figures 1–3). Out of various concentrations of nanoparticles, 8 mg/L shows the maximum increased in the growth, photosynthetic rate, and antioxidant level. Whereas at higher level (i.e., 16 mg/L), the results are less effective but still better than the control (Figures 1–3).

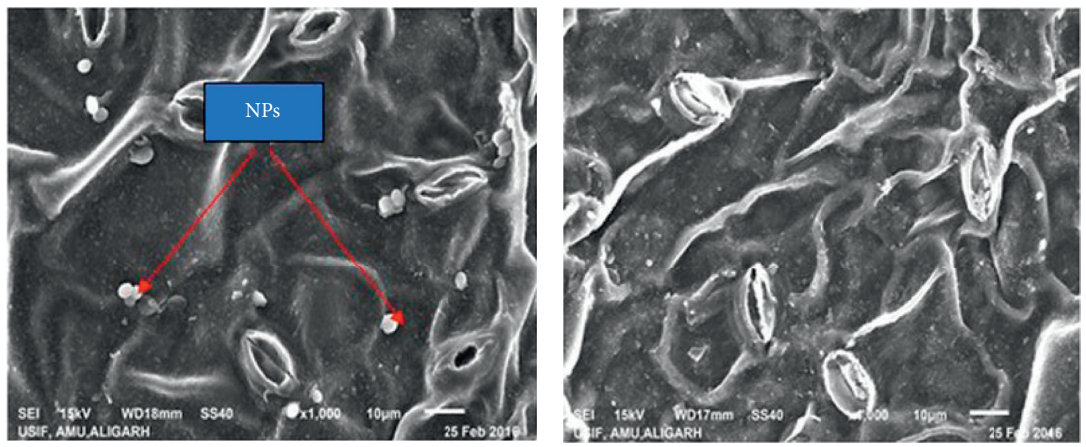
Our finding is also in accordance with the finding of other nanoparticles in which NPs improved the overall growth of plants, so these finding suggested that CuO NPs can be used in plants for enhancing their growth and development. Earlier research conducted on the application of nano-SiO_2 found that when seeds of changbai larch soaked in nano-SiO_2, it enhanced the quality and the growth of seedling and the parameters such as mean height, root length, collar diameter, lateral roots in number, and length of the root [20]. Raliya and Tarafdar reported that ZnO NPs can be useful in plant growth. They reported that when ZnO NPs were given as a foliar spray in cluster bean plants, the treatment improved overall growth of the plant [21]. Arora et al. found that gold nanoparticles (AuNPs) on *Brassica juncea* made improvement in growth and seed yield and increased the number of leaves, area of the leaf, plant height, chlorophyll content which leads to the better crop yield [22]. All these results clearly indicate that nanoparticles act as a growth regulator in plants and defend our finding in the case of CuO NPs.

Photosynthesis, respiration, and transpiration are the indicators of a healthy plant. Out of all radiation coming from the sun, plants utilize only 2–4% of this radiation during photosynthesis. Therefore, there is an immense need to increase these values so that plants can maximize the photosynthetic rate, which ultimately leads to higher biomass production. In our study, foliar-applied CuO NPs increased leaf area (LA), total chlorophyll content (SPAD), leaf net photosynthetic rate (P_N), transpiration rate (E), stomatal conductance (g_s), and internal CO_2 concentration (Figures 2(a)–2(f)). All these gas exchange traits increase gradually with CuO NPs. CuO NPs of 8 mg/L concentration found to be best when it comes to the untreated plant. The results of our experiments are similar to other previous reports where nanoparticles showed improved photosynthetic rate [23]. When carbon nanotubes (CNTs) were embedded in isolated chloroplast of *A. thaliana* leaves, it increases the photosynthetic rate by three times in comparison to plant without SWCNTs [24]. Illumination of TiO_2 NPs to the chloroplast of the spinach plant protect from aging which may be a possible cause for higher photosynthetic rate [25]. Increased activity of Rubisco was reported with TiO_2 nanoanatase [23]. Increased activity of Rubisco enhances the photosynthetic carbon assimilation, so there is increased growth in plants, which also satisfies the finding of our study. Feizi et al. in wheat and Zheng et al. in spinach have also reported the same result of improved photosynthesis by nanoparticles [26, 27]. They found that the use of nano-TiO_2 improves seedling growth or promote germination and photosynthesis in comparison to untreated control plants and stabilized our experimental result of positivity of CuO NPs. Moreover, the size/density of stomata also plays a major role in coordinating the process of diffusion of gases. In the present study, foliar application of CuO NPs increased the density of the stomata (Figure 4) which may be the possible reason behind the increase in gas exchange between the plant tissues and the atmosphere which ultimately increased the photosynthetic attributes. All these findings suggest that our results are good enough to show the positive character of CuO NPs in plants and improve photosynthetic efficiency.

Carbonic anhydrase and nitrate reductase are the features that determine the health of the plants and responsible for growth related physiological reactions. Both these traits improved by the exogenous application of CuO NPs in the form of a foliar spray, which confers the overall plant's growth and development (Figures 3(a) and 3(b)). Our finding is similar to another study where positive result by the application of others nanoparticles has been found on these enzymes. Increased nitrate reductase level has been reported with the use of nano-SiO_2 and nano-titanium dioxide (nano-TiO_2) which finally improve the seed germination in soybean [4].

Plants have adapted two types of defense mechanism, one is enzymatic (superoxide dismutase, peroxidase, and catalase) and another one is non-enzymatic (proline). Both this mechanism, i.e., proline (Figure 3(c)) and antioxidant level such as POX, SOD, and CAT (Figures 3(c)–3(e)) improved by the treatment of CuO NPs when given in the form of a foliar spray, these increased levels mitigate any ill effect caused by these nanoparticles and improve the overall plant's growth. Our result is also similar to that of Costa and Sharma, who used CuO NPs in *Oryza sativa* and reports increased expression of enzymatic antioxidants, APX and SOD at 10 and 100 mg L^{-1} of CuO NPs, respectively in which also protect plants from oxidative stress [10]. Lei et al. work on spinach plants and reported that nanoanatase TiO_2 diminished the accumulation of H_2O_2, MDA content and increased the level of SOD, CAT, and POX thus improved the antioxidant system under abiotic stress [28]. *Brassica juncea* showed improved activity of antioxidant enzyme such as APX, POX, and CAT when treated with Ag NPs. This increased level of antioxidant enzyme reduces the reactive oxygen species activity [29]. Recently, SOD activity was checked under CuO nanoparticles in wheat, and it was found that its activity was increased significantly with 25 mg/L CuO NPs when compared to untreated plants [30].

Proline is an important amino acid that stores in plants as compatible solutes and helps the plant against oxidative stress by removing the reactive oxygen species which had been released during that period of oxidative stress [31]. During oxidative stress accumulation of ROS increased the levels of antioxidative enzymes which are helpful to maintain homeostasis [32]. Accumulation of proline was reported at the highest level in plants treated with NPs with different concentrations as compared to control plants. Plants treated with 8 mg/L of CuO NPs possess 43% higher proline content than control at 45 DAS. Increased proline content was observed upon exposure to different concentrations of CuO NPs [11, 33, 34].



(a) (b)

Figure 4: Scanning electron microscope (SEM) image of treated with CuO NPs (a) and control (b).

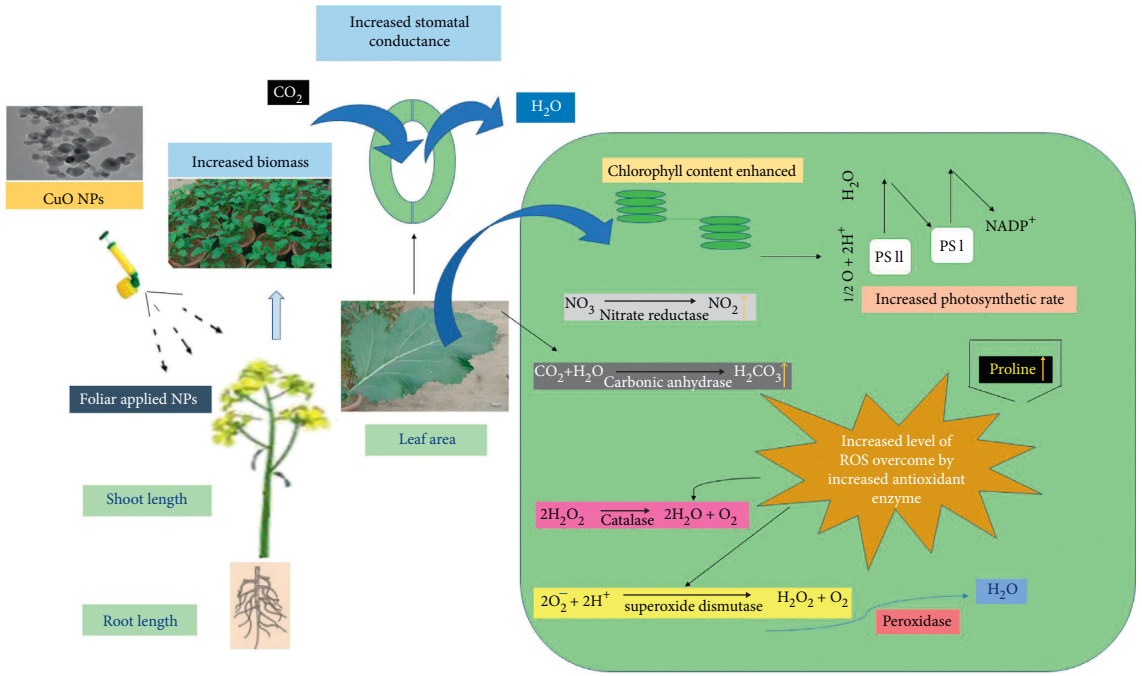


Figure 5: A simplified view of the foliar application of CuO NPs and their effect on the physiological and biochemical parameters in *Brassica juncea*.

Our finding with CuO NPs shows a positive response as we can see in the aforementioned paragraph. It has been reported that CuO NPs at all the concentration, when applied on foliage, enhanced almost all the growth, biochemical, and physiological parameters. This result can be applied in the field to see their further outcome on a large scale before making any recommendation to farmers. CuO NPs then can be utilized as micronutrients to enhance the production of mustard and their growth which ultimately results in higher yields. A simplified view of foliar application of CuO NPs in *B. juncea* has been shown in Figure 5, where we can see how NPs enters into plant cells and affect different growth and physiological parameters in plants.

5. Conclusions

It can be concluded from the present study that CuO NPs-mediated response was concentration-dependent. Moreover, the foliage of mustard plants treated with 8 mg/L showed the most promising response, increased the growth, and enhanced the photosynthetic efficiency of plants. NPs can get attached/entered to the cell surface through the pores and lenticels. This attachment increases the gaseous exchange and causes improved growth and development of *B. juncea*. The physiological parameters were improved by the application of CuO NPs which led to overall growth improvement in mustard plants. Increased stomatal conductance increases the gas exchange rate which further

improves photosynthesis and results in the production of higher biomass and crop yield. However, studies at the field level using different crops and soil types are needed before recommendations can be confirmed.

Data Availability

All the related files are included in the manuscript.

Conflicts of Interest

The authors declare that there are no conflicts of interest amongst them.

Authors' Contributions

All the authors contributed to the study conception and design. Ahmad Faraz and Mohammad Faizan prepared the material preparation, collected the data, and analysed the data. Ahmad Faraz wrote the first draft of the manuscript. All authors commented on the previous version of the manuscript and read and approved the final manuscript.

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Research Article

Foliar Application of Leaf Extracts of *Glycyrrhiza uralensis* Increases Growth and Nutritional Value of Chinese Flowering Cabbage Plants under Field Conditions

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This study was conducted to explore the effects of ethanolic extracts of leaves of *Glycyrrhiza uralensis* on the growth and nutritional quality of Chinese flowering cabbage (*Brassica rapa* subsp. *parachinensis*) under field conditions. Preliminary greenhouse experiments were carried out with different concentrations of *G. uralensis* extracts to select the suitable dose for field studies. An extract concentration of 12.5 g/L was selected based on relative growth rate analysis and increase in photosynthetic pigment biosynthesis. Shoot length, shoot fresh weight and dry weight, number of leaves, and marketable value of Chinese flowering cabbage plants were significantly increased in the field trials with foliar application of *G. uralensis* leaves extracts at a concentration of 12.5 g/L. This biotic elicitor also enhanced the total phenolic and flavonoid contents, with optimal values increasing by 18.76% and 22.43%, respectively, compared with the control under field conditions. The total glucosinolate content was effectively increased (from 11.21 to 15.37 μmol·g^{-1} DW sinigrin equivalents), particularly 4-methoxyglucobrassicin (from 4.31 to 6.72 μmol·g^{-1} DW sinigrin equivalents) and glucoalyssin (from 0.14 to 0.19 μmol·g^{-1} DW sinigrin equivalents) compared with the control plants in field trials. Overall, foliar application of leaf extracts of *G. uralensis* can markedly increase the growth of Chinese flowering cabbage and enhance its medicinal and nutritional quality in the fields.

1. Introduction

Vegetables belonging to the Brassicaceae family show valuable health benefits owing to the presence of biologically active and strong antioxidant substances. Chinese flowering cabbage (*Brassica rapa* subsp. *parachinensis*) is an annual vegetable crop belonging to the Brassicaceae family. Its growth period is short and multiple cropping index is high, with only 40–56 days from germination to flowering [1]. Chinese flowering cabbage has valuable biological and nutritional properties [2]. Its above ground parts, including leaves, stem, and inflorescence, can be cooked or consumed raw in salads. Its leaves contain adequate amounts of glucosinolates and polyphenolic compounds [3]. This rich chemical composition and scientifically proven biological activity have made Chinese flowering cabbage a famous culinary plant [4].

Glucosinolates (GLS) are primarily found in plants of the genus *Brassica*, which include crops of economic and nutritional importance. GLS are rich in sulfur and anionic secondary metabolites [5]. GLS have been extensively studied for their protective effect against herbivory in plants and chemotherapeutic activity in humans [6]. The consumption of vegetables containing glucosinolates may confer protection against cancer in humans [5]. The hydrolytic breakdown products of glucosinolates have

beneficial effects on human health, including cytotoxic and apoptotic effects in damaged cells and reducing risks of degenerative diseases [5, 6].

Lethal effects of synthetics pharmaceuticals have augmented the discovery and large-scale production of natural bioactive molecules. However, the supply of natural bioactive compounds is limited for various reasons. On the other hand, the consumer demand for these compounds is increasing progressively. Hence, application of novel strategies to meet the current growing demand for natural bioactive compounds is of immense relevance. The use of conventional approaches to accelerate natural biosynthetic pathways in plants is shown to produce high levels of bioactive compounds, without the need of genetic engineering applications [7]. Furthermore, advances in technology have augmented the discovery of new biotic elicitors capable of increasing production of secondary metabolites in plants [8, 9].

Glycyrrhiza uralensis Fisch (Fabaceae), commonly known as licorice, is a traditional plant recognized through the ages for its multiple health benefits and medicinal uses [10, 11]. The roots of this plant are used to treat influenza, coughs, and liver damage in traditional medicinal formulations [10]. However, the roots or rhizomes correspond to merely one fourth of the whole biomass of the plant. The aerial portion of licorice is of lesser importance to cultivators and usually constitutes as agroindustrial waste after the harvest. The purpose of this study was to investigate the effect of using alcoholic extracts of licorice leaves as a foliar spray to improve plant vegetative growth and concentration of specific biologically active substances such as glucosinolates, phenolics, and flavonoids in Chinese flowering cabbage under field conditions.

2. Materials and Methods

2.1. Plant Material and Preparation of the Extracts. Aerial parts of *G. uralensis* were obtained from Qinghaihu Pharmaceutical Co., Ltd. (Qinghai, China). Prof. Dr. Xuebo Hu from College of Plant Sciences and Technology, Huazhong Agricultural University, China, verified the identity of plant material. The plant material was extracted as described in our previous publication [12]. Briefly, the leaves of *G. uralensis* were air-dried and ground to fine powder. This powdered material was extracted twice with ethanol/water solution (70 : 30, v/v) for 2 h at 80°C at 80 revolutions per minute under reflux. The solvent was removed by the rotary evaporator followed by freeze drying under vacuum. The dried extracts were stored in a refrigerator until use.

2.2. Treatment Optimization of Foliar Elicitors under Growth Room Conditions. A preliminary study was preformed to optimize the dose of elicitor for field studies. Plants of Chinese flowering cabbage were raised in plastic pots of 4-inch diameter containing sterilized commercial potting mix. Foliar formulations of the elicitor were prepared in 2.5% dimethyl sulfoxide (DMSO) solution at different concentrations, e.g., 0, 2.5, 5.0, 7.5, 1.0, 1.25, 1.5 g/L. Control plants received 2.5% DMSO solution alone. Application was performed at the trifoliate stage. The relative growth rate (RGR) was calculated over 5 d time spans, after 1 week of elicitor application using the following formula:

$$RGR = \frac{InW2 - InW1}{t2 - t1}, \quad (1)$$

where $W1$ is the initial shoot DW, $W2$ is the final shoot DW, and $t2 - t1$ represents the growth period.

2.3. Determination of Photosynthetic Pigments. Quantification of chlorophyll and carotenoid contents was performed in accordance with the standard method of Arnon [13].

2.4. Field Experiment. Field studies were performed in a high tunnel at the experimental station of the Vegetable Research Institute, Guangdong Academy of Agricultural Sciences, Guangzhou city of Guangdong Province, China. Chinese flowering cabbage (*Brassica rapa* subsp. *parachinensis*) were planted in September 2019. The plants were grown on raised beds. The distance between the beds was 0.3 m. Equal amounts of water were supplied, and fertilizers were applied uniformly to all treatments during the trial. Plant protection was carried out by the timely spraying of agricultural pesticides. The trial was carried out in a randomized complete-block design containing three technical replicates of each treatment. Two independent field trials were conducted. No less than 100 plants were included in each treatment.

The elicitor was applied as foliar application starting at the four-leaf stage and repeated on a fortnight basis. The optimized dose of extract (12.5 g L^{-1}) was prepared in 2.5% DMSO solution. Control plants received 2.5% DMSO solution. Plant growth parameters such as shoot length, shoot biomass, and number of leaves were evaluated after 2 months of first foliar spray. The marketable value of plants was calculated using the following formula:

Marketable value (%) = 100 – (100 × percentage of injured or diseased plants/percentage of healthy plants).

A minimum of 20 plants were harvested from each treatment and were used for morphological and metabolomic analyses. Plant samples intended for metabolomics analysis were frozen in liquid nitrogen and stored at −80°C until analysis.

2.5. Analysis of the Nutritional Values of Leaves. Leaf samples for nutritional analysis were prepared as described by Mahmoud et al. [14]. Leaf samples were washed, spread on paper towels, and air dried for 60 minutes at room temperature. Thereafter, leaf samples were oven dried at 70°C to ensure constant weight. These dried samples were ground in a stainless-steel grinder, and the following nutritional analyses were performed.

2.6. Estimation of Total Phenolic, Flavonoid, and Anthocyanin Contents. The total phenolic content was determined using the standard Folin–Ciocalteu method [15] and expressed as

milligrams gallic acid equivalent per gram of dry weight tissue (mg GAE/g DW). The total flavonoid content was estimated using the aluminum chloride colorimetric method of Chang et al. [16] and expressed as mg quercetin equivalent per gram of dry weight tissue (mg QCE/g DW).

2.7. Quantification of Glucosinolate Content. We used our recently devised method [17] for the identification and quantification of different types of glucosinolates from the leaves of Chinese flowering cabbage plants. Leaves from 10 plants were taken from each treatment and pooled together. Analysis was performed on an API 4000 QTrap mass spectrometer equipped with a TurboIonSpray probe (AB Sciex; Foster city, CA, USA) connected to a Shimadzu UFLC (Shimadzu, Kyoto, Japan). Chromatographic separations were performed with a Luna C18 column (250 mm × 4.6 mm, 5 μm particle size; Phenomenex, Macclesfield, UK). The mobile phase was a mixture of (A) trifluoroacetic acid (0.1%) and (B) acetonitrile/trifluoroacetic acid (99.9 : 0.1), with a gradient elution program of 2% B (0–5 min), 2–80% B (4–20 min), 80–95% B (20–30 min), and reconditioning with 2% B (30–38 min). The mass spectrometer worked with the triple quadrupole analyzer in the multiple reaction monitoring (MRM) mode in the negative ionisation mode for all analysed compounds. Applied Biosystems Analyst software was used to control the UFLC-MS/MS system and applied for data acquisition and processing. Sinigrin does not exist in *B. rapa* and *B. napus* [18]. In this study, sinigrin was used as an internal standard for quantitative analysis of glucosinolates [19].

2.8. Statistical Analysis. For data interpretation, analysis of variance (ANOVA) was performed using DSAASTAT (Onofri, Italy). Duncan's new multiple range test (DNMRT) was used to find a significant difference among means of different treatments. All experiments were repeated twice, and mean values are presented.

3. Results and Discussion

In the past few years, several studies have shown that exogenous elicitors, including plant extracts, can mediate plant growth and productivity [20–22]. To the best of our knowledge, the present study is the first to report the positive effects of alcoholic extract of nontraditional parts (leaves) of *G. uralensis* on the growth and health-promoting elements of Chinese flowering cabbage.

3.1. Effect of Biotic Elicitors on Growth of Chinese Flowering Cabbage. A preliminary study was conducted to optimize elicitor dose for field application based on the effects on relative growth rates and levels of photosynthetic pigments. The relative growth rate of Chinese flowering cabbage was greatly influenced by exogenous elicitor application (Figure 1). As leaves and shoots of Chinese flowering cabbage are mainly consumed, we focused on the above ground plant biomass. Generally, the relative growth rate (RGR) of Chinese flowering cabbage plants increased in an extract concentration-dependent manner but showed no significant difference when extract concentration was increased from 12.5 to 15 g/L (Figure 1). Plants receiving 12.5 and 15.0 g/L elicitor treatments showed highest RGR with no significant difference between them (Figure 1). An RGR of >0.2 d^{-1} was obtained when plants were sprayed with these two concentrations compared to other treatments. On the basis of these findings, the treatment containing 12.5 g/L of elicitor was selected for field application.

The effect of foliar application of the elicitor (12.5 g/L) was assessed on the growth and nutritional attributes of Chinese flowering cabbage in the experimental station of Guangdong Academy of Agricultural Sciences, Guangzhou, China. We observed that plant height of Chinese flowering cabbage treated with the elicitor was 31.06% higher than that of control plants. In addition, the fresh (39.72%) and dry (28.31%) biomass of Chinese flowering cabbage treated with the elicitor was significantly higher than that of the untreated plants on average in both field trials (Table 1). The number of leaves in the plants treated with the elicitor was 19.28% higher than that of the control plants (Table 1). Overall, the marketable value of the Chinese flowering cabbage plants improved by 18.32% after receiving elicitor treatment (Table 1).

The increased growth rate of Chinese flowering cabbage plants observed under the influence of the foliar elicitor could be attributed to the presence of phytohormones, soluble sugars, amino acids, and mineral elements in the ethanolic extracts of leaves of *G. uralensis* [23]. The constituents of *G. uralensis* leaf extracts may improve the yield and quality of Chinese flowering cabbage by affecting the cellular metabolism [17]. For example, it is known that sugars act as signaling molecules and improve plant growth and development [24]. Amino acids provide improved stress tolerance in plants [25]. Some organic acids present in plant extracts can chelate metal ions to stimulate root growth [26]. All these together could supply nutrition for cell growth, with resulting increase in growth and vigor.

3.2. Analysis of Leaf Pigment Content. Foliar application of *G. uralensis* extracts positively affected total chlorophyll and carotenoids contents in a dose-dependent manner (Table 2). Increasing the elicitor concentration from 2.5 to 15.0 g/L sharply increased the total chlorophyll and carotenoid contents, with no remarkable differences between the elicitor treatments at concentrations of 12.5 and 15.0 g/L (Table 2).

The positive effect of *G. uralensis* leaf extracts on leaf pigment content could be attributed to the delay in leaf senescence or enhancement in leaf pigment biosynthesis [27, 28]. These beneficial effects are possibly due to the presence of natural phytohormones in *G. uralensis* leaf extracts. The physiological parameter of leaf pigment content also acts as indicators of improved quality of Chinese flowering cabbage that can be obtained by the application of exogenous elicitors.

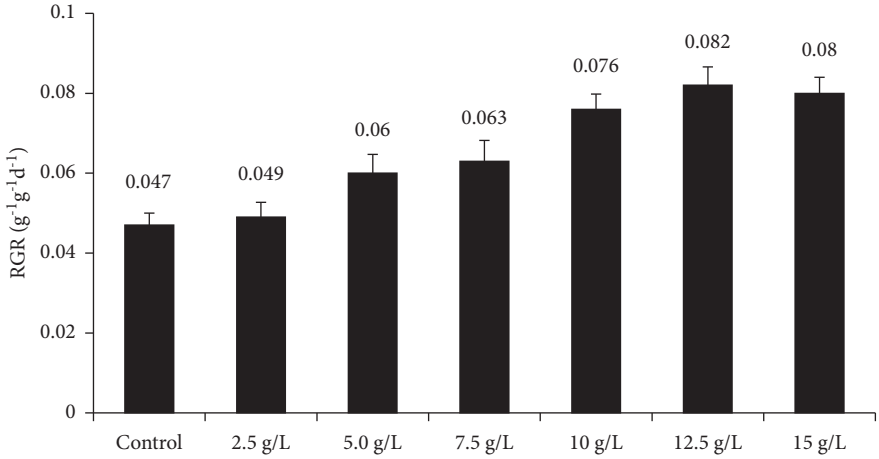

Figure 1: Effect of different concentration of leaf extracts of *Glycyrrhiza uralensis* on the relative growth rate of Chinese flowering cabbage. Vertical bars show standard error, whereas small letters show levels of significance among different treatments as governed by ANOVA and DNMRT at $p = 0.05$.

Table 1: Changes in growth attributes of *Brassica rapa* under field conditions.

Plant traits	Trial 1		Trial 2	
	Control	Treated	Control	Treated
Plant height (cm)	26.32 ± 2.1^{a}	33.57 ± 2.9^{b}	29.07 ± 1.7^{a}	31.18 ± 2.6^{b}
Shoot fresh biomass (g)	88.29 ± 5.3^{a}	141.68 ± 9.1^{b}	91.21 ± 6.2^{a}	123.17 ± 8.7^{b}
Shoot dry biomass (g)	71.91 ± 5.7^{a}	92.27 ± 6.0^{b}	76.93 ± 4.4^{a}	88.43 ± 5.9^{b}
Number of leaves	8.04 ± 0.4^{a}	10.62 ± 0.22^{b}	7.31 ± 0.36^{a}	11.31 ± 0.49^{b}
Marketable value (% age)	91.78 ± 6.24^{a}	83.54 ± 9.37^{b}	88.23 ± 7.31^{a}	81.52 ± 6.27^{b}

Data presented here are mean values of two independent field trials. Letters with ± shows standard error, whereas small letters show levels of significance among different treatments as governed by ANOVA and DNMRT at $p = 0.05$.

Table 2: Effect of *Glycyrrhiza* leaf extracts on leaf photosynthetic pigments.

Concentration (g/L)	Chlorophyll (mg/g F.W)	Carotenoids (mg/g F.W)
Control	08.52 ± 07.31^{de}	3.42 ± 02.74^{ef}
2.5	09.93 ± 05.04^{cd}	3.56 ± 01.91^{ef}
5.0	$10.85 \pm 06.19^{b\text{-}d}$	4.18 ± 02.17^{e}
7.5	12.07 ± 09.54^{bc}	5.02 ± 03.86^{cd}
1.0	14.13 ± 01.27^{ab}	5.84 ± 02.21^{bc}
12.5	14.92 ± 01.09^{a}	6.34 ± 03.87^{ab}
15.0	15.34 ± 01.58^{a}	6.86 ± 04.17^{a}

Data presented here are mean values of triplicates. Letters with ± shows standard error, whereas small letters show levels of significance among different treatments as governed by ANOVA and DNMRT at $p = 0.05$.

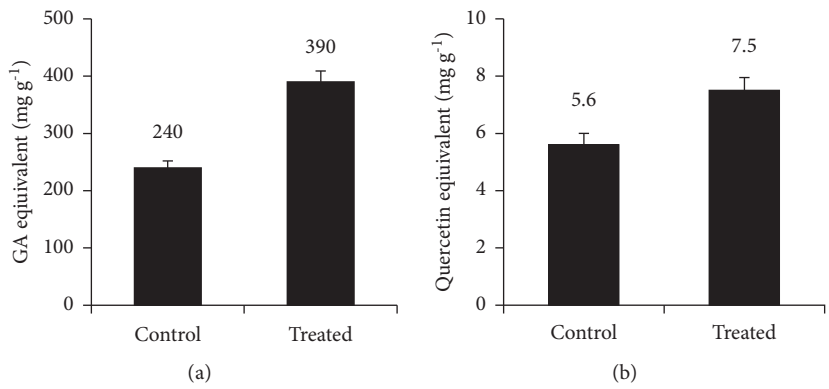

Figure 2: Effect of leaf extracts of *Glycyrrhiza uralensis* on total phenolic (a) and flavonoid (b) contents of Chinese flowering cabbage under field conditions. Data presented here are mean values of two independent field trials. Vertical bars show standard error, whereas small letters show levels of significance among different treatments as governed by ANOVA and DNMRT at $p = 0.05$.

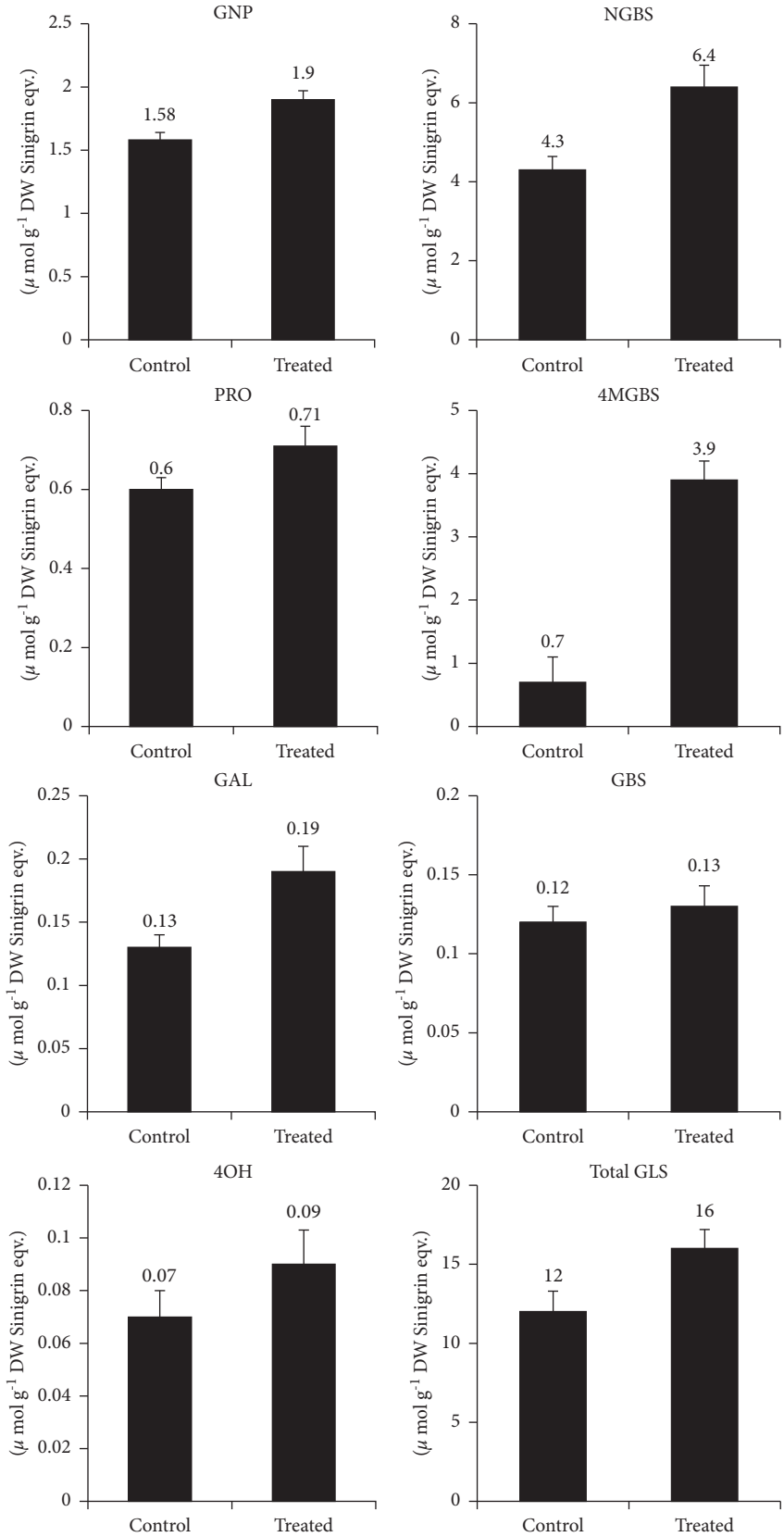

Figure 3: Effect of leaf extracts of *Glycyrrhiza uralensis* on glucosinolate content of Chinese flowering cabbage under field conditions. Data presented here are mean values of two independent field trials. Vertical bars show standard error, whereas small letters show levels of significance among different treatments as governed by ANOVA and DNMRT at $p = 0.05$.

Table 3: Details of glucosinolates quantified in of Chinese flowering cabbage.

ID	Compound	Type	Q1	Q2
Sin	Sinigrin	Aliphatic	358	195
PRO	Progoitrin	Aliphatic	388	259
GAL	Glucoalyssin	Aliphatic	450	259
GNP	Gluconapin	Aliphatic	372	371
GBS	Glucobrassicin	Aliphatic	446	259
NGBS	Neoglucobrassicin	Indolic	477	466
4OH	4-Hydroxyglucobrassicin	Indolic	463	267
4MGBS	4-Methoxyglucobrassicin	Indolic	477	259

Sinigrin was used as an internal standard for quantitative analysis of glucosinolates.

3.3. Increase in Nutritional and Medicinal Quality of Chinese Flowering Cabbage under Field Conditions. Natural bioactive compounds are isolated from a wide variety of plants. Their demand is increasing due to high therapeutic potentials and nutritional values. Data shown in Figures 2 and 3 indicate that the elicitor treatment significantly improved the production of total phenolic, flavonoid, and GLS contents in leaves of Chinese flowering cabbage plants in field trials. The foliar elicitor increased the total phenolic and flavonoid contents by 18.76% and 22.43%, respectively, compared to the control plants on average across both the field trials (Figure 2).

GLSs in Chinese flowering cabbage plants was analysed using the LC–MS/MS in MRM mode. A total of seven different GLS were quantified (Table 3). Same type of GLS has been reported in Chinese flowering cabbage in previous studies [29, 30]. Statistical analysis showed that the levels of total GLS in Chinese flowering cabbage plants was increased up to 32.62% under the influence of the foliar elicitor (Figure 3). Among different GLSs quantified, 4-methoxyglucobrassicin and glucoalyssin were sharply increased in the leaves of the Chinese flowering cabbage plants (Figure 3). Similar significant increases were also seen for other GLS such as gluconapin, neoglucobrassin and 4-hydroxyglucobrassicin (Figure 3).

The results presented in this study are consistent with the findings of Ashraf et al. [31] who reported that foliar application of plant extracts increased total phenolic and flavonoid contents of *Raphanus sativus* plants. Baenas et al. [32] showed that the nutritional quality of sprouts of *Brassica* vegetables was improved by foliar application of biotic elicitors.

In this study, application of *G. uralensis* leaf extracts caused an improvement in nutritional and medicinal values of Chinese flowering cabbage plants. This could be attributed to the presence of various nutrients and phytohormones in the leaf extracts of *G. uralensis*, which increase the production of precursor/intermediate compounds employed in biosynthetic pathways of secondary metabolites [33].

4. Conclusion

This study demonstrates that leaf extracts of *G. uralensis* could be used as an effective plant growth biostimulant. Our findings indicate that the nutritional and medicinal contents in leaves of Chinese flowering cabbage plants can be increased by foliar application of leaf extracts of *G. uralensis* at a rate of 12.5 g/L. Further study is required to understand the mechanism underlying the bioeffect on crop growth, which will be helpful in promoting the use of this biotic elicitor in organic agriculture.

Data Availability

The data used to support the study are included within the article.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

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Research Article

A New Reference Plasmid "pGMT27" Provides an Efficient Transgenic Detection Method for Flue-Cured Tobacco

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Owing to the economic value of its foliage, tobacco (*Nicotiana tabacum*) is cultivated all across the world. For the detection of genetically modified (GM) tobacco, there is a lack of universal standard material which ultimately limits the detection methods because the accuracy and comparability of the results cannot be ensured. Here, we prepared a reference plasmid "pGMT27" for the detection of GM tobacco, which was 18,296 bp in length harboring two of the tobacco endogenous and seven exogenous genes. By using qualitative PCR test for the nine genes, 10 copies were used for plasmid sensitivity. In the quantitative real-time PCR (qPCR) assays with pGMT27 as a calibrator, the reaction efficiencies for *P-35S* and *NR* were 101.427% and 98.036%, respectively, whereas the limit of detection (LOD) and limit of quantification (LOQ) were 5 copies and 10 copies per reaction. For standard deviation (SD) and relative standard deviation (RSD) of the Ct values, the repeatability values were from 0.04 to 0.42 and from 0.18% to 1.29%, respectively; and the reproducibility values were from 0.04 to 0.39 and from 0.18% to 1.14%, respectively. For the unknown sample test, the average conversion factor (Cf) was 0.39, and the accuracy bias was from −15.55% to 1.93%; for precision, the SD values ranged from 0.02 to 0.62, while RSD values were from 1.34% to 10.6%. We concluded that using the pGMT27 plasmid as a calibrator provided a highly efficient transgenic detection method for flue-cured tobacco.

1. Introduction

Tobacco is one of the most widely cultivated nonfood crops and has emerged as an extensively investigated model plant with the accelerated development of molecular biology research. Attributable to its agronomic importance, tobacco is grown in over 125 countries for its foliage, mainly consumed as cigarettes, cigars, and snuff. According to the FAO (Food and Agriculture Organization) data, approximately 4 million hectares of lands are used for cultivating tobacco, one-third of which is in China alone [1]. As smoking is health related [2–4], consumers appear to have more cautious attitudes to tobacco than other crops and GM tobacco, as well as other GM crops. Though applications from biotechnology to tobacco products have been limited, unintentional release of GM tobacco seeds and leaves of nonpermissible sort to the market is possible [5]. Now, GM composition detection in tobacco is becoming an international trade barrier for monitoring the presence of adventitious GM tobacco. So, when entering into the market, it is indispensable for the tobacco companies to enhance controls on import and export of the tobacco product. In many countries, respecting public's right to information and education, the GMO threshold levels of food and feed products have already been established [6–8], Consequently, to ensure the accurate and comparable measurements, selection of standard material for GMO detection becomes critical [9]. The standard reference material includes matrix and plasmid. Plasmid has

many advantages, such as ease of availability, straightforward manipulation, repeatable production, and low cost [10]. Some plasmids as calibrators have been used for transgenic soybean, maize, cotton, and other crops, including ERM-AD413, ERM-AD415, ERM-AD427, and ERM-AD425. They are also used to calibrate the transgenic maize MON810, NK603, 98140, and transgenic soybean 356043 [11].

However, the lack of standard material has limited the GM tobacco detection methods. Up until now, no standard material as the calibrator for detecting GM tobacco has been found, and no corresponding products have been reported. In the tobacco trade market, tobacco is usually sold in the form of tobacco product, including flue-cured tobacco, redried leaf tobacco, and cigarettes. Here, we reported a plasmid for the GM tobacco test, which was designated as pGMT27. It was constructed based on plant expression vector pBI121. pGMT27 contained two tobacco endogenous genes (*actin* and *NR*), genetic elements of the *P-35S* promoter, *T-NOS* terminators, selective marker genes of *NPT II*, *HPT*, *Bar*, and *aadA*, and *GUS* reporter gene. These seven exogenous genes could make the screening rate of transgenic tobacco reach 100% theoretically in our previous screening strategy [12]. Accordingly, a qualitative PCR detection method for detecting the nine targets was developed. Furthermore, a qPCR assay for *P-35S* and *NR* was also developed by using plasmid DNA as the calibrator. Thereby, we concluded that using the pGMT27 plasmid as a calibrator provided a highly efficient transgenic detection method for flue-cured tobacco.

2. Materials and Methods

2.1. Flue-Cured Tobacco and Genomic DNA Isolation. Transgenic flue-cured tobacco tested by the FAPAS (Food Analysis Performance Assessment Scheme) and CORESTA (Cooperation Centre for Scientific Research Relative to Tobacco) proficiency test in 2014 was defined as 100% positive GM with *P-35S* and *T-NOS*. Nontransgenic flue-cured tobacco was collected in our laboratory. All materials were leaves' flour. Plant genome extraction kit (TIANGEN Biotech, China) was employed to extract DNA according to the manufacturer's protocol. Total DNA quantification was performed using a NanoDrop ND-2000 Spectrophotometer (NanoDrop Technologies, USA), OD260/OD280 ≈ 1.80–1.90, and it was diluted to a 100 ng/μL working stock with sterile distilled water, which was stored at −80°C.

2.2. Construction of the pGMT27 Plasmid. According to the screening strategy previously reported [12], we synthesized a 3,818 bp fragment of *HPT* + *Bar* + *aadA* + *actin* + *NR* junction and subcloned it into pMD18-T simple vector by restriction enzyme sites, EcoR I and Sac I. Furthermore, the full-length fragment of five targets was digested with EcoR I/Sac I and cloned into the plant expression vector pBI121 instead of "*T-NOS*" with the same enzyme sites, so as to create the recombinant pGMT27 plasmid, which includes nine target DNA fragments consisting of seven exogenous genes and two endogenous. Then, pGMT27 plasmid was isolated and purified by using TIANGEN Plasmid Miniprep Kit (TIANGEN Biotech, China) according to the manufacturer's guidelines. Afterwards, it was identified by Sac II and EcoR I restriction digests and DNA sequencing (Shanghai Generay, China). Total DNA quantification was performed on a Qubit® 2.0 Fluorometer (Invitrogen Life Technologies, USA) by using the Qubit® dsDNA BR Assay Kit (Invitrogen Life Technologies, USA) following the manufacturer's directions. The copy numbers were calculated on the basis of pGMT27 plasmid size (18,296 bp) by using Avogadro's constant (6.023×10^{23}). The plasmid DNA molecular weight (660 g/mol/dp) was calculated with formula (A) proposed by Lee et al. [13].

$$\text{Formula (A): DNA copies} = \frac{6.02 \times 10^{23}\,(\text{copy/mol}) \times \text{DNA amount (g)}}{\text{DNA length (dp)} \times 660\,(\text{g/mol/dp})}. \quad (1)$$

2.3. Qualitative PCR Assay for the pGMT27 Plasmid. For the LOD test of the qualitative PCR, the plasmid copy number was prepared by 0.1 × TE buffer (10 mM Tris, 0.1 mM EDTA, and pH 8.0). All samples were prepared by serially diluting 10^6 copies/μL to 1 copy/μL, and all the reactions were performed in 20 μL volume containing 1.0 U of rTaq DNA polymerase (TAKARA, Japan) and 2 μL of 10 × PCR buffer along with 125 μM dNTP, 0.5 μM of primers, and 1 μL of plasmid DNA. PCR amplifications were performed in C1000 Touch PCR (Bio-Rad, USA) with the program as follows: one step of 5 min at 95°C; 40 cycles of 30 s at 94°C, 45 s at 63–65°C (*T-NOS* annealing temperature was 63°C, and the others were 65°C), and 45 s at 72°C; and 5 min at 72°C. Amplification products were electrophoresed in 2% agarose gels for approximately 40 min at 100 V. Primers were synthesized and purified by Shanghai Generay Biotech Co., Ltd. (Table S1).

2.4. Quantitative PCR Assay for the pGMT27 Plasmid

2.4.1. Reaction System and Conditions. All qPCR amplification reactions were performed in a 20 μL final volume containing 10 μL of Premix Ex Taq Mix (TAKARA, Japan), 0.5 μM of each primer, 0.2 μM of probe, 0.4 μL of ROX Dye II (50×), and 1 μL of plasmid DNA. PCR was in accordance with the following cycling conditions: 95°C 30 s, 40 cycles of 5 s at 95°C, and 34 s at 60°C. Reactions were run in a ViiA™ 7 Real-Time PCR System (Invitrogen Life Technologies, USA), and the data were analyzed by ViiA 7 software v1.2. PCR

primer and probes for *P-35S* and *NR* were synthesized by Shanghai Generay Biotech Co., Ltd. (Table S2).

2.5. Dilution for Standard Curves, Reproducibility, Repeatability, and Sensitivity. To test the availability of pGMT27, the plasmid was prepared as a calibrator and was serially diluted to 10^6, 10^5, 10^4, 10^3, and 10^2 copies/μL with $0.1 \times$ TE buffer to construct the standard curves. For the repeatability and reproducibility test, the same five dilution points were also used. Each dilution point was amplified in three replicates. For the reproducibility test, the amplification was finished in three different days. For sensitivity, it included LOD and LOQ, and the pGMT27 plasmid was diluted to 50, 25, 10, and 5 copies/μL with $0.1 \times$ TE buffer.

2.6. Conversion Factor (Cf) Calculation and Unknown Samples' Test. In order to minimize the differences between plasmid DNA and plant genomic DNA in PCR efficiencies, calculating the Cf value is a prerequisite [14]. For the unknown samples' test, 100% transgenic flue-cured tobacco DNA was diluted into a gradient of 100%, 50%, 25%, 12.5%, and 6.25% (V/V) with the content of 0% transgenic flue-cured tobacco DNA, reactions were run in ViiA^{TM} 7 Real-Time PCR System, and then target gene and endogenous gene copy number were calculated according to the pGMT27 standard curve. The calculations were performed according to formula (B) suggested by Pi et al. [15], and the final Cf value represented the mean of five calculated Cfs. The unknown sample GM content (%) was calculated by using formula (C) according to Pi et al. [15]. Each reaction was repeated thrice.

$$\text{Formula (B): Cf} = \frac{\text{copy number of the exogenous gene}}{\text{copy number of the endogenous gene}},$$
$$\text{Formula (C): GM content (\%)} = \frac{\text{copy number of the exogenous gene}}{\text{copy number of the endogenous gene} \times \text{Cf}}. \quad (2)$$

3. Results

3.1. Construction of the pGMT27 Plasmid and LOD Test by Qualitative PCR. Figure 1 represents a plasmid pGMT27 (18,296 bp), constructed in this study for the qualitative detection of GM tobacco. It harbored nine targets including seven exogenous and two endogenous targets shown in Table 1. Sac II and EcoR I restriction digests (Figure S1) and DNA sequencing (data not given) validated the acquisition of expected plasmids. For testing sensitivity in the qualitative PCR, nine targets by using 10^6, 10^5, 10^4, 10^3, 10^2, 50, 10, 5, and 1 copies of the pGMT27 plasmid as templates were measured by the LOD method. Nine targets were detected as low as 5 copies, which meant that the lowest testing level was 5 copies (Figure 2). However, we recommend that the template should be more than 10 copies if pGMT27 is a positive control plasmid in the practice test work because some groups have no amplification signals in the three repeats when only 5 copies of the templates were present (data not shown); therefore, the uncertainty is obviously greater because of the low template amount.

3.2. Preparation of the pGMT27 Plasmid as the Calibrator. The pGMT27 plasmid was diluted to 10^6, 10^5, 10^4, 10^3, and 10^2 copies/μL, so as to establish standard curves for *P-35S* and *NR* targets for qPCR. Standard curves of the two reactions are displayed in Figure 3. The correlation coefficient (R^2) values of the standard curves were 0.997 and 0.998 for both *P-35S* and *NR* which indicated excellent linearity. PCR efficiencies were 101.427% and 98.036%, respectively, and data were generated by equation $E = 10^{-1/\text{slope}} - 1$ [16] and analyzed by ViiA 7 software v1.2, and standard curves of the two targets showed excellent linearity.

3.3. Repeatability, Reproducibility, LOD, and LOQ for qPCR Assays. For repeatability and reproducibility of Ct from *P-35S* and *NR*, targets of the pGMT27 plasmid were diluted and amplified for one day and three different days, respectively, and the data are shown and analyzed in Table 2. For the repeatability test, the SD of the Ct values ranged from 0.04 to 0.42, and the RSD was less than 1.29%. For the reproducibility test, the SD and RSD values were in the range of 0.04 to 0.39 and 0.18% to 1.14%, respectively. For qPCR assays, usually, repeatability of RSD less than 25% and reproducibility below 35% were accepted according to the European Network of GMO Laboratories (ENGL) guideline in 2015 [17], and our results indicated good repeatability and reproducibility, which were all in the acceptable range. To test the LOD and LOQ of plasmid pGMT27 in the qPCR, four kinds of concentrations (50, 25, 10, and 5 copies/μL) were prepared. According to ENGL [17], the acceptance criterion of LOD and LOQ should be less than 25 copies and 50 copies, respectively. In the assays, the results determined that the LOD and LOQ were 5 copies and 10 copies per reaction for *P-35S* and *NR*, respectively (Table 3).

3.4. Estimation of Cf Values and Quantitative Analysis of Unknown Samples. In order to analyze the GM tobacco content of unknown samples by using the pGMT27 plasmid as a calibrator in qPCR, the Cf values were calculated by the copy ratios of *P-35S* to *NR*. The endogenous gene *NR* was a single-copy gene in the tobacco genome [5]. In Table 4, Cf values for each time, including three parallel reactions, were 0.34, 0.39, 0.40, 0.40, and 0.41, and the mean value of the Cfs was 0.39. The SD and RSD values of the Cf were 0.03 and 6.77%, respectively, which were all in the acceptable range. With the aim of testing the GM content of the unknown

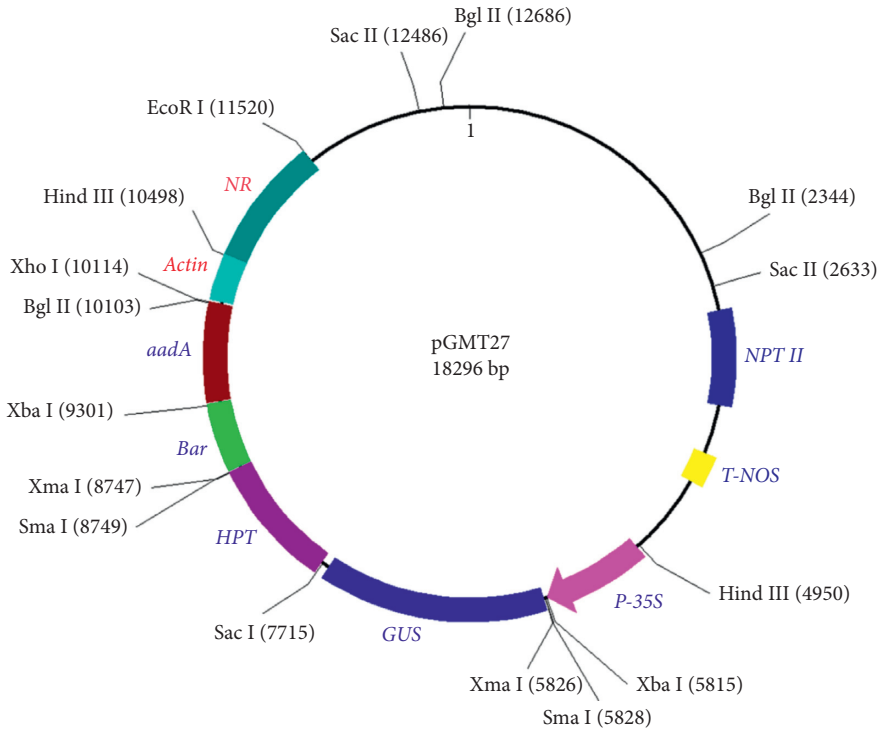

Figure 1: Schematic diagram of pGMT27 for the seven GM tobacco events. *P-35S*, *T-NOS*, *NPT II*, *GUS*, *HPT*, *Bar*, and *aadA*: fragment of the tobacco exogenous gene in red font; *actin* and *NR*: fragment of the tobacco endogenous gene in blue font.

Table 1: Description of the fragments present in the pGMT27 plasmid (18,296 bp).

Targets		Description of genetic elements	NCBI (accession no. and position of corresponding bp on the sequence of the accession)	Size (bp)
Exogenous genes	*P-35S*[A]	Cauliflower mosaic virus 35S promoter	AF485783 (4974..5808)	835
	NPT II[A]	Neomycin phosphotransferase II	AF485783 (2838..3632)	795
	T-NOS[A]	*Agrobacterium tumefaciens* nopaline synthase terminator	AF485783 (4022..4277)	256
	GUS[A]	Beta-glucuronidase (uidA) gene	AF485783 (5845..7656)	1,812
	HPT[B]	Hygromycin phosphotransferase gene	AF354045 (8948..9973)	1,026
	Bar[B]	Herbicide-resistant gene	KF840400 (2381..2932)	552
	aadA[B]	Spectinomycin-resistant gene	EU497669 (3544..4335)	803
Endogenous genes	*Actin*[B]	*N. tabacum* gene for actin	X63603 (4269..4640)	372
	NR[B]	*N. tabacum* gene for nitrate reductase (nia-1)	JN384019 (445..1455)	1,011

A: from plant expression vector pBI121; B: synthesis.

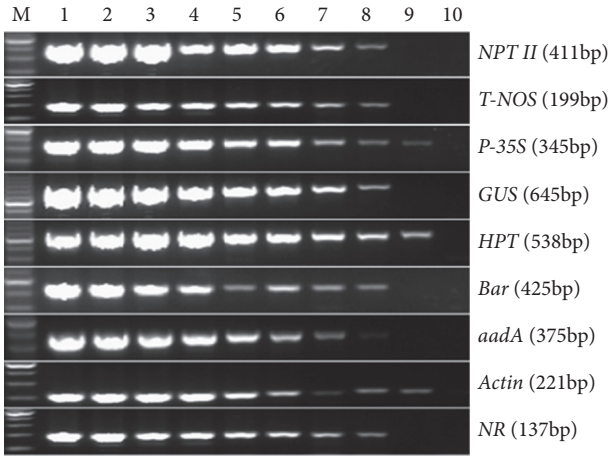

Figure 2: LOD of pGMT27 using qualitative PCR for nine targets. Lane M: TAKARA 100 bp DNA ladder; lanes 1–9: 10^6, 10^5, 10^4, 10^3, 10^2, 50, 10, 5, and 1 copies/μL; lane 10: NTC.

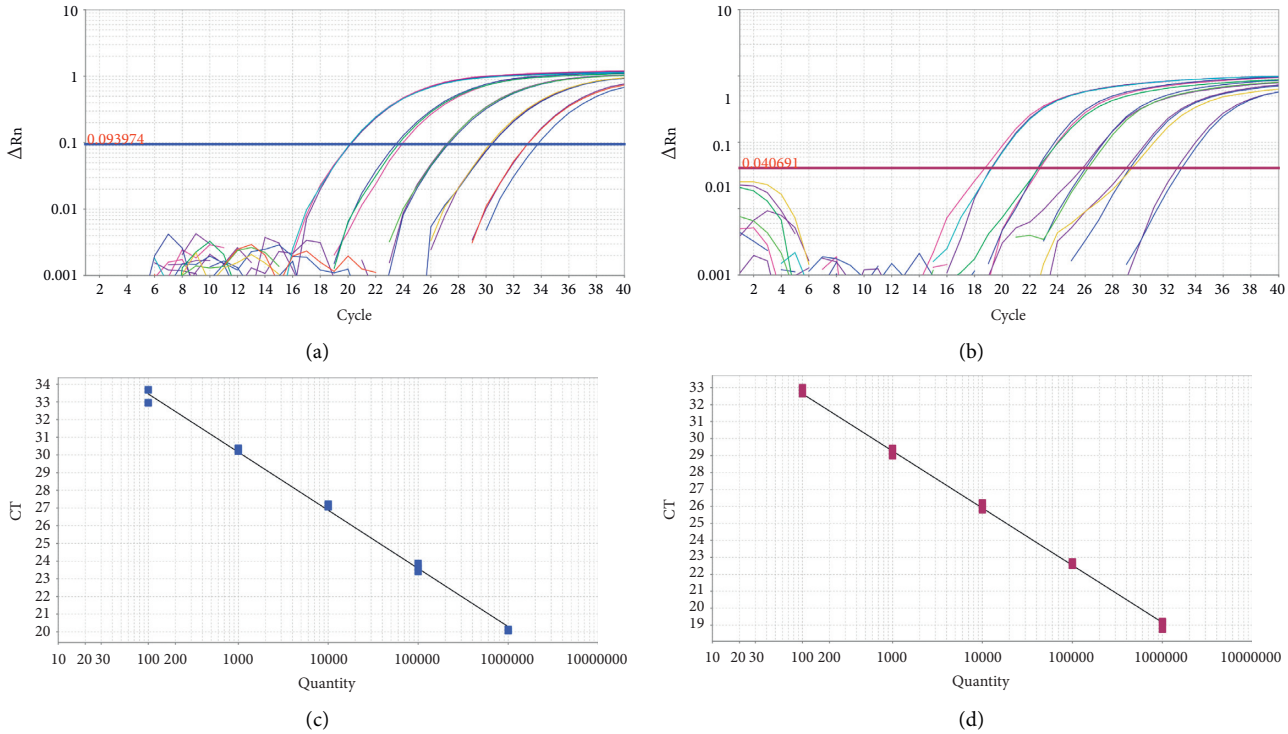

Figure 3: Calibration curves for *P-35S* and *NR* in qPCR. The dilutions contained approximately 10^6, 10^5, 10^4, 10^3, and 10^2 copies per PCR. (a) Amplification plot of *P-35S*. (b) Amplification plot of *NR*. (c) Standard curve of *P-35S* ($y = -3.288x + 40.022$, $R^2 = 0.997$, Eff% = 101.427). (d) Standard curve of *NR* ($y = -3.37x + 39.38$, $R^2 = 0.998$, Eff% = 98.036).

Table 2: Repeatability and reproducibility of the pGMT27 plasmid as a calibrator in qPCR.

Targets	True copy number	Ct repeatability (one day)				SD	RSD (%)	True copy number	Ct reproducibility (different days)				SD	RSD (%)
		1	2	3	Mean				1	2	3	Mean		
P-35S	100	32.96	33.68	32.94	33.19	0.42	1.28	100	33.24	32.90	33.26	33.14	0.20	0.62
	1,000	30.21	30.37	30.33	30.31	0.08	0.27	1,000	30.66	30.47	30.41	30.51	0.13	0.43
	10,000	27.10	27.22	27.07	27.13	0.08	0.29	10,000	27.38	27.33	27.11	27.27	0.14	0.52
	100,000	23.60	23.41	23.86	23.62	0.23	0.97	100,000	23.71	23.77	23.54	23.67	0.12	0.50
	1,000,000	20.07	20.14	20.07	20.09	0.04	0.18	1,000,000	20.20	20.14	20.13	20.16	0.04	0.18
NR	100	32.16	32.99	32.65	32.60	0.42	1.29	100	33.66	33.72	34.35	33.91	0.39	1.14
	1,000	29.42	29.25	28.99	29.22	0.22	0.74	1,000	29.99	29.88	30.04	29.97	0.08	0.27
	10,000	26.20	26.00	25.80	26.00	0.20	0.76	10,000	26.77	26.59	26.82	26.73	0.12	0.46
	100,000	22.61	22.54	22.72	22.63	0.09	0.41	100,000	23.05	22.93	23.12	23.03	0.09	0.40
	1,000,000	19.21	19.18	18.77	19.05	0.25	1.29	1,000,000	19.70	19.57	19.59	19.62	0.07	0.35

samples, six concentrations were used for the analysis, and these samples were 0.5%, 2%, 5%, 6.25%, 12.5%, and 25% (v/v), which were prepared by mixing the samples of several GM and non-GM flue-cured tobacco DNA. GM percentages of unknown samples were calculated by formula (C). The qPCR analysis is shown in Table 5, where the accuracy of bias ranged from −15.55% to 1.93%; however, our bias values were lower than the acceptance criteria (−25% to 25%) according to ENGL [17]. Precision of SD and RSD ranged from 0.02 to 0.62 and from 1.34% to 10.6%. All these results indicated good repeatability, which were all in the acceptable range.

4. Discussion

On a global scale, the number of genetically engineered plants has risen exponentially in recent years, adding up to the complexity of efforts taken by the enforcement laboratories to detect not only the authorized GMOs in food and feed samples but also the unauthorized ones. In order to produce reliable data, research laboratories need to harmonize their techniques to quantify GMOs or to demonstrate that different methods used for the quantification of GMOs are commutable [6, 7]. Quantitative examination of GMOs is usually carried out by the determination of the

Table 3: The LOD and LOQ of the pGMT27 plasmid as the calibrator in qPCR.

Target	Template copies	Positive signals/total repeats	Mean Ct	Ct SD
P-35S	50	9/9	34	0.33
	25	9/9	35.08	0.54
	10	9/9	36.63	0.89
	5	8/9	—	NA
NR	50	9/9	34.04	0.61
	25	9/9	35.22	0.38
	10	9/9	36.03	0.76
	5	8/9	—	NA

NA: not applicable.

Table 4: Conversion factor (Cf) estimation of the reference molecule pGMT27 by qPCR.

Quality of genomic DNA (ng)	Target copies		Cf	Mean Cf value	SD	RSD (%)
	P-35S	*NR*				
6.25	1675.12	4905.71	0.34			
12.5	3850.91	9848.56	0.39			
25	7782.50	19525.56	0.40	0.39	0.03	6.77
50	15455.08	38570.14	0.40			
100	30893.15	76172.85	0.41			

Table 5: Unknown sample analyses for GMO content (%) using the pGMT27 plasmid as the calibrator (Cf = 0.39).

True value (%)	Experimental value (%)			Accuracy		Precision	
	1	2	3	Mean (%)	Bias (%)	SD	RSD (%)
0.5	0.40	0.39	0.47	0.42	−15.55	0.04	9.84
2	1.71	1.75	1.75	1.73	−13.29	0.02	1.34
5	4.26	4.28	3.90	4.15	−17.08	0.21	5.16
6.25	6.25	5.16	5.32	5.57	−10.80	0.59	10.60
12.5	12.65	12.54	11.91	12.37	−1.07	0.40	3.26
25	26.18	25.28	24.99	25.48	1.93	0.62	2.42

Bias = [(mean experimental value − true value)/true value] ∗ 100.

amount of event-specific target with respect to the reference gene [8]. In the current study, seven exogenous ones were frequent, and the theoretical coverage reached 100% in the tobacco genetic transformation event [12]. Approaches for the detection and quantification of many event-specific targets of commercially available genetically modified organisms and reference genes are available. Additionally, there are many studies that demonstrate the development of methods and their in-house validation [6, 10]. Other studies indicated that the LOD was 5 and 20 copies, respectively, by using the plasmid as a reference molecule for detecting transgenic canola and soybean [18, 19]; correspondingly, in our study, nine targets were detected as low as 5 copies, which meant that the lowest testing level was 5 copies. Nonetheless, it is important to critically evaluate any validation status before implementing a method in the research laboratory for routine sample analyses. For reliable quantification of GMOs, laboratories need appropriate organization and quality management systems, and several critical points should be considered in the analytical procedures, such as sampling, sample preparation, and DNA extraction, which have been described recently [10]. Differential qPCR for the detection of unauthorized GMOs is based on the occurrence of several common elements in different GMOs (e.g., promoter and genes of interest). A statistical model was developed to study the difference between the number of targets of such a common sequence and the number of event-specific targets that can identify the approved GMO and the donor organism of the common sequence. When this difference statistically deviates from zero, the presence of an unauthorized GMO can be deduced [20]. However, this approach has low sensitivity, and it is reliable only if the presence of an unknown GMO exceeds 30%. Furthermore, a qPCR assay for *P-35S* and *NR* was also developed by using plasmid DNA as the calibrator. The R^2 values of the standard curves were 0.997 and 0.998 for both *P-35S* and *NR*, those R^2 values ≥ 0.98 were accepted [9], and other researchers' results ranged from 0.995 to 0.999 [18, 21–23]. Theoretically, the Cf score should be 1.0 for the quantitative PCR test when the exogenous gene and endogenous gene are both one copy [5, 15], but in this study, the Cf value was 0.39, which is far more below 1.0, so we could observe three potential reasons: (1) large differences between plasmid DNA and plant genomic DNA in quantitative PCR efficiencies, and some studies involving crops validate this as Cf values ranging from 0.53 to 0.83 have been reported [14, 21, 23]; (2) these samples' GM content was defined as 100% positive by the FAPAS, but the fact was there was less than 100% GM content; (3) flue-cured tobacco DNA partially degrades after curing [24]. The SD and RSD values of the Cf were 0.03 and 6.77%, respectively, which were all in the acceptable range. The advancement of new approaches that are suitable for the quantitative measurement of specific sequences is a promising solution that can overcome the drawbacks of the currently used ways/methods. In the past, several alternative methods for the detection of GMOs were developed that explored different aspects of amplification, detection, and

identification approaches [25, 26]. Recently, additional methods have been developed [27–31]. Consequently, in the current study, a qualitative PCR detection method for detecting the nine targets was developed. Highly linear and efficient reactions indicated that the standard curves of the pGMT27 plasmid were suitable for further quantitative test [32]. Meanwhile, using the pGMT27 plasmid as a calibrator provided a highly efficient method in the transgenic detection of flue-cured tobacco.

5. Conclusion

The newly developed screened plasmid pGMT27 provides better coverage of the GM elements that could be present in a sample and will facilitate advancements in the detection of unauthorized/unknown GM tobacco. It includes two tobacco endogenous genes (*actin* and *NR*), exogenous gene of the *P-35S* promoter and *T-NOS* terminators, selective marker genes of *NPT II*, *HPT*, *Bar*, and *aadA*, and *GUS* reporter gene. Overall, qualitative and quantitative PCR tests infer that the constructed plasmid is a suitable and cost-effective strategy for the detection and quantification of GM tobacco.

Data Availability

All the data generated or analyzed during this study are included in the main paper and supplementary information files.

Ethical Approval

Not applicable.

Consent

Not applicable.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Authors' Contributions

Jing Yu conceptualized and investigated the study, provided the methodology, performed formal analysis, and wrote the original draft. Xiaolian Zhang visualized the study and wrote the original draft. Muhammad Faheem Adil edited and reviewed the article. Bo Lei investigated the study and provided formal analysis. Mengao Jia provided the methodology and visualized the study. Huina Zhao contributed to data curation and edited and reviewed the article. Shizhou Yu visualized the study and performed formal analysis. Jiemin Liu edited and reviewed the article. Yushuang Guo conceptualized the study, contributed to funding acquisition, provided resources, and reviewed and edited the article. Imran Haider Shamsi conceptualized and supervised the study, contributed to funding acquisition, provided resources, and edited and reviewed the article.

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Supplementary Materials

Figure S1: Sac II and EcoR I restriction digests of pGMT27. Table S1: primers used in qualitative PCR. Table S2: primer and probe sequence of *P-35S* and *NR* in qPCR. (*Supplementary Materials*)

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Research Article

Growth and Carotenoid Contents of Intercropped Vegetables in Building-Integrated Urban Agriculture

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Sustainable food security due to climate and social change is more important than ever. This study was conducted to increase plant growth and bioactive contents using intercropping technology in urban agriculture. Tomatoes (*Solanum lycopersicum L.*) and sunflowers (*Helianthus annuus L.*) were intercropped in different ratios in outdoor rooftop, and lettuce *(Lactuca savita* L.) was grown with chicory (*Cichorium intybus.* L.) in the indoor LED plant growth chambers. Carotenoids in plant foods were analyzed using an ultraperformance liquid chromatography with photodiode array detection. Chlorophyll contents were determined by the soil analysis development chlorophyll meter. Tomatoes planted with sunflowers (3 : 1) had a significantly larger stem diameter ($p < 0.05$), a large number of leaves ($p < 0.05$), and significantly higher lycopene at d88 ($p < 0.05$), d102 ($p < 0.001$), and d115 ($p < 0.01$), and β-carotene contents at d102 ($p < 0.05$) as compared to those of monocultured tomatoes. Lettuce planted in a ratio of 1 : 3 with lettuce and chicory had significantly higher contents of chlorophyll ($p < 0.05$), β-carotene ($p < 0.05$), and lutein ($p < 0.01$), than lettuce planted alone. On the other hand, intercropping of chicory and lettuce did not have a beneficial effect on the growth and carotenoid content of chicory. The current study indicates that plant growth and carotenoid content can be substantially modified by cocultivation, and the effects may vary depending on the type of plant and the crop ratio.

1. Introduction

Urban agriculture, a food production system of inside city boundaries or densely populated areas, has become an attractive land use alternative in modern society [1]. Urban agriculture's social, economic, and environmental contributions are becoming more important than ever [2]. In particular, there is a new interest in urban agriculture as a means of securing food supply in the context of climate change and other impacts such as the COVID-19 pandemic in all parts of the world [3]. Rooftop gardens, providing an alternative controlled space for growing vegetables, have recently become an important part of urban agriculture revitalization [4, 5]. In addition, indoor farm that can produce plants year-round with controlled lighting is rapidly expanding in urban and suburban areas [6, 7]. Along with such building-integrated urban agriculture, interest in improving the nutritional and bioactive content of crops is increasing [8–10].

In recent years, growing mix crops has become an important component of sustainable organic farming [11]. Cocultivation is a cross-crop practice often associated with organic farming and can increase in productivity of vegetables per unit area [12]. Growing two or more plants together is usually a method of disease management for crops. However, it can positively change the microclimate conditions around the canopy as well as add organic matter and nitrogen to the soil to retain water and nutrients and control weeds [13]. Additionally, adding noncrop vegetation to a single cultivation helps preserve biodiversity through

resource diversification and reduced use of pesticides [14]. Although these practices can be beneficial to agriculture, they are not practically utilized [15]. Therefore, it is important to demonstrate beneficial effects of easy-to-implement companion planting for urban agricultural farmers and consumers.

Tomato species are a key component of nutrition in many countries and are of great economic importance worldwide [16, 17]. However, continuous single cropping and excessive fertilizer application of tomato plants led to soil acidification and salinization in many areas, reducing tomato yield and fruit quality [18–20]. It has been reported that the yields of tomatoes can be increased when grown with tall sunflower in regions with high light intensities [21]. However, the effect on the bioactive contents in tomatoes grown with sunflowers has not yet been reported.

Lettuce can be a good potential source of bioactive substances such as carotenoids [22], and as Hernandez et al. recently reported [23], their contents can be modified through fertilization management. In addition, LEDs have been reported to improve plant performance and carotenoid content in lettuce [24] and help overcome unpredictable weather conditions [25].

Therefore, we investigated the effects of cocultivation on plant growth and bioactive components by utilizing the outdoor rooftop garden of tomatoes with sunflowers and indoor LED growth chamber of lettuce and chicory in urban agriculture with limited farmland.

2. Materials and Methods

An outdoor roof garden and indoor LED plant growth chamber were utilized to produce plant foods mimicking urban agriculture. Tomatoes (*Solanum lycopersicum L.*) and sunflowers (*Helianthus annuus L.*) were intercropped in different proportions in the roof garden, and lettuce *(Lactuca savita* L.) was grown with chicory (*Cichorium intybus.* L.) in different ratios in LED plant growth chamber as summarized in Figure 1.

The overall conditions of outdoor rooftop gardens and indoor LED growth chamber are presented in Table 1.

2.1. Outdoor Experimental Design: Rooftop Garden. The outdoor experiments were carried out on the rooftop garden of a complex practice building at the university campus (35°49′N, 127°08 N), Chungju-si, South Korea. There were a total of 15 square plots (100 cm length × 100 cm width × 30 cm height) which were made of brick and mortar constructed in full sun at the field experiment rooftop. This plot consists of three layers (from bottom to top): a drainage layer, filter fabric, and growing substrate. The drainage layer had a 25 mm thick drainage board with a high impact polystyrene dimple sheet. A nonwoven geotextile fabric was bonded to the upper surface of the drainage board as the filter layer, which prevented the small particles from being washed from the substrate layer. The growing substrate was filled with a 25 cm depth of commercial substrate (Hansel Green Co., Ltd., Korea) for green roofs

Tomato (*Solanum lycopersicum* L.) and sunflower seedlings (*Helianthus annuus* L.) were obtained from a nursery under natural conditions nearby and transplanted together in the rooftop plots with a plant density of 12 plants per square meter. Experimental treatments were the four-companion planting at different tomato to sunflower ratios as follows: tomato control (T alone, tomato monoculture); tomato : sunflower = 1 : 1 (T1S1); 2 : 1 (T2S1); and 3 : 1 (T3S1). The outdoor experiments on the rooftop garden were conducted using a randomized complete plot design with four treatments and three replications (a total 12 plots) as shown in Figure 2. During the experiments, regular cultural practices were applied as needed, uniformly through all experimental plots, without any additional fertilization during the growing seasons.

Plant height, diameter of the stem, number of leaves, and leaf shape index were recorded for each treatment when plants were at their peak growth time. These parameters have been used to reflect plant growth rates, competitive ability, and resource use [26]. Plant height (cm) was measured with a meter measuring tape at the vertical distance between the upper boundary of the plant and ground level. Stem diameter was determined using Vernier digital calipers (500–153, Mitutoyo Co., Japan) at the plant base.

Tomato fruits were collected three times while fully ripened (early harvest (88 days), midharvest (102 days) and late harvest (115 days)). An average of 30 fully ripe tomatoes from each experimental plot were harvested at each time point and were randomly transferred to the laboratory in plastic bags (Ziploc, Johnson & Son, USA) within 1 h of harvest for carotenoid analysis.

2.2. Indoor Experimental Design: LED Plant Growth Chamber. The indoor experiment was set up in the laboratory using a randomized miniplastic container with nine replicates. Experimental treatments were seven different seed mixture ratios of lettuce seeds (*Lactuca savita* L., Jeilseed Bio Co., Ltd., Korea) and chicory seeds (*Cichorium intybus.* L., Asia Seed Co., Ltd., Koera) as follows: 40 lettuce seeds (L alone), 20 lettuce seeds + 20 chicory seeds (L1C1), 13 lettuce seeds + 27 chicory seeds (L1C2), 10 lettuce seeds + 30 chicory seeds (L1C3), 27 lettuce seeds + 13 chicory seeds (L2C1), 30 lettuce seeds + 10 chicory seeds (L3C1), and 40 chicory seeds (C alone). Companion sowing experiments were conducted in nine replicates for each of the seven seed mixture ratios (a total of 63 miniplastic containers).

Seeds of the seven different mixtures were sown in each miniplastic container (20 oz) containing 15 g perlite (New PerlShine No. 3, Green Fire Chemicals Co., Ltd., Korea) and 65 g of commercial horticultural substrates (Hanpanseung, Samhwa Greenwell Co., Ltd., Korea) from bottom to top. After sowing, miniplastic containers were irrigated with mist water (100 ml), and a thin layer of horticultural substrates is placed over the seed (~5 mm) with each container covered with a plastic cap to maintain moisture content. Seedlings were grown in environmentally controlled LED plant growth chambers (Masuda Co., Ltd., Korea), where three cultivation frames with 150 cm × 70 cm × 35 cm

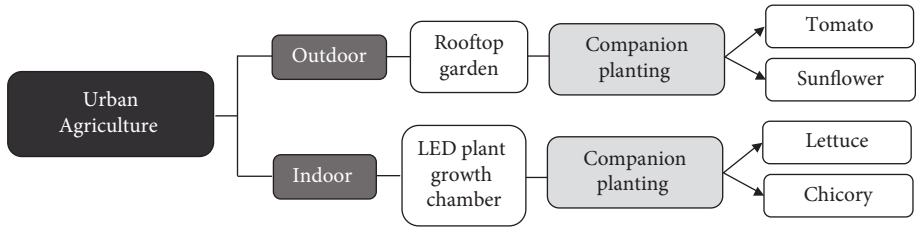

Figure 1: Flowchart of the study.

Table 1: Growth conditions for companion planting of urban agriculture.

Condition	Urban agriculture	
	Rooftop garden (tomato and sunflower)	LED plant growth chamber (green lettuce and chicory)
Temperature	23.8°C (11.2~32.1)[z]	27.7°C (24.7~31.6)
Humidity	66.6% (45.3~93.1)	37.3% (25.6~53.6)
Illuminance		10000 lux

[z]Mean (minimum~maximum).

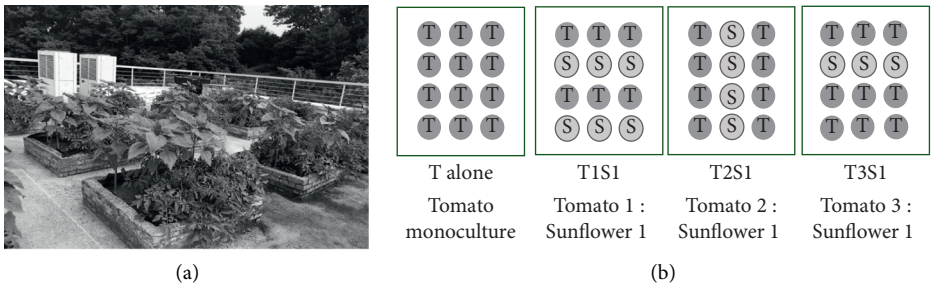

Figure 2: The outdoor experimental site (a) and schematic illustration (b) of the companion planting ratios between tomatoes and sunflowers in the plots on the rooftop garden.

(length × width × height) were installed providing the same light treatment as shown in Figure 3.

In the growth chamber, CO_2 concentration was the same as the ambient atmosphere, light intensity was 10,000 lux, the photoperiod was 16 h continuing from 06 : 00 to 22 : 00, day/night temperature was 25/21°C, and relative humidity was ~65%. The number of leaves, leaf shape index, chlorophyll contents, and fresh weights were evaluated for nine plants per treatment at day 23 immediately after sowing. The leaf shape index of the third leaf from the basal part was calculated by dividing leaf length by leaf width. Chlorophyll contents were determined by the soil analysis development chlorophyll meter (SPAD-502, Konica Minolta Co., Japan). It was measured in the middle of leaf per each treatment. The fresh weights of shoots and roots were measured using a digital scale (IP65, A&D Co., Japan). At 25 days after sowing (baby leaf stage), lettuce samples were collected in ~60 ml sample bags (LDPE, Cleanwrap Co., Ltd., Korea), wrapped in aluminum foil and immediately transported to the laboratory within 1 h of harvest for carotenoid analysis.

2.3. Carotenoid Analysis. The uniform, nonsenescent, and undamaged edible portion of plant samples were washed with d-H_2O, dewatered, and minced immediately after transport to the laboratory. Samples were extracted for carotenoids and aliquoted 1 g each in a freezer vial and stored at −80°C for further analysis. All procedures were conducted under red light.

One gram each of plant samples were placed in 50 mL glass vials, and 5 mL of methanol was added and vortexed for 30 sec. Plant samples and methanol mixtures were incubated for 1 hr at room temperature. Afterwards, the mixture was homogenized for 30 s in an ice bath. The mixture was centrifuged at 3000 rpm for 5 min. The methanol layer was transferred into a 50 mL volumetric flask, and the extraction was repeated four times with 10 mL of tetrahydrofuran (THF), followed by vortex and centrifugation. The THF layers were combined with the methanol layer and the volume was brought up to 50 mL. One mL of the extract was taken, dried under nitrogen, and resuspended in 100 μL of ethanol. All extraction procedures for carotenoid analysis were carried out under red light.

Carotenoids were analyzed using a previously reported UPLC-Photodiode Array Detector analysis [27] with minor modification. In brief, the UPLC (ACQUITY UPLC I-Class, Waters Co., Milford, MA, USA) system was equipped with a BEH C18 column (1.7 μm, 2.1 × 50 mm, Waters Co., Milford, MA, USA), binary pump delivery system, autosampler, and photodiode array detector. Mobile phase A was acetonitrile/methanol (7 : 3. v/v), and mobile phase B was water. Each sample was injected into the BEH C18 column (1.7 μm, 2.1 × 50 mm). The gradient condition was similar to that previously reported [27].

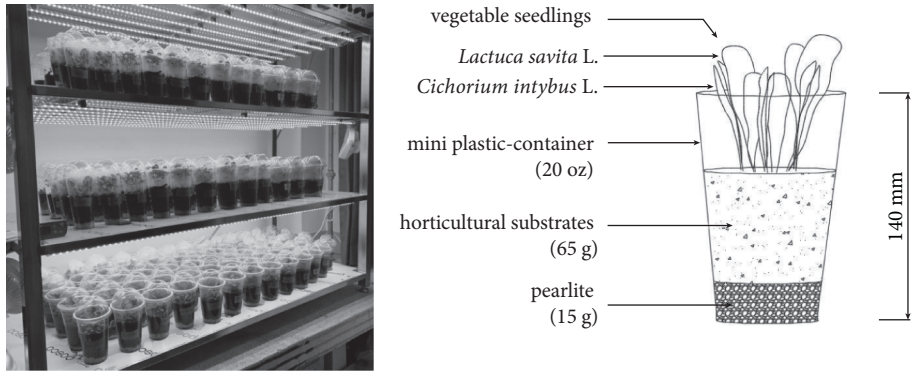

Figure 3: The indoor LED plant growth chamber experiments (a) and the cross section of miniplastic container (b) with different ratios of seed mixtures between lettuce and chicory.

β-Carotene, lutein, and lycopene (at 450 nm) were quantified by each standard curve. Each peak was confirmed by retention time and its unique spectrum. The interassay coefficient of variation (CV) was under 4% ($n = 10$), and the intra-assay CV was under 4% as well ($n = 10$).

Representative UPLC profiles of carotenoids from tomato, lettuce, and chicory, and spectra of corresponding carotenoids are shown in Figure 4.

2.4. Statistical Analysis. The experiment was designed as a completely randomized design to test the effect of companion plantings or sowings ratios on all measured parameters. One-way analysis of variance was conducted, and a mean comparison among treatments was conducted using Duncan's multiple range tests ($p < 0.05$). All statistical analyses were performed using SPSS statistical software version 18.0 (SPSS Inc., Chicago, USA).

3. Results and Discussion

The current study reports the companion planting, which was originally used for disease management [28, 29] and improving plant productivity [30], which can be utilized to improve plant growth and bioactive contents in building-integrated urban agriculture. We believe that increasing the availability of a nutritious food supplies through building-integrated urban agriculture can be a useful strategy to overcome food and nutrient instability caused by climate and environmental changes in modern society [31].

3.1. Growth and Carotenoid Contents in Intercropped Tomatoes with Sunflower. In the current study, there was no significant difference in the leaf shape index of tomatoes regardless of the cocultivation rate of tomato and sunflower. However, tomatoes grown in a ratio of 3 to 1 with tomatoes and sunflowers had significantly higher ($p < 0.05$) plant heights, stem diameters, and leaf counts compared to tomatoes grown alone (Table 2).

Considering the height of the sunflower plant was not less than 1.5 m and above the tomato stands 0.5 m, the tomato plant was protected from direct solar radiation by shady cool air columns. In addition, shading tomato plants creates an ambient microenvironment with high relative humidity, which clearly mitigates moisture loss in tomato plants [32]. On the other hand, the stem diameter and leaf count of tomatoes decreased as the percentage of sunflower cocultivation increased, indicating interspecies competition for sunlight. This suggests that although cocultivation of tomatoes and sunflowers may provide benefits against heat stress as previously reported [33], the increased number of sunflowers may provide tomatoes excessive shading, which can adversely affect their growth parameters.

A green roof with a variety of vegetation structures and colors is a system favored by the public [34]. In the current study, tomatoes and sunflowers were grown together using an outdoor rooftop garden, given that tomatoes are a major component of nutrition in many countries and of economic importance worldwide [35]. White mustard [35] and marigold [28] have also been reported as promising companion crops for tomatoes.

When tomatoes were planted with sunflowers, carotenoid contents in tomatoes were significantly different as compared to those of tomatoes planted alone as shown in Figure 5. Lycopene contents of fully ripened first harvest tomatoes were 5.5 mg and 6.0 mg/100 g FW Edible portion in monoculture and intercropping with lettuce and sunflower, respectively. This lycopene content is similar to the recently reported lycopene contents in cherry tomatoes that abscisic acid, a plant hormone, promoted lycopene accumulation from an undetectable level in green cherry tomatoes to 8 mg per 100 g FW of ripe tomatoes [36]. Companion cultivation of tomatoes with sunflowers at a 3 to 1 ratio yielded 9.6%, 26.9%, and 5.4% higher lycopene contents on days 88, 102, and 115, respectively, than monocultured tomatoes. In addition, the β-carotene content was significantly higher in tomatoes planted in a 3 : 1 (tomato:sunflower) ratio with sunflowers at 102 days compared to tomatoes grown alone. However, the increase in β-carotene contents by companion planting was not as pronounced as the effect of harvest time. Lutein content was significantly higher in the 1 : 1 and 3 : 1 (tomato : sunflower) ratios compared to tomatoes grown alone. On the other hand,

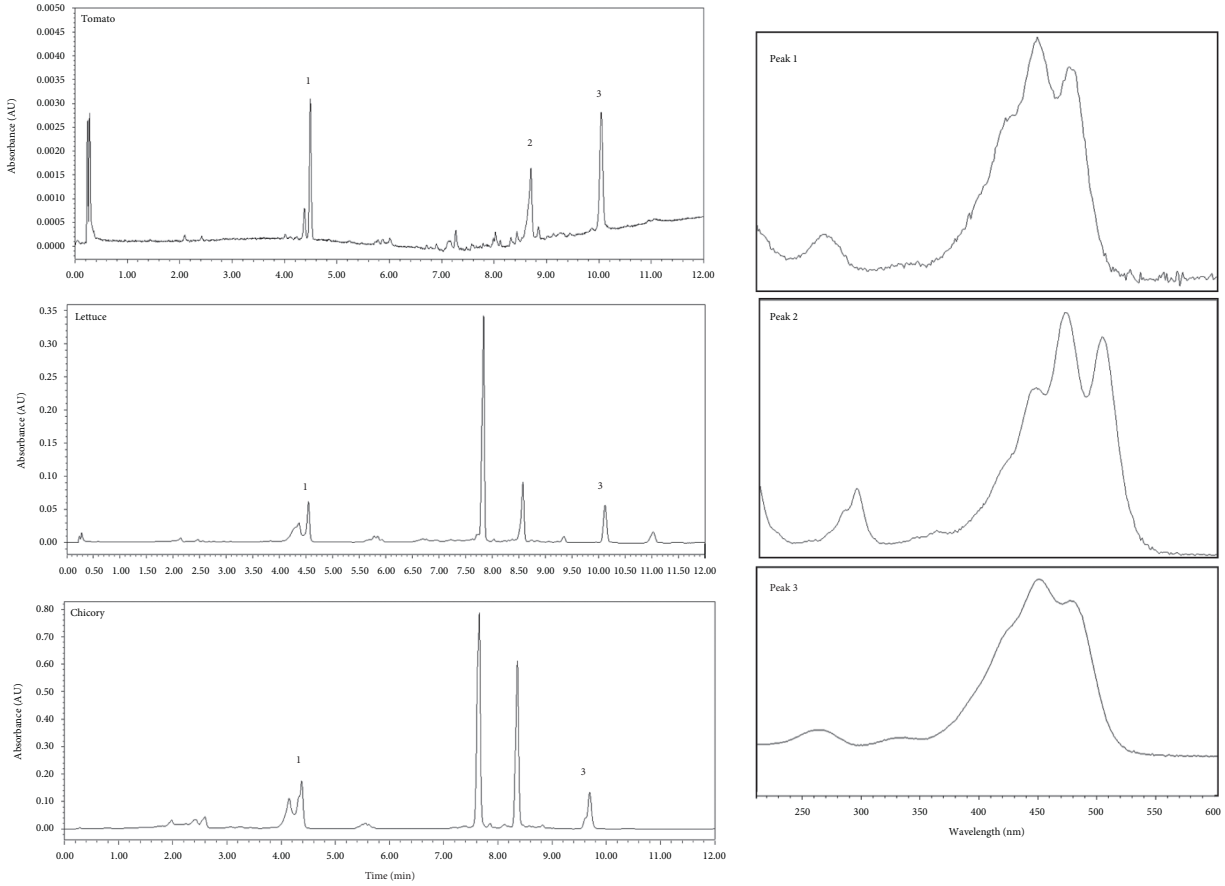

Figure 4: Representative UPLC profiles of carotenoids from tomato, lettuce, and chicory, and spectra of corresponding carotenoids.

Table 2: Plant growth parameters of tomatoes (*Solanum lycopersicum*) according to companion planting ratio with sunflower (*Helianthus annuus*) in green roofs at 88 days after planting.

Treatments	Plant height (cm)	Stem diameter (cm)	Number of leaves	Leaf shape index
T alone	80.33 ± 0.90c	17.71 ± 0.38bc	418.78 ± 9.06b	1.76 ± 0.03a
T1S1	105.55 ± 0.83a	14.75 ± 0.17c	326.44 ± 2.31c	1.68 ± 0.02a
T2S1	86.44 ± 0.68bc	20.67 ± 0.14ab	320.89 ± 3.35c	1.57 ± 0.02a
T3S1	92.89 ± 0.95b	23.50 ± 0.71a	673.33 ± 5.74a	1.68 ± 0.02a

T alone, tomatoes; T1S1, tomatoes : sunflower = 1 : 1; T2S1, tomatoes : sunflower = 2 : 1; T3S1, tomatoes : sunflower = 3 : 1. Data are means ± SE. The same letters are not significantly different within the same column according to Duncan's multiple range test at $p < 0.05$ ($n = 9$). Leaf shape index is leaf length/leaf width.

tomatoes grown by companion planting at a 2 : 1 (tomato: sunflower) ratio had significantly lower lutein and α-carotene contents than tomatoes grown alone.

The current study indicates that the carotenoid contents in plant foods can be affected by the intercropping system as well as harvest time. It is interesting to note that the lycopene contents in tomatoes gradually reduced by harvesting time, and β-carotene content increased instead. Considering that the β-carotene contents are ranging from 2.51 mg to 4.77 mg/100 g depending on the harvesting time, and that one cup of tomatoes (200 g) is one serving, one can theoretically obtain 418–795 μg of retinol by consuming one serving of tomatoes. These results suggest that the harvest time of tomatoes can not only be determined based on the needs of specific carotenoids, but can also be optimized, and that tomatoes can provide significant amounts of carotenoids.

It is well known that lipophilic plant pigments such as carotenoids provide valuable nutritional and health benefits [37]. In addition to provitamin A activity of β-carotene [38], nonprovitamin A carotenoids such as lycopene lower the risk of oxidative stress-associated chronic diseases [39] and lutein has beneficial effects on eye health [40]. Therefore, we believe the results of this study can be the first step in establishing a strategy to achieve food and nutritional security through urban agriculture using intercrop strategies.

3.2. Growth and Carotenoid Contents in Lettuce Intercropped with Chicory. In the LED plant chamber, when lettuce was grown alone or with chicory, there was no significant difference in the number of leaves and fresh weight. However, the leaf shape index of lettuce was significantly lower when

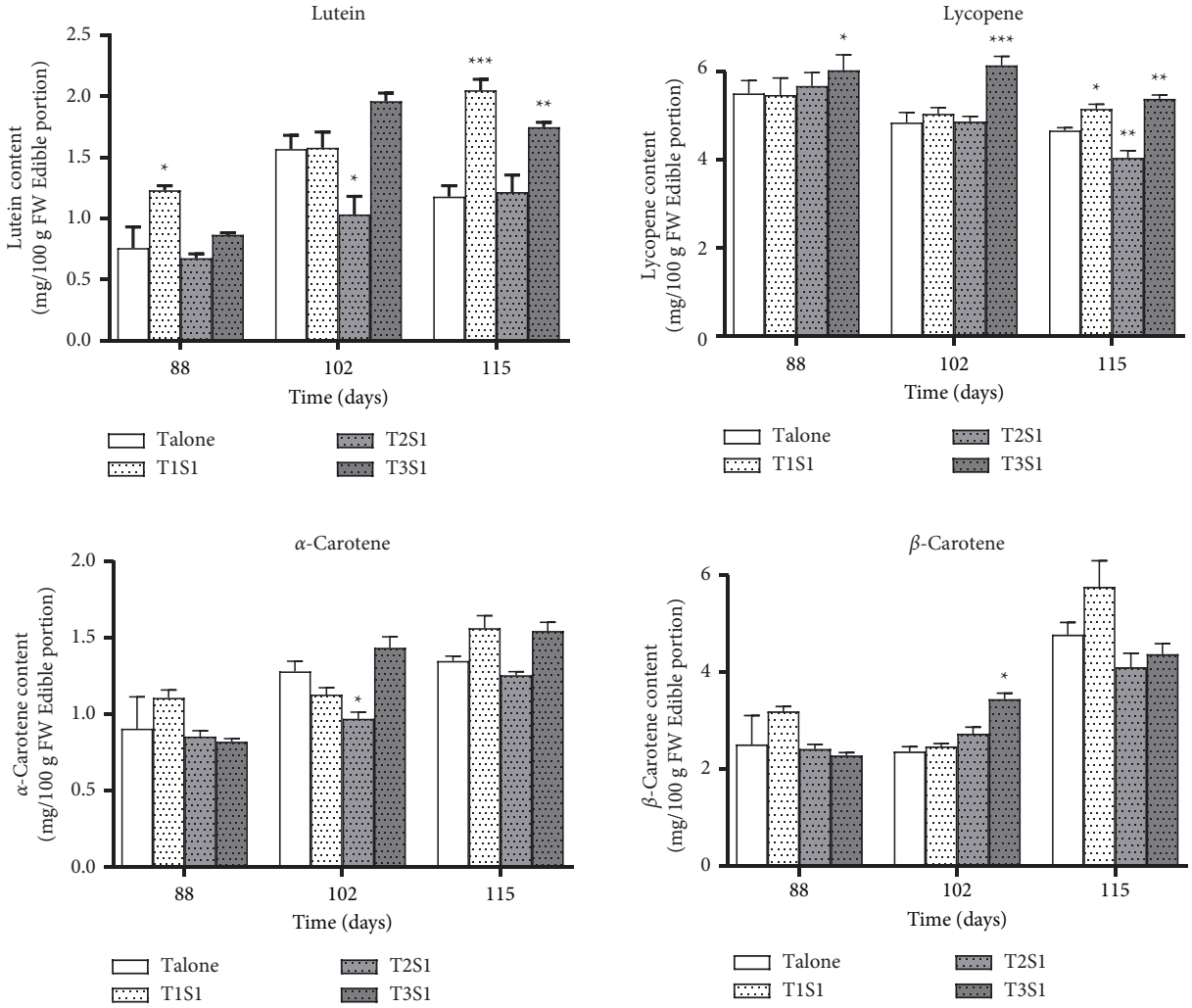

Figure 5: Effect of companion planting with sunflowers on carotenoid contents in tomatoes over time grown on the rooftop garden. Carotenoids were analyzed using an UPLC system (means ± SE, $n = 3$). Significantly different with the monoculture $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

lettuce was cultivated with chicory at a ratio of 1 : 3 than lettuce grown alone ($p < 0.05$). In addition, the chlorophyll content of lettuce was also significantly ($p < 0.05$) higher in lettuce intercropped with chicory at a ratio of 1 to 3 (Table 3).

It is probable that the companion planting of lettuce and chicory can be beneficial for the strong growth of lettuce as reported earlier [41]. The competition between lettuce and chicory may generate stress conditions, which may lead to the synthesis of secondary metabolites since the general response to (a) biotic stress can be an increase in the synthesis of compounds [42]. On the other hand, chicory intercropped with lettuce had significantly lower leaf shape index, chlorophyll contents, and fresh weight than those planted alone.

The effect of companion planting on carotenoid contents in lettuce are presented in Figure 6.

Lettuce were planted with chicory and harvested 25 days after sowing (baby leaf stage). Carotenoid contents tended to be higher in lettuce grown with chicory. In particular, lettuce grown with chicory at a 1 : 3 (lettuce:chicory) ratio had significantly higher β-carotene (6.96 mg/100 g FW edible portion) and lutein contents (6.89 mg/100 g FW edible portion) than lettuce grown alone (β-carotene, 3.66 and lutein, 4.0 mg/100 g FW edible portion, respectively). These carotenoid levels were relatively higher than those reported in Lithuania [43] and were within the range of carotenoid levels of various lettuce previously reported in Korea [44]. It should be noted that the carotenoid content of vegetables can vary depending on a number of factors, including the variety [44], harvest time [45], and extraction procedure [46]. On the other hand, chicory intercropped with lettuce at a ratio of 1 to 1 or 1 to 3 had significantly lower lutein and β-carotene contents than those grown alone as shown in Figure 7.

Lettuce along with chicory represents a fresh leafy vegetable crop with a global production of close to 27 million tons in 2017 [47]. In the cocultivation of lettuce and chicory, the content of β-carotene in lettuce was 15.8–90.1%, and lutein content was 13.5–72.3% higher than that of lettuce grown alone. Considering that β-carotene content of lettuce

Table 3: Plant growth parameters of lettuce (*Lactuca savita*) and chicory (*Cichorium intybus*) according to seed mixture ratio in LED plant growth chamber at 25 days after sowing.

Treatments[z]	Number of leaves[y]	Leaf shape index[x]	Chlorophyll contents (SPAD-value)	Fresh weight (g)
Lettuce				
L alone	7.1 ± 0.38a	4.1 ± 0.19a	17.8 ± 1.01bc	1.50 ± 0.14a
L1C1	6.7 ± 0.52a	2.2 ± 0.11c	17.1 ± 1.55bc	1.32 ± 0.26a
L1C2	7.9 ± 0.39a	2.8 ± 0.15b	14.8 ± 1.03c	2.08 ± 0.35a
L1C3	6.5 ± 0.26a	2.5 ± 0.10bc	22.8 ± 0.61a	2.26 ± 0.56a
L2C1	7.5 ± 0.33a	2.4 ± 0.08bc	20.2 ± 1.28ab	2.38 ± 0.21a
L3C1	7.3 ± 0.37a	3.9 ± 0.25a	17.9 ± 1.00bc	1.34 ± 0.23a
Chicory				
C alone	5.8 ± 1.13a	3.2 ± 0.19b	28.8 ± 2.97a	0.45 ± 0.17a
L1C1	4.3 ± 0.47ab	2.8 ± 0.19b	26.2 ± 1.04a	0.11 ± 0.02b
L1C2	5.5 ± 0.34a	2.5 ± 0.14b	25.7 ± 0.90a	0.23 ± 0.02a
L1C3	5.1 ± 0.41a	3.3 ± 0.19b	25.1 ± 1.70a	0.20 ± 0.03a
L2C1	4.6 ± 0.40a	2.5 ± 0.13b	25.7 ± 1.00a	0.11 ± 0.02b
L3C1	4.7 ± 0.24a	4.5 ± 0.78a	21.2 ± 1.37b	0.14 ± 0.02ab

Means ± standard error within column followed by the same letter are not significantly different. L alone, lettuce 40 seeds; C1L1, chicory 20 + lettuce 20; C1L2, chicory 13 + lettuce 27; C1L3, chicory 10 + lettuce 30; C2L1, chicory 27 + lettuce 13; C3L1, chicory 30 + lettuce 10; C alone, chicory 40 seeds. Data are means ± SE, the same letters are not significantly different within the same column according to Duncan's multiple range test at $p < 0.05$ ($n = 9$). Leaf shape index is leaf length/leaf width.

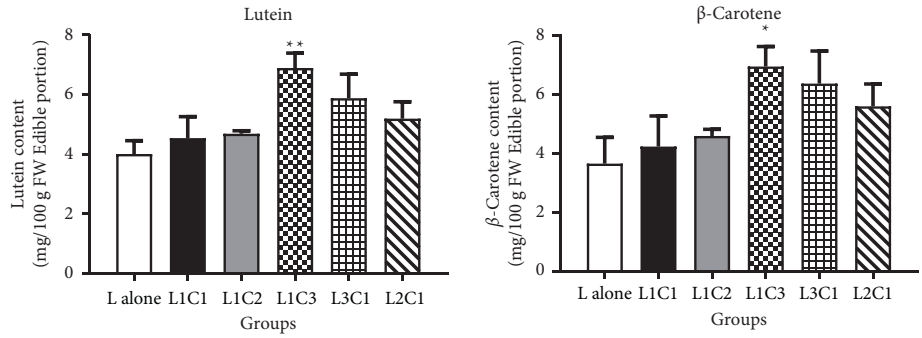

Figure 6: Effect of companion planting with chicory on carotenoid contents in lettuce grown in the indoor LED growth chamber for 25 days. Carotenoids were analyzed using an UPLC system (means ± SE, $n = 3$).

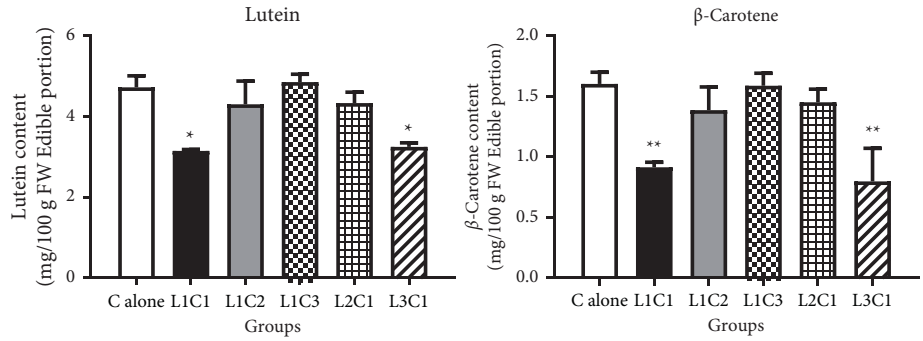

Figure 7: Effect of companion planting with lettuce on carotenoid contents in chicory grown in the indoor LED growth chamber for 25 days. Carotenoids were analyzed using an UPLC system (means ± SE, $n = 3$).

can be almost doubled from 3.66 mg/100 g to 6.96 mg/100 g of lettuce by companion planting, the β-carotene intake in one serving of 36 g lettuce can be increased from 1.32 mg to 2.51 mg. It should not be overlooked, however, that the intercropping of lettuce and chicory was not beneficial for chicory in terms of its growth and carotenoid contents.

4. Conclusions

This study evaluated the effect of cocultivation of plant foods widely used around the world using an outdoor rooftop garden and indoor LED plant growth chamber. The substantial modification of plant growth, as well as carotenoid contents by cocultivation, shown in this study suggests that

building-integrated urban agriculture combined with co-cultivation may be a good strategy to improve food and nutritional security.

Data Availability

All the relevant data have been provided in the manuscript. The authors will provide additional details if required.

Conflicts of Interest

The authors declare no conflicts of interest.

Acknowledgments

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Research Article

Frying Time and Temperature Conditions' Influences on Physicochemical, Texture, and Sensorial Quality Parameters of Barley-Soybean Chips

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Demand for innovative healthy snacks that achieve consumer satisfaction represents increased interest for competitive food producers. The aim of this work was the assessment of physicochemical and sensory quality of barley-soybean chips involving legume protein flours by studying the effects of different substitution levels (10, 20, and 30%) of defatted soybean (DSB) flour, frying temperatures (150, 170, and 190°C), and frying times (60, 90, and 120 sec). The chips' moisture content was significantly decreased with increased frying temperature and time. The moisture content (1.40%) was achieved at 10% DSB fried at 190°C for 120 sec. The least absorbed oil (29.25%) was achieved at the least substitution percentage (10% DSB), the least frying temperature (150°C), and the least frying time (60 sec). These results were reflected on sensorial parameters that revealed that the most preferred chips were barley-soybean chips with 10% DSB fried at 150 and 170°C. The amylose content was increased by 33.80% in chips substituted with 30% DSB, while it was decreased to 27.16% in chips substituted with 10% DSB, and vice versa for the amylopectin content. TPA revealed that DSB substitution levels were directly proportional with hardness and inversely proportional with elasticity and adhesiveness. From obtained results, substitution levels with 10% DSB fried at 150°C are recommended. These findings encourage the production of innovative enhanced snacks involving legume protein while maintaining consumer satisfaction.

1. Introduction

Snacking is the intake of foodstuff between main meals and/or consuming "snack foods" typically identified as energy-dense and nutrient-poor [1]. Healthy snacking became recommended for weight gain control, increased mental ability, and boosting energy. Awareness about the benefits of healthy snacks can be effective strategies for promoting healthy snack consumption and helping consumers adhere to healthful diets. Blending cereals and legumes enhance diets by increasing protein and carbohydrate contents. Regular intake of these biofunctional flours is an attractive option for people who are looking for healthier options [2–4].

Barley (*Hordeum vulgare*) took an important role in human nutrition and health due to its nutritional characteristics and adaptability to different environmental growing conditions [5, 6]. Barley contains high carbohydrate, moderate protein, and significant content of phosphorus and potassium [7]. Additionally, barley seeds contain rich amounts of dietary fiber, especially β-glucan, which has useful health influences, e.g., lowering cholesterol, reducing the inflammatory response, and boosting the immune system [8]. Soybean (*Glycine max*) is a protein-rich oilseed widely employed in the food industry. The global soy food market is expected to reach the value of 53.1 billion US$ by 2024 [9]. Soybean is supplied as a source of high-quality protein, low saturated fat content, and high in vitamins C

and B and a good source of calcium and potassium and provides many health benefits (lowering of the blood cholesterol level, increasing of bone density, and minimization of the risk of cancer development) [9]. Additionally, defatting improves protein interaction, organoleptic qualities, and acid hydrolysis of phenolic compounds [10–12].

Frying is one of the oldest processing and widely used technologies for many starch-based foods targeting crisp texture, aromatic flavor, and golden-brown color [13]. Frying is a complex process involving simultaneous heat, mass, and momentum transfers accompanied with a series of physical and chemical reactions [14]. During the frying process, heat is transferred from oil to fried foods leading to physical reactions (water evaporation and oil uptake) and chemical interactions within legume components (starch, reducing sugars, amino acids, and water) leading to structural changes [15]. The sensory quality represented in texture and flavor is a significant attribute which is always issued in the manufacturing of fried foods as it is related to processing conditions and variables, such as frying time, frying temperature, and moisture content [16].

The aim of this work was the assessment of physicochemical and sensory quality of barley-soybean chips targeting healthier snacks involving legume protein flours with enhanced properties. For achieving this aim, the impact of different substitution levels (10, 20, and 30%) of defatted soybean (DSB) flour, frying temperatures (150, 170, and 190°C), and frying times (60, 90, and 120 sec) on physicochemical characteristics (moisture, oil absorption, amylose, and amylopectin content), texture profile analysis (TPA), and sensorial acceptability of barley-soybean chips was studied.

2. Materials and Methods

2.1. Materials. Organic unhulled barley flour was obtained from Al-Hajraciyah Organic Farm, Saudi Organic Farming Association (SOFA), Kingdom of Saudi Arabia, while organic soybeans were obtained from Nature Oasis, origin: China, packed in Saudi Arabia.

2.2. Preparation of Defatted Soybean Flour. Clean soybean seeds were cracked in a rice sheller (6NF-9 (NF-400), China) and winnowed for hull removing. Cracked hulled soybeans were then milled in a hammer mill (Christy and Norris Ltd., England) with a speed of 8,000 rpm. Soybean flour was then defatted as described by Meyer [17], using hexane in a Soxhlet extractor for 6 h/50–55°C, dried at 40°C ± 5/24 h, and kept in polyethylene bags at −4°C.

2.3. Preparation of Barley-Soybean Chips. Barley-soybean chips were prepared according to Prakash et al. [18]. Based on preliminary sensory evaluation experiments, the production process was based on three treatments of barley substitution percentage with defatted soybean (DSB) flour (10, 20, and 30%) as a high-protein source, three treatments of frying temperature (150, 170, and 190°C), and three treatments of frying time (60, 90, and 120 sec) which make 27 treatment combinations which were examined. Masa, the dough used for making chips, was prepared by mixing barley/soybean flour with distilled water (41°C) (1000 mL/1 kg of dry flour) in a pilot-scale mixer at low speed for 5 min. The masa was allowed to rest in polyethylene bags for 10 min. The masa was then sheeted through model S-18-BNO 4458, Moline Machinery, Ltd., Duluth, MN, which was set at the lowest thickness (fourth lowest position: 0.2 cm). The masa was then rapidly cut into 5 cm side squares with a blade cutter, and each square was then cut diagonally with a pizza cutter.

2.4. Frying Process. The frying process was accomplished in a thermostatically temperature-controlled fryer (Crown Co., XB5356 model, China) containing 1.5 L frying maize oil. The fresh oil was preheated to frying temperature for 30 min before the barley-soybean chips were fried. Samples were immersed into the frying oil basket at the desired temperature (150, 170, or 190°C) for the desired frying time (60, 90, and 120 sec). The samples were then immediately removed from oil, blotted with a tissue paper to remove excess oil on the surface, and allowed to cool at room temperature (20°C ± 2) before analyses.

2.5. Quality Characteristics of Barley-Soybean Chips. The effect of substitution percentages, frying temperatures, and frying times on main physicochemical quality characteristics of fresh barley-soybean chips was studied.

2.5.1. Chemical Characteristics Affecting Chips' Quality. The moisture content of the fried chips was determined by drying preweighed samples (2.0 g) in a hot air oven (130 °C ± 1/h), and the moisture content in percentage was calculated from loss of weight (AACC 44-15.02) [19]. Fat content (for oil absorption determination) was estimated by extracting the sample with petroleum ether, and percent fat was calculated according to AOAC 990.19 [20]. The amylose and amylopectin contents were determined by the method prescribed by Hoover and Ratnayake [21].

2.5.2. Texture Profile Analysis of Barley-Soybean Chips. Instrumental texture measurements were conducted at room temperature (20°C ± 2) using Texture Analyzer (CNS Farnell Com, UK). Immediately after deep-fat frying and cooling, uniaxial compression test, by using a 35 mm-diameter plastic cylinder probe, was conducted on the barley-soybean chips after they were mounted over the platform. Samples were compressed to 30% strain at a constant speed of 25 mm/min [22]. The texture of the barley-soybean chips was expressed as fracture force (highest peak followed by a hardness peak compression force (N) at target deformation), apparent modulus of elasticity or initial target modulus (sample rigidity that is the linear part of the force-deformation curve (g/sec)), adhesiveness (negative force area during withdrawal (g/sec)), and compressive energy (area under the curve for the compression that is the work (g/sec) required to attain deformation indicative of internal strength of bonds within the product) [23].

2.5.3. Organoleptic Properties of Barley-Soybean Chips. Fresh barley-soybean chips' samples were examined for sensory evaluation parameters, flavor, color, crispiness, mouthfeel, and overall acceptability, by twenty panelists (14 men and 6 women) in Nutrition and Food Science Department, Faculty of Science, Taif University, Kingdom of Saudi Arabia, under the supervision and agreement of the Institutional Committee of Taif University, Kingdom of Saudi Arabia [24]. The evaluation was done under normal white fluorescent lighting at a temperature of 20°C ± 1. Rating of samples was carried out using a nine-point scale where 1 = nonexistent, imperceptible characteristic, and 9 = too intense. Throughout panel sessions, panelists were instructed to rinse their mouths with water before testing each sample.

2.6. Statistical Analysis. Data were expressed as means ± standard deviation (SD) and were analyzed using IBM SPSS Statistics 23 software program. Results were analyzed by multiple comparisons one-way analysis of variance (ANOVA) using Duncan's test where probability $p < 0.05$ was considered statistically significant. Pearson's correlation coefficient analyses (Pearson's r) were performed to determine the relationships between parameters where +1 indicates a perfect positive relationship, −1 indicates a perfect negative relationship, 1–0.7 indicates a strong relationship, 0.7–0.3 indicates a moderate relationship, 0.3–0 indicates a weak relationship, and 0 indicates no linear relationship [25].

3. Results and Discussion

3.1. Quality Parameters of Barley-Soybean Chips

3.1.1. Moisture Content and Oil Absorption. Moisture content is one of the most critical parameters as it affects the crisp texture that is a unique property of extruded snacks. Effects of frying temperature and frying time on the moisture content of different barley-soybean chips' blends are illustrated in Table 1. The moisture baseline of the masa was 3.61 ± 0.75%, while, after frying, it ranged from the highest of 2.2% in 10% DSB fried at 150°C/60 sec to the least of 1.4% in the same substitution percentage fried at 190°C/120 sec. This result reflects the significant effect of temperature and time of chips' properties. Obtained results agreed with those of Yadav et al. [26]. The differences in the moisture content of barley-soybean chips for the treatments can be attributed to a moderate negative correlation ($r = -0.68$, -0.66, and -0.63) between the frying temperature and frying time. An inverse relationship was detected when moisture contents of barley-soybean chips showed a significant decrease along with increasing frying temperature from 150°C to 190°C and frying time from 60 to 120 min. On the contrary, higher moisture contents lead to hard and chewy in chips. These findings are in agreement with those of Cruz et al. [15], who reported that when the final moisture content is less than 2%, it helps to obtain a crisp texture. Elevation of frying temperature and frying time engages the alteration of the moisture content in the chips, while nixtamalization (the process of cooking) results in a higher quality of cereal chips [15]. These effects were in consistency with decreased adhesiveness announced in texture profile analyses (Figure 1) and the sensory evaluation results that showed enhanced crispness of chips along with increased frying time.

Table 1 shows the effects of frying temperature and frying time on oil absorption of different barley-soybean chips' blends. The oil absorption showed an elevated pattern along with increased frying time, temperature, or DSB substitution percentage. The values of the oil absorption of barley-soybean chips with different treatments can be attributed to a moderate positive correlation ($r = 0.44$, 0.45, and 0.47) between the frying temperature and frying time. Elevation in frying temperature from 150 to 190°C for frying time 60, 90, and 120 sec of 10% DSB barley-soybean chips showed significant increase in oil absorption from 29.25, 29.50, and 29.75% to 30.50, 30.80, and 31.10%, respectively. Same patterns were observed in 20 and 30% DSB barley-soybean chips. On the contrary, increasing DSB flour substitution percentage significantly increased the chips' moisture content and oil absorption. The ability to absorb oil of soybean flour may be due to a high protein content and particle size that may have contributed to the uptake of excess frying oil [9]. Oil absorption capacity increasing with increased soybean flour percentage in blends was reported by Oladeji Alamu et al. [10]. Oil enters chips through the water replacement mechanism, which involves changes in cellular structures and the formation of pores during frying, to fill some of the spaces created by induced dehydration [27].

3.1.2. Amylose and Amylopectin Contents of Barley-Soybean Chips. The results in Table 2 showed the influence of frying temperature and frying time on the amylose content of barley-soybean chips. Results revealed that the amylose content of barley-soybean chips was significantly increased along with elevated frying temperature, frying time, and DSB substitution percentage. The amylose values showed a moderate positive correlation ($r = 0.41$, 0.39, and 0.37) between the frying temperature and frying time. The highest recorded amylose value (33.80%) was observed in barley-soybean chips fortified with 30% DSB at 190°C for 120 sec, whilst the lowest amylose content (27.16%) was observed in barley-soybean chips with 10% DSB at 150°C for 60 sec. The barley grain was reported to contain 25.39% amylose and 74.61% amylopectin [18]. Direct relationship was reported between apparent amylose, temperature, and moisture content [28]. Amylose has a crucial role in oil absorption of starch during frying, and more amylose molecules provide more hydrophobic helical cavities available for lipids [13], which supports the oil absorption results raised in Table 1.

The amylopectin content of barley-soybean chips with 10.0% DSB flour recorded the highest percentage (72.83%) at 150°C for 60 sec, while the barley-soybean chips with 30% DSB substitution recorded the lowest percentage (66.20%) at 190°C for 120 sec (Table 2). It showed an opposite pattern to the amylose content in the same values but with a negative correlation ($r = -0.41$, -0.39, and -0.37) between the frying

Table 1: Effects of frying temperature and frying time on the moisture content and oil absorption of barley-soybean chips.

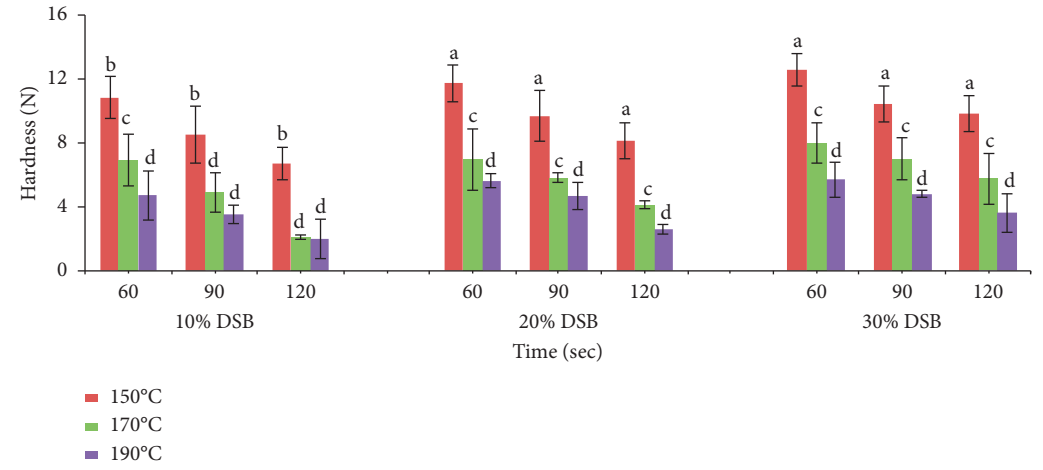
DSB substitution (%)	Frying temperature (°C)	Frying time (sec)		
		60	90	120
Moisture content				
10	150	2.20 ± 0.24^{ab}	2.10 ± 0.18^{b}	2.00 ± 0.15^{b}
	170	1.90 ± 0.21^{c}	180 ± 0.11^{cd}	1.70 ± 0.14^{b}
	190	1.80 ± 0.14^{c}	1.50 ± 0.14^{d}	1.40 ± 0.12^{d}
20	150	2.30 ± 0.23^{a}	2.20 ± 0.19^{ab}	2.10 ± 0.21^{b}
	170	2.00 ± 0.18^{b}	1.90 ± 0.15^{c}	1.80 ± 0.17^{c}
	190	1.90 ± 0.16^{c}	1.70 ± 0.12^{cd}	1.60 ± 0.13^{d}
30	150	2.50 ± 0.21^{a}	2.40 ± 0.21^{a}	2.30 ± 0.26^{a}
	170	2.30 ± 0.24^{a}	2.25 ± 0.24^{ab}	2.15 ± 0.26^{ab}
	190	2.20 ± 0.22^{ab}	2.15 ± 0.26^{ab}	2.10 ± 0.21^{b}
Pearson's correlation r		−0.680	−0.666	−0.633
Oil absorption				
10	150	29.25 ± 2.14^{d}	29.50 ± 2.14^{d}	29.75 ± 2.16^{d}
	170	30.00 ± 2.18^{c}	30.22 ± 2.18^{c}	30.40 ± 2.52^{c}
	190	30.50 ± 2.45^{c}	30.80 ± 2.41^{c}	31.10 ± 2.49^{b}
20	150	30.30 ± 2.35^{c}	30.40 ± 2.68^{c}	30.55 ± 2.46^{c}
	170	30.65 ± 2.37^{c}	30.80 ± 2.57^{c}	30.95 ± 2.74^{c}
	190	31.15 ± 2.49^{b}	31.25 ± 2.49^{b}	31.35 ± 2.61^{b}
30	150	31.45 ± 2.51^{b}	31.65 ± 2.94^{b}	31.80 ± 2.38^{b}
	170	31.95 ± 2.65^{b}	32.20 ± 2.76^{a}	32.35 ± 3.12^{a}
	190	32.45 ± 2.17^{a}	32.65 ± 3.01^{a}	32.85 ± 3.28^{a}
Pearson's correlation r		0.448	0.457	0.472

Data represent means ± SD. Means followed by different superscript letters in each column are significantly different ($p < 0.05$). DSB: defatted soybean; Pearson's correlation r: Pearson's correlation coefficient.

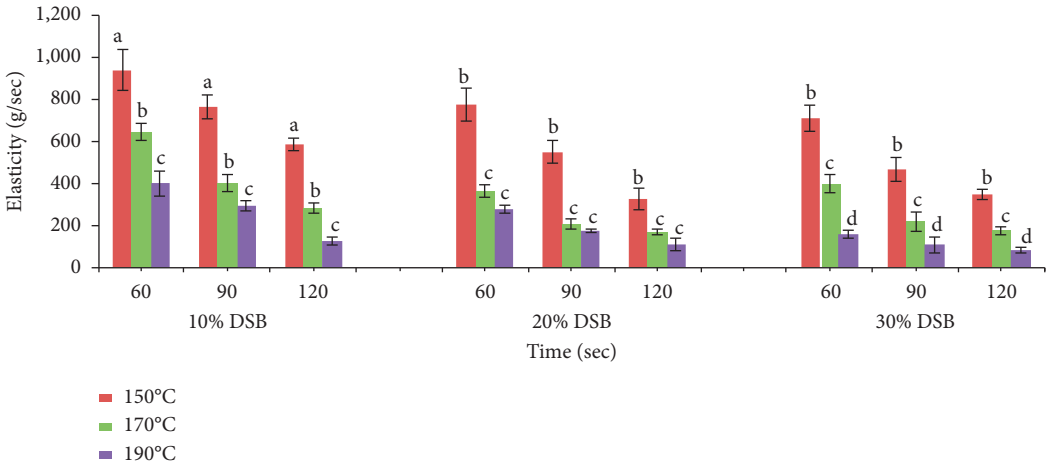
temperature and frying time. The significant decrease in the amylopectin content along with increased frying temperature, frying time, and DSB substitution percentage was previously reported by several authors due to heat stress [29–31]. The amylose-to-amylopectin ratio is one of the most important factors affecting the physicochemical and sensorial properties, processing performance, and functionality of starch [13]. Amylopectin is a glucose polymer in a large number of short-chain branches organized in semicrystalline granules [32]. This may introduce an explanation of the decreased scores of mouthfeel of barley-soybean chips in sensory evaluation results illustrated in Figure 2, along with the declined amylopectin content.

3.2. Texture Profile Analysis (TPA). The effect of treatments on texture profile analysis (TPA) of barley-soybean chips is presented in Figures 1(a)–1(d). Obtained results indicated a significant variation in all texture indices among different blends of barley-soybean chips. The hardness of barley-soybean chips (Figure 1(a)) was significantly increased by increasing the substitution percentage of DSB flour, whilst frying time and frying temperature showed an adverse effect on chips' hardness. The highest hardness value (12.57 N) was recorded for barley-soybean chips prepared with 30% DSB flour at 150°C fried for 60 sec followed by chips prepared with 20% DSB flour at 150°C for 60 sec (11.72 N) and then barley-soybean chips prepared with 10% DSB flour (10.82 N) at 150°C for 60 sec. An opposite pattern was observed for texture profile parameters elasticity and adhesiveness, while compression energy did not show any significant changes between different DSB substitution percentages in Figures 1(b)–1(d)). Modulus of elasticity is connected to the firmness of the sample. These results may be due to the intensive water evaporation during deep frying that promotes the expansion of the food matrix and allows oil absorption [32]. During the frying process, heat is transferred from oil to fried foods accompanied by mass transfer (water evaporation and oil uptake). Apart from heat, mass, and momentum transfers, chemical constituents such as starch, reducing sugars, amino acids, and water within the plant tissue react with each other during frying, and physical reactions occur accompanied by structural changes that consequently increase hardness [14]. Additionally, less force and work values were due to the foamy structure created by soybean flour in fried products along with the increasing level of DSB substitution. As the hardness increases, the elasticity and adhesiveness decrease for more crispness feeling which was observed in sensory evaluation (Figures 2–4).

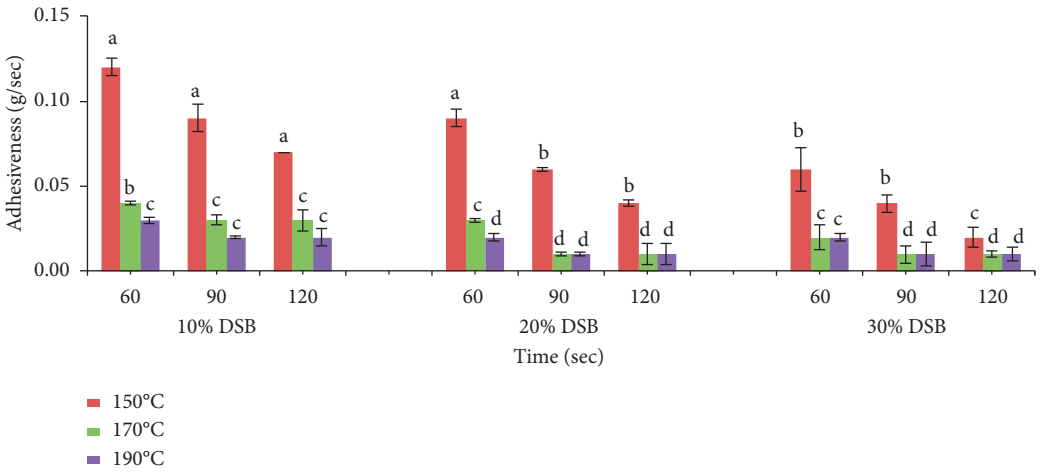
Frying time and frying temperature were inversely proportional with assessed texture parameter values. The elasticity of barley-soybean chips ranged from 124.8 to 940.0 g/sec at 10.0% DSB and 110.4 to 775.0 g/sec at 20.0% DSB flour to reach 85.4 to 709.9 g/sec in 30% DSB products. Adhesiveness indicates the quantity of work required for controlling the attractive power among the surface of the product and the material with which in contact [33]. The least adhesiveness was observed in 30% DSB flour fried at 170 and 190°C for 90 and 120 sec. Frying enhances the interactions between oil and fried food leading to increased oil absorption (Table 1). Surface-active agents can increase



(a)

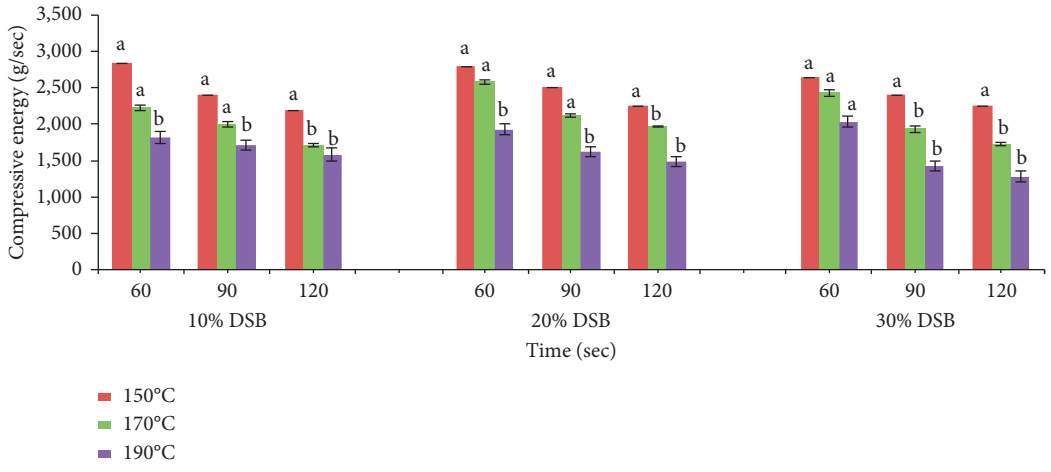


(b)



(c)

Figure 1: Continued.



(d)

Figure 1: Effect of treatments on the texture profile analysis (TPA) of barley-soybean chips. (a) Hardness (N). (b) Elasticity (g/sec). (c) Adhesiveness (g/sec). (d) Compression energy. Data represent the means ± SD. Means with different superscript letters are significantly different ($p < 0.05$). DSB: defatted soybean.

Table 2: Effects of frying temperature and frying time on the amylose content of barley-soybean chips.

DSB substitution (%)	Frying temperature (°C)	Frying time (sec)		
		60	90	120
Amylose content				
10	150	27.16 ± 1.90^{e}	27.50 ± 2.11^{e}	$28.00 \pm 2/18^{e}$
	170	28.35 ± 2.04^{d}	28.74 ± 2.17^{d}	29.07 ± 2.27^{d}
	190	29.44 ± 2.15^{d}	29.82 ± 2.21^{d}	30.11 ± 2.23^{c}
20	150	29.43 ± 2.35^{d}	29.77 ± 2.25^{d}	30.01 ± 2.61^{c}
	170	30.31 ± 2.58^{c}	30.86 ± 2.19^{c}	31.22 ± 2.43^{b}
	190	31.68 ± 2.67^{b}	32.12 ± 2.28^{b}	32.43 ± 2.57^{b}
30	150	31.90 ± 2.49^{b}	32.45 ± 2.94^{b}	32.95 ± 2.62^{b}
	170	32.55 ± 3.14^{a}	33.00 ± 3.16^{a}	33.50 ± 3.42^{a}
	190	33.15 ± 3.12^{a}	33.40 ± 3.25^{a}	33.80 ± 3.16^{a}
Pearson's correlation *r*		0.413	0.398	0.377
Amylopectin content				
10	150	72.83 ± 3.45^{a}	72.49 ± 4.35^{a}	71.90 ± 4.39^{ab}
	170	71.64 ± 3.68^{ab}	71.25 ± 4.16^{ab}	70.93 ± 4.16^{ab}
	190	70.55 ± 3.12^{ab}	70.17 ± 3.84^{b}	69.88 ± 3.48^{ab}
20	150	70.56 ± 3.69^{ab}	70.22 ± 3.68^{ab}	69.98 ± 3.59^{c}
	170	69.68 ± 3.11^{c}	69.13 ± 4.19^{c}	68.77 ± 2.97^{c}
	190	68.31 ± 3.19^{c}	67.87 ± 4.28^{d}	67.57 ± 3.28^{d}
30	150	68.10 ± 2.95^{c}	67.55 ± 3.79^{d}	67.05 ± 3.81^{d}
	170	67.45 ± 4.12^{d}	67.00 ± 3.81^{d}	66.50 ± 3.93^{e}
	190	66.85 ± 2.61^{e}	66.60 ± 2.99^{e}	66.20 ± 3.37^{e}
Pearson's correlation *r*		−0.414	−0.399	−0.373

Data represent means ± SD. Means followed by different superscript letters in each column are significantly different ($p < 0.05$). DSB: defatted soybean; Pearson's correlation *r*: Pearson's correlation coefficient.

the foaming tendency of oil and reduce the interfacial tension leading to the increase of surface hydrophobicity, leading to less elasticity and adhesiveness [14].

3.3. Sensory Evaluation of Barley-Soybean Chips. Sensory evaluation results of barley-soybean chips' attributes at different frying times (60, 90, and 120 sec) with different DSB substitution percentages (10, 20, and 30%) are exhibited in Figures 2–4, respectively. Obtained results revealed that the most preferred chips based on sensory evaluation were barley-soybean chips with 10% DSB fried at 150 and 170°C (Figures 4(a) and 4(b)) with no observed effects of frying time. At the same conditions of frying temperature and frying time, increased DSB flour substitution percentage scored less values of flavor, color, crispness, mouthfeel, and

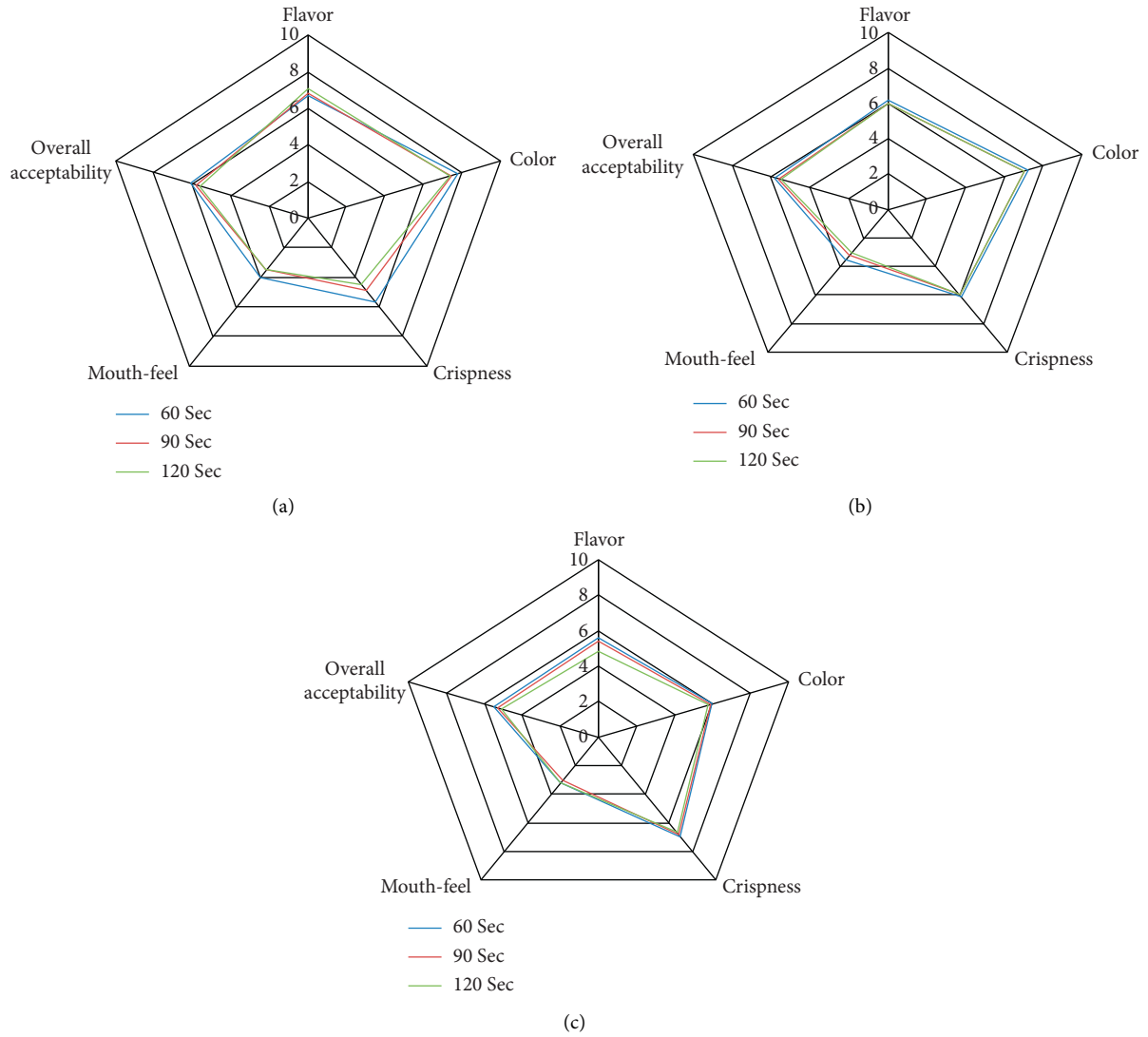


Figure 2: Sensory evaluation of barley-soybean chips (30% DSB) at different frying times (60, 90, and 120 sec). (a) Frying temperature 150°C (CF). (b) Frying temperature 170°C. (c) Frying temperature 190°C. DSB: defatted soybean.

consequently overall acceptability. This could be explained as soybean proteins are rich in lysine residues which are highly sensible to the Maillard reaction. Nutritional impairment of protein, as a consequence of the destruction of its essential amino acids or the decrease in their bioavailability, is one of the most known nutritional implications of the Maillard reaction [12].

Accordingly, barley-soybean chips with 30% DSB fried at 170 and 190°C received the least scores of all sensory parameters. Color is a significant quality parameter of fried snacks concerning consumer understanding and frequently is the foundation for their chosen fried snacks. Increased frying temperature or long frying time during the deep-frying process initiates nonenzymatic browning reactions (Millard reaction) that affect products' color [15]. Maillard reaction leads to the formation of brown polymers called melanoidins. They affect the color (as well as flavor) of heat-treated samples indicating the extent of the Maillard reaction. Gradual changes in color intensity starting with preferable cream to dark golden brown along with stages of the Maillard reaction should be controlled for accepted products. Another hypothesis expects that thermal oxidation of oil may also contribute to the color of the fried products [10].

Crispness is detected during a collection of different key texture advantages for snack products. Crispness is also connected to mastication which is based on break in crisp materialsthat generate sound participation in crispness sensation [34]. Increased crispness of barley-soybean chips showed to be related with increased frying temperature. It may be due to the spongy porous structures accompanied with losing moisture that plays a significant function in crispness [35]. These results are correlated with the moisture content (Table 1). Panelists experienced a fatty mouthfeel when the temperature and frying time increased, which may be due to greater oil absorption as indicated in Table 1.

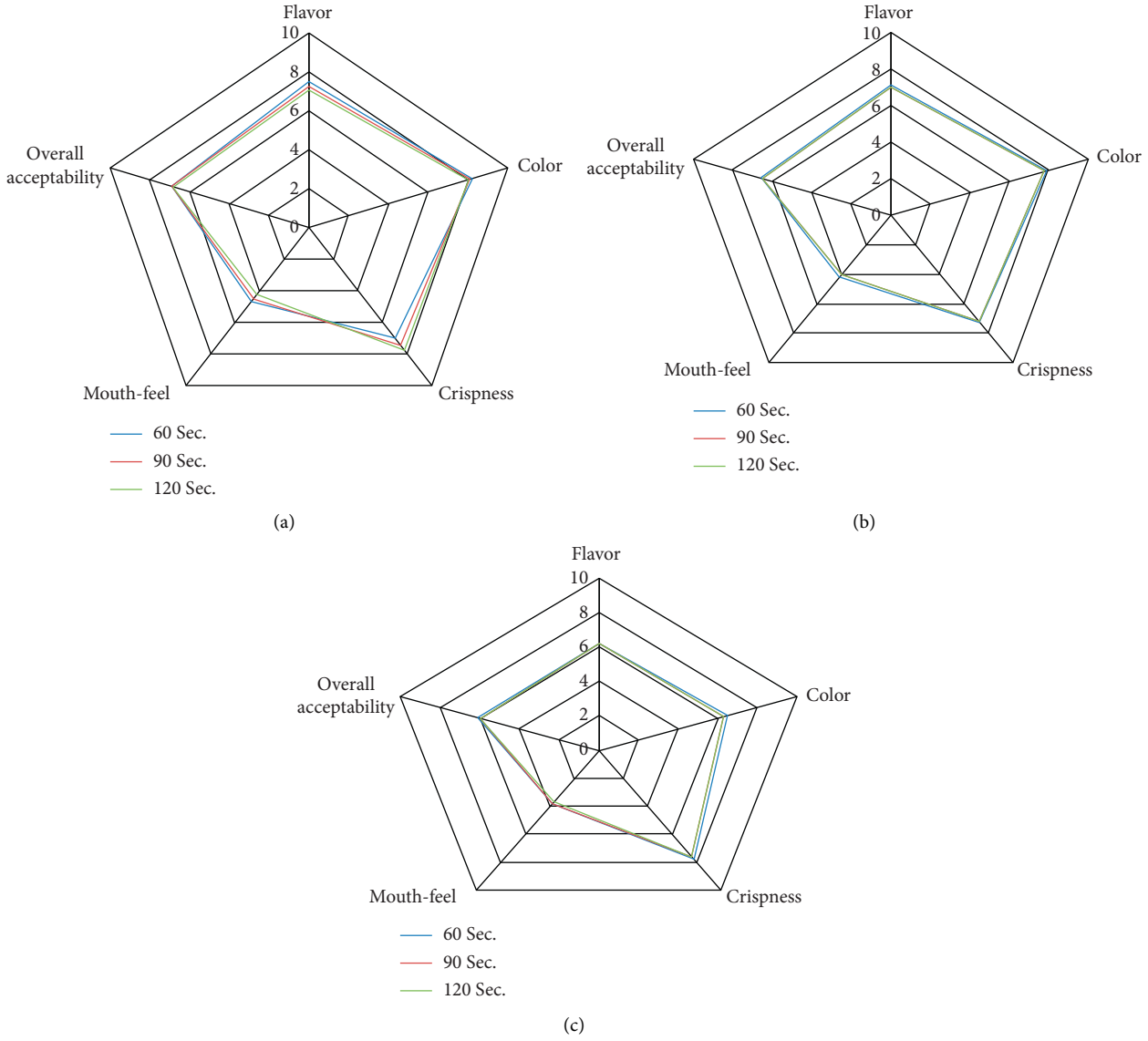


Figure 3: Sensory evaluation of barley-soybean chips (20% DSB) at different frying times (60, 90, and 120 sec). (a) Frying temperature 150°C (CF). (b) Frying temperature 170°C. (c) Frying temperature 190°C. DSB: defatted soybean.

Although less adhesiveness (Figure 1(c)) is correlated with enhanced mouthfeel [23], the greasy feeling caused the mouthfeel scores to decrease.

To sum sensory attributes' evaluation, panelists preferred barley-soybean chips at level 10 and 20% DSB substitution fried at 150 and 170°C as increased substitution percentage along with elevated frying temperature and frying time caused undesirable attributes either due to progressed Maillard reaction or excess oil absorption. These results are in agreement with what Oladeji Alamu et al. reported [10].

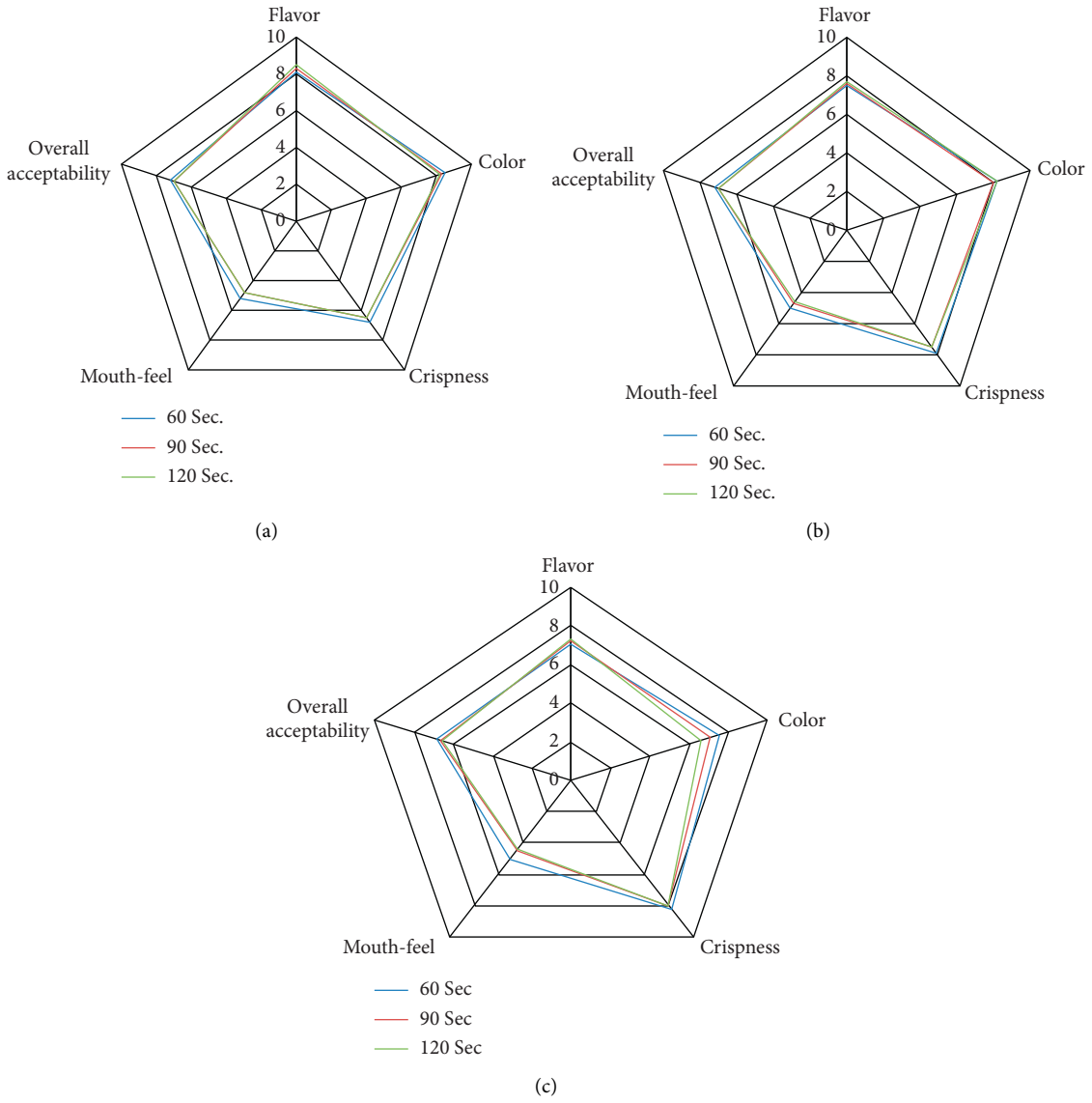


FIGURE 4: Sensory evaluation of barley-soybean chips (10% DSB) at different frying times (60, 90, and 120 sec). (a) Frying temperature 150°C (CF). (b) Frying temperature 170°C. (c) Frying temperature 190°C. DSB: defatted soybean.

4. Conclusion

Evaluation of the effects of different substitution levels (10, 20, and 30%) of defatted soybean (DSB) flour, frying temperatures (150, 170, and 190°C), and frying times (60, 90, and 120 sec) on physicochemical characteristics of barley-soybean chips revealed that the moisture content was substituted by oil absorption and amylose and amylopectin contents along with the increase in testing factors. These influences were reflected on the texture profile analysis (TPA) of barley-soybean chips resulting in increased hardness in parallel with decreased elasticity and adhesiveness. This in turn consequently affected sensorial acceptability to reveal that the most preferable substitution level was 10 and 20% DSB that were fried at temperature which did not exceed 170°C. These findings encourage snacks production with acceptable substitution levels of legume protein flour while maintaining consumer satisfaction which is the main pillar for innovative food preparations.

Data Availability

The supporting data are available from the corresponding author upon request.

Conflicts of Interest

The authors declare no conflicts of interest with respect to the research, authorship, and/or publication of this article.

Authors' Contributions

HAA and AMGD conceptualized the study, provided the methodology, investigated the study, and visualized the

study. HAA prepared and wrote the original draft. AMGD wrote, reviewed, and edited the final version of the manuscript. Both authors read and agreed to the published version of the manuscript.

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Research Article

Exogenous Application of Ascorbic Acid Enhances the Antimicrobial and Antioxidant Potential of *Ocimum sanctum* L. Grown under Salt Stress

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Ocimum sanctum L. (Tulsi) is the most important medicinal plant that has antimicrobial, antioxidants, and anticarcinogenic effects on human health. Plants, when under stress, gather several antioxidants and osmoprotectants. The present work focuses on the abiotic stress response of Tulsi and its mitigation by the application of ascorbic acid. In addition to this, an enhancement of antioxidant and antimicrobial activity was also analyzed using ascorbic acid. During the present work, when plants were grown under NaCl stress and ascorbic acid (AA) was provided with foliar applications, it ascertained encouraging effects on growth; likewise, its effect remains stable under salinity stress. The enzymatic antioxidants activity showed a significant change in response to AA alone or in combination. The highest catalase activity was recorded in plants subjected to 0.5 mM AA in combination with 100 mM NaCl (0.65 units/mL of enzyme). Likewise, a similar trend was recorded for the superoxide dismutase activity of Tulsi plants. The highest activity of SOD was recorded in plants subjected to 0.5 mM AA in combination with 100 mM NaCl (66.1 units/mg of protein). Flavonoid content showed its highest amount (27.41 mg/g) when plants were treated with 0.5 mM AA + 100 mM NaCl while the highest phenolic content (1.88 mg/g) was analyzed in salt treated plants sprayed with 0.5 mM AA. In the case of antimicrobial activity, 0.5 mM AA treated plants gave the highest value for the *Staphylococcus aureus* as 2.15 cm and in *Clostridium* species was 2.1 cm in the plants treated with 1 mM AA alone. Hence, the findings of the present study may lead to the conclusion that AA has a significant role in defense mechanisms of plants in response to salt stress. Further, it enhances the antimicrobial and antioxidant potential of Tulsi plants grown under salt stress.

1. Introduction

In the last few years, pharmacological industries became active in producing new antibiotics as microorganisms are becoming more and more resistant to the older drugs [1]. Folk medicines that include natural and herbal products have been utilized for centuries throughout the world. Along with a low adverse reaction rate, their cost is highly reduced [2]. Different parts of the plants are used for the extraction of different compounds that are efficient in curing cough, fever, bronchitis, cold, diarrhea, cholera, and dysentery, etc. [3]. Around the globe, one of the greatest economic value sources is a medicinal plant. Pakistan is one of the countries that are blessed with rich sources of botanical wealth [4]. *Ocimum sanctum* is one of the most important plants of genus *Ocimum* and is commonly known as "Tulsi" in Pakistan [5]. Generally, it possesses antifertility, anticancer compounds, antidiabetic, antifungal/antimicrobial, cardioprotective related compounds, analgesic, antispasmodic, and adaptogenic actions [6]. Eugenol (1-hydroxy-2-methoxy-4-allylbenzene), the active constituents present in *O. sanctum* L. have been found to be largely responsible for the therapeutic potentials [7]. The growth and productivity of medicinal plants are majorly hindered by abiotic and biotic stresses. Their medicinal properties are also severely affected by these environmental stresses. Whenever the

stress conditions prevail, several imbalances occur in the plant physiology that consequently may influence therapeutic traits as well as the growth of these medicinal plants [8]. Salt stress is specifically considered as one of the important abiotic factors that limit the production of crops due to different types of stresses like osmotic disturbance, ionic imbalance, oxidative stress, and hormonal irregularities [9]. Vitamin C, also known as "ascorbic acid" (AA), is one of the most important and most abundant growth promoters presents in plants [10].

A small amount of AA produced endogenously is involved in the promotion of the development and growth of plant cells. This AA is involved in the phytohormonal mediated signaling pathways and is bound towards a various number of environmental stress conditions along with development and growth [10]. Ascorbic acid applied as foliar application helped in the protection against toxic derivatives of oxygen that has an adverse effect on many enzyme activities [11]. According to the biochemical studies, under the salinity stress condition, plants usually accumulate metabolites that are known as compatible solutes. These compatible solutes do not hinder the biochemical process occurring in the cell [9]. During sodium chloride stress, AA displayed an efficient effect by the accumulation of different soluble proteins and antioxidant enzymes. Stress-induced proteins and enzymes are generally produced under stress conditions that play a vital role in tolerating stress conditions [10]. It is well reported in literature that antioxidants contribute to preventing several diseases like cancer, heart, hepatitis, etc., since these antioxidants are scavengers of reactive oxygen species in the body [12]. Several genes are responsible for controlling abiotic stress tolerance [13]. Salt stress has been reported to reduce germination percentage, seedling vigor, and biochemical parameters like carbohydrate, protein, catalase, total phenols, and proline of *Ocimum tenuiflorum* [14]. Keeping in view the importance of ascorbic acid and the significance of Tulsi plants, the present study was conducted to investigate the changes in phytochemical constituents of the Tulsi plants in response to NaCl and AA alone or in combination. Additionally, an enhancement in antimicrobial and antioxidant activities by exogenous application of ascorbic acid was also investigated.

2. Materials and Methods

2.1. Cultivation of Ocimum sanctum L. Seeds of Tulsi plants were obtained from the Botanical Garden University of the Punjab Lahore (Pakistan). Tulsi seedlings were grown in earthen pots (6×9 cm) having field soil mixture with Bhal and Manure in the ratio of 1 : 1 : 1. Plants were grown in a glasshouse at $26 \pm 2^{\circ}$C temperature and 70% relative humidity under natural sunlight conditions.

2.2. Application of Ascorbic Acid and NaCl Treatments. The experiment was laid out in a completely randomized design with three blocks. Each block consisted of five replicates for each concentration. All pots in experiments were given simple water for 30 days. NaCl (0 and 100 mM; Merk, USA) was treated through roots drenching for each concentration. Then plants were provided with different concentrations of AA (0.1, 0.5 and 1.0 mM; Sigma Aldrich; MW 176.5) through a foliar spray. Tween-20 (*polyoxyethylene sorbitan monolaurate*; Sigma Aldrich) was added as a surfactant for penetration of AA into leaf tissues. Eight Treatments of both AA and salt were given simultaneously after 7 days for 60 days. The treatments were as follows:

(1) Control (Simple water with foliar spray of distilled water). (2) Plants treated with 100 mM NaCl + foliar application of distilled water. (3) Plants treated with 0 mM NaCl + foliar application of 0.1 mM AA. (4) Plants treated with 0 mM NaCl + foliar application of 0.5 mM AA. (5) Plants treated with 0 mM NaCl + foliar application of 1.0 mM AA. (6) Plants treated with 100 mM NaCl + foliar application of 0.1 mM AA. (7) Plants treated with 100 mM NaCl + foliar application of 0.5 mM AA. (8) Plants treated with 100 mM NaCl + foliar application of 1.0 mM AA.

2.3. Collection of Plant Material and Estimation of Enzymatic Antioxidant Activity. The healthy and fresh leaves were separated, and inflated polythene bags were used for the packaging of plant materials. The plants were rinsed under running water and were dried by the Whatman filter paper no. 1. Fresh leaves of about 0.5 g were measured and crushed with mortar and pestle to form a paste. About 1 mL of PBS (Phosphate Buffer Saline) having a pH of 7.2 was added to the paste along with 0.05 g of PVP (Sigma Aldrich). Slurry so obtained was centrifuged at 4°C for 10 minutes at 14,000 rpm (wise spin CF-10, Germany). Supernatant was carefully collected for the estimation of antioxidant enzymes.

The method of Beers and Sizer [15] was used to estimate the catalase activity (E.C 1.11.1.6). Blank was prepared by adding 3 mL of 50 mM phosphate buffer. The test sample was prepared by adding 2.9 mL of 0.036% H_2O_2 solution prepared in phosphate buffer and 0.1 mL of plant extract. In this method, hydrogen peroxide disappeared was confirmed by taking absorbance at 240 nm. This procedure took place at optimum ($37 \pm 2^{\circ}$C) conditions.

Superoxide dismutase (SOD; E.C 1.15.1.1) assay was performed with little modification in the method proposed by Sevilla et al. [16]. The reaction mixture was prepared by 1 mL NaCN (Sigma Aldrich), 10 mL methionine, 10 mL EDTA, 1 mL NBT, and 1 mL of Riboflavin. The final volume was made up to 100 mL with buffer solution. Blank consists of 2 mL reaction mixture. The test was prepared by adding 5 μL of extract along with the reaction mixture. The absorbance of both samples was measured at 560 nm by using a spectrophotometer (UV-9000S).

The Guaiacol-H_2O_2 method was employed, followed by Luck [17], to determine the peroxidase (E.C. 1.11.1.7) activity. Two test tubes (15×150 mm), one having the reaction mixture consisted of 0.1 M phosphate buffer (pH 7.2; 3.0 mL; Sigma Aldrich), 20 mM guaiacol (2-methoxyphenol) 0.05, and 0.1 mL crude enzyme extract. While in control, crude enzyme extract was replaced by 0.1 mL distilled water. Both test tubes were placed at $25 \pm 2^{\circ}$C and then added to 0.03 mL of 12.3 mM H_2O_2 solution. Enzyme activity was determined

by the time required to increase the absorbance (UV-9000S) by a value of 0.1 (e.g., 0.4-0.5) at 240 nm and expressed as U/mL of the enzyme.

2.4. Quantitative Analysis of Nonenzymatic Antioxidants. The freshly obtained plant leaves were air-dried in the shade for 3-4 days at room temperature. The fully dried leaves were then ground in fine powder or dust by using mortar and pestle. Methanol (100 mL) and 20 g of air-dried powder were poured in a conical flask and kept on a rotary shaker for 24 hours (Shaker-A by lab watch enterprises) at 150 rpm. Supernatant was collected, solvent vaporized to make one-fourth out of original volume by evaporating in a water bath (Daeyang ETS, Korea) and stored in sealed bottles at 4°C [18]. The total flavonoids content of the Tulsi plants was measured by means of a colorimetric method [19]. The extract (250 μL) of Tulsi samples was mixed with 75 μL of 5% $NaNO_2$ solution and 1.25 mL of deionized water. Afterward, 150 μL of 10% $AlCl_3$ solution was added to this sample. The reaction was started by the addition of 0.5 ml of 1 M NaOH, making up volume up to 2.5 mL by adding deionized water. Absorbance was taken at 510 nm with the help of a spectrophotometer (UV-9000S).

The total phenolic components were examined with an improved method with slight modifications in the Folin-Ciocalteu reagent method [20]. Samples were prepared by adding 0.25 mL Folin-Ciocalteu (Merk) reagent, 0.25 mL of the plant extract in dilute form, and then 3.5 mL of distilled water was added to make a volume of 4.6 mL. After this, 1 mL of 20% Na_2CO_3 solution was added, followed by vortex mixing, and then samples were incubated at room temperature for two hours. For the blank reading, 0.25 mL of 80% methanol was added instead of the extract on a spectrophotometer (UV-9000S) at 765 nm.

2.5. Determination of DPPH Radical Scavenging Activity. The total antioxidant activity of Tulsi extract was measured by using DPPH 1, 1- diphenyl -2-picrylhyrdazyl method after Ghafoor et al. [21] with certain modifications. One mL of the Tulsi extract (100 μL/mL methanol) was thoroughly mixed in 2 mL of the solution of DPPH (10 mg DPPH was dissolved in 1 L methanol) and kept in this sample at 250°C for 5 minutes. The absorbance was measured at 517 nm.

2.6. Antimicrobial Activity. Pathogenic bacterial strains including *Clostridium* sp. and *Staphylococcus aureus* were used for the determination of antimicrobial activity. The strains were obtained from the laboratory of the Department of Biotechnology, Lahore College for Women University, Lahore, Pakistan. These strains were maintained in the nutrient agar slants and were stored at 4°C. The medium used for suspension bacterial culture was the nutrient broth. Strains of *Clostridium* and *Staphylococcus aureus* were inoculated at 37 ± 2°C for 16–18 hours in test tubes.

2.7. Statistical Analysis. All these experiments were set up in triplicate, and the data thus obtained was statistically analyzed by applying statistical software SPSS version 22.0.0 following the Duncan Multiple Range Test in order to compare the means for the selected parameters.

3. Results

3.1. Catalase (CAT, EC 1.11.1.6) Activity. The present study indicates that plants grown in the presence of salt stress gave a significant increase in the activity of catalase from 0.29 to 0.43 units/mL of enzyme in control and salt treated plants, respectively (Figure 1). At the same time, AA treated plants helped to alleviate the effect of salt stress and hence gave a much higher catalase activity value to cope with salt stress. The maximum catalase activity was recorded in the salt treated (100 mM NaCl) *Ocimum* plants when treated with 0.5 mM AA (0.65 units/mL of enzyme).

3.2. Superoxide dismutase (SOD, EC 1.15.1.1) Activity. The present study indicated that SOD activity generally had an increasing trend in response to salt treatment. The plants treated with salt stress gave an increase in value from 33.0 to 40.7 units/mL of the enzyme (Figure 2), whereas the plants treated with 0.1, 0.5 and 1 mM AA along with 100 mM NaCl showed a significant increase in the value of SOD from 50.2, 66.1 and 50.8 respectively, which indicated a positive effect in the inhibition of salt stress conditions.

3.3. Peroxidase (POD, EC 1.11.1.7) Activity. The present study indicated that when plants treated with salt stress gave an increase in peroxidase activity from 2.6 to 3.6 mg/g of tissue. POD activity generally had an increasing trend as a result of AA treatment given to the salt-stressed plants. The plants treated with 0.1, 0.5 and 1 mM AA along with 100 mM NaCl gave a significant increase in the value of peroxides activity as 4.7, 5.8, and 4.8, respectively (Figure 3).

3.4. Flavonoid Contents. During the present work, plants treated with salt stress showed an increase in the value of flavonoid contents from 10.38 to 22.39 mg/g, whereas the plants treated with 0.1, 0.5 and 1 mM AA along with 100 mM NaCl gave a significant increase in the value of flavonoid as 26.41, 27.41 and 25.39 respectively, which indicated a positive effect in the inhibition of salt stress conditions (Figure 4).

3.5. Phenolic Contents. During the present work, it was found that the total phenolic contents were significantly increased after the application of AA, which helped to alleviate the harmful effects of salt stress conditions (Figure 5). The plants treated with 100 mM NaCl gave an increase in value from 0.89 to 1.50 mg/g, whereas the plants treated with 0.1 mM, 0.5 mM and 1 mM ascorbic acid along with 100 mM NaCl gave a significant increase in the value of total phenolic content as 1.6, 1.88, and 1.53, respectively, which indicated a positive effect in the inhibition of salt stress conditions.

3.6. Antioxidant Activity. During the present study, a DPPH test was performed for the determination of the nonenzymatic antioxidant activity. The plants treated with 100 mM

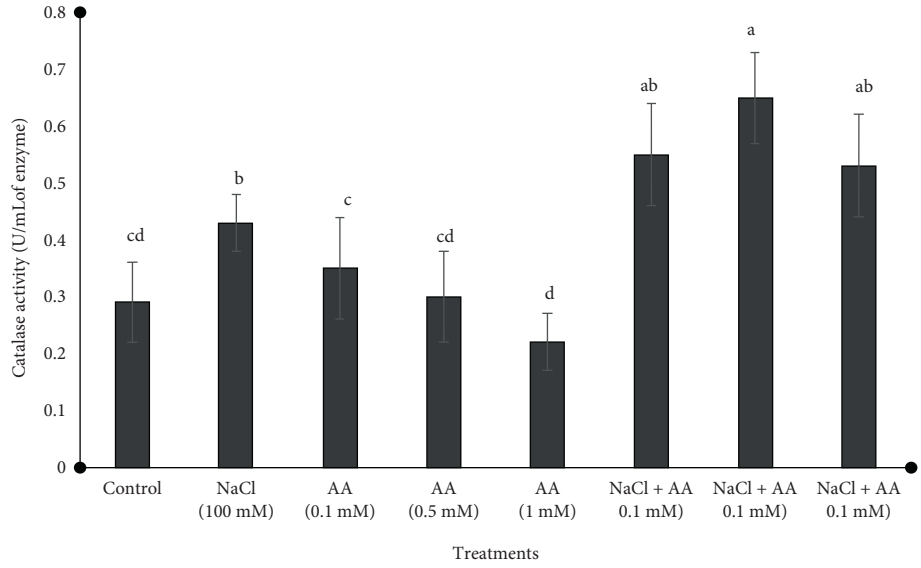

Figure 1: Effect of various ascorbic acid treatments on Tulsi plants subjected to salt stress on catalase activity.

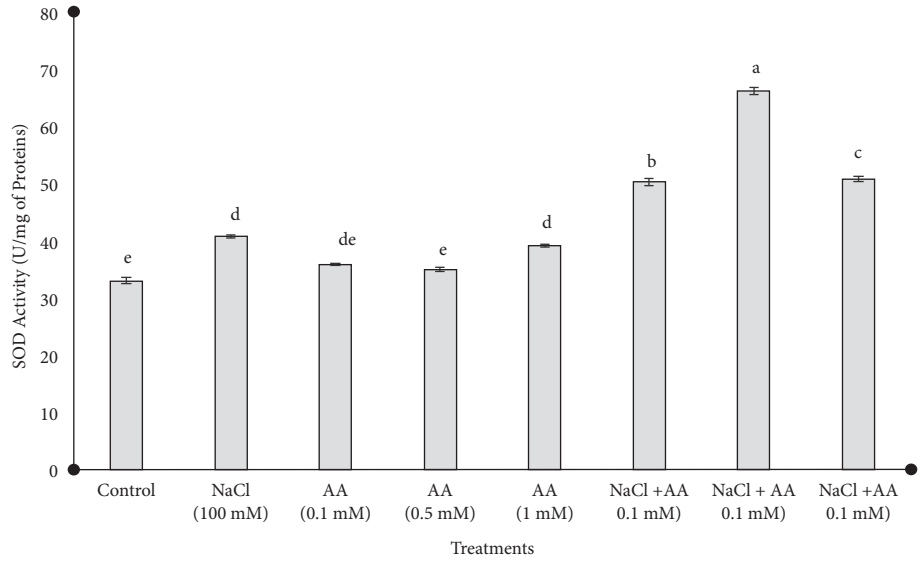

Figure 2: Effect of various ascorbic acid treatments on Tulsi plants subjected to salt stress on superoxide dismutase activity.

NaCl gave an increase in value from 36.8% to 40.03% (Figure 6), whereas the plants treated with 0.1 mM, 0.5 mM, and 1 mM ascorbic acid along with 100 mM NaCl showed a significant increase in the value of antioxidant activity as 51.67, 52.96, and 50.63%, respectively, which indicated a positive effect of treatment.

3.7. Antimicrobial Activity of Extracts of Ocimum sanctum. *Ocimum sanctum* is a medicinally important plant; hence, the antimicrobial effect has a great impact on its activity. It was used for the measurement of the effectiveness of treated plant extract against pathogenic bacteria. The plants treated with 100 mM NaCl gave an increase in the zone of inhibition from 1.9 to 2.1 cm, whereas the plants treated with 0.1, 0.5, and 1 mM AA along with 100 mM NaCl gave a significant increase in the zone of inhibition as 1.15, 2.12 and 1.6 cm as presented in Table 1. This indicated a positive effect on antimicrobial activity. In the case of *Clostridium* species, antimicrobial activity was increased from 1.6 cm to 2.0 cm in the plants treated with 100 mM NaCl, whereas the plants treated with 0.1, 0.5, and 1 mM AA along with 100 mM NaCl gave a significant increase in antimicrobial activity as 1.9, 1.25, and 1.0 cm, respectively.

4. Discussion

During the present work, an increase in catalase activity was observed in response to salinity. However, the negative results of salinity were reduced in response to ascorbic acid treatment. The activity of SOD was increased in response to AA application combined with the NaCl treatment indicating its possible role in salt tolerance mechanisms. These

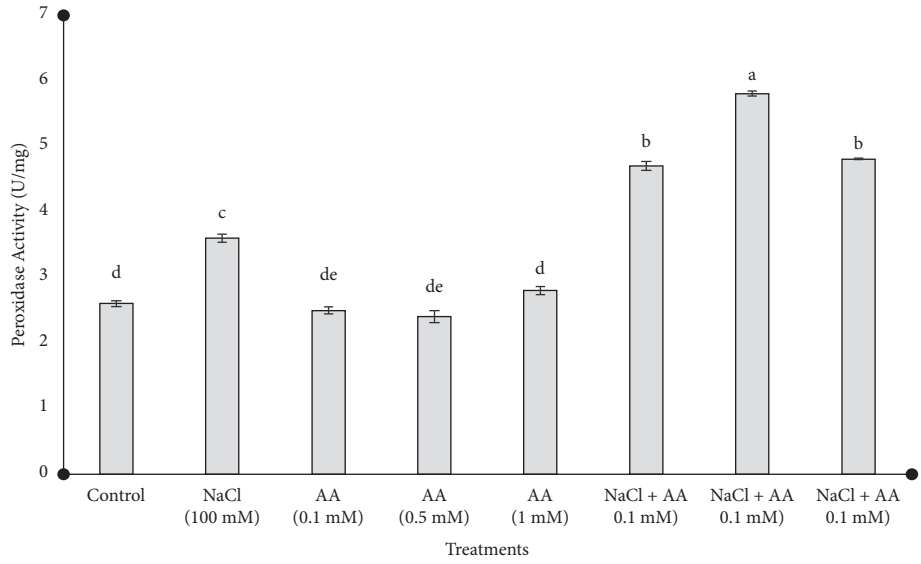

Figure 3: Effect of various ascorbic acid treatments on Tulsi plants subjected to salt stress on peroxidase activity.

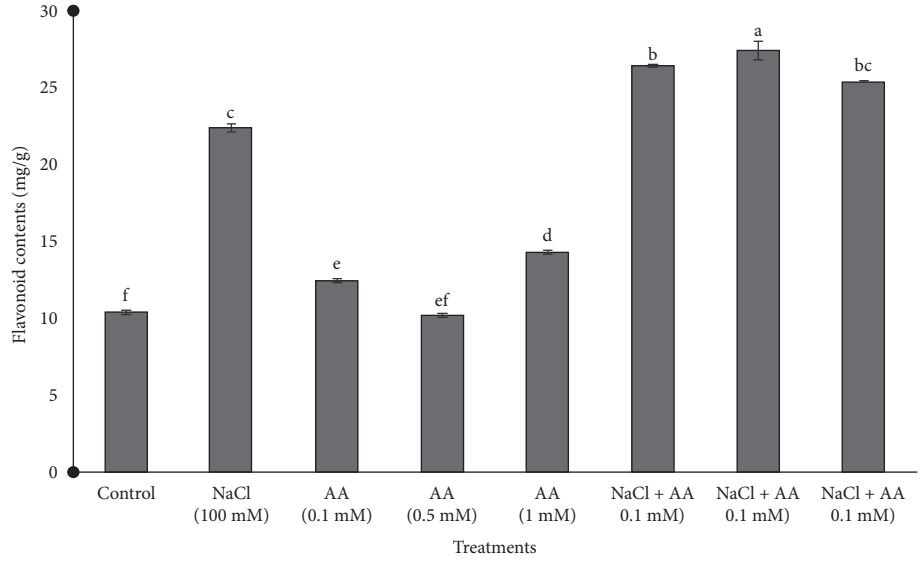

Figure 4: Effect of various ascorbic acid treatments on Tulsi plants subjected to salt stress on flavinoids contents.

findings are in accordance with findings of Nair et al. [22]; those observed positive effects of AA on *O. sanctum* for their antioxidant potential. Likewise, Gul et al. [23] also reported an increase in enzyme activity in response to salt stress when different concentrations of AA were applied, resulting in an increase in SOD activity in *Cymposis tetragonoloba*. In response to NaCl stress, an increase in catalase and SOD activities was linked to reducing the oxidative damage caused by reactive oxygen species (ROS) in potatoes and several other plants [24, 25].

An increase in peroxidase activity in response to different treatments of AA has indicated a positive effect in the inhibition of salt stress conditions during the present work. Peroxidase converts the H_2O_2 into water molecules. Likewise, Nair et al. [22] also reported an increase in peroxidase activity in response to AA. Flavonoids have radical scavenging activity; therefore, these are considered powerful antioxidants [26]. Phenols and flavonoids are important secondary metabolites present in medicinal plants. An increase in flavonoids during the present work is in line with the findings of Prasad et al. [27], who also reported an increase in flavonoid contents of five different medicinal plants in response to salt stress when different ascorbic acid levels were applied as a foliar spray. Similarly, Gaffar et al. [9] also reported an increase in phenolic, flavonoids, and tannins contents in response to foliar application of AA in common beans, growing under water stress conditions.

A positive effect of AA treatment in enhancing the antioxidant activity observed by DPPH assay indicated the role of AA in the inhibition of salt stress conditions.

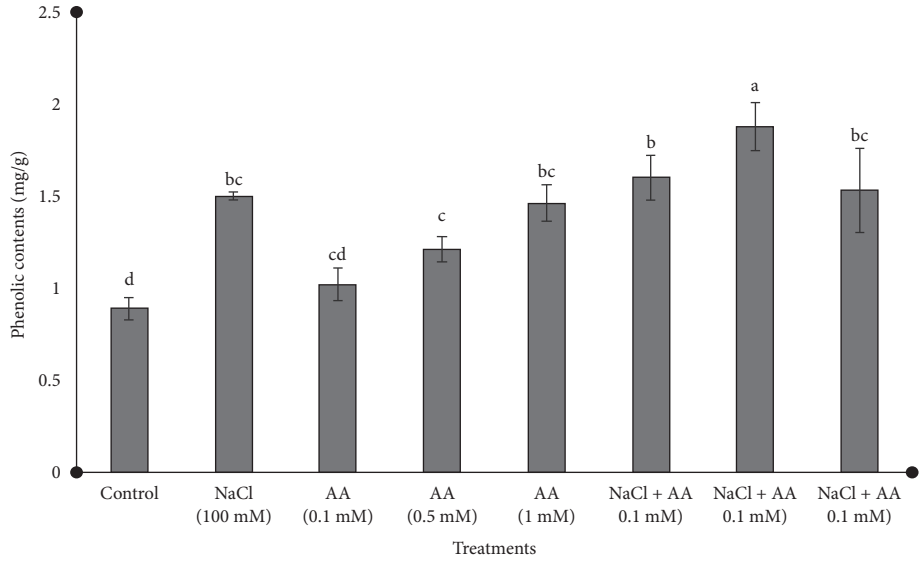

Figure 5: Effect of various ascorbic acid treatments on Tulsi plants subjected to salt stress on phenolics contents.

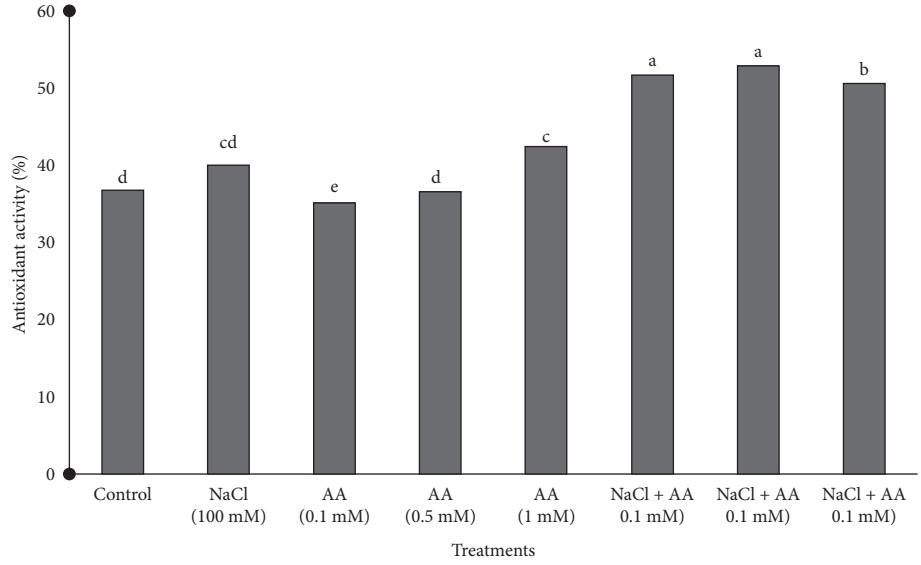

Figure 6: Effect of various ascorbic acid treatments on Tulsi plants subjected to salt stress on antioxidant activity.

Table 1: Effect of different treatments on the antimicrobial activity of leaf extracts of *Ocimum sanctum* against *Staphylococcus aureus and Clostridium* sp.

	Staphylococcus aureus	*Clostridium* sp.
Treatments	Zone of inhibition (cm)	
Control	0.6 ± 0.0^{e}	0.6 ± 0.0^{f}
Distilled water	1.9 ± 0.92^{b}	1.6 ± 0.71^{c}
100 mM NaCl	2.10 ± 1.06^{a}	2.0 ± 0.99^{ab}
0.1 mM AA	1.85 ± 0.88^{b}	1.37 ± 0.55^{cd}
0.5 mM AA	2.15 ± 1.10^{a}	1.65 ± 0.74^{c}
1.0 mM AA	1.65 ± 0.74^{c}	2.1 ± 1.06^{a}
0.1 mM AA + 100 mM NaCl	1.15 ± 0.39^{d}	1.9 ± 0.92^{b}
0.5 mM AA + 100 mM NaCl	2.12 ± 0.42^{a}	1.25 ± 0.46^{d}
1.0 mM AA + 100 mM NaCl	1.6 ± 0.71^{c}	1.0 ± 0.28^{e}

Means within a column followed by the same letter do not differ significantly ($P \leq 0.05$) according to Duncan's multiple range test. Results are mean ± S.E.

Previously, an increase in antioxidant activity has been reported in three different medicinal plants when different growth regulators were applied as foliar applications [28]. Likewise, Choudhary et al. [29] also reported an increase in antimicrobial activity in response to salt stress when different growth regulators were applied as a foliar application to *Ocimum sanctum*. The phytochemical present in methanol extract was found to be more active against all of the bacterial species tested. Furthermore, extracts prepared from leaves are shown to have better efficacy and demonstrated that the secondary metabolites and antimicrobial agents are highly effective against several pathogenic bacteria.

5. Conclusions

Ocimum sanctum plants were tested for their response to salt stress alone and in combination with foliar application of AA during the present study. The *Ocimum* plants showed significant changes in flavonoids, phenolic, catalase, peroxidase, and SOD as these compounds are very significant for plant defense mechanisms, so this increase is of great significance to see the possible role of AA application in the amelioration of salinity tolerance. The concentration of the antioxidants and antimicrobial activity differ among various treatments given to *O. sanctum* plants. Differently treated plants of *O. sanctum* exhibited both enzymatic and nonenzymatic antioxidant defense systems to cope with the ROS under the salt stress condition. Ascorbic acid treatments resulted in greater activities of antioxidant enzymes (catalase, SOD, and peroxidase). Further, an increase in antimicrobial activity was also observed by the exogenous application of ascorbic acid. The obtained results provided some clues as to which of these responses may be affected by the foliar application of AA that helps in the defense mechanism of plants against salt stress. Hence, the results of the present study may be helpful in the mitigation of the negative effects of salt stress on *Ocimum sanctum* plants. These results also confirmed the potential use of exogenous application of ascorbic acid to enhance the antimicrobial and antioxidant activities of Tulsi plant grown under stress conditions; however, this study necessitates further work to test the effectiveness of ascorbic acid on other plants.

Means followed by the same letter do not differ significantly ($P \leq 0.05$) according to Duncan's multiple range test in all figures.

Data Availability

The data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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Research Article

In Vitro Bioaccessibility of the Vitamin B Series from Thermally Processed Leafy African Indigenous Vegetables

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Thermal processing of leafy African indigenous vegetables (LAIVs), which are rich in nutrients, especially vitamin B series affects the levels and bioaccessibility of the vitamins. This study investigated the bioaccessibility of vitamin B series in fresh and thermally processed LAIVs. Five commonly consumed indigenous vegetables, *Cleome gynadra, Vigna unguilata, Amaranthus viridis, Basella alba,* and *Cucurbita maxima,* were processed by boiling and/or frying, treated to *in vitro* gastrointestinal digestion procedure, and levels of vitamin B series determined before and after treatment. The vitamin B series in fresh LAIVs ranged from 0.73 ± 0.01 mg/100 g (B_9; spider plant) to 174.16 ± 3.50 mg/100 g (B_2; vine spinach) and had both significant increase (ranging from +8.71% to +446.84%) and decrease (ranging from −0.44% to −100.00%) with thermal processing ($p < 0.001$). The *in vitro* digestion resulted in a significant increase ($p < 0.001$) of vitamins ranging from 5.18% (B_5; boiled cowpeas) to 100% (B_2, B_3, and B_6 in several processed vegetables). Where detected, the bioaccessible levels of vitamin B series in fresh, processed, and *in vitro* digested samples were sufficient to meet the Recommended Dietary Allowances (RDA) of children and adults. These findings support the promotion of a nutritional approach to malnutrition resulting from vitamin B series deficiency.

1. Introduction

Vitamins are a group of organic compounds which are important for the normal functioning of the body [1, 2]. Vitamin B, a series of vitamins, namely, thiamin (B_1), riboflavin (B_2), niacin (B_3), pantothenic acid (B_5), pyridoxine (B_6), and folate (B_9), are water-soluble and are found majorly in leafy African indigenous vegetables (LAIVs) and fruits. They play key roles in cellular metabolism and homeostasis, maintenance of healthy skin and muscle tone, enhancing immune and nervous function, and promoting healthy cell division and hematopoietic activity [3]. Consistent consumption of diets rich in these vitamins not only maintains good health but also addresses malnutrition [4, 5]. The recommended dietary allowances (RDAs) for the vitamin B series range from 0.1–20 mg/day [6].

Malnutrition affects more than two billion people globally [1], although it is more alarming in developing countries where young women and children are mostly affected [7]. The result of malnutrition is poor health, low worker productivity, high mortality rate, and morbidity [1]. The deficiency of vitamins B_1, B_2, B_3, B_5, B_6, and B_9 causes beriberi, cardiac disorder, pellagra, impaired coordination, neurological disturbances, and megaloblastic anemia, respectively [8]. Malnutrition is mainly addressed through the nutritional approach, supplementation, and food fortification [8, 9]. Consumption of LAIVs as a nutritional approach is promoted because they are rich sources of not only vi-

tamin B series but also other vitamins, minerals, and other phytochemicals [8]. Alam et al. [10], for instance, reported levels of vitamin B (B_1, B_2, B_3, and B_6) in unconventional leafy and nonleafy vegetables ranging from 0.06 ± 0.01 to 1.05 ± 0.13 mg/100 g and recommended that regular intake of the vegetables would help combat micronutrient deficiency.

There are more than 200 species of LAIVs in Kenya cultivated by over 60% of households in the rural and periurban parts of Kenya [11, 12]. Spider plant (*Cleome gynandra*), cowpeas (*Vigna unguiculata*), vegetable amaranth (*Amaranthus viridis*), vine spinach (*Basella alba*), and vegetable pumpkin (*Cucurbita maxima*) are among the common LAIVs in Western Kenya [13]. Cultivated by smallholders, the vegetables are commonly consumed due to their medicinal value, such as antibacterial and antioxidant properties, and rich micronutrient such as vitamins and microelements [14]. This makes them not only generally available but also inexpensive, although a number of them are unfortunately underutilized and unconsumed [15]. In addition to being promoted to address malnutrition, the LAIVs are a source of income for small-scale farmers [16, 17].

Vegetables are commonly processed, mainly by boiling, frying, and steaming [11, 18, 19]. However, thermal processing affects the levels of vitamins, though the process is necessary since it releases vitamins from the vegetable matrix [20]. Cooking water has been reported to reduce levels of thiamin by between 31% and 50%, riboflavin by 50%, and niacin by 80% [21, 22]. Factors that enhance the loss of vitamin B series in heat processing include the presence of water, oxygen, light, and pH [3, 8, 21, 23, 24]. These water-soluble vitamins are extractable into water (leaching) during blanching and cooking [21]. Oxidative cleaving is usually minimized during cooking by steaming the vegetables until the pathway between tender and crisp [3, 24]. Pantothenic acid is stable to heat at a pH of 5–7, although it can be lost during cooking [23].

Vitamins in vegetables are complexed in the nutrient matrix, requiring their breakdown to make them bioaccessible for absorption. Bioaccessibility, the amount of ingested nutrients that potentially become available for absorption in the gastrointestinal tract [25], is affected by food matrix, processing, interaction with other dietary compounds, and physicochemical properties [26, 27]. High dietary fiber in vegetables decreases the bioaccessibility of nutrients by slowing gastric emptying, digestion, and absorption of nutrients, mainly due to the water retained by pectin that forms a viscous solution in the gut [28–31]. Thermal processing is important in increasing the surface area and the interaction of hydrolytic enzymes and emulsifiers with food particles during the gastric and intestinal phases of digestion to release nutrients [28, 29, 31]. However, the released vitamin may undergo a chemical reaction due to pH change or from the oxidative reaction, as seen in beta-carotene in thermally processed LAIVs [32].

Studies of *in vitro* bioaccessibility using the gastrointestinal model can determine the digestibility of the food material, the fraction of food components that are transformed into potentially accessible matter through physical and chemical processes occurring in the ileum [25, 33] and inform the best food processing procedures that retain high levels of nutrients. There is little information on the bioaccessibility of vitamin B series from thermally processed LAIVs, which are important in addressing vitamin B deficiency. Hence, an *in vitro* method of digestion was employed to study the bioaccessibility of vitamin B series from thermally processed (boiled and boiled-fried) spider plant, cowpeas, amaranth, vine spinach, and pumpkin leaves.

2. Materials and Methods

2.1. Equipment and Chemicals. High-Performance Liquid Chromatography (HPLC), (Shimadzu SPD 20A) equipped with a photodiode array detector (PDA) and an autosampler was used to quantify the vitamins. The separation was achieved using a reversed-phase C-18 column (250 mm × 2 mm i.d, 5 μm) manufactured by Hamilton Company. The mobile phase used was potassium dihydrogen phosphate and methanol in the ratio of 90 : 10. All chemicals and reagents used were of analytical grade, and methanol was HPLC grade. The standards thiamin, riboflavin, niacin, pantothenic acid, pyridoxine, and folic acid were purchased from Sigma-Aldrich. Sep-Pak C18 (500 mg) cartridge from Sigma-Aldrich was used for precolumn separation.

The stock solutions of vitamin B_1, B_2, B_3, B_5, B_6, and B_9 were prepared by dissolving 0.01 g of each standard in 50 ml of 0.1 m HCl in a 100 ml volumetric flask and diluted to the mark. Following appropriate dilutions using 0.1 m HCl, serial standards were prepared in the following ranges in ppm; B_1 (0–10), B_2 (0–10), B_3 (0–8), B_5 (0–8), B_6 (0–8), and B_9 (0–8) then filtered using 0.45 μm. A calibration line was obtained by plotting the peak area values as a function of the concentration of the vitamin.

The LoDs, regression equations, and correlation coefficients are presented in Table 1.

The LoDs ranged from 0.02 to 0.12 and this is comparable to that reported by Cheruiyot [34] (0.03 to 0.17). The correlation coefficient values were above 0.9736, indicating at least a 97.36% relationship of absorbance against concentration, thus implying linearity [35]. The percentage recoveries were between 98.96 ± 0.15 and 101.27% implying that the methods had a conventionally acceptable precision and accuracy [36].

2.2. Sample Preparation. Five leafy vegetables cultivated in Africa samples, spider plant (*Cleome gynadra*), cowpeas (*Vigna unguiculata*), amaranth (*Amaranthus blitum*), vine spinach (*Basella alba*), and pumpkin leaves (*Cucurbita maxima*), were collected from an open market in Kisii County, Kenya in September (wet season). Kisii County is a

Table 1: Method validation parameters of vitamin B series.

Vitamins	LoD	Correlation coefficient	Regression equation	% recovery
Thiamin	0.12	0.9783	$y = 36499x - 2346.8$	101.27 ± 1.27
Riboflavin	0.04	0.9998	$y = 49440x - 496.57$	99.13 ± 0.47
Niacin	0.08	1	$y = 9441x - 72.8$	100.37 ± 0.38
Pantothenic acid	0.10	0.9736	$y = 2203.8x + 547.67$	100.18 ± 0.16
Pyridoxine	0.03	0.9960	$y = 14982x - 2931.8$	99.74 ± 1.38
Folate	0.03	0.9960	$y = 49747x + 132.5$	100.35 ± 0.35

major grown region in western Kenya [13] and sampling was done in Kisii Town because leafy African indigenous vegetables (LAIVs) from many planting areas in the County are brought there for sale since the town is central and has a large population. The LAIVs were purchased randomly from several venders and mixed to give 1 kg of each vegetable, and immediately sprayed with water to keep them moistened, packed in dark plastic polythene bags, and transported to the Department of Food Science and Technology laboratory, Jomo Kenyatta University of Agriculture and Technology (JKUAT) for analysis.

The vegetables were trimmed to remove inedible parts, washed under tap water, rinsed with distilled water, and flapped to remove water. Pumpkin leaves (*Cucurbita maxima*), which had broad leaves, were cut into small pieces after washing. Each vegetable type was divided into 2 portions, with the first portion of about 100 g used for fresh analysis and the second portion boiled. To prepare the boiled samples, 80 g of the fresh vegetables were boiled in 200 ml of distilled water for 10 minutes at 100°C and then cooled to room temperature. For the fried samples, 40 g of boiled vegetables were added to 40 ml of vegetable oil already heated in a cooking pan (100°C). This was fried for 10 minutes and then cooled to room temperature. The fresh, boiled, and boiled-fried samples were placed in zip-locked bags, frozen for five hours at −20°C, and then freeze-dried at −50°C for 96 hours. The freeze-dried samples were then wrapped in aluminum foil and kept in the refrigerator at 4°C awaiting determination of vitamin B series.

2.3. Extraction and Measurement of Vitamin B Series. Extraction of vitamin B series was performed according to [37]. To 5 g of each vegetable sample, 20 mL of deionized water was added and the mixture homogenized at medium speed for 1 minute before centrifuging for 10 minutes at 14×103 g (Sigma, Bio Block Scientific 2–16). A sample (10 mL) of the supernatant was then loaded to a Sep Pak C18 cartridge, flushed with 10 mL methanol and 10 mL water adjusted to pH 4.2, and was eluted with 5 mL acidified water, pH 4.2 (prepared by adding 0.005 m HCl) followed by 10 mL methanol at a flow rate of 1 mL/min. The eluent was collected in a bottle and evaporated to dryness, and then reconstituted using the mobile phase. Before HPLC analysis, all samples were filtered through 0.45 μm pore size (FP 30/45 CA-S filters, Schleicher and Schuell, Darmstadt, Germany) at 7 bar max. 20 μL of each sample solution was injected into the HPLC column by an autosampler.

The accuracy of HPLC was investigated by spiking samples with a known amount of standards. Analysis was performed thrice. The analysis of the samples was done simultaneously for thiamin (B_1), niacin (B_3), pantothenic acid (B_5), pyridoxine (B_6), and folate (B_9) using the mobile phase 0.1 mol/L KH_2PO_4 (pH 7) and methanol (90 : 10) as reported by Ekinci and Kadakal, while riboflavin (B_2) was analyzed separately. The column elute was monitored with a photodiode array (PDA) detector for thiamin (234 nm), riboflavin (266 nm), niacin (261 nm), pantothenic acid (204 nm), pyridoxine (324 nm), and folic acid (282 nm). The mobile phase was filtered through a 0.45 μm membrane and degassed by sonication before use. The flow rate was 1 mL/min and the column was operated at room temperature (25°C). Chromatographic peak data were integrated for up to 39 minutes. Identification of the compounds was achieved by comparing their retention times and UV spectra with those of the standards. Calibration curves were plotted for each vitamin and the concentrations of the vitamins were calculated from the integrated areas of the sample and the corresponding standards.

2.4. Simulated Gastrointestinal Digestion and Measurement of Bioaccessible Vitamin B Series. The simulated *in vitro* digestion method was adapted from [31] and was done in triplicate. The process involved two phases: the gastric phase and intestinal phase. The sample, 2 g, was subjected to simulated gastric digestion at pH 2.0 in the presence of pepsin at 37°C (16 g in 100 mL 0.1 m HCl) for 2 hrs, followed by digestion in the presence of pancreatin-bile extract mixture (4 g porcine pancreatin) and 25 g of bile extract (porcine) in 1000 ml of 0.1 m $NaHCO_3$ pH 7.5 at 37°C for 2 hrs. The micellar fraction containing the bioaccessible vitamin B was separated by ultracentrifugation at 70,000 ×g for 120 minutes using a Beck-man L7-65 ultracentrifuge. The supernatant aliquot was filtered using a 0.22 μm microfilter to obtain a micellar fraction, placed in an amber glass bottle, and the levels of vitamin B series measured as per Section 2.3. To obtain the percentage bioaccessibility (%), the bioaccessible levels were divided by the original levels and then multiplied by one hundred.

$$\%\,\text{bioaccessibility} = \frac{\text{bioaccessible levels}}{\text{original content}} \times 100. \quad (1)$$

2.5. Data Analysis. One-way ANOVA was used to compare the mean levels of vitamin B series in vegetables prepared using different thermal processes, at p value <0.05 significant difference. Mean separations were done by the standard error [38].

3. Results and Discussion

The mean levels (mg/100 g DW) and bioaccessible levels (%) of vitamin B series in fresh and processed vegetables are represented in Tables 2 and 3, respectively. The range of mean levels (mg/100 g) of thiamin (B_1), riboflavin (B_2), niacin (B_3), pantothenic acid (B_5), pyridoxine (B_6), and folate (B_9) in the fresh vegetables were as follows: 0.83 ± 0.01 (vine spinach)—4.56 ± 0.04 (pumpkin leaves), 35.44 ± 0.72 (pumpkin leaves)—174.16 ± 3.50 (vine spinach), 10.36 ± 0.87 (amaranth)—107.70 ± 1.80 (spider plant), 9.45 ± 0.20 (cowpeas)—14.00 ± 0.15 (pumpkin leaves), 5.88 ± 0.30 (spider plant)—83.10 ± 0.92 (amaranth), and 0.73 ± 0.01 (spider plant)—20.68 ± 0.12 (cowpeas), respectively. Fresh vegetables are known to contain different levels of vitamins, this being attributed to vegetable variety, plant age, soil, climate, loss during washing, and postharvest handling and storage [21, 39, 40]; Uraku et al.; [41]. However, the levels of some vitamins in vegetables have been documented to be lower than those reported in this study [41] and Kunyanga et al. [42] reported levels (mg/100 g) of vitamin B_1, B_2, B_3, and B_9 in amaranth to be 0.42 ± 0.09, 0.44 ± 0.03, 0.70 ± 0.00, and 0.83 ± 0.02, respectively, while in pumpkin leaves, the levels of vitamin B_1, B_2, and B_3 were 0.08 ± 0.12, 0.06 ± 0.01, and 0.32 ± 0.01, respectively. The levels of vitamin B_1, B_2, and B_6 reported by [41] in amaranth dried for two weeks were 9.731 ± 3.250, 7.161 ± 0.521, and 25.020 ± 2.667, respectively.

The levels of the vitamins in vegetables showed a significant change with thermal processing ($p < 0.001$). The levels both increased (+11.14 to +425.96) and decreased (−6.02 to −100.00), although the resultant levels in most processed vegetables except dried ones were found to be sufficient to meet the RDA of children and adults when the right amounts are consumed [6]. Boiling, for example, had an increase of thiamin in spider plants (+75.64%), amaranth (+129.52%), and vine spinach (+28.92%), but it reduced the same vitamin in cowpeas (−15.86%) and pumpkin leaves (−18.42%). Drying, on the other hand, reduced the vitamin (B_1) in all vegetables. Vitamins B_1, B_2, B_3, B_5, B_6, and B_9 were not detected in boiled, fried cowpeas, boiled, fried spider plant, boiled amaranth, dried pumpkin leaves, boiled vine spinach, and dried amaranth, respectively.

It was observed that specific vegetables responded differently and encountered different effects on the vitamin B series. Spider plants, for example, had the levels of B_1, B_3, and B_6 increasing with boiling, while vitamins B_2, B_5, and B_9 decreased. On the other hand, dried cowpeas, fried vine spinach, and dried pumpkin had levels of all the vitamins decrease. Factors including loss during washing and leaching during boiling are known to contribute to the effects of processing [21, 22, 41]. The reductions are attributed to the vitamin being water-soluble and sensitive to heat and oxidation, while the release of the vitamin from its protein matrix during cooking explains their increase [8, 20, 21].

Following *in vitro* digestion of the LAIVs, the resultant mean bioaccessible levels (mg/100 g) of the vitamin B series in fresh vegetables (Table 3) indicated that the lowest was 0.27 ± 0.01 for B_1 and the highest was 174.52 ± 3.57 for B_2 in vine spinach. There were significant differences in the mean bioaccessible levels, dependent on the original levels in the vegetables as a result of the thermal processing of the vegetables ($p < 0.001$). The increase in the vitamins ranged from 5.18% (B_5) in boiled cowpeas to 100% (B_2, B_3, and B_6) in several processed vegetables. Boiled and boiled-fried amaranth and vine spinach had undetectable levels of B_3, indicating 100% loss of the vitamin with processing (Table 2). Vitamins B_2 (boiled spider plant), B_1 (boiled vine spinach), and B_3 (boiled pumpkin and cowpeas), which, although increased with processing (Table 2), were undetected following the *in vitro* digestion. These findings underscore the fact that boiling alone does not encourage bioaccessibility despite the vegetable variety and plant age [21, 39].

The digestibility of the vegetables differs depending on the fiber content [25, 33]. Dietary fiber lowers bioaccessibility by physically entrapping nutrients and enhancing the viscosity of gastric fluids, thereby restricting the mixing process [30, 43]. Results from several studies report an increase in fiber content in the order spider plant < vine spinach < amaranth < pumpkin leaves < cowpeas [42, 44–46]. The findings show that the order of increasing fiber content did not necessarily have a decrease in the bioaccessibility of the vitamins [29, 31]. Thiamin (B_1), and riboflavin (B_2), for example, had increased bioaccessibility, with vine spinach < amaranth < spider plant < cowpeas < pumpkin leaves for B_1, and pumpkin leaves, cowpeas amaranth spider plant, vine spinach for B_2. These findings, therefore, may suggest other dynamics that led to the variation of vitamins in vegetables following both thermal processing and *in vitro* digestion [30, 43]. However, the difference in fiber content in the vegetables may not be significant to determine the changes. The variation in the bioaccessible levels found in this study partly arises from the difference in the alteration of the vegetable matrix, and in particular, vitamins are destroyed during cooking which then decreases their bioaccessible levels [28, 29]. Further, the physicochemical properties of the vitamin have also been used to support the bioaccessible levels of vitamins [26, 27].

Table 2: Mean levels of vitamin B series and the percent change in fresh and processed LAIVs.

LAIVs	Fresh/processed	Mean (±SD, $n = 3$) levels (mg/100 g DW) of vitamin B series (% change on processing)					
		B_1	B_2	B_3	B_5	B_6	B_9
Spider plant	Fresh	3.12 ± 0.06^{a}	127.64 ± 9.70^{b}	107.70 ± 1.80^{c}	9.98 ± 0.06^{d}	5.88 ± 0.03^{c}	0.73 ± 0.01^{d}
	Boiled	5.48 ± 0.11^{b} (+75.64)	73.19 ± 5.70^{a} (−42.66)	125.13 ± 1.55^{d} (+16.18)	5.45 ± 0.16^{b} (−45.39)	6.96 ± 0.01^{d} (+18.37)	0.42 ± 0.01^{c} (−49.32)
	Boiled-fried	16.41 ± 1.00^{c} (+425.96)	ND (−100.00)	40.16 ± 1.40^{a} (−62.71)	2.92 ± 0.11^{a} (−70.74)	2.59 ± 0.39^{b} (−55.95)	0.33 ± 0.01^{b} (−54.79)
	Dried	2.26 ± 0.08^{a} (−27.56)	ND (−100.0)	87.52 ± 2.54^{b} (−18.74)	6.82 ± 0.27^{c} (−31.66)	1.31 ± 0.43^{a} (−77.72)	0.20 ± 0.00^{a} (−72.60)
	p value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Cowpeas	Fresh	2.90 ± 0.09^{b}	60.83 ± 1.58^{b}	24.51 ± 1.22^{b}	9.45 ± 0.20^{a}	3.60 ± 0.04^{a}	20.68 ± 0.12^{c}
	Boiled	2.44 ± 0.10^{a} (−15.86)	1.30 ± 0.05^{a} (−97.86)	95.66 ± 2.23^{c} (+290.29)	24.56 ± 0.50^{b} (+159.89)	14.72 ± 0.51^{b} (+308.89)	0.36 ± 0.01^{a} (−98.26)
	Boiled-fried	ND (−100.00)	1.69 ± 0.02^{a} (−97.22)	ND (−100.00)	33.56 ± 0.90^{c}+225.13	16.20 ± 0.06^{c} (+350.00)	7.54 ± 0.02^{b} (−63.53)
	Dried	ND (−100.00)	ND (−100.00)	10.67 ± 0.99^{a} (−56.47)	ND (−100.00)	3.37 ± 0.34^{a} (−6.39)	19.39 ± 1.60^{c} (−6.24)
	p value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Amaranth	Fresh	3.76 ± 0.11^{a}	79.73 ± 5.41^{c}	10.36 ± 0.87^{b}	11.18 ± 0.24^{b}	83.10 ± 0.92^{d}	1.70 ± 0.02^{a}
	Boiled	8.63 ± 0.05^{c} (+129.52)	6.03 ± 0.18^{a} (−92.44)	ND (−100.00)	5.12 ± 0.70^{a} (−54.20)	21.18 ± 0.94^{b} (−74.51)	8.86 ± 0.12^{c} (+421.18)
	Boiled-fried	4.60 ± 0.58^{b} (+22.34)	6.31 ± 0.38^{a} (−92.09)	ND (−100.00)	46.69 ± 1.50^{c} (+317.63)	25.18 ± 0.40^{c} (−69.70)	3.71 ± 0.01^{b} (+118.24)
	Dried	ND (−100.00)	23.91 ± 1.45^{b} (−70.01)	8.81 ± 0.66^{a} (−14.96)	ND (−100.00)	2.49 ± 0.13^{a} (−97.00)	ND (−100.00)
	p value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Vine spinach	Fresh	0.83 ± 0.01^{b}	174.16 ± 3.50^{d}	23.25 ± 2.12^{b}	13.52 ± 0.51^{c}	5.97 ± 0.10^{b}	3.71 ± 0.09^{a}
	Boiled	1.07 ± 0.04^{c} (+28.92)	1.35 ± 0.03^{a} (−99.22)	127.14 ± 2.79^{d} (+446.84)	13.46 ± 0.66^{c} (−0.44)	18.68 ± 0.30^{c} (+212.09)	ND (−100.00)
	Boiled-fried	ND (−100.00)	18.45 ± 0.50^{b} (−89.40)	36.61 ± 2.20^{c} (+57.46)	4.49 ± 0.07^{a} (−66.79)	2.85 ± 0.06^{a} (−52.26)	ND (−100.00)
	Dried	0.78 ± 0.01^{a} (−6.02)	48.93 ± 3.68^{c} (−71.92)	14.66 ± 0.28^{a} (−36.95)	7.96 ± 0.09^{b} (−41.12)	ND (−100.00)	ND (−100.00)
	p value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Pumpkin leaves	Fresh	4.56 ± 0.04^{c}	35.44 ± 0.72^{c}	40.40 ± 3.49^{b}	14.00 ± 0.15^{a}	8.62 ± 0.10^{c}	2.46 ± 0.03^{c}
	Boiled	3.72 ± 0.06^{b} (−18.42)	0.86 ± 0.04^{a} (−97.57)	43.92 ± 2.62^{b} (+8.71)	20.75 ± 1.51^{b} (+48.21)	0.63 ± 0.01^{a} (−92.69)	1.35 ± 0.03^{b} (−45.12)
	Boiled-fried	10.26 ± 0.80^{d} (+125.00)	ND (−100.00)	26.49 ± 2.10^{a} (−34.43)	45.84 ± 2.00^{c} (+227.43)	7.24 ± 0.48^{b} (−16.01)	3.47 ± 0.20^{d} (+41.06)
	Dried	3.71 ± 0.01^{a} (−18.64)	13.34 ± 1.01 b (−62.36)	ND (−100.00)	ND (−100.00)	ND (−100.00)	0.98 ± 0.11^{a} (−60.16)
	p value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

Mean values followed by the same letters (superscript) within the same column of individual vegetables are not significantly different (SNK, $\alpha = 0.05$) ND- not detected.

Table 3: Mean bioaccessible levels of vitamin B series in fresh and processed LAIVs and the percent change with *in vitro* digestion.

LAIVs	Fresh/processed	Mean (±SD, $n = 3$) bioaccessible levels (mg/100 g DW) of vitamin B series (% change on processing)					
		B_1	B_2	B_3	B_5	B_6	B_9
Spider plant	Fresh	0.89 ± 0.10^{a} (28.61)	126.75 ± 9.77^{b} (99.30)	105.62 ± 1.86^{b} (98.07)	0.43 ± 0.02^{a} (4.38)	5.24 ± 0.03^{b} (89.10)	0.73 ± 0.0^{c} (100.00)
	Boiled	0.39 ± 0.06^{a} (7.08)	72.48 ± 5.65^{a} (99.30)	125.13 ± 1.55^{c} (100.00)	2.04 ± 0.26^{b} (37.35)	1.21 ± 0.25^{a} (12.30)	0.23 ± 0.01^{b} (55.04)
	Boiled-fried	16.13 ± 1.09^{b} (98.27)	ND	38.70 ± 1.55^{a} (96.34)	0.60 ± 0.12^{a} (22.89)	1.15 ± 0.37^{a} (39.78)	0.19 ± 0.01^{a} (55.80)
	p value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

Table 3: Continued.

LAIVs	Fresh/processed	Mean (±SD, $n = 3$) bioaccessible levels (mg/100 g DW) of vitamin B series (% change on processing)					
		B_1	B_2	B_3	B_5	B_6	B_9
Cowpeas	Fresh	2.49 ± 0.09^b (85.88)	60.83 ± 1.58^b (100.00)	24.51 ± 1.22^a (100.00)	1.18 ± 0.21^a (11.63)	2.12 ± 0.11^a (61.88)	20.68 ± 0.12^c (100.00)
	Boiled	0.42 ± 0.04^a (17.20)	1.30 ± 0.05^a (100.00)	95.66 ± 2.22^b (100.00)	0.99 ± 0.56^a (5.18)	16.50 ± 3.14^b (100.00)	0.24 ± 0.01^a (66.87)
	Boiled-fried	ND	1.60 ± 0.03^a (94.74)	ND	15.64 ± 0.99^b (46.57)	47.23 ± 0.86^c (98.74)	7.54 ± 0.20^b (100.00)
	p value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Amaranth	Fresh	0.77 ± 0.04^a (20.62)	79.73 ± 5.41^b (100.00)	6.93 ± 0.64^a (63.25)	12.72 ± 1.82^a (34.53)	75.23 ± 1.39^c (90.63)	3.71 ± 0.14^b (61.80)
	Boiled	1.99 ± 0.01^b (23.00)	3.70 ± 0.25^a (61.41)	ND	10.36 ± 0.64^a (18.15)	16.33 ± 0.88^a (75.21)	8.86 ± 0.12^c (100.00)
	Boiled-fried	3.67 ± 0.15^c (85.55)	2.73 ± 0.29^a (43.17)	ND	15.64 ± 0.99^b (46.57)	24.16 ± 3.14^b (95.83)	3.66 ± 0.12^a (98.72)
	p value	<0.001	<0.001	<0.001	<0.006	<0.006	<0.006
Vine spinach	Fresh	0.27 ± 0.01^b (32.31)	174.12 ± 3.57^c (99.98)	6.93 ± 0.64^a (98.50)	2.82 ± 0.60^b (22.94)	5.00 ± 0.30^b (83.66)	3.17 ± 0.10^a (100.00)
	Boiled	0.19 ± 0.00^a (18.09)	1.35 ± 0.03^a (100.00)	127.14 ± 7.79^c (100.00)	1.50 ± 0.17^a (9.12)	17.50 ± 0.33^c (93.67)	ND
	Boiled-fried	ND	18.17 ± 0.55^b (98.49)	36.22 ± 2.29^b (98.94)	0.64 ± 0.17^a (14.23)	2.71 ± 0.06^a (94.86)	ND
	p value	<0.001	<0.001	<0.001	<0.004	<0.004	<0.004
Pumpkin leaves	Fresh	3.06 ± 0.09^b (67.22)	34.67 ± 0.72^b (97.82)	40.40 ± 3.49^b (100.00)	0.93 ± 0.38^a (5.17)	1.58 ± 0.01^b (59.45)	2.24 ± 0.04^b (90.87)
	Boiled	2.04 ± 0.18^a (54.94)	0.19 ± 0.01^a (22.28)	37.27 ± 4.71^b (100.00)	4.75 ± 2.35^b (16.97)	0.63 ± 0.01^a (100.00)	0.95 ± 0.03^a (70.81)
	Boiled-fried	9.74 ± 0.84^c (95.02)	ND	24.87 ± 2.13^a (93.85)	25.51 ± 2.79^c (55.58)	3.86 ± 0.05^c (100.00)	2.98 ± 0.30^c (85.79)
	p value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

Mean values followed by the same letters of the same vegetable within the same column are not significantly different (SNK, $\alpha = 0.05$).

4. Conclusion

The levels of vitamin B series (B_1, B_2, B_3, B_5, B_6, and B_9) in *Cleome gynandra, Vigna unguiculata, Amaranthus viridis, Basella alba,* and *Cucurbita maxima* were affected by processing, with most reduction experienced in dried samples. Boiling and boiled-frying processes resulted in both increases and decreases in the bioaccessible levels of the vitamins. The changes in the bioaccessible levels did not reflect the changes in the fiber content of the vegetables. The levels in both the fresh and processed vegetables and further their *in vitro* bioaccessible levels are sufficient to meet the WHO recommended dietary allowances (RDA) levels for children and adults. These findings can be used to promote the nutritional approach to address malnutrition.

Data Availability

The data used to support the findings of the study have been deposited within the article.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this article.

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Research Article

Magnetic Field Stimulation Effect on Germination and Antioxidant Activities of Presown Hybrid Seeds of Sunflower and Its Seedlings

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Magnetic field biostimulation plays a significant role in enhancing the germination of seeds and increasing the metabolic rate. The low magnetic field effect for long exposure time and its effect on antioxidant profiling have not been studied. Therefore, in the recent findings, the static magnetic field's impact on sunflower seeds subjected to the magnetic field at varying intensity (millitesla) for different exposure times was examined. The effectiveness of magnetic biostimulation on presown sunflower seeds, growth parameters of seedlings (biomass, root and shoot length, fresh and dry weight of roots, shoots, leaf, and height of plants), and antioxidant activities were also studied. It has been revealed that magnetic treatment at 50 mT/45 min greatly influenced the growth parameters, including mean germination growth (100 ± 0.02) and final emergence rate. Concerning the antioxidant parameters, seed variety FH620 at 500 μg/μL concentration showed significant results compared to other varieties. FTIR was employed to determine the conformational changes and functional groups of organic compounds from sunflower seedlings. Tocopherol analysis by HPLC showed that magnetic treatment at 50 mT/45 min had a higher concentration of vitamin E compared to the control group. These modifications indicated that magnetic field induction enhanced seeds' inner energy that led to seedlings' growth and development enhancement. Besides, magnetic field pretreatment has been shown to have a beneficial influence on sunflower seeds and their bioactive compounds. Future studies should be focused on growth characteristics at the field level and yield attributes.

1. Introduction

Presown magnetic biostimulation of sunflower seeds has a significant effect on germination parameters. Because of magnetic treatment, the mobilization of stored protein contents plays a significant role in antioxidant activity. In the present time, to support the agriculture system that has been considered as a backbone of the global economy and food chain, scientists are working on technologies including physical treatments, biotic and abiotic stress that have a great effect on the enhancement of crop production and seedling vigor [1, 2].

Therefore, seed priming is the most accessible approach that improves the germination and the nutritional value of seeds. This technique has replaced the old methods for germination of seeds as they cause environmental pollution [3]. For more than 20 years, several techniques have been used for seed improvement. These techniques comprise seed priming, magnetic induction, seed spray drying, and coating [4, 5]. It was hypothesized that treatment with magnetic stimulation results in the increase of free radicals in irradiated seeds that react with free oxygen and leads to hydrogen peroxide formation, which increases the mobilization of stored nutrients.

Pakistan is an agricultural country, and importance is also being given to ethnobotany [6–9]. Sunflower (*Helianthus annuus* L.) is an essential permissive oilseed crop. However, unfortunately, Pakistan is chronically low in edible oil and invests a significant portion of its foreign exchange imports. It is the second-largest food supply in Pakistan, with profitability rising from 0.30 million tons to 2.79 million tons in current history. Sunflowers in Pakistan have two seasons (i.e., spring and winter). The growth of sunflower planted in spring is reasonably slower compared to autumn crop, but the response to the agronomic parameters remains significant [10].

Physical priming is an easy and safe way to increase the germination of seeds using the magnetic field. Magnetic seed treatment brings numerous valuable biochemical, cellular, and molecular processes [11]. Presowing magnetic seed treatment also increases ascorbic acid contents [12]. Magnetic seed stimulation could be employed to recover seed potency by increasing enzymes and proteins' activity [13]. Consequently, without any alteration in the seed's chemical composition, biochemical functions such as ion concentration, electrical charges, and free radicals are enhanced and can make the membrane more permeable. Due to the increased metabolic pathway via ions' free movement, physiological and biochemical properties are also increased [14]. The remarkable increase in plant growth and productivity has been postulated in response to magnetic fields. The germination of seeds under magnetic fields has been studied under several levels of biostimulation. Magnetic field treatment significantly improves seed output in terms of germination velocity [15], seedling length [16], and seedling dry weight compared to unexposed seeds (control). Suitable magnetic field strength increases the germination potential of plant seeds [17].

The response of crops towards the magnetic field is associated with the intensity and exposure time of the magnetic field. It has been hypothesized that the magnetic field treatment would enhance the sunflower plant's nutritional and pharmaceutical value. Therefore, the present study was conducted to evaluate the effect of magnetic field on presown hybrid seeds of sunflower to check the growth parameters of seedlings and antioxidant activities. Treated and controlled seeds (i.e., untreated) were compared for this purpose. Moreover, the impact of treatment on the germination of sunflower seeds was also explored.

2. Materials and Methods

2.1. Chemicals and Reagents. The chemicals used were of analytical grade. Folin-Ciocalteu (FC) reagent, 1,1-dipheny l-2-picrylhydrazyl (DPPH), and trichloroacetic acid (TCA) were purchased from Merck (Darmstadt, Germany), whereas aluminum chloride, sodium carbonate, potassium ferrocyanide, and sodium hydroxide were purchased from Bio-Rad (USA), respectively.

2.2. Magnetic Field Setup. The presowing magnetic treatment of seeds was carried out using an electromagnetic seed stimulator specially designed in the Department of Physics, University of Agriculture, Faisalabad, which was similar to Pietruszewski and Martínez's [18] stimulator in construction. The seed stimulator consisted of two pairs of cylindrical shape coils, each of which consisted of 4000 turns that were coated with copper. The seeds were exposed to magnetic field treatment with various intensities (50, 80, and 100 mT) at different exposure times (45, 30, and 15 min), respectively.

2.3. Seed and Treatment Procedure. Healthy and uniform seeds of different sunflower varieties (FH620, FH615, and FH545) were taken from Oil Seed Department, Ayub Agriculture Research Institute, Faisalabad, Pakistan. For magnetic treatment, seeds were treated with different field intensities (50 mT, 80 mT, and 100 mT) at various exposure times (45 min, 30 min, and 15 min), respectively. An intensiometer was used to measure the intensity of the magnetic field. The seeds were treated and sown in replicates for each dose. Untreated seeds were used as a control.

2.4. Petri Dish Experiment. The treated seeds were sown in sterilized Petri dishes having a diameter of 9 mm in three replicates (100 seeds of each variety and in each Petri plate 18 seeds were kept). A double-layer Whatman filter paper was used in each Petri dish. The filter paper was wet with 2.5 mL distilled water. Water was given daily for seven days [19].

2.5. Antioxidant Activities. The antioxidant activities, including total phenolic contents, total flavonoid contents, DPPH radical scavenging activity, reducing power ability, and total antioxidant activity, were performed.

2.5.1. Extraction Method. Extraction was done by taking (1 : 10 w/v) of the ground sample in the respective solvent (distilled water) in a sterile flask and put on an orbital shaker for three days at 120 rpm to homogenize the mixture. After three days, the filtrate was concentrated using a rotatory evaporator and placed in a refrigerator for 4°C for further study [20].

2.5.2. Determination of Total Phenolic Contents. The total phenolic contents of extracts of seedlings were measured by using Folin-Ciocalteu reagent. The reaction mixture comprised sample extract (200 μL), 10% Folin-Ciocalteu reagent (800 μL), and 7.5% sodium carbonate (200 μL). Mixtures were incubated in the dark at room temperature for 2 hours. The absorbance was taken at 765 nm by spectrophotometer [21]. Gallic acid was used as a standard [22].

2.5.3. Determination of Total Flavonoid Contents. Seedling extract (0.1 mL) was mixed with 0.3 mL distilled water, 0.3 mL (5%) $NaNO_2$ and 0.3 mL of $AlCl_3$ (10%). After incubation of 5 minutes, 0.3 mL of (1 mM) NaOH was added, and the absorbance was taken at 510 nm. The results were expressed as quercetin equivalent per dry matter (mg/g) [23].

2.5.4. DPPH Radical Scavenging Assay. The free radical scavenging activity of the extract was measured *in vitro* by 1, 1-diphenyl-2-picrylhydrazyl (DPPH) assay. The DPPH powder (0.005 g) was added in 100 mL (99.9%) methanol. DPPH solution (5 mL) was mixed with 50 μL of sample extract. The mixture was incubated at room temperature for 30 min, and the absorbance was taken at 517 nm against a blank. A reaction mixture without sample extract served as a control. Ascorbic acid was used as a standard. The percentage of scavenging activity was calculated by the following equation [23]:

$$\%\ \text{scavenging activity} = \frac{\text{control}_{\text{Abs}} - \text{test sample}_{\text{Abs}}}{\text{control}_{\text{Abs}}} \times 100. \quad (1)$$

2.5.5. Reducing Power Ability. The reducing power assay of extracts was measured by mixing 5 mL phosphate buffer (2 M, pH 6.6), 5 mL (1%) potassium ferricyanide, 5 mL trichloroacetic acid (10%), and 0.1 mL of sample extract. The mixture was incubated at 50°C for 20 min and then centrifuged at 3,000 rpm for 10 min. The upper layer of the solution (5 mL) was mixed with 5 mL distilled water and 1 mL ferric chloride (0.1%). The absorbance was taken at 700 nm. Increased absorbance of the mixture indicates increased reducing power [24]:

$$\%\ \text{reducing power} = \frac{\text{test sample}_{\text{Abs}} - \text{control}_{\text{Abs}}}{\text{control}_{\text{Abs}}} \times 100. \quad (2)$$

2.5.6. Total Antioxidant Activity. The total antioxidant activity of the extract was calculated by the method of Murthy et al. [25]. Ammonium molybdate reagent solution was prepared in 0.6 M of H_2SO_4 (1 mL). Sodium phosphate buffer (20 mM, pH 6.8) and ammonium molybdate (4 mM) were added in 20 mL of distilled water, and the final volume was made to 50 mL The testing sample (1 mL) was mixed with 2 mL of ammonium molybdate reagent and incubated at 30°C for 60 min. The absorbance was taken at 665 nm against a blank, which contained reagent only. Total antioxidant activity (%) of the standard and extracts was determined using the following formula:

$$\text{total antioxidant activity}\ \% = \frac{\text{control}_{\text{Abs}} - \text{sample}_{\text{Abs}}}{\text{control}_{\text{Abs}}} \times 100. \quad (3)$$

2.6. Fourier Transforms Infrared Spectroscopy (FTIR) Analysis. By using an FTIR spectrometer, an assessment was conducted out. The KBr/Germanium beam splitter was found within this instrument. At a range of 4000–1000 cm^{-1}, FTIR spectra have been acquired. ATR plates were washed thoroughly with *n*-hexane solution to avoid the traces of the previous samples. ATR cleanliness was monitored by collecting previous sample spectra that were compared with previous ones [26].

2.7. HPLC Analysis. The HPLC analysis (Jasco PU-980 intelligent HPLC pump) was done by Sayban Group of Pharmaceuticals, Lahore, Pakistan, to detect the functional groups. The UV was used as a detector at 295 nm. The experimental settings were improved by column HSA chiral C18 (250 mm × 4.6 mm, 5 μm). Acetonitrile and methanol were used as the mobile phase with a flow rate of 0.8 mL/min [27].

2.8. Statistical Analysis. All determinations were made in complete randomized designs. The presence or absence of significant difference among different factors was curtained with the analysis of variances (ANOVA). The means were compared with least significant difference (LSD) at the level of significance of 0.01. Overall interaction of all the factors was checked for significance using computer software CoStat CoHort (6.4).

3. Results and Discussion

3.1. Germination Parameters. The application of different intensities of the magnetic field plays a significant role in sunflower seedling growth parameters. The effect of magnetic field stimulation on selected sunflower seed varieties' germination parameters is given in Table 1. Generally, response to the magnetic field induces the plant growth of sunflower seeds. Similarly, the treatment of seeds with different magnetic field intensities improves germination [28, 29]. The results of the present work showed that the magnetic treatments led to a slight increase in the germination of some varieties, which is in accordance with the results of Iqbal et al. [30]. The magnetic field interface and disclosure time, such as 50 mT/45 min and 80 mT/30 min, were most effective. It was found that the weak magnetic field at longer exposure (50 mT/45 min) resulted in an increased percentage of mean germination time when seeds were placed in a magnetic field [18]. As the magnetic field strength increases (e.g., 80 mT/30 min and 100 mT/15 min), the germination rate gets reduced [28, 29].

Similarly, in the case of final emergence percentage (FEP), the suitable combination of magnetic field stimulation with time exposure influenced greatly the final emergence percentage. Seed variety of FH620 showed a significantly high percentage of mean germination time and final emergence percentage.

3.2. Effect of Magnetic Field on Phenolic and Flavonoid Contents of Sunflower Seed Varieties. Total phenolic and flavonoid contents of selected sunflower seed varieties are given in Table 2. It was revealed that the treatment 80 mT/30 min of FH620 variety and control of FH615 variety gave the highest phenolic contents, while equal amounts of phenolic contents were found at treatments 50 mT/45 min and 100 mT/15 min for FH545 variety. Similarly, the highest

Table 1: Effect of magnetic field stimulation on germination parameters of sunflower seeds.

Sunflower seed varieties	Treatments	Final emergence % age	% age of mean germination time
FH620	50 mT/45 min	100 ± 1.00^{a}	50 ± 0.06^{a}
	80 mT/30 min	80 ± 1.3^{b}	40 ± 0.46^{b}
	100 mT/15 min	94 ± 0.89^{c}	47 ± 0.59^{c}
	Control	100 ± 1.00^{d}	50 ± 0.82^{d}
FH615	50 mT/45 min	80 ± 1.3^{d}	40 ± 0.24^{d}
	80 mT/30 min	80 ± 1.4^{e}	40 ± 0.83^{f}
	100 mT/15 min	80 ± 1.7^{f}	40 ± 0.40^{g}
	Control	93 ± 0.89^{d}	34 ± 0.24^{e}
FH545	50 mT/45 min	68 ± 1.67^{d}	47 ± 0.51^{e}
	80 mT/30 min	68 ± 1.64^{g}	34 ± 0.83^{f}
	100 mT/15 min	68 ± 1.76^{g}	34 ± 0.44^{h}
	Control	86 ± 0.97^{h}	43 ± 0.87^{h}

The data presented in the table are the mean values of three replications ± stander error. Level of significance is indicated by different alphabetical letters.

Table 2: Effect of magnetic field on total phenolic and total flavonoid contents of sunflower seed variety extracts.

Sunflower seed varieties	Treatments	Total phenolic contents (mg/g of dry matter)	Total flavonoid contents (mg/g of dry matter)
FH620	50 mT/45 min	76.22 ± 0.21^{b}	61.31 ± 0.71^{c}
	80 mT/30 min	81.68 ± 0.51^{a}	49.78 ± 0.46^{e}
	100 mT/15 min	71.68 ± 0.32^{c}	67.80 ± 0.51^{b}
	Control	70.77 ± 0.34^{c}	60.1 ± 0.82^{c}
FH615	50 mT/45 min	48.05 ± 0.30^{h}	67.80 ± 0.24^{b}
	80 mT/30 min	63.04 ± 0.11^{e}	51.31 ± 0.83^{d}
	100 mT/15 min	57.59 ± 0.54^{f}	78.79 ± 0.11^{a}
	Control	63.5 ± 0.89^{e}	68.90 ± 0.24^{b}
FH545	50 mT/45 min	68.95 ± 0.61^{d}	51.38 ± 0.51^{d}
	80 mT/30 min	58.95 ± 0.54^{f}	51.31 ± 0.83^{d}
	100 mT/15 min	68.95 ± 0.61^{d}	78.79 ± 0.11^{a}
	Control	58.95 ± 0.54^{f}	66.70 ± 0.68^{b}

Level of significance indicated by different alphabetical letters.

amounts of total flavonoid contents were observed at treatment 100 mT/15 min for all three selected varieties. Polyphenols are chemical substances that contain aromatic compounds with hydroxyl groups. Phenolic compounds found in plants act as natural sources of antioxidants. Phenolic compounds inhibit auto-oxidation and prevent lipid oxidation by inhibiting the lipoxygenase enzymes [31, 32]. Phenolic and polyphenolic compounds contribute directly to antioxidative action and are found as natural antioxidants present in plants [33, 34]. Therefore, it is necessary to calculate the total phenolic contents in plant species. From the findings, it is clear that there is a clear relationship between the total phenolic composition of various sunflower hybrids and their antioxidant capacity. It indicates that seeds also have potent antioxidant activity if they have more phenolic content, and likewise. Numerous findings on the relationship between phenolic content and antioxidant properties have been recorded [29].

Plants contain a complex of antioxidant system including phenolic and flavonoid contents that help to protect the reactive oxygen species. Usually, antioxidant activities are high at a lower concentration of sample that decreases with increasing concentration of the sample and becomes pro-oxidant that inhibits the reactive oxygen species strongly. Flavonoid components and tocopherol are the most important antioxidants for storage stability in sunflower seeds [34].

The diffusion of charged biological particles in a solution can be oriented with a magnetic field current under the effect of Lorentz force or Maxwell stress. The interaction between the external magnetic field and the internal magnetic field resulting from free radicals' nonpaired electrons has a significant impact on the biological system [35]. The impact of magnetic field energy excitation can be directed positively by distributing energy that accelerates metabolism and leads to better germination [36]. Magnetic field treatment induces molecular transformation to provide cells with better conditions for growth and further development. Flux and intensity and exposure period affect different plant systems positively or negatively [11].

3.3. Effect of Magnetic Field on Antioxidant Parameters. Effect of magnetic field on antioxidant parameters such as DPPH activity, reducing power assay, and total antioxidant activity of selected sunflower seed verities is given in Table 3. 1, 1, 1-Diphenyl-2-picryl-hydrazyl (DPPH) is a strong oxidant

Table 3: Effect of magnetic field on antioxidant parameters of sunflower seed varieties.

Sunflower seed varieties	Treatments	DPPH activity (%)	Reducing power assay (%)	Total antioxidant activity (%)
FH620	50 mT/45 min	59.17 ± 0.21	76.85 ± 0.71**	40.38 ± 0.21
	80 mT/30 min	59.55 ± 0.31	23.14 ± 0.21	62.30 ± 0.21
	100 mT/15 min	60.67 ± 0.41**	42.00 ± 0.32	55.76 ± 0.61
	Control	40.07 ± 0.21	58.85 ± 0.21	64.61 ± 0.82**
FH615	50 mT/45 min	59.17 ± 0.21	49.41 ± 0.30	45.76 ± 0.24
	80 mT/30 min	59.92 ± 0.11	77.42 ± 0.61**	53.07 ± 0.21
	100 mT/15 min	62.17 ± 0.41**	64.85 ± 0.21	55.38 ± 0.61
	Control	58.42 ± 0.21	56.57 ± 0.21	58.84 ± 0.21**
FH545	50 mT/45 min	53.93 ± 0.61	74.85 ± 0.61	60.76 ± 0.51**
	80 mT/30 min	55.80 ± 0.21	56.00 ± 0.21	58.07 ± 0.21
	100 mT/15 min	61.17 ± 0.41**	55.14 ± 0.76	48.46 ± 0.61
	Control	59.17 ± 0.21	83.14 ± 0.21**	60.76 ± 0.21**

Level of significance indicated by "**."

at one atom of the Nitrogen Bridge with an unbound valence electron. Free radical scavenging activity is the basis of the common DPPH antioxidant assay [37]. In sunflower oil, as well as in its shell, antioxidant compounds are found [38]. Many other investigations using the DPPH assay to determine the radical scavenging activity of oilseeds, particularly sunflower, have found significant antioxidant potential values for these seed extracts [39, 40], and these studies are in accordance with the findings for the DPPH assay. The aqueous extract's radical scavenging activity was found to be 58.8 percent by DPPH in striped sunflower seeds.

The yellow color of the reaction mixture changed to different shades of green in the reduction power assay, which is dependent on the sample's reduction power. The antioxidant radicals modify the ferrous form of the Fe3+/ferricyanide complex. An enhanced absorption spectrum at a wavelength of 700 nm is observed with rising ascorbic acid and perillaldehyde amounts. This shows the reduction (electron donation) potential of the test samples, which is a property of the sample concentration [41].

The reducing power of active compounds has been reported to be correlated with antioxidant activity. Therefore, the reducing power of polyphenolic compounds must be calculated to illustrate the connection between their antioxidant effects and the reducing power [42]. The magnetic field has also been documented to stimulate the action of enzymes such as alpha-amylase, dehydrogenase, and protease in seeds [43]. De Souza et al. [44] suggested that when the seeds were handled with a magnetic field, the mean fruit weight, fruit yield, and tomatoes' biological yield were enhanced. When the seeds were subjected to a magnetic field, enhanced germination rate, fresh shoot weight, and seedling length of maize were recorded.

Huang et al. [45] examined the reduction of the strength of sunflower extracts as neutralization of DPPH radicals. By changing the sample's color from purple to yellow because of the electron donation, neutralization of the DPPH radicals could be seen and calculated. By accepting a hydrogen atom from the hydroxyl group of phenolic compounds, the stable DPPH is neutralized, resulting in a reduced shape (DPPH-H). Complete phosphomolybdenum-dependent antioxidant activity based on sample analyte reduction of Mo (VI) to Mo (V) and subsequent formation of a green phosphate/Mo (V) complex at acidic pH typically detects antioxidants such as certain phenolics, ascorbic acid, alpha-tocopherol, and carotenoids [46]. Sunflower is a potential source of natural antioxidants, closely associated with a reduced risk of chronic diseases and protection against harmful free radicals. These natural plant products' antioxidant activity is due to their antioxidant capacity, which enables them to act as reducing agents, donors of hydrogen, and quenchers of single oxygen and chelators of metal [3, 47–49].

3.4. Characterization of Sunflower Hybrid Seed Varieties

3.4.1. Fourier Transforms Infrared Spectroscopy Studies. Fourier Transform Infrared Spectroscopy (FTIR) spectra have been used to evaluate the structural changes and functional groups of sunflower seedling organic compounds. The average wavelength of the spectrum was 1000–3500 cm^{-1}, as shown in Figure 1. The FTIR profiling of untreated sunflower seed extracts shows different functional groups, such as the characteristic absorption band at a broad signal of 3500 cm^{-1} (N-H stretching from amine superimposed to C-H stretching), while C-H (Arene) stretching at 3000 cm^{-1} has been observed. In the seed extract, the abovementioned data indicated the separation of C=O into two peaks. The carboxylic group O-H expanding was overlapped by a 2500 cm^{-1} spectral range that emerged from the alkane group as a band comparable to C-H bending. The FTIR seed extract spectrum showed negligible variations compared to the control group at the most efficient (50 mT/45 min) magnetic field strength (Figure 1). In Table 4, the identified organic groups were described.

These spectra typically consist of several bands that originate from the vibration of carbohydrates, proteins, lipids, and nucleic acids of different functional groups and give different configurations. Generally, spectra consist of many bands originating from the biochemical arrangement of plant species. In general, the band centered around 3500 cm^{-1} illustrates N-H stretching vibrations induced primarily by proteins. The bands among 3000 and 2500 cm^{-1} significantly influence lipid-caused C-H stretching

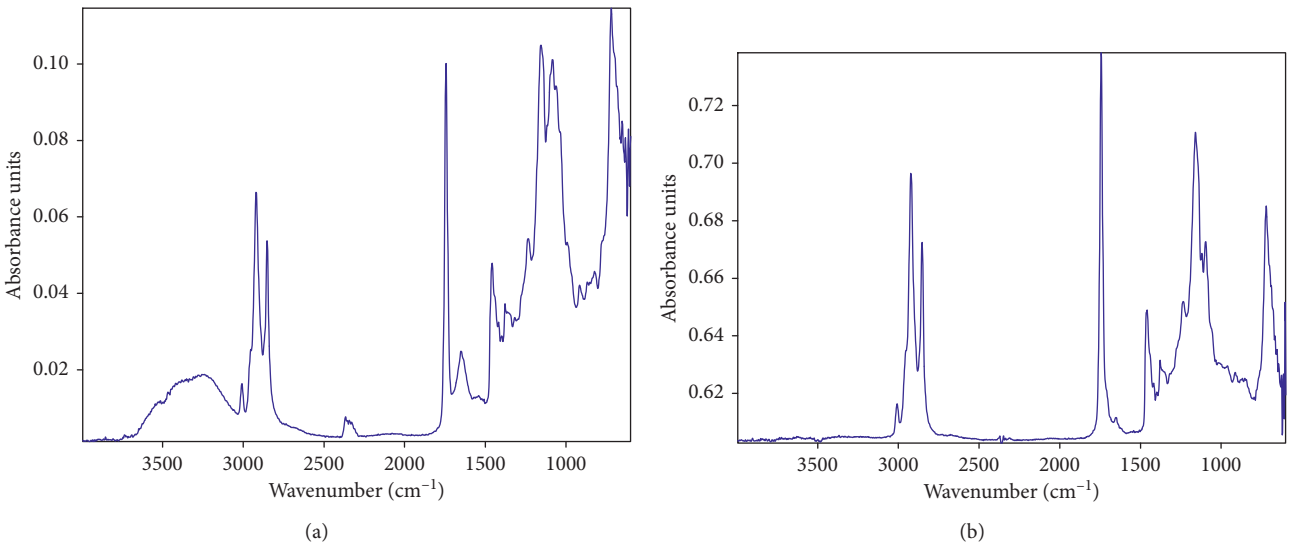

Figure 1: FTIR profile of sunflower seed variety FH620 at 50 mT/45 min magnetic field intensity, (a) control group, (b) magnetically treated.

Table 4: Functional groups detected by FTIR spectra.

Control group		Seed variety FH620 treated at (50 mT/45 min)	
Functional group	Wavenumber (cm^{-1})	Functional group	Wavenumber (cm^{-1})
Amine (N-H group)	3500	—	3500
Arene (C-H)	3000	Arene (C-H)	3000
Caarboxylic acid (O-H)	2500	—	2500
—	2000	—	2000
Areene (C=C)	1500	Areene (C=C)	1500
Ester sp^3 (C-O)	1000	Ester sp^3 (C-O)	1000

vibrations. Overall, in the absorption range, the control spectrum and magnetic field handled samples vary, suggesting apparent differences in the structure and quality of the biological spectrum due to the static magnetic field [35].

3.4.2. Tocopherol (Vitamin E) Analysis by HPLC. HPLC analysis of tocopherol from the most effective sunflower seed variety FH620 is shown in Figure 2. Vitamin E is a major lipophilic antioxidant that has remarkable scavenging activity due to alkoxyl and peroxyl radicals. It protects other plant tissues and the cell membrane from oxidation and helps protect from damage caused by free radicals.

Peak areas of α- and β-tocopherols detected by HPLC are given in Table 5. It was revealed that the concentration of tocopherol had been increased compared to the control group. It was further noticed that by treating seeds with low magnetic field intensity (i.e., 50 mT/45 min) the mobilization of organic molecules increased as many of them play roles in antioxidant activity. Therefore, it has been observed that the antioxidant potential increases as seeds are treated with low magnetic field intensity.

The low intensity of the static magnetic field at a longer exposure time (50 mT/45 min) significantly influenced the biochemical changes, especially antioxidant profiling. A low magnetic field improved the cellular leakage and electrical conductivity that improved the antioxidant potential, including tocopherol contents in sunflower oil extracted from the seeds treated with (50 mT/45 min) magnetic field intensity. The plant growth and the germination of the sunflower were significantly enhanced to respond to the magnetic field. In a study, increased seed germination and seedling growth was observed at a low temperature of 15°C after the application of gamma radiation and magnetic field strength [50]. Similarly, Bahadira et al. [51] reported that seed tubers of potato treated with 150 mT magnetic field strength for 72 h gave the best results for different parameters including plant height, total chlorophyll content, tuber number/plant, and mean tuber weight. An increase in the yield and antioxidant activity was also observed in this study. The magnetic field treatment enhanced the plant's overall growth, and its nutritional value was also found to be improved.

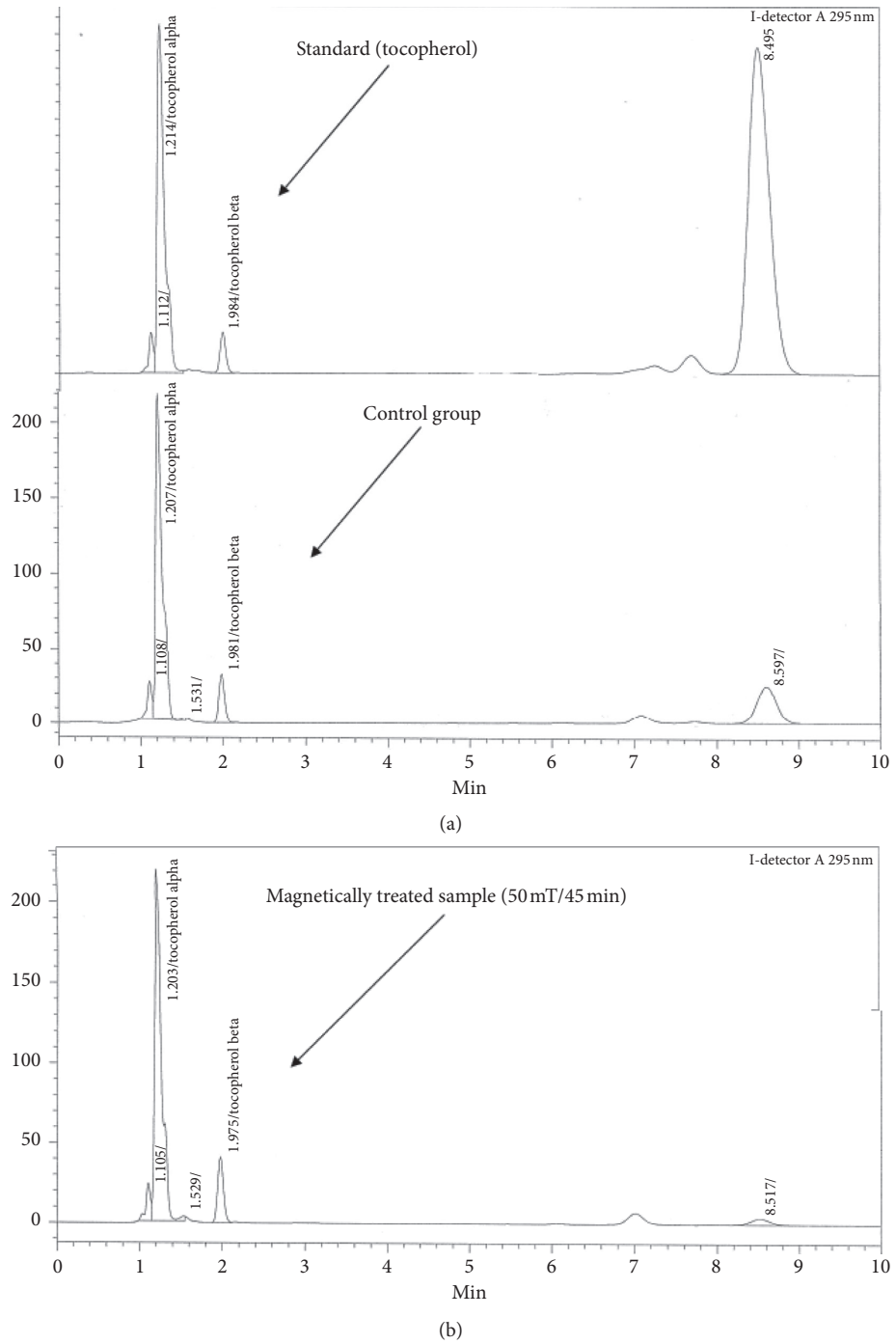

Figure 2: HPLC analysis of tocopherol from sunflower seed variety FH620, (a) control group, (b) at 50 mT/45 min magnetic field intensity.

Table 5: Peak area α-tocopherol and β-tocopherol of FH620 seed variety detected by HPLC.

Sr. no.	Sample	α-Tocopherol			β-Tocopherol		
		Ret. time	Peak area	% age	Ret. time	Peak area	% age
1	Control	1.207	1242937	27.33	1.98	155590	14.02
2	Magnetic treatment (50 mT/45 min)	1.203	1239891	27.05	1.97	200922	48.32

4. Conclusion

The effects of magnetic field treatment on presowing sunflower seed varieties were checked on total phenolic contents, total flavonoid contents, DPPH radical scavenging activity, reducing power, total antioxidant activity, FTIR, and HPLC of tocopherol analyses. This study concluded that the magnetic field intensity (50 mT and 80 mT) is more effective for sunflower seeds. Magnetic field effects at different intensities showed variable effects on the measured parameters. The biostimulation of presowing sunflower seeds positively affected the seed germination and development stages, which led to accelerated germination, yield, and an increase in the percentage of antioxidant activity. Treatment of low magnetic field (50 mT/45 min) improved the antioxidant profile of sunflower seeds that were not studied before and therefore opened a new path to study the metabolic changes. The magnetic field treatment also improved vitamin E, inner energy of seeds, and bioactive compounds present in the plant.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Research Article

Fruit Waste Substrates to Produce Single-Cell Proteins as Alternative Human Food Supplements and Animal Feeds Using Baker's Yeast (Saccharomyces cerevisiae)

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Production of single-cell proteins (SCP) utilizing food wastes is an alternative solution to meet the global protein shortage and minimize pollution problems. Utilization of fruit wastes to produce SCP via fermentation using *Saccharomyces cerevisiae* for animal feed and potential human food was studied. The waste materials such as Mango (*Mangifera indica*), Prickly Custard Apple (*Annona muricata*), Pineapple (*Ananas comosus*), Papaya (*Carica papaya*), Banana (*Musa accuminara Colla*), Mangosteen (*Garcinia mangostana*), Cashew apple (*Anacardium occidentale*), Cacao (*Theobroma cacao*), Jackfruit (*Artocarpus heterophyllus*), and Pomegranate (*Punica granatum*) were used as the substrates for SCP production. Maximum biomass production yield and protein production were significantly higher on the fourth day ($P \leq 0.05$) in all the fruit waste substrates. The maximum dried biomass and the protein production were significantly higher ($P \leq 0.05$) in the PAM substrate (0.429 ± 0.004 g and $48.32 \pm 2.84\%$ resp.) than the others, and PGM substrate yielded significantly lower biomass and protein. Considering the moisture content and ash content, the highest values were observed in JM and BM substrates, respectively, while the least values were observed in CM and PGM substrates. The bulk density values were ranging from 0.31 to 0.61 g/cm^3. The values for water absorption capacity and oil absorption capacity (mL/g) were high in all substrates, and they were comparable to each of them.

1. Introduction

The world population is overgrowing, but the individual dietary protein requirement is not fulfilled consistently since industrialization increases without full fill human needs and requirements towards a healthy life. Protein supply poses a problem because essential amino acids cannot be replaced [1]. Although the developed countries have sorted out different ways to fulfill their protein supplementations, the developing countries are unable to build up their production capacity and economy to meet the demand for protein manufacturing. This results in the malnourishment of a vast population, especially infants and children [2]. The specific protein deficiency disorders in the people suffering from malnutrition are Kwashiorkor and Marasmus [3]. Proteins are essential biomolecules, function as structural components of cells, tissues, muscles, and organs, and are necessary to carry out the metabolic process and production of enzymes and some hormones [4].

SCP is dried cells of microorganisms used as a protein supplement in human foods or animal feeds by extracting the total amount of proteins from pure cultures or cocultures of bacteria, yeasts, fungi, and microscopic algae [5]. Most SCPs are used as an animal feed supplement, and very few are documented as food for human consumption [6]. Fats, carbohydrates, nucleic acids, vitamins, and minerals are present in SCPs [7]. Lysine and methionine are the abundant amino acids present in SCPs, which are limiting in most plant and animal-based diets [8]. There are essential requirements when producing SCPs, such as physically or

chemically pretreated carbon source, nitrogen, phosphorus, and other nutrients. Those components are needed for the optimal growth of the selected microorganism. Also, maintenance of sterile conditions or hygienic conditions is essential to prevent contamination, pure microorganism culture, and adequate aeration must be provided due to the high aerated process [9].

Although to produce biomass of SCPs, bacteria, yeast, fungi, and algae are used widely, yeast SCP is a high-nutrient feed substitute [10]. The advantages of yeast to use as a common microorganism to produce SCPs are being easy to harvest because they are larger than bacteria, high level of malic acid content, high lysine content, the ability to grow at acidic pH, and long history of traditional use [11]. Common substrate for SCP production is starch, molasses, fruit, and vegetable wastes.

Using plant-based substrates to produce SCPs becomes a more effective method to overcome the problem of waste management and leads to minimum cost for raw materials [12]. Further, agricultural waste as a source for SCPs could contribute to conserving the fixed carbon resources produced. The present investigation was carried out to assess the potential of various fruit wastes for cost-effective yeast biomass production. In this study, the waste materials (peels/mesocarps) of Mango (*Mangifera indica*), Prickly Custard Apple (*Annona muricata*), Pineapple (*Ananas comosus*), Papaya (*Carica papaya*), Banana (*Musa* sp.), Mangosteen (*Garcinia mangostana*), Cashew apple (*Anacardium occidentale*), Cacao (*Theobroma cacao*), Jackfruit (*Artocarpus heterophyllus*), and Pomegranate (*Punica granatum*) were introduced as a potential substrate for fermentation to produce bio protein which can be used in food as such or as animal feed.

2. Materials and Methods

2.1. Materials. In this study, waste materials of Mango (*Mangifera indica*), Prickly Custard Apple (*Annona muricata*), Pineapple (*Ananas comosus*), Papaya (*Carica papaya*), Banana (*Musa* sp.), Mangosteen (*Garcinia mangostana*), Cashew apple (*Anacardium occidentale*), Cacao (*Theobroma cacao*), Jackfruit (*Artocarpus heterophyllus*), and Pomegranate (*Punica granatum*) were used as a carbon source for fermentation by Baker's yeast. All the fruits and Baker's yeast (Mayuripan brand) were purchased from the supermarket in Colombo (Kiribathgoda), Sri Lanka.

2.2. Preparation of Fruit Waste Media. The fruits were washed five times with regular tap water and twice with sterile distilled water, and the remaining water was removed by sterile cotton. The peels of fruits were collected to further experiments.

They were blended separately with sterile distilled water in a 1 : 1 ratio (g/g) to obtain a pulp. Then, the blended fruit waste pulps were filtered through a muslin cloth to trap the solid residues. Then, 100 ml of the above pulps were separately added to 250 mL Erlenmeyer flasks and autoclaved at 121°C for 15 psi and 15 minutes. Samples were prepared in triplicate.

2.3. Preparation of Baker's Yeast Culture. Baker's yeast was soaked in sterile sugar water (1 : 4 w/w) overnight. Then, it was cultured on Yeast Peptone Dextrose Agar media (YPDA), which contains Yeast Extract 10 gL^{-1}, Peptone 20 gL^{-1}, Dextrose 20 gL^{-1}, Agar 15 g/L to confirm it as a pure culture by observing the pure yeast colonies. The cultures were maintained in YPDA plates and as glycerol stocks.

2.4. Media Preparation for Fermentation. The fruit waste pulp media was prepared with the following compositions [13]. The fruit waste pulp 100 mL/L was mixed separately with end concentration of KH_2PO_4 1.0 g/L, $MgSO_4.7H_2O$

0.5 g/L, NaCl 0.1 g/L, $CaCl_2$ 0.1 g/L, and the total volume of the mixture were maintained to 1000 mL by adding distilled water. The pH of the media was maintained at 5.0 by adding 1.0 N.

H2SO4 or 1.0 N NaOH: The mixture was autoclaved at 121°C for 15 psi and 15 minutes. Then, 50 mL of each mixture was inoculated with baker's yeast in sterile 1X PBS (500 μL), at $OD_{600} = 0.5$. Then, the inoculated flask was placed on a shaking incubator at 100 rpm at room temperature. After fermentation, the mixture in the flask was poured into a centrifuged tube and centrifuged at 4000 rpm for 20 minutes. Then, the sediment was collected and weighed before drying. After that, collected sediment was oven-dried at 50 °C for 16 hours until getting constant weight. According to the Kjeldahl method, dry weight was measured, and protein content was estimated at every two days' interval for eight days.

2.5. Moisture Content. The moisture content was calculated according to the following formula.

$$\text{Moisture content} = \frac{(\text{Initial weight} - \text{Final weight})}{\text{Initial weight}} \times 100\%. \quad (1)$$

2.6. Ash Content. Two grams (2 g) of SCP was kept in a muffle furnace at 500 ± 5°C for 24 hours, and the final weight was measured after cooling the mass in a desiccator. The ash content was calculated according to the following formula.

$$\text{Ash content} = \frac{(\text{Final weight of ash left})}{\text{Initial weight before drying}} \times 100\%. \quad (2)$$

2.7. Bulk Density. Five grams (5 g) of SCP obtained from different fermentation media were separately placed in a polypropylene measuring cylinder and taped until no apparent reduction of the volume was observed. The bulk density was expressed as $g\,cm^{-3}$.

2.8. Water Absorption Capacity. Water absorption capacity (WAC) was measured according to the procedure described by [14]; ten milliliters (10 mL) of distilled water was added to 2 g of SCP from each fermentation media and allowed in a static position. After 30 minutes, each mixture was centrifuged at 2000 rpm for 45 min. WAC was expressed as percent water bound with each SCP.

2.9. Oil Absorption Capacity. Two grams (2 g) of SCP was mixed with soybean oil (specific gravity: 0.902) and allowed in a static position for 30 minutes at room temperature. Then, each mixture was centrifuged at 2000 rpm for 45 min and expressed as percent oil bound with SCP [14].

Media for each fruit were denoted as MM, PCAM, PAM, PM, BM, MSM, CAM, CM, JM, and PGM for Mango (*Mangifera indica*) media, Prickly Custard Apple (*Annona muricata*) media, Pineapple (*Ananas comosus*) media, Papaya (*Carica papaya*) media, Banana (*Musa*) media, Mangosteen (*Garcinia mangostana*) media, Cashew apple (*Anacardium occidentale*) media, Cacao (*Theobroma cacao*) media, Jackfruit (*Artocarpus heterophyllus*) media, and Pomegranate (*Punica granatum*) media, respectively.

2.10. Statistical Analysis. All the experiments were done in triplicate, and biological replicates were carried out unless otherwise indicated. The mean values and standard deviations were used to plot the graphical representations. A paired sample *t*-test was carried out for the determination of significant differences ($P \leq 0.05$) between the mean values with IBM SPSS 23 software.

3. Results and Discussion

Proteins, fats, carbohydrates, ash ingredients, water, and other elements such as phosphorus and potassium are rich in SCPs [15]. Aside from the nutritional benefits of SCP, another benefit of SCP technology is constant production throughout the year. Water management strategy plays a vital role by using the waste material as the substrates [16]. A small area of land is required, and SCP is made in less time. In this study, the above fruit wastes were used according to bioavailability and affordability. These fruit wastes are one of the locally available agrowaste rich in organic matter that can be used as carbon and energy sources for microorganisms to grow to SCP production. The study investigated the viable potential of selecting peels/mesocarps of Mango (*Mangifera indica*), Prickly Custard Apple (*Annona muricata*), Pineapple (*Ananas comosus*), Papaya (*Carica papaya*), Banana (*Musa*), Mangosteen (*Garcinia mangostana*), Cashew apple (*Anacardium occidentale*), Cacao (*Theobroma cacao*), Jackfruit (*Artocarpus heterophyllus*) and Pomegranate (*Punica granatum*) as a substrate for SCP production. Through heat treatments, these wastes were converted to fermentable sugar.

Yeasts are active in an extensive temperature range with an optimum temperature of 32°C–35°C [17]. It is essential to maintain the pH when growing yeast cultures [18]. To assess the nutritional value of SCP, it is essential to consider factors such as nutrient composition, amino acid profile, and vitamin and nucleic acid content, as well as palatability, allergies, and gastrointestinal effects [19].

Batch fermentation can be considered as a closed system [20]. Initially, when the time is zero, the sterile nutrient solution in the fermenter is inoculated with microorganisms and incubated to ferment. As a result of the metabolism of the cells, the composition of the culture medium generally changes (the biomass concentration and the metabolite concentration). Under optimum physiological conditions, the inoculation of a sterile nutrient solution with microorganisms and cultivation can undergo four typical phases of growth, namely, the lag phase, log phase, stationary phase, and death phase [21]. The growth of the newly inoculated batch typically follows these mentioned phases. Initially, in the lag phase, the cell concentration does not increase very much [22]. But, when the composition of the medium and the environmental conditions in the seed culture is identical with no need for adaptation, a short lag phase occurs.

Figure 1 illustrates the dried biomass with the days' interval of each media. Considering each media overall, the dried biomass increased with the increasing four days suggesting that fermentation progressed gradually in those days. The optimum biomass was obtained on the 4^{th} day in all the fruit waste media, and the biomass yields were decreased gradually, suggesting nutrient depletion in the growth media. Those values are 0.383 ± 0.002, 0.342 ± 0.004, 0.429 ± 0.004, 0.400 ± 0.003, 0.321 ± 0.004, $0.301 \pm .002$, $0.391 \pm .001$, 0.360 ± 0.003, 0.370 ± 0.002, and $0.244 \pm .002$ g for MM, PCAM, PAM, PM, BM, MSM, CAM, CM, JM, and PGM, respectively. Though the biomass yield was higher ($P \leq 0.05$) in all days in PAM comparing to other media, the least biomass yield ($P \leq 0.05$) was in PGM in all the days' intervals. This may be the high sugar content and nutrients in PAM. Over the decades, several studies have focused on exploiting pineapple waste as animal feed. However, some researchers have reported the unattractive nature of by-products from the pineapple processing industry due to its high fiber content and soluble carbohydrates with low protein content. This investigation agrees with [23] reporting that pineapple waste is the best substrate for the production of yeast biomass. The growth of fungi depends on the nutritional composition of the waste material and that can help to increase the mass [24]. Since Mango contains more nutrients, it can also be used to produce a sufficient amount of SCP [25]. Further, considering the carbohydrates and other nutrients, banana waste also contains a high amount of chemical compositions than other waste, and it supports faster growth of the fungus [26]. Although the carbohydrate composition in waste of Mangosteen and Pomegranate is not comparable to Pineapple waste, that media also contain considerable biomass in dry weight [27, 28]. Jack fruit peels also contained a high amount of carbohydrates and protein amount, which is a good source to grow yeast or other fungi to produce SCPs [29]. Although Cashew apple is rich in minerals and vitamins, it contains a few amounts of carbohydrates and proteins, which can be used as a substrate to yeasts [30].

Measuring protein content is very important when considering the SCPs. Figure 2 illustrates the total protein percentages of each fruit waste pulp media with the days' intervals. Overall, the protein percentages increase in the first four days in each sample and then decrease gradually. This is because the nitrogen supplementation/dissolved nitrogen in the media is limited to the increasing growth rate. Further, other nutrient factors also are affected by the final production of nitrogen mass. According to Figure 2, the significantly ($P \leq 0.05$) highest protein percentage was observed in PAM, and the significantly ($P \leq 0.05$) lowest

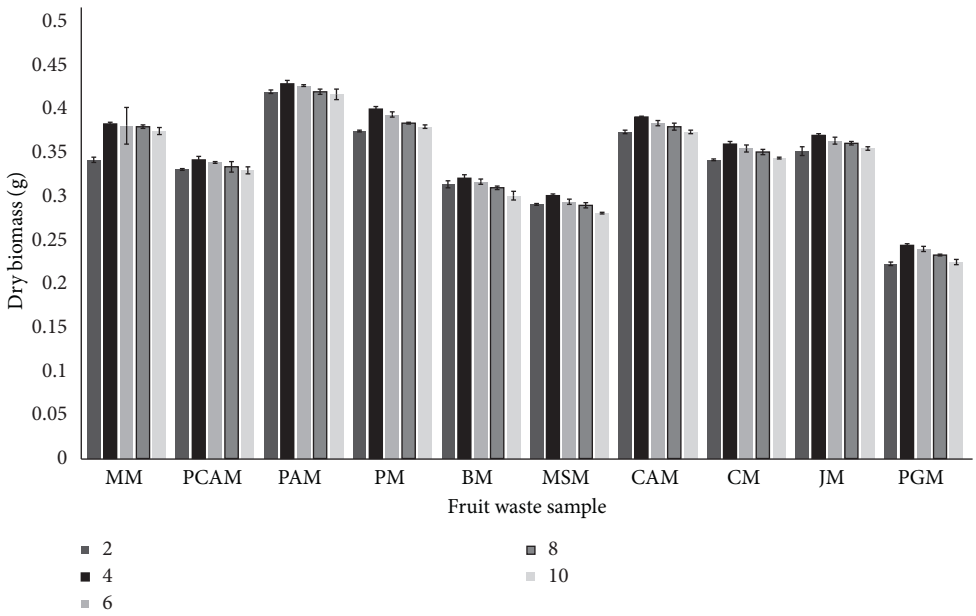

Figure 1: Dried biomass (g) of Bakers' yeast yield, produced in the various fruit wastes media in submerged fermentation at 100 rpm on a time course basis. Each data point represents the mean ± standard deviation ($n = 3$, $^*P \leq 0.05$).

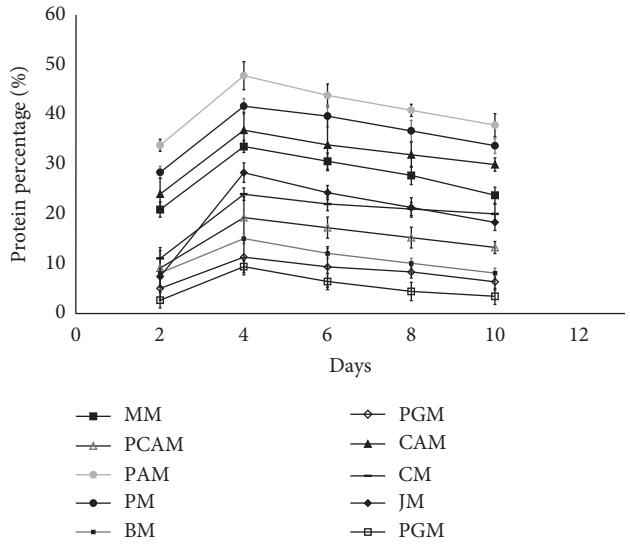

Figure 2: Protein content (%) of Bakers' yeast biomass using various fruit wastes media in submerged fermentation at 100 rpm on a time course basis. Each data point represents the mean ± standard deviation ($n = 3$, $^*P \leq 0.05$).

protein percentage was in PM. This is maybe due to the high nitrogen content in the PAM [31] and low content of nitrogen in the PM [32]. The protein percentages on the fourth day are 33.98 ± 2.21, 19.58 ± 4.21, 48.32 ± 2.84, 42.14 ± 1.56, 15.32 ± 3.64, 11.57 ± 3.58, 37.28 ± 3.64, 24.31 ± 1.28, 28.68 ± 1.98, and 9.64 ± 1.22% for MM, PCAM, PAM, PM, BM, MSM, CAM, CM, JM, and PGM, respectively. Production of yeast protein is correlated with the nitrogen compound used in the culture medium [33]. Although protein content was calculated by multiplying total nitrogen by 6.25, Figure 2 obtained for protein must consider other nitrogenous compounds such as purine, pyrimidine, nucleic acid, and amino sugars, in addition to true protein. Therefore, the amino acid profile of the yeast protein is more important if we consider it in amino acid levels. This suggests that amino acid content takes on considerable importance if yeasts are to be utilized as an inexpensive source of protein to supplement poor quality proteins or to serve as components of protein-rich foods.

In food processing and testing, measuring moisture content is one of the most commonly used measurements. It is due to the relation of moisture content with the alteration of food during the storage and processing and hence affects the final quality. The moisture content in percent was calculated from the weight loss, and the results are shown in Table 1. The highest moisture content was observed in the JM media significantly ($P \leq 0.05$) while the least value was in CM significantly ($P \leq 0.05$). The values in MM, PCAM, PAM, PM, MSM, and PGM for moisture content were significantly ($P \leq 0.05$) different from other values, while the values of CM were different from all the other samples significantly ($P \leq 0.05$). Overall, the values of moisture content are varying from 6 to 9%. Measuring ash content refers to the inorganic residue remaining after either ignition or complete oxidation of organic matter in a food sample. The inorganic residue of the food sample refers to the total mineral present in the sample. So, the total ash content represents the proximate analysis for nutritional evaluation. The ash content of SCP was expressed as percentages of the dry weight Table 1. The significantly ($P \leq 0.05$) highest ash content was observed in BM, and the least value was observed in PGM, suggesting that the highest total mineral content was in BM while the least value was in PGM. The values of MM, CAM, JM, and PGM were significantly ($P \leq 0.05$) different from all other samples, while the value of BM is significantly ($P \leq 0.05$) different from all other

Table 1: Proximate composition and functional properties of SCP from various substrates.

	Moisture content (%)	Ash content (%)	Bulk density g/cm^3	Water absorption capacity mL/g	Oil absorption capacity mL/g
MM	7.12 ± 0.62^a	6.23 ± 0.66^a	0.53 ± 0.12^a	2.14 ± 1.01^a	1.89 ± 0.12^a
PCAM	7.52 ± 0.88^a	7.45 ± 0.35^b	0.45 ± 0.52^a	1.98 ± 0.12^a	1.71 ± 0.15^a
PAM	7.08 ± 0.32^a	7.86 ± 0.51^b	0.43 ± 0.14^a	1.96 ± 0.37^a	1.70 ± 0.21^a
PM	6.94 ± 0.41^a	7.51 ± 0.42^b	0.44 ± 0.47^a	2.02 ± 0.52^a	1.68 ± 0.23^a
BM	7.58 ± 0.56^b	8.91 ± 0.12^c	0.54 ± 0.09^a	2.20 ± 0.67^a	1.78 ± 0.39^a
MSM	6.97 ± 0.21^a	8.11 ± 0.28^b	0.61 ± 0.31^a	2.51 ± 0.97^a	1.81 ± 0.12^a
CAM	8.02 ± 0.88^b	6.30 ± 0.75^a	0.48 ± 0.12^a	1.80 ± 0.12^a	1.71 ± 0.74^a
CM	6.34 ± 0.45^c	5.64 ± 0.33^d	0.31 ± 0.19^a	1.74 ± 0.37^a	1.59 ± 0.39^a
JM	8.75 ± 0.30^d	6.23 ± 0.15^a	0.53 ± 0.44^a	2.02 ± 0.41^a	1.83 ± 0.16^a
PGM	7.35 ± 0.55^a	6.21 ± 0.48^a	0.54 ± 0.67^a	2.06 ± 0.09^a	1.90 ± 0.12^a

samples. Functional properties of a food sample describe how ingredients behave during preparation and cooking and how they affect the finished food product in terms of how it looks, tastes, and feels. The bulk density is described as the mass of many particles of the material divided by the total volume they occupy, which includes interparticle void volume, particle volume, and internal pore volume. Considering overall bulk density values of SCP, the values were ranging from 0.310.61 g/cm3, and they were comparable. SCP produced in fruit waste has higher water absorption and oil absorption capacity comparatively which indicate the implementation in bakery industries; thus, it is an important functional property such as the consistency, mouthfeel and flavor stability, and enhancing and preservation.

Letters a, *b*, and *c* were used to compare statistical significance ($P \leq 0.05$) in the same column. Each data point represents the mean ± standard deviation ($n = 3$).

These results can be compared with [34], which is a similar work that has been done with pineapple wastes. Further, proper aeration of the growth medium is very important in fermentation. Inadequate aeration in baker's yeasts propagation results in the production of ethyl alcohol rather than cell substance, which is reflected as a loss in cellular yield, suggesting that overaeration, however, serves no useful purpose and hence maintaining the proper growth conditions are required.

4. Conclusions

The above-mentioned fruit waste substrates are readily soluble in the growth medium, and the problem of contact of the yeast with the substrate is not encountered. The oxygen requirements in such systems are high due to the fact that the hydrocarbons are in a lower state of oxidation than the usual carbohydrates. These substrates are easily available, do not require separation or solvent removal, and require less agitation and cooling than hydrocarbon fermentation because these substrates release lower heat than hydrocarbons. The SCPs by yeast mainly depend on the cost of the substrate and the capital investment, including all associated costs such as collection, transportation, purification or separation, and sterilization. Since tremendous amounts of fruit wastes are available, the cost of the raw material will be low or negligible.

Since fruit waste substrates do not need purification and separation, preparation costs would be minimal compared with the use of hydrocarbons or other wastes. But if cellulose is used as a substrate, pretreatment, that is, acids, alkalis, and size reduction, is necessary to improve enzymatic hydrolysis rates. These considerations are of considerable significance in cases where the incorporation of low-technology approaches is essential.

Data Availability

All the data have been indicated in the paper either as tables or graphs (figures).

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Research Article

Effects of Seed Priming with Zinc Sulfate on Nutritional Enrichment and Biochemical Fingerprints of *Momordica charantia*

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Zinc is an essential element for plant growth and development as it plays an important role in various metabolic processes with nutritional enrichment. The treatment with zinc sulfate is also economic. *Momordica charantia* is an economically important medicinal plant reported for a range of pharmaceutical and pharmacological properties. In this study, nutripriming with zinc sulfate (0.1%, 0.2%, and 0.3% solution) was applied to *M. charantia* seeds to optimize better dose. Based upon seedling establishment, 0.3% zinc sulfate was selected for final field experiment with randomized complete block design (RCBD) with five replications. Improved germination percentage, vigor, total soluble sugars, chlorophyll-a, chlorophyll-b, total chlorophyll content, and peroxidase activity were observed variably in leaves, fruit, and peel. Other nutritive components showed maintenance in fruits of treated plants indicating that the treatment did not cause any nutritive loss. Antimicrobial activity of leaves (in terms of the minimum inhibitory concentration) against *Staphylococcus aureus* and *Pseudomonas aeruginosa* was positively correlated with sinapic acid, vanillic acid, cinnamic acid, syringic acid, chlorogenic acid, benzoic acid, and ferulic acid. It has been concluded from this study that seed priming with zinc sulfate can improve seedling establishment, photosynthetic pigments, and stable nutritive value. Therefore, zinc from zinc sulfate priming has been proved as a beneficial fertilizer for *M. charantia* plant growth, yield, and nutraceutical potential.

1. Introduction

Seed priming is one of the promising techniques that impart positive effect upon plant growth, yield and metabolism. Pre-sowing seed treatments range from hydropriming, chemical, and biological to physical ones [1]. Improved seed performance in the field has been observed in response to these priming agents in terms of improved germination rate, metabolic pool, and stress-tolerance amongst all halopriming being comparatively easy to manage by a farmer is practiced more than other strategies [2, 3].

Zinc is a micronutrient with versatile contributions in plant physiology. It participates in regulation of different enzymes of primary and secondary metabolism and particularly related to photosynthesis and hormonal regulation. For years, plant scientists and farmers have been working on zinc supplementation. Major emphasis has been upon addition of zinc salts in rooting medium [4]. In recent literature, there has been focus upon different zinc nanoparticles too [5]. Root supplementation needs bulk of chemicals and therefore becomes expensive and non-friendly for environment. Nanoparticles need expensive series of technical

work before plant growth. Hence, seed priming with zinc salts may be a better option being economic and ecofriendly.

Momordica charantia (bitter gourd) has God-gifted nutraceuticals and antioxidants. Therefore, extracts of *M. charantia* have been reported with decreasing blood sugar level in diabetes type II patients. Bitter gourd improves the metabolic problems and has positive effects on the glucose metabolism. Because of high antioxidant concentration, the bitter gourd extract has the ability to inhibit the growth of cancer cells as antioxidants protect cells from the damage caused by free radicals, environmental toxins, and poor nutrition. *a* and *ß* carotene, zeaxanthin, and lutein are the antioxidants which are present in great amounts in bitter gourd [6]. A rich quantity of vitamin A also occurs with these antioxidants that protects the body from free radicals and prevents premature aging and other problems [7–9]. Our research group has formerly explored the antimicrobial potential of *M. charantia* leaf extracts against *Staphylococcus aureus* and *Pseudomonas aeruginosa* under the influence of magneto priming [8]. It was hypothesized that the seed priming with zinc sulfate could modulate primary and secondary metabolism of *M. charantia* and hence improves nutraceutical value of plants in terms of antimicrobial, antioxidant, and antiglycation potential.

2. Materials and Methods

The seeds of a local race of *M. charantia* were collected from Ayub Agricultural Research Institute (AARI), Faisalabad, Pakistan. Seeds were taxonomically identified and confirmed from the Department of Botany, Government College University Faisalabad, Pakistan.

2.1. Pilot Experiment. A lab experiment was planned for screening better dose of $ZnSO_4.7H_2O$ (Sigma-Aldrich, analytical grade, purity >99%) as priming agent. *M. charantia* seeds (approx. 30 g) were subjected for presowing seed treatments with zinc sulfate (unprimed, hydroprimed, 0.1%, 0.2%, and 0.3% solution) for 12 hours. The seeds were completely dipped in the solutions. Seeds were sown in soil filled glass pots, five replicates, and completely randomized design. Selection was made on the basis of seedling establishment in terms of seed fresh and dry biomass and germination percentage.

2.1.1. Germination Percentage. Incubation proportion of emergence was calculated at the end of 7th day of incubation test by the process designated by Ijaz et al. [10].

$$Gp = \left(\frac{Ng}{Np}\right) \times 100, \tag{1}$$

where Ng is the last number of emerged seeds and Np is the total number of seeds sown.

2.1.2. Mean Germination Time. Mean growth time (MGT) in days was calculated as follows:

$$MGT = \frac{\sum (Dn)}{\sum n}, \tag{2}$$

where n is number of seeds germinated on day D and n is number of days counted from the beginning of the germination test.

2.1.3. Vigor Index Measurement. Seedling vigor was calculated by following Vashisth and Nagarajan [11].

$$\begin{aligned} &\text{Vigor index I} = \text{germination \%} \times \text{seedling length (root + shoot)},\\ &\text{Vigor index II} = \text{germination \%} \times \text{seedling dry weight (root + shoot)}. \end{aligned} \tag{3}$$

Similarly, number of leaves per plant was manually counted of all plants in each row in the field and mean was calculated. Ten plants were used for the estimation of these traits. Shoot length was calculated using measuring tape from the ground to the ligule of upper most leaf of plants of each pot and then average length of each plant/shoot was calculated. At the time of harvest (7th day), the plants were uprooted form land and root length was recorded with the help of scale and average was calculated. Total fresh weight per plant was calculated by adding shoot and root fresh weight of each plant. Plants were shade-dried and total dry weight per plant was calculated based on dry weight of root and shoot.

2.2. Final Field Experiment. Final field experiment with selected dose (3% $ZnSO_4$) was planned with Randomized Complete Block Design (RCBD) with three replicates and two harvests (at vegetative stage and final maturity stage). Hydroprimed seeds were used as control. A distance of 2.5 to 3.5 m was maintained between rows and there was a gap of 90 to120 cm between two plants. Experiment was conducted in two consecutive growing seasons under natural field conditions. Presented data is based upon average of both experiments.

2.2.1. Photosynthetic Pigments. Photosynthetic pigments were determined following the method of Bukhari et al. [8]. Fresh leaves (0.5 g) were ground in 80% acetone with pestle and mortar. The filtrate was made up to 10 mL and absorbance was taken at 645 nm, 663 nm, and 480 nm using a spectrophotometer. Estimations for quantity of chlorophylls a, b, and total were made by the following formulas:

$$Chl.a\left(\frac{mg}{g}\right) = [12.7O\,D663 - 2.69\,(O\,D645)] \times \frac{V}{1000} \times W,$$
$$Chl.b\left(\frac{mg}{g}\right) = [22.9O\,D645 - 4.68\,(O\,D663)] \times \frac{V}{1000} \times W, \quad (4)$$
$$Total\,Chl.\left(\frac{mg}{g}\right) = [20.2O\,D645 + 8.02\,(O\,D663)] \times \frac{V}{1000} \times W,$$

where V is volume of the acetone used and W is weight of leaf used.

2.2.2. Antimicrobial Activity. Using water, acetone, and methanol as solvents, three extractions of fresh leaf sample (0.1 g for each extraction) were prepared. Then, antimicrobial activities were measured by Broth Micro Dilution Method [12]. Briefly, the microdilution trays were first prepared using 2–6-fold dilutions of each sample extract volumetrically in broth. A new pipette was used for each subsequent dilution step. The extracts were dispensed into the plastic microdilution trays. The bacterial cultures of *S. aureus* and *P. aeruginosa* were grown in proper growth medium to prepare inoculum and 0.01 mL of the suspension was inoculated carefully into the broth. The bacterial growth was maintained approximately at 5×105 CFU/mL (or 5×104 CFU/well in the microdilution method). Using growth method, the standardization of the inoculum was prepared within 15 min. The standardized inoculum was inoculated within 15 min in each well of a microdilution tray using an inoculator device to deliver a volume that would not exceed 10% of the volume in the well. Colony counts of inoculum suspensions were done followed by incubation at $35 \pm 2^{\circ}$C for 16 to 20 h in an ambient air incubator. Finally, the lowest concentration of leaf extracts that completely inhibited the bacterial growth in the microdilution wells was detected expressing the minimal inhibitory concentration (MIC).

2.2.3. Catalase Activity. Catalase activity was assayed following the methods described by Bukhari et al. [8] and Sharma [13] with minor modifications. Briefly, 0.5 g of *M. charantia* samples (each of leaves, peel, and fruit) was taken and homogenized in 1.5 mL of 1 M phosphate buffer (pH 7.0) by grinding in a pre-chilled mortar. The homogenate was centrifuged at 15,000 rpm for 15 min at 40°C and supernatant was used for catalase activity analysis. Hydrogen peroxide (H_2O_2) and phosphate buffer (3.0 mL) were taken in a cuvette, followed by the rapid addition of 40 μL of enzyme extract and mixed thoroughly. The time required for a decrease in absorbance by 0.05 units was recorded at 240 nm in a spectrophotometer (Genesys 10-S, USA). One enzyme unit was calculated as the amount of enzyme required to decrease the absorbance at 240 nm by 0.05 units.

2.2.4. Peroxidase Activity. POD was determined by the methods of Bukhari et al. [8] and Bhargavi et al. [14] using 20 mM guaiacol and H_2O_2 as a substrate. Briefly, 0.5 g of plant material was extracted in 3 mL of 0.1 M phosphate buffer of pH.7.0 by grinding in a pre-chiller mortar. The homogenate was then centrifuged at 18,000 rpm for 15 min at 5°C. Supernatant was used as enzyme source within 2-4 hours, stored on ice till the assay was carried out. Then, 3 mL of the buffer solution, 0.05 mL guaiacol solution, 0.1 mL enzyme extract, and 0.03 mL hydrogen peroxide (H_2O_2) solution were pipetted out in a cuvette. The mixture was well shaken and placed in the spectrophotometer. The time required for the mixture to increase absorbance by 0.1 (Δt) at 430 nm was recorded and used in calculations.

$$\text{The enzyme specific activity units}\left(g^{-1} f.wt.\right) = \left[\frac{500}{\Delta t}\right] \times \left[\frac{1}{1000}\right] \times \left[\frac{TV}{VU}\right] \times \left[\frac{1}{f.\,wt.}\right],$$
$$\Delta t = \text{change in time (min)},$$
$$TV = \text{total volume of extract } (mL), \quad (5)$$
$$UV = \text{volume used } (mL),$$
$$f.wt. = \text{weight of fresh leaf tissues } (g).$$

2.2.5. Total Soluble Proteins. For total soluble proteins, the method of Bradford [14] was followed with some modifications as described by Bukhari et al. [8]. Briefly, 0.5 g of *M. charantia* sample was homogenized with 1.5 mL of 1 M phosphate buffer of pH 7.0 by grinding in a pre-chiller mortar. The homogenate was centrifuged at 12,000 rpm for

15 min at 40°C and supernatant was used for total soluble protein determination. The spectrophotometer was warmed up before use. First of all, Bradford reagent was prepared by treating 0.1 g of Coomassie blue dye with 50 mL of ethanol and 100 mL of orthophosphoric acid. The total volume was made up to 1,000 mL and the mixture was filtered carefully. Then, 0.1 mL of the extract was taken and placed for 15 min at room temperature for proper reaction. A spectrophotometer was used to measure wavelength at 595 nm (Jenway, 6700; Thermo Fisher Scientific; USA). Bovine serum albumin was taken as a standard and calculations were made using the standard curve.

2.2.6. Total Free Amino Acids. Total free amino acids were determined following Noreen et al. [15] with some modifications as described previously by Bukhari et al. [8]. Briefly, 0.5 g of *M. charantia* sample was homogenized with 1.5 mL of 1 M phosphate buffer (pH 7.0) by grinding in a pre-chiller mortar. The homogenate was centrifuged at 12,000 rpm for 15 min at 40°C and supernatant was used for total free amino acids analysis. The extract (1 mL) was taken in a test tube and 10% pyrimidine solution and 1 mL of 2% ninhydrin solution (solution was prepared by dissolving 2 g ninhydrin in 100 mL dist. water) were added. The test tubes containing the mixture were heated at 100°C for 30 min in a water bath and then mixture (i.e., 2.7 mL) was transferred into a 10 mL volumetric flask. The final volume of the mixture was made up to 10 mL with distilled water. The optical density of the solution was measured at 570 nm with the help of a spectrophotometer (Jenway 6700; Thermo Fisher Scientific; USA). Calculations were made using the standard curve prepared with proline.

2.2.7. Total Soluble Sugar. Total soluble sugar was determined by the method defined by Bukhari et al. [8] and Van Handel [16]. Briefly, 0.5 g of *M. charantia* samples (each of leaves, peel, and fruit) was taken and homogenized in 1.5 mL of 1 M phosphate buffer (pH 7) by grinding in a pre-chilled mortar. The homogenate was centrifuged at 15,000 rpm for 15 min at 40°C and supernatant was used for total soluble sugar analysis. The extract (100 μL) was taken in a test tube and 900 μL of dist. H_2O was added. After that, 3 mL of anthrone reagent was added and the test tube was incubated in a shaker for 5 min at 120 rpm and then set in a water bath at 95°C for 10 min. The reaction mixture was cooled and absorbance was taken at 625 nm. The amount of soluble sugars in the sample was calculated using glucose standard curve prepared by plotting concentration of the standard on the *X*-axis versus absorbance on the *Y*-axis.

2.2.8. Total Phenolic Contents. Contents of total phenols were determined as reported by Ustaömer et al. [17] using Folin–Ciocalteu reagent method. The dilutions were made to ensure the oxidation of 1 mL of Folin-Ciocalteu reagent with 200 μL of water followed by the neutralization with 2 mL of 7.5% sodium carbonate (w/v). Finally, this volume was made up to 7 mL with distilled water. The absorbance of the resulting blue color was measured at 765 nm on spectrophotometer with a 1 cm cell after incubation for 2 hours in the dark at room temperature. Gallic acid was used as a standard for the calibration curve.

2.2.9. Total Flavonoid Contents. Total flavonoid contents were determined by colorimetric assay [18] with minor modifications. The diluted sample (1 mL) from the above was added to a 10 mL volumetric flask containing 4 mL of distilled water followed by immediate addition of 0.6 mL of 5% $NaNO_2$, 0.5 mL of 10% $AlCl_3$ after 5 min, and 2 mL of 1 M NaOH after 1 min. Furthermore, each reaction flask was then immediately diluted with 2.4 mL of distilled water and mixed. The absorbance of the pink color solution was noted at 510 nm. The quercetin (μg/g) was used as a standard for the calibration curve.

2.2.10. Antiglycation Activity. In 1 g of sample, 1.5 mL methanol (50%) was added and centrifuged at 1500 rpm for 10 min. The supernatant was used for analysis of antiglycation activity. The advanced glycation end-products (AGEs) formation was assessed by characteristic absorbance [19]. Briefly, the reaction mixture of 150 μL D-glucose, 150 μL bovine serum albumin (BSA in 1 mL sodium phosphate buffer, pH 7.2), and 150 μL sample was incubated at room temperature for 7 days. The absorbance was measured using a spectrophotometer at a wavelength of 440 nm. The reaction mixture without D-glucose was used as a blank. Measurements were performed in duplicate.

2.2.11. Calculation of 50% Inhibitory Concentration (IC50%). The IC_{50} value is a quantitative measure and used to show how much of a particular inhibitory substance will be required for *in vitro* inhibition of a specific biological process or component by 50%. The % inhibition was calculated using the following equation:

$$\%\ \text{inhibition} = \frac{\text{absorbance of control} - \text{absorbance of sample}}{\text{absorbance of control}} \times 100. \quad (6)$$

2.2.12. Quantitative Analysis of Phenolic Profile through HPLC. HPLC was done for the investigation of individual phenolic profile following the procedure reported by Hussain et al. [20] with slight modifications previously described by Bukhari et al. [8]. Leaves and fruit samples (i.e., 1 g of each) of *M. charantia* were preserved in liquid nitrogen and 0.5 g of each sample was taken and homogenized with 1.5 mL of HPLC grade three extraction solvent combinations

(80 : 20 v/v ethanol:H_2O, 70 : 25 : 0.5 v/v/v methanol:H_2O : HCl, and 50 : 50 v/v dimethyl sulfoxide (DMSO):methanol). Freeze-dried homogenate was centrifuged at 18,000 rpm for 15 min at 4°C and supernatant was used for HPLC analysis. Varian HPLC using ODS (C_{18}) reversed phase column was used for the identification of phenolic acids present in extracts. To separate different phenolic acids, two solvents (i.e., solvent 1, acetonitrile (70): methanol (30) and solvent 2, 0.5% glacial acetic acid) were used as mobile phase with a constant flow rate of 1 mL/min in gradient mode. Microsyringe was used to inject the sample in the column and volume of sample injected was 20 μL. Detection was carried out at 275 nm. Identification of phenolic acids was performed by correlating their relative retention times with those of standard mixture chromatogram. The amounts of individual compounds were measured based on peak area measurement.

2.3. Statistical Analysis. MS Excel was used for graphical presentation of the data. All determinations were made in complete randomized designs. The presence or absence of significant difference among different factors was as curtained with the analysis of variances (ANOVA). The means were compared with least significant difference (LSD) at the level of significance of 0.05. Overall interaction of all the factors was checked for significance using computer software CoStat CoHort (6.4).

3. Results

3.1. Pilot Experiment

3.1.1. Percentage of Final Emergence Rate. It was found that the final emergence percentage rate of *M. charantia* seeds treated with 0.3% of zinc sulfate solution had a higher percentage of emergence as compared to 0.1% and 0.2% solutions (Figure 1(a)). Moreover, it was also found that control group with hydropriming had higher final emergence rate (i.e., 22%) as compared to unprimed (18%). Therefore, a positive correlation for final emergence rate was observed between zinc sulfate treated and water treated plants and the percentage of final emergence rate in zinc sulfate treated seeds of *M. charantia* was significant ($p < 0.05$) as compared to untreated samples.

3.1.2. Emergence Index. It was found that 0.3% zinc sulfate solution has higher emergence index that was comparable with the control groups (Figure 1(b)), so that emergence index in zinc sulfate treated *M. charantia* seeds was significant ($p \leq 0.05$) as compared to untreated samples.

3.1.3. Vigor Indices I and II. The vigor indices I and II in zinc sulfate treated *M. charantia* seeds were significant ($p \leq 0.05$) as compared to untreated samples (Figures 1(c) and 1(d)).

3.2. Growth Attributes

3.2.1. Root Length and Shoot Length. Statistical analysis of plant root length and shoot length revealed a significant effect of treatment according to the analysis of variance data ($p \leq 0.05$). In plants treated with zinc, root length remained unaffected as compared to untreated plants (Figure 2(a)), while shoot length of zinc-treated plants was improved as compared to control plants (Figure 2(b)).

3.2.2. Root Fresh Weight and Shoot Fresh Weight. The root fresh weight indicated nonsignificant results ($p \leq 0.05$) of the treatment in plants (Figure 2(c)) whereas shoot fresh weight showed highly significant effect of treatment according to the analysis of variance data ($p \leq 0.05$). Plants treated with zinc showed higher shoot fresh biomass as compared to control plants (Figure 2(d)).

3.2.3. Leaf Fresh Weight and Leaf Area. Results of statistical analysis of leaf fresh weight indicated highly significant effect of treatment according to variance data ($p \leq 0.05$). Plants treated with zinc had leaf fresh weight greatly increased as compared to control plant (Figure 2(e)) whereas leaf area by statistical analysis represented non-significance ($p \leq 0.05$) for the treatment (Figure 2(f)).

3.2.4. Number of Flowers and Fruit Weight. Statistical analysis of fruit weight indicated nonsignificant effects ($p \leq 0.05$) of treatment (Figure 2(g)) whereas number of flowers indicated highly significant effects of treatment according to the variance data ($p \leq 0.05$). The number of flowers was increased in the plants treated with zinc as compared to the untreated plants (Figure 2(h)).

The data presented is the mean of three replicates. Values are mentioned as mean (±SD). Treatments with significant differences ($p \leq 0.05$) are mentioned as 'a' and 'b'.

3.2.5. Germination %Age. Statistical analysis of germination percentage revealed significant effect ($p \leq 0.05$) of the treatment as zinc sulfate treated plants had shown higher germination percentage (55.76 ± 5.72) as compared to control plants (26.67 ± 5.77).

3.3. Field Experiment

3.3.1. Total Soluble Sugar and Protein. Results of statistical analysis of total soluble sugar expressed nonsignificant effects ($p \leq 0.05$) of treatments in leaves (Table 1) and whole fruit (Table 2). However, there was a decline in peel (Table 2). Protein in leaves had a significant effect of treatment according to the variance data ($p \leq 0.05$). Plants treated with zinc had reduced protein in leaves as compared to control plants (Table 1) whereas in fruit it showed marked increase and in peel it was with insignificant difference (Table 2).

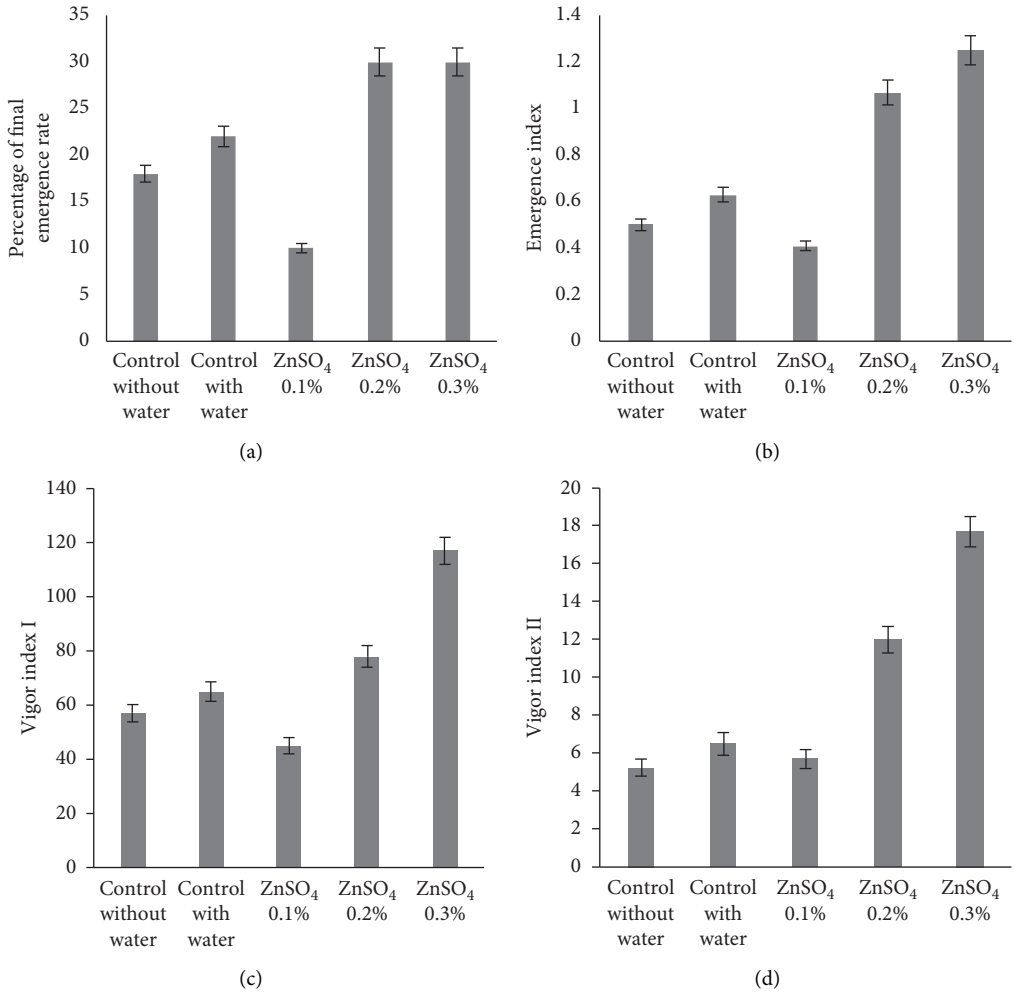


Figure 1: Optimization of zinc sulfate level as priming agent for *M. charantia* in terms of seedling establishment.

3.3.2. Phenolic and Flavonoid Contents. Plant phenolic and flavonoid contents in leaves revealed significant results of treatment according to analysis of variance data ($p \leq 0.05$). Plants treated with zinc sulfate had a gradual decrease in these secondary metabolites (Table 1).

3.3.3. Free Amino Acids. Free amino acids revealed non-significant results of treatment according to analysis of variance data ($p \leq 0.05$). Plants treated with zinc sulfate had a gradual increase in free amino acids of fruit peel (Table 2) whereas it was nonsignificant for other two parts (Table 1).

3.3.4. Photosynthetic Pigments. Statistical analysis of plant chlorophyll-a and chlorophyll-b and total chlorophyll in leaves indicated significant effects ($p \leq 0.05$) of the treatment as their levels were increased as compared to those of control plants (Table 1).

The data presented is the mean of three replicates. Values are mentioned as mean (±SD). Treatments with significant difference ($p \leq 0.05$) are mentioned as 'a' and 'b'.

3.3.5. Antiglycation. Zinc sulfate treatment on seeds of *M. charantia* has no effect on the antiglycation level in its fruits.

3.3.6. Phenolic Profile. Freeze-dried samples (leaf, fruit) of *M. charantia* showed significant difference between zinc sulfate treated and untreated plants (i.e., represented by analysis of variance). In leaves, the concentrations of quercetin and cinnamic acid were decreased after zinc sulfate treatment (Figure 3). In fruits, the concentration of quercetin was decreased after zinc sulfate treatment while concentrations of caffeic acid and syringic acid were found to be 8.64 ppm and 3.77 ppm, respectively, and both phenolics were not detected in control *M. charantia* plants (Figure 4). Fruit phenolics (Table 3) showed a significant correlation with metabolizable energy (the physiologically useful energy obtained when protein, fat, or carbohydrate is catabolized), peroxidase, and free amino acids while they showed a nonsignificant correlation with nutritive parameters. The HPLC chromatogram of phenolic profile of standards is shown in Fig. S1.

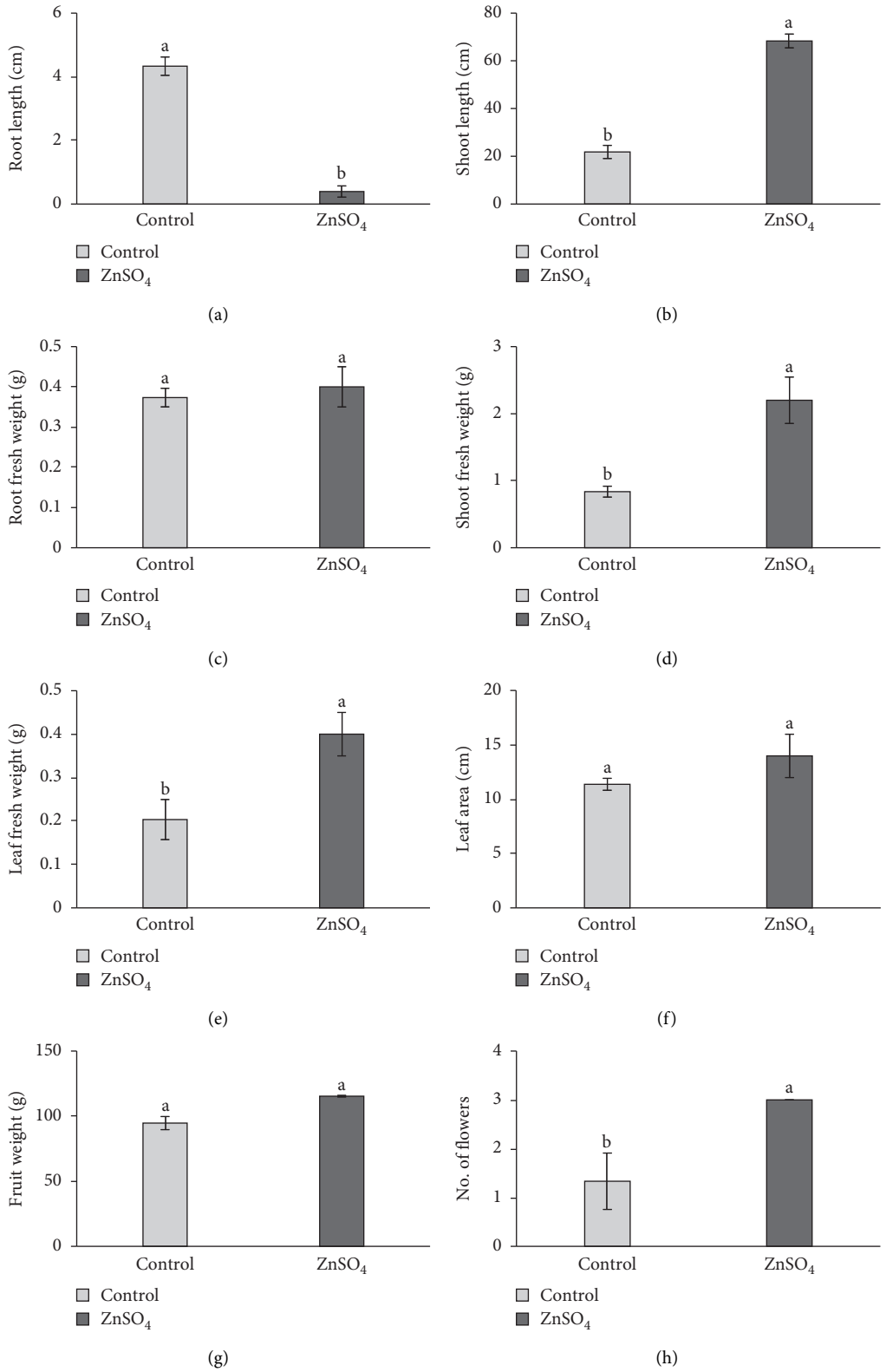


Figure 2: Effect of zinc sulfate seeds priming upon morphological parameters of (M) charantia. (a) Root length, (b) shoot length, (c) root fresh weight, (d) shoot fresh weight, (e) leaf fresh weight, (f) leaf area, (g) fruit weight, (h) no. of flowers.

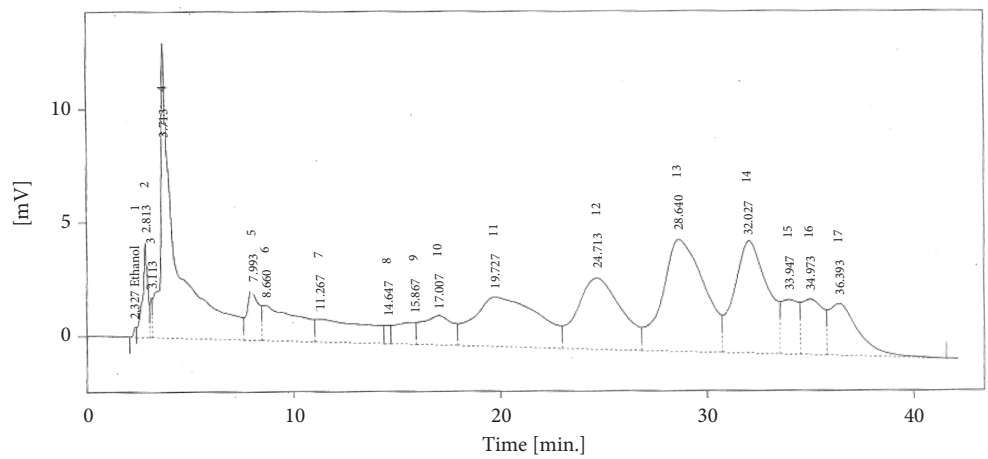
3.3.7. Antimicrobial Activity. At the vegetative growth stage, non-treated leaf extracts exhibited better activity in methanol extract followed by acetone and water. Zinc sulfate treatment improved antimicrobial activity in each extraction against *P. aeruginosa* but only in methanol extraction against *S. aureus* (Figure 5). Both bacterial species showed positive

Table 1: Biochemical analysis of zinc sulfate treatment on seed priming of *M. charantia* at biochemical attributes of vegetative tissues in leaves.

Biochemical analysis	Control *M. charantia*	Treated *M. charantia*
Total soluble sugar (%)	28.43 ± 3.11^a	30.54 ± 2.26^a
Protein (mg/g)	27.4 ± 1.162^a	20.16 ± 0.04^b
Phenolic content (mg/g)	10.21 ± 0.01^a	4.76 ± 0.02^b
Free amino acids (mg/g)	4.07 ± 0.06^a	3.6 ± 0.36^a
Chlorophyll-a (mg/g)	2.85 ± 0.01^b	6.34 ± 0.58^a
Chlorophyll-b (mg/g)	3.78 ± 0.66^b	7.33 ± 0.68^a
Flavonoid (mg/g)	6.86 ± 0.01^a	3.07 ± 0.06^b
Total chlorophyll content (mg/g)	7.16 ± 1.03^b	17.67 ± 0.58^a
Catalase (U/g)	542.4 ± 17.86^a	466.87 ± 57.39^a
Peroxidase (U/g)	5.68 ± 0.01^a	6.44 ± 0.33^a
Carotenoids (μg/g)	5.03 ± 0.06^a	3.28 ± 0.49^a
Anthocyanin (mg/g)	0.66 ± 0.002^a	0.54 ± 0.001^a

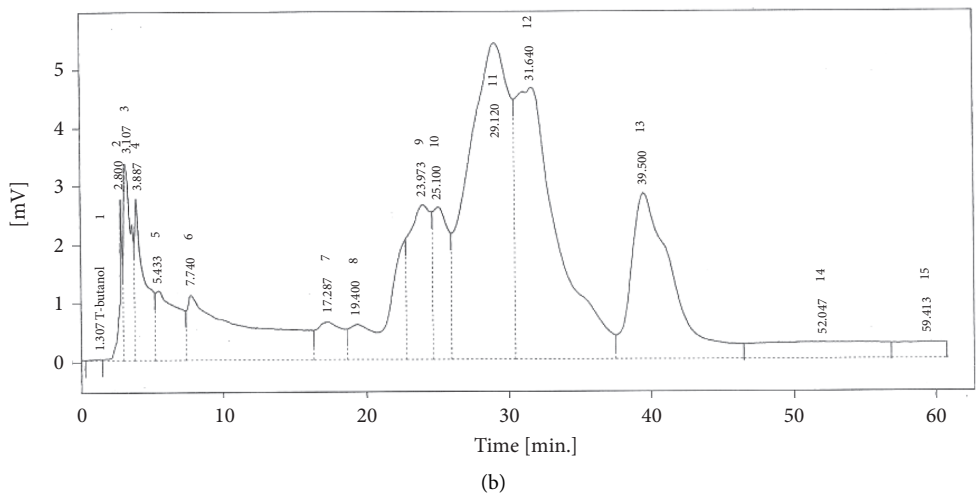
Table 2: Biochemical attributes of zinc sulfate treatment on seed priming of *M. charantia* for nutritive analysis in fruit and peel.

	Fruit		Peel	
Proximate analysis	Control	Treated	Control	Treated
Free amino acid (mg/g)	10.44 ± 0.05^a	8.43 ± 0.02^a	0.5 ± 1.05^b	1.96 ± 0.31^a
Crude fiber (%)	3.08 ± 0.04^a	2.81 ± 0.02^a	3.4 ± 0.44^a	3.62 ± 0.10^a
Protein (mg/g)	26.62 ± 1.57^b	76.20 ± 3.56^a	26.78 ± 0.68^a	28.63 ± 0.15^a
Total soluble sugar (%)	39 ± 3.46^a	40.53 ± 1.65^a	31.6 ± 0.07^a	30.05 ± 0.2^b
Ash (%)	14.93 ± 0.46^a	20.53 ± 2.57^b	15.82 ± 0.75^a	15.38 ± 0.75^a
Peroxidase (U/g)	2.24 ± 0.41^a	3.04 ± 0.56^a	2.43 ± 0.57^b	5.64 ± 0.25^a
Catalase (U/g)	322.9 ± 48.52^a	347.47 ± 34.76^a	374.3 ± 17.31^a	290.13 ± 4.69^a
Oil (%)	0.96 ± 0.01^a	0.96 ± 0.01^a	0.97 ± 0.01^a	0.96 ± 0.01^a
Metabolize energy (kcal/100 g)	17.05 ± 0.77^b	30.94 ± 0.86^a	17.05 ± 0.77^b	28.94 ± 0.86^a



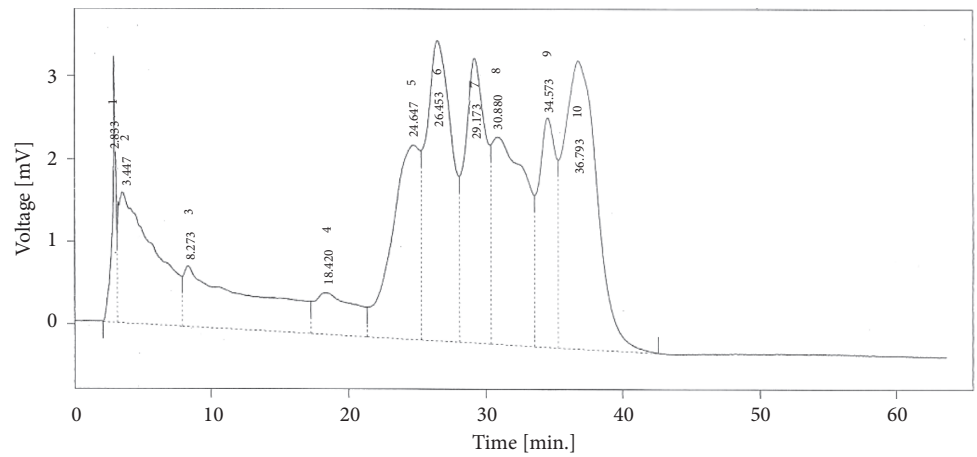
(a)

Figure 3: Continued.

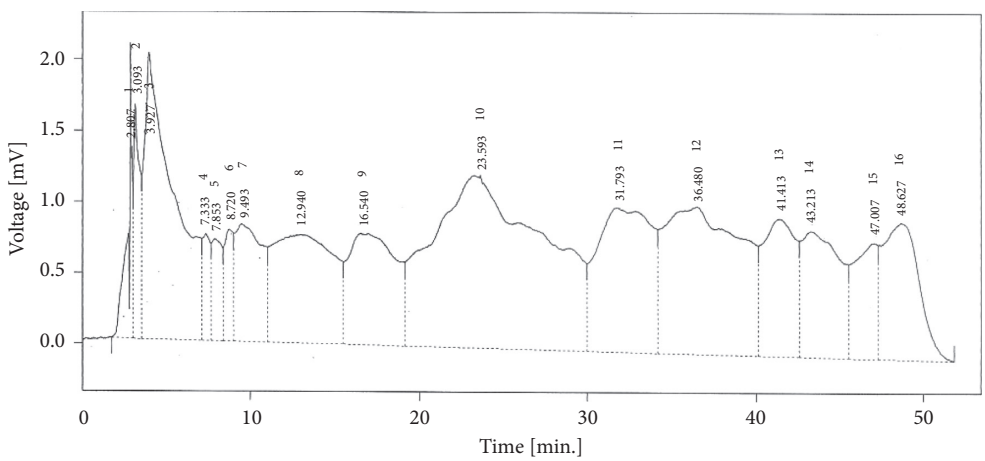


(b)

Figure 3: HPLC chromatograms of (M) charantia (L) leaves for phenolic profile. (a) Control, (b) zinc sulfate priming.



(a)



(b)

Figure 4: HPLC chromatograms of (M) charantia (L) fruit for phenolic profile. (a) Control, (b) zinc sulfate priming.

Table 3: Correlation matrix of leaf antimicrobial activity with enzymes, and primary and secondary metabolites of *M. charantia*.

Pathogen	Enzymes		Primary metabolites						Secondary metabolites	
	Catalase	Peroxidase	Chl-a	Chl-b	Chl-tot	Proteins	Soluble sugar	Amino acids	Carotenoid	Anthocyanin
P. aeruginosa	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
S. aureus	ns	0.7***	ns	ns	ns	ns	ns	ns	ns	ns

Pathogen	Secondary metabolites									
	Flavonoid	Sinapic acid	Vanillic acid	Cinnamic acid	Coumaric acid	Syringic acid	Chlorogenic acid	Benzoic acid	Quercetin	Ferulic acid
P. aeruginosa	ns	1***	1***	1***	0.5*	1***	1***	1***	ns	1***
S. aureus	ns	1***	1***	1***	1***	1***	1***	1***	ns	1***

ns: non-significance at $p < 0.05$ and stearic. The symbols *, **, and *** indicate significant correlation.

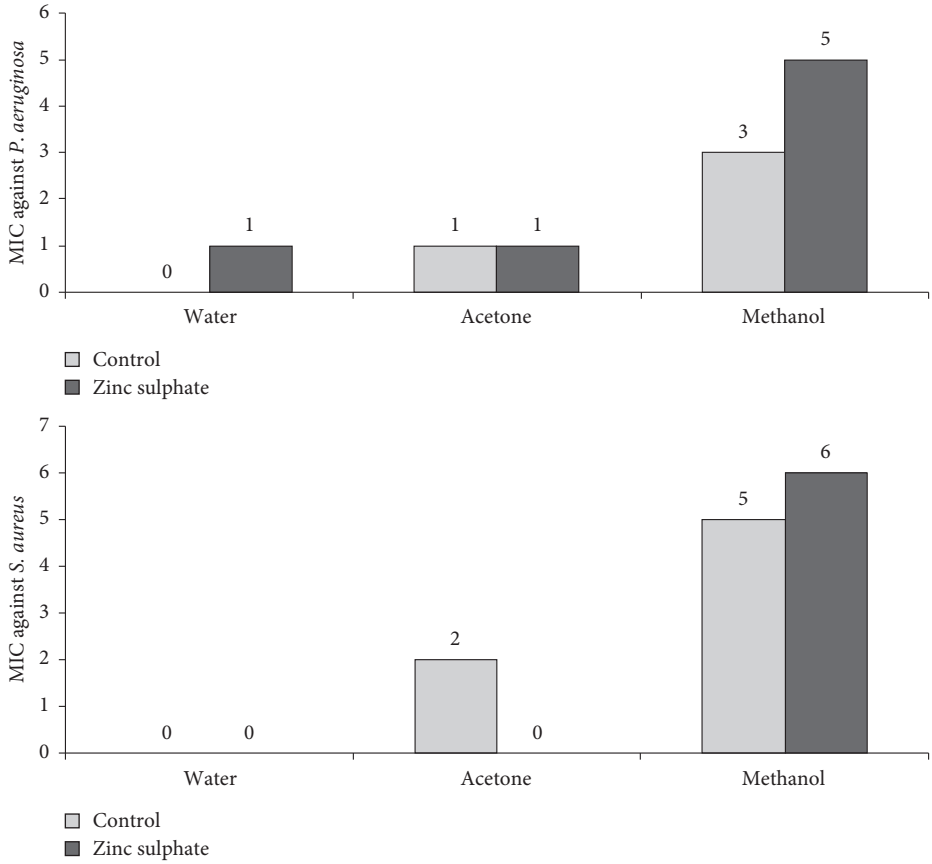


Figure 5: Antimicrobial activity of leaves extractions of (M) charantia against (P) aeruginosa (above) and (S) aureus (below) expressed as minimum inhibitor concentration to inhibit growth of bacterial species.

correlations to peroxidase, sinapic acid, vanillic acid, coumaric acid, syringic acid, chlorogenic acid, benzoic acid, and ferulic acid (Table 3). Both bacterial species revealed a nonsignificant correlation with catalase, chlorophyll-a, chlorophyll-b, total chlorophyll content, proteins, soluble sugar, amino acids, carotenoids, anthocyanin, flavonoid, and quercetin (Table 3).

Results of correlation among fruit phenolics with nutritive parameters of *M. charantia* are given in Table 4. The ferulic acid and coumaric acid in zinc treated fruit of *M. charantia* showed a direct correlation to metabolizable energy, peroxidase, and free amino acids while an indirect correlation to catalase, soluble sugar, and proteins. Benzoic acid, quercetin, and phenolic acid directly correlated to catalase and soluble sugar and indirectly correlated to metabolizable energy (i.e., the physiologically useful energy obtained when protein, fat, or carbohydrate is catabolized), peroxidase, protein, and amino acids. Sinapic acid and

Table 4: Correlation matrix of fruit phenolics with nutritive parameters of *M. charantia*.

Acids	Metabolizable energy	Catalase	Peroxidase	Ash	Soluble sugar	Protein	Crude fiber	Oil	Amino acid
Ferulic acid	0.98***	−0.97**	0.97***	ns	−0.93**	−0.91**	ns	ns	0.9***
Coumaric acid	0.98***	−0.97**	0.97***	ns	−0.93**	−0.91**	ns	ns	0.9***
Benzoic acid	−0.98***	0.97**	−0.97***	ns	0.93**	-0.91**	ns	ns	−0.9***
Sinapic acid	0.98***	-0.97**	0.97***	ns	−0.93**	0.91**	ns	ns	0.9***
Cinnamic acid	0.98***	-0.97**	0.97***	ns	−0.93**	0.91**	ns	ns	0.9***
Quercetin	−0.98***	0.97**	−0.97***	ns	0.93**	-0.91**	ns	ns	−0.9***
Phenolic acid	−0.98***	0.97**	−0.97***	ns	0.93**	−0.91*	ns	ns	−0.9***

ns: non-significance at $p < 0.05$. Thee symbols *, **, and *** indicate significant correlation.

cinnamic acid were directly correlated to metabolizable energy, peroxidase, proteins, and amino acids while they were indirectly correlated to catalase and soluble sugars.

4. Discussion

Zn priming in the form of halopriming [21] and nanoparticle [5] has been reported with positive outcomes in a number of crops including wheat [22] and maize [23]. Currently, zinc sulfate priming has showed its potential to effect upon growth and metabolism of *M. charantia*.

In this study, increase in percentage of final emergence rate, emergence index, vigor indices I and II, shoot length, shoot fresh weight, leaf fresh weight, and number of flowers was observed. These findings are in line with Rouhi et al.[24]. Zinc induced betterment of growth, yield, and seedling establishment could be justified by the role of zinc in enzymatic activities associated with auxin metabolism. Zinc plays a crucial role in the efficiency of growth regulation as a structural component and cofactor [25]. Mallikarjuna et al. [26] have given molecular evidences about direct correlation of zinc availability of plants with regulation of ethylene, auxin, gibberellins, and cytokinin like growth regulators hence implying the role in growth and development of plants as depicted by current findings.

Photosynthesis is related to shoot biomass and it varies with the provision of zinc. The limited zinc availability may cause structural damage on photosynthetic, particularly, bundle sheath cells and chloroplast structure. It is related to the possible inhibition of the gene expression responsible for chlorophyll biosynthesis. Additionally, the genes responsible for thylakoid structural organization are noted to be downregulated in case of limited zinc supply hence indicating role of zinc provision to photosynthesis. The correlation of photosynthetic rate and zinc supply is in line with our work [27]. Here, we have noted enhanced chlorophyll-a and total chlorophyll. Photosynthetic pigments have a vital role in biological metabolism ranging from their effects upon plants to the metabolism of consumers. For years, chlorophyll-a to chlorophyll-b ratio and total chlorophyll content were considered as an index for the betterment of plants and their consumers [28]. There was a substantial enhancement of chlorophyll-a, chlorophyll-b, and total chlorophyll contents in zinc sulfate primed *M. charantia* leaves samples. Better photosynthetic activity is always found related to better yield [29].

In the present study, though the fruit weight remained ineffective by zinc sulfate priming, however positive effect of priming upon number of flowers indicated that individual fruit weight even being the same may affect total yield in response to improved chlorophyll contents. Previously, both the positive and negative effects of zinc sulfate seed treatments have been reported which were varied with treatment doses and crop species. It has been suggested that zinc sulfate priming could change free radical or ionic oscillations and movements and improve levels of photosynthetic pigments along with some other metabolic variations [30].

Similarly, seed priming of *Phaseolus vulgaris* (L.) with zinc improved the yield significantly [31]. Ahmad et al. [32] discussed that seed priming with novel synthetic zinc fertilizers such as $[Zn(Arg)_2]$ and $[Zn(His)_2]$ could be more suitable alternatives in relation to commercial applications of zinc sulfate in the soil. The seed priming with zinc is an affordable and practical way for increasing zinc amount in seeds before their sowing which benefits seedling growth and plants show enhanced yield and biomass [33]. Pengel and Graham [34] showed that zinc contents when increased from 0.25 μg to 0.70 μg per seed significantly improved the growth of root and shoot and therefore it could be concluded that high contents of zinc in seeds could act as starter fertilizer.

Formerly, in a number of studies, zinc application has proved its importance with reference to protein contents in wheat [35] and mungbean [36] where dose-dependent effect of zinc was noted upon crude protein. They observed that zinc deficiency affects nitrogen metabolism in the corn plant. In contrast, Sagardoy et al. [37] observed the antagonistic effect of zinc along with nitrogen in the sugar beet (*Beta vulgaris* L.) hydroponically grown. In the current study, *M. charantia* manifested marked enhancement in whole fruit of plant grown from zinc-primed seeds. Recent proteomic studies related such zinc based changes in protein with the possible role of zinc in chlorophyll production and related membranous structures [27].

Plant phenolics are one of the blessings for herbal consumers. Phenolics with their nutraceutical role in human being and stress tolerance in plants have made their eminent position in literature. Gąsecka et al. [38] found variable pattern of phenolics in zinc treated plants. They noted enhancement of syringic acid, ferulic acid, p-coumaric acid, caffeic acid, t-cinnamic acid, vanillic acid, and naringenin. Our findings partially agree with them where primed plants showed enhanced accumulation of syringic acid, sinapic

acid, vanillic acid, ferulic acid, coumaric acid, and benzoic acid contents in *M. charantia* leaves. In the same way, reduction of some phenolics (i.e., quercetin and cinnamic acid) in *M. charantia* leaves indicated that zinc has some metabolic link with phenylpropanoid pathway responsible to upregulation and downregulation of some genes associated with the above-mentioned phenolics. It indicates that the effect of zinc in modulation of phenolic metabolism varies from species to species. Positive correlation of phenolics with antimicrobial activities against *P. aeruginosa* and *S. aureus* has confirmed the enhanced nutraceutical value of treated plants. Positive correlation of phenolics with antimicrobial activity is in accordance with previous literature. As the zinc priming showed its potential to enhance phenolics content, hence it can be related to the findings of Afonso et al. [39] as scientific justification.

5. Conclusion

Products of phenylpropanoid pathway are enhanced with zinc priming of *M. charantia* that improved pharmaceutically important phenolics (e.g., syringic acid, sinapic acid vanillic acid, ferulic acid, coumaric acid, and benzoic acid). Additionally, zinc treated plants were found with better seedling establishment, photosynthetic pigments, and stable nutritive value and therefore proving it as a beneficial fertilizer for *M. charantia* plant growth, yield, and nutraceutical potential.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Supplementary Materials

Figure S1: HPLC chromatograms of phenolic profile of standards. (*Supplementary Materials*)

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