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Antimicrobial Activity of Methanolic Extract and Ether Extract of *Ageratum conyzoides*

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Abstract

Ageratum conyzoides, a weed prevalent in India, is known for its several therapeutic uses to control infections. In the present study we compared the antimicrobial potential of its ether extract and methanolic extract with ciprofloxacin on 294 strains of Gram positive bacteria (GPBs), 575 strains of Gram negative bacteria (GNBs), 15 yeast and 5 mould strains *of clinical and nonclinical origin belonging to* 49 genera and more than 155 species using disc diffusion assay. The microbial strains in the study were isolated from samples of abiotic (41) and biotic (101) environment, foods (81), clinically sick (441), dead (108) and healthy (75) animals and human beings, and 42 were reference strains. The study revealed that there was no appreciable difference in antimicrobial activity of ether extract (ACEE) or methanolic extract (ACME) of *A. conyzoides*. A total of 214 (24.1%) strains were sensitive to ACME while of the 697 strains tested for ciprofloxacin 551 (79.1%) were sensitive. Sensitivity to ACME among 294 GPBs (44.9%) was significantly ($p<0.0001$) higher than among 575 strains of GNBs (12.4%). There was no significant difference among GPBs and GNBs for ciprofloxacin (one of the most commonly used antibiotics in India) sensitivity, but oxidase negative GNBs (385) as well as GPBs (238) were about two times more commonly sensitive to ciprofloxacin than 190 oxidase positive GNBs ($p = 0.001$) and 56 oxidase positive GPBs (p, 0.03), respectively. For ACME oxidase positive strains had 2.4 times more odds ($p < 0.0001$) in their favour of being sensitive to ACME (53.4%) than oxidase negative strains (18.6%). The most sensitive strains to ACME belonged to oxidase positive GPBs (62.5%) followed by oxidase negative GPBs (40.8%), oxidase positive GNBs (27.4%) and oxidase negative GNBs (4.9%). All Aeromonas, Alcaligenes, Klebsiella, and Proteus species strains were resistant to ACME irrespective of source of isolation or association with illness. In contrast, majority of the strains of Burkholderia (76.9%), Bacillus (66.7%) and Brucella (53.8%) species were sensitive to ACME. The study revealed that *A. conyzoides* might be containing useful antimicrobial component(s) more active against oxidase positive potentially pathogenic strains often associated with systemic and deadly infections in animals as well as in humans.

Keywords: Herbal antimicrobials; Animal pathogens; Zoonotic pathogens; Aeromonas; Burkholderia; Brucella; Ciprofloxacin

Introduction

Ageratum conyzoides (Billygoat-weed, Chick weed, Goatweed, Whiteweed Cut-lon or Pig faeces) also known as *Ageratum conycoides* L., *Ageratum obtusifolium* Lam., *Cacalia mentrasto* Vell, is an invasive weedy herb having 0.5 m – 1 m height with 2 cm – 6 cm long ovate leaves and blue white to mauve flowers, it is found throughout India [1]. Though it has several medicinal properties, due to its potent hepatotoxic and carcinogenic nature associated with pyrrolizidine alkaloids lycopsamine and echinatine [2], it can be used only externally. It possesses insecticidal and nematodicidal potency [3]. In Central Africa and some parts of Asia fresh extract from leaves is used to treat pneumonia, common cold, wounds and burns, diarrhoea, dysentery, fever, rheumatism, headache, and colic [4]. Wound healing properties of leaf extract of *A. conyzoides* is reported to be enhanced when mixed with *Ficus religiosa, Curcuma longa* and *Tamarindus indica* [5,6]. Essential oil of Ageratum has been reported to inhibit several bacteria and fungi having more potent antimicrobial activity than citronella and geranium oils [7]. Essential oil of *A. houstonianum*, a close relative of *A. conyzoides*, has antibacterial activity against *Micrococcus luteus* and *Rhodococcus rhodochrous* with minimum bactericidal concentration (MBC) of 100 mg/ml and 12.5 mg/ml, but not against *Arthrobacter protophormiae*, *Escherichia coli* and *Staphylococcus aureus* [8]. However in another study [9] hexane extract, aqueous extract and methanolic leaf extract of *Ageratum conyzoides* inhibited one strain each of *S. aureus, Yersinia enterocolitica*, *Salmonella gallinarum* and *E. coli*. In the study [9], aqueous leaf extracts gave minimum bactericidal concentration (MBC) of 50, 25 and 25mg/ml for *S. aureus, Y. enterocolitica* and *E. coli*, respectively, and methanolic leaf extract gave MBC of 25 mg/ml and 50 mg/ml for *S. aureus* and *E. coli*, respectively [9]. In another similar study on a few strains of bacteria, *A. conyzoides* aqueous leaf extract inhibited the growth all 5 strains of *S. aureus* but had little or no activity against *E. coli* and *Pseudomonas aeruginosa*, methanolic extract inhibited all but *P. aeruginosa* while ether extracts did not show any antibacterial activity against the test organisms [10]. Considering the lacunae in the current understanding of the antimicrobial potential of *A. conyzoides* this study was undertaken on large number of microbes of clinical and environmental origin so that the true antimicrobial potential of the herb can be assessed. Sensitivity of bacterial strains to

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A. conyzoides extracts was compared with sensitivity to ciprofloxacin, a most widely used antibiotics in veterinary practice in India [11].

Materials and Methods

Methanolic extract of *A. conyzoides* leaves (ACME) and preparation of discs

For ACME, green leaves of *A. conyzoides* were collected at ICAR Research Complex for NEH Regional, Nagaland Centre, Jharnapani, Nagaland, from lush green mature plants ready to bloom in the campus. The leaves were washed gently with sterile distilled water to remove any dirt and dried in shade for a week. The leaves were powdered through pounding and 250 g of powder was mixed with 500 ml of methanol (99.9% pure, Merck, India Ltd.) in a 2 L conical flask and allowed to stand overnight over rotary shaker (50 rpm) at 25°C. Next morning, the flask contents were filtered through glass wool and filtrate was allowed to dry in crystallization bowls at 45°C for 24 h - 36 h, till the entire methanol got evaporated and contents were weighed [9]. The ACME was dissolved in methanol to contain 100 mg/mL of solution. On the basis of earlier studies [12], 20 µL of solution containing 2 mg of dry weight of ACME was poured on to individual 5 mm sterile discs and discs were dried in air at 45°C for 3 h [13]. The ACME discs were stored at 4°C throughout the study.

Ether extract of *A. conyzoides* leaves (ACEE) and preparation of discs

For ACEE, same procedure as used for ACME was followed except the use of di-ethyl ether as solvent instead of methanol [9]. Discs of ACEE were prepared as described earlier for ACME.

Microbial strains

A total of 889 strains including 294 strains of Gram positive bacteria (GPBs), 575 strains of Gram negative bacteria (GNBs), 15 yeast and 5 mould strains were included in the study. The strains belonging to 49 genera (Table 1) isolated from abiotic (41) or biotic (101) environment, food samples (81), clinically sick (441), dead (108) or healthy (75) animals and human beings and 42 reference strains (Table 2) were tested. The isolates in the study belonged to more than 155 species (Table 3). All the strains were available in glycerol stocks at Division of Epidemiology Laboratory of the Institute and were revived and checked for purity and identity using phenotypic, growth and biochemical characteristics [14,15]. Test strains were kept on tryptic soy agar (TSA, BD, BBL Difco) slants for the period of the study and were grown overnight in tryptic soy broth (TSB, BD, BBL Difco) for susceptibility testing.

Testing antimicrobial activity

Disc diffusion assay using ACME and ACEE discs was performed as described earlier [13,16] for different bacteria in triplicate on Mueller Hinton agar (MHA) plates inoculated (with swab) with overnight broth culture adjusted to 0.1 OD_{590}. All strains were tested for sensitivity on MHA plates but Moraxella, Streptococcus, Brucella, Bordetella and Pasteurella strains, all slow growing and fastidious bacteria, were tested on brain heart infusion (BHI) agar (BD BBL Difco) instead of MHA [16]. All strains were tested at 37°C aerobically except Brucella, which were incubated in 5% CO_2 enriched environment at 37°C and yeast and mould strains were incubated at 25°C for 3 to 7 days before reading. Ciprofloxacin (10 µg) discs (BD BBL Difco) were used as standard antimicrobial discs and blank discs {first soaked in methanol and diethyl ether (1:1) and dried in similar way as for ACME discs} were used as control discs.

Statistical analysis

To determine correlation between sensitivity (zone of inhibition in mm) of test strains to ciprofloxacin, ACME and ACEE discs correlation coefficient was calculated using MS Office Excel-7. To estimate association between sensitivity of microbes to ciprofloxacin and ACME with respect to species and source of microbes, odds ratio analysis and χ^2 tests were performed in MS Office Excel-2007. The statistical comparison was done for only those genera or sources of microbes where number (n) of strains tested was ≥ 10.

Results

Of the 889 microbial strains of different origin (Table 1), 214 (24.1%) were sensitive to ACME while of the 697 strains tested for ciprofloxacin sensitivity, 551 (79.1%) were inhibited.

Antimicrobial activity of ether extract versus methanolic extract

A total of 103 strains belonging 25 species of 11 genera were tested for their sensitivity to *A. conyzoides* ether extract (ACEE) and methanolic extract (ACME). Of the 103 only 34 (33%) strains were sensitive to ACME as well as the ACEE. The results matched perfectly after testing in triplicate for sensitivity of the strains to both of the extracts, i.e., ACME and ACEE. For further studies only ACME was preferred being more economic in preparation.

Gram staining and oxidase reaction versus antimicrobial drug sensitivity

Sensitivity to ACME among 294 GPBs (44.9%) was significantly ($p < 0.0001$) higher than among 575 strains of GNBs (12.4%). GNBs were even more resistant to ACME than 15 yeast ($p = 0.018$) and 5 mould ($p < 0.0001$) strain tested in the study.

Though there was no significant difference among GPBs and GNBs for ciprofloxacin sensitivity, oxidase negative GNBs (385) and GPBs (238) were about two times more commonly sensitive to ciprofloxacin than 190 oxidase positive GNBs ($p = 0.001$) and 56 oxidase positive GPBs ($p = 0.03$), respectively. In general oxidase positive strains (246) were significantly ($p = 0.0007$) more often (~ 2 times) ciprofloxacin resistant (26.4%) than 623 oxidase negative strain (15.4%). However, oxidase positive strains had 2.4 times more odds ($p < 0.0001$) in their favour for being sensitive to ACME (53.4%) than oxidase negative strains (18.6%). The most sensitive strains to ACME belonged to oxidase positive GPBs (62.5%) followed by oxidase negative GPBs (40.8%), oxidase positive GNBs (27.4%) and oxidase negative GNBs (4.9%). All the four groups differed significantly from each other for ACME sensitivity ($p < 0.01$).

Source of microbe and their sensitivity of ACME and ciprofloxacin

Among all the strains (Figure 1) tested in the study, microbes of food origin were more commonly sensitive to ACME ($p < 0.0001$) than strains originating from abiotic or biotic environment, clinical samples, healthy stocks, dead animals or the reference strains. However, among all non-food origin microbes there was hardly any significant ($p > 0.05$) difference to their ACME sensitivity except isolates from healthy animals, which were relatively ($p = 0.02$) more resistant than those from abiotic environment (Table 2).

The effect of source was evident with respect to ACME sensitivity among GPBs (Table 2 and Figure 1), foodborne isolates were more

Genus of the isolates	Strains from clinical illness and mortality			Strains from environment, food, healthy animals, etc.			Total strains tested		
	Strains	ACS (%)	CipS (%)	Strains	ACS (%)	CipS (%)	Strains	ACS (%)	CipS (%)
Achromobacter	2	0 (0.0)	2 (100.0)	1	0 (0.0)	1 (100.0)	3	0 (0.0)	3 (100.0)
Acinetobacter	5	3 (60.0)	5 (100.0)	3	0 (0.0)	2 (66.7)	8	3 (37.5)	7 (78.5)
Actinobacillus	1	0 (0.0)	1 (100.0)	1	1 (100.0)	1 (100.0)	2	1 (50.0)	2 (100.0)
Aerococcus	2	0 (0.0)	1 (50.0)	0	0 (0.0)	0 (0.0)	2	0 (0.0)	1 (50.0)
Aeromonas	9	0 (0.0)	7 (77.8)	3	0 (0.0)	2 (66.7)	12	0 (0.0)	9 (75.0)
Agrobacterium	0	0 (0.0)	0 (0.0)	1	0 (0.0)	1 (100.0)	1	0 (0.0)	1 (100.0)
Alcaligenes	9	0 (0.0)	7 (77.8)	2	0 (0.0)	2 (100.0)	11	0 (0.0)	9 (81.8)
Aspergillus	0	0 (0.0)	0 (0.0)	5	4 (80.0)	0 (0.0)	5	4 (80.0)	0 (0.0)
Bacillus	20	11 (55.0)	16 (80.0)	31[27]	23 (74.2)	3 (75.0)	51[27]	34 (66.7)	19 (79.2)
Bordetella	1	1 (100.0)	1 (100.0)	7	1 (14.3)	7 (100.0)	8	2 (250.0)	8 (100.0)
Brucella	22	12 (54.5)	16 (72.7)	4	2 (50.0)	2 (50.0)	26	14 (53.8)	18 (69.2)
Budvicia	1	0 (0.0)	1 (100.0)	0	0 (0.0)	0 (0.0)	1	0 (0.0)	1 (100.0)
Burkholderia	9	9 (100.0)	7 (77.8)	4	1 (25.0)	4 (100.0)	13	10	11
Campylobacter	0	0 (0.0)	0 (0.0)	4	0 (0.0)	4 (100.0)	4	0 (0.0)	4 (100.0)
Candida	8	3 (37.5)	0 (0.0)	4	2 (50.0)	0 (0.0)	12	5 (41.7)	0 (0.0)
Citrobacter	3	0 (0.0)	3 (100.0)	19[17]	4 (21.1)	2 (100.0)	22[17]	4 (18.2)	5 (100.0)
Corynebacterium	2	2 (100.0)	1 (50.0)	0	0 (0.0)	0 (0.0)	2	2 (100.0)	1 (50.0)
Dermatophilus	2	2 (100.0)	1 (50.0)	0	0 (0.0)	0 (0.0)	2	2 (100.0)	1 (50.0)
Edwardsilella	2	0 (0.0)	2 (100.0)	2[2]	2 (100.0)	NT	4[2]	2 (50.0)	2 (100.0)
Enterobacter	30	3 (10.0)	25 (83.3)	22[8]	0 (0.0)	12 (85.7)	52[8]	3 (5.8)	37 (84.1)
Enterococcus	11	1 (9.1)	9 (81.8)	64[64]	27 (42.2)	NT	75[64]	28 (37.3)	9 (81.8)
Erwinia	6	0 (0.0)	5 (83.3)	4[1]	2 (50.0)	3 (100.0)	10[1]	2 (20.0)	8 (88.9)
Escherichia	136	2 (1.5)	111 (81.6)	24[12]	2 (8.3)	11 (91.7)	160[12]	4 (2.5)	122 (82.4)
Geotrichum	0	0 (0.0)	0 (0.0)	1	0 (0.0)	0 (0.0)	1	0 (0.0)	0 (0.0)
Hafnia	3	0 (0.0)	2 (66.7)	0	0 (0.0)	0 (0.0)	3	0 (0.0)	2 (66.7)
Klebsiella	25	0 (0.0)	22 (88.0)	15[7]	0 (0.0)	8 (100.0)	40[7]	0 (0.0)	30 (90.9)
Kluyvera	0	0 (0.0)	0 (0.0)	2	0 (0.0)	2 (100.0)	2	0 (0.0)	2 (100.0)
Listeria	0	0 (0.0)	0 (0.0)	1	1 (100.0)	1 (100.0)	1	1 (100.0)	1 (100.0)
Micrococcus	1	0 (0.0)	0 (0.0)	2[2]	2 (100.0)	NT	3[2]	2 (66.7)	0 (0.0)
Moraxella	0	0 (0.0)	0 (0.0)	2	1 (50.0)	2 (100.0)	2	1 (50.0)	2 (100.0)
Morganella	1	0 (0.0)	0 (0.0)	0	0 (0.0)	0 (0.0)	1	0 (0.0)	0 (0.0)
Mycoplasma	0	0 (0.0)	0 (0.0)	2	0 (0.0)	2 (100.0)	2	0 (0.0)	2 (100.0)
Pasteurella	25	11 (44.0)	15 (60.0)	12	2	8 (66.7)	37	13 (35.1)	23 (62.2)
Pediococcus	1	0 (0.0)	0 (0.0)	0	0 (0.0)	0 (0.0)	1	0 (0.0)	0 (0.0)
Pragia	2	0 (0.0)	2 (100.0)	9[9]	3 (33.3)	NT	11[9]	3 (27.3)	2 (100.0)
Proteus	20	0 (0.0)	18 (90.0)	2	0 (0.0)	2 (100.0)	22	0 (0.0)	20 (90.9)
Pseudomonas	48	6 (12.5)	34 (70.8)	11[1]	2 (18.2)	9 (90.0)	59[1]	8 (13.6)	43 (74.1)
Raoultella	3	0 (0.0)	2 (66.7)	0	0 (0.0)	0 (0.0)	3	0 (0.0)	2 (66.7)
Rhodotorula	0	0 (0.0)	0 (0.0)	1	0 (0.0)	1 (100.0)	1	0 (0.0)	1 (100.0)
Salmonella	18	0 (0.0)	14 (77.8)	30[23]	1 (3.3)	7 (100.0)	48[23]	1 (2.1)	21 (84.0)
Serratia	1	0 (0.0)	1 (100.0)	1[1]	0 (0.0)	NT	2[1]	0 (0.0)	1 (100.0)
Shingomonas	2	0 (0.0)	1 (50.0)	0	0 (0.0)	0 (0.0)	2	0 (0.0)	1 (50.0)
Staphylococcus	45	10 (22.2)	40 (88.9)	32[13]	17 (53.1)	15 (78.9)	77[13]	27 (35.1)	55 (85.9)
Streptobacillus	1	1 (100.0)	1 (100.0)	0	0 (0.0)	0 (0.0)	1	1 (100.0)	1 (100.0)
Streptococcus	69	30 (43.5)	58 (84.1)	10[5]	6 (60.0)	4 (80.0)	79[5]	36 (45.6)	62 (83.8)
Trichosporum	0	0 (0.0)	0 (0.0)	1	0 (0.0)	0 (0.0)	1	0 (0.0)	0 (0.0)
Trichophyton	1	0 (0.0)	0 (0.0)	0	0 (0.0)	0 (0.0)	1	0 (0.0)	0 (0.0)
Vibrio	2	1 (50.0)	1 (50.0)	0	0 (0.0)	0 (0.0)	2	1 (100.0)	1 (100.0)
Yersinia	0	0 (0.0)	0 (0.0)	1	0 (0.0)	1 (100.0)	1	0 (0.0)	1 (100.0)
All	549	108 (19.7)	432 (78.7)	340[192]	106 (31.2)	119 (80.4)	889[192]	214 (24.1)	551 (79.1)

ACS, sensitive to ACME; CipS, sensitive to ciprofloxacin; superscript numbers shows strains not tested for ciprofloxacin sensitivity.
Isolates from mortal and clinical cases were almost two times more often resistant to ACME than those isolated from non-clinical sources (p = 0.0001).

Table 1: Microbes of different genera isolated from clinical and nonclinical sources and their sensitivity to 2 mg *Ageratum conyzoides* methanolic extract (ACME) and 10 µg ciprofloxacin (Cip) discs.

Source of microbes	Gram +ve bacteria		Gram –ve bacteria		Yeasts		Moulds		Total	
	Tested	ACS	Tested	ACS	Tested	ACS	Tested	ACS	Tested	ACS
Abiotic environment	9	3 (33.3)	32	9 (28.1)	0	0	0	0	41	12 (29.3)
Biotic Environment	48	12 (25.0)	53	6 (11.3)	0	0	0	0	101	18 (17.8)
Clinical illness	132	49 (37.1)	300	36 (12.0)	9	3 (33.3)	0	0	441	88 (20.0)
Healthy animals/ human	10	0 (0.0)	59	4 (6.8)	1	1 (100.0)	5	4 (80.0)	75	9 (12.0)
Post-mortem cases	22	7 (31.8)	86	11 (12.8)	0	0	0	0	108	18 (16.7)
Reference strains	8	3 (37.5)	29	4 (13.8)	5	1 (20.0)	0	0	42	8 (19.0)
Food (Axone)	65	58 (89.2)	16	1 (6.3)	0	0	0	0	81	59 (72.8)
Total	294	132 (44.9)	575	71 (12.3)	15	5 (33.3)	5	4 (80.0)	889	212 (23.8)

Figures in parentheses indicate percent sensitive strains.

Table 2: Sensitivity of Gram positive bacteria (GPBs) and Gram negative bacteria (GNBs) from different sources to 2 mg *Ageratum conyzoides* methanolic extract (ACME).

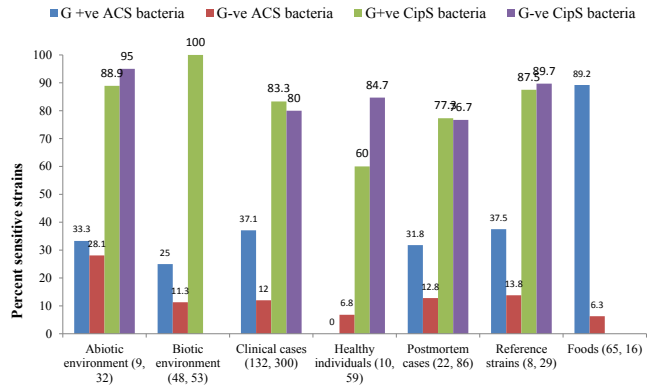

Figure 1: Sensitivity patterns of G +ve and G –ve bacteria of different origin to 2 mg *Ageratum conyzoides* methanolic extract and 10 µg ciprofloxacin discs.

often sensitive than strains from other sources. Among GPBs of non-food origin, strains from healthy animals were more commonly resistant than strains from abiotic (p, 0.08) and biotic (p = 0.08) environment, from clinical (p = 0.017), post-mortem (p = 0.04) cases and the reference (p = 0.03) strains.

In contrast, GNBs isolated from food samples or non-food samples (Table 2) had no significant (p > 0.08) difference in sensitivity to ACME. However, GNBs from abiotic environment were comparatively more often ACME sensitive (18.2 times more) than strains from other sources and specifically more than the strains from biotic environment (p = 0.05), clinical samples (p = 0.01) or strains associated with mortality in animals (p = 0.05).

With respect to ciprofloxacin sensitivity (Figure 1), effect of source was not evidently significant irrespective of their staining characteristics viz., GPBs (p > 0.2) or GNBs (p > 0.07)

Though in general non-food origin microbes appeared similar in sensitivity to ACME (Figure 1) but on further analysing the data and further specifying the sources (Figure 2) results indicated that isolates from swamp buffaloes and pigs were more often resistant to ACME than other sources except those isolated from mithuns, laboratory animals and sheep and goats (p > 0.05)

Isolates from pigs were more often (p < 0.01) ACME resistant than isolates from cattle, dogs, birds, wild animals, horses, mules and humans. Similarly isolates from swamp buffaloes were more commonly ACME resistant than isolates from buffaloes (p = 0.05), cattle (p = 0.02), dogs (p = 0.008), birds (p = 0.002), wild animals (p = 0.008), laboratory animals, (p = 0.05), horses and mules (p = 0.001) and human beings (p = 0.005).

Isolates from cattle had 19.5 times odds for being ACME sensitive than those from pigs (p = 0.01). Isolates from birds have 11.7 times higher odds for being ACME sensitive than strains of pig origin (p = 0.05). Bacterial isolates from wild animals have 9.75 times higher odds of being ACME sensitive than those of pig (p = 0.05) origin.

Though there was little association of source with ciprofloxacin resistance, isolates from humans had almost 2.5 times higher odds of being ciprofloxacin resistant than those from dogs (p = 0.04), mithun (p = 0.02), pigs (0.0010, swamp buffaloes (p = 0.005) and horse and mules (p = 0.007).

Association of microbes with health or illness and their drug sensitivity

Though clinical and nonclinical isolates differed in their sensitivity to ACME, the difference was more often due to type of the pathogen. Among the strains of the same species or the same genus of bacteria difference in sensitivity to ACME was not apparent except for Gram positive bacteria. GPBs including Enterococcus and Staphylococcus species strains, bacteria isolated from food (p < 0.0001) samples were more often sensitive to ACME than those isolated from clinical samples or from cases of mortality or from healthy animals. Streptococci from biotic environment were more often ACME sensitive (p = 0.02) than isolates from domestic animals irrespective of their association with health, illness or death. The intra-species variation among strains associated with health, disease or mortality in animals and those isolated from food or environment was not evident for ciprofloxacin sensitivity except the *E. coli* isolated from abiotic environment (air, water and soil) were significantly (p = 0.003) more commonly sensitive to ciprofloxacin than *E. coli* isolates from clinical cases.

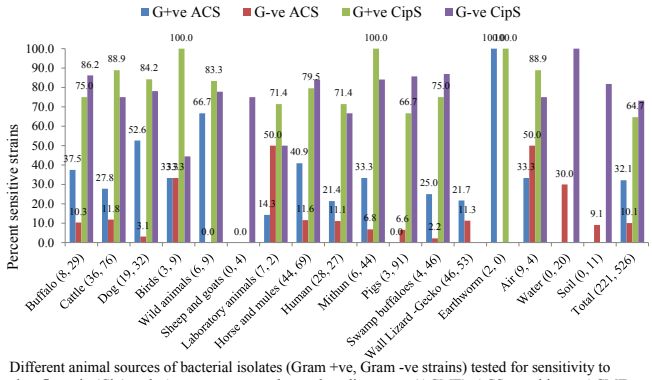

Figure 2: *Ageratum conyzoides* mthanolic extract (2 mg) and 10 µg ciprofloxacin sensitivity patterns of G +ve and G –ve bacteria isolated from different animals and their environment.

Genus	N	ACS	CipS	Species of the microbe, number of strains (ACS, CipS)
Achromobacter	3	0	3	*A. xylosoxidans* 2 (2, CipS), UI 1 (1CipS)
Acinetobacter	8	3	6	*A. boumanni* 1, *A. schindleri* 2 (both ACS and CipS), UI 5 (1 ACS, 4 CipS)
Actinobacillus	2	1	2	*A. seminis* 1 (ACS and CipS), UI 1 (CipS)
Aerococcus	2	0	1	UI 2 (1 CipS)
Aeromonas	12	0		*A. salmonicida* ssp. *masoucida* 1 (CipS), *A. caviae*1 (CipS), *A. eucranophila* 1, *A. media* 7 (5 CipS), *Aeromonas salmonicida* ssp. *salmonicida* 1 (CipS), *A sobria* 1 (CipS)
Agrobacterium	1	0	1	*A. tumefaciens* 1 (CipS)
Alcaligenes	11	0	10	*A. denitrificans* 1 (CipS), *A. faecalis* 10 (9 CipS)
Apergillus	5	4	0	*A. niger* 1 (ACS), UI 4 (3 ACS)
Bacillus	51	34	NT	UI 20 (8 ACS, 16 CipS), *B. coagulans* 9 (9 ACS), *B. Laterosporus* 1 (ACS), *B. lentus* 1 (ACS), *B. licheniformis 6* (1 ACS), *B. mycoides* 2 (ACS), *B. stearothermophilus* 5 (ACS), *B. subtilis* 3 (ACS), *B. marcerans* 1 (ACS, CipS), *B. plymyxa* 2 (ACS and CipS)
Bordetella	8	1	8	*B. bronchiseptica* 8 (1 ACS, 8 CipS)
Brucella	26	14	18	*B. abortus* 17 (5 ACS, 9 CipS), *B. melitensis* 9 (all ACS and CipS)
Budvicia	1	0	1	*B. aquatica* 1 (CipS)
Burkholderia	13	10	11	*B. cepacia* 1 (ACS, CipS), *B. gladoli* 1 (CipS), *B. pseudomallei* 2 (CipS), *B. mallei* 9 (9 ACS and 7 CipS)
Campylobacter	4	0	4	*C. jejunii* 4 (CipS)
Candida	12	5	0	UI 9 (4 ACS), *C. albicans* 1, *C. crusei* 1 (ACS), *C. tropicalis* 1
Citrobacter	22	4	5	*C. amalonaticus* 6 (2 ACS, 1 CipS), *C. diversus* 1, *C. freundii* 15 (4 ACS, 4 CipS)
Corynebacterium	2	2	1	*C. stationis* 2 (2ACS, 1 CipS)
Dermatophilus	2	2	1	*D. congolensis* 2 (2 ACS, 1 CipS)
Edwardsiella	4	2	2	*E. tarda* (2 ACS, 2 CipS)
Enterobacter	52	3	37	*E. agglomerans* 33 (2 ACS, 26 CipS), *E. amnigenus* 6 (3 CipS), *E. cloacae* 1, *E. gregoviae* 2 (1 CipS), *E. intermedius* 1 (CipS), *E. sakazaki* 1, UI 8 (1 ACS)
Enterococcus	75	28	9	*E. avium* 4 (4 ACS), *E. cecorum* 11 (11 ACS), *E. casseliflavus* 13, *E. dispar* 14 (5 ACS), *E. faecalis* 9 (1 ACS, 8 CipS), *E. hirae* 14, *E. malodoratus* 2 (2ACS), *E. mundatti* 3 (3 ACS), *E. raffinosus* 3 (3 ACS), *E. solitarus* 2 (1 CipS)
Erwinia	10	2	9	*E. ananas* 3 (1 ACS, 2 CipS), *E. caratovora* 1 (CipS), *E. chrysanthemi* 4 (1 ACS, 4 CipS), *E. cyperipedii* 1, *E. tracheiphila* 1 (CipS)
Escherichia	160	4	NT	*E. coli* 156 (4 ACS, 130 CipS), *E. fergusonii* 4 (CipS)
Geotrichum	1	0	0	UI 1
Hafnia	3	0	3	*H. alvei* 3 (CipS)
Klebsiella	40	0	37	*K. oxytoca* 3 (CipS), *K. pneumoniae* 37 (34 CipS)
Kluyvera	2	0	2	*K. cryocrescens* 1 (CipS), *K. ascorbata* 1 (CipS)
Listeria	1	1	1	*L. monocytogenes* 1 (ACS, CipS)
Micrococcus	3	1	NT	*M. agilis* 2 (ACS), UI 1
Moraxella	2	1	2	M. osloensis 2 (1 ACS, 2 CipS)
Morganella	1	0	0	*M. morganii* 1
Mycoplasma	2	0	2	*M. capri* 2 (CipS)
Pasteurella	37	13	23	*P. canis* 10 (1 ACS, 8 CipS), *P. dagmatis* 1 (1 ACS), *P. langaaensis* 1 (1ACS), *P. multocida* B2 15 (8 ACS, 9 CipS), *P. multocida* D 8 (1 ACS, 5 CipS), *P. pneumotropica* 2 (1 ACS, 1 CipS). All Type B strains resistant to AC were resistance to Cip too.
Pediococcus	1	0	0	UI 1
Pragia	11	3	2	*P. fontium* 11 (3 ACS, 2 CipS)
Proteus	22	0	20	*P. mirabilis* 16 (14 CipS), *P. penneri* 2 (CipS), *P. vulgaris* 4 (CipS)
Pseudomonas	59	8	44	*P. aeruginosa* 42 (5 ACS, 30 cipS), *P. alkaligenes* 1 (CipS), *P. paucibacillus* 5 (2 ACS, 4 CipS), *P. pseudoalkaligenes* 6 (4 CipS), *P. stutzeri* 2 (CipS), *P. testosteronii* 2 (CipS), *P. vesicularis* 1 (ACS, CipS)
Raoultella	3	0	2	*R. terrigena* 3 (2 CipS)
Rhodotorulla	1	0	0	UI 1
Salmonella	48	1	21	*S. enterica* 25 (21 CipS), *S. indica* 13 (1 ACS), *S. salamae* 10
Serratia	2	0	1	*S. odorifera* 1 (CipS), *S. plymuthica* 1
Sphingomonas	2	0	1	*S. echinoides* 2 (1 CipS)
Staphylococcus	77	27	55	S. aureus 21 (8 ACS, 20 CipS), S. capitis ssp. capitis 3 (3 CipS), *S. capitis* ssp. *urealyticus* 3 (CipS), *S. carnosus* 5 (2 CipS), *S. caseolyticus* 3 (2 ACS, 3 CipS), *S. chromogenes* 1 (CipS), *S. delphini* 1 (CipS), *S. epidermidis* 4 (CipS), *S. felis* 1 (CipS), *S. gallinarum* 1 (CipS), *S. haemolyticus* 5 (2 ACS, CipS), *S. hominis* 2 (CipS), *S. hyicus* 1 (CipS), *S. intermedious* 5 (1 ACS, 5 CipS), *S. lentus* 1 (ACS, CipS), *S. sciuri* 20 (14 ACS, 7 CipS)
Streptobacillus	1	1	1	*S. moniliformis* (ACS, CipS)
Streptococcus	79	36	63	*S. agalactiae* 2 (2 ACS, 1 CipS), *S. alactolyticus* 1 (ACS), *S. caseolyticus* 1 (ACS), *S. equi* ssp. *equi* 2 (ACS, CipS), *S. equi* ssp. *zooepidemicus* 11 (6 ACS, 8 CipS), *S. intestinais* 5 (2 ACS, 5 CipS), *S. macacae* 1 (ACS, CipS), *S. milleri* 43 (14 ACS, 30 CipS), *S. pneumoniae* 1 (ACS, CipS), *S. porcinus* 4 (1 ACS, 2 CipS), *S. pyogenes* 6 (3 ACS, 5 CipS), *S. rattus* 1 (CipS), *S. suis* 1 (ACS, CipS)

Trichosporum	1	0	0	UI 1
Trichphyton	1	1	0	UI 1
Vibrio	2	1	1	*V. anguillarum* 1 (CipS), UI 1 (ACS)
Yersinia	1	0	1	*Y. enterocolitica* 1 (CipS)

NT: Not Tested; ACS: Sensitive to 2 mg *Ageratum conyzoides* methanolic extract; CipS: Sensitive to 10 µg ciprofloxacin; UI: Unidentified Species.

Table 3: Microbes of different genera species tested for their sensitivity to 2 mg *Ageratum conyzoides* methanolic extract (ACME) and 10 µg ciprofloxacin discs.

Among isolates from clinical samples, pig origin strains were more often resistant to ACME than isolates from clinical cases of cattle ($p = 0.05$), dog ($p = 0.005$), horse and mules ($p = 0.001$) and humans ($p = 0.03$) but less than isolates of avian origin ($p = 0.05$). Among clinical isolates of other than pig origin sensitivity to ACME did not differed significantly ($p > 0.2$) except the clinical isolates of horse and mules being more sensitive than strains of cattle ($p = 0.007$) and mithun ($p = 0.03$) origin.

Microbes associated with mortality in different animals had no significant variation with respect to ACME sensitivity except isolates of cattle origin being more sensitive than isolates from horses and mules ($p = 0.04$) and pigs (0.0002). Bacteria causing mortality in pig were more commonly ACME resistant than those causing death in bird ($p = 0.005$), wild animals ($p = 0.01$) and cattle ($p = 0.0002$).

Among the isolates from healthy human or animals, isolates from swamp buffaloes were significantly more commonly resistant to ACME than those from healthy human ($p = 0.00004$) and healthy pigs ($p = 0.007$).

With respect to sensitivity to ciprofloxacin among clinical isolates, bacteria associated with pig infection had more probability of being ciprofloxacin sensitive than isolates causing illness in buffaloes ($p = 0.007$), cattle ($p = 0.05$), birds ($p = 0.03$), laboratory animals ($p = 0.02$) and horses and mules ($p = 0.0007$).

Ciprofloxacin resistance was not significantly associated with host with respect to microbes associated with mortality except the isolates from dead horses which were more sensitive to ciprofloxacin than those from the dead birds ($p = 0.04$).

Bacteria isolated from healthy human beings were more often sensitive to ciprofloxacin than those from healthy dogs ($p = 0.02$), horses and mules ($p = 0.003$), pigs ($p = 0.002$) and swamp buffaloes ($p = 0.0003$).

Effect of genus and species of microbes on their sensitivity to ciprofloxacin and ACME

All Aeromonas, Alcaligenes, Klebsiella and Proteus species strains were resistant to ACME irrespective of source of isolation or association with illness. In contrast, majority of the strains of Burkholderia (76.9%), Bacillus (66.7%) and Brucella (53.8%) species were sensitive to ACME. For ACME, sensitivity significantly differed among strains of different genera (Table 4 Supplementary). Escherichia coli and Klebsiella, often the most studied potentially pathogenic bacteria for drug resistance were more commonly ($p < 0.01$) resistant to ACME than Bacillus, Brucella, Burkholderia, Candida, Citrobacter, Enterococcus, Erwinia, Pasteurella, Pragia, Pseudomonas, Staphylococcus and Streptococcus species strains (Table 4 Supplementary). However, these were much similar in ACME resistance to Enterobacter, Proteus and Salmonella species strains belonging to the same family (Enterobacteriaceae). In general, genera having oxidase positive strains including Burkholderia, Brucella and Bacillus (66.7%) were significantly more often sensitive to ACME than strains belonging to other genera (Table 4 Supplementary). Though not the majority but significantly more number or strains of Pasteurella (35.1%), Staphylococcus (35.1%), Enterococcus (37.3%), Candida (41.7%) and Streptococcus (45.6%) species were ACME sensitive than many of the Enterobacteriaceae and Vibrionaceae members (Table 4 Supplementary).

Among strains of the same genus belonging to different species, for most of the bacteria no apparent effect of species of strains was evident ($p > 0.05$) on their sensitivity to ciprofloxacin and ACME leaving only a few exceptions. *Staphylococcus sciuri* strains were more often ACME sensitive than *S. aureus* strains ($p = 0.04$); *E. avium* ($p = 0.01$) and *E. cecorum* ($p = 0.0001$) strains were significantly more ACME sensitive while *E. casseliflavus* ($p = 0.008$), and *E. hirae* ($p = 0.006$) were more often ACME resistant than strains of other species of *Enterococcus*. Among GNBs, *B. meletensis* strains were significantly more commonly sensitive to ACME ($p = 0.0006$) and ciprofloxacin ($p = 0.01$) than *B. abortus* strains. Besides, *P. multocida* type B strains were more commonly ACME sensitive than *P. canis* ($p = 0.027$) and *P. multocida* type D ($p = 0.06$) strains.

All yeasts and moulds strains were resistant to ciprofloxacin while all the Citrobacter species strains tested were ciprofloxacin sensitive. The sensitivity to ciprofloxacin among bacterial strains of different genera ranged between 18.2% for Pragia to 100% for Citrobacter strains, for other bacteria there was only little effect of genus of microbes on their sensitivity to ciprofloxacin (Tables 1 and 3) with a few exceptions. Pragia species strains were more often ($p < 0.01$) resistant to ciprofloxacin than strains of Aeromonas, Alcaligenes, Bacillus, Brucella, Burkholderia, Citrobacter, Enterobacter, Enterococcus, Erwinia, Escherichia, Klebsiella, Proteus, Pseudomonas, Salmonella, Staphylococcus and Streptococcus species. Next to Pragia, Pasteurella strains were more commonly ciprofloxacin resistance (37.8%) than strains of Escherichia ($p = 0.008$), Klebsiella ($p = 0.005$), Proteus ($p = 0.02$), Staphylococcus ($p = 0.006$) and Streptococcus ($p = 0.01$) species.

Discussion

In the current era of emergence of multiple drug resistant (MDR) and total drug resistant (TDR) microbial strains causing difficult to cure infections in animals and human beings [17] have attracted lot of researchers to look into herbarium for effective antimicrobials and lot of research has been reported from all parts of the world [12]. Recent studies have indicated that resistance among microbes is not only limited to antibiotics but other antimicrobials too including those of herbal origin [18]. Therefore, it is pertinent to test any putative antimicrobial not only on a few reference or laboratory strains but on large number of strains of diverse origin as has been attempted in the present study to evaluate the antimicrobial potential of *A. conyzoides* methanolic and ether extracts.

Of the 889 microbial strains tested, sensitivity to 24.1% ACME while 79.1% strains were sensitive to ciprofloxacin indicated that antibiotics are still not ruined hope. The observation further revealed that there was no significant difference in antimicrobial activity of ACEE and ACME, indicating that the antimicrobial moiety of *A. conyzoides* might be an organic solvent soluble substance like a component of its essential oil. Essential oil of Ageratum has been reported earlier to

possess antimicrobial activity [7] but in contrast to observations some of the earlier observations [10,19,20]. Dayie et al. [10] reported ether extracts ineffective while methanolic extract as effective antimicrobial against *E. coli* and *S. aureus*, however, Garg and Grewal [19] found ether extract more inhibitory than methanolic and chloroform extracts to several GNBs and GPBs including *E. coli* and *S. aureus*. In contrast, Prajapati and co-workers [20] testing hexane and methanolic extracts of *A. conyzoides* reported no significant in MIC of the two for *Candida albicans* and *S. aureus* strains and methanolic extract being twice more inhibitory to *E. coli* than hexane extract. Observations are in concurrence to observations of Dayie et al. [10,19] for *A. conyzoides* extracts being more effective against Staphylococcus strains than *E. coli* ($p < 0.001$) and Pseudomonas ($p = 0.004$) strains. However earlier studies [10] indicated that Pseudomonas were the most resistant bacteria tested against Ageratum oil. Our observation on comparatively large set of strains indicated that pseudomonad were more often sensitive to ACME than *E. coli* ($p = 0.001$) and klebsiellae ($p = 0.015$), it might be due to variation in sensitivity of strains of the same species of bacteria as observed in the present study. The few strains tested in earlier studies [7,10,19,20] might have belonged to some resistant / sensitive clones. Besides, variation in antimicrobial activity and spectrum of activity of *A. conyzoides* extracts might be due to variation in herbal quality, Garg and Grewal [19] collected the herb from western Himalayas, Prajapati et al. [20] from plains of central Uttar Pradesh, while in the present study the Herb was collected from Eastern Himalayas, but more studies are required to reach at the conclusion.

In the study, though only 24.1% strains of microbes were sensitive to ACME this is much higher than several other potential herbs including citronella and geranium oils reported earlier [7,21,22] inhibiting only about 10% of the strains tested. However, Lalfakjuala et al. [23] reported methanolic extract of *A. conyzoides* much inferior in antimicrobial activity against phosphate degrading bacteria than other weedy herbs including *Eupatorium odoratum*, *Mikania micrantha* and *Centella asiatic*.

In earlier studies on *A. conyzoides* or other species of Ageratum no statistical comparison has been reported for GNBs and GPBs or with respect to oxidase reaction of the strains because of the small number of strains tested [19,20,23]. In the present study, the most sensitive strains to ACME belonged to oxidase positive GPBs (62.5%) followed by oxidase negative GPBs (40.8%), oxidase positive GNBs (27.4%) and oxidase negative GNBs (4.9%). The role of oxidase production ability might be important in herbal drug resistance, similar observation have been made earlier with lemongrass oil [24], *Artemesia vulgaris* oil [25,26], geranium oil [21] and citronella oil [22].

Microbes of food origin were more commonly sensitive to ACME ($p < 0.0001$) than strains originating from abiotic or biotic environment, clinical samples, healthy stocks, dead animals or the reference strains. Similar pattern of higher sensitivity of microbes of food (vegetable as well as animal origin) has also been reported earlier for essential oils of *Artemisia vulgaris* [25,26], lemon grass oil [24] indicating that variables responsible for persistence of microbes in animal system might be associated with herbal drug resistance or vice-versa, but needs more targeted research to lucidly understand.

Bacteria isolated from swamp buffaloes and pigs were more often resistant to ACME than other sources except those isolated from mithuns and sheep and goats ($p > 0.05$). The pig being a scavenger might harbour a wide variety of resistant organism, resistance in bacteria from semi-wild (swamp buffaloes, mithun) or grazing (sheep and goat) animals indicated that exposure to different herbs during their natural feeding (grazing) might be responsible for ACME resistance in microbes isolated from all such animals. Though *A. conyzoides* is usually not consumed by animals but in scarcity, it may be. Similar views have also been expressed earlier for higher resistance in bacterial isolates from such kinds of animals for *A. vulgaris*, lemon grass and citronella oil [21,22,24-26].

Ciprofloxacin resistance in bacteria isolated from humans was much more than in those isolated from dogs ($p = 0.04$), mithun ($p = 0.02$), pigs (0.0010, swamp buffaloes ($p = 0.005$) and horses and mules ($p = 0.007$) but not significantly more than bacteria of cattle, buffalo, sheep and goat origin ($p > 0.05$). Although ciprofloxacin or its equivalent enrofloxacin is not recommended in food and dairy animals, its incorporation as preservative in FMD vaccine in India [27] intended to be used in these animals might have led to emergence of ciprofloxacin resistance in ruminants vaccinated against FMD but not in dogs and pigs. Interestingly bacteria from healthy human beings were more often sensitive to ciprofloxacin than isolates from healthy animals ($p = 0.02$) indicating that ciprofloxacin resistant strains from animals might be associated with illness in human-beings on accidental transfer. However, more molecular epidemiological studies are needed to understand the trend.

Strains from clinical samples of pig were more often ACME resistant than bacteria from clinically sick cattle ($p = 0.05$), dogs ($p = 0.005$), horses and mules ($p = 0.001$), and humans ($p = 0.03$) but less than bacteria of avian origin ($p = 0.05$). Though this variation in sensitivity of bacteria cannot be explained on the basis of the present study, the diversity of pathogens might be responsible for the variation, and needs more studies to establish the actual reason.

Resistance in all Aeromonas, Alcaligenes, Klebsiella and Proteus species strains to ACME irrespective of source of isolation or association with illness and sensitivity of majority of the strains of Burkholderia (76.9%), Bacillus (66.7%) and Brucella (53.8%) species to ACME indicated role of genetic heritance of resistance to *A. conyzoides* as observed earlier for other herbal antimicrobials [12,18].

Among strains of different species of the same genus no significant difference was evident ($p > 0.05$) for their sensitivity to ciprofloxacin and ACME except a few viz., *S. sciuri* strains among staphylococci ($p = 0.04$), *E. avium* ($p = 0.01$) and *E. cecorum* ($p = 0.0001$) among strains of enterococci, *B. meletensis* among different strains of brucellae and *P. multocida* type B among Pasteurella species were more sensitive to ACME. There seems to be no association of ACME resistance with pathogenicity potential as *S. sciuri*, *E. avium* and *E. cecorum* are rarely reported to be pathogenic while *B. meletensis* and *P. multocida* type B strains are considered to be the most pathogenic strains among the respective genus [15].

All yeasts and moulds strains were resistant to ciprofloxacin as expected [16]. Among bacteria, at one extreme all the Citrobacter species strains were sensitive and at other end all Pragia species strains were resistant to ciprofloxacin, irrespective of source and species of the strains. Both of the species of bacteria belong to Enterobacteriaceae family, and reasons for this wide variation among genera of the same family may not be explained on the basis of our observations. Pasteurella strains, often associated with severe illness and mortality in animals, were more often resistant (37.8%) to ciprofloxacin than Escherichia ($p = 0.008$), Klebsiella ($p = 0.005$), Proteus ($p = 0.02$), Staphylococcus ($p = 0.006$) and Streptococcus ($p = 0.01$) species strains. Pasteurella strains are more often associated with systemic infections in ruminants while others listed are more often associated with colonization on skin or in

intestine [15]. It is premature to say that use of injectable enerofloxacin / ciprofloxacin or incorporation of enerofloxacin as preservative in FMD vaccine in India [27], inoculated twice every year in ruminants, might be responsible for the observed increase in resistance in bacteria often residing or infecting different internal organs or system.

The study concludes that *A. conyzoides* extract might be containing useful antimicrobial component(s) which were usually more active against oxidase positive, potentially pathogenic, strains, often associated with systemic and deadly infections in animals as well as in humans. Further studies may reveal the chemical nature of antimicrobial components present in methanolic / ether extract of *A. conyzoides*.

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