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Anti-bacterial and β-Lactamase inhibitory effects of *Anchusa azurea and Globularia alypum* extracts.

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ABSTRACT

Resistance to antibiotics has emerged following their widespread use; the important mechanism of beta-lactam resistance in bacteria is the production of beta-lactamases. In order to find new bioactive beta-lactamase inhibitors, this study investigated the inhibition effect of the extracts of *Anchusa azurea (*AA*)* and *Globularia alypum (*GA*)* on a beta-lactamase from *Bacillus cereus*. The extracts exerted inhibitory effects on beta-lactamase in a dose-dependent manner, the results showed that The crude extract (CrE) and the ethyl acetate extract (AcE) of *Anchusa azurea* showed a very high inhibitory activity at a concentration of 10 mg / mg, the percentage of inhibition was between 58 % and 68 %. For *Globularia alypum,* the percentage of inhibition is between 63 % and 70 %. CrE-GA exhibited the most potent inhibitory activity at the concentration of 1.25 mg / ml with inhibition percentage of 38 %. The anti-beta-lactamase activity of chloroform extract *(*ChE) was very weak. All extracts were not as potent as the original inhibitors such as clavulanic acid, the isolation and the structural elucidation of the active constituents in these extracts will provide useful means in the development of beta -lactamase inhibitors. The extracts of GA were screened for their antimicrobial activity against 11 bacterial strains. ChE and AcE were the most active against both gram-positive and gram-negative bacteria.

Keywords: *Anchusa azurea, Globularia Alypum,* Beta-lactamase inhibitors, Antibacterial activity.

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INTRODUCTION

Beta-lactams constitute one of the most important families of antibiotics, but resistance to these drugs has emerged following their widespread use. Resistance to this antibiotic family can be attributed to several contributing factors. However, the important mechanism of beta-lactam resistance in bacteria is the production of beta-lactamase (Livermore, 1995). These enzymes are divided into four classes A, B, C and D, on the basis of their amino acid sequence homologies. In contrast to the class B of β-lactamases, which are metallo-enzymes, all others are active-site serine enzymes (Sykes and Matthew, 1976; Bush et *al.,* 1995).

The use of beta-lactamase inhibitors coupled with beta-lactam antibiotics is currently the most successful strategy combination to treat a variety of infections. The function of this enzyme inhibitors is the inactivation of the beta-lactamase in the periplasmic space. These compounds have also been called "suicide inhibitors" because they irreversibly acylate the beta-lactamase enzyme. Beta-lactamase inhibitors such as tazobactam, clavulanic acid, sulbactam (Miller et *al.,* 2001) isolated from natural products or synthesized for the development of medicines. However, bacterial resistance to these suicide inhibitors has significantly increased in recent years (Cantón, 2008).

Medicinal plants reveal an important pharmacological activities used in developing a novel therapeutic agents, extracts from various medicinal plants containing favonoids. Flavonoids have been reported to possess antimicrobial activity (Tim Cushnie and Lamb, 2005). Flavonoids possess antibacterial properties; they may be important tools in antimicrobial strategies (Rong-Dih et *al.,* 2005).

The purpose of this study was to investigate the effect of some Algerian medicinal plants used by the population to cure various diseases by screening their potential to inhibit beta-lactamase activity.

MATERIALS AND METHODS

Plant material:

Anchusa azurea (AA) and *Globularia Alypum (GA)* were used in this study. These plants were collected from various places in Algeria in the period starting from 2010 to 2012. Samples were identified by Pr. H. LAOUER, Department of Ecology and Vegetal Biology, University Setif 1.

Extraction procedure

The extraction was carried out using various solvents of different polarity. According to Markham (1982), the powdered plant material was extracted with methanol (MeOH), at room temperature overnight. The MeOH extracts were combined and concentrated under reduced pressure on a rotary evaporator. MeOH extract (CrE) successively extracted with

hexane, chloroform and ethyl acetate. Each fraction was evaporated to dryness under reduced pressure to give hexane (HE), chloroform (ChE), ethyl acetate (AcE), and the remaining aqueous (AqE) extracts.

Determination of total flavonoid and polyphenols contents

Total flavonoid content of each extract was determined by a colorimetric method as described by Bahorun et *al.,* (1996). Total phenolic content was determined by the Folin-Ciocalteu reagent (Li et *al.,* 2007).

Beta-latamase inhibition assays

Elimination of tannins

The crude plant extracts were treated with bovine serum albumin in order to remove tannins according to Berboucha et *al* (2010). Briefly, A mixture prepared by mixing 2 ml of a solution of bovine serum albumin and 1 ml (1 mg / mL) of extract solution. The mixture was incubated for 24 hours at 4 °C. After centrifugation at 3,000 g for 15 minutes, the precipitate was eliminated and the supernatant obtained was used.

Enzyme assays

Plant extracts at several concentrations (1.25, 2.5, 5 and 10 mg / mg) were tested for their ability to inhibit the hydrolysis of nitrocefin by β-lactamase. 80 µl of each plant extract solution was dispensed into the wells of micro dilution plates. 10µl of diluted β-lactamase was added to each well. An incubation for 15 min was performed at room temperature. The assay was started by the addition of 10 µl of nitrocefin (250 µM). Measurement of the activity of β- lactamase was performed using a plate reading spectrophotometer at 482 nm for at least 20 min. Inhibition of beta-lactamase was calculated as follows:

$$\text{Inhibition \%} = 100x\ (A_c - A_e)/A_c$$

Where A_c: beta-lactamase activity without extracts or clavulanic acid, and A_e: beta-lactamase activity in the presence of plant extracts or clavulanic acid.

Antibacterial activity

The following bacterial strains were used in the bioassays **(Gram-negative)** :*Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 25922, *Salmonella typhimurium* ATCC 13311, *Acinetobacter baumanii* ATCC 19606, *Citrobacter freundii* ATCC 8090, *Proteus mirabilis* ATCC 35659*, Klebsiella pneumoniae* ATCC 700603. (**Gram-positive)**: *Staphylococcus aureus* ATCC 25923, *Bacillus cereus* ATCC
10876, *Enterococcus faecalis* ATCC 49452, *Lysteria monocytogenes* ATCC 15313,. The micro-organisms were cultured overnight at 37°C in nutrient agar. Suspensions of the bacterial strains

with an optical density of 0.5 McFarlend were made in isotonic sodium chloride solution. Petri dishes of sterile Mueller-Hinton agar were seeded with the appropriate bacterial suspension. Sterile, 6 mm diameter filter paper disc were impregnated with the extract. Two other sterile blank discs, one impregnated with water and one in DMSO, were used as negative controls. After incubation for 24 h at 37 °C, all plates were observed for zones of growth inhibition, and the diameter of these zones was measured in millimeters. Additionally, for comparative purposes, standard gentamicin (10 mg / disc) were included in the test as positive controls. For data analysis, the clear zone of inhibition around the discs was measured in mm and compared to known sensitivity drug (Rahal, 2005).

RESULTS AND DISCUSSION

Total Polyphenols and flavonoids contents in the extracts

The determination of total phenolic and flavonoid contents of different extracts of *Anchusa azurea* and *Globularia Alypum* were conducted using Folin-Ciocalteu and aluminium chloride respectively. Phenolic content was expressed as mg gallic acid equivalent per gram dried extract (EGA/ g E). Total flavonoid contents of different fractions were expressed as mg quercetin and rutin equivalent per gram dried extract (EQ and ER / g E). Values in different extracts showed that the EAc is the richest fraction in polyphenols for both plants followed by ECh for *Anchusa azurea* and CrE for *Globularia alypum.*

The determination of total flavonoids showed that ChE is the richest fraction followed by AcE for *Anchusa azurea*. The AcE is the richest fraction in flavonoids for *Globularia alypum* followed by CrE and the ECh represents the poorest fraction of flavonoids for this plant (Table 1).

Table 1: Total polyphenols and flavonoids contents of different extracts of *Anchusa azurea and Globularia alypum* .

	Anchusa azurea		*Globularia alypum*	
Extracts	**Polyphenols**	**Flavonoids**	**Polyphenols**	**Flavonids**
CrE	9.94 ± 0.008	1.88 ± 0.43	140.24 ± 4.18	2.64 ± 0.08
ChE	49.11 ± 0.17	8.98 ± 1.74	81.01 ± 1.91	1.12 ± 0.15
AcE	60.31 ± 0.70	3.78 ± 0.78	157.74± 5.27	8.56 ± 0.22

Inhibition of Beta-latamase by Plants extracts

The objective of our study was to evaluate the inhibitory effect of flavonoids and polyphenols extracted from plants on the β-lactamase "penicillinase from *Bacillus cereus*". The enzyme activity was tested in the presence of increasing concentrations of three extracts for each plant. These extracts were subjected to treatment with albumin to remove tannins and thus avoid the inhibition of beta-lactamases by the precipitation action of tannins. This enzyme has the specific substrate nitrocefin, clavulanic acid was used as suicide specific inhibitors of β-lactamase.

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Plants extracts at different concentrations showed that they inhibit the enzyme activity in a dose dependent manner. The inhibition percentage was determined for each concentration, in the range of 1.25 to 10 mg / mg, the inhibition increased from 8% to 68% for *Anchusa azurea* and 19% to 70% for *Globularia alypum* (Figure 1).

The CrE, AcE of *Anchusa azurea* showed a very good inhibitory activity at a concentration of 10 mg / mg, the percentage of inhibition varied between 58% and 68%. The AcE and the CrE of *Globularia alypum* exhibited good inhibition as indicating in figure 1. The inhibitory activity of ChE-GA and ChE-AA at a concentration of 10 mg / ml is 46 % and 48 % respectively, this is the poorest inhibition percentage comparing to other extracts.

Flavonoids are a group of polyphenolic compounds which have been reported to possess antibacterial activity (Basile et *al.,* 1999; Tim Cushnie and Lamb, 2005 ; Rong-Dih et *al.,* 2005). In the present study, we observed that there is a correlation between total polyphenols and flavonoid contents and beta-lactamase inhibition activity. For *Globularia alypum*, AcE and CrE are the richest fractions in flavonoids and plolyphenols, showed a strong inhibitory activity. In addition, our results showed that the beta-lactamase inhibition could be linked not only to polyphenols and flavonoids contents, but also to the nature of these compounds, for exemple ChE of *Anchusa azurea* witch is rich in flavonoids, presents a poor inhibitory activity compared with the other extracts.

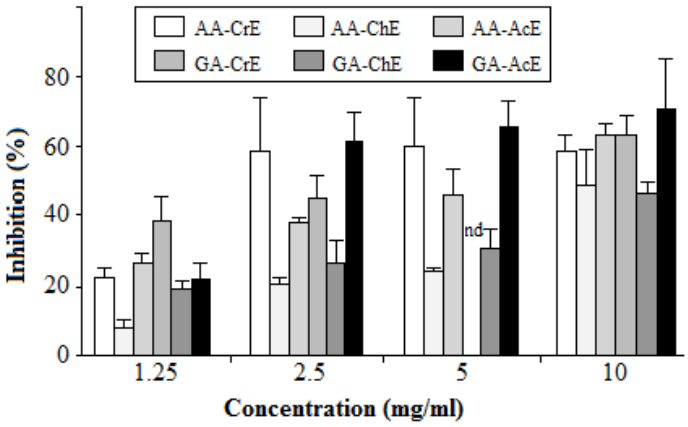

Figure 1: Inhibition percentage of beta-lactamase by *Anchusa azurea* (AA) and *Globularia Alypum* (GA) extracts. nd = not determined.

Yang et *al.*, (2007) tested the inhibitory effect of the salicylsalicylic acid on beta-lactamase extracted from a clinical strain of *Pseudomonas aeruginosa* G19, he showed that this molecule (IC_{50} = 71.77 mM) is lightly potent beta-lactamase inhibitor as the original inhibitors such as clavulanic acid, sulbactam, and tazobactam. The epigallocatechin gallate inhibited penicillinase activity, thus restoring the antibacterial activity of penicillin against penicillinase-producing *S. aureus* (Zhao et *al.,* 2002).

Gangoué-Piéboj et *al* (2007) evaluated the anti-beta-lactamase activity of 16 Cameroonian plants. This investigation showed that there are several extracts presented strong inhibition properties on TEM-1, OXA-10, IMP-1 and P99 with values around 90%. These plants belong to five plant families (Euphorbiaceae, Clusiaceae, Ochnaceae, Passifloraceae and Rosaceae). The family of Clusiaceae was the most important with three plants (*Garcinia lucida*, *G. kola* and *Mammea africana*).

Other studies by Denny et *al*. (2002) on the inhibition of flavonoids on a partially purified metallo-beta-lactamase from *Stenotrophomonas maltophilia*, showed that galangin and querectin have an average inhibitory activity.The effect was not reversed by the addition of $ZnCl_2$ suggesting that the inhibitory effect is not related to metal chelating.

In a recent publication by our laboratory (Boussoualim *et al*., 2011), we have tested the inhibitory effect of 17 flavonoids and polyphenols on a TRI beta-lactamase resistant to clavulanic acid isolated from a clinical strain of *E.coli*. The results showed that fisetin, flavone, quercetin, catechin and gossypin are non-competitive inhibitors. Myricetin, quercitrin, naringenin, morin, kaempferol and rutin are non-competitive inhibitors. The K_I of the most flavonoïds tested are above 100 µM, which are 200 folds less effective than clavulanic acid (K_I = 0.5 µM). We concluded that the position of OH in the structures of molecules has a major influence.

Comparing the inhibitory effect of all extracts tested in this study by inhibitory effect of the clavulanic acid, for the concentration of 2.5 mg / ml of CrE-AA (22 %) for instance, the clavulanic acid in a concentration of 0.025 mg / ml (100 times lower) inhibit the beta-lactamase two times higher (38 %). The data indicated that all extracts were not as potent as the original inhibitors clavulanic acid. In conclusion, the tested molecules have a low inhibitory activity and the discovery of a candidate molecule inevitably evaluation of a large number of other plant and purified products. The isolation and the structural elucidation of the active constituents of these extracts will provide useful tools in the development of beta-lactamase inhibitors.

Phytochemical information about the plants tested is limited; there is no previous report on the anti-beta-lactamase activities, or the chemical natures of the potentially inhibitory compounds. However, studies on spices in other families, revealed the presence of coumarins, flavonoids, polyphenols, xanthones, cycloartane derivatives, anthocyane, saponins and triterpenes (Gangoué-Piéboj et *al.,* 2007). The isolation and the structural elucidation of the active constituents in these extracts will be beneficial.

Antibacterial activity

The extracts of GA were screened for antimicrobial activity against 11 bacterial strains. CrE, ChE and AcE have the highly inhibitory activity (Table 2). ChE and AcE were the most active against both Gram-positive and Gram-negative bacteria. AqE extracts inhibited few bacteria. The result showed that the extract, which had a strong antibacterial activity are potentially a rich source of antimicrobial agents (flavonoids and polyphenols). The most active extracts were AcE

and CrE on *Staphylococcus aureus*, ChE on the strains of *Acinetobacter baumanii*, *Staphylococcus aureus* and CrE on *Pseudomonas aeruginosa*. Whereas, the least active extract was AqE on the most of strains but present a strong antibacterial activity on *Klebsiella pneumoniae*. The antimicrobial activity profile of all extracts against the tested strains indicated that *Staphylococcus aureus* was the most susceptible bacterium of all tested bacterial strains.

Table 2: Screening of the antimicrobial activity of the extracts of GA against gram positive and negative bacteria

	Zone of inhibition (mm)			
	CrE	**ChE**	**AcE**	**AqE**
Pseudomonas aeruginosa ATCC 27853	15	09	16	10
Escherichia coli ATCC 25922	-	09	10	-
Salmonella typhimurium ATCC 13311	11	11	12	09
Acinetobacter baumanii ATCC 19606	09	12	09	11
Citrobacter freundii ATCC 8090	09	08	08	09
Proteus mirabilis ATCC 35659	10	09	12	-
Klebsiella pneumoniae ATCC 700603	-	-	-	15
Staphylococcus aureus ATCC 25923	16	13	20	12
Bacillus cereus ATCC 10876	08	11	09	-
Enterococcus faecalis ATCC 49452	-	09	10	-
Lysteria monocytogenes ATCC 15313	-	0.7	0.9	-

The antibacterial activity was more pronounced on the Gram-positive than Gram-negative bacteria. The reason for the difference in sensitivity between these bacteria is the differences in morphological constitutions between these microorganisms, Gram-negative bacteria having an outer phospholipidic membrane carrying the structural lipopolysaccharide components. This makes the cell wall impermeable to antimicrobial chemical substances. The Gram-positive bacteria on the other hand are more susceptible having only an outer peptidoglycan layer which is not an effective permeability barrier. Therefore, the cell walls of Gram-negative organisms which are more complex than the Gram-positive ones act as a diffusion barrier and making them less susceptible to the antimicrobial agents than Gram-positive bacteria (Nostro et *al.,* 2000; Hailu et *al.,* 2007).

The most active studied plants seem to possess similar antimicrobial active compounds including essential oils, flavonoids and terpenoids and other compounds of phenolic nature, which are classified as active antimicrobial compounds (Rios, 2005). Several high-quality investigations have examined the relationship between flavonoid structure and antibacterial activity and these are in close agreement. In addition, numerous research groups elucidated the antibacterial mechanisms of selected flavonoids. The activity of quercetin, for example, has been at least partially attributed to inhibition of DNA gyrase. It has also been proposed that licochalcones A and C inhibit energy metabolism (Cushnie and Lamb, 2005). In another study, six flavonoids were tested against several strains of *K. pneumoniae*, all these flavonoids, showed antimicrobial activity similar to that produced by the control antibacterial (ofloxacin) (Özçelik et *al.,* 2008).

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