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Phenolic Contents, Antioxidant Capacity and Antibacterial Activity of Extracts from *Bacillus* spp. Associated with The Leaves of Some Medicinal Plants

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ABSTRACT

Bacteria living inside the plant tissues produce an array of bioactive phenolic compounds similar to their host plants with a broad spectrum uses in medicinal and pharmacological industries. In this study, five species related to genus *Bacillus* i.e., *Bacillus cereus* Mp1, *Bacillus zhangzhouensis* Mp2, *Bacillus drentensis* Mp3, *Bacillus vallismortis* Mp4, and *Bacillus velezensis* CLT81 were isolated from the leaf tissue of *Solenostema argel, Calotropis procera,* and *Hibiscus sabdariffa*. The ethyl acetate extracts of these species were subjected to the evaluation of phenolic contents, antioxidant capacity and antibacterial activity. Results revealed that Mp1, Mp2, Mp3, Mp4, and CLT81 are potent source of natural bioactive phenolic compounds i.e., phenolic acids, flavonoids and tannins with considerable antioxidant capacity, and antibacterial activity against common pathogenic bacteria i.e., *Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa, Klebsiella pneumonia, Salmonella typhi,* and *Streptococcus agalactiae*. Therefore, these *Bacillus* species may gain great benefit in the pharmaceutical industry as potential candidates for the development of new drugs.

INTRODUCTION

Phenolic compounds are a group of natural bioactive secondary metabolites that ubiquitously distributed in plants and medicinal herbs particularly the edible parts such as fruits, seeds, leaves, stems, and roots (Cheynier *et al.,* 2013). The human body cannot synthesize such compounds, they are supplied as food additives from the natural sources. Flavonoids and phenolic acids are the most common types used as additives in edible products (Martillanes *et al.,* 2017). In the plant, the phenolic compounds support defense against pathogens, attract pollinators and reduce the growth of neighboring plants (Balasundram *et al.,* 2006; Cheynier *et al.,* 2013).

In human life, phenolic compounds are involved in numerous industrial and therapeutic applications (Özeker, 1999). They are extensively used in the photography, petroleum, tanning, paint, explosive, rubber and plastics industries (Özeker, 1999; Carlsen *et al.,* 2010; Ribas-Agustí *et al.,* 2014; Martillanes *et al.,* 2017).

Moreover, they extensively used as antioxidant, anticarcinogenic and anti-inflammatory agents in medicine (Del Rio *et al.,* 2013; Sharma, 2014; Sen *et al.,* 2015; Zhou *et al.,* 2015; Saravanan *et al.,* 2020).

Free radicals and oxidative stress in biological systems play role in the development of chronic and degenerative diseases such as cancer, ageing, diabetes, and neurodegeneration (Chae *et al.,* 2013; Bhuyan and Basu, 2017). Antioxidants act to limit the chain of oxidation reactions via modifying the reactions and removing the free radical intermediates (Heim *et al.,* 2002). Phenolic compounds have been recognized as potential antioxidants because of their redox properties and hydroxyl groups (-OH) that enable them to act as reducing agents and hydrogen donors that scavenging reactive oxygen species (ROS) and preventing the generation of new radicals (Rice-Evans *et al.,* 1997; Galleano *et al.,* 2012; Chae *et al.,* 2013; Pourreza, 2013; Bhuyan and Basu, 2017).

Several phenolic compounds have broad-spectrum antibacterial activity against a wide range of pathogens (Erdemoglu *et al.,* 2007; Milovanović *et al.,* 2007; Xia *et al.,* 2011a). The toxic impact of phenolic compounds on microorganisms attributed to their interaction with cell walls, cell membranes and enzymes (Fattouch *et al.,* 2007; Xia *et al.,* 2011a).

Medicinal plants are populated with diversity of bacterial species that enhance the biological activity of the host through the production of secondary metabolites. These bacteria represent vital source of bioactive compounds that have a wide variety of exploitation in medicine, pharmacy, agriculture, and industry (Joseph and Mini Priya, 2011).

Genus *Bacillus* comprises a large number of heterogeneous rod-shaped, Gram-positive, endospores- forming bacteria (Zeigler and Perkins, 2008). *Bacillus* spp. revealed their potential in the production of of secondary metabolites including phenolic compounds that are crucial in many aspects of their lives in the natural environment (Sansinenea and Ortiz, 2011). The broad structural variability and biological activities of these compounds attract the concern of chemists and pharmacists for their applications in pharmaceutical and drug discovery (Sansinenea and Ortiz, 2011; Bhuyan and Basu, 2017).

In the last years, since the formulation of food products or drugs has impact on human health and consumption, phenolic compounds from natural sources have received increasing interest. A broad spectrum of structurally and biologically active variation of secondary metabolites has been provided from nature (Verpoorte, 1998; Maier *et al.,* 1999).

Although phenolic compounds produced by the plants are physiologically active, they suffer from many problems in their production as the quality level and insufficient quantities (Singh *et al.,* 2017). In contrast, the production of bioactive secondary metabolites from microorganisms perform under controlled conditions which achieve high quality and maximum amounts (Sato and Kumagai, 2013). Therefore, in this study, the antioxidant and antibacterial evaluations of the phenolic compounds produced by various *Bacillus* spp. isolated from the leaves of different medicinal plants were highlighted.

MATERIALS AND METHODS

Isolation, Phenotypic and Genotypic Characterization:

Healthy leaves of different medicinal plants i.e. *Solenostema argel, Calotropis procera* and *Hibiscus sabdariffa* growing in Aswan University campus, Aswan governorate, Egypt were collected and immediately transferred into the laboratory for bacterial isolation. Leaves were carefully surface-sterilized with 70 % ethanol (1 min) followed by 5 % sodium hypochlorite solution (5 min) and then washed three times with sterilized distilled water (Hallmann *et al.,* 1997). One gram of fresh leaf tissue was macerated in 9 ml sterilized saline solution under aseptic conditions. One milliliter of the

prepared suspension was spread onto nutrient agar plates. One milliliter of the sterilized distilled water from the final step of the surface sterilization process was also used for confirmation. Plates were incubated for 72 hr at 37 ^{o}C. Colonies were picked up according to the diversity in their morphological characteristics, sub-cultured and stained by Gram staining. The Gram positive, rod shaped, spore forming Bacilli were selected and stored in the refrigerator for further bioassays.

Colony and cell characterization, Gram and spore staining, as well as the biochemical tests of the isolates, were investigated followed the methods in Bergey's Manual of Determinative Bacteriology (1994).

DNA was extracted and 16S rRNA was amplified according to the protocol edited by Ausubel *et al.,* (1995) using the universal primers 27F and 1492R (Wilson *et al.,* 1990). PCR products were sent commercially via National Biolab for Trade Company to Macrogen, Korea (http://www.macrogen.com/eng/) for sequencing. The NCBI website (https://www.ncbi.nlm.nih.gov/) was used for analyzing the obtained sequences. Sequences were deposited into NCBI database to get accession numbers. The phylogenetic relationships between the isolates and closely related species in genus *Bacillus* were revealed by a neighbor-joining tree using MEGA X software (Kumar *et al.,* 2018).

Fermentation and Preparation of Crude Extract:

The bacterial strains were inoculated into 1L Erlenmeyer flask contained 500 ml nutrient broth (Difco™). Flasks were incubated for 72 hr at 37 ^{o}C and 150 rpm. The cell culture supernatants were extracted with ethyl acetate (1:1v/v) using separation funnel at room temperature. The solvent was then removed by vacuum evaporator and the obtained crude extracts were dried and re-dissolved in ethyl acetate for further analysis (Monowar *et al.,* 2019).

Determination of Total Phenolics Content:

Folin-Ciocalteu reagent method of Ainsworth and Gillespie (2007) was used for the estimation of the total phenolics content. Briefly, 1 ml of the bacterial extract, 2 ml of diluted Folin-Ciocalteu reagent with de-ionized water (1:16, v/v) and 1 ml of sodium carbonate solution (10 %, w/v) were mixed and incubated with shaking at room temperature for 1 hr. The appeared blue color was measured spectrophotometrically at 700 nm. The content of total phenolic compounds was calculated in mg/g dry extract using the calibration curve of standard gallic acid.

Determination of Total flavonoids Content:

The content of the total flavonoids was estimated using the aluminum chloride colorimetric assay (Zhishen *et al.,* 1999). In brief, to 1 ml of the bacterial extract, 0.3 ml of $NaNO_2$ (5 %, w/v) was added and incubated for 6 min, then 0.3 ml of $AlCl_3$ (10 %, w/v) was added and allowed to settle for 6 min at the room temperature, followed by the addition of 2 ml of NaOH (1 M). After 12 min, the absorbance was measured at 510 nm. The total flavonoid content was expressed as mg quercetin equivalent per g dry extract.

Determination of Total Condensed Tannins:

The assay method described by Sun *et al.,* (1998) was followed to determine the total condensed tannins. Volumes of 3 ml methanolic solution of vanillin (4 %, v/v) and 1.5 ml of concentrated HCl were mixed with 50 µl of the bacterial extract. The mixture was vortexed and kept at room temperature for 20 min. The absorbance was measured at 550 nm and the amount of the total condensed tannins was calculated in mg using catechol calibration curve.

Determination of Total Antioxidant Capacity (TAC):

The phosphomolybdenum assay according to Prieto *et al.,* (1999) was used to estimate the total antioxidant capacity. To 1 ml of the bacterial extract, 1 ml of the

reagent solution [Na_3PO_4 (28 mmol L^{-1}), H_2SO_4 (0.6 mol L^{-1}) and $(NH_4)_2MoO_4$ (4 mmol L^{-1})] was added. The mixture was incubated in a water bath at 90 °C for 90 min. Then, the mixture was cooled and the absorbance was recorded at 695 nm. The total antioxidant capacity was expressed as mg ascorbic acid equivalent per g dry extract using standard ascorbic acid.

In Vitro* Antibacterial Assay*:

The antibacterial activity of the bacterial extracts was evaluated by agar well diffusion technique as described by Valgas *et al.,* (2007). Six common pathogenic bacteria i.e. *Escherichia coli* (ATCC 35218), *Staphylococcus aureus* (ATCC 25923), *Pseudomonas aeruginosa*, *Klebsiella pneumonia* (ATCC 13882), *Salmonella typhi* (ATCC 14028) and *Streptococcus agalactiae* were used in this investigation. Muller-Hinton agar plates were inoculated with 1 ml of each bacterial suspension (1×10^7 CFU/ml), and 6-mm holes were aseptically made with a sterile cork borer. Into each hole, 100 µl of 1 mg/ml of the bacterial extract was introduced. Standard ampicillin (30µg/ml) and ethyl acetate solutions were served as positive and negative control respectively. Plates were incubated for 24 hr at 37 ºC. The diameters of inhibition zones were recorded. Triplicates were done for each test.

Statistical Analysis:

The significant statistical differences ($P < 0.05$) of the obtained data were evaluated using analysis of variance (ANOVA) from Minitab (version 12.21; Minitab, Coventry, UK).

RESULTS AND DISCUSSION

Initially, all the obtained isolates were stained with Gram staining. The Gram-positive, rod-shaped and spore-forming isolates that coded as Mp1, Mp2, Mp3, Mp4 and CLT81were selected for further characterization and identification. The morphological and biochemical characteristics of the isolates were investigated and recorded (Table 1).

Table 1. Morphological and biochemical characteristics of *Bacillus* isolates.

Characteristics	**Isolates**				
	Mp1	**Mp2**	**Mp3**	**Mp4**	**CLT81**
Colony	Circular, entire, raised	Circular, irregular, raised	Circular, entire, flat	Circular, irregular, flat	Circular, undulate, flat
Pigmentation	Off white	Off white	Off white	Off white	White
Cell shape	Rods	Rods	Rods	**Rods**	Rods
Gram staining	+	+	+	+	+
Spore formation	+	+	+	+	+
Motility	+	+	-	+	+
H_2S production	+	+	-	+	+
indole formation	-	-	+	+	+
citrate utilization	+	+	+	+	+
Methyl red test	+	+	+	-	+
Voges Proskauer test	+	+	-	+	+
Carbohydrate fermentation:					
Glucose	+	+	+	-	-
Fructose	+	+	+	+	-
Sucrose	-	+	+	-	-
Maltose	-	+	+	-	-
Lactose	+	+	-	-	-
Dextrose	-	-	-	-	-
Galactose	+	-	-	-	-
Mannose	+	+	-	+	+
xylose	-	+	+	+	+

-: negative; +: positive

The isolates were further identified by 16S rRNA gene sequencing. The blast analysis of Mp1, Mp2, Mp3, Mp4 and CLT81 sequences with the NCBI reference sequence database revealed E-value (0.0) and percent identity (100 %) to *Bacillus cereus, Bacillus zhangzhouensis, Bacillus drentensis, Bacillus vallismortis,* and *Bacillus velezensis* respectively. The sequences of isolate Mp1, Mp2, Mp3, Mp4, and CLT81 are available in NCBI database with accession numbers MT145941, MT145942, MT145943, MT145944, and MT012197 respectively. Neighbor-Joining tree with 1000 bootstrap replicates was constructed by MEGA X software to illustrate the evolutionary relationships between the present isolates and the more closely related members of the genus *Bacillus* from NCBI Genbank (Fig.1).

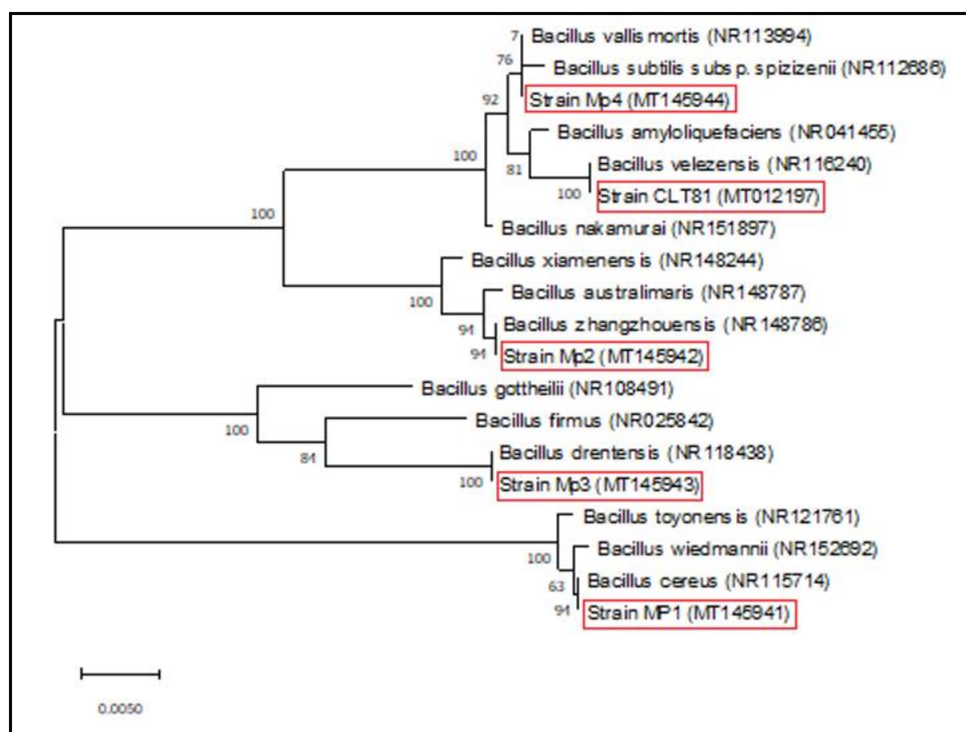

Fig.1. Neighbor-Joining tree with 1000 bootstrap replicates constructed by MEGA X software illustrating the evolutionary relationships between the present isolates and the more closely related members of the genus *Bacillus* from NCBI Genbank.

A wide diversity of structurally varied natural compounds that have a broad spectrum of biological activities has been extracted from bacteria (Akinsanya *et al.,* 2015 and Beiranvand *et al.,* 2017). Phenolic compounds are among the most diverse groups of secondary metabolites derived from microorganisms (Strobel, 2003). In this study, the contents of total phenolics, total flavonoids and total condensed tannins of the crude ethyl acetate extracts of isolate Mp1, Mp2, Mp3, Mp4, and CLT81 were estimated (Fig.2). Significantly, all the isolates revealed potentiality to produce considerable amounts of phenolics, flavonoids, and tannins. The bioactive secondary metabolites production by plant-associated bacteria was previously reported (Strobel, 2003; Qin *et al.,* 2011; Passari *et al.,* 2017; Ek-Ramos *et al.,* 2019).

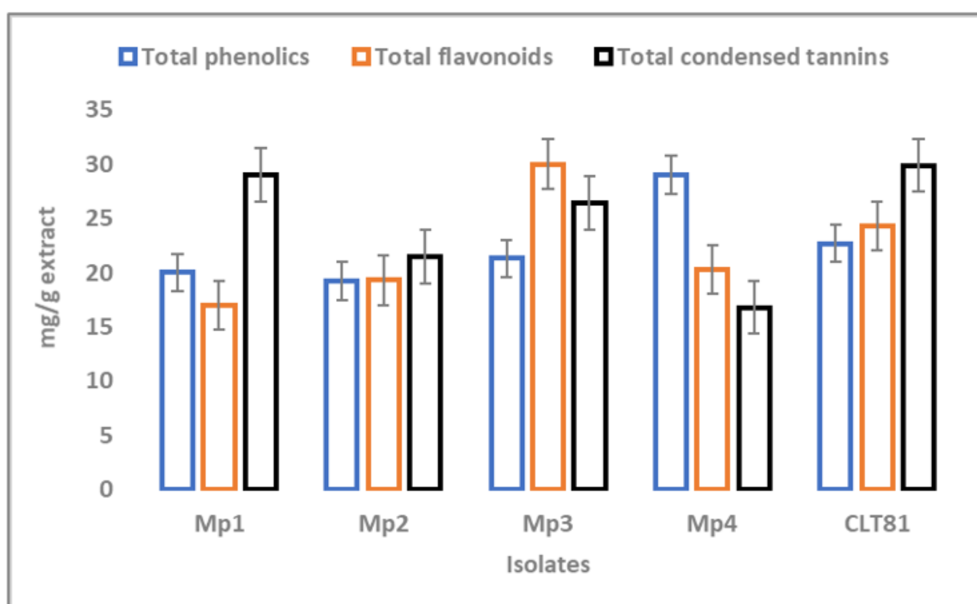

Fig.2. Contents of total phenolics, total flavonoids and total condensed tannins of the ethyl acetate extracts from *Bacillus* isolates. Values are mean ± standard errors (SEs) of three replicates (n =3).

Due to the important biological and pharmacological properties of phenolic compounds, they have a broad array of health-promoting benefits especially as antioxidants and antimicrobial agents (Stalikas, 2007). Whereas, the synthetic antioxidants have toxic effects as well as the consumption of natural products constantly increases, so the screening for effective antioxidants from natural sources is an urgent approach (Barlow, 1990). In this study, the antioxidant capacity of the extracts from the present *Bacillus* *i*solates was evaluated (Fig.3). Meaningfully, the extracts from the present isolates exhibited antioxidant capacity ranged from 50.2 to 80.4 mg AA equivalent/g dry extract. Isolate Mp3 revealed the strongest antioxidant capacity followed by the CLT81, Mp4, Mp1, and Mp2 respectively. It was reported that phenolics and flavonoid molecules can deactivate free radicals by donating hydrogen atoms (Amarowicz *et al.,* 2004). Literatures indicated that the total phenolic and flavonoid content has a linear correlation with antioxidant capacity (Shrestha and Dhillion, 2006). This agreed with the findings of other researchers, Swarnalatha *et al.,* (2015) reported the antioxidant activity of extract from *Lactobacillus* sp. that has been isolated from *Adhathoda Beddomei* leaves. Moreover, *Bacillus cereus* SZ1 extract isolated from the medicinal plant *Artemisia annua* was found to possess antioxidant activity (Zheng *et al.,* 2016). On the other hand, extracts of various endophytic bacteria isolated from *Aloe vera* i.e. *Pseudomonas hibiscicola, Macrococcus caseolyticus, Enterobacter ludwigii,* and *Bacillus anthracis* exhibited antioxidant capacity (Akinsanya *et al.,* 2015). Monowar *et al.,* (2019) reported the presence of phenolic compounds with antioxidant activities in the extracts from the endophytic *Acinetobacter baumannii* associated with *Capsicum annuum* leaves.

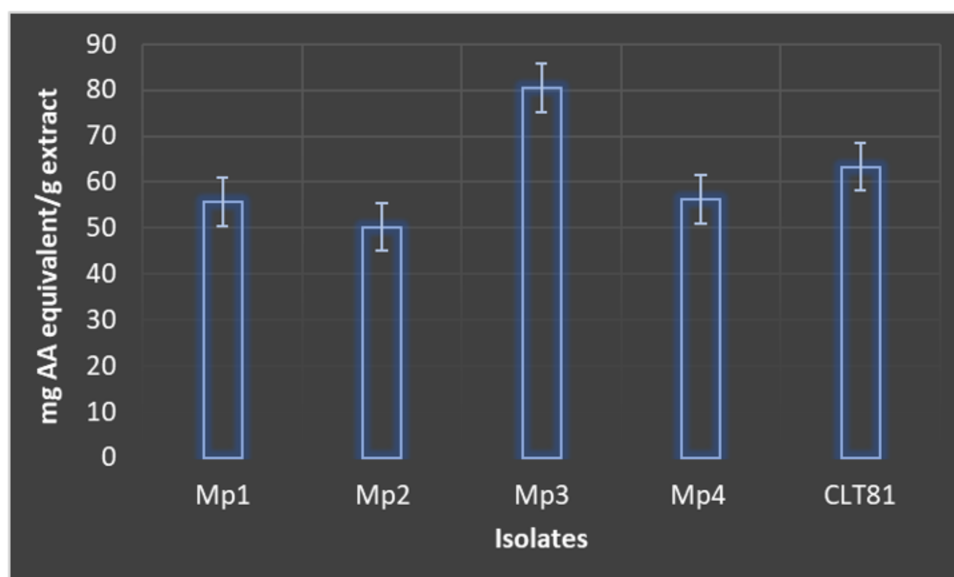

Fig.3. The total antioxidant capacity (TAC) of the ethyl acetate extracts from *Bacillus* isolates. Values are mean ± standard errors (SEs) of three replicates (n =3).

Besides their antioxidant capacity, many microbial extracts that are rich in phenolic compounds exhibit significant antibacterial activity (Cushnie and Lamb, 2011; Li *et al.,* 2014). With increasing the prevalence of antibiotic-resistance of human pathogens, screening for natural sources of antimicrobials is an increasing demand (Dewick, 2002). In this study, the crude ethyl acetate extracts of Mp1, Mp2, Mp3, Mp4, and CLT81 revealed antibacterial efficacy against *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Klebsiella pneumonia*, *Salmonella typhi* and *Streptococcus agalactiae* (Table 2). The maximum antibacterial activity was detected in Mp3 against *Streptococcus agalactiae* (29 mm), CLT81 against *Klebsiella pneumonia* (29 mm), Mp1 against *Pseudomonas aeruginosa* (28 mm), Mp2 against *Streptococcus agalactiae* (27 mm), CLT81 against *Staphylococcus aureus* (27 mm), Mp1 against *Klebsiella pneumonia* (25 mm), Mp3 against *Staphylococcus aureus* and *Salmonella typhi* (25 mm), Mp4 against *Streptococcus agalactiae* (25 mm) and CLT81 against *Escherichia coli* and *Salmonella typhi* (25 mm). The phenolic compounds act against bacterial pathogens through many mechanisms at the cellular level (Sikkema *et al.,* 1995). Several authors reported that the antibacterial effect of phenolic compounds can be attributed to the modification in cell membrane permeability, inactivation of cellular enzymes and subsequently membrane disruption, loss of cellular integrity and eventual cell death (Ikigai *et al.,* 1993; Stapleton *et al.,* 2004; Moreno *et al.,* 2006; Taguri *et al.,* 2006; Cushnie and Lamb, 2011; Srivastava *et al.,* 2013). Previous studies indicated that numerous endophytic bacteria belong to *Arthrobacter, Achromobacter, Bacillus, Enterobacter, Erwinia, Pseudomonas, Pantoea* and *Serratia* associated with medicinal plants exhibited significant antibacterial activity (Stein, 2005; Guo *et al.,* 2008; Egamberdieva *et al.,* 2017).

Table 2. Antibacterial activity of ethyl acetate extracts from *Bacillus* isolates.

	Pathogens					
Isolates	***Escherichia coli* (ATCC35218)**	***Staphylococcus aureus* (ATCC 25923)**	***Pseudomonas aeruginosa***	***Klebsiella pneumonia* (ATCC 13882)**	***Salmonella typhi* (ATCC 14028)**	***Streptococcus agalactiae***
Mp1	17±1	-	28±1	25±1	13±1.15	-
Mp2	11±1.1	19±1.15	-	17±0.57	25±1	27±1.04
Mp3	23±0.57	25±0.57	21±1.04	23±0.5	25±1	٢٩±0.5
Mp4	15±1	-	15±0.5	15±1.15	19±0.57	25±1
CLT81	25±0.57	27±1	22±1.15	29±0.57	25±1.04	23±0.57

Conclusion

Five potent species of genus *Bacillus*; *Bacillus cereus* Mp1, *Bacillus zhangzhouensis* Mp2, *Bacillus drentensis* Mp3, *Bacillus vallismortis* Mp4, and *Bacillus velezensis* CLT81 were isolated from the leaf tissue of three medicinal plants i.e. *Solenostema argel, Calotropis procera* and *Hibiscus sabdariffa*. Ethyl acetate extracts derived from these species contained significant amounts of phenolic compounds including phenolic acids, flavonoids, and tannins. All species possess considerable total antioxidant capacity and effective antibacterial action against *Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa, Klebsiella pneumonia, Salmonella typhi* and *Streptococcus agalactiae*. Therefore, the secondary metabolites from Mp1, Mp2, Mp3, Mp4, and CLT81 could be used as potent antioxidant and antibacterial candidates for pharmaceutical purposes.

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