Biological Forum – An International Journal **15(3): 298-304(2023)**

ISSN No. (Print):0975-1130
ISSN No. (Online):2249-3239

Phytochemical and Antimicrobial Assessment of Leafy Vegetables Collected from Solid Waste Dumping Ground and Normal Ground - A Comparative Study

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(Received: 08 January 2023; Revised: 12 February 2023; Accepted: 20 February 2023; Published: 22 March 2023)
(Published by Research Trend)

ABSTRACT: Vegetables having antimicrobial activities play significant role against some pathogenic microorganisms. The study aims to assess and compare moisture content, antioxidant and antimicrobial activity of some selected leafy vegetables collected from Solid Waste Dumping Ground (SWDG) at Titagarh, Kolkata, West Bengal, India and from Normal ground (NG) namely Bottle gourd (*Lagenaria siceraria*), Red Amaranth (*Amaranthus cruentus*) and Ceylon Spinach (*Basella alba*). Matured, disease free and fresh leafy samples were collected from different SWDG and NG. Vegetables collected from NG showed significant higher values of all parameters compared to SWDG (P< 0.05). Disc diffusion method was applied for antimicrobial assessment at different concentrations (50µl and 100µl) against Gram negative organisms. The inhibitory action was indicated in almost all raw leafy vegetables but, vegetables having concentrations of 50µl and 100µl, showed no inhibitory effect on the tested organism except leaves of *L. siceraria* (100µl). Among these vegetables collected from SWDG, showed maximum inhibitory effect in leaves of *L. siceraria* followed by *B. alba* and in *A. cruentus* against Gram negative organism. It has been concluded that, cultivation on solid waste have significant positive impact on the antioxidant and antimicrobial activities of leafy vegetables.

Keywords: Antioxidant activity, phytochemical screening, disc diffusion method, antimicrobial activity, antibacterial susceptibility test, SWDG, NG and CFU.

INTRODUCTION

Disposal of wastes to landfill areas generated by human activities play important roles for improving the soil property when it is mixed with surface soil. These wastes not only provide essential nutrients to the soil crops, but also protect the environment from hazardous effects (Phung *et al.,* 1978; Hossain *et al.,* 2017). After investigation by the experts, it was found that 62 million tons of municipal solid waste is generated per annum in 7,935 towns and cities by 377 million of urban people. Among these 43 million tonnes (MT) waste is collected, 31 MT is disposed in landfill areas and 11.9 MT deal with in certain way (Lahiry *et al.,* 2017). Some green leafy vegetables for e.g. Ceylon Spinach (*B. alba*), bottle gourd (*L. siceraria*) and Red Amaranth (*A. cruentus)* play significant roles in maintaining of human health as well as improving wealth of human life. These vegetables are rich in vitamins, minerals, phytochemicals and have antimicrobial, antioxidant and anticancer properties and also can protect against some chronic diseases like cancer, metabolic syndrome, diabetes, obesity and many others (Ülger *et al.,* 2018).Various studies have been showed that these bioactive antimicrobial agents are better as compared to other synthetic drugs which cause reaction like sensitization (Mahendranathan *et al.,* 2021; Yadav *et al.,* 2016; Jaiswal *et al.,* 2011; Cherian *et al.,* 2016). When plants are subjected to any stress condition, they secrete secondary metabolites which are known as phytochemicals (Ramakrishna *et al.,* 2011; Thakur *et al.,* 2020).

Antioxidants are the substances which at a low concentration prevent or retard the oxidation of easily oxidizable biomolecules such as lipids, proteins and DNA (Burgos *et al.,* 2021; Ratnam *et al.,* 2006). Antioxidants reduce damage by neutralizing the free radicals before they can attack cells and prevent damage to lipids, proteins, enzymes, carbohydrates and DNA (Fang *et al.,* 2002). This study emphasizes to assess the effects of secondary metabolites on living organism by Antibiotic susceptibility test method. AST is used to assess antimicrobial agent which could treat an infection caused by a particular organism (Wanger *et al.,* 2017). Disc diffusion method and Dilution methods are generally belonging to phenotype susceptibility testing, disk diffusion method generate qualitative

result based on zone of inhibition and the dilution method generate a quantitative result such as minimum inhibitory concentration. Results are reported as "susceptible" "intermediate" and "Resistant" (Banaei *et al.,* 2016).

AIMS AND OBJECTRIVE

- To assess and compare the moisture content of the selected green leafy vegetables collected from SWDG and NG.
- To assess and compare the antioxidant content in terms of total phenolic and flavonoids, of selected green leafy vegetables in ethanolic extract collected from SWDG and NG.
- To assess and compare the quality of selected green leafy vegetables in extract (aqueous and methanol) collected from SWDG and NG in terms of phytochemical screening.
- To assess and compare the antimicrobial activity of the selected green leafy vegetables in different extracts (raw, aqueous) collected from SWDG and NG.

METHODOLOGY (Olasupo *et al.,* 2018)

I. Collection of plant

3 type of vegetables like, Ceylon Spinach (*B. alba*), Bottle gourd leaves (*L. siceraria*) and Red Amaranth (*A. cruentus*) were collected from solid waste dumping ground SWDG Titagarh, Kolkata (WB), India and Normal Ground (NG). The vegetables leaves were collected in plastic zip lock bags and put on to the ice bag and brought to the laboratory which thereafter washed with distilled water with the help of tissue paper to remove any debris and used for the extraction.

II. Preparation of plant extracts

1 g each of the sample was crushed into mortar pestle, diluted with 10 ml of distilled water and 80% ethanol (v/v) separately and, the mixture was allowed to incubate at 4°C for 48 h. After the incubation period was over, the mixture was centrifuged (high speed cold centrifuge, superspin R-V/FA, PLASTO CRAFTS) at 10,000 rpm at 4° C for 10 min. The supernatant was filtered and collected in the test tube.

III. Phytochemical Screening of the Extracts

a) **Test for alkaloid (Mayer test):** The vegetable extract was stirred with 5 ml of diluted HCl and was filtered. The filtrate was tested carefully with addition of two drops of Mayer's regent by the sides of the test tube. A white creamy precipitate was recorded as positive indication (Tamrakar *et al.,* 2017).

b) **Test for coumarins**: 0.5 ml of diluted extracts was treated with 1ml of 2N NaOH. Formation of yellow color indicated the presence of coumarins. Further, 1ml of 5N HCl was added. A colorless solution was formed at the upper layer and it confirmed the presence of coumarin (Jacob *et al.,* 2011).

c) **Test for flavanoids**: The various sample extracts were treated with amyl alcohol and sodium acetate. After 5 min, the reaction mixture was treated with $FeCl_3$. The appearance of pink or red color confirms the presence of flavonoids (Mahendrarajah *et al.,* 2011).

d) **Test for tannins:** 1 ml of diluted extracts treated with 2 ml of 10% lead acetate. White color precipitation indicated the presence of tannins (Jacob *et al.,* 2011).

e) **Saponins (for methanol extract):** 2 ml of methanolic extract (v/v) was shaken vigorously to obtain a stable persistent froth. The frothing was then mixed with 3 drops of olive oil and observed for the formation of emulsion, which indicated the presence of saponins (Mahendrarajah *et al.,* 2011).

f) **Saponins (for water extract):** 1 g of each sample was boiled with 5 ml of distilled water separately and then filtered. To each filtrate, about 3 ml of distilled water was further added and shaken vigorously for about 5 min, frothing which persisted on warming was taken as an evidence for the presence of saponins (Bhatt *et al.,* 2014).

IV. Analysis of moisture content

a) **Instrumentation:** Hot air woven model no OU-1001/1S was used for drying the samples.

b) **Drying method:** For determination of moisture content, simply drying method was used, in which 5 g each of vegetable samples taken from SWDG and NG were allowed to dry in hot air woven after taking the initial weight of the samples. After drying for 3-4 h at 70° C final weight of the samples were taken. The experiments were carried out in triplicate for three consecutive days (AOAC, 1990).

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V. Determination of antioxidant content

a) **Instrumentation**

Spectrophotometer (Cystonic UV vis 118) was used to ascertainment the content of antioxidant.

b) **Biochemical assay of phenolics**

For determination of total phenolic content present in the leafy vegetables collected from SWDG and NG, FC (Folin-Ciocalteau) method was used. In this process, each of vegetables' extract solution was prepared using 70% of ethanol (w/v), and makes the concentration of each sample (50 mg/ml). 1 ml of the each plant extract was mixed with the 1ml of FC reagent (Folin-Ciocalteau 10%) and with 7.5% aqueous solution of 0.8 ml Sodium Carbonate ($NaCO_3$). The volume was made upto 10 ml using distilled water. After 30 min of incubation in dark room, blue color was formed due to the reduction of folin- ciocalteau in the presence of phenolics and the absorbence of the reaction was measured using spectrophotometer at 765 nm (Cystonic UV vis 118). The total phenolic content of vegetables was calculated as galic acid equivalent (GAE/g of dry weight of the sample) by using gallic acid as standard (Singleton *et al.,* 1999).

c) **Biochemical assay of flavonoids**

For determination of total flavonoid content present in the leafy vegetables collected from SWDG and Normal Ground (NG), AC method (Aluminum chloride method) was used in this process Each of the vegetables, extract solution was prepared using 70% of ethanol, and make the concentration of each sample (50

mg\ml). 1 ml of the each plant extract were mixed with 0.15ml of a 5% Sodium Nitrate ($NaNO_2$). After 6 min 0.15ml of 10% Aluminium Chloride solution ($AlCl_3$) was added after addition of Aluminium chloride a yellow color complex is formed with keto group of flavonoid Again after 6 minutes 2ml of 4% Sodium Hydroxide (NaOH) was added (turned immediately into red) and volume was made up to 10ml. The solutions were mixed well and kept in dark for 15 min at room temperature and the absorbance of reaction compound at 510nm was measured in spectrophotometer (Cystonic UV vis 118). The overall flavonoid content was expressed as mg of Quercetin equivalents (QE/g of dry weight of the sample) by using Quercetin as standard (Zhzng *et al.,* 2014; Pękal *et al.,* 2014).

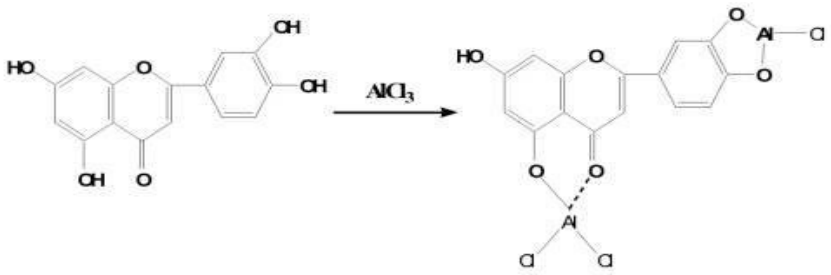

Fig. 1. (Makuasa *et al.,* 2020).

VI. Antibacterial susceptibility test (Cherian *et al.,* 2016)

a) Chemicals and Reagents

Streptomycin (25 mcg/disc), kanamycin (30 mcg/disc) and Penicilin (10 units/disc) Ampicilin (25 mcg/disc) were obtained from Thermofisher chemicals, West Bengal, India. Along with media like, nutrient agar (Hi media) and bacterial strains were collected from the pond water of cultivation area Titagarh, Kolkata, India.

b) Instrumentation

Laminar air flow chamber of model MFIH3X2 MICROFILT, India (Pune) was used for the sub-culturing of microbes, incubator model O-CIS 6 and autoclave model 220/230 VOLT/1/CY-AC, India.

c) Preparation of leaf extract concentrations

2 g of each plant extracts was crushed by mortar pestle, diluted with 10 ml of distilled water and crushed raw sample was used as sample.

d) Sterilization of Glass wares

All the glass wares (test tubes, pipettes, petri dishes, beakers and conical flasks etc.) nutrient agar and glass spreader were sterilized in a hot air oven at 120°C for 10 min at 15 psi of pressure.

VII. Differentiation of bacterial strains

a) Sample collection

Water sample collected from Ganga, West Bengal, India for the isolation of bacteria which could use in antimicrobial susceptibility test. 200ml of water was taken from Ganga river, and samples were brought to the laboratory of Food And Nutrition Department, Barrackpore Rastraguru Surendranath College (WB, India).

b) Preparation of media

14 g of nutrient agar (Hi media) dissolved in 500 ml of distilled water in a sterile conical flask. The medium was sterilized by autoclaving at 120°C for 10 min under 15 psi of pressure. Melted, sterile nutrient agar was poured into a series of sterile Petriplates following strict aseptic conditions and allowed to solidify for 30 min. The plates were filled to about two-third capacity of the molten agar that is about 20 ml per plate. Sterile nutrient agar medium in petri plate was incubated at fridge for 24 h to check for any sort of contamination and the plates were sealed with brown paper.

c) Isolation of bacteria from Ganga water

In order to get the pure colonies by reducing the number of bacterial colonies, this method was used. In this process, the collected water sample was taken and serially diluted with distilled water upto 10^9. 10 ml of each dilution was spread into the agar plate. Plates were incubated at 37°C for 24 h. After successfully growth of the microorganism, the pure colonies were sub cultured into nutrient agar slant, incubated at 37° C to achieve vigorous growth.

d) Characterization of bacterial culture

i) Morphological studies

In order to differentiate the bacteria whether it belongs to Gram positive or Gram negative, Gram staining was done.

ii) Shape, Size, Color

Morphological characteristics such as shape, size and color were studied by microscopic observation. The shape of the colony can studied by observing their margin and elevation and the colonies may fall into either round, rod or coccid shape. The size of the bacteria was studied by microscopic observation and was calculated in mili-microns (mμ). The size of the colony was measured by scale. The color of the bacteria was identified by observing the colony under microscope (Bhumbla *et al.,* 2022; Bruckner *et al.,* 2021). From this methods we separated Gram negative microorganisms.

iii) Preparation of culture media

In agar disc diffusion method, 28 g of nutrient agar (Himedia) was dissolved 1000 ml of distilled water in a sterile conical flask. The medium was sterilized by autoclaving at 120°C for 10 min under 15 psi of pressure. Melted, sterile nutrient agar was poured into a series of sterile petri plates following aseptic conditions and allowed to solidify for 30 min. The plates were filled to about two-third capacity of the molten agar i.e., about 20 ml per plate. Sterile nutrient agar medium in Petri plate was incubated at refrigerator for 24 h to check for any sort of contamination and the plates were sealed with brown paper. Gram negative bacteria cell

suspensions were adjusted to turbidity standard (10^{-5}) by serial dilution 0.1 ml of bacterial suspension was inoculated on the sterile nutrient agar in petri dishes at approximately 3.2×10^7 colony forming unit through spread plate method.

iv) Disc diffusion method

Antimicrobial activity of prepared plant extracts were determined by disc diffusion method. After spreading the bacterial strain it was allowed to dry for about 3 min. The sterile filter paper discs were impregnated with each test aqueous plant extracts (0.1ml) and placed on the surface of the inoculated agar plates. Streptomycin (25mcg/disc), kanamycin (30 mcg/disc) and penicillin (10 units/disc) ampicillin (25 mcg/disc) were used as positive control. The plates were then incubated at 37°C for 24 h after which microbial growth was determined by measuring the diameter of the zone of inhibition (mm) by using a transparent scale (Bhatt *et al.,* 2014).

RESULTS AND DISCUSSION

A. Preliminary phytochemical analysis in different plant leaf extracts (Mahendranathan et al., 2021)

Qualitative analysis of phytochemicals present in methanol and aqueous extracts of selected vegetables were indicated in below tables. As per the results, preliminary phytochemical analysis revealed the presence of totally 5 active compounds such as alkaloids, flavonoids, tannins, saponins, and Coumarins in different plant leaf extracts. Out of them, alkaloids, saponins, and flavonoids were present in almost all plant extracts.

Table 1.

Parameter	Bottle gourd leaves (*L. siceraria*)		Red Amaranth (*A. cruentus*)		Ceylon Spinach (*B. alba*)	
Solid Waste Dumping Ground(SWDG)						
	Aqueous extract	Methanolic extract	Aqueous extract	Methanolic extract	Aqueous extract	Methanolic extract
Saponins	+	+	-	+	-	+
Tannins	+	+	+	+	+	-
Flavonoid	+	+	+	+	+	+
Alkaloid	-	+	+	-	+	-
Coumarins	+	-	+	-	+	-
Normal Ground(NG)						
Saponins	-	+	-	-	-	+
Tannins	+	+	+	+	+	+
Flavonoid	+	+	+	-	+	+
Alkaloid	-	-	-	-	+	-
Coumarins	+	-	-	-	+	-

The experiments were carried out in triplicate.
(+) Presence of phytochemicals (-) Absence of phytochemicals

In case of Solid waste dumping ground (SWDG), alkaloids were absent in aqueous extract of leaves of *L. siceraria* and methanol extract of *A. cruentus* and *B. alba*.

Tannins were present in all leaf extracts. Saponins were only absent in aqueous extracts of *B. alba* and *A. cruentus* flavooids were present in all plant methanol and aqueous extracts.

Vegetables cultivated in normal ground (NG), saponins were only present in methanol extract of *B. alba* and leaves of *L. siceraria*. Tannins are present in all the leaf extract. Flavonoids were only absent in the methanol extract of *A. cruentus.*

As per the results, maximum number of phytochemicals was identified in aqueous extract of leaves of *L. siceraria* (except alkaloid in aqueous extract and cumarins in methanol extracts) collected from SWDG.

B. Analysis of moisture content

Table 2: Mean±SD of Moisture Content in Leafy Vegetables Collected from Solid Waste Dumping Ground (Titagarh) & Normal Ground.

Parameter	Moisture content (gm%)	Mean SD (n=3)
	SWDG	NG
Bottle gourd leaves (*L. siceraria*)	70.842.39	82.912.44
Red Amaranth(*A. cruentus*)	72.781.69	73.881.62
Ceylon Spinach(*B. alba*)	71.092.57	71.722.58

* SWDG(T)= Solid Waste Dumping Ground (Titagarh) **NG= Normal Ground

Table 3: Analysis of Variance of Moisture Content.

Conditions	Sum of square	Df	Mean square	F value	P value	Significance
Between places	31.74	1	31.74	1.515	0.034	S*
Between samples	30.79	2	15.40	0.735	0.576	NS

***P< 0.05, **P<0.01, ***P<0.001**

Table 3 revealed Moisture Content (gm %) of the studied leafy vegetables of SWDG (T) and NG. Result showed that moisture content is maximum in case of *A. cruentus* (72.78 g %) in solid waste dumping area but in Normal ground highest amount of moisture was found in leaves of *L. siceraria* (82.91gm%). *B. alba* (SWDG-71.09 gm%, NG- 71.72 gm%) had lowest amount of moisture irrespective of the locations.

Statistical analysis showed no significant differences (P>0.05) between samples but significant variations exit (P< 0.05) between two places.

C. Determination of antioxidant content Biochemical assay of phenolics

Table 4: Mean±Sd of Total Polyphenol Content in Leafy Vegetables Collected from Solid Waste Dumping Ground (Titagarh) & Normal Ground.

Parameter	**POLYPHENOL CONTENT (mg GAE/g) MEAN±SD, n=3**	
	SWDG(T)*	**NG****
Bottle gourd leaves (*L. siceraria*)	13.820.21	7.070.21
Red Amaranth(*A. cruentus*)	30.260.32	23.670.33
Ceylon Spinach(*B. alba*)	19.530.41	6.930.56

Table 5: Analysis of Variance of phenolic Content.

Conditions	Sum of square	Df	Mean square	F value	P value	Significance
Between places	32.713	1	32.713	0.104	0.008	S*
Between samples	1655.372	2	827.686	2.636	0.275	NS

*P< 0.05, **P<0.01, ***P<0.001

Polyphenbol content was found in the range of 13.8 – 30.26 mg GAE/g in case of SWDG (T) and 7 -23.67 mg GAE/g in case of NG. Statistical analysis showed (Table 5) significant variations did not exist (P<0.001) between samples but exist between two places (P <0.05). The amount of polyphenol contents for the samples of SWDG(T) were 13.82±0.21, 30.26±0.32, and 19.53±0.41 GAE/g whereas in case of NG it was found to be 7.07±0.21, 23.67±0.33 and 6.93±0.56 mg GAE/ in case of leaves of *l. siceraria*, *A. cruentus*, and *B. alba* respectively.

D. Biochemical assay of flavonoids

Table 6: Mean±SD of Total Flavonoid Content (mg QA/ gm of DW) In Leafy Vegetables Collected From Solid Waste Dumping Ground (Titagarh) & Normal Ground.

Parameter	**Flavonoid content (mgQA/g) Mean±SD, n=3**	
	SWDG(T)*	**NG****
Bottle gourd leaves (*L. siceraria*)	11.53±0.28	10.32±0.13
Red Amaranth (*A. cruentus*)	20.24±0.81	12.06±0.71
Ceylon Spinach (*B. alba*)	17.35±0.52	13.91±0.22

Table 7: Analysis of Variance of Total Flavonoid Content.

Conditions	Sum of square	Df	Mean square	F value	P value	Significance
Between places	24.923	1	24.923	4.754	0.016	S*
Between samples	35.116	2	17.558	3.348	0.230	NS

*P< 0.05, **P<0.01, ***P<0.001

Table 7 showed that the flavonoid contents (mg QA/g) in Leafy Vegetables Collected from Solid Waste Dumping Ground (Titagarh) & Normal Ground varied significantly (P <0.05). Flavonoid content was found in the range of 11.53 – 17.3 mg QA/g in case of SWDG (T) and 10.3 – 13.9 mg QA/g in case of NG. Statistical

analysis showed (**Table 3.1**) significant variations did not found between three different samples but found between two places (P< 0.05). The amount of flavonoid content for SWDG(T) were 11.53±0.28, 20.24±0.81, and 17.35±0.52 mg QA/g whereas in case of NG the flavonoids content were 10.32±0.13, 12.06±0.71, and 13.91±0.22 mg QA/g in case of leaves of *l. siceraria*, *A. cruentus*, and *B. alba* respectively.

E. Determination of Antimicrobial Activity

Table 8: Mean±SD diameter of zone of inhibition, caused by extracts of leaf samples against gram negative bacteria at different test concentrations collected from SWDG and NG.

Plant species	**Zone of inhibition (mm)**		
	Test concentration		
SWDG	**0.05ml(50µl)**	**0.1ml(100 µl)**	**Raw extract**
Bottle gourd leaves (*L. siceraria*)	12.330.57	15.330.57	20.331.52
Red Amaranth (*A. cruentus*)	8.33	9.660.57	17.33
Ceylon Spinach (*B. alba*)	7.770.57	8.660.57	19.33
NG			
Bottle gourd leaves (*L. siceraria*)	5.330.57	14.330.57	17.33
Red Amaranth (*A. cruentus*)	6.661.15	9.660.57	11.66
Ceylon Spinach (*B. alba*)	6.330.57	7.020.03	14.661.15
	Antibiotics Zone of inhibition (mm)		
Penicilin	8.912.64		
Ampicilin	19.66		
Streptomycin	29.333.05		
Kanamycin	25.66		

The experiments were carried out in triplicate.

Table 9: Analysis of variance of antimicrobial activity of Raw Extract.

Conditions	Sum of square	Df	Mean square	F value	P value	Significance
Between places	29.659	1	29.659	32.599	0.003	S***
Between samples	18.940	2	9.470	10.408	0.0176	S**

***P< 0.05, **P<0.01, ***P<0.001**

The result revealed that all the raw extract of vegetables from the places mentioned were potentially effective against Gram negative bacterial strain than the applied test concentration of different level of leaf extracts. There were no any inhibitory effect on gram negative organism at 50µl of all the tested vegetables, but (leaves of *L. siceraria*) collected from SWDG show inhibitory effect (intermediate) of 0.1ml (100 µl) against tested bacterial strain. But the collected samples leaves of *L. siceraria, A. cruentus* & *B. alba* from NG of different amount (50µl and 100 µl) except Raw Extract show no any inhibitory effect on tested bacterial strains (compared as per the standard given by CLSI 2020).

Statistical analysis showed Raw Extract of the vegetables significant variations found (P<0.01) between three different samples and also between two places (P<0.001).

Here, Ampicilin (beta-lactam), streptomycin, kanamycin are broad spectrum antibiotic and penicillin used to treat a number of bacterial infections, was used as positive control (penicillin show no inhibitory effect on Gram negative organisms) and various vegetables of different amount and concentration were used to compare the antimicrobial effect. There were significant difference in inhibitory with different concentrations has been observed here.

CONCLUSION AND FUTURE SCOPE

The results obtained from this study provided evidence that ethanol extracts of three types of commonly consumed green leafy vegetables collected from SWDG Titagrah (Kolkata, West Bengal) and NG contained substantial amount of phytochemicals, polyphenols and flavonoids representing antioxidant properties. From the study it has been concluded that the polyphenol and flavonoid contents were found to be significantly higher (P< 0.05) in the samples collected from SWDG as compared to those that were collected from NG which might be due to the higher concentration of heavy metals coming from the contaminated soil with solid wastes as reported by several previous studies. Higher amount of flavonoids may be due to the presence of tea leaves as waste in SWDG.

It has also been concluded that, raw extract of vegetables from different places of SWDG and NG exhibited beneficial antibacterial activity against Gram negative bacterial strains with varying degrees of potency. Leafy vegetables collected from SWDG, significantly had more antimicrobial property than vegetables from NG (P< 0.05). Bioactive compounds are normally accumulated as secondary metabolites in all plant cells and presence of these bio-active compounds might be a reason to indicate high antibacterial activity of these leafy extracts.

Acknowledgement: We acknowledge our college authority to carry on this research work properly.

Application: This research work indicates that these vegetables could be included in the diet as its plays beneficial role in maintaining healthy gut system.

Conflict of Interest: We do not have any conflict of interest regarding our research work.

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How to cite this article: Paramita Barman, Debasree Ghosh and Ayan Saha (2023). Phytochemical and Antimicrobial Assessment of Leafy Vegetables Collected From Solid Waste Dumping Ground and Normal Ground - A Comparative Study. *Biological Forum – An International Journal, 15*(3): 298-304.