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Original Research

Anti-methicillin-resistant *Staphylococcus aureus* (MRSA) activity of Rubiaceae, Fabaceae and Poaceae plants: A search for new sources of useful alternative antibacterials against MRSA infections

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Abstract: In this study, we evaluated the effects of the extracts of the leaves of species from the Rubiaceae (*Galium aparine* L. and *Asperula arvensis* L.), Fabaceae (*Lathyrus aphaca* L. and *Vicia narbonensis* L.) and Poaceae (*Digitaria sanguinalis* (L.) Scop. and *Hordeum murinum* L.) plant families on a wide and extensive panel of isolated methicillin-resistant *Staphylococcus aureus* strains (MRSA). The effects of the methanolic leaf extracts of Rubiaceae, Fabaceae and Poaceae plants on MRSA were evaluated by the disc diffusion assay and the broth dilution method. Among a total of 177 *S. aureus* isolates, 92 (51.97%) were found to be methicillin-resistant in an antibiogram and this was confirmed by the presence of the *mec*A gene in polymerase chain reaction method. All MRSA isolates were sensitive to all extracts. There were dose-dependent inhibitions on tested microorganisms for all plant extracts which showed maximum inhibition zones at a concentration of 300 mg/L. *L. aphaca, G. aparine* and *H. murinum* exhibited the highest antibacterial activity on the MRSA strains compared to the positive control ($P < 0.05$), as well as higher total polyphenol and flavonoid contents than other plant extracts. Minimum inhibitory concentrations on MRSA isolates ranged from 388.4 ± 0.2 mg/L, in *D. sanguinalis*, to 5.5 ± 0.1 mg/L, in *L. aphaca*. The methanolic extracts of *L. aphaca* (Fabaceae), *G. aparine* (Rubiaceae), and *H. murinum* (Poaceae) proved to have high antibacterial activity on MRSA isolates, thus representing promising antimicrobial agents in clinical settings.

Key words: Antibiotic resistance, antibiotic therapy, nosocomial infections, medicinal plants, total polyphenol content, total flavonoid content.

Introduction

Methicillin resistant *Staphylococcus aureus* (MRSA) was first identified almost 50 years ago shortly after the introduction of the antibiotic methicillin (1). MRSA infections are caused by *S. aureus* strains resistant to a number of antibiotics; they are highly contagious and can be spread through direct contact with an infected person. Infection can also be contracted by coming into contact with an object or surface that an infected person has touched. MRSA infections are classified as either hospital-acquired (HA-MRSA) or community-acquired (CA-MRSA). HA-MRSA is associated with infections that are contracted in healthcare settings such as hospitals or nursing homes. It can cause severe problems, such as septicemia and pneumonia. CA-MRSA is associated with infections that are transmitted through close personal contact with an infected person or through direct contact with an infected wound. This type of MRSA infection may also develop as a result of poor hygiene such as infrequent or improper hand-washing (2). In spite of the fact that superficial skin and soft tissue infections remain the most common manifestation of CA-MRSA, severe diseases such as necrotizing fasciitis, necrotizing pneumonia, pyomyositis, septic embolism, venous thrombosis, and osteomyelitis have been described and previously caused death in healthy children (3,4). Despite the availability of new antibiotics, MRSA continues to threaten the world population. Therefore, there is a continuous necessity for novel antimicrobial compounds due to the rapid appearance of MRSA (5, 6).

In developing countries, due to economic constraints, nearly 80% of the population still depends on plant extracts as a source of natural remedies (7). Nevertheless, though plants are greatly exploited in traditional healing systems, only in some cases their therapeutic potential in human has been substantiated (8-10). The need of herb-based medicines, cosmetics, food supplements, pharmaceuticals and health products is progressively increasing all over the world, because, in some cases, natural products *i*) are non or low toxic, *ii*) show low side effects and *iii*) are available at affordable costs (11). Not the least, plant extracts can reduce the occurrence of resistant microbial strains because they consist of many bioactive phytochemicals with different molecular and biochemical targets.

Plants are historically used to treat infectious diseases. In addition, antimicrobial compounds from

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plants may be effective in counteracting infections caused by antibiotic-resistant bacterial strains (12-14). Due to the increasing phenomenon of bacterial resistance to antibiotics, in this study, we investigated the *in vitro* antibacterial activity of Rubiaceae (*Galium aparine* L. and *Asperula arvensis* L.), Fabaceae (*Lathyrus aphaca* L. and *Vicia narbonensis* L.) and Poaceae (*Digitaria sanguinalis* (L.) Scop. and *Hordeum murinum* L.) plants against MRSA bacterial strains, in order to find new strategies to control antibiotic-resistant bacteria.

Materials and Methods

Plants and extraction

The leaves of Rubiaceae (*Galium aparine* L. and *Asperula arvensis* L.), Fabaceae (*Lathyrus aphaca* L. and *Vicia narbonensis* L.) and Poaceae (*Digitaria sanguinalis* (L.) Scop. and *Hordeum murinum* L.) plants (Table 1) were collected between April-May 2013 from the area of Hamun Lake of Zabol, Sistan and Baluchestan Province, Iran. The specimens were identified by a plant taxonomist at the Department of Pharmacognosy, Faculty of Pharmacy, Zabol University of Medical Sciences, Zabol, Iran. The plant leaves were dried in an oven at 60 °C for 72 h. Twenty grams of leaves for each plant were powdered separately and then suspended in 200 mL of methanol 80%, water 20% using a shaker water bath for 24 h at 25 °C. After filtration with Whatman No. 1 filter paper, the resulting solutions were concentrated by a rotary evaporator at 40 °C for 35 min to remove solvent from the extracts. Solid extracts (residues of plant extracts) were stored at -20 °C until further analyses.

Total phenol content

Total phenols were assayed based on the method of Dewanto *et al*. (15). An aliquot of each diluted extract was added to 0.5 mL of distilled water and 0.125 mL of Folin-Ciocalteu reagent. The mixture was shaken and allowed to stand for 10 min, before addition of 1.25 mL of 5% Na_2CO_3. The solutions were then adjusted with distilled water to a final volume of 4 mL and mixed thoroughly. Absorbance at 760 nm was read versus blank after incubation in the dark. The total phenol concentration of each plant was expressed as milligrams of gallic acid equivalents per gram of dry weight (mg GAE/g DW) from a calibration curve with gallic acid.

Total flavonoid content

The colorimetric assay was used for the assay of total flavonoids according to Dewanto *et al.* (15). An aliquot of diluted sample or standard solution of (+)-catechin was added to 50 mL of $NaNO_2$ solution (5%) and mixed for 5 min before the addition of 0.15 mL $AlCl_3$ (10%). After 5 min, 0.5 mL of NaOH was added. The final volume was adjusted to 2.5 mL with distilled water and mixed thoroughly. Absorbance was determined at 510 nm against blank. The total flavonoid concentration was expressed as milligrams of catechin per gram of dry weight (mg CE/g DW) against the calibration curve of (+)-catechin, from 0 to 400 mg/L.

Bacterial isolation and culturing

One hundred and seventy-seven clinical specimens including burns, wounds, urines, pus, and throat swabs were collected from patients who attended the emergency Hospital and Internal Laboratory of Hospital and Central Laboratory in Zabol, Iran, for different infections. Standard isolation protocols were used for all the samples. Identification of *Staphylococcus aureus* was approved by standard techniques accord to diagnostic tests, such as catalase test, culturing on mannitol salt agar, coagulase tube test and DNase.

Determination of MRSA isolates and antibiotic susceptibility

For MRSA differentiation from other *S. aureus* isolates, Muller Hinton Agar medium (Oxoid Oxoid Ltd, UK) was used. The strains in a liquid medium of 0.5 McFarland standard concentrations were grown in Muller Hinton Agar medium and antibiotic discs with ampicillin (10 µg), erythromycin (15 µg), penicillin (10 U), ciprofloxacin (5 µg), clindamycin (2 µg), vancomycin (30 µg), methicillin (5 µg) and gentamycin (10 µg) (Mast Group Ltd, UK) were placed on the medium and incubated for 24 h at 37 °C (16). Then, the diameter of the clear zone around the discs was measured by standards of Clinical Laboratory Standard Institute (17).

DNA extraction, polymerase chain reaction (PCR) assay and electrophoresis

For DNA extraction the phenol-chloroform method was used (18). DNA extracted samples were dissolved in Tris acetate-ethylenediaminetetraacetic acid (Tris-EDTA) buffer (HCl 10 mM Tris, 1 mM EDTA, pH = 7.4), and DNA concentration (µg/mL) was determined by a spectrophotometer at A_{260}. The quantity of DNA samples used ranged from 10 to 1000 ng. DNA obtained was conserved at -20 °C for further assays.

All MRSA isolates were tested for the presence of the *mec*A gene by polymerase chain reaction (PCR) using previously described primers (19, 20). The standard strain used for MRSA was *S. aureus* ATCC14458; distilled water was used as the negative control. For PCR, forward and reverse primers (*mec*A-1 GTGAAGATATACCAAGTGATT; *mec*A-2 ATGCGCTATAGATTGAAAGGAT) were diluted to reach a concentration of 100 pM. After preparing the PCR mix, amplifications were performed. PCR products were mixed with 1 µL of loading buffer solution and carefully loaded in the wells of the agarose gel (1.5%) and electrophoresed at 75 V for 90 min. The gel was then stained with ethidium bromide (Merck, Germany) solution for 15 min and observed under a UV transilluminator (UV doc, England).

Disc diffusion assay

Antimicrobial tests were carried out by the disc diffusion method using 100 µL of bacteria suspension (containing 2.0×10^8 CFU/mL of bacteria) dispersed on Mueller-Hinton agar in sterilized Petri dishes (60 mm in diameter). To the discs (6 mm in diameter, HI Media Laboratories Pvt. Ltd., Mumbai, India) placed on the inoculated agar 10, 25, 50, 100, 150, and 300 mg/L of each leaf plants extracts were added. The inoculated plates were maintained at 4 °C for 2 h and later incubated at 37 °C for 24 h. Antimicrobial activity was determined by measuring the zone of inhibition (mm) against the test bacterial (MRSA isolates and MRSA ATCC14458).

Table 1. Species names, common names, synonyms and botanic families of the plants used in the study.

Plant name	Family	Common names	Synonyms
Galium aparine L.	Rubiaceae	Cleavers, Clivers, Goosegrass, Catchweed, Stickyweed, Robin-run-the-hedge, Sticky willy, Sticky willow, Velcro weed, Grip grass	*Aparine hispida* Moench nom. illeg./ *Aparine vulgaris* Hill/ *Asperula aparine* (L.) Besser nom. illeg./ *Asterophyllum aparine* (L.) Schimp. & Spenn./ *Crucianella purpurea* Wulff ex Steud./ *Galion aparinum* (L.) St.-Lag./ *Galium aculeatissimum* Kit. ex Kanitz/ *Galium adhaerens* Gilib. nom. inval./ *Galium asperum* Honck. nom. illeg./ *Galium australe* Reiche nom. illeg./ *Galium charoides* Rusby/ *Galium chilense* Hook.f./ *Galium chonosense* Clos nom. illeg./ *Galium hispidum* Willd./ *Galium horridum* Eckl. & Zeyh. nom. illeg./ *Galium intermedium* Mérat nom. illeg./ *Galium lappaceum* Salisb. nom. illeg./ *Galium larecajense* Wernham/ *Galium parviflorum* Maxim. nom. illeg./ *Galium pseudoaparine* Griseb./ *Galium scaberrimum* Vahl ex Hornem./ *Galium segetum* K.Koch/ *Galium tenerrimum* Schur/ *Galium uliginosum* Thunb. nom. illeg./ *Galium uncinatum* Gray/ *Rubia aparine* (L.) Baill.
Asperula arvensis L.	Rubiaceae	Blue woodruff	-
Lathyrus aphaca L.	Fabaceae	Yellow pea, Yellow vetchling	*Orobus aphaca* (L.) Doll
Vicia narbonensis L.	Fabaceae	Narbon bean, Moor's pea	*Vicia serratifolia sensu auct. non* Jacq.
Digitaria sanguinalis (L.) Scop.	Poaceae	Hairy crabgrass, Hairy finger-grass, Large crabgrass, Crab finger-grass, Purple crabgrass	*Asperella digitaria* Lam./ *Cynodon praecox* (Walter) Roem. & Schult./ *Dactilon sanguinale* (L.) Vill./ *Digitaria aegyptiaca* Willd./ *Digitaria australis* Willd. ex Trin. nom. inval./ *Digitaria caucasica* Henrard/ *Digitaria eriogona* (Schrad.) Link/ *Digitaria gracilis* Guss./ *Digitaria intermedia* Gennari/ *Digitaria nealleyi* Henrard/ *Digitaria nervosa* (Rottb.) Roem. & Schult./ *Digitaria panacea* Willd. ex Steud. nom. inval./ *Digitaria pectiniformis* (Henrard) Tzvelev/ *Digitaria pilosa* Pieri nom. illeg./ *Digitaria plebeian* Phil./ *Digitaria praecox* (Walter) Willd./ *Digitaria sabulosa* Tzvelev/ *Digitaria sanguinaria* Steud. nom. inval./ *Digitaria sanguinea* Weber [Spelling variant]/ *Digitaria sanguinolenta* Edgew. ex Aitch. nom. inval./ *Digitaria sienitica* Trin. nom. inval./ *Digitaria stricta* Willd. ex Steud. nom. inval./ *Digitaria tristachya* Willd. ex Steud. nom. inval./ *Digitaria vulgaris* (Schrad.) Besser/ *Leptochloa fascicularis* Griseb. ex Benth. nom. inval./ *Milium membranaceum* Moench/ *Panicum aegyptiacum* Retz. nom. illeg./ *Panicum aegyptium* J.F.Gmel. [Spelling variant]/ *Panicum ambiguum* Lapeyr. nom. inval./ *Panicum eriogonum* Schrad./ *Panicum fallax* Spreng./ *Panicum gracile* (Guss.) Nyman nom. illeg./ *Panicum gussonii* K.Richt./ *Panicum nervosum* Rottler nom. illeg./ *Panicum rottleri* Kunth/ *Panicum sanguinale* L./ *Panicum sanguineum* Gueldenst./ *Panicum sanguinolentum* Edgew. ex Aitch. nom. inval./ *Panicum trichostachyum* Steud.nom. inval./ *Paspalum aegyptiacum* (Willd.) Poir./ *Paspalum filiforme* Steud. nom. inval./ *Paspalum oxyanthum* Steud./ *Paspalum sanguinale* (L.) Lam./ *Sanguinaria nevenarae* Bubani/ *Syntherisma praecox* Walter/ *Syntherisma sanguinale* (L.) Dulac/ *Syntherisma sanguinalis* (L.) Dulac/ *Syntherisma vulgaris* Schrad.
Hordeum murinum L.	Poaceae	Wall barley, False barley	-

Distilled water and vancomycin (30 µg) were used as negative control and positive control, respectively.

Determination of minimum inhibitory concentration

For determination of minimum inhibitory concentration (MIC) the broth dilution method was used: 500 µL of 24 h culture of the test organisms (10^8 CFU/mL) adjusted to McFarland's turbidity standard, were incubated in distilled water for 24 h at 37 °C in serial dilutions ranging from 3.9 to 500 mg/L of each plant extract. The concentration of the lowest dilution with no detectable bacterial growth was considered as the MIC. Growth absence was confirmed by the absence of turbidity and inoculation into agar (21).

Statistical analysis

Diameters of inhibition zones caused by each plant extracts and MICs on different bacteria were analyzed by SPSS v11. Data were compared by one-way ANOVA and Dunnett's *post hoc* test. *P* values less than 0.05 were considered as statistically significant. Each test was performed with three replicates.

Results

The total polyphenol and flavonoid contents in methanol leaf extracts are shown in Table 2. Total polyphenols in *G. aparine*, *A. arvensis*, *L. aphaca*, *V. narbonensis*, *D. sanguinalis* and *H. murinum* leaf extract were 76.4 ± 0.2, 8.4 ± 1.1, 160.2 ± 0.5, 6.75 ± 0.2, 4.8 ± 0.3, and 65.9 ± 0.5 GAE/g DW, respectively. Flavonoid contents were 83.7 ± 1.2, 5.5 ± 0.2, 94.6 ± 0.4, 3.9 ± 0.4, 2.3 ± 0.1, and 31.3 ± 0.2 GAE/g DW for *G. aparine*, *A. arvensis*, *L. aphaca*, *V. narbonensis*, *D. sanguinalis* and *H. murinum* leaf extracts, respectively.

Antibiotic resistance/sensitivity of MRSA isolates is reported in Table 3. Ninety-two (51.97%) out of 177 isolates were found to be methicillin-resistant in the antibiogram. In the antibiotic profile of MRSA strains, very high resistance was observed against ampicillin, penicillin and methicillin (100%). A lower degree of resistance was observed against the other antibiotics (ciprofloxacin, clindamycin, erythromycin and gentamycin). In addition, 6 isolates were resistant to vancomycin (Table 3). These results were confirmed by the presence of *mec*A gene in the 92 isolates, as indicated by the 147 bp fragment observed in electrophoresis (Figure 1). The results of the disk diffusion assay, i.e. the inhibition zones of each plant extract, negative control (water) and positive control (vancomycin 30 µg/disc) are shown in Tables 4, 5, and 6. There were

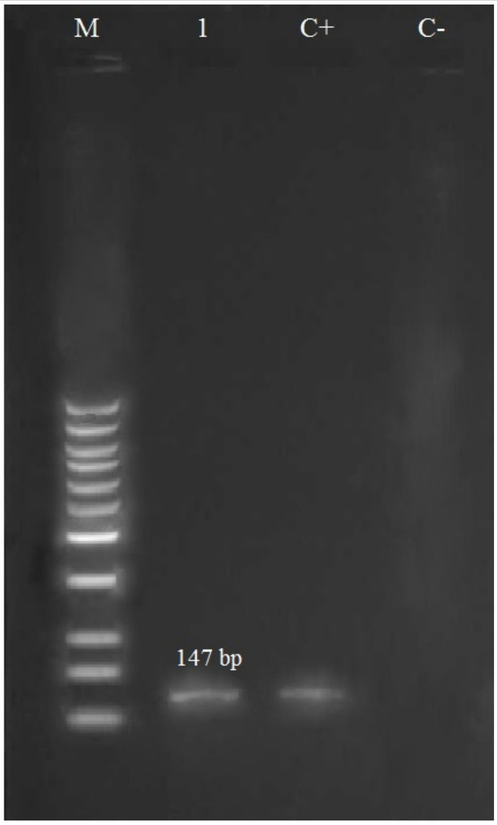

Figure 1. Patterns of agarose gel electrophoresis showing PCR amplification products for methicillin-resistant *Staphylococcus aureus* (MRSA) isolates. Lane M: DNA 100 basepair molecular size marker; lane 1: MRSA isolate; lane C+: positive control (reference strain *S. aureus* ATCC14458); lane C-: negative control.

Table 2. Total polyphenol and flavonoid contents of leaf methanolic extracts.

Plant name	Total phenolic content (GAE/g DW)[#]	Total flavonoid content (mg CE/g DW)[†]
Galium aparine L.	76.4 ± 0.2 b[§]	83.7 ± 1.2 b
Asperula arvensis L.	8.4 ± 1.1 d	5.5 ± 0.2 d
Lathyrus aphaca L.	160.2 ± 0.5 a	94.6 ± 0.4 a
Vicia narbonensis L.	6.75 ± 0.2 e	3.9 ± 0.4 e
Digitaria sanguinalis (L.) Scop.	4.8 ± 0.3 f	2.3 ± 0.1 f
Hordeum murinum L.	65.9 ± 0.5 c	31.3 ± 0.2 c

[#]Gallic acid equivalents per gram of dry weight.

[†]Milligrams of catechin per gram of dry weight.

[§]Values are expressed as mean ± SD; different letters show significant differences for each plant in each column at a $P < 0.05$.

Table 3. Antibiotic susceptibility of *Staphylococcus aureus* isolates.

Antibiotics	Number of isolates		
	Sensitive	Moderate	Resistant
Ampicillin	0	0	92
Ciprofloxacin	60	0	32
Penicillin	0	0	92
Clindamycin	44	2	46
Methicillin	0	0	92
Erythromycin	65	0	27
Vancomycin	86	0	6
Gentamycin	52	0	40

Table 4. Inhibition zones of Rubiaceae plant extracts on MRSA isolates.

Extract concentration (mg/L)		MRSA isolates	*S. aureus* (ATCC14458)
Galium aparine L.	10	6.3 ± 0.0 g	5.5 ± 0.4 g
	25	14.5 ± 0.2 f	12.4 ± 0.1 f
	50	16.5 ± 0.0 e	15.8 ± 0.5 e
	100	21.4 ± 0.1 c	22.1 ± 0.0 c
	150	25.9 ± 0.4 b	24.5 ± 0.3 b
	300	29.3 ± 0.2 a	27.8 ± 0.5 a
Negative control (distilled water)		0.0 ± 0.0 h	0.0 ± 0.0 h
Positive control (vancomycin 30 µg)		18.5 ± 0.1 d	19.8 ± 0.5 d
MIC (mg/L)		65.5 ± 0.3	77.9 ± 0.5
Asperula arvensis L.	10	0.0 ± 0.0 f	0.0 ± 0.0 f
	25	0.0 ± 0.0 f	0.0 ± 0.0 f
	50	3.5 ± 0.1 e	2.2 ± 0.2 e
	100	6.6 ± 0.4 d	5.5 ± 0.3 d
	150	8.7 ± 0.5 c	7.9 ± 0.6 c
	300	9.9 ± 0.6 b	8.5 ± 0.4 b
Negative control (distilled water)		0.0 ± 0.0 f	0.0 ± 0.0 f
Positive control (vancomycin 30 µg)		18.5 ± 0.1 a	19.8 ± 0.5 a
MIC (mg/L)		245.4 ± 0.2	264.3 ± 0.2

Data are expressed as mean ± SD of the inhibition zone diameter (mm) for different concentrations of plant extracts and controls; the values with different letters within a column are significantly different ($P < 0.05$); MIC: minimum inhibitory concentration.

Table 5. Inhibition zones of Fabaceae plant extracts on MRSA isolates.

Extract concentration (mg/L)		MRSA isolates	*S. aureus* (ATCC14458)
Lathyrus aphaca L.	10	11.5 ± 0.3 g	10.3 ± 0.2 g
	25	17.7 ± 0.3 f	16.9 ± 0.1 f
	50	28.4 ± 0.1 d	26.8 ± 0.5 d
	100	37.5 ± 0.2 c	37. 6 ± 0.3 c
	150	48.6 ± 0.5 b	47.5 ± 0.2 b
	300	65.5 ± 0.4 a	63.3 ± 0.1 a
Negative control (distilled water)		0.0 ± 0.0 h	0.0 ± 0.0 h
Positive control (vancomycin 30 µg)		18.5 ± 0.1 e	19.8 ± 0.5 e
MIC (mg/L)		5.5 ± 0.1	7.8 ± 0.3
Vicia narbonensis L.	10	0.0 ± 0.0 e	0.0 ± 0.0 e
	25	0.0 ± 0.0 e	0.0 ± 0.0 e
	50	0.0 ± 0.0 e	0.0 ± 0.0 e
	100	4.3 ± 0.1 d	3.3 ± 0.2 d
	150	5.5 ± 0.3 c	4.7 ± 0.5 c
	300	8.8 ± 0.2 b	7.8 ± 0.5 b
Negative control (distilled water)		0.0 ± 0.0 e	0.0 ± 0.0 e
Positive control (vancomycin 30 µg)		18.5 ± 0.1 a	19.8 ± 0.5 a
MIC (mg/L)		288.3 ± 0.1	299.5 ± 0.4

Data are expressed as mean ± SD of the inhibition zone diameter (mm) for different concentrations of plant extracts and controls; the values with different letters within a column are significantly different ($P < 0.05$); MIC: minimum inhibitory concentration.

Table 6. Inhibition zones of Poaceae plant extracts on MRSA isolates.

	Extract concentration (mg/L)	MRSA isolates	S. aureus (ATCC14458)
Digitaria sanguinalis (L.) Scop.	10	0.0 ± 0.0 e	0.0 ± 0.0 e
	25	0.0 ± 0.0 e	0.0 ± 0.0 e
	50	0.0 ± 0.0 e	0.0 ± 0.0 e
	100	3.5 ± 0.2 d	3.2 ± 0.1 d
	150	4.8 ± 0.3 c	3.5 ± 0.2 c
	300	5.4 ± 0.1 b	4.8 ± 0.5 b
Negative control (distilled water)		0.0 ± 0.0 e	0.0 ± 0.0 e
Positive control (vancomycin 30 µg)		18.5 ± 0.1 a	19.8 ± 0.5 a
MIC (mg/L)		388.4 ± 0.2	394.9 ± 0.5
Hordeum murinum L.	10	4.5 ± 0.3 g	4.4 ± 0.2 g
	25	12.9 ± 0.3 f	11.5 ± 0.0 f
	50	14.7 ± 0.5 e	14.8 ± 0.3 e
	100	19.5 ± 0.3 c	18.8 ± 0.5 d
	150	22.7 ± 0.4 b	20.4 ± 0.2 b
	300	25.5 ± 0.3 a	24.5 ± 0.1 a
Negative control (distilled water)		0.0 ± 0.0 h	0.0 ± 0.0 h
Positive control (vancomycin 30 µg)		18.5 ± 0.1 d	19.8 ± 0.5 c
MIC (mg/L)		83.8 ± 0.2	96.7 ± 0.8

Data are expressed as mean ± SD of the inhibition zone diameter (mm) for different concentrations of plant extracts and controls; the values with different letters within a column are significantly different ($P < 0.05$); MIC: minimum inhibitory concentration.

dose-dependent inhibitions on tested microorganisms for all plant extracts. All extracts exhibited maximum inhibition at 300 mg/L on MRSA isolates and standard strain (*S. aureus* ATCC14458). Inhibition haloes on MRSA isolates at 300 mg/L of *G. aparine*, *A. arvensis*, *L. aphaca*, *V. narbonensis*, *D. sanguinalis* and *H. murinum* leaf extracts were 29.3 ± 0.2, 9.9 ± 0.6, 65.5 ± 0.4, 8.8± 0.2, 5.4 ± 0.1 and 25.5 ± 0.3 mm in diameter, respectively. The standard strain showed smaller zones of inhibition than the MRSA isolates, and diameters of

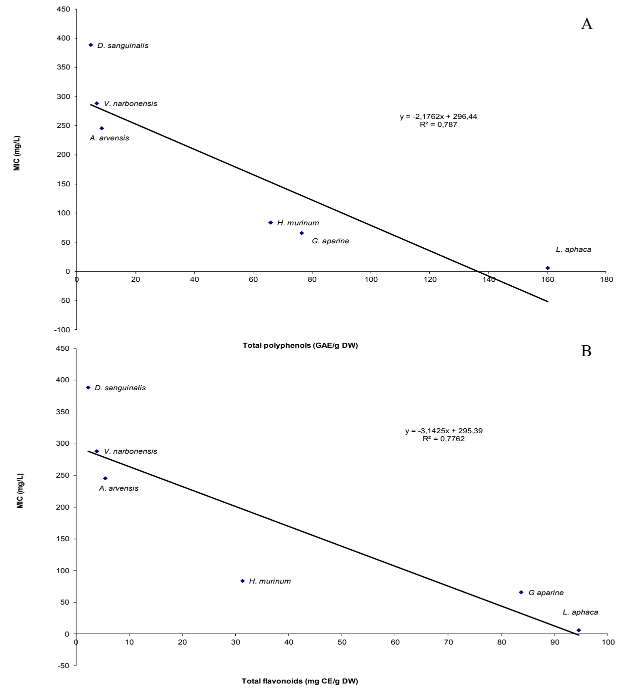

Figure 2. Correlation analysis based on simple linear regression at the 95% confidence level between (A) total polyphenol content expressed as milligrams of gallic acid equivalents per gram of dry weight (mg GAE/g DW) or (B) total flavonoid content expressed as milligrams of catechin per gram of dry weight (mg CE/g DW) and minimum inhibitory concentration (MIC, mg/L) of methanol leaf extracts of *Galium aparine* L., *Asperula arvensis* L., *Lathyrus aphaca* L., *Vicia narbonensis* L., *Digitaria sanguinalis* (L.) Scop. and *Hordeum murinum* L. on methicillin-resistant *Staphylococcus aureus* (MRSA) strains.

inhibition haloes were 27.8 ± 0.5, 8.5 ± 0.4, 63.3 ± 0.1, 7.8 ± 0.5, 4.8 ± 0.5 and 24.5 ± 0.1 mm for *G. aparine*, *A. arvensis*, *L. aphaca*, *V. narbonensis*, *D. sanguinalis* and *H. murinum* leaf extracts, respectively. The positive control (vancomycin) showed inhibition zones of 18.5 ± 0.1 and 19.8 ± 0.5 mm on MRSA isolates and the standard strain, respectively. Among all plants, the inhibition zones of *G. aparine, L. aphaca* and *H. murinum* were more significantly different than the positive control ($P < 0.05$).

The results of the broth dilution test of plant extracts are shown in Tables 4, 5, and 6. MICs on MRSA isolates were 65.5 ± 0.3, 245.4 ± 0.2, 5.5 ± 0.1, 288.3 ± 0.1, 388.4 ± 0.2 and 83.8 ± 0.2 mg/L for *G. aparine*, *A. arvensis*, *L. aphaca*, *V. narbonensis*, *D. sanguinalis* and *H. murinum* leaf extracts, respectively. The MICs of the extracts against the standard *S. aureus* strain were 77.9 ± 0.5, 264.3 ± 0.2, 7.8 ± 0.3, 299.5 ± 0.4, 394.9 ± 0.5 and 96.7 ± 0.8 mg/L for *G. aparine*, *A. arvensis*, *L. aphaca*, *V. narbonensis*, *D. sanguinalis* and *H. murinum* leaf extract, respectively and these values were higher than the MICs of the MRSA isolates.

For all the plant extracts, a correlation analysis based on simple linear regression was performed on the assayed variables (total polyphenol content and total flavonoid content vs. MIC) at the 95% confidence level (Figure 2A,B). Both polyphenols and flavonoids were highly correlated with MIC values, with high linear correlation coefficients ($R^2 = 0.787$ and $R^2 = 0.776$, respectively).

Discussion

The results of our study illustrated that plants with higher levels of total polyphenols and flavonoids in the methanol leaf extracts, i.e. *L. aphaca* (Fabaceae), *G. aparine* (Rubiaceae) and *H. murinum* (Poaceae), showed higher antibacterial activity against the MRSA strains (Figure 2). Antimicrobial activity of plant phenolics, including flavonoids, has been demonstrated since decades (22, 23). Eight flavonoids were isolated in the whole plant of *G. aparine*: chrysoeriol, apigenin, luteolin, quercetin, chrysoeriol-7-*O*-β-D-glucoside, apigenin-7-*O*-β-D-glucoside,luteolin-4′-*O*-β-D-glucoside and luteolin-7-*O*-β-D-glucoside (24). More recently, other phenolic compounds were identified in this species, such as coumarin, esculetin, chlorogenic acid, cinnamic acid, p-coumaric acid and quercitrin (25). Two iridoid glycosides were also isolated from the aerial parts of *G. aparine*, asperulosidic acid and 10-deacetylasperulosidic acid (26), as well as lipophilic compounds including fatty acids, phytosterols, sesquiterpenoids and other isoprenoids (27, 25, 28). In a previous study, a tincture of *G. aparine* showed a mild inhibitory activity on *S. aureus*, producing an average zone of inhibition of 2 mm (29). Conversely, *S. aureus* and *Pseudomonas aeruginosa* were highly sensitive to the lipophilic extract of this plant (27). Anticancer activity of *G. aparine* was also reported in leukemia and breast cancer cells (25, 28). A number of flavonoid, namely larycitrin, syringetin-3-*O*-rutinoside-7-*O*-β-D-glucoside, quercetin and quercetin-3-*O*-β-D-glucoside were found in different tissues (leaves, flowers, seeds and pod) of *L. aphaca* (30, 31). Noteworthy, the butanol extract of *L. aphaca* seeds exhibited inhibitory activity on *S. aureus*, *Escherichia coli* and *Klebsiella pneumoniae*, with MICs of 76.25, 90.50 and 98.50 µg/mL, respectively (32). To the best of our knowledge, no information is available on phytochemical profile (in terms of polyphenols or flavonoids) and biological activities of *H. murinum*.

As regards other species, flavonoids isoquercitrin, hyperin, quercetin-7-*O*-β-D-galactoside, quercetin-4’-*O*-β-D-galactoside, isorhamnetin-3-*O*-β-D-galactoside, isorhamnetin-5-*O*-β-D-galactoside, dihydrokaempfero-7-4’-dimethylether-3-*O*-β-D-glucoside and isorhamnetin-3-*O*-α-D-ramnosil-(1’’’→6’’)-β-D-glucoside were identified in the aerial parts of *A. arvensis* (33). Similarly, a number of quercetin and kaempferol glycosides were isolated from *V. narbonensis* fruits and leaves (34), whereas veratric acid (a phenolic compounds), maltol and loliolide (a monoterpene lactone) were found in *D. sanguinalis* (35).

In conclusion, this study provides new information on the antimicrobial potential of *L. aphaca* (Fabaceae), *G. aparine* (Rubiaceae), and *H. murinum* (Poaceae) to MRSA strains. As known, *mec*A gene encodes the protein PBP2A (penicillin binding protein 2A) in the bacterial cell wall with a low affinity for β-lactam antibiotics (36). In this view, it will be pivotal to investigate the mechanism(s) involved in the observed phenomena, in order to develop new, promising antibacterial agents.

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