DOI: 10.47743/achi-2025-2-0010 *ACTA CHEMICA IASI*, **33_2**, 183-216 (2025)

OPTIMIZING SEWAGE SLUDGE PERCENTAGE BY MIXING WITH SOIL TO OBTAIN PROMISING CHARACTERISTICS IN *MOMORDICA CHARANTIA L.*

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Abstract: Sewage sludge generated by wastewater regulatory plants is an environmental hazard in Pakistan. Along with the soil, if this sewage sludge is mixed in the right percentage during plant growth, it can help in improving the characteristics of the growing plant along with eradicating hazardous sewage sludge. The enhancement of germination with increased chlorophyll, carotenoid, protein, phenolic, flavonoid, and tocopherol contents of *Momordica charantia* L. is achieved by conducting pot experiments in pure soil and in several soil amendments with varying sewage sludge proportions. Appropriate percentages of sewage sludge in soil are evaluated for plant growth which may enhance not only the above-described factors but also increased antioxidant potential. The plant was grown in separate containers with varying proportions of sewage sludge (20 %, 40 %, 60 %, and 80 %) in the soil while pure soil was taken as control. Growth parameters were highest along with increased phytochemical content and decreased toxicity in 40 % and 60 % sewage sludge soil amendments whereas at higher percentages of sewage sludge in soil, increased the toxicity due to a higher metal content and resulted in less promising results. The proposed sewage sludge percentages in soil for the growth of *Momordica charantia* L. are 40 % and 60 %.

***Keywords*:** Agriculture; antioxidant activity; contents in plant; sewage sludge; soil.

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Introduction

Sewage sludge, being a solid residue generated by wastewater regulatory plants, has gained immense importance in the field of environmental science. Utilization of this solid residue is a crucial question for environmentalists because it is being produced in massive amounts throughout the world. Its nutritional attributes along with the presence of unwanted hazardous organic pollutants as well as heavy metals content make sewage sludge management an important assignment .[1] The potential for its utilization in various regions of the world depends upon the local law and acts of the environmental protection agencies.[2,3] For example, in the USA, 47 % of the sewage sludge is applied on the land for agricultural purposes. On the other hand, in Japan, only 2.4 % of sewage sludge is used in land applications due to the strict legislation in the health sector.[4] Other means for the management of sewage sludge include dumping, incineration, landfilling, and anaerobic digestion.[5] In developing countries such as Pakistan, the most appropriate and economical method for sewage sludge utilization can be its addition to the soil for making suitable soil amendments for agricultural purposes. The selection of plants to be grown in such sewage sludge soil amendments is an important task. Generally, the selection of a plant should be based on its therapeutic value.

It is well known that humans have been using plants for the treatment of several infectious diseases since ancient times. Chemical profiling has proved the efficacy of the plants for their therapeutic uses. It is a common practice in many countries to treat different health-related problems including infectious diseases of the gastrointestinal, urinary, and respiratory tracts by using natural compounds derived from plants.[6] Widespread public health problems can be controlled by the discovery of novel bioactive components from medicinal plants using frontier technologies.[7] People residing in remote areas of Pakistan such as the South

Waziristan Agency and Bajaur Agency have reported using traditional herbal extracts? for the treatment of domestic animals. The most frequently used herbal plants are *Solanum nigrum* and *Sophora mollis*.[8] In the northeastern region of Algeria, people also use medicinal plants for the treatment of a wide range of diseases especially those related to the digestive tract. A native Brazilian species named *Echinodorus grandiflours* is reported to be used against certain inflammatory diseases, arthritis, and hypertension.[9] Several compounds isolated from African medicinal plants extracts are reported to show significant anticancer effects.[10] In Saudi Arabia, many plant species showed prominent effects in the treatment of the victims from the sting of the scorpion species. Hence, across the globe, medicinal plants are considered a valuable resource, and they can provide a suitable alternative to primary health care.[11]

One of such plant possessing promising medicinal characteristics is *Momordica charantia* which belongs to the family Cucurbitaceous and generally grows in tropical and subtropical regions including India, the Caribbean, South Asia, Africa, and China. It is an annual plant which is commonly known as 'Bitter melon'. It is well known that this plant contains certain bioactive components which possess promising sugar regulatory effects in human beings and therefore, it is being used for medicinal purposes around the world.[12] The unripe fruits, seeds, and aerial parts of the plant in question are used to treat diabetes. Bitter melon also enhances the nutritional value of the food as it contains several phenolic compounds.[13] In addition, this plant is also used to banish intestinal gas, for wound treatment, and against rheumatism and malaria. Furthermore, fruits and leaves of the plant possess laxative, emetic, and stomachic effects.[14] The enhancement of the biological potential of this particular plant by making soil amendments using varying proportions of sewage sludge was the objective of this study. The sewage sludge contains several vital nutrients essential for the good growth of plants. In this way, by making soil amendments with sewage sludge, not only the problem of devising ways to get rid of the sewage sludge can be solved but also the medicinal importance of plants will be enhanced.

Result s and Discussion

Germination index of *M. charantia*

Germination index calculation is a potential indicator to check the influence of soil characteristics on plant growth.[27] Germination index calculation of *M. charantia* showed maximum growth of the plant in soil with 60 % sewage sludge amendment. This proves that an increase in sewage sludge content in soil can enhance soil fertility. However, at higher proportions (80 % and 100 %) of sewage sludge, the plant showed a decreasing trend towards growth. The decrease in plant growth can be attributed to increasing metal content with an increasing percentage of sewage sludge in the soil. This metal content increment may serve as toxicity and eventually could result in growth reduction. The fact that the germination index is highly variable from species to species limits the scope of maximum growth of plants in soil with 60 % sewage sludge amendment to *M. charantia* and may be extended to similar plants of the same species. Therefore, optimization of sewage sludge mixing with the soil is required for every type of plant grown in amended soil (Table 1).

Table 1. Comparative germination index of *Momordica charantia* L.

Sludge %	0	20	40	60	80	100
***Momordica charantia* L.**	2.00±0.57	4.33±0.88	4.00±0.58	7.33±0.33	6.00±0.58	3.33±0.33

Effect on total biomass of the plant grown in pure and amended soil

The determination of total biomass obtained from a plant grown in various amendments of the sewage sludge and soil is an efficient indicator to estimate the effect of sewage sludge on plant growth. Sewage sludge is known as an important nutrient resource for the soil. However, pathogens as well as heavy metals content in the sewage sludge may pose serious environmental and public health risks. Therefore, optimization of the percentage of sewage sludge in soil is required for the better and more reliable growth of the plants.[28] Total biomass is not only a growth parameter of the plant, but it also indicates the yield of the crop under discussion. Mixing sewage sludge with soil increased the number of leaves on the plant as well as the plant's height. Comparison among natural and sewage sludge-amended soil showed significant differences in the biomass of the plant.[29] However, this increasing biomass was not in a linear fashion with an increasing percentage of sewage sludge in the soil. A decrease in biomass at higher percentages of sewage sludge-amended soil was also observed. This decrease in biomass can again be attributed to the increased content of heavy metals present in the higher percentages of sewage sludge-amended soil. These heavy metals in higher quantities may create oxidative stress and inhibition of many enzymatic processes occurring in plants.[30] Therefore, a controlled and optimized amount of sewage sludge is suggested for acquiring better quantities of biomass. This optimized quantity of sewage sludge in the soil may vary from species to species depending upon the heavy metal content tolerance levels of the plant. Results of the determination of the total biomass of the *M. charantia* grown in pure and amended soil showed marked differences. The biomass of the plant was measured by determining FW and DW of the plant taken in grams. Results showed (Figure 1) that the plant grown in amended soil with 20 % sewage sludge yielded 525 g FW whereas 40 % sewage sludge soil amendment yielded maximum biomass i.e. 623 g per plant as compared to the control soil as well as other soil amendments.

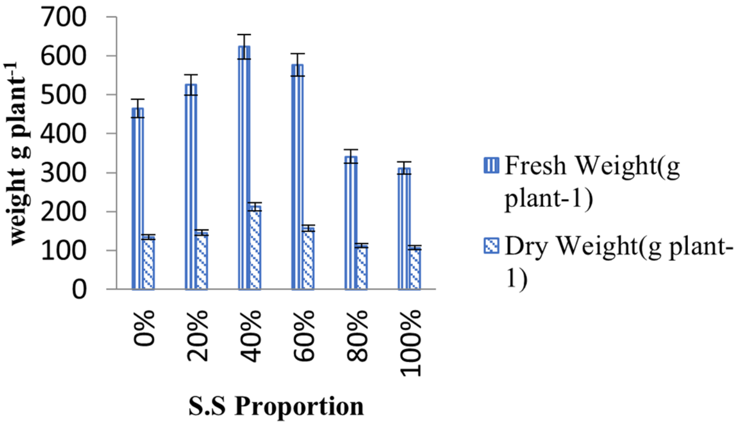

Figure 1. Total plant biomass (fresh weight and dry weight in grams/plant) of *Momordica charantia* L.

The least quantity of biomass was obtained when plants were grown in pure sewage sludge. The DW of the plant also showed similar trends. It signifies that higher amendment levels of soil with sewage sludge are not favorable for plant growth as the increased heavy metal content along with the free radicals presence may impair the growth of plant. These free radicals can cause oxidative stress and, therefore, may inhibit plant growth.

Chlorophyll content

Chlorophyll is a photosynthetic pigment which has been reported to increase quantitatively in the mustard plant when grown in the soil amended with mixed industrial effluents. It was reported that chlorophyll content increased after 30 and 60 days of cultivation but decreased predominantly after 90 days.[31] Chlorophyll content has also been reported to increase in

Brassica nigra when the plant was grown in amended soil with sewage sludge. However, chlorophyll content decreased when more than 50 % sewage sludge were added to soil.[32] The total chlorophyll content in the leaves of *M. charantia* was found to increase when the plant was grown in the higher percentage of the sewage sludge in soil. Maximum quantity of photosynthetic pigments appeared in the plant when it was grown in soil with 40 % and 60 % amendment of the sewage sludge.

The plant leaves grown in soil with 80 % sewage sludge amendment as well as in pure sewage sludge showed a decrease in photosynthetic pigment. The results presented in Table 2 reveal that 4.15 $mg \cdot g^{-1}$ of chlorophyll in the leaves of *M. charantia* grown in soil with 40 % sewage sludge and 4.98 $mg \cdot g^{-1}$ of chlorophyll in the leaves of *M. charantia* grown in soil 60 % sewage sludge are the two considerably higher chlorophyll content values as compared to the control (pure soil) as well as other higher amendments of soil and sewage sludge.

Carotenoid content

The results of carotenoid content determination are also presented in table 2. The optimum growth of the *M. charantia* was observed in soil with 40 % and 60 % amendments of sewage sludge. The results showed 2.73 $mg \cdot g^{-1}$ and 2.46 $mg \cdot g^{-1}$ carotenoid content in plant grown in soil with 40 % and 60 % sewage sludge amendment, respectively. The effect of effluents has been reported to enhance the carotenoid content in the presence of sewage sludge. However, increased concentrations of sewage sludge in soil are known to decrease the growth of the plant which can be attributed once again to the presence of higher amounts of heavy metals which may increase the production of free radicals and reactive oxygen species and, as a result, may reduce the metabolic functioning of the plant.[32]

Table 2. Chlorophyll content (Chl *a* and Chl *b*), Chl *a* / Chl *b* ratio, and carotenoid content in *Momordica charantia* L. plant (Leaves).

Sludge %	Chl *a* (mg·g^{-1} FW)	Chl *b* (mg·g^{-1} FW)	Chl *a* / Chl *b*	Carotenoids (mg·g^{-1} FW)
0 %	3.14±0.11	0.56±0.44	2.35±0.56	0.78±0.11
20 %	3.56±0.43	0.78±0.53	2.74±0.33	1.63±0.21
40 %	4.15±0.19	0.87±0.12	3.15±0.21	2.73±0.16
60 %	4.98±0.44	1.43±0.77	3.66±0.19	2.46±0.27
80 %	4.66±0.32	0.94±0.88	3.31±0.14	2.31±0.15
100 %	3.64±0.22	0.88±0.32	3.12±0.11	2.05±0.24

Protein content

According to literature, the protein content in several parts of mustard plant have increased in 60 days of the growth period with 50 % sewage sludge and soil amendment.[33] On similar basis, the protein contents in roots, shoots, leaves and fruits of *M. charantia* grown in different sewage sludge and soil amendments were calculated. The results (Table 3) prove that the protein content is highest in plant sample grown in soil with 60 % sewage sludge amendment. Therefore, the optimum soil amendment of sewage sludge is 60 % in the case of *M. charantia.*

Table 3. Protein content (mg·g^{-1}) in various parts of *Momordica charantia* L.:

Sludge	Roots	Shoots	Leaves	Fruits
0 %	51±0.32	47±0.43	51±0.44	77±0.44
20 %	66±0.64	54±0.76	57±0.92	79±0.13
40 %	72±0.31	58±0.12	62±0.43	87±0.44
60 %	73±0.42	58±0.32	67±0.13	84±0.11
80 %	68±0.55	53±0.22	59±0.18	74±0.55
100 %	55±0.19	44±0.65	52±0.41	66±0.77

Toxicity evaluation of the plant

Heavy metals in the edible parts (fruit) of the plant were estimated using an atomic absorption spectrophotometer. All estimated heavy metals (Figure 2) in all plant samples are found to be present within the permissible limits within the fruit. So, there has been no toxicity found in the plant. Hence, an edible part of the plant grown in the amended soil with sewage sludge is recommended for human use. However, repeated application of sewage sludge in the soil may contribute towards accumulation of heavy metals in the soil more than the threshold values. Therefore, an optimized and controlled amount of the sewage sludge should be mixed in soil for better results with minimum toxicity.[34]

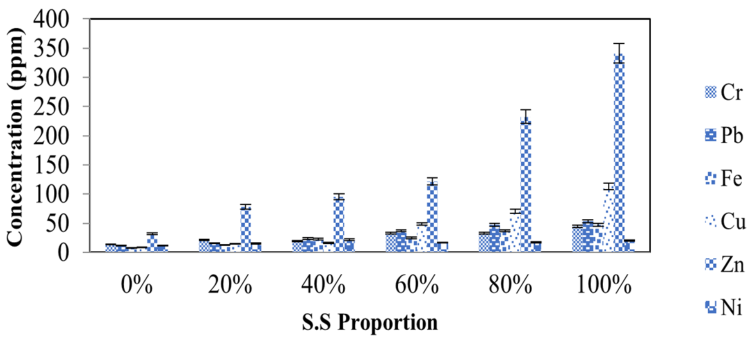

Figure 2. Concentration of heavy metals (ppm) present in different samples of *Momordica charantia* L (Fruit) grown in several sewage sludge soil amendments.

Quantitative determination of ascorbic acid

Ascorbic acid is considered an important antioxidant because it is known to possess the ability to scavenge peroxyl radicals and can inhibit cytotoxicity occurring in the plant by oxidants. Therefore, metabolic disorders may occur in the plant due to the deficiency of ascorbic acid. The amendment of soil with tannery sludge (35 %) is reported to enhance the ascorbic acid content in the root of the Fenugreek plant after 30 days of cultivation. However, in the same study, ascorbic acid content decreased after 60 days of cultivation.[35] The variation in the ascorbic acid content was also evaluated in this study in all parts of *M. charantia* grown in pure soil, and pure sewage sludge as well as in various amendments of the soil and sewage sludge. The results presented (Figure 3) reveal that the quantity of ascorbic acid varies greatly in different soil amendments with sewage sludge.

The maximum ascorbic acid content in *M. charantia* was found to be present in soil with 60 % and 40 % sewage sludge amendments. This variation in soil due to sewage sludge amendments brought diverse features that changed the soil characteristics as well as its fertility. The changing amount of sewage sludge in the soil revealed the effect of soil on the ascorbic acid content of the plant under investigation.

Total phenolic content

The reactive oxygen species are reported to cause damage to biological systems such as introducing cardiovascular diseases, cancer, diabetes mellitus, and aging in the human body.[36] The phenolic compounds in the plants can be taken as a quantitative measure of their antioxidant potential as phenolic compounds are known for their ability to quench singlet oxygen atoms, scavenge free radicals, and play a vital role in the decomposition of peroxides. It has been reported that the methanol extract of a plant sample generally contains the major portion of phenolic content.[37]

Therefore, the FC reagent was used to determine the total phenolic content present in the plant sample. The reduction of the reagent occurs during the reaction; the greater the phenolic content present in the plant sample, the greater the reduction will be.

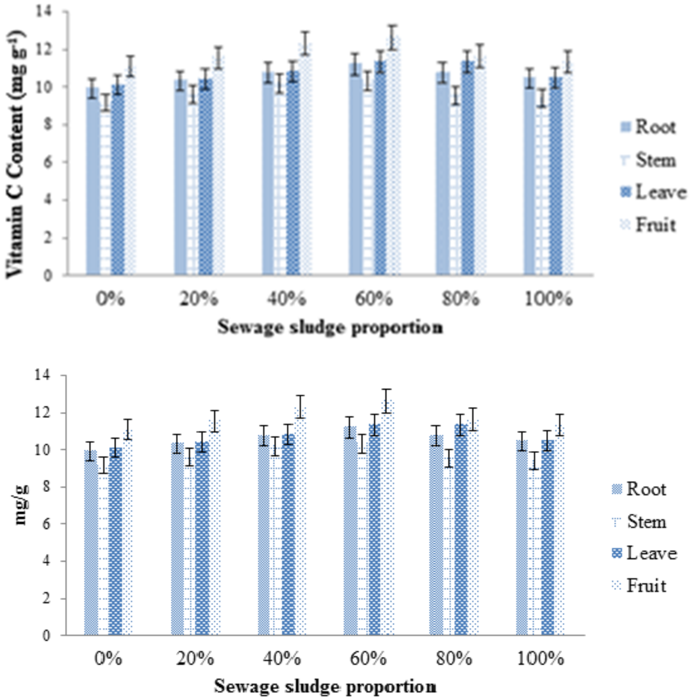

Figure 3. Ascorbic acid (Vitamin C) content (mg·g^{-1}) in *Momordica charantia* L. was s tudied under varying sewage sludge amendments in the soil.

The results of the present study reveal that sewage sludge treatment of the soil up to a certain level can increase the total phenolic content significantly.[38] However, a considerable reduction in content was observed at higher percentages of sewage sludge in the soil. Figure 4 represents the phenolic content of *M. charantia.* The maximum phenolic content was recorded in soil with 60 % amendment of the sewage sludge. The plant samples grown in this sewage sludge and soil amendment presented higher phenolic content compared to the control (pure soil).

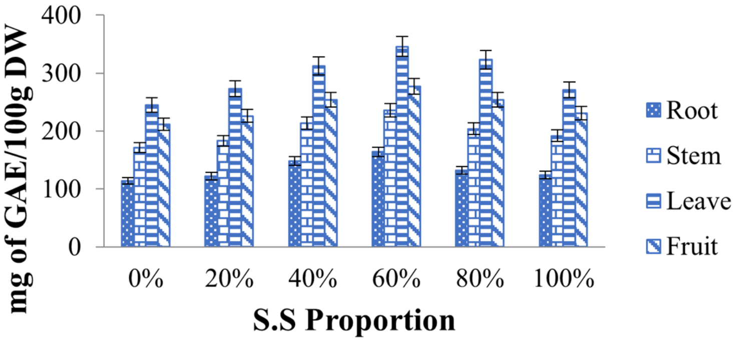

Figure 4. Total phenolic content presented in *Momordica charantia* L. in varying percentage amendments of sewage sludge with soil.

However, increasing the sewage sludge percentage further resulted in a decrease in phenolic content quantity with the minimum phenolic content found in plant samples grown in 100 % sewage sludge. This increase in total phenolic content at certain soil amendments with sewage sludge can be due to the presence of organic matter along with the higher quantity of macronutrients such as nitrogen, phosphorous, and potassium in the amended soil.[39] Conversely, elevated quantities of heavy metals in soil amended with higher percentages of sewage sludge may inhibit the production of phenolic compounds due to producing of a higher concentration of free radicals and other related reactive oxygen species. Hence, an optimized quantity of sewage sludge for a particular plant species depending upon its tolerance level should be mixed with the soil.[40]

Total flavonoid content

Flavonoids are secondary metabolites that possess low molecular weight. Production of flavonoids in plant cells occurs within the vacuoles and cytosols. Flavonoids are important molecules as they possess the ability to inhibit lipid peroxidation and can lessen oxidative stress triggered by reactive oxygen species as well as free radicals in the biological systems.

The quality of food can be greatly attributed to the flavonoid content present in it. Flavonoids are also useful in several pharmaceutical applications as they can improve blood circulation and possess some anticancer properties.[41] Therefore, determining the flavonoid content in plant samples under discussion grown in different soil amendments with sewage sludge was one of the tasks. The results presented (Figure 5) reveal that the flavonoid content increases in the leaves of the *M. charantia* with increasing sewage sludge proportion in the soil amendment. Analogously, other parts such as roots, shoots, and fruits display a similar trend in flavonoid content with the increase in sewage sludge proportion in soil. Flavonoid content has also been reported to increase under drought conditions as well as is known to be affected by the duration of light exposure. The increased flavonoid content in this study might be attributed to the presence of soluble carbohydrates as well as increased organic matter content in the soil with an increase in sewage sludge proportion in soil amendment.

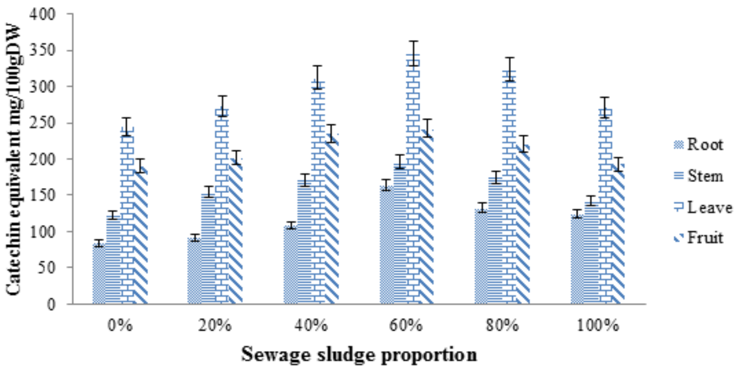

Figure 5. The total flavonoid content of *Momordica charantia* presented as catechin equivalents (mg/100g dry weight) in different sewage sludge soil amendments.

Total tocopherol content

The reactive oxygen species (ROS) create oxidative stress which adversely affects the plant productivity. In this regard, plants possess strong antioxidant defense system which helps them to reduce these ROS. One of such useful antioxidant compounds produced by plants is named tocopherol. It is commonly called vitamin E which is synthesized in photosynthetic pigments of the plants.[42] The tocopherols play a vital part in defending plants from singlet oxygen species as well as from the peroxidation of lipids. The compounds can increase the tolerance of the plant against oxidative stress brought by metal content. They usually occur in one of four forms such as α-, β-, γ- and δ-tocopherol. These tocopherols differ in position and number of methyl groups on chromanol ring moiety.[43] Amongst all the four forms of tocopherols, α-tocopherol is predominant and has the maximum vitamin E activity.[44] The tocopherol content can be measured using HPLC in μg g^{-1} dry weight of the plant sample. During this study, this content was found maximum in all the parts (roots, shoots, leaves, and fruits) of *M. charantia* grown in soil with 40 % sewage sludge amendment (Figure 6).

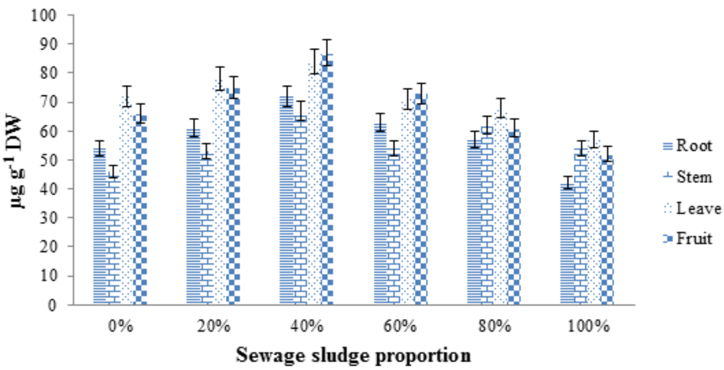

Figure 6. The total tocopherol content of *Momordica charantia* presented as μg g^{-1} dry weight in different sewage sludge soil amendments.

However, the tocopherol content decreased significantly in the plant growing in higher percentages of sewage sludge soil amendments. This reduction in tocopherol content might be related to the production of ROS due to the presence of heavy metals in the sewage sludge itself. Hence, a controlled quantity of sewage sludge in the soil can be a potent source for the biosynthesis of secondary metabolites such as tocopherols.[45]

DPPH free radical scavenging activity

The antioxidant potential of any edible plant determines its quality and utility. Edible plants with higher antioxidant potential can play a vital role against various diseases not only in human beings but also in other members of the food chain. Antioxidants inhibit the oxidative stress caused by the free radicals or heavy metals. The DPPH scavenging activity is among the oldest as well as most used methods for the determination of antioxidant potential in plant extracts.[46] The DPPH is a famous scavenger or trapper of the free radicals that are responsible for causing oxidative stress. It is an organic radical that is nitrogen-based and is commonly used to determine *in vitro* antioxidant potential of the plant. This radical provides the highest absorbance from 515 nm to 528 nm. Its original color is violet which vanished away because of reduction. The capacity of radical scavenging of any sample is evaluated by the color change of the original DPPH solution and ultimately it shows the antioxidant activity of the sample.[47] Hydrogen donor species in the sample determines the scavenging ability as DPPH possesses the capability to take either an electron or hydrogen radical and, as a result, it becomes diamagnetic with enhanced stability. Phenolic compounds in the sample provide protons to the radical. Hence, the increasing concentration of phenolic content in the sample results in increased DPPH scavenging ability.[48] Therefore, the concentration of phenolic compounds could be an indicator of antioxidant activity. The DPPH radical is highly sensitive to the active components even at lower concentrations and therefore, is useful to analyze multiple samples in less amount of time.[49] Hence, it is generally used to determine the scavenging ability of various extracts. The results of this study have been shown as IC_{50} (mg mL^{-1}) values. The DPPH scavenging capacity IC_{50} (mg mL^{-1}) values were found maximum (Figure 7) in the extracts of *M. charantia* grown in the soil with 60 % amendment of sewage sludge. On the other hand, minimum scavenging ability was found in the extracts grown in 100 % sewage sludge proportion.[50] These results reveal the existence of metallic toxicity in the sewage sludge as a high concentration of sewage sludge inhibits the production of phenolic compounds in the extracts. Characterization of sewage sludge also indicates the presence of nutrients and organic matter which may help in the plant growth.[51] Therefore, the maximum IC_{50} value in the extracts of plants grown in soil with 60 % sewage sludge proportion showed enhanced radical scavenging ability.

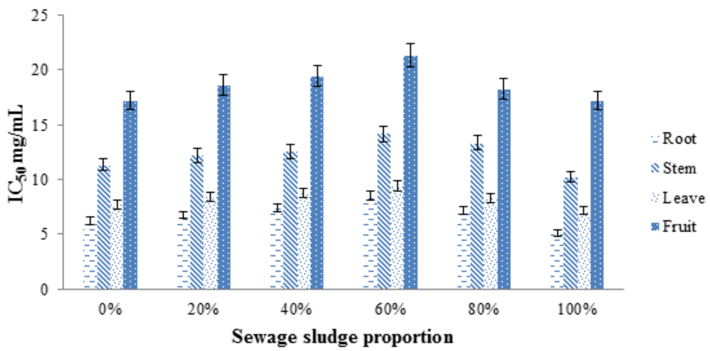

Figure 7. The DPPH free radical scavenging activity (IC_{50} mg/mL) of *Momordica charantia* extracts when plants were grown in different sewage sludge soil amendments.

ABTS ± radical scavenging assay

Evaluation of the antioxidant activity of the plants can also be carried out by using ABTS ± radical scavenging assay *i.e.* 2,2-azino-*bis* (3-ethylbenzo thiazoline-6-sulfonic acid). In this method, a metastable radical cation is produced by using H_2O_2 which oxidizes 2,2-azino-*bis*

(3-ethylbenzo thiazoline-6-sulfonic acid). The scavenging ability of antioxidants is measured spectrophotometrically at 734 nm as the antioxidants can donate hydrogen to 2,2- azino-*bis* (3-ethylbenzo thiazoline-6-sulfonic acid) radical cation. The extent of plant extract to scavenge this radical is an indication of the plant's ability to show a similar type of activity if any ROS enters the biological system.[52] The scavenging ability of methanol-based extracts of *M. charantia* is presented (Figure 8). It has been established that $ABTS^{\pm}$ scavenging ability increases in a linear fashion in plants grown in soil with 20 % to 60 % sewage sludge amendment. However, at higher proportions of sewage sludge-amended soil, the scavenging ability decreases markedly.

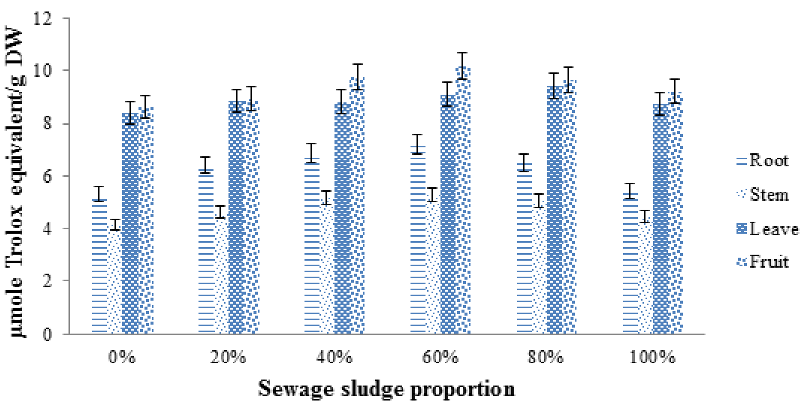

Figure 8. The variation in $ABTS^{\pm}$ radical scavenging activity (µmole Trolox equivalent/g dry weight) of *Momordica charantia* extracts. The plants were grown in pure and sewage sludge amended soil.

Inhibition of linoleic acid oxidation

Linoleic acid is an example of omega-6 fatty acid which is polyunsaturated. This is used in the biosynthesis of prostaglandins and cell membranes. Linoleic acid is an 18-carbon chain structure with two *cis* double bonds which can predominantly be found in plant oils. Determination of oxidation inhibition in the lipid system is considered an important assay for the assessment of the antioxidant potential of plant extracts. The peroxidation inhibition in the linoleic acid assay is related to the antioxidant potential of the plant extracts, and it was performed by using the thiocyanate method.[53] Oxidation of linoleic acid produced peroxides and these peroxides oxidized Fe (II) ions to Fe (III) ions, which eventually formed complexes with thiocyanate. Afterward, the concentration of the complex was evaluated spectrophotometrically. For this purpose, the absorbance could be measured at 500 nm. Greater absorbance accounts for the increased concentration of peroxides generated during reactions. Therefore, the higher the absorbance, the lesser will be the antioxidant potential. Results revealed that extracts of the *M. charantia* grown in the soil with 60 % sewage sludge amendment showed good antioxidant activity (Figure 9).

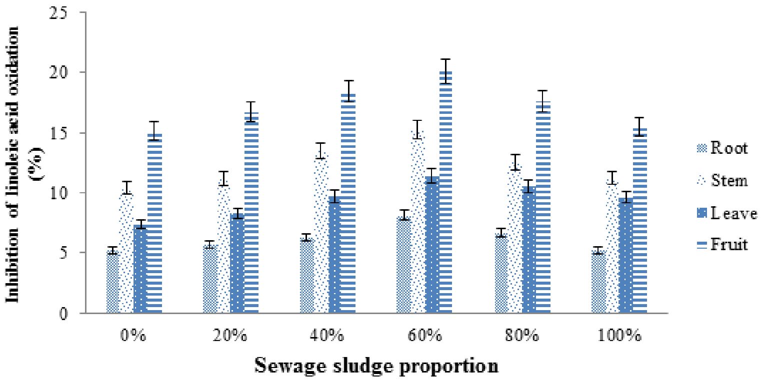

Figure 9. Inhibition of linoleic acid oxidation (%) by *Momordica charantia* extracts resulted from plants grown in several sewage sludge soil amendments.

The absorbance was lowest in the plant sample grown in soil with 60 % sewage sludge amendment as compared to the other sewage sludge and soil amendments.

The lower absorbance is attributed to the least quantity of complex formation with thiocyanate and it points out towards less peroxide formation by the oxidation of linoleic acid. Thus, antioxidant potential is considered higher in plant samples grown in soil with 60 % sewage sludge amendment.[25]

Ferric reducing antioxidant power (FRAP)

Free radicals in the living systems can oxidize biomolecules which may result in tissue damage, and cell death and can lead to several health-related problems such as cardiovascular-related issues, arteriosclerosis, cancer, inflammation, and neural disorders. Antioxidants can hinder and deactivate these free radicals. Reducing power is a noteworthy reflection of antioxidant activity. Compounds with reducing power can donate electrons which can result in the reduction of lipid peroxidation intermediate products.[54] Therefore, these compounds behave as primary and secondary antioxidants. The reducing power of any sample depends upon its capability to convert Fe (III) ion to Fe (II) ion. Estimation of the reducing power of the plant under discussion was performed spectrophotometrically by measuring absorbance at 700 nm. The conversion of the Fe (III) ion to the Fe (II) ion results in the formation of blue colored complex. A change of color, from yellowish to green or blue, of the test solution, occurs and it all depends upon the reducing ability of the test samples.[55] An increase in absorbance of the samples proves their higher ferric-reducing ability. Results of the present study showed the effect of soil amendments with varying proportions of sewage sludge on the reducing ability of the plant extracts. Changes in sewage sludge composition of the soil altered the reducing capabilities of the plant samples grown in it. Optimum reducing power was shown (Figure 10) by all the parts (roots, shoots, leaves, and fruits) of *M. charantia* grown in the soil with 60 % sewage sludge amendment. On the other hand, the reducing power markedly decreased in the plant samples grown in soil amendments with higher percentage of sewage sludge. This variation might be correlated with the amount of the total phenolic content and flavonoid content in the plant samples.

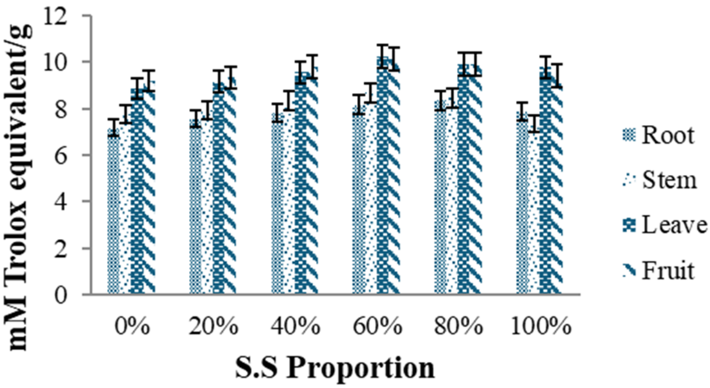

Figure 10. Ferric reducing antioxidant power (mM Trolox equivalent/g) of *Momordica charantia* extracts. The plants were grown in different sewage sludge soil amendments.

Experimental

Materials and Methods

All chemicals and solvents used in this study were of analytical grade and purchased from Sigma Aldrich/Fluka. The chemicals and solvents were used without further purification. The absorbance measurements were carried out in a 10 mm quartz cuvette on T80 + UV-*Vis* spectrophotometer (PG instruments, Leicester, UK). For HPLC separations a Shimadzu Kyoto Japan LC-20A system equipped with a LC-20AT pump and a RF-20A fluorescent detector was used.

Preparing soil amendments by using sewage sludge

The experimental setup was designed using 18 earthen pots of the same size. For this purpose, various combinations of sewage sludge and soil (20 %, 40 %, 60 %, 80 %) were prepared along with two pure forms of soil and sewage sludge (10 kg weight per pot) respectively in fields of

University College of Agriculture, Khushab Road, University of Sargodha, Sargodha, Pakistan. All amendments, pure soil, and sludge were taken in triplicates.

Measurement of physical growth parameters

The parameters associated with plant growth were measured using conventional methods. The determination of the total biomass of the plant was carried out for both fresh weight (FW) of plant and dry weight (DW) of the plant by measuring stem length along with the size and number of leaves. Biomass (FW, DW) of the plant grown in various amendments of soil and sludge were measured (g·plant^{-1}).[15]

Measurement of physical growth parameters

The parameters associated with plant growth were measured using conventional methods. The determination of the total biomass of the plant was carried out for both fresh weight (FW) of plant and dry weight (DW) of the plant by measuring stem length along with the size and number of leaves. Biomass (FW, DW) of the plant grown in various amendments of soil and sludge were measured (g·plant^{-1}).[15]

Estimation of biochemical parameters of plants

Biochemical and nutritional attributes of plant samples grown in various amendments of soil and sludge were estimated using the following methods;

Chlorophyll contents

A total weight of 1 g of leaves from plants grown under different soil amendments was used to measure the chlorophyll contents. The weight of mature leaves from each sample was determined and these leaves were immersed in cold acetone (80 %) for 72 hours in shade at 4 ºC. The quantification of chlorophyll was carried out by measuring absorbance at 645 and 663 nm.[16]

Carotenoid contents

A total weight of 1 g from each sample grown under different sewage sludge and soil amendments was used to determine carotenoid content in leaves. The leaves were chopped and immersed in cold acetone (80 %) for 72 hours in shade at 4 ºC. For carotenoid contents determination, the absorbance of each sample was recorded at 470 nm.

Protein content determination

Protein content was determined by taking 500 mg from each sample of plant grown under different sewage sludge and soil amendments. All samples were powdered into fine particles. Afterward, 5-10 mL of buffer solution (pH = 9) was added to each sample followed by centrifugation. The supernatants from samples were used for the measurement of variation in protein content in each grown sample. Five test tubes were taken and 0.2, 0.4, 0.6, 0.8, and 1 mL of working standards were added to these test tubes. Each sample extract (0.1 mL) was also taken in individual test tubes. Distilled water was used to make a volume of up to 1 mL for each sample as well as for the working standard. A mixture (50 mL of 2 % Na_2CO_3 in 0.1 N NaOH, 1 mL of 0.5 % $CuSO_4$ in 1 % Rochelle salt, and 5 mL of Alkaline Copper solution) was added in each test tube, mixed properly, and left on the shelf for 10 minutes. The addition of Folin-Ciocalteau (FC) reagent (0.5 mL) was also carried out in all samples followed by room temperature incubation for 30 minutes in the dark. The absorbance was measured at 600 nm.

Determination of heavy metal content

Atomic absorption spectrophotometry was employed to determine heavy metal content in the fruit of the plant being studied grown under different sewage sludge and soil amendments. This study was carried out to evaluate the toxicity buildup in plant samples due to the presence of heavy metals in sewage sludge. For this purpose, the fruit of *M. charantia* grown in various amendments, was used. Each fruit sample (1g) from different sewage sludge and soil amendments was digested using a mixture H_2O_2 and HNO_3 (1:3/v:v). All samples were diluted using distilled water. The heavy metals were estimated using standard solutions of each metal. Results were obtained in triplicates and presented in ppm for each sample in comparison to standard curves.[17,18]

Antioxidant studies in selected plant parts

The determination of antioxidant potential was carried out by several methods which are as follows.

Quantitative determination of ascorbic acid

The indophenol titration method was used for the determination of ascorbic acid content in all parts of the plant grown under different sewage sludge and soil amendments. The methanolic extracts (0.3 mL) from selected plant parts were homogenized in a 10 mL of metaphosphoric acid (8 %) followed by the addition of 20 mL of glacial acetic acid (3 %) solution to each sample. The titration of this mixture was carried out against 2,6-dichloro-indophenol till the pink color was sustained. Ascorbic acid content was measured using a standard curve and results were presented as mg ascorbic acid per g of the powdered samples.[19]

Extraction and determination of total phenolic contents

To determine the total phenolic content in all parts of plant extracts grown under different sewage sludge and soil amendments, the FC reagent was used. For this purpose, 142 mL of the plant extract from each sewage sludge and soil amendment (25 mg) was taken in a flask and mixed initially with 20 mL of distilled water and freshly prepared FC reagent (800 μL) was added to it with vigorous shaking for 3 minutes followed by the addition of 3 mL of Na_2CO_3 (7.5 %) to the mixture. The final volume of the solution was precisely adjusted to 50 mL. Solutions were allowed to stand for 2 h in the shade under normal conditions. The absorbance of each sample was measured at 765 nm spectrophotometrically. Gallic acid was used as the standard, and the mean value of triplicate results was presented as gallic acid equivalents. Results were obtained in triplicate and were averaged.[20]

Extraction and determination of total flavonoid contents

Total flavonoid content (TFC) in all parts of *M. charantia* grown under different soil amendments with sewage sludge was determined using a reported method. Each grown sample (1 g) was extracted with 80 % methanol (20 mL) using Soxhlet unit followed by filtration and centrifugation at 3000 rpm for 20 minutes. The resultant supernatant layer was subjected to the estimation of TFC.[21]

For this purpose, each sample (1 mL) was taken in a flask, and doubly distilled water was added to each sample for diluting it five times. Afterward, 0.6 mL of $NaNO_2$ (5 %) was added followed by the addition of 0.5 mL of $AlCl_3$ (10 %). After a time, interval of 5 minutes, 2 mL of 1 M NaOH were added. After a thorough mixing of the above ingredients, each sample was diluted with 2.4 mL of distilled water. The absorbance of each sample was measured at 510 nm spectrophotometrically. The mean value of triplicate results for each sample was presented as $mg \cdot g^{-1}$ epicatechin equivalents.

Total tocopherol content

The determination of total tocopherol content in all plant parts grown under different soil amendments with sewage sludge was carried out by using HPLC. The sample injection was carried out by using a Rheodyne injection valve with a 20 μL loop. A mobile phase (acetonitrile/methanol/isopropanol) with a volume ratio (10:9:1) was used. After a time, an interval of 30 minutes elution, the chromatograms displayed several sharp tocopherol signals. For the quantification of tocopherols, areas under the curves and retention times were calculated in comparison to the standards used. The total sum of α, β, γ, and δ isomers constituted the total tocopherol content.[22]

Quantitative determination of free radical scavenging ability

A previously documented method was applied to determine the free radical scavenging ability of all parts of the plant grown under different sewage sludge and soil amendments. For this purpose, each plant extract (2 mL) was added to 5 mL of freshly prepared ethanolic solution of 2, 2-diphenyl-1-picrylhydrazyl (DPPH). The variation in absorbance at 515 nm was recorded at different intervals of time such as 0, 0.5, 1.0, 2.0, 5.0, and 10 minutes (up to 50 %). The remaining concentration of DPPH stable radicals was determined using the standard curve. Capability of each plant extract was evaluated by recording the absorbance at 515 nm after 5 minutes. Results were expressed in IC_{50} (mg mL^{-1}).[23]

Quantitative determination of antioxidant potential by $ABTS^{\pm}$ scavenging assay

The validity of the antioxidant potential of all parts of the plants grown under different soil amendments was also checked by using $ABTS^{\pm}$ radical cation scavenging assay. A filtrate was prepared by passing

2, 2′-azino-bis (3- ethylbenzthiazoline-6 sulphonic acid) aqueous solution (5 mM) through manganese oxide (MnO_2 which is an oxidizing agent) on filter paper. Excess MnO_2 was removed from the filtrate using a fisher band membrane of 0.2 mm. Methanolic extracts of all the plant samples were diluted with 5 mM phosphate buffered saline (pH = 7.4) to attain an absorbance of 0.700 at 734 nm spectrophotometrically. The absorbance of all samples was measured after 10 minutes of mixing each extract (1 mL) with $ABTS^{\pm}$ radical cation solution (5 mL) at ambient temperature. The control used in this study was phosphate buffered saline solution (pH = 7.4). Results were expressed as μmole Trolox equivalent per g DW for each sample. All readings were taken in triplicates.[24]

Inhibition of linoleic acid oxidation

The inhibition study of linoleic acid oxidation was performed on selected plant parts grown under different sewage sludge and soil amendments by a previously reported method. Methanolic extracts of all samples of *M. charantia* were prepared. Linoleic acid (0.13 mL), ethanol 99.8 % (10 mL), and 0.2 M sodium phosphate buffer (10 mL) with pH 7.0 were mixed with 1.0 mL from each sample extract. Distilled water was added to the sample solutions to acquire a total volume of 25 mL each. The incubation of all samples was carried out at 40 ^{0}C and the degree of oxidation was measured using the thiocyanate method. Thiocyanate solution was prepared by mixing ethanol 75 % (10 mL), ammonium thiocyanate 30 % (0.2 mL), and ferrous chloride solution (0.2 mL). The ferrous chloride solution was 20 mM and was prepared in 3.5 % HCl. The resultant thiocyanate solution was mixed with 0.2 mL of each sample. The absorbance of all the samples along with blank linoleic acid was measured at 500 nm after stirring them for 3 minutes. A synthetic antioxidant, BHT α-tocopherol was used as a positive control. The antioxidant activity of samples was expressed as percent inhibition of linoleic acid peroxidation.[25]

Ferric reducing antioxidant power (FRAP)

Another method used for validation of antioxidant activity of the plant samples grown under different soil and sewage sludge amendments was by using FRAP assay. Determination was carried out by mixing 40 mM acetate buffer (pH 3.6), 20 mM ferric (III) chloride and 10 mM tripyridyltriazine (prepared in 40 mM HCl) in a ratio of 10:1:1, respectively. Crude methanolic extracts (50 µL) from all grown samples of *M. charantia* were added to the separate flasks containing 2.0 mL of above-mentioned FRAP reagent for each extract sample. Similar quantity of FRAP reagent was added to the methanol taken as blank. Absorbance of each sample was recorded at 593 nm. All measurements were taken in triplicates and results were expressed in mM Trolox equivalents in accordance to standard curve of Trolox.[26]

Conclusions

The present study covers the determination of growth patterns of *Momordica charantia* L. in pure as well as amended soil with varying percentages of sewage sludge. The amendment of soil with sewage sludge not only solves the problem of sewage sludge disposal, as it is known to pose several health-related risks for mankind, but also helps effectively in enhancing germination and total biomass along with improvements in total carotenoid, chlorophyll, and protein contents. The soil amendments with certain percentages of sewage sludge also showed a marked increase in phenolic, flavonoid, and tocopherol contents along with better radical scavenging ability of the plant. Certain protocols used in the study reveal that the antioxidant capacity of the plant grown in amended soil with sewage sludge increases compared to pure soil. The suitable percentages of sewage sludge in soil are 40 % and 60 %; beyond these percentages of soil amendments with sewage sludge the results started to show negative impacts which can possibly be attributed to the increased metal toxicity in the plant with increasing percentage of sewage sludge. The suitable percentages of sewage sludge soil amendments are purely for *Momordica charantia* L. and may vary for other plants.

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