BIO-CONTROL OF BACTERIAL PATHOGENS WITH SOLVENT EXTRACTS OF WEEDS OF AMARANTHACEAE FAMILY

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ABSTRACT
Biologically active substances have been isolated from weedy plants and investigated against bacterial pathogens for their antimicrobial potential. Medicinal value and biological functions of weeds are being tapped in order to find out novel substances against microorganisms, which have developed resistance to current antibiotics and pesticides. Major objective of the current study was to investigate the antiphytopathogenic potential of the organic extracts of root, stem and leaves of weeds from the family Amaranthaceae (*Amaranthus viridis* L. and *chenopodium murale*) against seven bacterial species viz, *Bordetella pertussis, Kurthia gibsonii, Burkholderia pseudomallei, Azotobacter nigricans, Phenylobacterium immobile, Azomonas agilis, Enterobacter intermedius.* We observed significant results with the application of organic leaf and stem extracts on tested pathogens using well diffusion method whereas aqueous extracts exhibited lower efficacy. Methanolic stem extract (MSE) of *A. viridis* showed maximum inhibition against *Azotobacter nigricans* (40±0.08), *Enterobacter intermedius* (39±0.24), *Phenylobacterium immobile* (37±0.04), *Azomonas agilis* (34±0.19) respectively. Methanolic root extract (MLE) of *A. viridis* were more effective to inhibit the growth of *Bordetella pertussis* (42±0.24), *Kurthia gibsonii* (37±0.09). Whereas methanolic leaf extracts were significantly effective against *Burkholderia pseudomallei* (40±0.13). Significant growth inhibition was also observed in bacterial strains when treated with methanolic stem extract (MSE) of *C. murale.* against *Azotobacter nigricans* (38±0.15), *Bordetella pertussis* (36±0.24) and *Phenylobacterium immobile* (34±0.04) respectively. Methanolic leaf extracts of *C. murale* showed maximum inhibition against *Kurthia gibsonii* (40±0.19), *Burkholderia pseudomallei* (36±0.19) and *Enterobacter intermedius (*38±0.08) and root extracts was effective against *Azomonas agilis* (30±0.19). Both of the species exhibited diverse biochemical compounds in aqueous and organic extracts. Results obtained clearly indicates that antimicrobial constituents of *Amaranthus viridis* L. and *Chenopodium murale* weeds can be screened for the isolation of alternative antibacterial compounds to develop novel biopesticides against resistant bacteria.

Keywords: *Amaranthus viridis* L., *Chenopodium murale* L., antimicrobial activity, growth inhibition, organic solvent extracts.

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INTRODUCTION

Plants represents nature's excellence and vegetation of Pakistan is exceptionally blessed with incredible restorative plants. Plants have influenced the human being since dawn of civilization to provide them with food, shelter and medicines. The medicinal importance and variety of biological functions of many plants is exhibited by their phytochemical nature (Huseini *et al.*, 2005). Several herbal plants are the potential source of compounds (phenolics, anthocyanins and

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carotenoids) of biological importance to enhance the flavor and storage life of food products (Nisar *et al.*, 2011; Qayum *et al.*, 2012) and their potential microbial growth inhibition properties (Abukakar *et al.*, 2010) Phytochemical components of weedy annual herbs determine their medicinal and biological functionalities (Huseini *et al.*, 2005). Phytopathogens are major threat for agriculture dependent economies (Strange *et al.*, 2005). Bacteria and fungi are the major phytopathogens for crop destruction (Wang *et al.*, 2017).

Amaranthus viridis L. and *Chenopodium murale* (Amaranthaceae, subfamily Chenopodiacea) are fast growing weedy, annual eudicot herbs of medicinal importance being cultivated and utilized as herbal medicines worldwide (Amin *et al.*, 2006; Moghadam *et al.*, 2021). These two species distributed worldwide in many agricultural areas. Their competitive and allelopathic activities highly reduce crop yield and quality (Bajwa *et al.*, 2019). These weeds are extensively used as diuretic, antirheumatic, antiulcer, anti-leprotic, anti-diabetic, anti-inflammatory, laxative, and hyperlipidemia, cardiotonic, carminative, and digestive (Usman *et al.*, 2010). Plants contains variety of secondary metabolites (Girija *et al.*, 2011). *A. virids* has medicinal, nutritional and antimicrobial properties (Bokaeian *et al.*, 2013). Phytochemical analysis of *Amaranthus spp*. has shown the presence of various secondary products such as aldehydes (Cutillo *et al.*, 2004; Horio *et al.*, 1993; Tahara *et al.*, 1994), apo-carotenoids (DellaGreca *et al.*, 2004), flavonoids (Gohara *et al.*, 1997), phyto-ecdysteroids and an unusual xylosidic compund (DellaGreca *et al.*, 2005; DellaGreca *et al.*, 2004).

World Health Organization reported that approximately 80% global population depends on medicinal plants for their basic healthcare needs. A number of phytochemicals are components of herbal medicines (Stace, 1991). It is estimated that 20% of the plant species from the global flora have been screened for their medicinal and biological potential (Suffredini *et al.*, 2004). For many decades, a number of plant species are excellent source of antimicrobial substances to overcome antibiotic resistance in pathogen, which is major futuristic problem. This approach to develop and utilize medicinal plants as a traditional medicine system is also encouraged and promoted by WHO (Mickymaray *et al.*, 2016). Studies with extracts obtained from *A. hypochondriacus* showed potent antifungal properties against pathogenic genera of *Alternaria, Fusarium*, *Candida* and *Aspergillus* (Bahrami-Teimoori *et al.*, 2017; Rivillas-Acevedo *et al.*, 2007). Spore germination of *Phakopsora pachyrhizi* was inhibited by potential antifungal compounds isolated from the root extracts of *Amaranthus spinosus* (Yusnawan, 2015).

Evaluation of the methanolic stem extract of *Salicornia herbacea* (Chenopodiaceae) exhibited antioxidant, antibacterial effect against several pathogens. Ethanolic extracts of *Chenopodium ambrosioides* showed synergistic antimicrobial action in combination with conventional antimicrobials against tested strains of bacteria. Currently, pathogen management is linked with the active screening of natural phytoprotectants in sustainable agriculture and efforts to control phytopathogens by utilizing aqueous or organic plant extracts (Chaudhary *et al.*, 2013; Elsharkawy *et al.*, 2015). Secondary metabolites in the weeds, which has antimicrobial properties, can be extracted with aqueous or organic solvents (Sales *et al.*, 2016). These metabolites also helps weeds to invade in various agricultural ecosystems (Dhankhar *et al.*, 2013).

Bacterial species viz, *Bordetella pertussis* is causative pathogen of whooping cough (Hozbor, 2018), *Kurthia gibsonii* causative agent of sexually transmitted zoonosis,(Kövesdi et al., 2016) *Burkholderia pseudomallei,* causative agent of melioidosis (Titball *et al.*, 2008) *azatobacter nigricans* is a nigrogen fixing bacteria having antifungal potential (Nagaraja *et al.*, 2016)*, Phenylobacterium immobile* is an aerobic, gram-negative, coccus pathogen that causes cutaneous infectious granuloma (Zhu *et al.*, 2010), *Azomonas agilis is a* gram-negative nitrogen fixing bacteria (Sehrish *et al.*, 2018) and *kluyvera (enterobacter) intermedius* is a urinary tract infectious agent (Pavan *et al.*, 2005). These *bacterial strains were* isolated from specimens of various kinds of fruits and

vegetables collected from local markets of Lahore district, Pakistan (Ali *et al.*, 2014). These phytopathogens exhibits diverse physiology to tolerate harsh environmental conditions as they are capable to resist biotic and abiotic stress induced by heavy metals, herbicides, pesticides (Bajwa *et al.* 2009).

The present study aims to screen aqueous, ethanolic and methanolic extracts obtained from various plants parts of both *Amaranthus viridis. L* and *Chenopodium morale* for their antibacterial potential against seven important bacterial strains i.e *Bordetella pertussis, Kurthia gibsonii, Burkholderia pseudomallei, Azotobacter nigricans, Phenylobacterium immobile, Azomonas agilis* and *Enterobacter intermedius* isolated from the fruit and vegetables (apple potato, radish, spinach, ginger, mint, cucumber, turnip, and lemon) collected from the famer market of Lahore, Pakistan.

MATERIALS AND METHODS

Bacterial cultures

The bacterial species were isolated from diseased citrus soil by using Luria Bertani Agar (L.B.A.) and Nutrient Agar (N.A.) media (Sehrish *et al.*, 2018). Plates were inoculated and incubated at 37 °C till appearance of colonies. Next day, streak plate technique was used to transfer the colonies on fresh media petri plate aseptically (Beishir, 1996). Identification of bacterial strain was carried by observing morphological parameters (cell shape, Gram type, capsule stain, motility and pigmentation) under higher magnification power of light microscope Olympus CH300 (Leck, 1999). Detection of biochemical enzymes (nitrate reductase, oxidase, catalase, urease, malonate and gelatinase) was carried out through standard tests (Benson, 1994; Holt *et al.*, 1994).

Weed collection

Amaranthus viridis L. and C*henopodium murale* (Amaranthaceae, subfamily Chenopodiacea) were identified systematically and collected from wild places and botanical garden of the University of the Punjab, Lahore. Plant organs (root, stem and leaves) were washed and dried under shade to avoid loss of compounds due to exposure to sunlight. The dried parts were added to portable kitchen electric grinder (Panasonic) and a fine powder was obtained.

Extraction with Water

In extraction with water, 5 gm of weed powder (leaf, stem and root) was soaked separately in 50 ml of distilled water for 48 hours under lab conditions. The mixture was filtered through cheesecloth and 5 ml aliquots of filtrate were condensed in a rotary evaporator. The extract was preserved aseptically in an amber bottle and preserved at 4 ºC for future use.

Extraction with Organic Solvents

Organic solvent extracts were prepared by adding 5 gm dried weed powder (leaf, stem and root) in 100 ml of organic solvents separately (Methanol and Ethanol) in 100 ml glass flasks placed on constant shaking (100 rpm) and filtered after 48 hours. Organic solvent was evaporated by in a rotary vacuum evaporator. Aliquots of the extracts were preserved in 10 ml screw capped Eppendorf vials at 4 ºC for future use.

Bacterial Growth Assays

Antibacterial potential of aqueous and organic solvents of selected members of Amaranthaceae family was investigated by well-diffusion method (Mushatq *et al.*, 2012). Sterile cork borer (8.0 mm) was used to made wells in the L.B. agar plate and loaded with 60 µl each of all solvents (aqueous/organic). Penicillin (5µg/ml) was used as control. Bacterial colonies were suspended in Tween 80 and number of colonies per ml were counted with a haemocytometer. The agar plates with inoculated with bacterial suspension containing 10^4 CFU/ml. Wells in the control plates were loaded with similar amount of sterile distilled water or organic solvent. Test and control plates were incubated at 37 ºC and bacterial growth inhibition zone (IZ) was measured in mm

under aseptic conditions for the assessment of antibacterial activity. Each treatment consists of three replicates for each of the bacterial strain. Antibacterial activity of the extracts was expressed as the mean of triplicates ± SE. Each set of experiment was repeated twice.

RESULTS AND DISCUSSION

Morphological and biochemical characters for all bacterial strains were recorded and used to identify through Bergey's Manual of Determinative Bacteriology (9th Edition). The bacterial community included: *Bordetella pertussis, Kurthia gibsonii, Burkholderia pseudomallei, Azotobacter nigricans, Phenylobacterium immobile, Azomonas agilis* and *Enterobacter intermedius*. Out of seven bacterial species, *Kurthia gibsonii* was a rod shaped (bacillus) gram positive bacteria, while other bacterial strains were gram negative cocci (Table 1). Results of the current study revealed significant antimicrobial potential of aqueous, methanolic and ethanolic extracts of *A. viridis* and *chenopodium murale* weed plants against different bacterial pathogens. *Azotobacter nigricans* was highly sensitive to methanolic stem extract of *A. viridis* (40±0.08) and *chenopodium murale* (38±0.15). Methanolic stem extract (MSE) of *A. viridis* showed maximum inhibition against *Azotobacter nigricans* (40±0.08), *Enterobacter intermedius* (39±0.24), *Phenylobacterium immobile* (37±0.04), *Azomonas agilis* (34±0.19) respectively. Methanolic root extract (MRE) of *A. viridis* were more effective to inhibit the growth of *Bordetella pertussis* (42±0.24), *Kurthia gibsonii* (37±0.09), as compared to MSE (Table 2), whereas methanolic leaf extracts (MLE) were significantly effective against *Burkholderia pseudomallei* (40±0.13).

Amaranthaceae family showed varied results against pathogenic bacterial strains (Table 2 and 3). The antibacterial efficacy of aqueous and two solvent extracts of weeds against pathogenic bacteria showed variable level of inhibition. It was observed that aqueous extracts of *A. viridis* and *chenopodium murale* has lower antibacterial activity as compared to organic solvents of same part. However, methanolic extracts showed significant results as compared to ethanolic extract. When comparison was made (Figure 1) between the extracts of various parts, it was observed that ethanolic extract of leaves of both weeds exhibited minimum percentage of inhibition as compared to other parts (stems and roots).

Current findings are supported by a previous trail conducted with organic (ethanol, methanol and chloroform) extracts of *A. viridis* to test their antimicrobial activity against selected gram positive and gram-negative bacteria using disk diffusion assay. They reported that ethanolic extracts of leaves and stem are more potent antimicrobial when compared with methanolic and chloroform extracts of same tissue (Malik *et al.*, 2016).

Significant growth inhibition was also observed in bacterial strains when treated with methanolic stem extract (MSE) of *C. murale.* Data given in Table 3 represents significant results against all selected bacterial strains as in *Azotobacter nigricans* (38±0.15), *Bordetella pertussis* (36±0.24) and *Phenylobacterium immobile* (34±0.04) respectively. Methanolic leaf extracts of *C. murale* showed maximum inhibition against *Kurthia gibsonii* (40±0.19), *Burkholderia pseudomallei* (36±0.19) and *Enterobacter intermedius (*38±0.08) and root extracts was effective against *Azomonas agilis* (30±0.19). Leaf extracts of *C. morale* exhibited weak antimicrobial potential when applied against *B. subtilis* (13±0.16)*, E. coli* (11±0.02), *P. fluorescens* (*12*±0.01)*, S. aureus* (13±0.24) *and X. axonopodis* (15±.001), when compared with other selected plants was reported earlier (Arif *et al.*, 2018). Eextracts of some medicinal plants against wound causing bacteria showed higher efficacy of methanol extracts due to stability of plant secondary metabolites as compared to the aqueous counterpart (Hussain *et al.*, 2013).

Antibacterial activity of the phytochemicals extracted in methanol from the dried seed of *A. viridis* against fungal and bacterial pathogens was determined by measuring minimum inhibitory concentration (MIC) and zone inhibition (ZI). The trend for antifungal activity was 100% methanol leaf > 100

methanol seed > 80% methanol leaf > 80% methanol seed (Ahmed *et al.*, 2013). Our results are in accordance with previous findings which has ascertained the presence of phytochemicals in the members of Amaranthaceae to be used as agents of biological control. These secondary metabolites can be used as phyto-protectant to repel harmful organisms (Lipkin *et al.*, 2005). A number of research studies have shown that weeds can resist the microbial attack due the presence of the certain phytochemicals which possess antimicrobial and antioxidant properties (Bhuvaneswari *et al.*, 2011; Dhankhar *et al.*, 2013). Weeds are easily available and inexpensive to manage the microbial infections in crop plants (Khan, 2014). Results of this research has clearly shown that *A. viridis* and *C. murale* are candidate weeds as a source of antimicrobial compounds suitable to be used as biocontrol agents.

CONCLUSION

Results obtained from present study clearly indicates presence of pharmacologically active substances of antimicrobial and antioxidant potential in the leaves of *A. viridis* and *C. murale*. Further research is needed for the identification of the active components in the organic extracts responsible for the antimicrobial action. Detailed toxicological evaluation should also be carried out to determine their role as food preservative for the processed foods.

Table 1. List of Bacterial strains according to their morphological and biochemical characters

S. No.	Colony characters	G	C	H	I	CU	N	O	CT	U	L	GL	IN	SB	GO	MN	Identified Bacterial species
B-1	Raised smooth, spherical entire, creamy opaque	-ve	cocci	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	*Bordetella pertussis*
B-2	Raised smooth, Spherical entire, Yellowish translucent	+ve	rod	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	*Kurthia gibsonii*
B-3	Flat smooth spherical entire creamy opaque	-ve	cocci	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	*Burkholderia pseudomallei*
B-4	Flat smooth long rodsrhizoid creamy opaque	-ve	cocci	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	*Azotobacter nigricans*
B-5	Raised, smooth, spherical, entire creamy,Transluce nt	-ve	cocci	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	*Phenylobacterium immobile*
B-6	Slimy smooth, short rods, entire yellowish, opaque	-ve	cocci	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	*Azomonas agilis*
B-7	Flat, rough spherical, rhizoid creamy, opaque	-ve	cocci	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	*Enterobacter intermedius*

NOTE: G: Gram type, C: Cell shape, H: Hydrogen sulfide test, I: Indole test, CU: Citrate utilization test, N: Nitrate reduction test, O: oxidase test, CT: Catalase test, U: Urease test, L: Lysine test, GL: Gelatin test, IN: Inositol test, SB: Sorbitol test, GO: Glucose test, MN: Mannitol test

Table 2. Antibacterial Activity of solvent and aqueous extracts of *A. viridis* against citrus pathogens

Bacterial species	Control	Aqueous Extract (mm)			Methanolic extract (mm)			Ethanolic Extract (mm)		
		Leaf	Stem	Root	Leaf	Stem	Root	Leaf	Stem	Root
Bordetella pertussis	55±0.0	25±1.19	27±0.35	24±0.19	40±0.13	39±0.29	42±0.24	33±0.09	37±0.24	33±0.14
Kurthia gibsonii	55±0.0	20±0.39	19±1.29	20±0.09	32±0.19	35±0.34	37±0.09	25±0.05	29±0.34	33±0.24
Burkholderia pseudomallei	55±0.0	29±1.49	25±1.19	19±0.14	30±0.19	29±0.09	30±0.05	30±0.08	33±0.19	31±0.09
Azotobacter nigricans	55±0.0	25±0.25	24±0.19	20±0.16	35±0.24	40±0.08	32±0.08	38±0.07	35±0.09	37±0.08
Phenylobacterium immobile	55±0.0	20±0.28	26±0.08	21±0.15	36±0.09	37±0.04	30±0.08	32±0.24	32±0.06	35±0.15
Azomonas agilis	55±0.0	20±0.29	19±0.98	17±0.14	30±0.04	34±0.19	33±0.24	32±0.11	33±0.08	30±0.16
Enterobacter intermedius	55±0.0	29±0.16	27±0.87	21±0.16	30±0.08	39±0.24	34±0.09	30±0.19	35±0.19	33±0.24

Table 3. Antibacterial Activity of solvent and aqueous extracts of *C. morale* against citrus pathogens

Bacterial species	Control	Aqueous Extract (mm)			Methanolic extract (mm)			Ethanolic Extract (mm)		
		Leaf	Stem	Root	Leaf	Stem	Root	Leaf	Stem	Root
Bordetella pertussis	55±0.0	23±1.19	24±0.35	24±0.19	32±0.13	36±0.24	37±0.24	30±0.09	30±0.24	35±0.14
Kurthia gibsonii	55±0.0	21±0.39	23±1.29	22±0.09	40±0.19	39±0.34	32±0.09	32±0.05	34±0.34	40±0.24
Burkholderia pseudomallei	55±0.0	30±1.49	29±1.19	21±0.14	36±0.19	35±0.09	30±0.05	36±0.08	36±0.19	36±0.09
Azotobacter nigricans	55±0.0	27±0.25	24±0.19	25±0.16	37±0.24	38±0.15	32±0.08	35±0.07	36±0.09	37±0.08
Phenylobacterium immobile	55±0.0	24±0.28	23±0.08	24±0.15	30±0.09	34±0.04	32±0.08	30±0.24	32±0.06	30±0.15
Azomonas agilis	55±0.0	19±0.29	19±0.98	17±0.14	28±0.04	30±0.19	32±0.24	25±0.11	29±0.08	30±0.16
Enterobacter intermedius	55±0.0	26±0.16	26±0.87	27±0.16	38±0.08	35±0.24	34±0.09	33±0.19	37±0.19	33±0.24

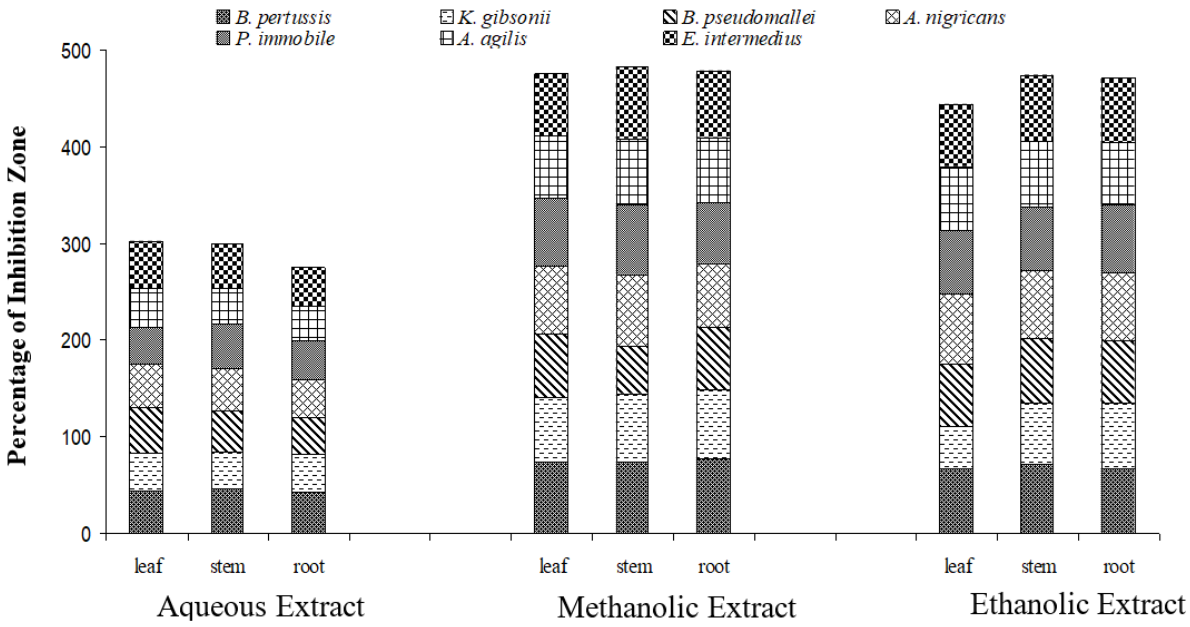

Figure 1. Zone Inhibition percentage of *A. viridis* extracts against bacterial strains

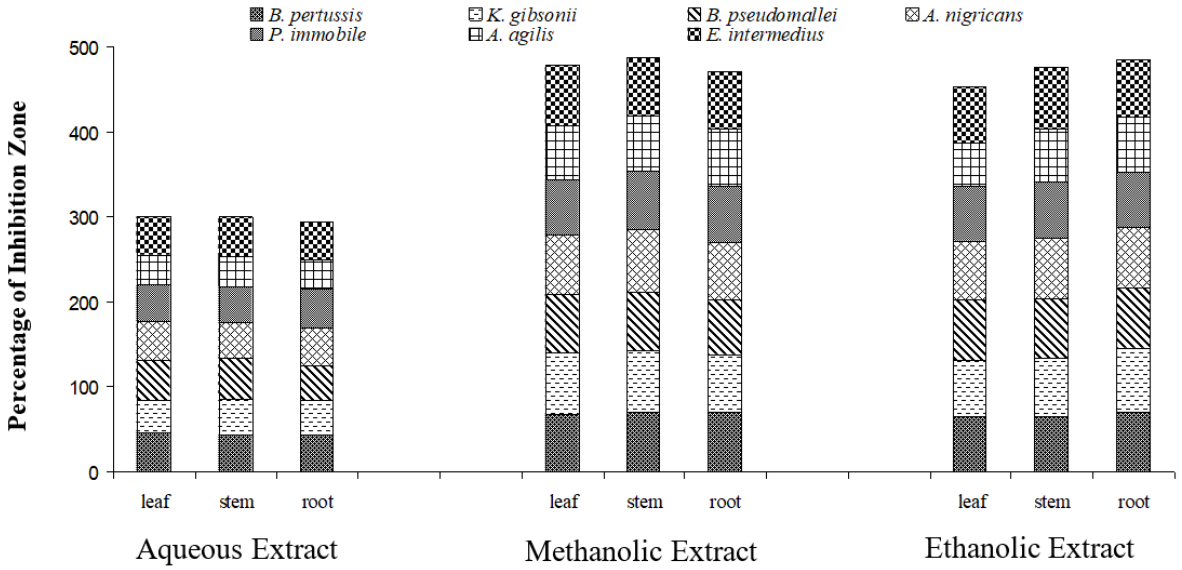

Figure 2. Zone Inhibition percentage of *C. morale* extracts against bacterial strains

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