Research Article CODEN: IJPRNK ISSN: 2277-8713
F Beddou, IJPRBS, 2014; Volume 3(1): 172- 187 IJPRBS

INTERNATIONAL JOURNAL OF
PHARMACEUTICAL RESEARCH AND BIO-SCIENCE

EVALUATION OF NATURAL ANTIMICROBIAL PHENOLIC COMPOUNDS FROM *ANVILLEA RADIATA* COSS. & DUR.

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Accepted Date: 13/01/2014; Published Date: 27/02/2014

Abstract: Objective: To evaluate the possible *in vitro* antimicrobial activity of *Anvillea radiata* Coss. & Dur. organs (Asteraceae) against a panel of microorganisms and to characterize the putative compounds responsible for this activity. **Methods:** Hydroalcoholic extract and its fractions (petroleum ether, chloroform, ethyl acetate, n-butanol and water fractions) were tested against eleven microorganisms including six bacteria species, two yeasts and three filamentous fungi using disc diffusion and broth microdilution methods. Qualitative screening of the highly active extracts was done using thin layer chromatography (TLC) analysis. **Results:** Crude methanolic extracts and fractions of *A. radiata* organs showed *in vitro* antimicrobial activity against one or more tested pathogens. Among the tested fractions, chloroform and ethyl acetate fractions derived from methanol extract of leaves demonstrated maximum activity against *Bacillus cereus* (MIC: 0.156 mg/ml) followed by n-butanol fraction (0.312 mg/ml). No antifungal activity was seen with any of the extracts tested. TLC analysis revealed the presence of high levels of some secondary metabolites in the leaf active extracts of *A. radiata*, which have been linked to antimicrobial properties. **Conclusion:** The obtained results provide justification for the use of *A. radiata* in folk medicine to treat various infectious diseases and may contribute to the development of novel antimicrobial agents for the treatment of infections caused by these drug-resistant microogranisms.

Keywords: *Anvillea radiata*, Antimicrobial activity, TLC, disc-diffusion, Broth microdilution.

PAPER-QR CODE

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How to Cite This Article:

F Beddou, IJPRBS, 2014; Volume 3(1): 172- 187

INTRODUCTION

Infectious diseases caused by microorganisms are one of the main reasons of death in the world. Even though pharmaceutical industries have produced a number of new antimicrobial drugs in recent years, the resistance to these drugs by microorganisms has increased. The abusive and indiscriminate use of antimicrobial drugs is the main factor responsible for the appearance of bacterial resistance [1]. This reason imposes severe limitations on therapeutic options, implying a threat to public health. New antimicrobial agents prototypes are needed to address this situation and plants are among the most important common sources of potentially valuable new drugs. They are effective in the treatment of infectious diseases while simultaneously mitigating many of the side effects that are often associated with conventional antimicrobials [2]. Therefore, it is of great interest to search new antimicrobials from plants in order to validate their use in folk medicine and to reveal the active principle by isolation and characterization of active constituents [3]. *Anvillea radiata* Coss. & Dur., commonly known as nougd l'hoor [4], belong to the Asteraceae family. This family is well marked in their characteristics and cannot be confused with any other. A large majority of the plants belonging to this family are herbaceous while trees and shrubs are comparatively rare [5]. The genus *Anvillea* is found to contain two species which occur in North Africa and the Middle East. *Anvilleina* Maire is reduced to a synonym of *Anvillea* and the following two new combinations are made: *A. platycarpa* (Maire) and *A. garcini*i (Burm. f.) DC. ssp. radiata (Coss. & Dur.) [6]. Due to their bio-active properties plants from Asteraceae family are commonly used in treatment of various diseases. *Anvillea radiata* Coss. & Dur. is widely used in the folk medicine as excellent heating, for the treatment of dysentery, gastrointestinal disorders and has been reported to have hypoglycemic activity [4, 7]. In previous phytochemical study [8], parthenolid-9-one, 9α-hydroxyparthenolide, 8α, 9α-epoxyparthenolide from *A. radiata* were described. Some other germacranolides, 9α-hydroxy-1β,10α-epoxyparthenolide, 9β-hydroxyparthenolide, 9β-hydroxy-1β,10α-epoxyparthenolide, cis-partenolid-9-one [9-12] and flavonols, quercetin 3-rhamnoglucoside 3', 4'dimethyl ether, 6-methoxykaempferol 3-galactoside, 6-methoxykaempferol 3-galactoside 7,4-dimethylether, kaempferol 3-rhamnoglucoside, spinacetin 3-rhamnoglucoside [13] were detected in *A. garcinii*. Thirteen flavonoids from which, four aglycones and nine flavonol glycosides were also isolated from *A. radiata* [14]. Till date no reports exist on the antimicrobial activity of *Anvillea radiata* organs. The present paper has therefore been designed to study the *in vitro* antimicrobial potential of methanol extract/fractions from *A. radiata* organs against a panel of microorganisms including Gram-positive bacteria, Gram-negative bacteria, yeasts and filamentous fungi and also to characterize the putative compounds responsible for this activity.

MATERIAL AND METHODS

Plant materials

Stems, flowers and leaves of *Anvillea radiata* were collected during April 2010, in Aïn Sefra, province of Naâma (Algeria). Identification of plant material was made by Dr. Mahboubi (Department of Ecology and Environment, University of Tlemcen, Algeria). A voucher specimen has been deposited at the Laboratory of Natural Products, (Department of Biology, University of Tlemcen, Algeria), under the accession No. A. 2788. Collected plant material was dried in a shaded and well-ventilated place, pulverized then kept refrigerated in glass containers before further processing.

Preparation of plant extract

Organ extracts were obtained by refluxing of 5 g of dry organ powder in 70% methanol (1:10, w/v) at temperature no higher than 65°C, using three cycles of 2h. The extracts were filtered through filter paper and concentrated with a vacuum evaporator (HAHNVAPOR HS-2005 V-N) to obtain a crude methanolic extract (ME). The ME was resuspended in warm water and then partitioned sequentially with petroleum ether, chloroform, ethyl acetate, n-butanol and water. Petroleum ether fraction (PF), chloroform fraction (CF), ethyl acetate fraction (AF), n-butanol fraction (BF) and water fraction (WF) were collected separately and concentrated using a vacuum evaporator to remove the solvent. Residues were dissolved in pure dimethyl sulfoxide (DMSO) to a final concentration of 100 mg/ml and stored at 4°C until analysis. The yield (%) of evaporated dried extracts was calculated as 100 DWext/DWsamp, where DWext is the weight of extract after evaporation of solvent, and DWsamp is the dry weight of organ sample.

Antimicrobial activity

Microbial strains

The extracts were individually tested against a panel of 11 microorganisms including six bacteria species, three Gram-positive (*Staphylococcus aureus* ATCC 25923, *Bacillus cereus* ATCC 10876 and *Micrococcus luteus* ATCC 9341), three Gram-negative (*Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853 and *Klebsiella pneumoniae* ATCC 700603), two yeasts (*Candida albicans* ATCC 10231, and *Candida albicans* IP 444) and three filamentous fungi (*Aspergillus flavus* MNHN 994294, *Aspergillus fumigatus* MNHN 566 and *Fusarium oxysporum* MNHN 963917).

Inhibition zone determination by disc diffusion assay

Antimicrobial activities of extracts were first screened for their inhibitory zone by the agar disc-diffusion method. The inoculums for the assays were prepared by diluting cell mass in 0.9% NaCl solution, adjusted to 0.5 McFarland scale, confirmed by spectrophotometrical reading on Specord 200 Analytikjena (Germany) at 625nm (λ = 0.08 - 0.1, corresponding to 10^8 CFU/ml) for bacteria and 530nm (λ = 0.12 - 0.15, corresponding to 1-5 x 10^6 CFU/ml) for yeasts. For filamentous fungi, spore suspensions were prepared from 1 week old cultures on PDA plates at 25°C and standardized by adjusting the transmittance to 68 to 82% at 530nm [15]. One milliliter of standardized suspension of the tested microorganisms (10^6 CFU/ml for yeasts and bacteria except, *S. aureus* at 10^7 CFU/ml and filamentous fungi at 10^4 spores/ml) was spread on the solid media plates, using Mueller–Hinton agar (Sigma, India) for Bacteria, Mueller Hinton agar supplemented with 2% glucose (to support growth) and 0.5 µg/ml of methylene blue (to improve zone edge definition) for yeasts [16] and PDA (Sigma, Spain) for filamentous fungi. They were "flood-inoculated" onto the surface of the solid media plates. After drying, a sterile paper discs (6mm in diameter) impregnated with 10 µl of the plant extracts (1mg/disc) were applied in the Petri dish. A disc prepared in the same condition with only the corresponding volume of DMSO (Sigma, France) was used as a negative control. The activity was determined by measuring the inhibitory zone diameter in mm after incubation at 37°C/24h for bacteria, at 30°C/48h for yeasts and at 25°C/72h for fungi. Nystatin (Ny. 30µg/disc) was used as reference antifungal against yeasts and filamentous fungi and Ceftazidime (CAZ. 30µg/disc), Amikacin (AK. 30µg/disc), Cefotaxime (CTX. 30µg/disc), Ampicillin (AMP. 10µg/disc) and Oxacillin (OX. 1µg/disc) were used as positive controls against bacteria. The data used was the mean of three replicates.

Minimum inhibitory concentration (MIC) and minimum microbicide concentration (MBC) determinations

The minimum inhibitory concentration (MIC) was determined by a broth microdilution method as described by Okusa et al. [17] with minor modifications. Plant extracts were dissolved in DMSO (25mg/250µl) and diluted to 5ml with Muller Hinton Broth (Sigma, India) (for antibacterial test) or Sabouraud Dextrose Broth (Sigma, India) (for anticandidal test), the final DMSO concentration being 5%. This solution was transferred in 96-wells plates (200 µl/well) and serially diluted (base 2 logarithmic dilutions) with Muller Hinton Broth or with Sabouraud Dextrose Broth. 24h cultures of microbial strains were stirred with 0.9% NaCl to achieve 0.5 McFarland (10^8 cells/ml for bacteria and 10^6 cells/ml for yeasts), diluted for 1/100 to achieve 10^6 and 10^4 cells/ml for bacteria and yeasts, respectively and inoculated in the 96-wells plates (100 µl/well). The cultures were incubated at 37°C (bacteria) and 30°C (yeasts) for 24h. The minimal inhibitory concentration (MIC) was defined as the lowest concentration of the sample

which prevents visible growth of microorganisms in the microdilution wells. Extracts with MIC less than 100 µg/ml were considered as significantly active, 100<MIC<625 µg/ml were considered as moderately active, and when MIC is higher than 625 µg/ml extracts were considered as weakly active [18]. As detected by the unaided eye, the minimal bactericidal/fungicidal concentration (MBC/MFC) was determined by sub-culturing the negative wells on Muller Hinton Agar plate. After 16-20 h of incubation at 37°C, the number of surviving organisms was determined. MBC/MFC was defined as the lowest extract concentration at which 99.9% of the bacteria/fungi were killed [19]. Each experiment was repeated twice.

TLC screening for phytochemical analysis

Qualitative screening of the highly active extracts was done using a number of physicochemical methods, including qualitative analysis and thin layer chromatography (TLC) analysis. Ten microlitres of each sample (10 mg/ml in methanol) was loaded on Merck TLC 60 F_{254} silica gel sheets (Germany). Development was carried out with various solvent systems viz.: n-Hexane:Ethyl acetate (8:1.9, v/v), Cyclohexan:Ethyl acetate (10:8, v/v), Ethyl acetate:Chloroform:Acetic acid (8:7:0.5, v/v/v) and Toluene:Acetic acid:Formic acid (3:5:1, v/v/v) [20]. After drying, the plates were sprayed with (KOH, (CH3CO2)2Pb, NH3) to detect coumarin; AlCl3 and NH3 to detect flavonoids, Libermann-Burchard and vanillin in the presence of sulfuric acid to detect sterols and polyterpens, Dragendorf's reagent to detect alcaloïdes and ferric chloride to detect tannins [21-24]. Detection was carried out visually in visible light and under UV light (λ = 366 nm). The retention factor (Rf) was calculated using the following equation:

Rf = (Distance move by the substance (cm))/(Distance move by the solvent (cm))

RESULTS

Inhibition zone determination by disc diffusion assay

In the present study, the in vitro antimicrobial activity of 18 extracts against eleven reference cultures and their potential activity were qualitatively assessed by the presence or absence of inhibition zones (IZ) (Table 1).

According to Tekwu et al. [19], diameters of inhibition zone less than 7 mm were recorded as non-active, between 7 and 10 mm were recorded as weakly active, more than 10 mm and less than 15 mm were recorded as moderately active and significantly active when diameters of growth inhibition were more than 16 mm. It could be observed that the extracts of *A. radiata* organs showed in vitro antimicrobial activity against one or more tested pathogens. No activity was seen against Gram-negative bacteria or fungi. The aqueous fraction prepared from the organs of *A. radiata* had not antimicrobial activity at all. The tested extracts demonstrated

varying degrees of antimicrobial activity against Gram-positive bacteria and yeasts. The inhibition zones against the standard type strains tested ranged from 7mm to 16mm. Chloroform fraction derived from the methanol extract of *A. radiata* was found to be most effective followed by ethyl acetate and n-butanol fractions. Among the two Gram-positive bacteria, *B. cereus* was found to be more sensitive than *S. aureus* on 9 vs. 6 extracts, respectively. Some extracts showed weakly anticandidal activity. However, the petroleum ether fraction of leaves has the best anticandidal activity with IZ diameter of 10mm. Impregnated paper discs containing only DMSO used as negative control did not show any inhibition zone. Ampicillin and Oxacillin were found to be resistant against most bacteria tested while Amikacin, Cefotaxime and Ceftazidime were sensitive.

Minimum inhibitory concentration (MIC) and minimum microbicide concentration (MBC) determinations

The agar diffusion assay is a qualitative, non standardized method useful only for the screening of large numbers of samples [25]. Activities discovered with disc diffusion assay were confirmed using the microdilution broth method and the antimicrobial activity was expressed as MIC and minimum bactericidal or fungicidal concentration (MBC/MFC) of the extracts. The MIC and MBC/MFC values were used to compare the antimicrobial activity of extracts. The selection of active extracts for this assay was made based on the size of inhibition zones (higher than 7mm) formed in the agar disc diffusion method. The results of MIC and MBC or MFC values of methanol/fractions of pathogenic strains are shown in Table 2. The data indicate that the extracts exhibited variable levels of antimicrobial activity against the investigated pathogens. The inhibitory property of the extracts was observed within a range of concentrations from 0.156 to 1.25mg/ml. Among the tested fractions, chloroform and ethyl acetate fractions of leaves demonstrated maximum activity against *B. cereus* (MIC: 0.156 mg/ml) followed by n-butanol fraction (0.312 mg/ml). This indicated the powerful and very interesting antibacterial potential of *A. radiata* (leaves). The petroleum and water fractions of *A. radiata* organs failed to show promising inhibitory activity against all tested strains. These findings supported the results of agar disc diffusion method (Table 1). Among the remaining extracts organs, some showed a moderate activity (MIC 0.312– 0.625 mg/ml) and others showed lowest activity (MIC 1.25 mg/ml).

TLC screening for phytochemical analysis

Qualitative TLC analysis revealed the presence of high levels of some secondary metabolites in the leaf active extracts of *A. radiata*. Spots were characterized by Rf-values and color under UV light before and after spraying of reagents (Tables 3 - 6). It is interesting to note that all the important phytochemicals were present in *A. radiata*. The presence and the absence of these

phytochemicals indicate the effectiveness of antimicrobial activity. No positive result was obtained with Dragendorf's reagent.

DISCUSSION

Antimicrobial agents are chemical compounds present in, or added to, foods that retard microbial growth or cause microbial death. In the last decades, there has been particular interest in the use of abundant naturally occurring antimicrobials (herbs, spices and plants) [26]. Among the different pathogenic microorganisms of relevance as food poisoning agents, there are two Gram-positive bacteria: *Staphylococcus aureus* and *Bacillus cereus*. *S. aureus* is considered as one of the most prevalent causes of gastroenteritis worldwide [27]. Whereas, *B. cereus* is considered as an endospore forming bacteria, which is involved in foodborne outbreaks with variable but increasing incidence (1-22% of all outbreaks) in industrialized countries [28, 29]. In the present study, the results showed that chloroform and ethyl acetate fractions of leaves revealed maximum activity against *B. cereus* and *S. aureus* (Tables 1 - 2) followed by n-butanol fraction. This indicated the powerful and very interesting antibacterial potential of *A. radiata* (leaves). Traditional use of *A. radiata* to treat infectious diseases such as dysentery and gastrointestinal disorders stimulated this investigation. The absence of antimicrobial activity in the water fraction indicates the insolubility of the active substances in this solvent. The resistance of Gram-negative bacteria may be due to their morphological constitutions, they have an outer phospholipidic membrane carrying the structural lipopolysaccharide components. This makes the cell wall impermeable to antimicrobial chemical substances. However, Gram-positive bacteria are more susceptible, because they have only an outer peptidoglycan layer which is not an effective permeability barrier [30]. The therapy of fungal infections caused by opportunistic pathogens such as *C. albicans* remains a major medical challenge [31]. Infection by *C. albicans* leads to the formation of a biofilm which is resistant to the penetration of antifungal agents [31]. The most active extract was the petroleum ether fraction of leaves of *A. radiata* (MIC = 0.625mg/ml, MFC = 0.625mg/ml). Using an ultra structural approach, Sangetha et al. [31] showed that *Cassia spectabilis* leaf extract prevented the formation of *C. albicans* biofilm at the MIC of 6.25 mg/ml. The findings of the present study with MICs lower than 6.25 mg/ml suggest that *A. radiata* extracts may also function by interfering with formation of a *C. albicans* biofilm. Very few studies reported bio-guided isolation of the active principles responsible for the activity observed, or at least a fractionation of the active extracts in order to determine more precisely the nature of the active constituents [32]. From the literature it is clear that the chemical structure of the antimicrobial agents found in higher plants belong to the most commonly encountered classes of higher plant secondary metabolites [32]. TLC-based techniques aimed at separation, purification and identification of individual biomolecules in mixtures are conventional tools and

represent mostly the first step in the analysis [33]. Combined with both biological and chemical detection methods, thin-layer chromatography is an effective and inexpensive technique for the study of plant extracts [34]. The leaf extracts of *A. radiata* when subjected to TLC showed the presence of Steroids, Triterpenic genins, Anthraquinones, Oleanane- and Ursane-type triterpenoids in petroleum ether fraction, Lupane-type triterpenoids, Phenolic compounds, Oleanane- and Ursane-type triterpenoids, Steroids and Coumarins in chloroform fraction, Xanthonic compounds, Coumarins, Phenolic compounds, Anthracene and Tannins in ethyl acetate fraction, Flavonoids, Tannins and Phenolic compounds in n-butanol fraction. Some unidentified compounds are present in the chloroform, ethyl acetate and n-butanol fractions (Tables 3 - 6). In previous phytochemical study [8], parthenolid-9-one, 9α-hydroxyparthenolide, 8α, 9α-epoxyparthenolide from *A. radiata* were described and thirteen flavonoids from which, four aglycones and nine flavonol glycosides were isolated by Dendougui et al. [14]. The presence of these constituents may be useful in establishing a relationship between chemical composition of the leaf extracts and previously reported activity of *A. radiata*. According to the findings of the present study, leaf extracts of *A. radiata* showed better antibacterial activity against certain Gram-positive bacteria and weakly anticandidal activity. These results provide justification for the use of *A. radiata* in folk medicine to treat various infectious diseases and may allow the development of potent natural antimicrobial agents with new or modified mode of action to overcome the problem of microbial resistance to the chemical antibiotics. Further bioassays, purification, structural characterization and toxicological properties of extracts will yield noteworthy information about the use of this plant material in pharmaceuticals, cosmetics and food industry.

CONFLICT OF INTEREST STATEMENT

We declare that we have no conflict of interest.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge Dr. Mahboubi (University of Tlemcen) for his help in the plant identification.

Table 1 Extraction yields and antimicrobial activity of different extracts through agar disc-diffusion method.

Used parts		Extracts (1mg/disc)	Extraction yield (%)	Inhibition zone diameter (mm)*										
				Gram-positive bacteria			Gram-negative bacteria			Yeasts		Fungi		
				Sa	*Bc*	*Ml*	*Ec*	*Pa*	*Kp*	*Ca*	*Ca.IP*	*A. flavus*	*A. fumigatus*	*F. oxysporum*
Anvillea radiata Coss. & Dur.	Leaves	ME	30.72	8	12	6	6	6	6	6	6	6	6	6
		PF	1.14	6	6	6	6	6	6	10	7	6	6	6
		CF	8.59	12	16	6	6	6	6	7	8	6	6	6
		AF	2.94	14	15	6	6	6	6	7	7	6	6	6
		BF	2.41	8	14	6	6	6	6	7	6	6	6	6
		WF	15.36	6	6	6	6	6	6	6	6	6	6	6
	Flowers	ME	28.75	6	7	6	6	6	6	6	6	6	6	6
		PF	0.85	6	6	6	6	6	6	8	7	6	6	6
		CF	2.24	8	14	6	6	6	6	7	8	6	6	6
		AF	2.43	6	8	6	6	6	6	8	7	6	6	6
		BF	3.11	6	6	6	6	6	6	6	6	6	6	6
		WF	16.77	6	6	6	6	6	6	6	6	6	6	6
	Stems	ME	14.41	6	8	6	6	6	6	6	6	6	6	6
		PF	0.46	6	6	6	6	6	6	8	7	6	6	6
		CF	1.17	11	15	6	6	6	6	7	7	6	6	6
		AF	1.10	6	8	6	6	6	6	7	7	6	6	6
		BF	1.00	6	6	6	6	6	6	6	6	6	6	6
		WF	8.46	6	6	6	6	6	6	6	6	6	6	6
AK (30µg/disc)				27	26	25	25	29	24	nt	nt	nt	nt	nt
CTX (30µg/disc)				29	8	33	30	23	19	nt	nt	nt	nt	nt
CAZ (30µg/disc)				16	6	21	23	24	10	nt	nt	nt	nt	nt
AMP (10µg/disc)				27	6	nt	15	6	6	nt	nt	nt	nt	nt
OX (1µg/disc)				16	6	nt	6	6	6	nt	nt	nt	nt	nt
Ny (30µg/disc)				nt	nt	nt	nt	nt	nt	16	20	22	35	14

* The diameter of the filter paper discs (6 mm) is included; (nt): Not tested; ME: Methanolic extract; PF: Petroleum ether fraction; CF: Chloroform fraction; AF: Ethyl acetate fraction; BF: n-butanol fraction; WF: water fraction; AK: Amikacin; CTX: Cefotaxime; CAZ: Ceftazidime; AMP: Ampicillin; OX: Oxacillin; Ny: Nystatin.

Table 2 Minimum inhibitory concentration (MIC) and minimum bactericidal/fungicidal concentration (MBC/MFC) of the most efficacious extracts

Used parts		Extracts	MICs / MBCs (mg/ml)				MICs / MFCs (mg/ml)			
			Gram-positive bacteria				Yeasts			
			S. aureus		*B. cereus*		*C. albicans*		*C. albicans IP*	
			MIC	MBC	MIC	MBC	MIC	MFC	MIC	MFC
Anvillea radiata Coss. & Dur.	Leaves	ME	1.25	> 1.25	0.625	0.625	nt	nt	nt	nt
		PF	nt	nt	nt	nt	0.625	0.625	0.625	1.25
		CF	0.312	0.625	0.156	0.156	1.25	> 1.25	1.25	1.25
		AF	0.312	0.625	0.156	0.312	≥ 1.25	> 1.25	1.25	> 1.25
		BF	0.625	1.25	0.312	0.625	≥ 1.25	> 1.25	≥ 1.25	> 1.25
	Flowers	ME	≥ 1.25	> 1.25	1.25	> 1.25	nt	nt	nt	nt
		PF	nt	nt	nt	nt	1.25	> 1.25	1.25	> 1.25
		CF	0.625	1.25	0.312	0.625	≥ 1.25	> 1.25	1.25	> 1.25
		AF	≥ 1.25	1.25	0.625	1.25	≥ 1.25	> 1.25	≥ 1.25	> 1.25
		BF	≥ 1.25	> 1.25	≥ 1.25	> 1.25	nt	nt	nt	nt
	Stems	ME	≥ 1.25	> 1.25	0.625	1.25	nt	nt	nt	nt
		PF	nt	nt	nt	nt	1.25	> 1.25	1.25	1.25
		CF	0.625	1.25	0.312	0.312	≥ 1.25	> 1.25	1.25	> 1.25
		AF	≥ 1.25	1.25	1.25	1.25	≥ 1.25	> 1.25	≥ 1.25	> 1.25
		BF	≥ 1.25	> 1.25	1.25	1.25	nt	nt	nt	nt

nt: Not tested because plant extracts were not active by disc diffusion assay.

Table 3 *Rf*-values and color of spots of the petroleum ether fraction of leaves

Mobile phase: n-Hexane:Ethyl acetate (8:1.9, v/v)

Extracts	Without revelation a			With revelation								Identified compounds
				NH_3 A			Liebermann-Burchard B			Vanillin-H_2SO_4 C		
	Rf	Visible	UV/366nm	Rf	Visible	UV/366nm	Rf	Visible	UV/366nm	Rf	Visible	
PF	-	-	-	-	-	-	0.00	violet	Yellow[f]	0.00	Grey	Steroids B **[35]**, Triterpenic genins C **[20]**
	0.30	-	Red[f]	0.30	-	Red[f]	0.27	violet	Yellow[f]	0.34	Grey	Anthraquinones a, A **[36]**, Steroids B **[35]**, Triterpenic genins C **[20]**
	0.40	-	Red[f]	0.40	-	Red[f]	0.39	violet	Pink[l]	0.36	Grey	Anthraquinones a, A **[36]**, Triterpenic genins C **[20]**
	-	-	-	-	-	-	0.64	-	Red[o]	0.77	Grey	Oleanane- and Ursane-type triterpenoids B **[35]**, Triterpenic genins C **[20]**
	-	-	-	-	-	-	0.89	-	Pink[l]	0.81	Grey	Triterpenic genins C **[20]**
	-	-	-	-	-	-	0.96	-	Red[o]	-	-	Oleanane- and Ursane-type triterpenoids B **[35]**

o: orange, l: light, f: fluorescent

Table 4 *Rf*-values and color of spots of the chloroform fraction of leaves

Mobile phase: Cyclohexan:Ethyl acetate (10:8, v/v)

Extracts	Without revelation a			With revelation														Identified compounds
				NH_3 A			Liebermann-Burchard B			$FeCl_3$ C		KOH D			$(CH_3CO_2)_2Pb$ E			
	Rf	Visible	UV/366 nm	Rf	Visible	UV/366 nm	Rf	Visible	UV/366 nm	Rf	Visible	Rf	Visible	UV/366 nm	Rf	Visible	UV/366 nm	
CF	0.00	Brown	Orange	0.00	Green	Orange	0.00	Brown[l]	Yellow[o]	-	-	0.00	Green	Red	0.00	Yellow[g]	Orange	Lupane-type triterpenoids B **[35]**
	0.03	Green	Red[f]	0.03	Green	Orange	0.03	Brown[l]	Orange	0.03	Green	-	-	-	0.04	Green	Grey[g]	Phenolic compounds C **[23]**
	0.06	Green[y]	Pink[f]	0.07	-	Pink	0.07	Violet[d]	Brown[g]	-	-	0.08	Yellow	Violet[d]	0.07	-	Pink	Coumarins A **[20]**, D **[37]**
	0.10	-	Pink[l]	0.11	-	Pink	0.14	Grey	Yellow[g]	-	-	0.15	Yellow	Green	0.11	-	Violet[d]	Coumarins A **[20]**, Steroids B **[35]**, D **[37]**
	0.18	-	Brown	0.17	-	Violet[d]	0.20	-	Yellow	-	-	-	-	-	-	-	-	Steroids B **[35]**
	-	-	-	0.23	-	violet	0.22	-	Yellow	-	-	-	-	-	-	-	-	Steroids B **[35]**
	-	-	-	0.45	-	Violet[d]	0.65	-	violet	-	-	-	-	-	-	-	-	Unidentified
	-	-	-	-	-	-	0.68	-	Yellow[o]	-	-	-	-	-	-	-	-	Lupane-type triterpenoids B **[35]**
	-	-	-	-	-	-	0.78	Yellow	Pink	-	-	-	-	-	-	-	-	Unidentified
	0.86	Yellow	Pink[l]	-	-	-	0.82	Green	Red[f]	-	-	-	-	-	0.86	-	Pink	Oleanane- and Ursane-type triterpenoids B **[20, 35]**
	0.89	Yellow[g]	Red[f]	0.90	Yellow[g]	Pink	0.88	Grey	Yellow[f]	-	-	-	-	-	0.89	-	Pink	Coumarins A **[20]**, Steroids B **[35]**

	0.93	Green	Red[f]	0.94	Yellow[g]	Red	0.93	Grey	Yellow[l]	-	-	0.94	Yellow	Red	0.94	Yellow	Red	Steroids B **[35]**, Coumarins D **[37]**

d: dark, o: orange, g: greenish, y: yellowish, l: light, f: fluorescent

Table 5 *Rf*-values and color of spots of the ethyl acetate fraction of leaves

Mobile phase : Ethyl acetate:Chloroform:Acetic acid (8:7:0.5, v/v/v)

Extracts	Without revelation a			With revelation												Identified compounds
				NH_3 A			KOH B			$AlCl_3$ C			$FeCl_3$ D			
	Rf	Visible	UV/366 nm	Rf	Visible	UV/366 nm	Rf	Visible	UV/366 nm	Rf	Visible	UV/366 nm	Rf	Visible		
AF	0.00	Brown	Orange	0.00	Brown	Orange	0.00	Green	Red	0.00	Green	Orange	-	-		Xanthonic compounds a **[38]**
	0.03	Green[y]	Red	0.03	Green	Pink	-	-	-	0.03	Yellow	Pink	-	-		Coumarins A **[20]**
	0.09	Yellow	Pink[f]	-	-	-	-	-	-	0.09	Yellow	Pink	-	-		Unidentified
	0.15	Green	Red	0.14	Green	Red	0.15	Green	Red	0.15	Green	Red	0.14	Green		Phenolic compounds D **[23]**
	0.2	Yellow[g]	Red	0.2	Yellow	Brown	0.23	-	Red	0.23	-	Pink	0.21	Grey		Tannins D **[23]**, Anthracene **[38]**
	-	-	-	0.68	-	purple	0.74	-	violet	-	-	-	-	-		Anthracene **[38]**, Coumarins A **[20, 39, 40]**
	0.9	-	Pink	0.9	-	purple		-	-	-	-	-	-	-		Coumarins A **[20, 39, 40]**
	0.94	Green	Red[f]	0.95	Green	Red	0.98	Green	Red	0.93	Yellow[g]	Red	0.94	Green		Phenolic compounds D **[23]**

g: greenish, y: yellowish, f: fluorescent

Table 6 *Rf*-values and color of spots of the n-butanol fraction of leavesMobile phase: Toluene:Acetic acid:Formic acid (3:5:1, v/v/v)

Extracts	Without revelation a			With revelation					Identified compounds
				$AlCl_3$ A			$FeCl_3$ B		
BF	Rf	Visible	UV/366nm	Rf	Visible	UV/366nm	Rf	Visible	
	0.76	Yellow[l]	violet	0.79	Yellow[l]	Blue[f]	0.76	Grey	Flavonoids A **[41]**, Tannins B **[23]**
	0.87	Yellow	Pink	-	-	-	-	-	Unidentified
	0.89	Green	Red	-	-	-	0.89	Green	Phenolic compounds B **[23]**
	-	-	-	0.94	Green	Orange	-	-	Unidentified

l: light, f: fluorescent

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