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ANTIMICROBIAL EFFECTS OF THE DIFFERENT EXTRACTS FROM *AMARANTHUS RETROFLEXUS* L.

ANTONIA POIATĂ[1], CRISTINA LUNGU[2]*, BIANCA IVĂNESCU[2]

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Abstract: The emergence of drug-resistant bacteria strains encouraged the study of natural products from plants with potential promise for clinical use. The purpose of this study was to investigate *in vitro* the antibacterial and antifungal effects of aqueous, ethanol and chloroform extracts from different parts of *Amaranthus retroflexus* L.. The antimicrobial activity was evaluated using the agar diffusion method. Each extract exhibited antimicrobial activity against Gram-positive strains (*Staphylococcus aureus, Sarcina lutea*) and *Candida albicans*. In addition, the chloroform extracts from *A. retroflexus* dry seeds had significant antibacterial (against both Gram-positive and Gram-negative species) and antifungal activity. The results of the combined action of the different antibiotics and chloroform extract did not display synergism. This study indicates that different amaranth extracts are antibacterial and mostly antifungal and may represent a future therapeutic strategy.

INTRODUCTION

Plants have been exploited for their food value and for their therapeutic effect in the traditional systems of medicine. Some plants have the potential to be useful antibacterial and antifungal agents. The development of resistance among Gram-positive and Gram-negative species to many antimicrobial agents can seriously compromise the effectiveness of antibiotic treatment and there is clearly an urgent need for the discovery of new synthetic or plant-derived drugs. Thus, a number of bioactive molecules from various plants have been exploited, due to their therapeutic effects. Over 150.000 of novel natural molecules derived from various herbs and microorganisms are mentioned in the literature (Taneja and Qazi, 2007).

Amaranthus retroflexus (redroot pigweed, *Amaranthaceae* family) is an annual herb with a shallow reddish taproot, stem up to 2 m high, simple or branched, and often hairy in the upper part. Leaves are alternate, long-stalked, ovate to rhombic-ovate and sparsely hairy. Flowers are small, green and unisexual, grouped into dense, blunt spikes. Each flower is enclosed by 1-3 stiff awl-shaped bracts, giving bristly appearance to spikes. The fruit is a capsule, transversely dehiscent, enclosing one black shiny seed, broadly elliptic, 1 mm in diameter (Costea et al, 2004). Amaranth seeds have high nutrition value due to elevated concentrations of proteins, carbohydrates, lipids and minerals (Toader et al, 2011).

Amaranthus retroflexus includes around 50 varieties, forms and sub-forms and it is considered to be among the world's worst weeds. Nonetheless, *Amaranthus retroflexus* can be used as vegetable, forage, grain crop and medicinal plant and for the phytoremediation of contaminated sites (Costea et al, 2004). Moreover, weeds are highly resistant to plant pathogens so it is possible that some plant metabolites are also active against human pathogens.

Previous studies (Zhanh et al, 2013, Tharun et al, 2012, Jin et al, 2013, Maiyo et al, 2010) reported the antimicrobial activity of the *Amaranthus* species. To the best of our knowledge, just one paper focused on the antimicrobial activity of *A. retroflexus* seeds on phytopathogenic fungi (Lipkin et al, 2005). Considering the potential application in medicine, the present study evaluates the activity of different extracts from roots, stems, leaves and seeds of *A. retroflexus* against human pathogens.

In the ongoing search for novel antimicrobial agents from Romanian plants, we report the evaluation of antimicrobial activity of different extracts from *A. retroflexus*.

MATERIALS AND METHODS

Plant material and preparation of extracts

The plants were collected at fruiting stage from the surroundings of Iasi city in October 2015. The plant material was authenticated and a voucher specimen was deposited in the Herbarium of Pharmaceutical Botany Department, Faculty of Phrmacy. The roots, stems and leaves, and the seeds were pulverized and extracted (1:10) with water, 80% ethanol and chloroform by mixing for 3 hours on a magnetic stirrer at room temperature, then filtrated. The chloroform extract was evaporated to dryness and the residue dissolved in DMSO before the antimicrobial test.

Susceptibility testing

The antimicrobial activity of different aerial parts of *A. retroflexus* was tested by the disc diffusion method in Mueller-Hinton agar, according to the guidelines recommended by the National Committee of Clinical Laboratory Standards (NCCLS, 2000).

The *in vitro* antimicrobial profile of *A. retroflexus* was determined against five reference strains: *Staphylococcus aureus* ATCC 25923, *Sarcina lutea* ATCC 9341, *Escherichia coli* ATCC 25a22, *Pseudomonas aeruginosa* ATC 27853 and *Candida albicans* ATCC 10231. Mueller-Hinton broth and Mueller-Hinton agar (respectively Sabourand media for *Candida*) were used for microbial growth and susceptibility testing.

The cylinder in plate bioassay technique was used. A standard suspension of each strain was prepared from fresh overnight culture and was mixed with 15 ml portions of molten Mueller-Hinton agar, in a sterile petri plates resulting in a final concentration of about 10^6 cells/ml. When the plates were solid, metal cylinders (6 mm diameter) were placed on the medium surface and the samples (0,2 ml) were pipetted into each well. A standard commercially disks of Ampicillin (10µg), Chloramphenicol (30µg) and Nystatin (100µg) were used for comparison.

The size of the zone of inhibition around the wells was measured after incubation at 37°C for 24 h. The values of diameter of the inhibition zones are expressed as mean of triplicates ± SD.

Test of combined antibacterial action

Antimicrobial synergy and antagonism were demonstrated in agar diffusion tests that offer a visually method to detect antimicrobial interactions. In the ”two disc method” antimicrobial agents are placed on the surface of seeded plates, at distances from each other that are predicted upon optimum demonstration of antagonistic or synergistic action (Acar,1981).

Results of the “two disc method” were correlated with another experiment designed to determine coactions, described by Natarajan et al. (2008). In order to detect combined antimicrobial activity in this type of test, the samples, at defined concentrations, are added into the molten agar and after the solidification, different antibiotic discs are placed on the plate surfaces. If the inhibition zone remained unchanged after incubation at 37^0 C for 24 h, there was no synergic effect.

RESULTS AND DISCUSSION

The agar disk diffusion procedure was one of the first methods for determining the *in vitro* microbial susceptibility to antimicrobial agents. In this microbiological assay the antimicrobial agent placed in a reservoir (sterile paper discs, cylinders) diffuses directly against seeded bacteria. We used the cylinder technique that is more sensitive by comparison with the method using paper discs (Thornsberry et al, 1977). The filter discs may act as chromatography paper and separate the components of the mixture which may result in irregular inhibition zones. We tested the ability of different extracts from *A. retroflexus* to inhibit microbial growth and the results are shown in table 1.

All the extracts obtained from different plant material showed antimicrobial activity only against Gram-positive strains. These plant extracts showed a moderate antibacterial activity against *Staphylococcus aureus* and *Sarcina lutea* when compared to the positive control ampicillin and chloramphenicol. In contrast, Gram-negative bacteria were not inhibited by either aqueous or ethanol extracts. This can be explained by the presence of the permeability barrier – the outer membrane which acts as a barrier to the penetration of antimicrobial molecules and the periplasmic space which contains enzymes that destroy the molecules introduced from outside.

It can be seen that the chloroform extract from dry seeds exhibited the strongest antifungal activity, even higher than the antimicrobial agent Nystatin. Also the aqueous extracts from leaves plus stem and root showed high activity against *Candida albicans*.

Unlike the other *A. retroflexus* tested samples, the chloroform extract from seeds exhibited at concentration of 16 mg/ml, excellent activity against both, Gram-positive and Gram-negative bacteria. The results demonstrate the similar potential between the chloroform extract from seeds and ampicillin, or chloramphemicol against standard strains of *Escherichia coli* and *Pseudomonas aeruginosa*.

Table 1. Antimicrobial activity of different extracts of *A. retroflexus* plant material

Nr. crt.	**Sample** (0.2 ml volume)	**Diameter of inhibition zone (mm±SD)**				
		S. aureus *ATCC 25923	**S. lutea ATCC 9341**	**E.coli ATCC 25922**	**P. aerugi nosa ATCC 27853**	**C. albicans ATCC 10231**
1.	Root - aqueous extract	12±0.1	16±0.3	0	0	20±0.5
2.	Leaves and stems- aqueous extract	13±0.2	13±0.1	0	0	23±0.4
3.	Leaves and stems - ethanol extract (80 %)	13±0.4	13±0.5	0	0	12±0.1
4.	Root – ethanol extract (80%)	15±0.1	15±0.3	0	0	16±0.3
5.	Seeds - ethanol extract (80%)	12±0.3	15±0.2	0	0	12±0.5
6.	Seeds - chloroform extract	18±0.4	20±0.1	20±0.3	19±0.4	28±0.4
7.	Ampicillin (10 µg)	27±0.6	40±0.5	21±0.5	19±0.5	nt **
8.	Chloramphenicol (30 µg)	25±0.3	38±0.3	24±0.3	18±0.2	nt
9.	Nystatin (100 µg)	nt	nt	nt	nt	20±0.4

*ATCC – American Type Culture Collection; **nt – not tested

The possibility that chloroform extract from *A. retroflexus* seeds may have synergistic or antagonistic interaction with different therapeutic antimicrobial agents has been explored by the *in vitro* susceptibility test methods. Analysis of our data did not demonstrate a significant relationship between these samples and different antimicrobial agents (Table 2). The enhancement of antibacterial activity of chloroform extract from *A. retroflexus* seeds by the addition of ampicillin, chloramphenicol, tetracycline and ciprofloxacin to form a potent combination in inhibiting both Gram-positive and Gram-negative bacteria, is not relevant.

The combined antibacterial effect may be greater than that which each agent alone could achieve and indicates antimicrobial synergy. Together these two compounds may potentiate each other activity at a biochemical level or one may assist the other to penetrate into the microbial cell or may protect from destruction or the agents may act separately against the microorganism. When the effect of one agent is reduced by the presence of another, the combination is antagonistic. In our case, these two agents, the chloroform extract of seeds and ampicillin, chloramphenicol, tetracycline or ciprofloxacin tested together demonstrated indifference, because the combined action was no greater than that of the individual compounds.

As show in Table 1, *A. retroflexus* extracts exhibited antibacterial and antifungal abilities. All extract types were clearly less effective than the chloroform extract from the plant seeds, which was the most active against all tested strains, with the largest inhibition zones.

Table 2. *In vitro* combined effect of chloroform extract from seeds of *A. retroflexus* and some antimicrobial agents

Organism	Diameter of inhibition zone (mm)							
	Antimicrobial agents alone				The combined effect			
	A	C	T	Cip	A+Sce	C+Sce	T+Sce	Cip+ Sce
Staphylococcus aureus ATCC 25923	27.0	25.5	26.0	30.0	26.5	26.0	27.0	30.5
Sarcina lutea ATCC 9341	40.0	38.0	35.5	38.0	40.0	40.0	36.0	40.0
Escherichia coli ATCC 25922	21.0	24.0	24.0	32.5	21.0	24.0	24.5	32.0
Pseudomanos aeruginosa ATCC 27853	19.0	18.0	20.0	32.0	19.5	18.0	20.0	32.0

A- ampicillin (10µg); C-chloramphenicol(30µg); T- tetracycline (30µg); Cip – ciprofloxacin (30µg); Sce – seeds chloroform extract

Data available today confirm that plants produce different natural compounds which are constitutive or inducible, capable to protect against pathogen organisms (Morrissey and Osbourn, 1999, Boman, 2000). Various secondary metabolites, high molecular defense proteins and antimicrobial peptides (AMP) have been isolated from different plant parts and they are mostly antifungal (Broekaert et al, 1992; Broekaert et al, 1995, Selitrennikoff, 2011, Garcia-Olmedo et al, 2001, Cammue et al, 1994). The mechanisms of peptide action are diverse: fungal cell wall polymer destruction, membrane channel and pore formation, action against ribosomes, inhibition of DNA synthesis and alteration of the cell cycle (Broekaert et al, 1997).

The literature data show that from the seeds of different amaranth species have been isolated peptides with antimicrobial activity (Broekaert et al, 1992, Rivillas-Acevedo and Soriano-Garcia, 2007). The antimicrobial peptides Ac-AMP1 and Ac-AMP2 were isolated from *A. caudatus* seeds (Broekaert et al, 1992). The gene encoding Ac-AMP2 peptide was also found in *A. albus, A. cruentus, A. blitum, A. hybridus, A. retroflexus, A. tricolor* and *A. hypocondriacus* (Pribylova et al., 2008).

The different extracts of *A. retroflexus* tested in our study showed antimicrobial activity due probably to the presence of active constituents like antimicrobial peptides (recognized as an important component of the innate defense system). An antimicrobial peptide with significant activity against five phytopathogenic fungi has been identified in *A. retroflexus* and named Ar-AMP. This peptide has a characteristic high content of cysteine residues which have the ability to form disulfide bridges and the amino-acids sequence demonstrates its homology with previously described chitin-binding proteins (Lipkin et al.2005).

Antimicrobial peptides isolated from amaranth act probably similar to plant defensins which have the capacity to bind in a reversible way to chitin from fungal cell wall resulting in a change of membrane permeability (Thevissen et al, 1999). Besides, it has been shown that antimicrobial peptides from amaranth species inhibit the growth of different Gram-positive bacteria (Broekaert et al, 1992). Our results indicate that the active compounds from seeds of *A. retroflexus* have also the ability to inhibit *in vitro* Gram-negative strains (*Escherichia coli* and

Pseudomanos aeruginosa). At concentrations of 16 mg/ml, the seeds chloroform extract had a good antibacterial activity, while the antifungal effect was remarkable, the extract strongly inhibiting the growth of *Candida albicans*.

The results are in agreement with other reports which showed that bioactive principles of *Amaranthus* species manifest antimicrobial activity. It has been shown that *A. tricolor* leaf extracts and the whole grass extracts from *A. viridis* have significant antimicrobial activity (Tharun et al, 2012, Jin et al, 2013). The ethyl acetate extracts (40-100 mg/ml) from the leaves and stems of *A. mangostanum* inhibited the growth of *Pseudomonas solanacearum, Acidovorax avenae, Rhizoctonia solani, Collelotrichum capsici, Pseudomonas aeruginosa, Bacillus cereus* and *Escherichia coli* (Zhang et al, 2013). A good antifungal action is presented in the previous study by Rivillas-Acevedo and Soriano-Garcia (2007) for the protean extract of *A. hypochondriacus* seeds.

Mayo et al. (2010) report that different extracts from the leaves of *A. hybridus, A. spinosus* and *A. caudatus* showed a broad spectrum of antibacterial activity against Gram-positive and Gram-negative bacteria, but resistance to the fungus *Candida albicans*. According to these authors, the minimum inhibitory concentrations (MICs) of amaranth extracts against Gram-negative species (*Escherichia coli, Salmonella typhi, Pseudomonas aeruginosa, Proteus mirabilis* and *Klebsiella pneumoniae*) ranged between 129-755 mg/ml (Mayo et al, 2010). In our experiments the chloroform extract obtained from the seeds of *A. retroflexus* is active against tested strains at significant lower concentrations.

CONCLUSIONS

In summary, the main result of the present study indicates that all extract types from *A. retroflexus* exhibited antimicrobial activity against Gram-positive bacteria and *Candida albicans*. The agar diffusion method indicated the highest antifungal activity for the chloroform extract from seeds of *A. retroflexus*, which was also the most active against Gram-negative and Gram-positive bacteria species. These data, in combination with other studies on amaranth suggests that *A. retroflexus* antimicrobial compounds, probably peptides, may be a useful source for the discovery of new antimicrobial agents.

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[1] Department of Microbiology, Faculty of Pharmacy, "Grigore T. Popa" University of Medicine and Pharmacy, Iasi, Romania
[2] Department of Pharmaceutical Botany, Faculty of Pharmacy, "Grigore T. Popa" University of Medicine and Pharmacy, Iasi, Romania
[*] Corresponding address: "Grigore T. Popa" University of Medicine and Pharmacy, 16 Universitatii Street, 700150, Iaşi, e-mail: ape3_c@yahoo.com, tel: +40.232.301.820, fax: +40.232.211.820