African Journal of Pharmacy and Pharmacology

Volume 9 Number 2, 15 January, 2015

ISSN 1996-0816

Academic Journals

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academicJournals

Vol. 9(2), pp. 19-25, 15 January, 2015
DOI: 10.5897/AJPP2014.4162
Article Number: 3DF67A949748
ISSN 1996-0816

http://www.academicjournals.org/AJPP

African Journal of Pharmacy and Pharmacology

Full Length Research Paper

Phytochemical profile, antibacterial, antioxidant and cytotoxicity activities of *Euphorbia cotinifolia*

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Received 27 August, 2014; Accepted 9 December, 2014

The aim of this study was to determine the content of phenolic compounds and flavonoids, the antibacterial, antioxidant and cytotoxicity activities of hydroethanolic extracts of root, stem, leaf and fruit of *Euphorbia cotinifolia*. Phytochemical screening was performed using spectrophotometric methods (phenolic and flavonoid content) and liquid cromatography. The antibacterial activity was determined by agar diffusion and broth microdilution technique. Additionally, antioxidant activity was determined by diphenylpicrylhydrazyl (DPPH) radical scavenging method and cytotoxicity by the MTT method using BHK-21 (newborn hamster's kidney) cells. All extracts presented notable content of phenolic compounds, flavonoids and tannins. The high-performance liquid chromatography with photodiode array detection (HPLC-DAD) analysis showed higher concentration of phenolic compounds in dried leaves than in fresh leaves and it indicated the presence of caffeic acid. The extracts of leaf, stem, root and fruit showed activity against five gram-positive bacteria, six gram-negative bacteria and two yeasts, but not for mycobacterial. The highest antioxidant activity was exhibited in the extract of dried leaf (EC_{50} = 7.32 µg/ml). Extracts showed no cytotoxicity at the concentrations tested. All extracts showed antibacterial, antifungal and antioxidant activities, phenolic compounds, tannins and flavonoids. The results provided evidence that the studied plant might indeed be potential sources of natural antioxidant and antimicrobial agents.

Key words: *Euphorbia cotinifolia*, antimibacterial, antioxidant, phenolic compounds, flavonoids.

INTRODUCTION

The use of medicinal plants to treat diseases is virtually an old strategy used by all populations in the world. Between 25 to 30% of all medicines available in therapeutics are derived from natural products (plants, microorganisms and animals) (Ramos et al., 2008). The increased resistance to the available antimicrobials has

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attracted the attention of the scientific community to investigate new effective drugs of natural origin (Bitu et al., 2012). Natural products are an important source of bioactive compounds that can be used as an alternative to the treatment of diseases and also as a natural preservative in the food industry. Studies report that many biological activities, as well as antioxidant and antimicrobial, are due to the content of total phenols, tannins and flavonoids (Einbond et al., 2004; Banerjee and Dasgupta, 2005; Choi et al., 2006). The largest genus in the euphorbiaceae family is the *Euphorbia*. Plants from this genus have a preference for dried areas and they are found in South America and also in African and Asian continents. They are found as weeds in gardens or as ornamental elements at homes. The plants of the *Euphorbia* genus are traditionally used as medicinal plants, since they have several bioactive compounds, such as flavonoids, alkaloids, tannins and terpenes (Wang et al., 2006; Pusztai et al., 2007; Zhang et al., 2008; Shlamovitz et al., 2009). *E. cotinifolia* is used in folk medicine to cauterize wounds and also as a laxative (Mortan, 1962). The *E. cotinifolia* presents molluscicide (Pereira et al., 1978), antiviral (Betancur-Galvis et al., 2002) and antimicrobial activity (Jayalakshmi et al., 2011). In this context, this study aimed to determine the content of phenolic compounds and flavonoids and also determine the presence of antimicrobial, antioxidant and cytotoxicity activities of hydroethanolic extracts of root, stem, leaf and flower of dried and fresh *E. cotinifolia*.

MATERIALS AND METHODS

Plant identification and extract production

E. cotinifolia root, stem, leaf and flower were obtained in the city of Alfenas - MG (21° 27 ' 50.70 " S and 45° 55' 33.48 " W) at an elevation of 908 m, on November, 2012. The plant was identified, registered and filed in the Herbarium of the Federal University of Alfenas, thus, getting the specimen voucher number 2337. Fresh plant parts (root, stem, leaf and fruit) were washed under non-sterile water and cut into small pieces using non-sterile scissors. To prepare extracts from fresh plant parts, 200 grams of the fragments were weighed and added to a flask containing 800 ml of ethanol at 70%. To prepare dry extracts from plant parts, the samples were dehydrated at 37°C for seven days to reach constant weight. The samples were grounded into a powder and particle size was determined according to the Brazilian Pharmacopoeia 5th ed. using the Sieve Shaker – Electromagnetic method (Ertel ®) (Brazilian Pharmacopoeia, 2010). The obtained dried powder was used to prepare the extracts as described previsiously. The extracts were macerated for seven days in dark and after maceration, the extracts were filtered using filterpaper. Subsequently, the extracts were concentrated by rotaevaporator apparatus using negative pressure of 500 mmHg at a temperature of 60°C and lyophilized. All extracts were ressuspendend at final concentration of 50 mg/ml using dimethylsulfoxide (DMSO) and sterilized using 0.22 µm filters.

Evaluation of the phytochemical profile of the extracts

The extracts of root, stem, leaf and flower of *E. cotinifolia* were subjected to phytochemical tests based on colorimetry and precipitation for detection of the major bioactive constituents: anthraquinones, flavonoids, tannins, steroids, saponins and alkaloids (Costa, 1994). For the determination of phenolic compounds, an aliquot of each extract (0.5 ml) at 0.1 mg/ml was mixed with 2.5 ml of Folin-Ciocalteu reagent (diluted 1:10 in distilled water) and 2.0 ml of Na_2CO_3 4% (w/v) in distilled water. After two hours incubation in the dark at room temperature, the absorbance was measured at 750 nm in a spectrophotometer. The results were expressed as gallic acid equivalents (mg GAE/g) calculated by a curve constructed with concentrations ranging from 5 to 100 mg/ml (Singleton, 1999). The flavonoid content was determined according to Kalia et al. (2008). An aliquot of 0.5 ml of the extracts (at a concentration of 1.5 mg/ml) was mixed with 1.5 ml of ethanol, 0.1 ml of aluminum chloride ($AlCl_3$.6H2O) 10% (w/v), 0.1 ml 1 M potassium acetate, 2.8 ml of distilled water and 5 ml of total reaction. After 30 minutes, the absorbance of the mixture was measured at 425 nm. The total flavonoid standard curve was made using quercetin. The total flavonoids was expressed as quercetin equivalents (mg QE/g), and the values were presented by mean of a triple analysis. High Performance Liquid Chromatography (HPLC) analyses were performed on Shimadzu chromatograph matched with a diode array detector (DAD) and Shimadzu C18 ODS column (250 x 4.5 mm, 5 mm in particle size). The eluents used were acetic acid solution 5% v/v (eluent A) and methanol (eluent B), the injection volume was 25 µl and a flow rate of 1.0 ml/min. The analysis started at 10% B, and the linear gradient followed by 100% B in 35 min. The concentration of B was maintained until 50 min. Analysed extracts were dissolved in the mobile phase. Ascorbic acid, caffeic acid, quercetin, benzoic acid, and gallic acid were used as standards in the HPLC analysis.

Evaluation of antibacterial activity of the extracts

The antimicrobial activity was evaluated by agar diffusion according to the methodology proposed in document M7-A6 (CLSI, 2003) for bacteria, M24-A2 (CLSI, 2008b) for mycobacteria and M44-A2 (CLSI, 2009) for fungi. The strains used in antimicrobial tests were *Candida albicans* ATCC 10231, *Saccharomyces cerevisiae* ATCC 2601, *Bacillus subtilis* ATCC 6633, *Bacillus cereus* ATCC 11778, *Micrococcus luteus* ATCC 9341, *Enterococcus faecalis* ATCC51299, *Staphylococcus aureus* ATCC 6538, *Escherichia coli* ATCC 25922, *Serratia marcescens* LMI - UNIFAL, *Pseudomonas aeruginosa* ATCC 27853, *Proteus mirabilis* ATCC 25922, *Salmonella typhimurium* ATCC 14028, *Enterobacter aerogenes* LMI - UNIFAL, ATCC 25177 *Mycobacterium tuberculosis* (H37Ra) and *Mycobacterium bovis* (BCG) ATCC 27289. For the agar diffusion test, chlorhexidine 0.12% (v/v) was used as positive control and distilled water as negative control for gram positive, gram negative and yeasts. The antibiotic Rifamycin was used as positive control for antimicobacterial test. The minimum inhibitory concentration (MIC) was performed by broth microdilution according to the methodology proposed in document M27A3 (CLSI, 2008a) using 96 well plates. The extracts were diluted in Mueller Hinton broth at concentrations of 25 mg/ml to 0.05 mg/ml. It was used Mueller Hinton broth inoculated with the tested organism as positive control, Mueller Hinton broth uninoculated as a negative control and Mueller Hinton broth with only the extract as sterility control of the extract. To determinate the minimum microbicidal concentration (MMC), 10 µL of each well was inoculated into nutrient agar plates. After an incubation of 24 h at 37°C, the MMC was considered as the lowest concentration where no visible growth and was detected on nutrient agar. All experiments were performed in triplicate.

Antioxidant activity of extracts

Different concentrations of the extracts (from 400 to 1.56 mg/ml) in

Table 1. Content of total phenolics and flavonoids in the extracts of *Euphorbia cotinifolia.*

Plant part	Phenolic compounds* (mg GAE/g)**	Flavonoids compounds* (mg QE/g)***
Leaf		
Fresh	73.8±5.0[a]	9.7±0.7[b]
Dry	190.2±7.9[b]	18.5±0.5[a]
Stem		
Fresh	224.3±7.7[d]	2.1±0.1[f]
Dry	335.6±8.1[g]	3.6±0.1[e]
Root		
Fresh	204.4±6.5[c]	1.0±0.2[g]
Dry	316.9±12.0[f]	4.9±0.5[d]
Fruit		
Fresh	237.4±6.8[e]	6.7±0.7[c]
Dry	71.7±1.7[a]	7.1±0.9[c]

*Results expressed as mean ± standard deviation (n = 3). Means with different letters are statistically different in the same column or compound (Scott -Knott $p < 0.05$). ** Milligrams of gallic acid (GA) per gram of sample. *** Milligrams of quercetin per gram of sample.

an ethanolic solution (2 ml) were mixed with 0.5 ml of 2,2-diphenyl-1-picrylhydrazyl (DPPH) (0.5 mM, diluted in ethanol). After incubation for 30 min in the dark, the absorbance was measured at 517 nm. The blank test was composed of all reagents except extracts. Ascorbic acid, butylated hydroxytoluene (BHT) and quercetin were used as positive controls. The abduction property was calculated as the percentage of abducted DPPH radical by using the following equation: Seizure of DPPH (%) = [(absorbance of blank – absorbance sample)/(absorbance blank)] x 100 and the EC50 was determined for each extract. All experiments were performed in triplicate (Yen, 2005).

Evaluation of the cytotoxic activity of extracts on cell culture

Cytotoxicity was assessed by 3 - (4,5-dimethylthiazol-2YL) -2,5-diphenyltetrazolium bromide (MTT) method. In this test, 1×10^4 BHK-21 cells (baby hamster's kidney cells) were seeded per well in 96-well plates containing the medium Eagle's Minimum Essential (MEM) with 10% fetal bovine serum and antibiotics. After 24 h, the medium was discarded and then 0.1 ml of MEM containing 1% fetal bovine serum with decreasing dilutions of the extracts (5 to 0.039 mg/ml) was added to the cultures for 48 h. After incubation, 10 µL of MTT at a concentration of 5 mg/ml was added and incubated for 4 h at 37°C for the MTT incorporation and for the formation of formazan crystals. To solubilize the formazan crystals, the medium was discarded and 0.1 ml of DMSO was added to wells. Spectrophotometric analysis was performed on a microplate reader at 570 nm. The percentage of cytotoxicity was calculated by using the formula [(A-B)/ Ax100], where A and B are values of the optical densities of the control and treated cells, respectively. All experiments were performed in triplicates (Araújo, 2008).

Statistical analysis

Statistical analysis of the results was performed by the SISVAR 5.3 software, using the analysis of variance (ANOVA) and the Scott-Knott test to observe significant differences between average values ($p < 0.05$) (Scott and Knott, 1964)

RESULTS AND DISCUSSION

The granulometric assessment of the ground material is impostant, as it has direct influence on the efficiency of the extraction process. The powders were classified as moderately thick according to the Brazilian Pharmacopeia (2010). The average partical size for leaf, stem, root and fruit were 204.43, 218.04, 216.39 and 200.20 mm, respectively. Particles with homogeneous dimensions increase the contact area between the solid material and extractor liquid, making the extraction more efficient (Migliato et al., 2007). Phytochemical screening revealed the presence of flavonoids and tannins for all extracts. The phenolic content ranged from 71.66 to 335.64 mg GAE/g. The dried stem extract showed the highest content of phenolic compounds (335.64 mg GAE/g) and dried fruit showed the lowest (71.66 mg GAE/g) (Table 1). Regarding the content of flavonoids, the dried leaf extract showed the highest content (18.52 mg QE/g) and the fresh root showed the lowest (1.01 mg QE/g) (Table 1). The dried extracts showed statistically higher values than fresh extracts. In studies conducted by Jayalakshmi et al. (2011) and Jayalakshmi et al. (2012), leaf extract of *E. cotinifolia* also presented flavonoids and tannins, corroborating our results.

Taking the higher biological activity into account, the extracts of fresh and dried plant were selected for

Tabel 2. Antimicrobial activity extracts of *Euphorbia cotinifolia* (inhibition areas in millimeters).

Microorganism	Leaf		Stem		Root		Fruit		Chlo*0,12%	Rifamycin
	Fresh	Dry	Fresh	Dry	Fresh	Seco	Fresh	Dry		
Gram-positive										
B. subtilis ATCC 6633	9.5	9	10	8	0	5	9	0	15	NT
B. cereus ATCC 11778	7	7.5	7	0	0	7	0	0	12	NT
M. luteus ATCC 9341	17.5	20	20	0	10	20	17.5	15	19	NT
E. faecalis ATCC 51299	0	0	0	0	0	0	0	0	19	NT
S. aureus ATCC 6538	10	10	9	0	0	10	0	0	18	NT
Gram-negative										
E. coli ATCC 25922	0	0	0	0	0	0	0	0	9	NT
E. aerogenes LMI-UNIFAL	0	0	0	0	0	0	0	0	6	NT
S. marcescens LMI-UNIFAL	0	0	0	0	0	0	0	0	12	NT
P. aeruginosa ATCC 27853	0	0	0	0	0	0	0	0	10	NT
P. mirabilis ATCC 25922	12	13	10	12.5	10	13	12.5	12	-	NT
S. typhimurium ATCC 14028	0	0	0	0	0	0	0	0	9	NT
Yeasts										
C. albicans ATCC 10231	0	0	0	0	0	0	0	0	14	NT
S. cerevisae ATCC 2601	0	0	0	0	0	0	0	0	18	NT
Mycobacteria										
M. tuberculosis ATCC 25177 (H37Ra)	0	0	0	0	0	0	0	0	NT	20
M. bovis ATCC 27289 (BCG)	0	0	0	0	0	0	0	0	NT	20

*Chlo: chlorhexidine NT: not tested

chemical analysis by high performance liquid chromatography (HPLC). It can be observed in the chromatograms that the first eluted compounds exhibit more polarity characteristics than later compounds. Thus, a higher methanol concentration for the drag of these substances were required. Considering the polar characteristics of phenolic compounds, it is suggested that first eluted peaks correspond to phenolic compounds, since they have spectra with absorption in the ultraviolet region. Comparing the peaks of around retention time 18 min of fresh leaf (peak 2; Figure 1A) and the dried leaf (peak 6; Figure 1B,), the area of the peak in the dried leaf is about 10 times larger than in the fresh leaf. According to the used standards, it was possible to identificated the presence of caffeic acid in leaf extracts (for fresh peak 2, Figure 1A, for dry peak 6, Figure 1B). The result obtained by liquid chromatography confirms the results expressed in the analysis of phenolic compounds by the Folin-Ciocalteau method and flavonoids by the aluminum chloride method, where it was also observed a highest concentration of phenolic and flavonoid compounds in the dried leaf extract when compared to fresh leaf extract.

In previous studies, 17 polyphenols were isolated using spectrometry, mass spectrometry (ESI-MS) and nuclear magnetic resonance (1H NMR) and 1D/2D NMR from the leaf of *E. cotinifolia* (Marzouk et al., 2012). Highlighting the presence of phenolic compounds in *E. cotinifolia*. Results of agar diffusion test showed an antibacterial activity against *B.subtilis, B.cereus, M. luteus, S. aureus* and *P. mirabilis*. *M. luteus* was the most sensitive bacteria and presented

Table 3. Minimum inhibitory concentration (MIC) and minimum concentration microbicide (MMC) in mg/ml extracts of *Euphorbia cotinifolia.*

Microorganisms	Leaf				Stem				Root				Fruit			
	Fresh		Dry		Fresh		Dry		Fresh		Dry		Fresh		Dry	
	MIC	MMC	MIC	MMC	MIC	MMC	MIC	MMC	MIC	MMC	MIC	MMC	MIC	MMC	MIC	MMC
Gram-positives																
Bacillus subtilis ATCC 6633	6.25	25.00	3.12	3.12	3.12	3.12	6.25	ND	6.25	12.50	6.25	25.0	6.25	12.50	3.12	6.25
Bacillus cereus ATCC 11778	3.12	ND	0.78	0.78	1.56	12.5	3.12	ND	3.12	ND	3.12	ND	3.12	25.00	1.56	ND
Micrococcus luteus ATCC 9341	0.09	12.50	0.05	0.78	0.05	6.25	0.19	0.78	1.56	12.50	3.12	ND	0.09	12.50	3.12	12.50
Enterococcus faecalis ATCC 51299	6.25	12.50	6.25	12.50	6.25	12.50	12.50	12.50	12.50	ND	6.25	ND	6.25	6.25	6.25	6.25
Staphylococcus aureus ATCC 6538	3.12	12.50	3.12	12.50	6.25	12.5	6.25	6.25	3.12	12.50	3.12	ND	6.25	12.50	6.25	6.25
Gram-negatives																
Escherichia coli ATCC 25922	3.12	25.00	6.25	12.50	6.25	12.5	6.25	25.00	6.25	6.25	3.12	ND	6.25	12.50	3.12	6.25
Enterobacter aerogenes LMI-UNIFAL	3.12	6.25	6.25	12.50	6.25	6.25	6.25	12.50	6.25	12.50	6.25	ND	6.25	12.50	6.25	6.25
Serratia marcesnces LMI-UNIFAL	3.12	6.25	3.12	12.50	3.12	ND	3.12	ND	6.25	ND	6.25	ND	6.25	12.50	6.25	12.50
Pseudomonas aeruginosa ATCC 27853	6.25	25.00	6.25	25.00	6.25	25.00	6.25	25.00	6.25	ND	6.25	ND	6.25	25.00	6.25	12.50
Proteus mirabilis ATCC 25922	6.25	25.00	3.12	1.56	6.25	25.00	6.25	12.50	3.12	ND	6.25	ND	6.25	12.50	6.25	12.50
Salmonella typhimurium ATCC 14028	3.12	25.00	6.25	25.00	6.25	25.00	6.25	6.25	6.25	ND	6.25	ND	6.25	25.00	6.25	25
Yeasts																
Candida albicans ATCC 10231	3.12	6.25	3.12	6.25	3.12	12.5	3.12	625	3.12	3.12	3.12	ND	3.12	6.25	3.12	6.25
S. cerevisae ATCC 2601	6.25	6.25	6.25	6.25	6.25	5.00	6.25	6.25	12.50	12.50	6.25	ND	3.12	12.50	6.25	12.5

ND: Not detected in the tested concentrations.

areas of inhibition between 15 and 20 mm (Table 2). The *P. mirabilis* was inhibited by all extracts and inhibition areas ranged between 10 to 13 mm. No inhibition was observed for *E. faecalis, E. aerogenes, E. coli, P. aeruginosa, S. typhimurium, S. marcescens, M. bovis, M. tuberculosis, C. albicans* and *S. cerevisiae*. The leaf extracts (fresh or dry) showed greater spectrum of activity followed by the stem, root and fruit extracts, respectively. The MIC values were below 6.25 mg/ml and the MMC between 3.12 and 25 mg/ml (Table 3). The leaf extracts presented the lowest MICs, followed by stem, root and fruit extracts, respectively. The dried extracts showed lower MICs and MMCs when compared to fresh extracts and the highest concentration of phenolics and flavonoids for leaf extract may be closely related to the antimicrobial activity (Choi et al., 2006). Phenolic compounds act on the cytoplasmic membrane, changing its structure and function, change the active transport and coagulate the cellular content (Burt, 2004). *E. coli*, *E. aerogenes, S. marcescens, P.aeruginosa, S. typhimurium, C. albicans* and *S. cerevisiae* were not inhibitied by any extract in agar diffusion test, but it is observed an antimicrobial activity in the broth microdilution test. The agar diffusion test is a qualitative test, in which nonpolar substances can not diffuse well in the medium, since the broth microdilution is a quantitative test therefore, more sensitive.

In the study carried out by Jayalakshmi et al. (2011) the methanolic leaf extract of *E. cotinifolia* showed activity against the phytopathogenic bacteria and *Xanthomonas* sp., *Agrobacterium* sp., *Erwinia* sp. and *Pseudomonas* sp. The methanolic and ethyl acetate leaf extracts of *E. cotinifolia* also showed antimicrobial activity against *E. coli* (MTCC 7410), *Klebsiella pneumoniae* (MTCC 7407), *B. subtilis* (MTCC 121), *B. cereus* (MTCC 1272), *S. typhi* (MTCC 733), *E. aerogenes* (MTCC 7325) and *S. aureus* (MTCC 7443). The MIC ranged from 0.312 to 1.25 mg/ml (Jayalakshmi et al., 2014). Although the same bacteria had been used, they showed lower

Table 4. Antioxidant activity of hydroethanolic extracts of *Euphorbia cotinifolia.*

Hydroethanolic extracts	% DPPH radical deviation (100 µg/ml)*	EC_{50} (µg/ml)
Fresh leaf	86,65±0,2^{f}	11.6
Dry leaf	85,9±0,1^{f}	7.3
Fresh stem	74,5±0,9^{d}	66.7
Dry stem	54,3±2,1^{a}	103.5
Fresh root	62,8±1,9^{c}	82.2
Dry root	85,6±0,1^{f}	25.0
Fresh fruit	59,2±0,8^{b}	78.2
Dry Fruit	80,3±1,1^{e}	39.2
Quercetin	81,4±0,2^{e}	4.6
Ascorbic acid	90,4±0,3^{g}	6.5
BHT**	63,7±0,1^{c}	70.1

*Results expressed as mean ± standard deviation (n = 3). Means with different letters are statistically different (Scott -Knott $p < 0.05$). ** BHT: Butylated hydroxytoluene.

MIC, but they are different strains, which may lead to these variations. Differences arising from the soil, climate and seasonality can influence the chemical composition of the plant, resulting in a higher concentration of compounds, thus influencing the biological activity. For the DPPH free radical scavenging activity, the highest antioxidant potential was shown on the extract of dried leaf, which had the lowest concentration capable of sequestering 50% of DPPH radicals, with EC_{50} of 7.32 µg/ml (Table 4). Correlation between phenolic compounds and antioxidant activity was positive (r^2= 0.32). Extracts showed the highest concentration of phenolic compounds showed higher antioxidant activity.

Several biological activities such as the antioxidant and antimicrobial may be related to phenol contents such as tannins and flavonoids. The dried extracts showed higher concentration of the total phenolics and flavonoids and this could explain the fact that there is greater antioxidant activity in the dry extracts, since the phenolic compounds are closely linked to antioxidant activity (Einbond et al., 2004; Banerjee and Dasgupta, 2008; Choi et al., 2006). Although the dried leaf extract did not present the highest concentration of phenolic compounds, this extract has the highest concentration of flavonoids among all tested extracts. This differential flavonoid concentration could justify the high antioxidant activity observed. Marzouk et al. (2012) isolate polyphenols, including two new ellagitannins and one trigaloil glucosilcampferol from *E.cotinifolia,* and they showed high activity in the DPPH assay, with EC_{50} values lower than the ascorbic acid.

In a study by Jayalakshmi et al. (2014) the *E.cotinifolia* chloroform extract was the most active with an EC_{50} of 15 µg/ml followed by petroleum ether, ethyl acetate and methanol, with EC_{50} values of 17, 18 and 19 mg/ml, respectively. These scavenging activities of the extracts were considered higher when compared with the standard. These studies corroborate with our results, since the leaf extracts leaf (dry and fresh), dry root, dry fruit, fresh stem, presented better results for the BHT, emphasizing the potential antioxidant activity of *E. cotinifolia.* The extracts showed no citoxicity against BHK-21 cells in all concentrations tested. Betancur-Galvis et al. (2002) valuated the cytotoxic activity using the MTT method of several species of *Euhorbia* genus, among them, the *E. cotinifolia*. The hydromethanolic extracts of leaf and stem also showed no cytotoxicity, corroborating our results.

Conclusion

All extracts of *E. cotinifolia* showed antibacterial, antifungal and antioxidant activities, high levels of phenolic compounds and the presence of secondary metabolites. The HPLC-DAD analysis suggests the presence of caffeic acid in leaf extracts. The extract of dried leaves showed lower MIC and MMC for antimicrobial activity and lower EC_{50} values for antioxidant activity. Extracts showed no activity against mycobacterial. Extracts showed no significant citoxicity. The results provided evidence that the studied plant might indeed be potential sources of natural antioxidant and antimicrobial agents.

Conflicts of interest

The authors declare that they have no conflicts of interest.

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academicJournals

Vol. 9(2), pp. 26-32, 15 January, 2015
DOI: 10.5897/AJPP2014.4215
Article Number: B667B9349749
ISSN 1996-0816

http://www.academicjournals.org/AJPP

African Journal of Pharmacy and Pharmacology

Full Length Research Paper

Impact of pharmacy residents in pharmaceutical hospital care

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Received 27 October, 2014; Accepted 22 December, 2014

The study aimed to identify and analyze drug-related problems (DRP), negative outcomes associated with medication (NOM) and the impact of pharmaceutical performance through interventions by pharmacy residents at the University Hospital of Campo Grande, Mato Grosso do Sul, Brazil. This retrospective, cross-sectional single-center study analyzed data registered in Pharmaceutical Intervention forms recorded by pharmacy residents between March 2011 and February 2012. DRP and NOM were classified according to the definitions proposed by the Third Consensus of Granada (2007). A total of 256 pharmaceutical interventions from 155 patients were registered, of which 50.78% were from patients of 60 years of age or above. Majority of interventions took place in the surgery wards, medical clinic and adult intensive care unit, with 89.06% of interventions being accepted. Among these interventions, 401 DRP, of which 21.07% were related to the probability of adverse effects, and 298 NOM, of which 33.87% were related to non-quantitative safety problems, were observed. Anti-infectives for systemic use were shown to be the group most often involved with DRP. Treatment effectiveness was the reason for intervention in 80.23% of forms. A close relationship between physicians and pharmacists ensures more rapid identification of prescription errors, possible adverse effects, DRP and NOM. Despite of the issue importance, the published studies on the topic remains scarce. The results of studies that evaluate DRP and NOM collaborate with the analysis of the pharmaceutical service provided to hospitalized patients in the present study.

Key words: Drug-related problems, negative outcomes associated with medication, pharmacotherapeutic monitoring, hospital pharmaceutical care, third consensus of Granada.

INTRODUCTION

According to Hepler and Strand (1990), the practice of pharmaceutical care involves the pharmacist's participation in healthcare interventions, including pharmaceutical interventions (PhI). This conduct is characterized as a planned, documented act involving the user and health professionals, which aims to solve or prevent issues that

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may interfere with pharmacotherapy and is included as part of the monitoring of the pharmacotherapeutic process (OPAS, 2002).

Several studies have shown that the participation of the pharmacist in reviewing patient pharmacotherapy can result in significant reduction in drug-related problems (DRP), negative outcomes associated with medication (NOM), patient length of stay, and treatment costs (Conde et al., 2006; Gandhi et al., 2001; García et al., 2002; Soria et al., 2011). DRP pertains to situations in which the medication use process may cause a negative result, while NOM refer to inadequate results from these situations related to medication use (Comitê de Consenso, 2007).

The main objectives of therapeutic monitoring include ensuring the rational and proper use of drugs to achieve the desired pharmacotherapeutic outcomes, maximizing the beneficial effects of drugs, preventing or minimizing undesirable effects and promoting collaboration for the reduction of spending on patient care (Farré Riba et al., 2000). In order to prevent errors or prevent errors from reaching the patient, the healthcare team's performance must be effective. Healthcare professionals should perform in an integrated manner during the steps of selection, management, prescription, dispensation, administration of drugs and post-administration monitoring (Nunes et al., 2008).

Thus, this study aimed to identify and analyze DRP, NOM, and the impact of pharmaceutical performance through interventions by pharmacy residents at the University Hospital of Campo Grande in the state of Mato Grosso do Sul, Brazil.

METHODOLOGY

Design, setting and subjects

This single-center, retrospective cross-sectional study was conducted at the Maria Aparecida Pedrossian University Hospital Center of the Federal University of Mato Grosso do Sul (NHU/UFMS). It is a tertiary teaching hospital with 280 bed capacity and a member of the Brazilian Unified Health System (Sistema Único de Saúde - SUS).

There was no selection of subjects. The NHU/UFMS pharmacy residents registered all pharmaceutical interventions performed in a specific form developed by the hospital pharmacy service during the study period.

Data collection

The analysis of DRP, NOM and the impact on pharmaceutical performance was based on information registered in Pharmaceutical Intervention forms completed by five pharmacy residents from March 2011 to February 2012.

Data on patient gender, age group and hospital sector of admission were recorded in the form. Data exposed in Table 1 were also registered. In addition to intervention result, a summary of the intervention performed and the results of the intervention were recorded. These data were used as a base to identify DRP and NOM, according to the proposal of the Third Consensus of Granada (Comitê de Consenso, 2007).

Multiple causes were accepted in data collection, as the patient may have been subjected to more than one DRP. The DRP related to personal characteristics of the patient included any impediment the patient may have presented due to the administration of any medication, that is, allergies, ideological/religious beliefs and/or refusal of treatment. The classification “other” was defined by the authors of this study as interventions related to prescription confirmation, change of prescribed drug formulation and replacement of a non-standardized drug for a standardized drug in the hospital’s pharmacotherapeutic guide.

After identifying the DRP and NOM and conducting the intervention, the process was evaluated by the application of the adapted pharmaceutical performance impact code proposed by Farré Riba et al. (2000) (Table 2).

Statistical analysis

Data was stored in Excel® 7.0 spreadsheets and statistical analyses were performed using Epi Info 3.5.1 (CDC, Atlanta, Georgia, USA). Results were presented in tables and included the description of absolute and relative frequency.

RESULTS

During the study period, 256 pharmaceutical interventions involving 155 patients were registered. Patients aged 60 years or older and male patients were involved in 50.78 and 51.56% of pharmaceutical interventions, respectively, while the young adult (17 to 29 years of age) age group underwent fewer interventions (8.30%) (Figure 1).

Most interventions took place in the Surgical Clinic Wards, Clinic Wards, and Adult Intensive Care Unit. The remainder hospital sectors had a lower rate of care, particularly the Maternity Ward which only performed two interventions for one patient (Table 3). The pharmaceutical interventions were mainly active, drug related, communicated verbally, had the physician as interlocutor and were accepted. Interventions that were not accepted mainly involved the indication for treatment initiation, suggestion of exchange and/or reduction of antimicrobial agent dose and dilution of drugs, most notably fentanyl, tramadol and amphotericin B deoxycholate.

Table 4 shows the distribution of DRP and NOM by hospital sectors. In interventions related to drugs (193/256), 401 DRP were identified, with an average of 1.60 ± 1.189 SD (minimum zero and maximum five) DRP per patient attended. Most frequent DRP were the likelihood of adverse effects and inadequate specified strength, dose and/or treatment length. For NOM, 248 events were observed, with an average of 1.0 ± 0.724 SD (minimum zero and maximum three) NOM per patient. Most frequent NOM were non-quantitative safety issues and untreated health issues.

Drugs related to DRP were classified using levels 1 and 2 of the Anatomical Therapeutic Chemical (ATC) classification

Table 1. Pharmaceutical interventions (PhI).

Form of detection	Active: when the pharmacy resident was the first professional to find the problem Passive: when the pharmacy resident was alerted by any other professional about a possible existing problem
Type	Related to medications taken Not related to medications taken
Interlocutor	Physician or nursing staff Other health professionals
Form of communication	Verbal Written Verbal and written
Intervention result	Accepted: change in the conduct of the interlocutor involved up to 72 hours after the PhI, Not accepted: interlocutor does not change their behavior within 72 hours after the PhI or non-responsive PhI.

Table 2. Pharmaceutical performance impact code (Farré Riba et al., 2000).

Optimization of pharmacological treatment (effectiveness)	
Indication	Indicate drug Discontinue drug Change to more effective drug
Dosage	Change treatment duration Change equivalent strength/interval of dosage
Route	Change to a more effective route Recommend the administration method
Preventive pharmaceutical interventions (toxicity)	
Adverse effects	Prevent allergic reaction Prevent adverse effects
Interactions	Confirm prescription Prevent pharmacological interaction
Route	Change to a safer route

system (WHO, 2012) and the main group involved in interventions was anti-infectives for systemic use. For the most frequent subgroup, classification was extended up to level 3 and the main subgroup was anti-bacterial for systemic use, most notably glycopeptides (Table 5).

The impact code (Table 6) was applied only in accepted pharmaceutical interventions related to drugs. Of these, 80.23% addressed the effectiveness of treatment, while 19.77% were preventive interventions for toxicity. Most interventions for effectiveness aimed at changing the specified strength or interval, while interventions related to toxicity were focused on confirming the prescription.

DISCUSSION

The high acceptance of pharmaceutical interventions shows pharmacists can perform effectively in patient care by taking responsibility for pharmacotherapy, ensuring compliance with the established treatment plan and

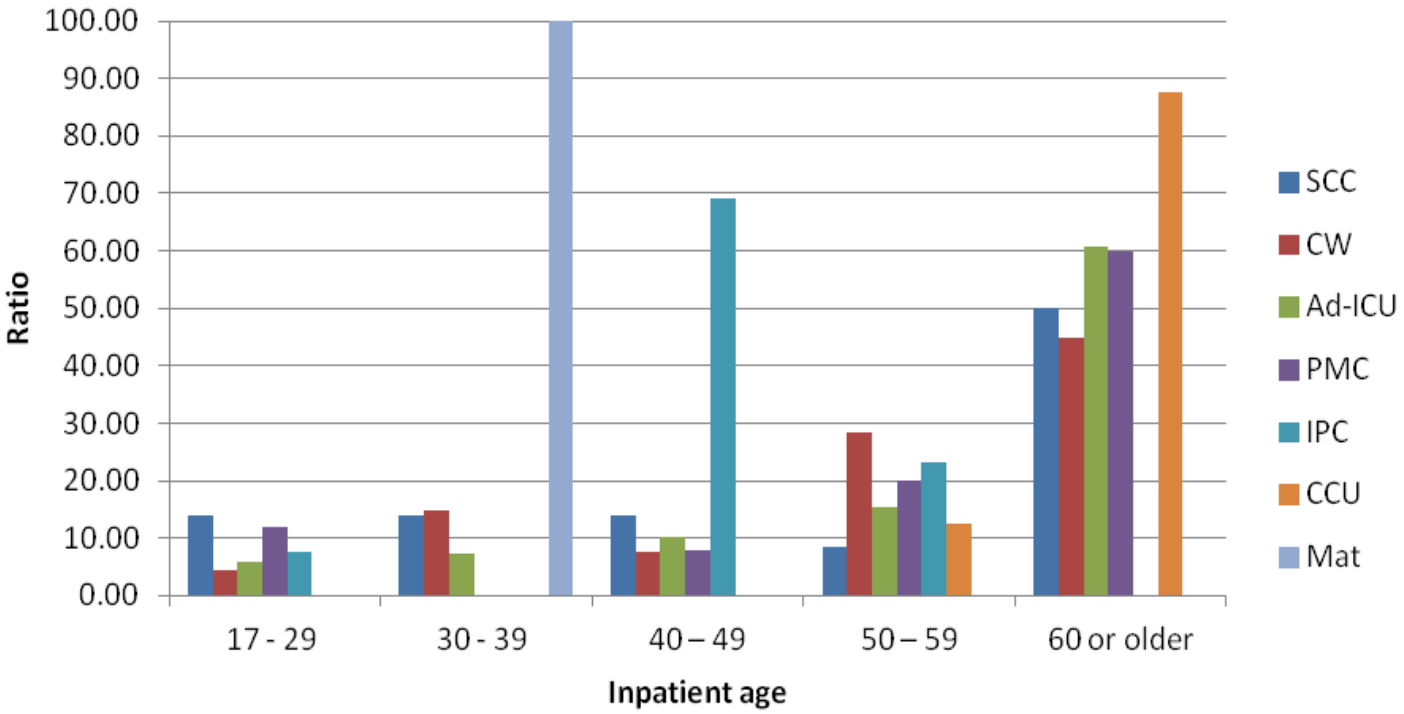

Figure 1. Age distribution of inpatients involved in 256 interventions according to the hospital sector. SCC: Surgical Clinic Wards; CW: Clinical Wards; Ad-ICU: Adult Intensive Care Unit; PMC: Prompt Medical Care; IPC: Infectious and Parasitic Disease; CCU: Coronary Care Unit; MAT: Maternity.

Table 3. Distribution of pharmaceutical interventions according to clinical.

Phl	SCC (%)	CW (%)	Ad-ICU (%)	PMC (%)	IPC (%)	CCU (%)	MAT (%)	Total	
								n	%
Detection	**28.13**	**26.17**	**26.95**	**9.77**	**5.08**	**3.13**	**0.78**	**256**	**100**
Active	72.22	80.60	84.06	84.00	38.46	87.50	50.00	198	77.34
Passive	27.78	19.40	15.94	16.00	61.54	12.50	50.00	58	22.66
Type	**28.13**	**26.17**	**26.95**	**9.77**	**5.08**	**3.13**	**0.78**	**256**	**100**
Drug related	59.72	80.6	79.71	88.00	84.61	75.00	100.0	193	75.39
Not drug related	40.28	19.4	20.29	12.00	15.39	25.00	-	63	24.61
Contact	**28.13**	**26.17**	**26.95**	**9.77**	**5.08**	**3.13**	**0.78**	**256**	**100**
Verbal	100.0	95.53	97.10	96.00	84.61	100.0	-	246	96.09
Other	-	4.47	2.90	4.00	15.39	-	100.00	10	3.91
Interlocutor	**28.13**	**26.17**	**26.95**	**9.77**	**5.08**	**3.13**	**0.78**	**256**	**100**
Physician	83.33	89.55	98.55	100.0	84.62	100.0	50.00	233	91.02
Other	16.67	10.45	1.45	-	15.38	-	50.00	23	8.99
Result	**28.13**	**26.17**	**26.95**	**9.77**	**5.08**	**3.13**	**0.78**	**256**	**100**
Accepted	81.94	89.55	91.30	92.00	100.0	100.0	100.0	228	89.06
Not accepted	18.06	10.45	8.70	8.00	-	-	-	28	10.94

SCC: Surgical Clinic Wards; CW: Clinical Wards; Ad-ICU: Adult Intensive Care Unit; PMC: Prompt Medical Care; IPC: Infectious and Parasitic Disease; CCU: Coronary Care Unit; MAT: Maternity.

Table 4. Distribution of DRP and NOM identified in pharmaceutical interventions.

Distribution	SCC (%)	CW (%)	Ad-ICU (%)	PMC (%)	IPC (%)	CCU (%)	MAT (%)	Total	
								n	%
DRP	**19.70**	**26.18**	**30.67**	**9.98**	**9.23**	**3.74**	**0.50**	**401**	**100**
Incorrect drug administration	0.75	2.49	3.99	0.75	2.00	0	0	40	9.98
Personal patient characteristics	0.25	0.50	0.25	0.25	0.25	0	0	6	1.50
Improper storage	0.25	0	0	0	0.25	0	0	2	0.50
Contraindication	0.25	0	0	0	0.50	0	0	3	0.75
ISDTD	4.49	4.99	6.73	2.24	1.50	0.75	0	83	20.70
Duplicity	0.50	0	0.50	0	0	0	0	4	1.00
Non-compliance with protocols	0.50	0	0	0	0.25	0.25	0	4	1.00
Drug interactions	1.25	0.25	2.24	0.50	0.75	0	0.25	21	5.24
Other health issues affecting treatment	0.50	2.74	1.75	0.25	0.50	0.50	0	25	6.23
Likelihood of adverse effects	4.24	4.74	7.98	1.75	2.00	0.75	0.25	87	21.70
Insufficiently treated health	4.74	6.98	2.99	2.49	0.25	1	0	74	18.45
Other	2.00	3.49	4.24	1.75	1.00	0.50	0	52	12.97
NOM	**22.98**	**28.23**	**29.44**	**10.08**	**6.05**	**2.82**	**0.40**	**248**	**100**
Untreated health issue	6.45	10.89	5.65	3.63	0.40	0.81	0	69	27.82
Effect of unnecessary drug	3.63	3.63	2.82	0.40	0.81	0.40	0	29	11.69
Non-quantitative ineffectiveness	2.02	2.42	4.03	1.61	1.21	0	0	28	11.29
Quantitative ineffectiveness	3.23	2.82	2.42	0	0	0.40	0	22	8.87
Non-quantitative safety issue	5.24	6.05	13.71	4.44	3.23	0.81	0.40	84	33.87
Quantitative safety issue	2.42	2.42	0.81	0	0.40	0.40	0	16	6.45

SCC: Surgical Clinic Wards; CW: Clinical Wards; Ad-ICU: Adult Intensive Care Unit; PMC: Prompt Medical Care; IPC: Infectious and Parasitic Disease; CCU: Coronary Care Unit; MAT: Maternity; ISDTD: Inadequate specified strength, dose and/or treatment duration.

preventing the occurrence of DRP and NOM. Most patients were aged 60 years or above, a characteristic that was also found in studies conducted by Al-Hajje et al. (2012), Jiang et al. (2012) and López et al. (2011). Elderly patients are known to be more vulnerable to DRP due to age-related physiological changes that may change pharmacokinetics and pharmacodynamic properties of drugs and frequent comorbidities that require the prescription of multiple drugs (Chan et al., 2012). These factors generate polypharmacy, which is one of the major determinants of many DRP aspects, such as adverse drug events, drug interactions and inappropriate drug selection (Chan et al., 2012; Elliot et al., 2012; Hanlon et al., 2004).

The hospital sector in which most interventions took place was the Adult Intensive Care Unit. In addition to greater interaction among pharmacists and other members of the healthcare team, the greater number of interventions in this clinic is justified by the characteristics of hospitalized patients, which comprise a greater number of related diseases and comorbidities, medication use, procedures and technologies, generating higher possibilities of pharmaceutical performance.

The pharmacy residents focused their activities in surgical clinics, medical clinic, infectious and parasitic disease clinic, prompt medical care, adult intensive care unit, and coronary care unit. Their participation in the related clinics of pediatric patients and maternity took place solely when requested for advice, thus justifying the few interventions taking place at maternity hospital.

Farrell et al. (2012) stated that a pharmacist who directly performs patient care tends to be more capable of initiating case discussions with physicians, conducting their own patient interview and even managing a multidisciplinary team. Also, the presence of the clinical pharmacist increases their contact with other healthcare professionals and allows for verbal interventions, as observed in this study.

Monitoring of patients conducted by the pharmacists enables them to make interventions in different areas of their duties. While the focus on medication related interventions was very clear, interventions not related to medications was divided into two parts. One of these parts involved performing biochemical and microbiological laboratory tests, which aided in the monitoring of patient clinical status and directing specific antibiotic therapy. This practice contributes to the rational use of antimicrobial agents and prevents the appearance of multi-resistant microorganisms. The other part was related to filling out specific documentation for dispensing medications, ensuring that the patient was given all drugs prescribed.

Pharmaceutical interventions should be documented in the patient medical record. However, this was rarely

Table 5. Therapeutic groups related to DRP identified in pharmaceutical interventions classified according to the Anatomical Therapeutical Chemical (ATC).

ATC	Therapeutic group levels 1 (N>10) and 2	N	%
A	Alimentary tract and metabolism	34	13.28
A.02	Drugs for acid disorders	22	8.59
B	Blood and blood forming organs	17	6.64
B.05	Blood substitutes and perfusion solutions	8	3.13
C	Cardiovascular system	33	12.89
C.09	Agents acting on the renin-angiotensin system	14	5.47
J	Anti-infectives for systemic use	122	47.66
J.01	Anti-bacterial for systemic use	92	35.94
J.02	Anti-mycotics for systemic use	30	11.72
N	Nervous system	31	12.11
N.02	Painkillers	10	3.91
N.03	Anti-epileptic	10	3.91
ATC	**Therapeutic group among anti-infectives for systemic use level 3 (N≥ 5)**	**N**	**%**
J.01.XA	Glycopeptides	37	40.22
J.01.DD	3rd generation cephalosporins	8	8.70
J.01.XB	Polymyxins	8	8.70
J.01.DH	Carbapenems	7	7.61
J.01.MA	Fluoroquilolones	7	7.61
J.01.XX	Oxazolidinones	5	5.43

observed in the present study. Only 10 cases had written interventions, four of them being medical records and six of them being registers in the prescription itself. This result can be explained in part by the newly instated practice of pharmacists making notes in medical records, added to the residents' lack of experience in documenting their actions. Another possible factor may be due to the resistance of professionals in registering pharmaceutical interventions in the patient record.

The professional involvement of physicians with the interlocutor of pharmaceutical interventions has already been demonstrated in other studies (Conce et al., 2006; Nunes et al., 2008; Torner et al., 2003). A close relationship between physicians and pharmacists ensures more rapid identification of prescription errors, possible adverse effects, DRP and NOM.

An 80% acceptance rate for pharmaceutical interventions was also noted in several studies (Al-Hajje et al., 2012; Arroyo et al., 2009; Conde et al., 2006; Soria et al., 2011; Torner et al., 2003). A study conducted by López et al. (2011) described a 100% acceptance rate. Significant acceptance rate values, such as the one found in this study, illustrate the role of the clinical pharmacists in ensuring compliance with the pharmacotherapeutic goals set by each patient. The reduction of antimicrobial specified strength was the target of unaccepted interventions in this study.

Inadequate specified strength, dosage and/or treatment duration (ISDTD) and probability of adverse effects (PAE) were the most frequently encountered DRP. This may occur because the study was conducted in a teaching hospital and most prescribers are resident doctors.

For a population similar to the one analyzed in this study, the group of drugs most associated with DRP was also the anti-infectives for systemic use (Arroyo et al., 2009; Conde et al., 2006; Farré Riba et al., 2000). The greater involvement of subgroup J.01.AX (glycopeptides) occurred due to frequent interventions in the adjustment of teicoplanin specified strength after three days of using double specified strength as the loading dose.

The impact code indicates the intervention rational and the benefit generated by the attention to patient regarding treatment effectiveness and/or toxicity. Pharmaceutical interventions enabling the optimization of pharmacological treatment provided to the patient influence the effectiveness. Effectiveness is considered to increase in events where intervention is motivated by subdosing, treatment omissions, improper drug selection, administration route or mode decreasing effectiveness, lack of treatment monitoring or existence of interactions impairing its effectiveness. Preventive pharmaceutical interventions enabling the risk reduction of medication use by the patient can lessen toxicity. Such risk is considered to exist if an intervention is motivated by overdosing, use of non-indicated drugs, modification of administration route to a safer one, detection of adverse reactions, allergies, interactions and prescription errors (Farré Riba et al., 2000).

Table 6. Impact of accepted pharmaceutical interventions.

Optimization of pharmacological treatment (effectiveness)	N (142)	% (80.23)
Indication	69	38.98
Indicate drug	31	17.51
Discontinue drug	17	9.60
Change to a more effective drug	21	11.86
Dose	55	31.07
Change specified strength/interval	53	29.94
Change treatment length	2	1.13
Route	18	10.17
Change to a more effective route	6	3.39
Recommend the administration method	12	6.78
Preventive pharmaceutical interventions (toxicity)	35	19.77
Adverse effects	19	10.73
Prevent allergic reaction	1	0.56
Prevent adverse effects	3	1.69
Confirm prescription	15	8.47
Interactions	8	4.52
Prevent drug interaction	8	4.52
Route	8	4.52
Change to a safer route	8	4.52

Despite the positive results presented here, in order to achieve a better analysis of the hospital pharmaceutical care service, issues such as cost reduction to the institution, hospitalized patient satisfaction and clinical outcome should be evaluated as well.

Conflict of interest

The authors declare no conflicts of interest.

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Vol. 9(2), pp. 33-37, 15 January, 2015
DOI: 10.5897/AJPP2014.4074
Article Number: B70583449877
ISSN 1996-0816

http://www.academicjournals.org/AJPP

African Journal of Pharmacy and Pharmacology

Full Length Research Paper

Vanillin reduces intestinal smooth muscle contractility

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Received 13 March, 2014; Accepted 7 January, 2015.

Vanillin consumption is common within the global population, often as a flavoring agent. Many vanillin-containing drinks claim a soothing effect on the body as a whole with some reports on their benefit in reducing gastrointestinal (GI) upset. In this study, vanillin was investigated for its ability to reduce rat ileal smooth muscle contractility induced by acetylcholine (Ach) and potassium chloride (KCl). A section of rat ileum was suspended in an organ bath containing Tyrode's solution. The tissue was stimulated using Ach or KCl and kept under 1 g tension at 37°C while continuously gassing with oxygen. Ileal smooth muscle contractility was studied in the absence and presence of vanillin. The results illustrated that vanillin ($1.4e^{-6}$ and $2.2e^{-6}$ M) hindered ileal smooth muscle contractility induced by both Ach and KCl. Vanillin ($1.4e^{-6}$ and $2.2e^{-6}$ M) also caused a rightward shift of the Ach concentration response curve and brought about a decrease (21.6 and 38.3%, respectively) in the maximum response. It also produced a rightward shift in the KCl dose response curve but without affecting the maximum response. These results indicate that vanillin counteracts Ach and KCl induced smooth muscle contractility in rat ileum. The results also suggest that vanillin prevented Ach induced contractility via non competitive inhibition kinetics. The reduction in KCl induced contractility also indicates that vanillin, at least partially, conveyed its effect through acting on ileal smooth muscle calcium channels.

Key words: Vanillin, acetylcholine, KCl, rat Ileum, smooth muscle contraction.

INTRODUCTION

Gastro intestinal (GI) motility results from complex interaction between the enteric nervous system, hormones and ileal smooth muscles (Andersson and Hedlund, 2002). Any imbalance between the interplay of these contributors will result in a loss of the normal physiologic rhythm, and in many cases, manifest clinically as a GI symptom (Kim et al., 2008). These symptoms include diarrhea, constipation, GI spasms and general GI discomfort (Samuels, 2009). In particular, GI spasms usually present as an unpleasant feeling of GI pain. Therapeutically, there are many remedies capable of relieving this pain, with the majority achieving their effect through inhibiting smooth muscle contractility (Kim et al., 2008; Samuels, 2009). Cellularly, smooth muscle

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contractions occur secondary to extra cellular stimulation when hormones or neurotransmitters bind membrane bound receptors. After initial receptor stimulation, the signal is relayed inside the cell were it transpires as an increase in free intracellular calcium ion (Ca^{+2}) leading to consequent activation of the actin and myosin fibers and hence muscle contraction (Andersson and Hedlund, 2002; Kim et al., 2008).

Vanillin is the primary component of the vanilla bean extract; it has a distinct flavour and aroma and is thus very often used as a flavouring agent. Therapeutically, vanillin has demonstrated an antioxidant and an antimicrobial effect (Burri et al., 1989). Another possible therapeutic benefit for vanillin is its use as an anti spasmodic remedy where individual reports have credited vanilla flavored drinks to possess such properties (Sinha et al., 2008). And while vanillin is not the only constituent in vanilla beans, it does present a suspect for this effect (Scharrer and Mosandl, 2001). One approach in determining whether plant extracts or chemical agents possess antispasmodic activity is to examine their effect on isolated ileal smooth muscle tissue. In this study, the clinical viability of vanillin as an antispasmodic remedy was investigated through examining its *in vitro* effect on rat ileum.

MATERIALS AND METHODS

Chemicals

Ach, KCl, vanillin and all tyrode solution constituents; NaCl, KCl, $CaCl_2$, $NaHCO_3$, NaH_2PO_4, $MgCl_2$ and glucose were obtained from Sigma-Aldrich.

Animals

Adult male Sprague Dawley rats (190 ± 10 g) were purchased from the animal facility at Jordan Applied University and housed at 20 to 24°C with free access to food and water. The rats were deprived of food (not water) for 24 h before the experiment to minimize ileal contents. All procedures concerning animals were carried out in accordance with Jordanian regulations for animal experimentation and care, and approved by the committee of institutional animal care and use (Protocol and Ethical approval memo number HS/KC/949 dated 7th of November, 2012). The study commenced on 1st of December, 2012 and lasted for a duration of 6 months. All experimentation was carried out at the Pharmacology research lab in the Faculty of Pharmacy at Isra University, Amman – Jordan.

Ileum preparation

On the day of experiment, rats were sacrificed by cervical displacement and one or two segments (1.5 to 2 cm) of ileum were dissected and freed of adhering mesentery. The pieces of ileum were cleaned from their luminal contents by flushing gently with a stream of Tyrode's solution using a 5 ml pipette. Tyrode solution contained 136.8 mM NaCl, 2.7 mM KCl, 1.3 mM $CaCl_2$, 0.14 mM NaH_2PO_4, 12 mM $NaHCO_3$, 0.5 mM $MgCl_2$ and 5.5 mM glucose. Ileal tissue was then mounted between two stainless steel hooks in a 40 ml tissue bath containing Tyrode solution being continuously gassed with oxygen. Temperature and pH were maintained at 37°C and 7.4. The lower hook was fixed at the bottom of the tissue bath and the upper one was connected to an isotonic transducer to measure forced smooth muscle contraction from base line (Harvard Transducer, UK). Each piece of tissue was placed under 1 g resting tension and equilibrated for 60 min prior to the execution of experimental protocols. During this period, the tissue was washed with Tyrode solution every 15 min. Ileum contractions were displayed and recorded on a Universal Harvard Oscillograph, (UK). The ileal contractions were initially induced by 15 mM of Ach or 10 mM of KCl. Dose response curves were then established in the presence of either Ach (0.1 to 2 mM) or KCl (1 mM to 1 M) non-cumulatively with a tissue contact time of one minute. Tissue was washed 3 times between each treatment. Further dose response curves were obtained after vanillin ($1.4\ e^{-6}$ and $2.2\ e^{-6}$) was added to the tissue bath.

Statistical analysis

All data were expressed as mean ± standard error of the mean (SEM). Results were analyzed using non linear regression (curve fit) with an extra sum-of squares F test comparison method for $logEC_{50}$ and hillslope values using GraphPad Prism 5 ® software.

RESULTS

Effect of vanillin on ileal contraction induced by Ach

The dose response curve for Ach was evaluated in the presence and absence of vanillin. Here, two concentrations of the vanillin were used, 1.4×10^{-6} and 2.2×10^{-6} M. The results clearly demonstrated that there was a concentration dependent rightward shift of the Ach dose response curve. Also, vanillin brought about a significant concentration dependent reduction in the maximal response (21.6 and 38.3% respectively; Figure 1).

Effect of vanillin on ileum's contraction induced by KCl

The dose response curve induced by KCl was also evaluated in the presence and absence of vanillin (1.4×10^{-6} and 2.2×10^{-6} M). Again, vanillin caused a concentration dependent rightward shift of the KCl dose response curve but this time without affecting the maximal response (Figure 2).

DISCUSSION

The results clearly showed that vanillin counteracted ileal smooth muscle contractility induced by both Ach and KCl, and thus indicated that vanillin could influence GI motility.

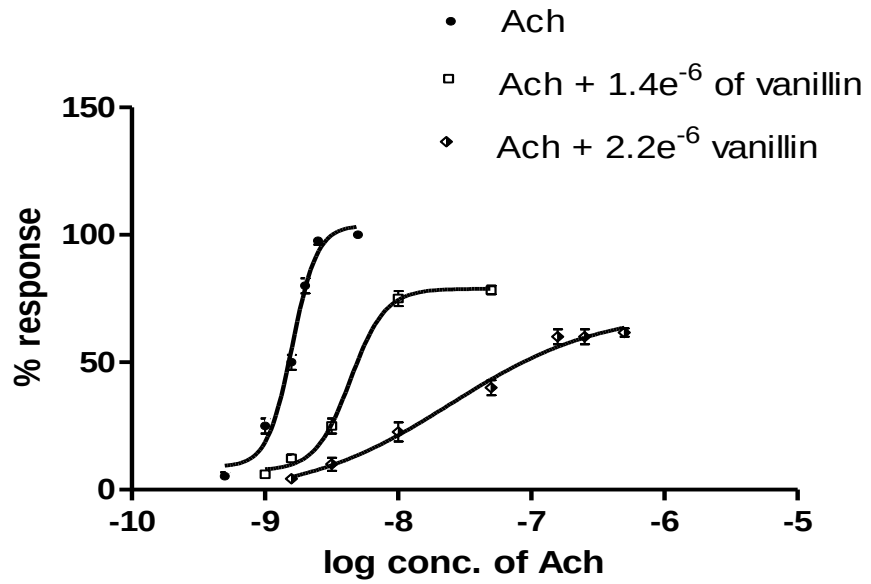

Figure 1. Effect of vanillin on rat ileum contraction induced Ach. Each point represents mean ± SEM of 3 observations. $LogEC_{50}$ for Ach = -8.840 for Ach alone, -8.221 in the presence of 1.4e-6 vanillin and -6.927 in the presence of 2.2e-6 vanillin. Hill Slope for Ach = 3.851 for Ach alone, 1.420 in the presence of 1.4e-6 vanillin and -6.927 in the presence of 2.2e-6 vanillin. $LogEC_{50}$ was significantly different for each data set ($P < 0.0001$). HillSlope was also significantly different for each data set ($P < 0.0001$).

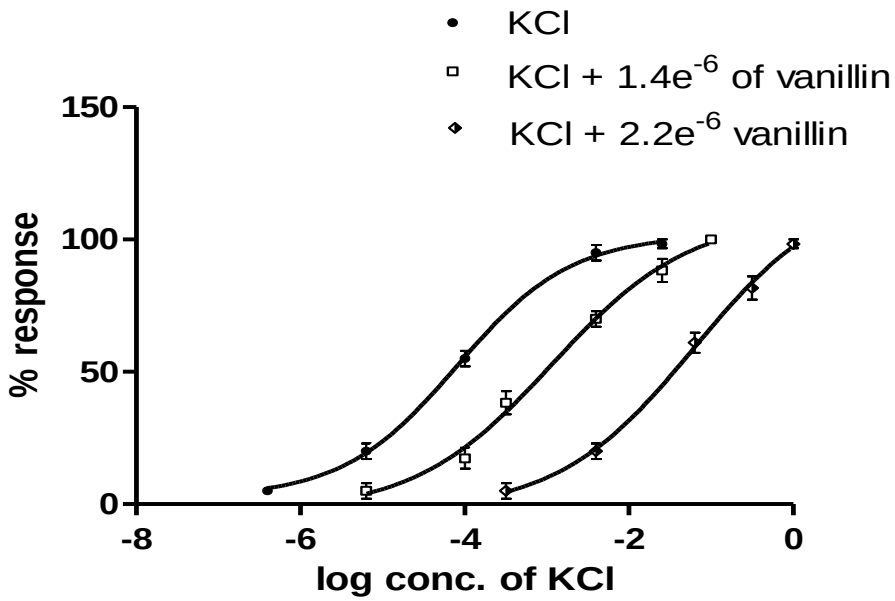

Figure 2. Effect of vanillin on rat ileum contraction induced by KCl. Each point represents mean ± SEM of 3 observations. $LogEC_{50}$ for KCl = -4.181 for KCl alone, -3.064 in the presence of 1.4e-6 vanillin and -1.511 in the presence of 2.2e-6 vanillin. $LogEC_{50}$ was significantly different for each data set ($P < 0.0001$). HillSlope was not significantly different for any of the data sets ($P > 0.05$).

To better place vanillin as an agent used for the treatment of gastro intestinal tract (GIT) disorders its exact mode of action needed to be determined. Vanillin's capacity to reduce ileal smooth muscle contractility displayed reversibility where washing allowed ileal tissue to regain normal responsiveness to the inducers (Gharib et al., 2007). This could be relevant to clinical application where reversibility allows obtaining desired antispasmodic effects without causing a sustained inhibition of contraction which may result in side effects. Also, the ability of vanillin to cause a rightward shift of the Ach dose response curve accompanied by a dose dependent decrease in the maximum response strongly suggests that it non-competitively inhibited Ach stimulation. This may perhaps occur through either indirectly blocking GI muscarinic receptors or through interfering with another down stream consequence of stimulating these receptors. Ach binds to M_2 and M_3 receptors in ileal tissue causing smooth muscle contraction through promoting calcium influx via receptor-operated calcium channels (Zhang et al., 2005). Ach also promotes inositol trisphosphate (IP_3) synthesis via phospholipase C activation which in turn increases calcium release from the sarcoplasmic reticulum (Coulson et al., 2004; Pacaud et al., 1996). Any step in this latter signal transduction pathway poses as a potential target for vanillin.

To further investigate how vanillin counteracted this ileal contraction, its effect was examined on KCl induced ileal smooth muscle contractility. Here, vanillin produced a rightward shift in the KCl dose response curve without affecting the maximum response. This indicated that vanillin was some how hindering the normal response of voltage gated Ca^{+2} channels where high potassium concentrations depolarizes smooth muscle cells with a resultant activation of the voltage dependent calcium channels present in rat ileum (Schneider et al., 2004). The opening of these channels in turn increases intracellular Ca^{+2} influx and causes smooth muscle contraction (Sadraei et al., 2013a; Godfraind et al., 1986; Sadraei et al., 2013b). It has been suggested that substances which inhibit KCl-induced smooth muscle contractility produce their effect via blocking voltage-gated Ca^{+2} channels (Gilani et al., 2001). Also, when high KCl concentration causes membrane depolarizing (Fujimoto and Mori, 2004), only sufficient intracellular Ca^{+2} levels will initiate muscle contraction (Zhang et al., 2005), which indicates that vanillin maybe interfering with final intracellular Ca^{2+} levels rather than preventing initial cell depolarizing. This notion is supported by the reversible non competitive inhibition displayed by vanillin on Ach induced ileal smooth muscle contractility. This suggests the involvement of voltage gated Ca^{2+} channels with the distant possibility of IP_3 and sarcoplasmic reticulum contribution.

Our findings are supported by Sadraei et al. (2013a), where they demonstrated that isovanillin, an isomer of vanillin, inhibited ileal contraction induced by both Ach and KCl and it was concluded that the mechanism of action of isovanillin was somehow different from that of simple muscarinic antagonism (Sadraei et al., 2013a). Although vanillin and isovanillin possess structural similarities as phenolic aldehydes, they may not necessarily behave similarly on ileal smooth muscles. This is verified by the fact that these isomers possess varying metabolic pathways where vanillin is metabolized by aldehyde oxidase and isovanillin is not, and rather metabolized by aldehyde dehydrogenase (Panoutsopoulos and Beedham, 2005). And although isovanillin may possess similar GI effects, vanillin remains the molecule of interest because of its wide use as a flavouring agent.

Conclusion

Taken together, our findings suggest that vanillin hinders ileal smooth muscle contractility. Vanillin is possibly causing this effect through interfering with the natural response of voltage gated Ca^{2+} channels and not via directly blocking muscarinic receptors. These results demonstrate that the potential usefulness of vanillin in the pharmaceutical industry is not limited to a flavouring agent but possibly as an antispasmodic remedy.

ACKNOWLEDGMENT

Acknowledgement and thanks goes to Al-Isra University for funding this research. Also, special thanks and gratitude to Mr. Mohammad Abu Shkhedem for all of his help with the animals used in this study.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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