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Optimization of sewage sludge mixing with soil to attain promising neutraceuticals attributes in the *Lagenaria siceraria* L. (Kaddu) Plant

ZOHAIB SAEED[1], SHAHID IQBAL*[1], UMER YOUNAS[1,2], MUHAMMAD PERVAIZ*[3], SYED MOHSIN ALI NAQVI[3] & RANA RASHAD MAHMOOD KHAN[3]

[1]*Department of Chemistry, University of Sargodha, Sargodha, Pakistan*
[2]*Department of Chemistry, The University of Lahore, Lahore, Pakistan*
[3]*Department of Chemistry, Government College University, Lahore, Pakistan*

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***Corresponding Author:**

Zohaib Saeed:
zohaibsaeed46@gmail.com

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ABSTRACT
Disposal of sewage sludge is a critical issue worldwide for the environmentalists. There are many methods for its disposal. The most suitable and economical method is its land application. *Lagenaria siceraria* L. plant was grown in various amendments of soil and sewage sludge to check the tolerance of the plant and to select the optimized mixing of sewage sludge with soil for this particular plant. Estimation of biochemical parameters like chlorophyll content, carotenoid content, and protein content along with total biomass of the plant showed that the soil mixed with 20% sewage sludge showed maximum growth of the plant. Meanwhile, antioxidants like TPC, Ascorbic acid content and antioxidant potential (DPPH, ABTS, FRAP) were also measured and maximum values were found in 20% to 40% of sewage sludge. Hence, sewage sludge may be exploited for the useful purposes in a controlled manner.
Keywords: Sewage Sludge, *Lagenaria siceraria* L., Antioxidant potential

INTRODUCTION

Lagenaria siceraria L. belongs to Cucurbitaceae family, its common name is bottle gourd (Urdu: Kaddu) and it is used as vegetable grown in Asia and Europe (Kubde *et al.,* 2010). There are 118 general and 825 species of this family (Clarke *et al.,* 2006). It contains many essential nutrients e.g, bottle gourd seeds are rich in protein (39.5 kg/100 kg of sample) and fat (46.1 kg/100 kg of sample). Moreover, minerals like potassium, magnesium and phosphorus are also present in this plant. Phenolic compounds such as, catechin, ascorbic acid and naringenin are also found in the fruit of this plant. (Ghule *et al.,* 2006). It was found that *Lagenaria siceraria* L. possessed antioxidant activity, central nervous system activity, anti-inflammatory activity, antidiarrheal activity, antimicrobial activity, cytotoxicity and anticancer activity (Palamthodi & Lele, 2014).

Rapid industrialization and massive increase in the population resulted in the generation of gigantic amounts of wastewater. Pretreatment of wastewater resulted in the generation of a solid residue called sewage sludge. Disposal of sewage sludge is an important issue for the environmental scientists. Utilization of sewage sludge for agricultural purposes is a very common practice worldwide. Sewage sludge has been reported to contain many useful contents which on mixing with soil enhance properties of the soil like bulk density, porosity, pH, mineral composition. On the other hand it may contain organic pollutants such as polycyclic aromatic hydrocarbons (PAHs), detergents and remains of pharmaceuticals discharged by household activities and toxic metals released by industries which limit its application for the agricultural purposes. Therefore, controlled and optimized amounts of sewage sludge must be used for each plant grown in it as various species of plants have different tolerance level.

Objective of this study is to enhance the growth, nutritional attributes and antioxidant potential of *Lagenaria siceraria* L. plant by optimizing the mixing of sewage sludge with soil.

MATERIALS AND METHODS

Preparation of soil amendments with sewage sludge (experimental setup)

Author's Contribution: Z.S., Did sampling, characterization, experimental designing and wrote manuscript; **S.I.**, Analyzed data, supervised project, revised and finalized manuscript; **U.Y.,** Did sampling, experimental designing and statistical analysis; **M.P**., Did experimentation, experimental Planning and manuscript revision; **S.M. & A.N**., Did sampling collection, experimentation and data analyzed; **R.R. & M.K.**, Revised manuscript and statistical analysis

Six different proportions of sewage sludge and soil (0% S.S, 20% S.S, 40% S.S, 60% S.S, 80% S.S and 100% S.S) were made (10 kg weight/pot) in triplicates respectively in the agricultural fields of University College of agriculture, Khushab Road, University of Sargodha, Sargodha. Seeds of *Lagenaria siceraria* L. (Kaddu) plant were sown in each pot and monitored for 75 days till harvesting.

Physical growth parameters of plant

Shoot length, number of leaves and size of leaves were measured for total biomass determination of fresh weight and dry weight as well. Biomass (FW, DW) of the plant *Lagenaria siceraria* L. grown in various compositions of soil and sludge were measured (g/plant) (Wang *et al.*, 2006).

Chlorophyll and carotenoid contents

For chlorophyll estimation about 1 g of mature leaves for each sample were taken and dipped in cold acetone (80%) for the period of 3 days in dark at 4ºC following method of *Lichtenthaler,* (1987). UV-Visible spectrometer was used for measuring absorbance of resulting extracts at 645 and 663 nm for the quantification of chlorophyll content and estimation of carotenoid was done at 470 nm (Wong, Li, & Wong, 1996).

Protein contents

500 mg of each plant sample was finely powdered and 5-10 mL of buffer (pH = 9) was added into each sample and centrifuged. Supernatant was used for measurements. Working standards were taken 0.2, 0.4, 0.6, 0.8 and 1 mL in five test tubes. Sample extract (0.1 mL) was also taken in each test tube. Make volume up to 1 mL for each sample and working standard with distilled water. A mixture (50 mL of 2% sodium carbonate in 0.1N Sodium hydroxide, 1 mL of 0.5% Copper sulphate in 1% potassium sodium tartrate and 5 mL of Alkaline Copper solution) was added in each test tube and allowed to stand for 10 min. after proper mixing. Folin-ciocalteau reagent (0.5 mL) was also added to each sample and incubated at room temperature for 30 min under dark. Protein content was estimated at 600 nm spectrophotometrically (Sadasivam & Manickam, 1992).

Heavy metals content in plant

Atomic absorption spectrophotometer (Shimadzu 7000 F) was used for the estimation of heavy metals in the fruit of *Lagenaria siceraria* L. for the toxicity evaluation of all the plants. (Sposito *et al.*, 1982) (Benbrahim *et al.*, 2006).

Quantitative determination of ascorbic acid

Indophenol titration method was used for the determination of ascorbic acid content in all parts of the plants (*Lagenaria siceraria* L.). Each methanolic extract (0.3 mL) was homogenized in metaphosphoric acid (8%) and 20 mL of glacial acetic acid (3%) solution. This mixture was titrated against 2, 6 dichloroindophenol till the pink color sustained. Results were presented as mg ascorbic acid/g of the powdered samples (Sims & Kline, 1991).

Extraction and estimation of total phenolic contents

For the determination of total phenolic content in all parts of plants (*Lagenaria siceraria* L.) extracts, FC reagent was used. For this purpose, 142 mL of Na_2CO_3 (7.5%) and freshly prepared FC reagent (800 µL) were mixed in diluted plant extract (200 µL) of each sample. All the mixtures were diluted with distilled water (7 mL). Solutions were kept for 2 h in dark under normal conditions for the completion of reaction. The absorbance of each sample was measured at 765 nm spectrophotometrically. Gallic acid was used as standard and results were presented as gallic acid equivalent. Results were obtained in triplicates and were averaged (Polshettiwar, Ganjiwale, Wadher, & Yeole, 2007).

DPPH- scavenging assay

A previously documented method was applied for the estimation of free radical scavenging activity of all the parts of plants (*Lagenaria siceraria* L.) For this purpose, each plant extract (2mL) was added to 5mL freshly prepared solution of DPPH (2, 2- diphenyl-1-picrylhydrazyl). Change in absorbance at 515 nm was recorded at different intervals of time like 0, 0.5, 1.0, 2.0, 5.0 and 10 min. (up to 50%). Remaining concentration of DPPH stable radicals was determined using standard curve. Capability of each plant extract was evaluated by noting absorbance at 515 nm after 5 min. Results were expressed in IC_{50} (mg/mL) (S. Iqbal, 2005).

ABTS $^{\pm}$ scavenging assay

Antioxidant activity of all parts of the plants was also measured using reported method called as ABTS$^{\pm}$ radical cation scavenging assay. A filtrate was prepared by passing 2, 2´-azino-bis (3-

ethylbenzthiazoline-6 sulphonic acid) aqueous solution (5mM) through manganese oxide (oxidizing agent) on filter paper. Excess MnO_2 was removed from filtrate using fisher band membrane of 0.2 mm. Methanolic extracts of all the parts of plant were diluted with 5mM phosphate buffered saline (pH=7.4) to attain absorbance of 0.700 at 734 nm spectrophotometrically. Absorbance of all samples was measured after 10 min of mixing each extract (1mL) and $ABTS^{\pm}$ radical cation solution (5mL) at ambient temperature. Phosphate buffered saline (pH=7.4) was used as a control. Results were expressed as µmole Trolox equivalent/g DW for each sample. All readings were taken in triplicates (S. Iqbal et al., 2012).

Ferric reducing antioxidant power (FRAP)

Antioxidant activity was estimated using FRAP assay. Evaluation was done by taking 40 mM acetate buffer (pH 3.6), 20 mM ferric (III) chloride and TPTZ- tripyridyltriazine (10 mM in 40 mM HCl) were taken and combined in a ratio of 10:1:1 respectively. Crude methanolic extract (50 microliter) of all parts of *Lagenaria siceraria* L. were added to the above mentioned FRAP reagent (2mL) for each sample. Similar amount of FRAP reagent were added to the blank (Methanol). Absorbance of each sample was recorded spectrophotometrically at 593nm. All readings were taken in triplicates and results were expressed in mM Trolox equivalents in accordance to standard curve of Trolox (S. Iqbal *et al.*, 2007).

RESULTS AND DISCUSSION

Effect on the total biomass of *Lagenaria siceraria* L. grown in pure and amended Soil

Lagenaria siceraria L. also showed prominent variation in total biomass with the change in sewage sludge proportion in soil. *Lagenaria siceraria* L. showed (Fig. 1) maximum yield in 20% amendment of sewage sludge with soil which is 677g fresh weight per plant and minimum fresh weight of the plant were estimated in the plant grown in pure sewage sludge. It reveals the sewage sludge as a potential nutrient resource along with its hazardous and toxic nature. (Gwenzi *et al.*, 2016).

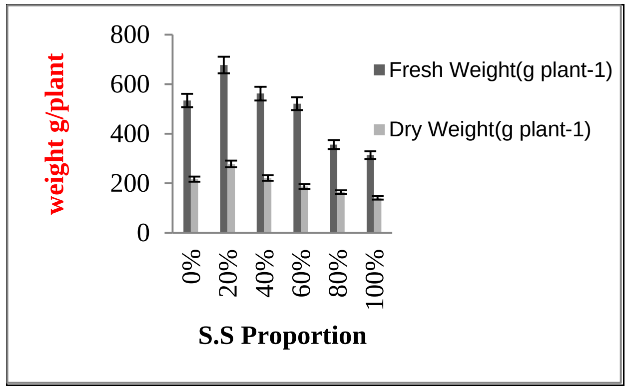

Fig. 1: Total biomass fresh weight (FW) and dry weight (DW) g/plant of *Lagenaria siceraria* L.

Chlorophyll and carotenoid contents

Chlorophyll a photosynthetic pigment has been reported to increase in the mustard plant when grown in the soil amended with mixed industrial effluents. (Singh & Rathore, 2018). Chlorophyll content has also been reported to increase in *Brassica nigra* L. when grown in amended soil (Karak *et al.*, 2013). In our results, (Table I) maximum amount of the photosynthetic pigments estimated in the plants grown in 20% to 40% amendment of sewage sludge with soil ranging from 4.75 to 4.88 mg/g for chlorophyll *a* and 1.45 to 1.52 mg/g for carotenoid. Higher amendments showed negative results as chlorophyll and carotenoid content decreased predominantly.

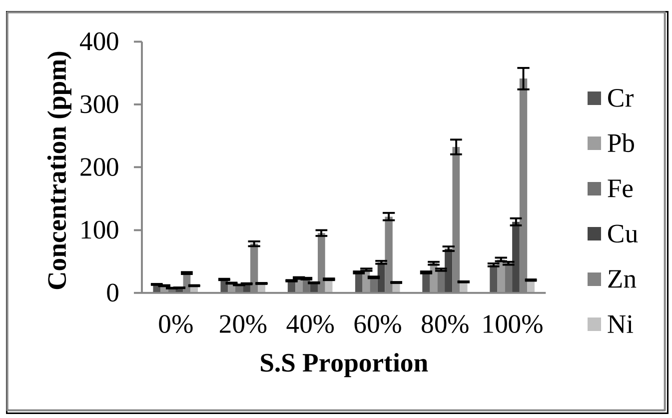

Fig. 2: Concentration (ppm) of heavy metals in fruit of *Lagenaria siceraria* L.

Quantitative determination of ascorbic Acid

Ascorbic acid content was found (Fig. 3) maximum in the 40% sewage sludge amendment with the soil in the *Lagenaria siceraria* L. plant ranges from 12 to 14 mg/g in all parts of the plant. This variation featured the change in the soil characteristics and its fertility with the change in the amount of the sewage sludge in the soil and revealed the effect of soil amendment with sewage sludge.

Table I Chlorophyll content (Chl *a* and Chl *b*), Chl *a* / Chl *b* ratio and Carotenoid content in *Lagenaria siceraria* L. (Leaves)

Sludge %	Chlorophyll *a* (mg/g FW)	Chlorophyll *b* (mg/g FW)	Chl *a* / Chl *b*	Carotenoids (mg/g FW)
0 %	3.34±0.44	1.14±0.11	2.65±0.03	1.27±0.07
20 %	3.46±0.24	0.88±0.14	2.44±0.07	1.38±0.11
40 %	4.75±0.61	0.97±0.12	2.14±0.02	1.45±0.04
60 %	4.88±0.01	1.23±0.11	2.26±0.12	1.52±0.08
80 %	3.81±0.08	0.84±0.09	1.92±0.17	1.64±0.05
100 %	3.73±0.02	0.78±0.05	2.12±0.19	1.55±0.02

Protein contents

Protein content in root shoot and leaves of the mustard plant have been reported to be increased at 50% amendment in 60 days of growth period (Ahmed et al., 2017). Protein content in roots, shoots, leaves and fruits of *Lagenaria siceraria* L. plant were found maximum in 60% amendment of sewage sludge with soil it was found 71, 88, 79 and 117 mg/g respectively in all parts (Table II). It drastically decreased at higher amendments of sewage sludge with soil such as at 80% and in pure sludge. This result proved the presence of toxic metal ions in the higher amendments of the sewage sludge (Wen et al., 2016).

Table II Protein content (mg/g) in various parts of the *Lagenaria siceraria* L.

Sludge %	Roots	Shoots	Leaves	Fruits
0%	55±0.66	57±0.55	64±1.12	97±0.54
20%	63±0.56	61±0.99	67±0.43	99±1.32
40%	71±0.32	88±0.45	79±0.66	117±0.57
60%	75±0.15	82±0.19	78±0.85	112±0.93
80%	69±0.66	71±0.12	67±0.43	94±0.45
100%	54±0.81	63±0.33	59±0.77	88±0.33

Toxicity evaluation of the plants

Heavy metals in the edible parts (Fruit) of the plant were quantified. All heavy metals estimated (Fig. 2) are in the permissible limit in the fruit of all the plants. So, there is no phytotoxicity found in the plant. Thus, edible part of the plants grown in the amended soil is recommended to be used for human being. However, repeated application of the sewage sludge in the soil may cause phytotoxicity. Therefore, optimized and controlled amount of the sewage sludge is required to apply for the best results with zero toxicity (Fu *et al.,* 2018).

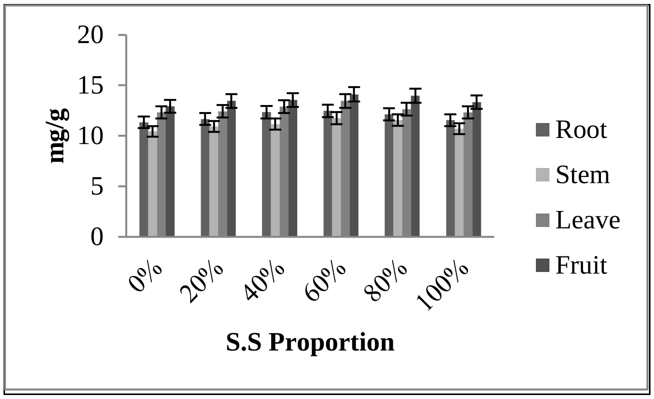

Fig. 3: Ascorbic acid (Vitamin C) content (mg/g) in *Lagenaria siceraria* L. in all parts of the plant

Total phenolic content

Total phenolic content was determined using Folin-ciocalteu based spectrophotometric assay. This F.C reagent reacts with phenolic compounds and get reduced. Total phenolic content in all the parts of *Lagenaria siceraria* was optimum in 60% proportion of the sewage sludge in soil ranges from 240 to 250 mg of GAE/100g DW in all parts of plant (Fig. 4). Results revealed that increase in total phenolic content at certain levels in both the plants is due to the presence of organic matter, and higher amounts of macronutrients like N, P, K in the amended soil (Valifard *et al.*, 2014). Conversely, elevated amount of heavy metals at higher rates of sewage sludge may inhibit the production of phenolic content by producing free radicals and other reactive oxygen species.

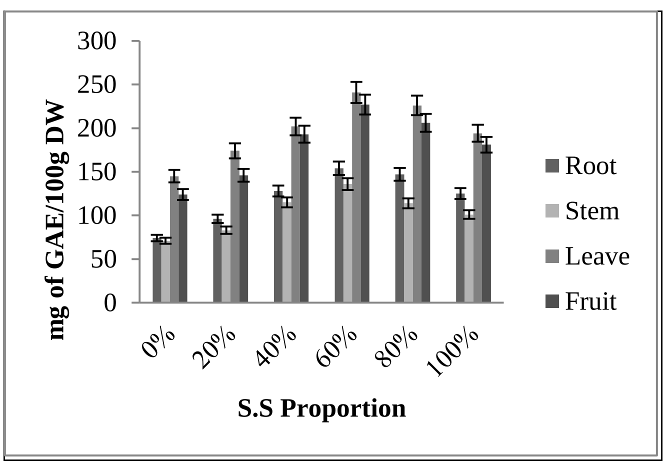

Fig. 4: Total phenolic content (mg of GAE/100g DW) in all parts of *Lagenaria siceraria* L.

DPPH free radical scavenging activity

Lagenaria siceraria L. showed (Fig. 5) maximum IC_{50} value in the extracts (root, shoot, stem, leave) grown in the soil with 60% sewage sludge and the results found in the range of 18 to 20 IC50 mg/mL and this value decreases with the increase in the sewage sludge proportion in the soil. Thus, we can deduce that sewage sludge mixing showed its impact on the antioxidant activity with the change in the sewage sludge proportion in the soil (Krol *et al.*, 2015).

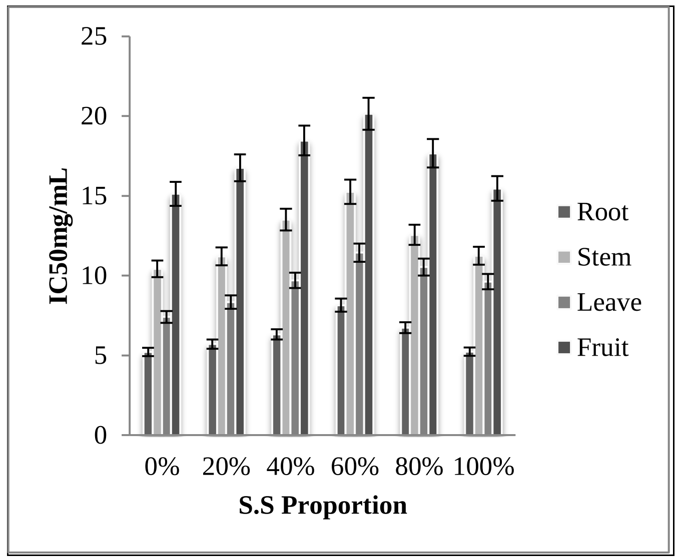

Fig. 5: DPPH free radical scavenging activity in all parts of (IC_{50}mg/mL) in *Lagenaria siceraria* L.

ABTS $^{\pm}$ Radical scavenging assay

The scavenging activity of the methanolic extracts of the various parts of the *Lagenaria siceraria* L. have been shown in the Fig. 6. It is clear that $ABTS^{\pm}$ scavenging activity in all the parts of the plants increased up to the 60% S.S in the soil (7 to 9 µmole Trolox equivalent/g DW) while at higher percentage of sewage sludge with soil it is found to be decreased markedly.

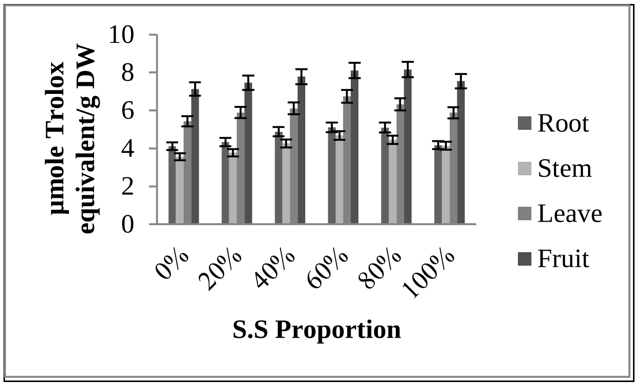

Fig. 6: ABTS± radical scavenging activity (µmole Trolox equivalent/g DW) in all parts of *Lagenaria siceraria* L.

Ferric Reducing Antioxidant Power (FRAP)

Change in sewage sludge composition in the soil altered the reducing capabilities of the plant samples grown in it. *Lagenaria siceraria* L. also showed variation in the reducing power (Fig. 7) in samples grown in different amendments of the soil and sludge. Maximum reducing power was shown in all parts of the *Lagenaria siceraria* L. grown in the 40% to 60% sewage sludge amendment as compared to the control and other higher amendments. Hence, sewage sludge amount in the soil is associated with the variation in the reducing power of both the plants. Characterization of sewage sludge attributes with this variation as higher percentage of the

sewage sludge in the soil enhance the amount of toxic heavy metals which in turn produce reactive oxygen species and these species inhibit the growth and productivity of the functional foods and other nutrients (Beghlal *et al.*, 2016).

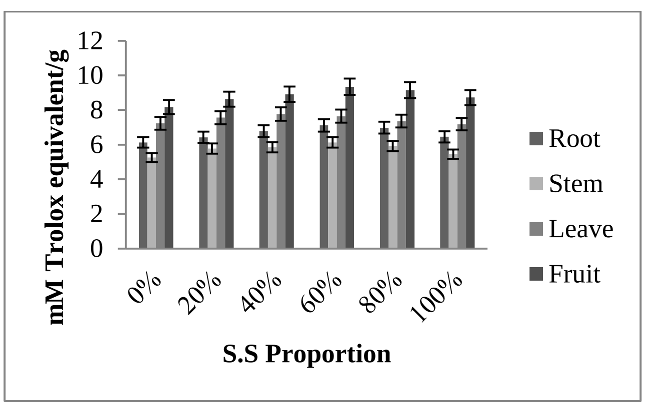

Fig. 7: Ferric Reducing Antioxidant Power (mM Trolox equivalent/g) in all parts of *Lagenaria siceraria* L.

CONCLUSION

Sewage sludge has been proved as a rich source of minerals and nutrients by altering the soil properties, which ultimately affects the neutraceuticals and antioxidant potential of the *Lagenaria siceraria* L. However, it is suggested that sewage sludge mixing with soil must be optimized as it may add phytotoxicity and other adverse effects to the plant. For *Lagenaria siceraria* L. 20% sewage sludge with soil is a best amendment.

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