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IMPROVING IN THE ANTIBACTERIAL ACTIVITY AND THE ANTIOXIDANT PROPERTIES OF CHEMICALLY MODIFIED EXTRACTS OF *ZINNIA PERUVIANA*

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Abstract

In the present work we investigate the changes in the antibacterial activity and the antioxidant properties of modified extracts of *Zinnia peruviana* (L.) L. campesterol, stigmasterol and β-sitosterol were identified as major triterpenoids in the natural extract, by CG-EIMS and NIST Mass Spectrometry Data Center. The acetonic extract was chemically modified by reactions of acid hydrolysis, sulfonylation, acetylation, methylation and silylation. The *in-vitro* antibacterial activity was evaluated against Gram-positive and Gram-negative bacteria. The acetonic extract showed significant activity only against Gram-positive bacteria (MIC/MBC = 2/9 mg/mL). The hydrolyzed extract improve the bioactivity to both, Gram-positive and Gram-negative (MIC/MBC = 2/8 mg/mL). Selectively, the acetylated extract was only effective toward *Staphylococcus* (MIC/MBC = 7/7 mg/mL). The tosylated extract was active against Gram-positive and *P. aeruginosa* (MIC/MBC = 24/24 mg/mL) and the silylated against Gram-positive and *E. coli.* (MIC/MBC = 15/15 mg/mL). The antioxidant activity was determining by DPPH and ABTS assays. Hydrolyzed extract increased the antioxidant properties (DPPH, $IC_{50,(mg/mL)}$ = 0.077 ± 0.007; ABTS $IC_{50,(mg/mL)}$ = 0.032 ± 0.005). The acylated and alkylated extracts reduce the bioactivity and silylated derivative eliminated the bioactivity. These results could be considered as a possible strategy to obtain antimicrobial agents from herbal medicines.

Keywords: *Zinnia peruviana*, modified extracts, phytosterols, antibacterial activity, antioxidant properties

Introduction

The therapeutic uses of herbs as well as the actions of plant extracts are either as old as human civilization and has evolved along with it[1]. In the last decade, the demand for antimicrobial agents is increasing due to emergent clinical microbial strains resistant to one or several antibiotics[2]. Plants are a promising source of natural antimicrobial agents. During the last years, special attention has been paid to a number of plants that also, could be used as potential sources of antioxidants[3].

Zinnia peruviana (L.) (Asteraceae) ("clavelillo", "chinita del campo") is an erect annual herb, native from South America. In folk medicine is employed for the treatment of malaria, stomach pain, as hepatoprotective and antiparasitic, antifungal and antibacterial agents[4-6]. Also, this traditional Argentinean herb is used as a garden ornament[7].

On the other hand, the hydroxyl group is commonly present in bioactive compounds derived from plants. According to the Dictionary of Natural Products, around 70% of the bioactive compounds isolated from plants, with antibacterial and antioxidant properties, contain at least a hydroxyl group in their molecular structure[8].

In addition, the World Health Organization estimates that the population of most developing countries regularly, uses traditional medicine. From a medicinal chemistry perspective, hydroxyl group provides specific properties and behaviors to bioactive compounds and plays a significant role in their bioavailability[9]. The chemical modification of plant extracts has been reported as a strategy to discover new active compounds or to increase a specific bioactivity[10,11].

In the present work we investigated the changes in the antibacterial bioactivity and antioxidant properties of chemically modified extracts of *Z. peruviana*. Modified extracts were obtained by chemical transformations of acetonic extract as a strategy to increase and to extend the bioactivities evaluated. Significant changes were observed.

Materials and Methods

Plant material

Aerial parts of *Zinnia peruviana* (L.) were collected in Rio Grande, San Luis, Argentina (latitude: 33° 7′ 0″ S; longitude: 65° 5′ 0″ W), in February 2016. A voucher specimen was identified by Ing. Luis del Vitto *et al*. and lodged in the University of San Luis (Argentina) Herbarium (L.A. del Vitto N° 8841).

Natural extracts

The aerial parts *Z. peruviana* (300 g) were dried in shade at room temperature, then chopped and ground to a fine powder in a mechanical blender. Dried aerial powder (80.5 g) was packed into a Soxhlet apparatus and extracted with 300 mL of *n*-hexane, acetone and methanol at 60-65 °C for 3-4 h, respectively. The organic extracts were filtered through Whatman filter paper N° 1, and the filtrates were concentrated under reduced pressure at 40 °C. The extracts were dried, weighed (32.7 g) and stored at 4 °C in storage vials for experimental use.

Phytochemical screening

Preliminary phytochemical analysis of acetonic extract was performed using TLC visualization reagents, according to Wagner and Bladt[12]. The chemical composition pattern of acetonic extract was characterized by CG-EIMS and NIST Mass Spectrometry Data Center. A CG-Mass spectrometer, GCQ Plus, with ion trap and MS/MS (Finnigan, Thermo-Quest, Austin, TX, USA) was used

Chemical derivatization of acetonic extract

Acid Hydrolysis of acetonic extract (50 mg) was performed with hydrochloric acid 2M, refluxing at 80 °C for 3 h. Sulfonylation reaction on acetonic extract (50 mg) was carried out with *p*-toluene sulfonyl chloride and K_2CO_3 in acetone refluxing for 24 h. *O*-methylation reaction of acetonic extract (50 mg) was carried out with equimolecular quantities of dimethyl sulfate (2 mL), in potassium carbonate. Acetylation reaction was performed on 50 mg of acetonic extract, dissolved anhydrous pyridine and then treated with acetic anhydride. Finally, silylated derivative was obtained treating the acetonic extract with equimolecular quantities hexamethyldisilazane and trymethylchlorosilane in pyridine. All reactions processes were monitored by TLC[13].

Antibacterial activity

Microorganisms

The antibacterial activity was assayed against a total of 5 of reference strains kept in the ceparium (maintained in the culture collection) of the Laboratory of Microbiology of the National University of San Luis: *Staphylococcus aureus* ATCC 43300, *Staphylococcus epidermidis* ATCC 12228, *Listeria monocytogenes* CLIP 74904, *Escherichia coli* ATCC 35218 and *Pseudomonas aeruginosa* ATCC 27853. Bacterial strains were maintained on trypticase soya broth supplemented with 20% glycerol at -80 °C until use.

Determination of Minimum Inhibitory Concentration (MIC)

The antibacterial activity was assayed *in vitro* using microplate method (microwell dilution) according to the CLSI method[14] in trypticase soya broth (Britania, Argentina) pH 7.2 supplemented with 0.01% (w/v) of 2,3,5

triphenyltetrazolium chloride (TTC) used as visual indicator of bacterial growth. The inoculum of each strain was prepared from 24 h broth culture and adjusted to concentration of 10^6 CFU/mL. All extracts were dissolved in dimethylsulfoxide (DMSO) and tested in appropriate concentrations. The 96-well plates were prepared by dispensing into each well 95 μL of nutrient broth and 5μL of the inoculum (final concentration of 10^5 CFU/mL). One hundred microliter aliquots from the serial dilutions of extracts were transferred into 8 consecutive wells. The final volume in each well was 200 μL. Controls of nutrient broth, strains, DMSO, and extracts were included. After 24 h incubation at 37 °C, the antibacterial activity of the extracts (MIC) was defined as the lowest concentration of the extract in the medium in which there is no visible grown. The experiments were performed in duplicate and replicated at least twice.

Determination of Minimal Bactericidal Concentration (MBC)

Extracts that showed inhibitory activity in the preliminary broth assay were submitted to a subculture on the surface of the trypticase soya agar plates, in order to evaluate bactericidal effect. The presence or absence of bacterial growth was determined by visual inspection. MBC was defined as the lowest concentration that showed no bacterial growth in the subcultures after 24 h of aerobic incubation at 37 °C.

Antioxidant activity

DPPH radical scavenging assay

The radical scavenging activity was evaluated using DPPH (2,2-diphenyl-1-picrylhydrazyl) assay. Experiments were carried out according to the method of Blois with a slight modification[15]. Briefly, 0.1 mM solution of DPPH in MeOH/H_2O (8:2) was prepared. Then 1 mL of this solution was mixed with sample solution (3 mL) (1mg/mL final conc.). Finally, after 30 min, the absorbance was measured at 517 nm. Tests were carried out in triplicate. As positive control were used caffeic acid and quercetin. DPPH radical scavenging activity was calculated by the equation: % DPPH radical scavenging = ($Abs_{control}$-Abs_{sample}/$Abs_{control}$) x 100. (Abs = absorbance). The IC_{50} was determined using GraphPad software.

ABTS radical scavenging assay

The free radical-scavenging activity of the extracts was determined by ABTS [2, 2′-azinobis (3-ethylbenzthiazoline-6-acid)] assay. Experiments were carried out according to the method of Cano with some modifications[16]. Radical cation ABTS•+ was produced by mixing 2 mM ABTS with 30 µM H_2O_2 and 6 µM horseradish peroxidase (HRP) enzyme in 50 mM phosphate buffered saline (PBS-pH 7.5). Immediately after the addition of the HRP enzyme, the contents were vigorously mixed, incubated at room temperature in the dark and the reaction was monitored at 730 nm until stable absorbance was obtained. Then, 10 µL of different extract concentrations were added in the reaction mixture and the decrease in absorbance at 730 nm was measured. Percent inhibition of ABTS radical was calculated by to the formula IC (%) = [($Abs_{control}$-Abs_{sample})/$Abs_{control}$)] x 100 (Abs = absorbance). Tests were carried out in triplicate. As positive control were used caffeic acid and quercetin. The IC_{50} was determined using GraphPad software.

Statistical analyses

Data was reported as mean ± standard deviation from triplicate determination. Analysis of variance (ANOVA) accompanied with LSD and Tukey tests (SPSS for Windows, Version 15) were conducted to identify the significant difference between samples ($p < 0.05$).

Results

A series of modified extracts of *Z. peruviana* were prepared in order to study the changes in the bioactivities. Organic extracts were obtained from aerial parts using *n*-hexane, acetone and methanol at room temperature. Previous studies reports significant antibacterial activity for the acetonic extract[17].

From this data, preliminary phytochemical analysis of acetonic extract was performed using TLC. Phytosterols were visualized under UV light after spraying the chromatograms with a 0.5% solution of berberine in 95% ethanol and phosphoric acid as visualization reagents[12].

As major secondary metabolites, present in the natural extract, were detected campesterol (**1**), stigmasterol (**2**) and β-sitosterol (**3**) (Figure 1). The phytosterols **1-3** were identified by CG-EIMS and NIST Mass Spectrometry Data Center (Figure 2).

On the other hand, it is well-accepted that the antibacterial activity and the antioxidant properties are markedly influenced by the presence of hydroxyl groups in phytosterols[18]. A series of chemically modified extracts were prepared through chemical transformations. Functional group interconversion reactions were performed, particularly of hydroxyl group, in order to improve the antioxidant and antimicrobial activity. In this sense, reactions of acid hydrolysis, sulfonylation, *O*-acetylation, *O*-methylation and *O*-silylation reactions were performed (Figure 3). Acid hydrolysis was made with HCl 2 M at 80 °C and sulfonylation reaction was carried out with *p*-toluene sulfonyl chloride and K_2CO_3 in refluxing acetone for 24 h. The *O*-acetylation reaction was made with acetic anhydride in pyridine. The *O*-silylation reaction was performed with hexamethyldisilazane and trymethylchlorosilane in pyridine and the *O*-methylation

with dimethyl sulfate in the presence of K_2CO_3. The changes produced were monitored by TLC. Then, the *in vitro* antibacterial activity was evaluated against Gram-positive and Gram-negative bacteria. The results are shown in Table 1. Acetonic extract of *Z. peruviana* presented antibacterial activity against *Gram* positive bacteria. It showed inhibitory activity with MIC values of 2 mg/mL for *S. epidermidis* and *S. aureus* and 9 mg/mL for *L. monocytogenes*, whereas bactericidal action was observed at a concentration of 9 mg/mL. No bacteriostatic or bactericidal effect was detected on Gram-negative bacteria. Also, significant changes in the antibacterial activity were observed in the chemically modified extracts. It can also be seen from Table 1 that, exceptionally, the hydrolyzed extract extended the antibacterial profile to Gram-negative bacteria (*E. coli* and *P. aeruginosa*) with MICs and MBCs values ≥ 8 mg/mL.

The acetylated extract had inhibitory effect for both species of *Staphylococcus* (MIC/MBC = 7/7 mg/mL), but not for *L. monocytogenes*, *E. coli* and *P. aeruginosa*. The alkylated extract, by *O*-methylation reaction, showed bacteriostatic and bactericidal effect for all the strains tested with values ≥ 7 mg/mL. In addition, *E. coli* was the only bacterium resistant to tosylated extract, the remaining strains were sensitive but high concentrations were required (MIC/MBC = 24/24 mg/mL). Finally, *P. aeruginosa*, a nosocomial opportunistic microorganism which is characterized by presenting multiple mechanisms of antimicrobial resistance and to produce serious infections in hosts with altered defenses, was resistant only to silylated extract. The remaining strains were sensitive but high concentrations were required (MIC/MBC = 15/15 mg/mL).

The minor susceptibility of Gram-negative bacteria of modified extract could be attributed to outer membrane surrounding the cell wall which restricts diffusion of hydrophobic compounds through the lipopolysaccharide. Moreover, the periplasmic space contains enzymes, which could be able of breaking down foreign molecules introduced from outside[19].

Further, the radical scavenging activity of acetonic and modified extracts were evaluated using DPPH and ABTS radical scavenging assays (Table 2). Natural extract show significant antioxidant activity (DPPH, $IC_{50,(mg/mL)}$ = 0.083 ± 0.014; ABTS $IC_{50,(mg/mL)}$ = 0.044 ± 0.001). As positive control were used caffeic acid (DPPH, $IC_{50,(mg/mL)}$ = 0.041 ± 0.009; ABTS $IC_{50,(mg/mL)}$ = 0.005 ± 0.001) and quercetin (DPPH, $IC_{50,(mg/mL)}$ = 0.071 ± 0.021; ABTS $IC_{50,(mg/mL)}$ = 0.017 ± 0.003). In the same way, significant changes in the antioxidant properties were observed in modified extracts. In particular, the hydrolysis reaction increased the antioxidant activity. Hydrolyzed extract showed the best antioxidants properties (DPPH, $IC_{50,(mg/mL)}$ = 0.077 ± 0.007; ABTS $IC_{50,(mg/mL)}$ = 0.032 ± 0.005). Further in, the acetylation, sulfonylation and methylation reactions reduce the antioxidant properties of natural extract. In contrast, the silylation reaction largely eliminated their antioxidant capacity.

This work provided a first effort to obtain new bioactive extracts, changing their bioactivities by chemical derivatization of natural extracts of *Z. peruviana.* These results show how antibacterial and antioxidant properties can be affected by chemical modification. The present investigations together with previous studies provide support to the antimicrobial properties of herbal extracts. They could be used as antimicrobial supplement in the developing countries, towards the development of new therapeutic agents. Additional *in-vivo* studies and clinical trials would be needed to justify and further evaluate the potential of these extracts as antimicrobial agents.

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Table 1. Antibacterial activity of natural extract and chemically modified extracts of *Z. peruviana* against Gram-positive and Gram-negative bacteria.

Extracts	*S. epidermidis*		*S. aureus*		*L. monocytogenes*		*E. coli*		*P. aeruginosa*	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Acetonic	2	9	2	9	9	9	NA	NA	NA	NA
Hydrolyzed	2	8	2	8	8	8	8	>8	8	>8
Acetylated	7	>7	7	>7	NA	NA	NA	NA	NA	NA
Methylated	7	>7	7	>7	7	>7	7	>7	7	>7
Tosylated	24	>24	24	>24	24	>24	NA	NA	24	>24
Silylated	15	>15	15	>15	15	>15	15	>15	NA	NA

MIC: Minimum inhibitory concentration (mg/mL); MBC: Minimun bactericidal concentration (mg/mL); NA: no activity.

Table 2. Antioxidant activity of natural extract and chemically modified extracts of *Z. peruviana.*

Extracts	DPPH IC_{50} (mg/mL)	ABTS IC_{50} (mg/mL)
Acetonic Extract	**0.083 ± 0.014**	**0.044 ± 0.001**
Hydrolyzed Extract	**0.077 ± 0.007**	**0.032 ± 0.005**
Acetylated Extract	**0.111 ± 0.011**	**0.062 ± 0.001**
Tosylated Extract	**0.125 ± 0.032**	**0.069 ± 0.002**
Methylated Extract	**0.136 ± 0.008**	**0.076 ± 0.004**
Silylated Extract	**0.141 ± 0.013**	**0.082 ± 0.013**
Quercetin	**0.071 ± 0.021**	**0.017 ± 0.003**
Caffeic Acid	**0.041 ± 0.009**	**0.005 ± 0.001**

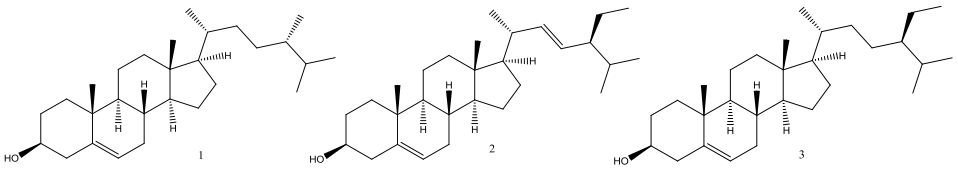

Figure 1. Compounds detected in acetonic extract of *Z. peruviana.*

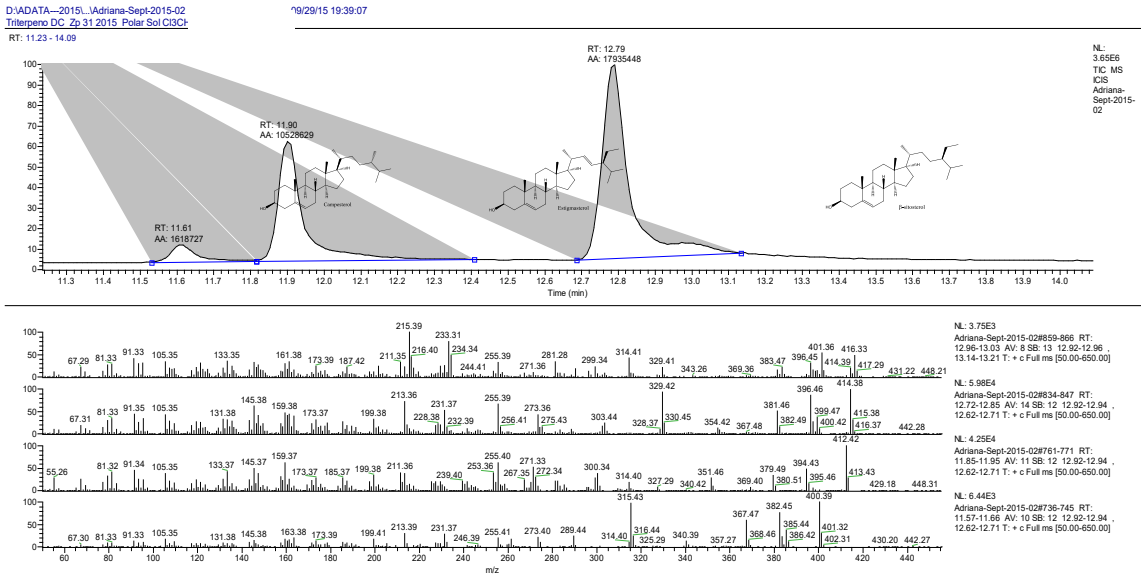

Figure 2. Phytosterols 1-3 identified by GC-EIMS as mayor secondary metabolites, in the acetonic extract of *Z. peruviana.*

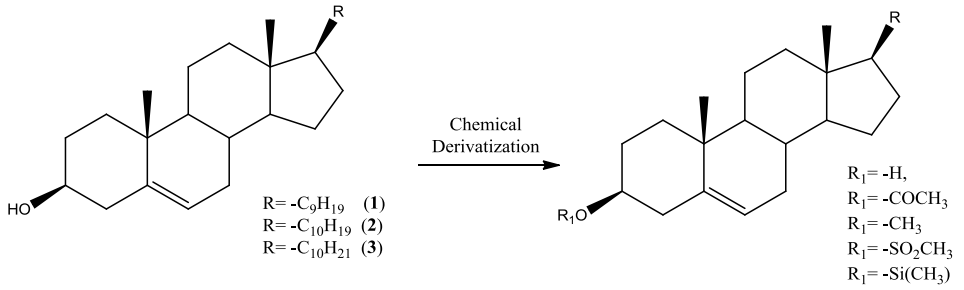

Figure 3. Chemical derivatization of compounds 1-3 present in acetonic extract of *Z. peruviana.*