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African Journal of Pharmacy and Pharmacology

Table of Contents: Volume 8, Number 5 8 February, 2014

ARTICLES

Research Articles

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Full Length Research Paper

Phytochemical screening, gas chromatography-mass spectrometry (GC-MS) analysis of phytochemical constituents and anti-bacterial activity of *Aerva lanata* (L.) leaves

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The present study is aimed to determine the phytochemical screening and anti-bacterial activity of the extracts (acetone, ethyl acetate and ethanol) of medicinal plant, *Aerva lanata* leaves against bacterial strains. Acetone, ethyl acetate and ethanol extracts of *A. lanata* leaves were prepared using Soxhlet apparatus. These extracts of *A. lanata* leaves were checked for their anti-bacterial activity by well diffusion, minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) techniques against bacterial strains. Preliminary phytochemical screening and gas chromatography-mass spectrometry (GC-MS) analysis of phytochemical constituents of *A. lanata* leaves were performed. Preliminary phytochemical screening of different extracts of *A. lanata* revealed the presence of alkaloids, proteins, amino acids, flavonoids, tannins, phenolic compounds, saponins, quinone, terpenes and coumarins. Among the three different extracts tested, acetone extract of *A. lanata* leaves showed maximum anti-bacterial activity. The bioactive components of acetone fraction of *A. lanata* leaves were evaluated by GC-MS analysis which showed the presence of sixteen chemical compounds. The extracts of *A. lanata* leaves have a broad spectrum of anti-bacterial activity and support the traditional use of these plants as medicines.

Key words: *Aerva lanata,* phytochemical screening, anti-bacterial activity, gas chromatography- mass spectrometry, minimal inhibitory concentration index.

INTRODUCTION

Plants have been widely used to treat various ailments, since ancient times. World Health Organization (WHO) has estimated that nearly 80% of the total population in the developing countries relies on medicinal plants for health care (Muruganantham et al., 2009). Therefore, such plants should be examined to understand their

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medicinal properties, safety and efficiency (Ellof, 1998).

Antibiotics are one of our most important weapons in fighting microbial infections and have greatly benefited the health-related quality of human life (Fransworth, 1993). Although, antibiotics have been widely used in last decades, the development of microbial resistance to them is also increased (Cohen, 1992). In order to overcome this problem, scientists deviated their research towards anti-microbial compounds of medicinal plants as an alternative solution. The demand for herbal medicine is due to their wide biological activities, higher safety compared to synthetic drugs and low cost (Grabley and Thiericke, 1999).

Aerva lanata is a medicinal plant and belongs to Amaranthaceae family. The whole plant of *A. lanata* is used as diuretic, anti-helminthic, anti-diabetic and expectorant and also used in the treatment of lithiasis (Gupta and Neeraj, 2004.). Traditionally, leaves of *A. lanata* are used as sap for eye-complaints; an infusion is given to cure diarrhea and kidney stone and the root is used in snake bite treatment. A leaf decoction preparation is used as gargle for treating sore throat and is also used in various complex treatments against guinea-worm. They are also used as an antidote for scorpion sting (Vijaya Kumar and Pulliah, 1998), spermatorrhoea and urinary troubles and as an anti-rheumatic (Kakrani and Saluja, 1994).

In addition to the traditional uses, *A. lanata* has a number of pharmacological activities including demulcent (Pullaiah and Naidu, 2003), immunomodulatory and anti-tumor activity (Nevin and Vijayammal, 2003.), anti-inflammatory (Vetrichelvan et al., 2000), diuretic (Udupihille and Jiffry, 1986), expectorant, hepatoprotective (Manokaran et al., 2008), nephroprotective (Shirwaikar et al., 2004), anti-diabetic (Vetrichelvan and Jegadeesan, 2002), anti-hyperglycemic (Deshmukh et al., 2008) , anti-microbial, cytotoxic (Chowdary et al., 2002), urolithiatic (Rao, 1985), hypoglycemic, anti-hyperlipidemic (Appia Krishnan et al., 2009), anti-helmintic and anti-parasitic (Anantha et al., 2010) and anti-asthmatic activities (Deepak Kumar et al., 2009; Rajesh et al., 2011). Considering the medicinal importance of *A. lanata*, an attempt has been made to investigate the phytochemical and anti-microbial activities of acetone, ethanol and ethyl acetate extracts from leaves of *A. lanata*. Furthermore, the phytochemical constituents were identified from acetone extract of this plant by using gas chromatography-mass spectrometry (GC-MS) analysis.

MATERIALS AND METHODS

Plant material collection

The plant *A. lanata* was collected in and around Salem, Tamil Nadu. The collected plant species was identified and confirmed (LOT.NO-68; A/series/F/AMA) by Dr. R. Selvaraj, Professor, Department of Botany, Annamalai University, Annamalai Nagar-608 002, Tamil Nadu, India. The plant was washed with running tap water and finally washed with distilled water to remove the dirt and dried under shade for 7 days.

Preparation of plant extracts

Thoroughly washed and dried leaves of *A. lanata* were then crushed gently to make it into powder by using mixer grinder. About 25 g of crushed powder was filled into the thimble and was extracted separately with different solvents such as acetone, ethyl acetate and ethanol in Soxhlet apparatus for 24 h. The solvent present in the extracts was allowed to evaporate in open air and the extracts were stored in refrigerator until further use.

Phytochemical screening

The different extracts of plant *A. lanata* were subjected to preliminary phytochemical screening by using standard procedures (Harborne, 1993; Trease and Evans, 2002) for the detection of alkaloids, proteins, amino acids, anthraquinone glycosides, flavonoids, carbohydrates, saponins, terpenes, coumarin, quinone, tannin and phenolic compounds.

Gas chromatography-mass spectrometry (GC-MS) analysis

GC-MS analysis of acetone extract of *A. lanata* leaves was performed by using Thermo GC-Trace ultra version 5.0 gas chromatography interfaced to Thermo MS DSQ II mass spectrometer instrument employing the following conditions: DB5-MS capillary standard non polar column (30 × 0.25 mm × 0.25 µm) and helium gas was used as a carrier gas at a constant flow rate of 1 ml/min. The oven temperature was kept at 70°C and was programmed to reach 260°C at a rate of 6°C/min. Mass range was 50 to 650 (m/z). The total running time was completed within 43 min. The chromatogram obtained from gas chromatography was then analyzed in mass spectrometry to get the mass of all fractions. The identification of phytochemical components was achieved through retention time and mass spectrometry by comparing the mass spectra of unknown peaks with those stored in Wiley 9 GC-MS library.

Anti-bacterial activity of plant extracts

Anti-bacterial activity of plant extracts was determined by agar well diffusion method against microbial type culture collection (MTCC) bacterial strains such as *Staphylococcus aureus* (MTCC 796), *Escherichia coli* (MTCC 443), *Klebsiella pneumoniae* (MTCC 109), *Salmonella typhi* (MTCC 733), *Proteus mirabilis* (MTCC 422), *Pseudomonas aeruginosa* (MTCC 6750) and *Bacillus subtilis* (MTCC 441). All the bacterial strains used in the present study were obtained from MTCC, Chandigarh, India. Muller-Hinton agar (MHA) (HiMedia, India) plates were prepared and 16 h old bacterial culture was swabbed uniformly and was allowed to dry for 5 min. Four wells of 5 mm in diameter were made in each of these plates by using a sterile cork borer. The extracts were prepared at a final concentration of 30 mg/ml by dissolving them in their respective solvents. The different concentrations of the plant extract (300, 600, 900 and 1200 µg) were loaded onto the wells and were allowed to

diffuse for 10 min. Then, the plates were incubated at 37°C for 24 h. After the incubation, diameter of inhibition zone around the well was measured and recorded.

Determination of minimal inhibitory concentration (MIC)

Each plant extracts (100 mg) were suspended in 1 ml of their respective solvents and were kept as stock solutions. These were subjected to double fold serial dilution in peptone water to obtain ten different concentrations ranging from 100 to 0.19 mg/ml and added to the respective culture tubes containing 2 ml of peptone water. These tubes were then inoculated with 100 µl of 16 h old broth culture of bacterial strains followed by incubation at 37°C for 24 h. Three tubes containing peptone water, peptone water and extract and peptone water and inoculum were used as control (John et al., 2011).

Determination of minimal bactericidal concentration (MBC)

The MBC of each extract was determined by sub-culturing 10 µl of the test dilutions from MIC tubes on to Muller Hinton agar plates. Plates were incubated for 24 h. The highest dilution that yielded low or no bacterial colony on the plate was recorded as MBC (Pavithra et al., 2010).

MIC index

The MIC Index (MBC/MIC) was calculated for each extract to determine whether an extract is bactericidal (MBC/MIC < 4) or bacteriostatic (MBC/MIC > 4) on growth of bacterial organisms (Chattopadhyay et al., 2007). The range of MIC Index value which is greater than 4 and less than 32 are considered as bacteriostatic (Cutler et al., 1994).

Statistical analysis

The experiments were repeated thrice and the average data were submitted to analysis of variance using ANOVA (Minitab version 15). A p value < 0.05 was considered statistically significant.

RESULTS

This study was designed to evaluate the phytochemical screening, GC-MS analysis of phytochemical constituents and anti-bacterial activity of *A. lanata*. Among the three extracts, acetone extract of leaves of *A. lanata* was found to have all the phytochemicals tested such as alkaloids, proteins and amino acids, anthraquinone glycosides, carbohydrates, flavonoids, saponins, coumarin, quinone, tannins and phenolic compounds. Ethyl acetate extract of leaf of plant showed the presence of flavonoids, terpenes, coumarin, tannins and phenolic compounds. Ethanol extract revealed the presence of alkaloids, proteins and amino acids, anthraquinone glycosides, flavonoids, terpenes, coumarin, quinone, tannins and phenolic compounds (Table 1).

GC-MS analysis of acetone extract of *A. lanata* leaves showed the presence of sixteen bioactive compounds that could contribute towards the medicinal properties to the plant (Figure 1 and Table 2). The first and predominant compound identified with less retention time (4.71) was (R)-(+)-ç-valerolactone (70.65%) whereas 5,14-di (*N*-butyl)-octadecane (1.47%) was the last compound identified which took longest retention time (44.83) for identification. The other prevalent compounds present in this extract were 9-octadecenoic acid (5.70%), 2-propynoic acid, methyl ester (5.46%) and 1-(2, 4, 6-trihydroxyphenyl)-3-(3-hydroxy-4-methoxy-6-ethylphenyl) propanone (4.24%). The remaining phytocompounds present in this extract were 2,3-trimethylsilyl-CC'-dimethyl-4,5-dicarbanido-hexaborane (1.67%), neophytadiene (1.62%), 5,14-di (N-butyl)-octadecane (1.47%), n-octacosane (1.31%), 2-hexadecen-1-ol, 3,7,11,15-tetramethyl-, [R-[R*,R*-(E)]]-phytol (1.28%), Trimethylamine borane (0.72%), 11-Hydroxy-8-oxo-13-tridecanolide (0.66%), n-heptacosane (0.58%), ethyl-2-bromo-3-hydroxy-3-methylpentanoate (0.43%), 1-hexadecene (0.43%), n-docosane (0.32%), and hexadecanoic acid, methyl ester (0.28%).

Acetone extract of *A. lanata* leaves exhibited the highest anti-bacterial activity against *P. mirabilis* followed by *S. aureus, S. typhi*, *B. subtilis, P. aeruginosa, K. pneumoniae* and *E. coli.* Ethyl acetate leaf extract of this plant showed anti-bacterial activity against *S. typhi, S. aureus* followed by *P. mirabilis, B. subtilis, E. coli, P.aeruginosa* and *K. pneumonia*e. Ethanol extract of leaf showed anti-bacterial activity against *E. coli* followed by *S. aureus*, *P. mirabilis, P. aeruginosa, S. typhi, K. pneumoniae* and *B. subtilis* (Table 3).

MIC value of acetone extract of *A. lanata* leaves was found to be 1.53 mg/ml against *S. aureus, P. mirabilis*, *S. typhi* and 3.06 mg/ml against *E. coli, K. pneumoniae, P. aeruginosa* and *B. subtilis.* MBC value of this extract was 6.12 mg/ml against *S. aureus, S. typhi, P. mirabilis* and 12.25 mg/ml against *B. subtilis* (Table 4). MIC index value of acetone extract of plant leaves showed that bactericidal activity against *S. aureus, S. typhi, P. mirabilis* and *B. subtilis* and bacteriostatic activity against *E. coli, K. pneumoniae* and *P. aeruginosa* (Table 7). MIC value of ethyl acetate extract of *A. lanata* leaves was found to be 1.53 mg/ml against *S. typhi* and 3.06 mg/ml against *S. aureus, P. mirabilis*, *B. subtilis* and 6.12 mg/ml against *E. coli, K. pneumoniae* and 12.25 mg/ml against *P. aeruginosa.* MBC value of this extract was 12.25 mg/ml against *S. aureus, S. typhi*, *B. subtilis* and 25 mg/ml against *K. pneumoniae, P. mirabilis* and 50 mg/ml against *E. coli* and *P. aeruginosa* (Table 5). MIC index value of this extract proved that they are found to be bactericidal against *S. aureus, K. pneumoniae, P. aeruginosa, B. subtilis* and *P. mirabilis* and bacteriostatic against *E. coli* and *S. typhi* (Table 7).

MIC value of ethanol extract of *A. lanata* leaves was

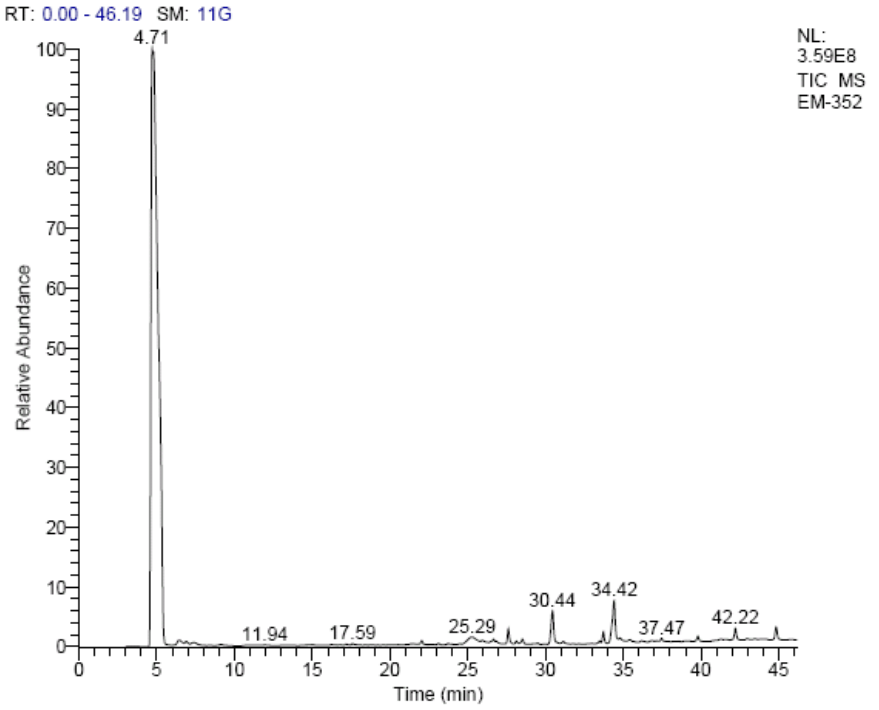


Figure 1. GC-MS chromatogram of acetone extract of *A. lanata* (L.) leaves.

found to be 1.53 mg/ml against *S. aureus, E. coli* and 3.06 mg/ml against *S. typhi, P. mirabilis* and 6.12 mg/ml against *K. pneumoniae* and *P. aeruginosa.* MBC value of this extract was 6.12 mg/ml against *S. aureus* and 12.25 mg/ml against *E. coli, S. typhi, P. mirabilis*, *B. subtilis* and 25 mg/ml against *K. pneumoniae* and *P. aeruginosa* (Table 6). MIC index value of this extract was also found to be bactericidal against *S. aureus, E. coli, P. aeruginosa, B. subtilis, S. typhi* and *P. mirabilis* and bacteriostatic against *K. pneumoniae* (Table 7). All these results proved that the acetone, ethyl acetate and ethanol extract of *A. lanata* leaves have bactericidal and also bacteriostatic effect on pathogenic microorganisms like against *S. aureus, E. coli, P. aeruginosa, B. subtilis, S. typhi* and *P. mirabilis* and *K. pneumoniae* and these effects were found to be varied from each solvent.

DISCUSSION

The antimicrobial activity of plants have been screened because of their great medicinal relevance with the recent years, infections have increased to a great extent and resistant against antibiotics becomes an ever in-creasing therapeutic problem. The presence of antifungal and antimicrobial substances in the higher plants are well established as they have provided a source of inspiration for novel drug compounds as plants derived medicines have made significant contribution towards human health.

Knowledge of the phytochemical constituents of plants is desirable, not only for the discovery of therapeutic agents, but also because such information may be of value in disclosing new sources of economic materials such as tannins, oils, gums, flavonoids, saponins, essential oils precursors for the synthesis of complex chemical substances (Akrout et al., 2010).

The results of phytochemical screening of *A. lanata* leaves clearly imply that the strength of active principle depends upon the use of solvent besides the type of plant species to achieve the positive results. The identified phytochemical compounds have many biological properties. For instance, hexadecanoic acid, methyl ester which is a palmitic acid compound found to be an anti-oxidant, hypocholesterolemic, nematicide, pesticide, lubricant activities and hemolytic 5-alpha is a reductase inhibitors. These results are strengthened by the findings of Sermakkani and Thangapandian (2012) who observed the presence of this compound in methanol extract of *Cassia italica* leaves.

9-Octadecenoic acid is a linoleic acid compound and reported to have an anti- inflammatory, nematicide, insectifuge, hypocholesterolemic, anti-cancer, hepatoprotective, anti-histaminic, anti-acne, anti-arthritic and anti-eczemic properties. Similarly, the presence of 9-octadecenoic acid was observed in the ethanolic root of *Plumbago zeylanica* by Ajayi et al. (2011). 2-Hexadecen-1-ol,

Table 1. Preliminary phytochemical screening of different solvent extracts of *A. lanata* (L.) leaves.

Active component /Test	Acetone	Ethyl acetate	Ethanol
Alkaloids			
Mayer's test	+	-	+
Dragendorff 's test	+	-	+
Hager's test	+	-	+
Wager's test	+	-	+
Protein and amino acid			
Millon's test	+	+	+
Ninhydrin test	+	+	+
Biuret test	+	+	+
Anthraquinone glycosides			
Borntrager's test	+	-	+
Flavonoids			
Shinoda 's test	+	+	+
Tannins and phenolic compounds			
Ferric chloride test	+	+	+
Lead acetate test	+	+	+
Gelatin test	+	+	+
Carbohydrates			
Molisch's test	+	-	-
Barfoed's test	+	-	-
Fehling's test	+	-	-
Saponins			
Frothing test	+	-	-
Terpenes			
Chloroform + Conc. H_2SO_4	+	-	+
Coumarin			
10% NaOH Solution	+	+	+
Quinone			
Conc. H_2SO_4	+	+	+

+ = Positive; - = Negative.

3, 7, 11, 15-tetramethyl-[R-[R*, R*-(E)]]- phytol is a diterpene compound which is reported to possess anti-microbial, anti-cancer, anti-inflammatory and diuretic agent (Praveen Kumar et al., 2010).

Similarly, the presence of phytol was observed in the leaves of *Lantana camara* (Mariai et al., 2011; Sathish and Manimegalai, 2008) and *Mimosa pudica* (Sridharan et al., 2011). Phytol was observed to have anti-bacterial activities against *S. aureus* by damaging the cell membranes which in turn causes leakage of potassium ions from bacterial cells. Phytol is a key acyclic diterpene alcohol that is a precursor for vitamins E and K_1. It is used along with simple sugar or corn syrup as a hardener in candies (Inoue et al., 2005). Plants are important source

Table 2. Phytochemical constituents of acetone extract of leaves of *A. lanata* (L.) by GC-MS spectra.

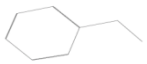
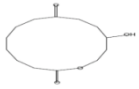
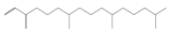
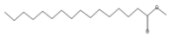
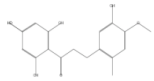
Retention time (min)	Name of the compound	Molecular formula	MW	Area (%)	Compound structure
4.71	(*R*)-(+)-ç-valerolactone	$C_5H_8O_2$	100	70.65	
5.26	2-Propynoic acid, methyl ester	$C_4H_4O_2$	84	5.46	
6.44	Trimethylamine borane	$C_3H_{12}BN$	73	0.72	
7.38	Ethyl 2-bromo-3-hydroxy-3-methylpentanoate	$C_8H_{15}BrO_3$	238	0.43	
22.05	1-Hexadecene	$C_{16}H_{32}$	224	0.43	
25.29	2,3-Trimethylsilyl-CC'-dimethyl-4,5-dicarbanido-hexaborane	$C_7H_{20}B_4Si$	176	1.67	
26.65	11-Hydroxy-8-oxo-13-tridecanolide	$C_{13}H_{22}O_4$	242	0.66	
27.61	Neophytadiene	$C_{20}H_{38}$	278	1.62	
29.49	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	270	0.28	
30.44	1-(2,4,6-Trihydroxyphenyl)-3-(3-hydroxy-4-methoxy-6 methylphenyl)propanone	$C_{17}H_{18}O_6$	318	4.24	
33.72	2-Hexadecen-1-ol, 3,7,11,15-tetramethyl-,[R-[R*,R*-(E)]]- phytol	$C_{20}H_{40}O$	296	1.28	
34.42	9-Octadecenoic acid	$C_{18}H_{34}O_2$	282	5.70	
37.47	n- Docosane	$C_{22}H_{46}$	310	0.32	
39.79	n- Heptacosane	$C_{27}H_{56}$	380	0.58	

Table 2. Contd.

42.22	n- Octacosane	$C_{28}H_{58}$	394	1.31	
44.83	5,14-Di (N-Butyl) octadecane	$C_{29}H_{60}$	408	1.47	

Table 3. Antibacterial activity of different solvent extracts of *A. lanata* (L.) leaves against bacterial (MTCC) strains by well diffusion method.

Bacterial strain	Zone of inhibition (mm)											
	Plant extracts concentration (µg/well)											
	Acetone				Ethyl acetate				Ethanol			
	300	600	900	1200	300	600	900	1200	300	600	900	1200
S. aureus	10	12	13	15	6	9	12	13	6	8	10	12
E. coli	6	7	8	9	6	8	10	11	8	10	12	14
K. pneumoniae	8	10	12	15	-	-	10	12	-	-	10	12
S. typhi	10	11	12	13	10	12	14	15	-	8	10	12
P. mirabilis	12	14	15	16	8	9	10	12	6	8	10	12
P. aeruginosa	8	10	12	14	-	-	8	10	6	8	10	11
B. subtilis	8	10	12	14	6	8	10	12	-	-	10	11

Table 4. Minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of acetone extracts of *A. lanata* (L.) leaves.

Bacterial strain	Minimal inhibitory concentration (MIC)									
	Plant extract concentration (mg/ml)									
	100	50	25	12.25	6.12	3.06	1.53	0.76	0.38	0.19
S. aureus	-	-	-	-	-	-	β	+	+	+
E. coli	-	-	-	-	-	β	+	+	+	+
K.pneumoniae	-	-	-	-	-	β	+	+	+	+
S. typhi	-	-	-	-	-	-	β	+	+	+
P. mirabilis	-	-	-	-	-	-	β	+	+	+
P.aeruginosa	-	-	-	-	-	β	+	+	+	+
B. subtilis	-	-	-	-	-	β	+	+	+	+
Minimal bactericidal concentration (MBC)										
S. aureus	-	-	-	-	**B**	+	+	+	+	+
E. coli	-	-	**B**	+	+	+	+	+	+	+
K. pneumoniae	-	-	**B**	+	+	+	+	+	+	+
S. typhi	-	-	-	-	**B**	+	+	+	+	+
P. mirabilis	-	-	-	-	**B**	+	+	+	+	+
P.aeruginosa	-	-	**B**	+	+	+	+	+	+	+
B. subtilis	-	-	-	**B**	+	+	+	+	+	+

Table 5. Minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of ethyl acetate extracts of *A. lanata* (L.) leaves.

Bacterial strain	Minimal inhibitory concentration (MIC)									
	Plant extract concentration (mg/ml)									
	100	50	25	12.25	6.12	3.06	1.53	0.76	0.38	0.19
S. aureus	-	-	-	-	-	β	+	+	+	+
E. coli	-	-	-	-	β	+	+	+	+	+
K.pneumoniae	-	-	-	-	β	+	+	+	+	+
S. typhi	-	-	-	-	-	-	β	+	+	+
P. mirabilis	-	-	-	-	-	β	+	+	+	+
P.aeruginosa	-	-	-	β	+	+	+	+	+	+
B. subtilis	-	-	-	-	-	β	+	+	+	+
Minimal bactericidal concentration (MBC)										
S. aureus	-	-	-	B	+	+	+	+	+	+
E. coli	-	B	+	+	+	+	+	+	+	+
K.pneumoniae	-	-	B	+	+	+	+	+	+	+
S. typhi	-	-	-	B	+	+	+	+	+	+
P. mirabilis	-	-	B	+	+	+	+	+	+	+
P.aeruginosa	-	B	+	+	+	+	+	+	+	+
B. subtilis	-	-	-	B	+	+	+	+	+	+

+ = Growth; - = no growth; β = MIC value and B = MBC value.

Table 6. Minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of ethanol extracts of *A. lanata* (L.) leaves.

Bacterial strain	Minimal inhibitory concentration (MIC)									
	Plant extract concentration (mg/ml)									
	100	50	25	12.25	6.12	3.06	1.53	0.76	0.38	0.19
S. aureus	-	-	-	-	-	-	β	+	+	+
E. coli	-	-	-	-	-	-	β	+	+	+
K.pneumoniae	-	-	-	-	β	+	+	+	+	+
S. typhi	-	-	-	-	-	β	+	+	+	+
P. mirabilis	-	-	-	-	-	β	+	+	+	+
P.aeruginosa	-	-	-	-	β	+	+	+	+	+
B. subtilis	-	-	-	-	-	β	+	+	+	+
Minimal bactericidal concentration (MBC)										
S. aureus	-	-	-	-	B	+	+	+	+	+
E. coli	-	-	-	B	+	+	+	+	+	+
K.pneumoniae	-	-	B	+	+	+	+	+	+	+
S. typhi	-	-	-	B	+	+	+	+	+	+
P. mirabilis	-	-	-	B	+	+	+	+	+	+
P.aeruginosa	-	-	B	+	+	+	+	+	+	+
B. subtilis	-	-	-	B	+	+	+	+	+	+

+ = Growth; - = no growth; β = MIC value and B = MBC value.

Table 7. Antibacterial activity of different solvent extracts of *A. lanata* (L.) in terms of MIC index (MBC/MIC).

Bacterial strain	MIC index		
	Acetone	Ethyl acetate	Ethanol
S. aureus	4.0	4.0	4.0
E. coli	8.16	8.16	4.0
K. pneumoniae	8.16	4.0	8.0
S. typhi	4.0	8.0	4.0
P. mirabilis	4.0	4.0	4.0
P.aeruginosa	8.0	4.0	4.0
B. subtilis	4.0	4.0	4.0

of potentially useful compounds for the development of new chemotherapeutic agents. *In vitro* evaluation of plants for anti-microbial property is the first step towards achieving the goal for developing eco-friendly management of infectious disease of humans by search for new biomolecules of plant origin (Samy et al., 2008; Mohana et al., 2008).

On comparison of three different *A. lanata* leaves extract, acetone extract showed the highest anti-bacterial activity against the bacterial strains. Senthilkumar et al. (2011) reported that the bark acetone, isopropanol and hexane extract of pomegranate possess anti-bacterial activity against *S. typhi* and *S. paratyphi* A isolates and also proved that among three solvents, acetone and hexane extracts showed better efficiency than isopropanol extract.

The increase of antibiotic resistance of microorganisms to conventional drugs has necessitated the search for new, efficient and cost effective ways for the control of infectious diseases. The results of different studies provide evidence that some medicinal plants might indeed be potential sources of new anti-bacterial agents (Kone et al., 2004). The use of medicinal plants is part of the Indian tradition. Many local regions all over India have a great variety of vegetation used by the local population to treat and prevent diseases (Bonyadi et al., 2009).

Conclusion

From this study it is concluded that leaves of *A. lanata* have a broad spectrum of anti-bacterial activity and supports the traditional use of these plants as medicines. This study demonstrated that herbal medicine can be as effective as modern medicine to combat pathogenic microorganisms and overwhelming the antibiotic resistance. Further studies are needed with this plant on its isolation, structural elucidation of bioactive compounds and about its *in vivo* toxic effects in experimental animals to formulate a new drug for regular practice.

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Full Length Research Paper

Evaluation of antioxidant capacity of the aqueous extract of *Cynara scolymus* L. (Asteraceae) *in vitro* and in *Saccharomyces cerevisiae*

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The present study evaluated the antioxidant capacity of the aqueous extract of the leaves of *Cynara scolymus* on *Saccharomyces cerevisiae* strains, proficient and deficient in antioxidant defenses, and by *in vitro* methods with 1,1-diphenyl-2-picrylhydrazyl (DPPH•), 2,2'-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS•$^{+}$), inhibition of hydrogen peroxide, lipid peroxidation, formation of nitric oxide, and removal of the hydroxyl radical. A simultaneous quantitative analysis to total phenolics, flavonoids and hydrolysable tannin was also performed, and according to the results obtained, phenolic compounds (661.15 ± 20.11 mg GAE/g of E), flavonoids (123.96 ± 11.47 mg R/g of E) and hydrolysable tannin (14.25 ± 3.18 mg GAE/g of E) were detected in the aqueous extract of *C. scolymus*. The aqueous extract at study showed high antioxidant capacity in all antioxidant methods *in vitro* and exhibited significant antioxidant capacity to protect strains of *S. cerevisiae* from oxidative damage induced by hydrogen peroxide. The analysis of the correlation made between the content of phenolic compounds and the different antioxidants *in vitro* methods, indicated that these compounds are mainly responsible for the antioxidant capacity of the aqueous extract of *C. scolymus*. Therefore, this study suggests that the aqueous extract of leaves of *C. scolymus* is a great natural source of compounds with antioxidant capacity.

Key words: Antioxidant capacity, *Cynara scolymus*, *Saccharomyces cerevisiae*, phenolic compounds.

INTRODUCTION

Reactive oxygen (ROS) and nitrogen (RNS) species, as well as other free radicals are potentially capable of producing damage in various biological macromolecules (DNA, proteins and lipids) playing an important role in the etiology of various diseases such as cancer, diabetes, cardiovascular diseases and premature aging (Kryston et al., 2011; Bhattacharya et al., 2011; Liochev, 2013). Some enzymatic antioxidant mechanisms promote the protection of human cells against the harmful effects of free radicals, but these antioxidant mechanisms may not be enough to combat oxidative stress, which results from the imbalance between production and elimination of free radicals (Jensen, 2003). Accordingly, certain amounts of antioxidant supplements are necessary to ensure the balance of reactive species derived from oxygen and nitrogen produced by pathophysiological metabol

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(Dizdaroglu et al., 2002; Sailaja Rao et al., 2011).

Over the last decade, interest in the search for antioxidants from natural sources has increased and various studies by the scientific community have reported the importance of fruits and vegetables in the prevention of chronic diseases resulting from oxidative stress (Gulcin, 2012). The plants have a rich source of molecules with antioxidant potential, as phenolic compounds, carotenoids, vitamins, flavonoids and terpenoids (Kumar et al., 2012; Ranilla et al., 2010). Therefore, greater attention is turned to the antioxidants of natural origin, which may act by inhibiting lipid peroxidation and/or neutralizing reactive oxygen species and nitrogen, resulting in the modulation of oxidative stress (Fadel et al., 2011).

Cynara scolymus L. (Asteraceae) is a native plant of the Mediterranean (North Africa and southern Europe) and popularly known in Brazil as artichoke. Regardless of its origin in regions of subtropical climates, the *C. scolymus* is cultivated worldwide because of its nutritional benefits and medicinal properties (Lattanzio et al., 2009). Since ancient times, the *C. scolymus* has been cited as a drug in traditional medicine and in much of the world it is attached to beneficial effects in diseases of the biliary tract, digestive action, and help in the treatment of scurvy, anemia and antiesclerotic effect (Gebhardt, 1997; Kraft, 1997; Kucukgergin et al., 2010). The *C. scolymus* is a plant used for medicinal purposes and is considered a healthy food because of its nutritional composition and phytochemistry, which contain minerals, proteins, phenolic derivatives including caffeoylquinic acid (chlorogenic acid and 1.5 dicaffeoylquinic cinarina), tannins, flavonoids and sesquiterpenes in fewer quantities, and various aliphatic acids, especially hydroxy acids (lactic, malic, glycolic) (Pandino et al., 2011; Bundy et al., 2008). Several pharmacological and preclinical stu-dies using the aqueous extract of leaves of *C. scolymus* demonstrated antibacterial, anti-HIV, hepato-protective, hepatostimulant, hypocholesterolemic, choleretic, diuretic and antioxidant effects (Joy and Haber, 2007; Miccadei et al., 2008; Lutz et al., 2011; Qiang et al., 2012).

The interest in working with medicinal plants is the therapeutic value they have and because there are few studies on the antioxidant capacity of *C. scolymus*. Thus, this study aimed at providing scientific evidence of the antioxidant capacity of the aqueous extract of leaves of *C. scolymus* through various tests. Tests were conducted to study the antioxidant capacity in *Saccharomyces cerevisiae* proficient and deficient in antioxidant defenses, evaluation of antioxidant capacity *in vitro* method for 1,1-diphenyl-2-picrylhydrazyl (DPPH•), 2,2'-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS•+), inhibition of hydrogen peroxide and lipid peroxidation (TBARS) removal of the hydroxyl radical (OH•), and inhibition of nitric oxide (NO). Furthermore, a quantitative study on the content of total phenolics, flavonoids and hydrolysable tannin was conducted to assess the contribution of these phenolics to the antioxidant capacity of the aqueous extract of *C. scolymus*.

MATERIALS AND METHODS

Reagents

Folin-ciocalteau reagent, aluminum chloride, potassium iodide, 2,2-diphenyl-1-picrylhydrazyl radical (DPPH•), 2,2'-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), hydrogen peroxide, thiobarbituric acid, 2,2'-azobis(2methylpropionamidine)dihydrochloride (AAPH), trichloroacetic acid, n-butanol, 2-deoxyribose, malonaldehyde, ferrous sulfate, phosphate buffer, phosphoric acid, sodium nitroprusside, Griess reagent, rutin, quercetin, gallic acid were purchased from Sigma (St. Louis, MO). All other reagents were of analytical grade.

Plant material and preparation of extract

The leaves of *C. scolymus* were provided by the processing industry of medicinal plants active life, located in the southeastern of Teresina city, Piauí, in August, 2012. The leaves of *C. scolymus* were dried at room temperature for 2 days and then crushed, milled in a knife mill to obtain 100 g and subsequently subjected to an extraction with water for 10 min by infusion (1/10, plant/solvent). After the treatment, the aqueous filtrate was frozen in liquid nitrogen and concentrated by lyophilization overnight for 3 days in order to obtain the aqueous extract of *C. scolymus* (AECS) in powder form. The yield of the extract under study was calculated by the following equation.

$$\text{Yield } (\%) = (A_1 \times 100) / A_2$$

Where A_1 corresponds to the AECS lyophilized and A_2 correspond to the powdered dried plant material used for extraction with water.

Determination of total phenols

The total phenolic content of AECS was determined according to Singleton et al. (1999) by Folin-Ciocalteu spectrophotometric method, which involves the reduction of Folin-Ciocalteu reagent by phenolic compounds with concomitant formation of a blue colored complex. An aliquot of 200 µl of AECS at 1000 µg/ml was mixed with 500 µl of Folin-Ciocalteu reagent. Then the solution was stirred for 1 min and added 200 µl of sodium carbonate (15%). The mixture was allowed to stand and the absorbance was measured after 2 h of reaction in spectrophotometer at wave length from 750 nm (nanometers). The analysis was performed in triplicate and results were expressed in mg gallic acid equivalents per gram of extract (mg GAE/g E) using the calibration curve of gallic acid

($y = 0.121x + 0.011$, $r^2 = 0.9819$).

Determination of total flavonoid

Total flavonoid content was determined by the formation of a complex of aluminum flavonoid using the methodology of Oyedemi et al. (2012). An aliquot of 200 µl of AECS (1000 µg/ml) was mixed with 200 µl of 20% aluminum chloride and two drops of acetic acid. The mixture was allowed to stand and the absorbance was measured after 40 min of reaction using spectrophotometer in length from 415 nm. The analysis was performed in triplicate and results were expressed as total flavonoid rutin milligram equivalent per gram of extract (mg R/g E) using the calibration curve of rutin

($y = 0.0182x - 0.004$, $r^2 = 0.9949$).

Determination of hydrolysable tannin

The hydrolysable tannin content was determined from AECS using the potassium iodide test (Saad et al., 2012). To 3 ml of the aqueous extract of AECS at 1000 µg/ml, 1 ml of a saturated solution of potassium iodide was added and the mixture left to stand at room temperature for 40 min. The absorbance was measured in a spectrophotometer at 550 nm. The analysis was performed in triplicate and results were expressed in mg equivalent of gallic acid per gram of extract (mg GAE/g E) using the calibration curve of gallic acid ($y = 0.121x + 0.011$, $r^2 = 0.9819$).

Antioxidant capacity by DPPH•

It was done as per the methodology described by Silva et al. (2005) with some modifications. Stock solution of AECS (250 µg/ml), of DPPH• (40 µg/ml), and of the standard solutions of gallic acid (250 µg/ml), quercetin (250 µg/ml) and rutin (250 µg/ml) were prepared. Concentrations of 25, 50, 100, 150 and 200 µg/ml of AECS and standards were prepared by dilution. The reaction mixture (0.3 ml of extract with 2.7 ml of a stock solution of DPPH•) was incubated at room temperature for 30 min and the absorbance was measured in a spectrophotometer at 517 nm. The antioxidant evaluation was performed in triplicate and the absorbance values were converted into percentage of antioxidant capacity (% AC) by the following equation.

% AC = [(Control Abs. – Extract Abs.) × 100] / Control Abs

Where, Control Abs. is the initial absorbance of the ethanol solution of DPPH•, and Extract Abs. is the absorbance of the reaction mixture (DPPH• + extract). The inhibitory effective concentration (EC_{50}) needed to extract the DPPH radical• reduced by 50% was determined by probit regression (Locatelli et al., 2009).

Antioxidant capacity by ABTS•+ method

In order to determine the antioxidant capacity by the method of $ABTS^{\bullet+}$, method described by Re et al. (1999) was used with modifications. Initially, a radical cation $ABTS^{\bullet+}$ was formed from the reaction of 5 ml of a 7 mM $ABTS^{\bullet+}$ solution with 88 µl of 2.45 mM potassium persulphate ($K_2S_2O_8$) solution and incubated at room temperature in absence of light for 16 h. After some time, a solution of $ABTS^{\bullet+}$ was diluted in ethanol to obtain a solution with absorbance of 0.70 (± 0.05) to 734 nm. The final concentrations of 25, 50, 100, 150 and 200 µg/ml of the AECS, gallic acid, quercetina and rutin were prepared. In dark environment, they were transferred at a rate of 40 µl of each sample for test tubes with 1960 µl of the radical $ABTS^{\bullet+}$. The absorbance readings, at times 1, 4 and 6 min, was done in a spectrophotometer at 734 nm and results were expressed in TEAC values (Trolox Equivalent Antioxidant Capacity) from a standard curve prepared with Trolox ($r^2 = 0.991$). The standard curve was prepared by measuring the decrease in absorbance of the solution $ABTS^{\bullet+}$ with different concentrations of the Trolox (0.1 and 2 mM).

Inhibition capacity of hydrogen peroxide (H_2O_2)

The ability to inhibit H_2O_2 was determined according to the method of Ruch et al. (1989) with some modifications. A solution of H_2O_2 (40 mM) and of AECS was formed in the same concentrations of DPPH• and ABTS•+ test in phosphate buffer (pH 7.4). 3.5 ml of AECS were added to 0.5 ml of H_2O_2 and the values of the absorbances at time 10 min was determined in a spectrophotometer at 230 nm. The absorbance of the mixture of the extract with H_2O_2 was converted to percent inhibition of hydrogen peroxide by the following equation.

% Inhibition of H_2O_2 = [(Control Abs. – Extract Abs.) × 100] / Control Abs.

Where, Control Abs. is the initial absorbance of the solution of H_2O_2 and Extract Abs. is the absorbance of the reaction mixture (H_2O_2 + extract).

Evaluation of the capacity to inhibit lipid peroxidation (TBARS)

The method used to evaluate the action of AECS in inhibiting lipid peroxidation, a major damage caused by reactive oxygen and nitrogen derivatives, was performed by the determination of substances reactive to thiobarbituric acid, known as TBARS method (Rosa et al., 2011). This method was used to measure the antioxidant capacity of AECS in a medium rich in lipids (Guimaraes et al., 2010). The lipid-rich substrate used was a homogenate of egg yolk 1% (w/v) in 50 mM phosphate buffer (pH 7.4). An aliquot of 0.5 ml of the substrate was sonicated and homogenized with 0.1 ml of AECS in different concentrations (25, 50, 100, 150 and 200 µg/ml). Lipid peroxidation was induced by adding 0.1 ml of AAPH (dihydrochloride of 2,2'-azobis-2 metilpropinamide 0.12 M). The reactions were performed for 30 min at 37°C. After cooling, samples (0.5 ml) were centrifuged in 0.5 ml of trichloroacetic acid (15%) at 1,200 × g for 10 min. An aliquot of 0.5 ml of the supernatant was added to 0.5 ml of thiobarbituric acid (0.67%) and heated at 40°C for 15 min. After heating, 2 ml of n-butanol were added to each test tube, then the tubes were shaken and centrifuged at 2000 rpm for 10 min. The absorbance of the samples was measured using a spectrophotometer at 532 nm. The results were expressed as a percentage of TBARS formed from AAPH only control (induced). Rutin, quercetin, and gallic acid were used as positive control at concentrations of 200 µg/ml.

Evaluation of the capacity of inhibiting the formation of hydroxyl radical (OH•)

The formation of hydroxyl radical from Fenton's reaction was measured using oxidative degradation of 2-deoxyribose (Moravia et al., 2011). The principle of the test is to quantify the degradation product of 2-deoxyribose in malonaldehyde (MDA) by its condensation with thiobarbituric acid (TBA). The reaction was initiated by the addition of Fe^{2+} (6 mM $FeSO_4$) to the solution containing 5 mM of 2-deoxyribose, 100 mM of H_2O_2 and 20 mM phosphate buffer (pH 7.4). To determine the antioxidant AECS against the hydroxyl radical, the AECS different concentrations (25, 50, 100, 150 and 200 µg/ml) were added to the system before addition of Fe^{2+}. The reactions were performed for 15 min at room temperature and stationed by adding phosphoric acid, 4% (v/v) followed by TBA 1% (w/v in 50 mM NaOH). The solutions were heated for 15 min at 95°C and then cooled to room temperature. The absorbance was measured at 532 nm and results were expressed as equivalents of MDA formed by Fe^{2+} and H_2O_2. Rutin, quercetin, and gallic acid were used as positive control at concentrations of 200 µg/ml.

Evaluation of ability to inhibit formation of nitric oxide (NO)

In this test, the nitric oxide (NO) is produced from the decomposition of sodium nitroprusside (SNP) in 20 mM phosphate buffer (pH 7.4). Once generated, NO interacts with oxygen to produce nitrite ions, which were measured by the Griess reaction (Basu and Hazra, 2006). The reaction mixture (1 ml) containing 10

Table 1. *Saccharomyces cerevisiae* strains used in antioxidant evaluation.

Description	Genotype	Deficiency in enzymatic defenses	Origin
EG103 (Sod wt)	MATa leu2-3,112 trp1-289 ura3-52	None	Edith Gralla, Los Angeles
EG118 (Sod1Δ)	Sod1::URA3 all other markers as EG103	Cu-Zn superoxide dismutase (cystosolic)	Edith Gralla, Los Angeles
EG110 (Sod2Δ)	Sod2::TRP1 all other markers as EG103	Mn superoxide dismutase (mitocondrial)	Edith Gralla, Los Angeles
EG133 (Sod1ΔSod2Δ)	Sod1::URA3 Sod2::TRP1 double mutant all other markers as EG103	Superoxide dismutase cystosolic and mitocondrial	Edith Gralla, Los Angeles
EG223 (Cat1Δ)	EG103, except Cat1:: TRP1	Catalase cystosolic	Edith Gralla, Los Angeles
EG (Sod1ΔCat1Δ)	EG103, except Sod1:: URA3 and Cat1::TRP1	Cu-Zn superóxido dismutase e catalase citosólica	Edith Gralla, Los Angeles

mM of SNP in phosphate buffer and EACS at concentrations of 25, 50, 100, 150 and 200 µg/ml, was incubated at 37°C for 1 h. An aliquot of 0.5 ml was taken and homogenized with 0.5 ml of Griess reagent. The absorbance of the chromophore was measured at 540 nm in a spectrophotometer. The percentage inhibition of NO production was determined by comparing the absorbance values of the negative control (only 10 mM SNP) and preparations of substances used in the test. The results were expressed as percentage of nitrite formed by SNP. Rutin, quercetin, and gallic acid were used as positive control at concentrations of 200 µg/ml.

Antioxidant capacity in *S. cerevisiae* cells

To evaluate the antioxidant capacity of the AECS against oxidative damage induced by hydrogen peroxide, strains of *S. cerevisiae* proficient (Sod wt) and deficient (Sod1Δ, Sod2Δ, Sod1ΔSod2Δ, Cat1Δ, Sod1ΔCat1Δ) were used in antioxidant defense system. The methodology used was of the central disk, according to Fragoso et al. (2008), with modifications. The strains of *S. cerevisiae* proficient and deficient in superoxide dismutase (Sod) and catalase (Cat) were kindly provided by the research group in Genetic toxicology of Federal University of Rio Grande do Sul (UFRGS) and are described in Table 1.

Concentrations of 25, 50, 100, 150 and 200 µg/ml of AECS were used in this study. Three types of treatment for antioxidant evaluation were performed using the strains in the stationary phase (2×10^8 cells/ml) of *S. cerevisiae*: pre-treatment, co-treatment and post-treatment. In the pre-treatment, the concentrations of the extract were first added to a hard filter paper in center of plate yeast extract-peptone-dextrose (YEPD) and three hours later stressor hydrogen peroxide was added. In co-treatment the concentrations were added simultaneously to stressor hydrogen peroxide in disk filter paper. In the post-treatment hydrogen peroxide was first added and three hours later, the drug concentration. After 48 h incubation at 37°C the halo of growth inhibition in millimeters (mm) was measured from the edge of the disc filter paper to the beginning of cell growth. The values can range from 0 mm (full growth) to 30 mm (no growth), these values being the size of a petri dish. All treatments were performed in triplicate. At each assessment, the strains were streaked on YEPD plate containing the center of a disc filter paper, to which 10 µl extract is added and hydrogen peroxide (40 mM). A control with only hydrogen peroxide was carried out for all strains of *S. cerevisiae*.

Statistical analysis

The antioxidant result is the value of mean ± standard deviation (SD) of three experiments, and each experiment was performed in triplicate for each treatment. Statistical analysis was performed using analysis of variance (ANOVA) and significant differences between means were determined by Tukey's test, using the GraphPad Prism 5.01. The values of $p < 0.05$ were considered significantly different. To evaluate the relationship between total phenolic content and antioxidant capacity, Pearson correlation coefficient was calculated with 95% confidence using the GraphPad Prism 5.01.

RESULTS AND DISCUSSION

Determination of phenols, flavonoids and hydrolysable tannin

With 18% of yield, the results of the phytochemical analysis showed the quantitative values of AECS 661.15 ± 20.11 mg GAE/g E to phenolic compound, 123.96 ± 12.47 mg R/g E to flavonoids and 14.25 ± 3.18 mg GAE/g E to hydrolysable tannin. These results demonstrate that the AECS has high quantities of phenolic compounds. Other studies have performed the quantification of phenolic compounds and flavonoids in methanolic and ethanolic extract of leaves of *C. scolymus*, however, in smaller quantities compared to our results for both phenolic and flavonoids compounds (Llorach et al., 2002; Wang et al., 2003; Gouveia and Castilho, 2012; Nassar et al., 2013).

The content of phenolic compounds found in plants may vary during processing steps such as growing, harvesting, storage and technological procedures used (Gil-Izquierdo et al., 2001; Fratianni et al., 2007; Lombardo et al., 2010). The *C. scolymus* is a plant rich in phenolic compounds among which are caffeic, coumarin, hydroxybenzoic and hydroxycinnamic, ferulic and gallic (Hammouda et al., 1991; Fratianni et al., 2007; Seneviratne and Kotuwegedara, 2009; Nassar et al., 2013; Abu-Reidah et al., 2013; Zan et al., 2013; Palermo et al., 2013). The phenolic compounds present in plants have received great attention because of their antioxidant properties and they can potentially interact with biological systems and play an important role in anticancer, anti-inflammatory and antimicrobial activity (Abu-Reidah et al., 2013; Wang et al., 2003). The

Table 2. Antioxidant capacity of the aqueous extract of *Cynara scolymus*, rutin, quercetin and gallic acid by the DPPH• method.

Concentration (µg/ml)	*Cynara scolymus*	Antioxidant capacity (%)		Gallic acid
		Rutin	Quercetin	
25	11.26±1.07[abc]	25.37±2.15	55.99±2.15	89.87±0.83
50	27.02±1.75[bc]	32.65±1.77	79.59±0.97	92.95±0.10
100	49.69±1.85[bc]	37.96±0.36	81.50±0.25	93.19±0.06
150	72.10±1.06[bc]	60.85±6.33	81.87±0.21	93.71±0.15
200	83.40±0.19[c]	64.22±1.76	82.18±0.15	93.95±0.47
CE_{50}	100.70	111.96	10.25	1.20

[a]significant (P<0.05) in relation to rutin. [b] significance (P<0.05) in relation to quercetin. [c]significance (P<0.05) in relation to gallic acid.

Table 3. Trolox equivalent antioxidant capacity (TEAC) of the aqueous extract of *Cynara scolymus*, rutin, quercetin and gallic acid.

Sample	TEAC, mM of Trolox		
	1 min	4 min	6 min
Cynara scolymus	3.45±0.26[c]	4.53±0.15[c]	5.88± 0.11[c]
Rutin	1.45±0.10	1.75±0.10	1.89±0.05
Quercetin	3.76±0.11	5.01±0.23	6.05±0.25
Gallic acid	8.03±0.13	12.84±0.08	13.21±0.03

[a]significance (P<0.05) in relation to rutin. [b]significance (P < 0.05) in relation to quercetin. [c]significance (P < 0.05) in relation to gallic acid.

antioxidant properties are attributed to flavonoids due to their hydroxyl groups that can act as free radical scavengers, reducing agents and metal chelation (Agati et al., 2012). Flavonoids are natural chemical constituents which may be found in larger quantities and play an important role in the pharmacological properties attributed to *C. scolymus* (Häusler et al., 2002; Zhu et al., 2004; Pandino et al., 2011). Among the flavonoids already found in extracts of *C. scolymus* may be cited luteolin, apigenin, myricetin, quercetin and rutin (Zan et al., 2013; Palermo et al., 2013).

Antioxidant capacity by DPPH• method

The DPPH• is a stable organic radical which has been widely used in studies to evaluate the antioxidant capacity of plant extracts or pure compounds. In this method, the antioxidant capacity is determined by the analysis of the decrease of absorbance of DPPH• solution at 517 nm in a spectrophotometer, in which the DPPH radical of purple color is reduced to form $DPPH_2$ of yellow (Mishra et al., 2012). Therefore, the AECS showed good antioxidant capacity by reducing DPPH• radical in a concentration-dependent manner (Table 2), wherein the values in percent of the antioxidant capacity of AECS at concentrations of 100, 150 and 200 µg/ml were significantly superior ($p < 0.05$) when compared to the values of antioxidant capacity of rutin at the same concentrations. When the antioxidant capacity of AECS is compared with the positive control quercetin at the concentrations of 25, 50, 100 and 150 µg/ml, it is observed that the values were significantly lower ($p < 0.05$). In contrast, at the concentration of 200 µg/ml, the AECS presented antioxidant capacity higher than quercetin. Compared to the control gallic acid, the values of antioxidant capacity at the concentrations of 25, 50, 100, 150 and 200 µg/ml were significantly lower ($p < 0.05$). The results of the EC_{50} of AECS, rutin, quercetin and gallic acid are shown in Table 2, being observed that the AECS was more effective in reducing of DPPH• radical than the control rutin.

The evaluation of antioxidant by DPPH• method has already been accomplished by Llorach et al. (2002) with the AECS using only the concentration of 10 µg/ml and with the reaction time of 1 h. In another study by Wang et al. (2003), it was shown that the extracts of three different varieties of *C. scolymus* presented good antioxidant capacity against DPPH• radical, which was attributed to the phenolic compounds with correlation $r^2 = 0.96$. Zan et al. (2013) using the AECS also achieved satisfactory results for the antioxidant capacity by the method of DPPH•.

Antioxidant capacity by the method of ABTS•+

Like DPPH•, the method ABTS•+ or TEAC is widely used to evaluate the antioxidant capacity of a variety of substances including plant extracts. The TEAC values of the AECS were obtained at three different times and compared to the TEAC of positive control rutin, quercetin and gallic acid (Table 3). As shown in Table 3, the values of TEAC of the AECS for the times of 1, 4 and 6 min were significantly ($p < 0.05$) higher when compared with the TEAC of the positive control rutin. Regarding the TEAC values of quercetin and gallic acid for the times 1, 4 and 6 min, it was observed that the TEAC values of the AECS

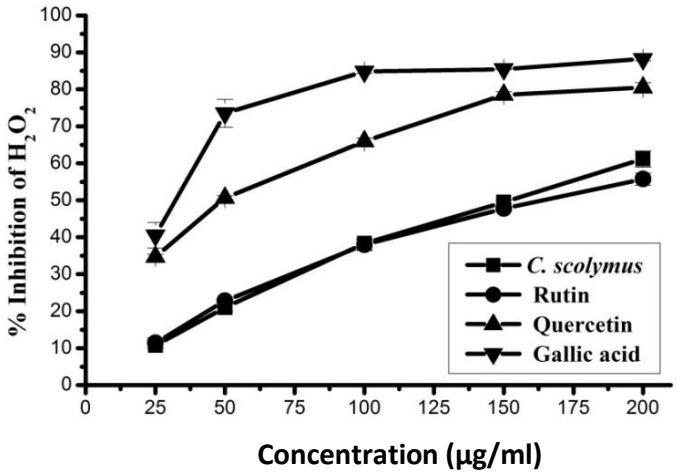

Figure 1. Percent inhibition of hydrogen peroxide from the aqueous extract of *Cynara scolymuse* of positive controls rutin, quercetin and gallic acid.
Each value represents the mean ± standard deviation (*n* = 3).

were lower, however, only relative to gallic acid was observed significant difference ($p < 0.05$). The TEAC value takes into account the capacity of a substance to react with ABTS•+ radical (Arts et al., 2004). Thus, this study demonstrated that the AECS was capable of reacting with the ABTS•+ radical, showing antioxidant capacity better than rutin. Wang et al. (2003) using only the concentration of 10 µg/ml also showed antioxidant activity against ABTS•+ radical. Betancor-Fernández et al. (2003) also evaluated the antioxidant capacity of the AECS by the TEAC method and the results obtained were similar to those in this study.

Inhibition capacity of H_2O_2

Hydrogen peroxide is a type of ROS which can be formed *in vivo* by many oxidizing enzymes, through the membranes and slowly oxidize a large number of biological molecules (Halliwell, 1991). The capacity of AECS to inhibit the hydrogen peroxide was determined according to the method of Ruch et al. (1989) and the results were 10.75 ± 0.61, 21.0 ± 0.97, 38.34 ± 0.89, 49.50 ± 0.46 and 61.18 ± 2.20% for concentrations of 25, 50, 50, 100, 150 and 200 µg/ml, respectively (Figure 1). The AECS was able to inhibit the hydrogen peroxide in a concentration-dependent manner, and the results of AECS were significantly lower ($p < 0.05$) in relation to results of quercetin and gallic acid. Furthermore, the results of AECS at concentrations higher than 100 µg/ml were superior to those of rutin, which showed values 11.47 ± 1.42, 22.83 ± 0.54, 37.98 ± 1.57, 47.78 ± 0.33 and 55.73 ± 1.60% of inhibition of hydrogen peroxide in concentrations of 25, 50, 50, 100, 150 and 200 µg/ml, respectively. No other study that addresses the action of AECS in the inhibition of the hydrogen peroxide was found, which makes this study the first.

Inhibition of lipid peroxidation (TBARS)

Free radicals are responsible for the chain reactions that cause lipid oxidation, inducing functional loss of biomembranes and various types of damage to essential biological molecules such as proteins and nucleic acid bases and, thus, having been implicated in the pathogenesis of various diseases (Niki, 2009). The TBARS method has been widely used to evaluate the extent of the *in vitro* lipid peroxidation, in which the oxidation of unsaturated fatty acids occurs from a source rich in lipids (Niki, 2010). Thus, the AECS at concentrations of 25, 50, 50, 100, 150 and 200 µg/ml was able to prevent lipid peroxidation by reduction of the levels of TBARS in 83.5, 83.95, 84.57, 86.56 and 87.6% (Figure 2). Similar results were obtained with the controls rutin, quercetin and gallic acid, which decreased the levels of TBARS in 85.25, 87.13 and 85.15%, respectively (Figure 2). As the positive controls, rutin, quercetin and gallic acid, the AECS significantly ($p < 0.05$) reduced the levels of TBARS formed by AAPH.

The percentage of inhibition of peroxidation shown in this study decreased according to the increase in the concentration of AECS. Besides, the concentration of 200 µg/ml presented a result of inhibition of lipid peroxidation greater than the controls rutin, quercetin, and gallic acid. Thus, the AECS may be considered as a good inhibitor of lipid peroxidation in this experimental model. In addition, studies that have used the TBARS method for evaluation of the inhibition of lipid peroxidation by AECS were not found. However, one study was conducted with AECS, whose capacity to inhibit lipid peroxidation was demonstrated through ferric thiocyanate method (Wang et al., 2013).

Inhibition of hydroxyl radical (OH•)

Among the ROS, hydroxyl radicals are the more reactive, being that these radicals can react with a wide variety of biomolecules and consequently induce severe cell damage and mutation, cell death, carcinogenesis and aging (Li et al., 2013). In the present study, the hydroxyl radicals were generated from the Fenton reaction, and as shown in Figure 3, the AECS at concentrations of 25, 50 , 100, 150 and 200 µg/ml was able to significantly remove the hydroxyl radical ($p < 0.05$) (Figure 3). At the concentration of 200 µg/ml, the positive controls rutin, quercetin and gallic acid presented the values 59.02, 52.77 and 64.75% of inhibition of the hydroxyl radical, respectively (Figure 3). According to these results, the AECS may be considered a good hydroxyl radical inhibitor.

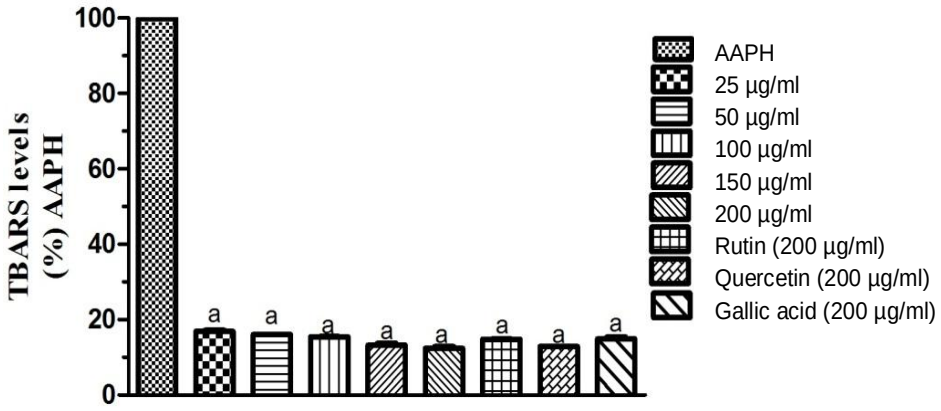

Figure 2. Inhibition of TBARS formed *in vitro* by AECS, rutin, quercetin, and gallic acid. Each value represents the mean ± standard deviation (n = 3). The significance (P <0.05) is relative to vehicle AAPH.

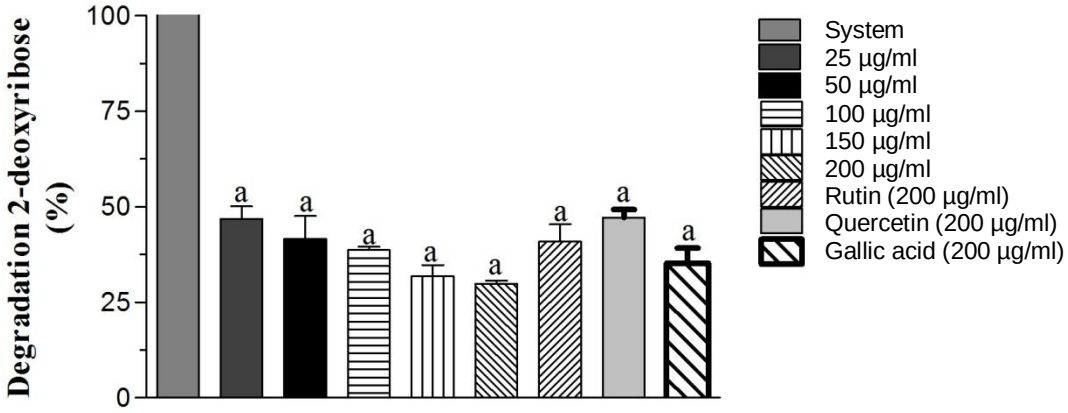

Figure 3. Inhibiting the formation of hydroxyl radical by AECS, rutin, quercetin and gallic acid. Each value represents the mean ± standard deviation (n = 3). The significance (P <0.05) compared to the System (does not contain the AECS).

inhibitor. The innovator character of this study can be expressed by the absence of reports that evaluate the antioxidant capacity of AECS in the inhibition of the hydroxyl radical.

Inhibition of nitric oxide (NO)

Nitric oxide is considered species reactive derivative of nitrogen, which if produced in excess can contribute to the development of pathologies such as chronic inflammation, cancer and autoimmune diseases (Muntane et al., 2013). The measure of antioxidant capa-city by the elimination of nitric oxide was based on the principle that the sodium nitroprusside (SNP) in aqueous solution at physiological pH spontaneously generates nitric oxide, which interacts with oxygen to produce nitrite ions (NO_2^-), which can be calculated by means of the Griess reagent. Thus, the AECS showed inhibitory capacity of nitric oxide generated by sodium nitroprusside, as for the concentrations of 25, 50, 100, 150 and 200 µg/ml, the values in percentage were 73.17, 77.46, 78.95, 79.42 and 80.18%, respectively (Figure 4). These outcomes also demonstrate a concentration-dependent and significantly ($p < 0.50$) different inhibitory capacity of AECS relative to SNP. When the inhibition of nitric oxide is compared with the controls rutin (79.25%), quercetin (76.8%) and gallic acid (74.62%), the concentrations of 150 and 200 mg/ml of AECS exhibited superior results than the positive controls. Thus, the extract of the *C. scolymus* at study can be considered as a good inhibitor of nitric oxide. Like the methods for evaluating of inhibition of

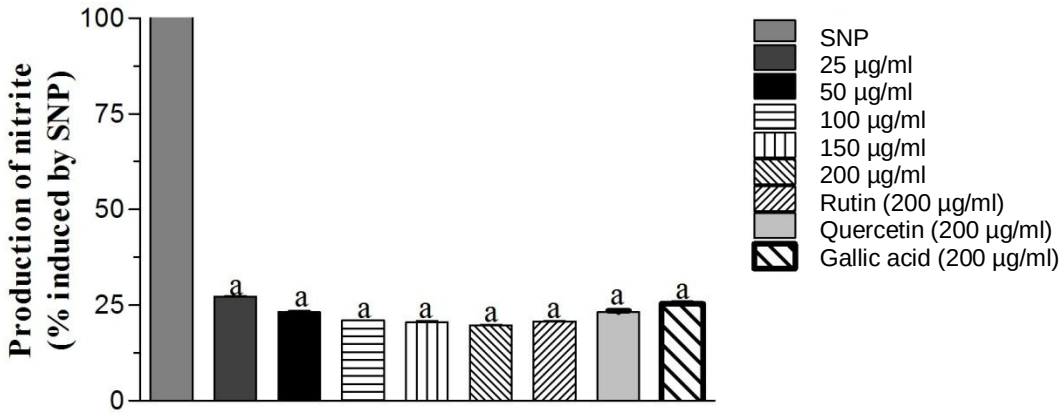

Figure 4. Inhibition of nitric oxide by AECS, rutin, quercetin, and gallic acid. Each value represents the mean ± standard deviation (n = 3). The significance (P <0.05) compared to SNP.

Table 4. Pearson correlation coefficient (r) between the content of phenolic compounds and antioxidant capacity.

Correlation	*r*	*r* (%)
Total phenols *vs*. DPPH•	0.989	98.9
Total phenols *vs*. ABTS•+	0.992	99.2
Total phenols *vs*. Inhibition of H_2O_2	0.983	98.3
Total phenols *vs*. TBARS	0.984	98.4
Total phenols *vs*. Hydroxyl	0.960	96.0
Total phenols *vs*. Nitrito	0.854	85.4

Table 5. Pearson correlation coefficient (r) between the different evaluation methodologies for antioxidant capacity.

Correlation	*r*	*r* (%)
DPPH• *vs*. ABTS•+	0.999	99.9
DPPH• *vs*. Nitrite	0.899	89.9
DPPH• *vs*. TBARS	0.966	96.6
DPPH• *vs*. Hydroxyl	0.971	97.1
DPPH• *vs*. Inibition of H_2O_2	0.996	99.6
ABTS•+ *vs*. Nitrito	0.943	94.3
ABTS•+ *vs*. TBARS	0.996	99.6
ABTS•+ *vs*. Hidroxila	0.875	87.5
ABTS•+ *vs*. Inhibition of H_2O_2	0.991	99.1
Nitrite *vs*. TBARS	0.785	78.5
Nitrite *vs*. Inibition of H_2O_2	0.925	92.5
Nitrite *vs*. Hidroxila	0.893	89.3
Hydroxyl *vs*. TBARS	0.964	96.4
Hydroxyl *vs*. Inhibition of H_2O_2	0.960	96.0

hydrogen peroxide and hydroxyl radical, the study presents itself as the first report on the evaluation of AECS in the inhibition of nitric oxide.

Correlation between the total phenolic content and antioxidant capacity

Phenolic compounds represent an important class of antioxidant compounds which are found in many plants. In this context, the correlation between the concentration of total phenolics and antioxidant capacity has been widely studied involving extracts of plants (Mulinacci et al., 2004; Wang et al., 2003; Llorach et al., 2002; Toma et al., 2013). Studies involving extracts of *C. scolymus* demonstrated that there is a linear correlation between phenolic content and antioxidant capacity (Mulinacci et al., 2004; Toma et al., 2013). In order to determine the contribution of the phenolic content in AECS on antioxidant capacity, the Pearson correlation coefficient (r) was determined. According to the results, strong positive correlations were observed between the concentration of phenolic content and antioxidant capacity of all antioxidants *in vitro* methods (Table 4). Therefore, the results suggest that phenolic compounds may exert an important contribution to *in vitro* antioxidant capacity of the AECS. The highest correlations were observed for the *in vitro* antioxidant methods DPPH• and ABTS•+, in which the two organic radicals are easily reduced in the presence of phenolic compounds. Phenolic compounds have been mainly responsible for the antioxidant capacity of natural extracts and probably due to their redox properties, they act as reducing agents, hydrogen donors and singlet oxygen scavengers (Letelier et al., 2008).

Although the mechanisms of reactions are different for the antioxidant evaluation methods employed in this work, a correlation between the *in vitro* results for the antioxidant capacity was determined and it is shown in Table 5. Positive correlations (r > 0) were observed between the antioxidants results, indicating that the antioxidants *in vitro* methods provide comparable values when they are used to determine the antioxidant capacity

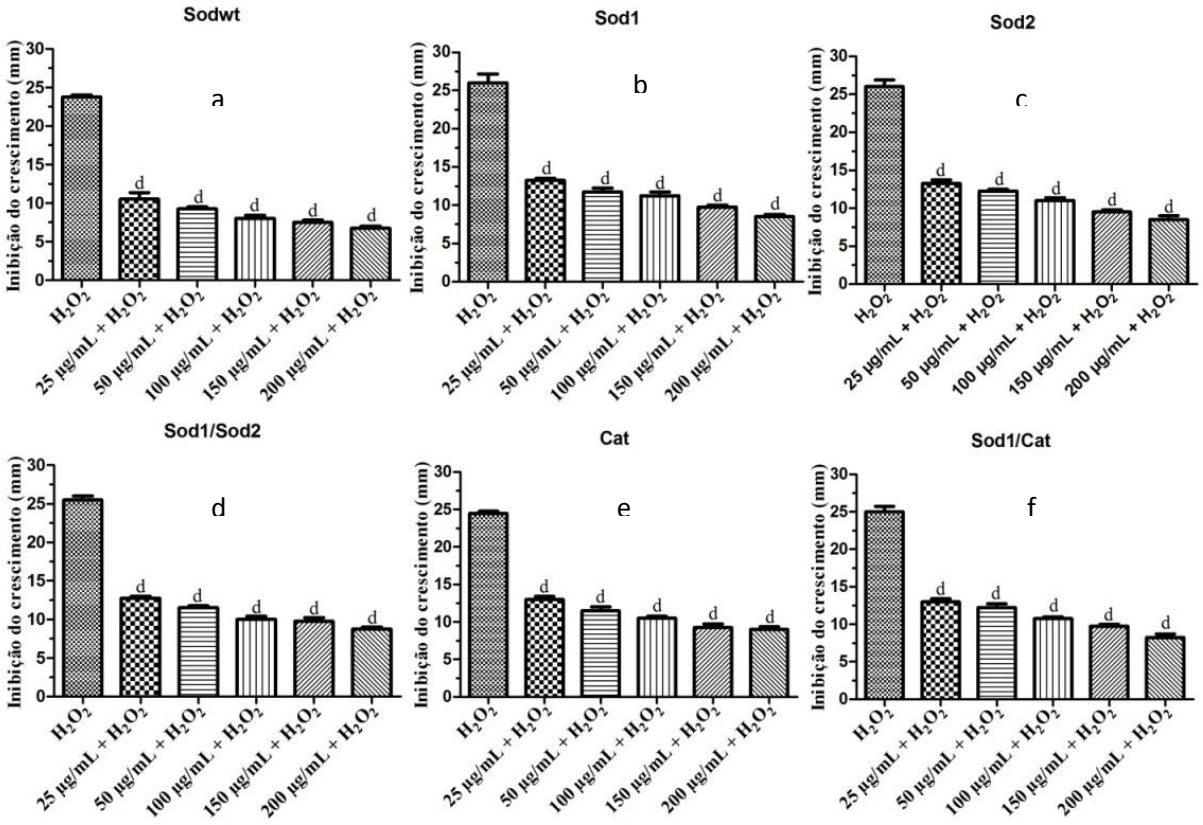

Figure 5. Antioxidant capacity of the aqueous extract *Cynara scolymus* against oxidative damage induced in strains of *Saccharomyces cerevisiae* pre-treatment. Significance (P <0.05) compared to the stressor (H_2O_2).

of AECS. The best correlation was for DPPH• vs. ABTS•+ in which the two tests have the same mechanism in a reaction with a substance which has antioxidant properties. The lowest correlation was found for inhibition of nitric oxide vs. TBARS, however, the results on AECS antioxidants are still considered a good correlation.

Antioxidant capacity in strains of *S. cerevisiae*

In the pre-treatment, co-treatment and post-treatment (Figures 5 and 6), it was possible to observe a trend to decrease in growth inhibition and the consequent increase in survival of strains of *S. cerevisiae* (wt Sod, Sod1Δ, Sod2Δ, Sod1ΔSod2Δ, Cat1Δ, Sod1ΔCat1Δ) at all concentrations of AECS was statistically significant ($p < 0.05$) compared to the results on stressor hydrogen peroxide. In the pre-treatment and co-treatment, the AECS showed significant antioxidant capacity for all strains of *S. cerevisiae* in a dose-dependent manner, inhibiting the oxidative effect of hydrogen peroxide and allowing the enhancement of the survival of strains tested. The concentration of 200 µg/ml was the most protective having the greatest antioxidant capacity, indicating that higher concentrations of AECS are required to nullify required to nullify the oxidizing effects of hydrogen peroxide. The strain of *S. cerevisiae* wild (Sodwt) showed a higher level of survival in the pre-treatment and co-treatment, while the strains of *S. cerevisiae* deficient in antioxidant defenses were more sensitive to hydrogen peroxide, suggesting the importance of superoxide dismutase in cellular protection against oxidative stress. Importantly, antioxidant capacity, was observed on AECS Sodwt lineage as well as in strains deficient in superoxide dismutase (Sod1Δ, Sod2Δ and Sod1ΔSod2Δ), which suggests that the protective effect may increase the activity of superoxide dismutase, demonstrating the key role of the aqueous extract of the leaves of *C. scolymus* in cellular antioxidant defense. In addition, the protection afforded against hydrogen peroxide seems to be also required for the double mutant strain and mutant Cat1Δ Sod1ΔCat1Δ because they were able to acquire tolerance to oxidative stress with decreasing inhibition of growth in pre-treatment and co-treatment.

In the post-treatment (Figure 7), the strains of *S. cerevisiae* were first exposed to oxidative damage caused by hydrogen peroxide, and after three hours the AECS was added at various concentrations for the verification of the behavior of strains of *S. cerevisiae*. The data obtained in the post-treatment showed a behavior similar to the pre-

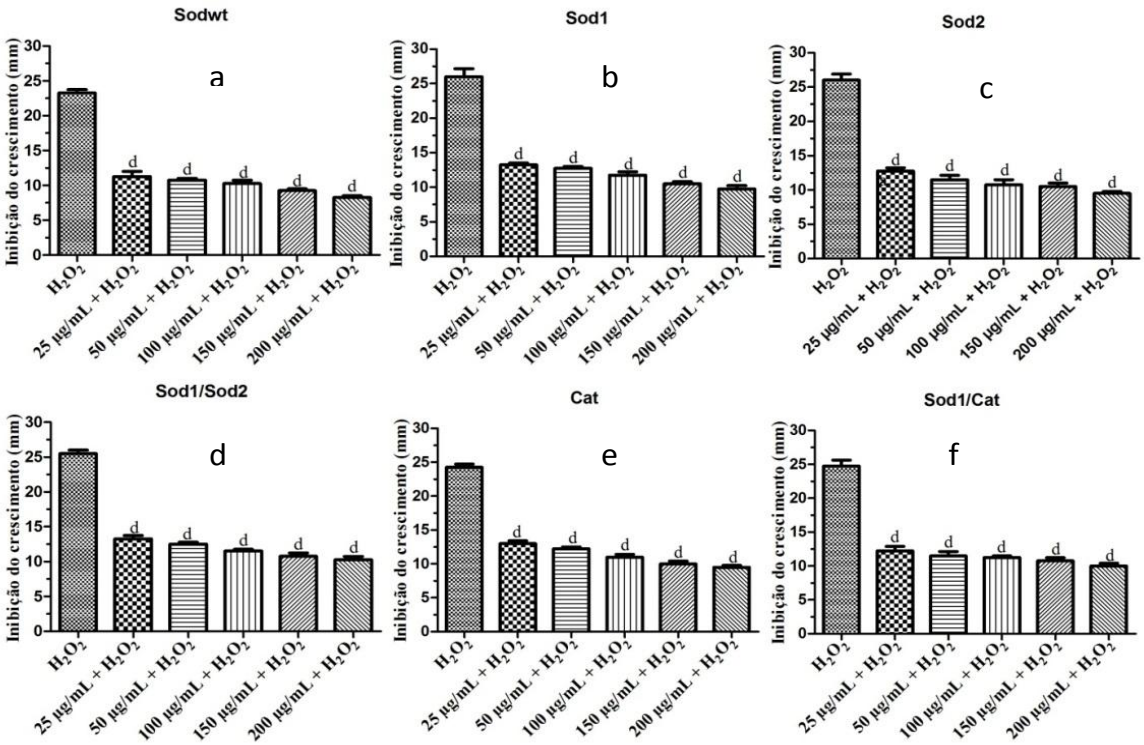

Figure 6. Antioxidant capacity of the aqueous extract of *Cynara scolymus* against oxidative damage induced in strains of *Saccharomyces cerevisiae* co-treatment. Significance (P <0.05) compared to the stressor (H_2O_2).

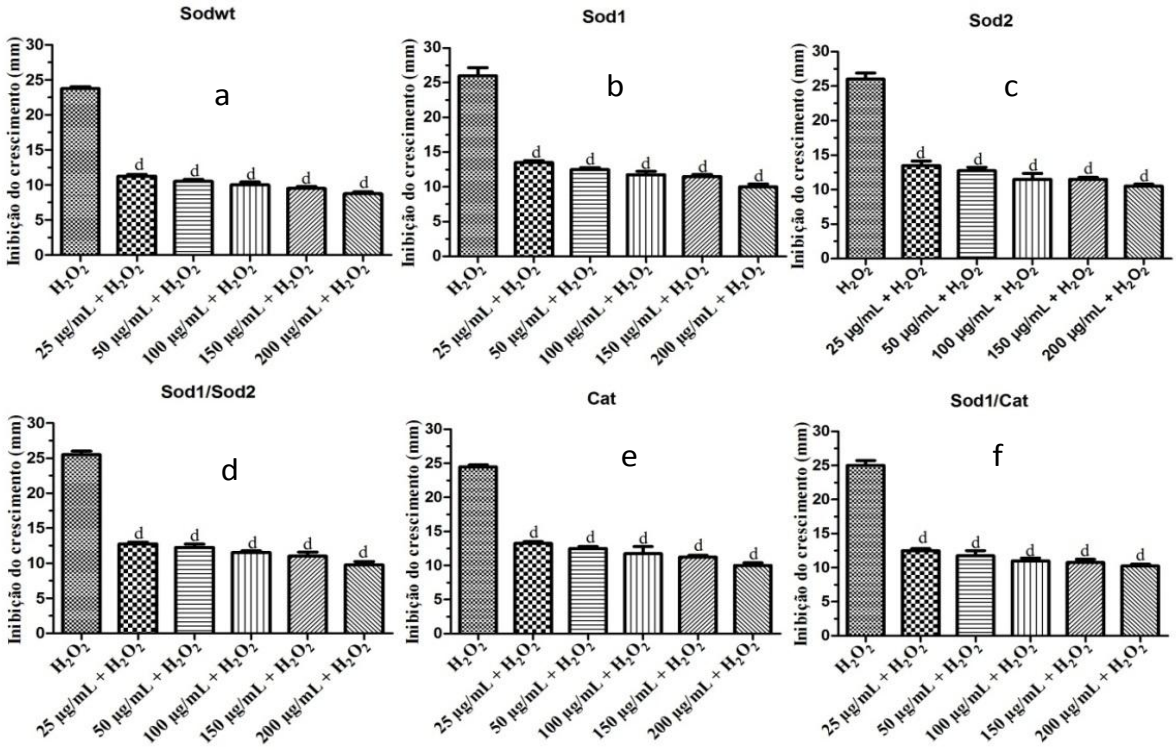

Figure 7. Antioxidant capacity of the aqueous extract of *Cynara scolymus* against induced oxidative damage in lines *Saccharomyces cerevisiae* after treatment. Significance (P <0.05) compared to the stressor (H_2O_2).

treatment and co-treatment, where the strains of *S. cerevisiae* in the presence of AECS showed an increase in survival rate with a decrease in growth inhibition at all concentrations tested as compared with the results of stressor hydrogen peroxide. It can also be observed that the strain of *S. cerevisiae* wild (Sodwt) was the one with the lowest rate of growth inhibition when compared with the mutant strains. The strains Sod1Δ and Sod2Δ Sod1ΔSod2Δ also had an increased survival with decreased growth inhibition when post-treated with the AECS at concentrations of 25, 50, 100, 150 and 200 µg/ml. This antioxidant effect is also evident for the mutant and double mutant Cat1Δ and Sod1ΔCat1Δ at all concentrations tested significantly ($p < 0.05$) in comparison to the results on hydrogen peroxide. In addition, all strains of *S. cerevisiae* showed a lower rate of inhibition at a concentration of 200 µg/ml compared with the inhibition induced by hydrogen peroxide.

Conclusion

The results obtained in the present work clearly indicate that AECS has considerable antioxidant capacity *in vitro* and in strains of *S. cerevisiae*. The antioxidant capacity of this extract can be attributed to the phenolic compounds in accordance with the correlations made with the *in vitro* evaluation of antioxidant capacity. Consequently, our results suggest that the aqueous extract may be used as a natural source of antioxidants, since there is a current need for the provision of new bioactive molecules derived from plants to the development of new drugs with antioxidant.

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Full Length Research Paper

Effects of volatile oils of the *Microlobius foetidus* on trypsin, chymotrypsin and acetylcholinesterase activities in *Aedes aegypti* (Diptera: Culicidae)

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Volatile oils of *Microlobius foetidus* were used for the evaluation of mortality, trypsin, chymotrypsin and acetylcholine inhibition and *Aedes aegypti* morphology. Bioassays were made with different concentrations (25, 50 and 100 µg/ml) and alterations in the gut of 4th stage larvae were observed. Volatile oils affected larvae in all stages, with 100% mortality for the 100 µg/ml concentration (LD_{50} >33.02). The acute toxic unit (2.7 µg/ml), chronic toxic unit (32.68 µg/ml) and toxic load (2.7 µg/ml) confirm the sensibility of the 4th stage larvae. These larvae and 3rd presented a lesser trypsin (0.176 µmol/min), chymotrypsin (0.110 µmol/min) and acetylcholinesterase (0.172 µmol/min) synthesis. Larvae of the 4th stage also had their internal morphology observed, and the main alterations were discontinuity of the peritrophic epithelium, thickening of the peritrophic membrane, decrease/increase of the subjacent epithelium and decrease of endoperitrophic space were observed in the mid gut. The results show the repellent activity of the volatile oils of *M. foetidus*, with a retardation of overall growth, that are associated with the inhibition of the trypsin and chymotrypsin synthesis. Mortality and enzymatic inhibition in all developmental stages confirm the insecticide potential of *M. foetidus*.

Key words: Dengue fever, digestive system, biological control agents, natural products.

INTRODUCTION

Classic and hemorrhagic dengue epidemics have been reemerging over the last 25 years in tropical regions of the planet which present warm and humid climate, as well as favorable socio-environmental conditions for the proliferation of the mosquito-vector, *Aedes aegypti* (Silva et al., 2008; Who, 2009).

In Brazil, adults and young people are the most affected by the disease, since the introduction of the virus. Since 2006, there has been an increase in the number of cases, in the number of severe forms, and of hospitalizations in children, mainly in the Northeastern Region of the country. In 2008, 585.769 cases were notified, and new epidemics occurred in various states of the country, configuring the worst national scenario of this disease yet regarding the total number of hospitalizations and deceases (Programa Nacional de Controle da Dengue, 2012).

These epidemics in Brazil also affect children of 1 to 10

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years of age, amounting to 50% of inpatients in the municipalities with a greater population quota, and 25% in smaller municipalities. In the year 2012, 266.285 cases of dengue were notified deceases (Programa Nacional de Controle da Dengue, 2012).

The control of *A. aegypti* is done with chemical synthetic insecticides with both larvicide and pesticide effects (Luna et al., 2004; Lima et al., 2006). However, its frequent use is costly, and may cause environmental pollution, damages to public health and the emergence of resistant mosquitoes (Oga, 2003; Braga and Valle, 2007). In view of this problematic, the use of bioactive natural products with insecticide potential surges as a viable alternative.

Microlobius foetidus (subsps. *paraguensis* (Benth.) M. Sousa et G. Andrade) belongs to the Fabaceae family, and is popularly known as pau-alho. The species has an arboreal constitution and can reach the height of 18 m. It occurs in the Pantanal region of the Brazilian state of Mato Grosso do Sul, in altered areas (pastures and roadsides), resprouting with great vigor after clear-cutting and burning. Due to the strong garlic aroma, it has potential for use as agriculture repellent and as a pioneer species, for the composition of heterogeneous reforestations destined to the recovery of the vegetation in degraded areas (Pott and Pott, 1994).

There are studies demonstrating the effects of vegetal extracts in larvae mortality by trypsin and chymotrypsin inhibition during larvae stages (Venâncio et al., 2009; Melo-Santos et al., 2010). These soluble enzymes are found in the intestinal lumen (Christofoletti et al., 2005) and its secretion occurs in the gut (Terra and Ferreira, 2005). In this regard, the middle portion of the gut of *A. aegypti* larvae contains great amounts of proteolytic enzymes that play a central role in the supply and replenishment of free aminoacids essential for normal larvae development (Michaud et al., 1995).

These enzymes are the most important enzymes for food digestion in *A. aegypti*, and take part in all phases of its biological cycle, being especially expressed in the larvae and pupa phases (Melo- Santos et al., 2010; Yang and Davies, 1971). Trypsin and chymotrypsin are synthesized by the intestinal epithelium cells and can cross the peritrophic membrane, reaching the endoperi-trophic space, where the initial phase of digestion, consisting on the breaking of the ingested polymers to oligomers, occurs (Terra et al., 1996). These fragments are now capable of crossing the peritrophic matrix so as to be degraded to smaller oligomers or dimers (intermediate phase). Finally, these molecules are broken into monomers and absorbed (Terra et al., 1996; Tellam, 1996).

Taking into account that the digestive and absorptive processes take place in the middle portion of the gut, the spatial organization of the digestion rely on the relations between each of its compartments (cell, esoperitrophic and subperitrophic spaces) such as the subperitrophic epithelium, epithelial tissue and the different phases of digestion and corresponding enzymes (Terra et al., 1996).

Thus, our research was aimed at identifying the volatile oils present in the leafs of *M. foetidus*, and evaluating the larvicide activity of the different growth stages, by means of mortality assays, trypsin, chymotrypsin and acetyl-cholinesterase inhibition, and alterations in the gut morphology of 4th-stage larvae.

MATERIALS AND METHODS

Plant

Leaves from *M. foetidus* were collected in the Pantanal region of the Brazilian state of Mato Grosso do Sul state, under the coordinates 19°29'16.20" S; 57°02'35.50" W and a voucher specimen was placed in the Federal University of Mato Grosso do Sul (CGMS) and Botanical Garden of Curitiba (MBM) herbariums, under number 21739 and 334776, respectively. The leaves were stored in an iced polystyrene box and taken to the laboratory for the volatile oil extraction.

Volatile oils extraction

Fresh leaves of *M. foetidus* were submitted to various hydrodistillation processes during four hours in a Clevenger-type apparatus, followed by exhaustive extraction of the distillated with hexane.

The gas chromatography–mass spectrometry (CG-MS) analyses were performed in a Varian GC-MS-MS system equipped with Varian – 3900 gaseous chromatographer equipped with a ZB-5 capillary column, a 1077 injector, a CP-8410 automatic injector coupled with a Varian Saturn 2100 mass spectrometer operating with an electron impact of 70 eV, at the same analysis conditions of CG/FID.

The utilized fiber was of NiTi-ZrO2-PDMS 35 µm, with 100 mg of leaf being used, with a 30-min extraction time and 40°C extraction temperature (Gebara et al., 2011). The analyses were performed in a gaseous chromatographer (GC 3900) coupled to the ion-trap (Saturn 2100) mass spectrometer, using a molten silica capillary column, VF-5 ms, measuring 30 m of length, internal diameter of 0.25 mm, film width of 0.25 mm and stationary phase with 5%-phenyl-95%-dimethylpolysiloxane of low bleed.

The chromatographic parameters used for the separation of the components were: Injector temperature: 250°C; Liner: Single gooseneck SPME liner for 1177 0.75 injector; Drag gas: Helium 99.999%. Outflow of drag gas in the column: constant of 1.0 minute, followed by a split rate of 50:1 for 15.0 min and of 20:1 for the remaining of the run. Temperature programming of the column oven: 50°C (2 min isotherm) and 50 to 250°C ramp with 3°C min^{-1} heating. The ion-trap, manifold and transference line temperatures were 200, 50 and 250°C, respectively. 70 eV ionization energy was employed with mass scanning from 40 to 450 m/z.

The identification of the oil components was based in comparisons of the retention times, by means of determination and comparison of the Kovats retention indexes and mass spectrums obtained from the NBS/NIST library (NIST Mass Spectral Library, 2002) with the indexes described by Adams (1995). A n-alkane (C_8-C_{32}) homologous series was used to calculate the Kovats retention indexes.

Larvicidal assays

The larvicide activity of the volatile oil against *A. aegypti* was evaluated according to World Health Organization (1981), with some modifications. Eggs from *A. aegypti,* Rockefeller strain, were provided by Oswaldo Cruz Foundation - RJ; the insecticide activity

being used as a susceptibility standard for the *A. aegypti* species (Hartberg and Craig, 1970). For the eclosion of the eggs, they were put in a plastic tray and 500 ml of water lacking chlorine was added, and then taken to a BOD incubator at a temperature of 27 ± 2°C and relative humidity of 80 ± 5%.

Larvae feeding was prepared with Fish's diet (Aldon Basic™, MEP 200 Complex) from the eclosion period to the 4th larvae stage, and solutions of volatile oils, in the concentration of 100 µg/ml, were prepared solubilizing the samples with 0.5% of dimethylsulphoxide (DMSO) and diluted in water lacking chloride in the concentrations of 100, 50 and 25 µg/ml for the assays.

Samples containing 15 1st, 2nd, 3rd and 4th stage larvae were put in plastic cups separately containing 5 ml of chlorine-free water. For each concentration, 45 larvae were used, in triplicate. A 0.5% DMSO aqueous solution was used in triplicate, as a negative control.

The protocol consists of the mortality response against the exposure in Diagnostic Concentration (DC) in the exposure to a concentration gradient (Multiple Concentrations – MC). The larvicide activity was evaluated after 24 h, by counting the number of dead larvae in each sample. Moribund larvae, unable to reach the water surface when touched, were considered dead (Who, 1981). The acute toxic unit (ATU), chronic toxic unit (CTU) and toxic load (TL) were calculated (Who, 1981).

The lethal concentration (LC_{50}) values, in µg/ml were determined using the Probit analysis method (Finney, 1971). For each evaluated sample, triplicates were used, and data were submitted to analysis of variance and when a difference was detected, the averages were compared by the Dunnet test, with 5% of probability.

Morphologic study of *A. aegypti* larvae

For the internal morphology evaluation, 4th stage larvae were selected, because they have more developed tissues. The collected larvae were immediately fixed in 2% glutaraldehyde, 2% paraformaldehyde, 3% saccharose in 0.1M Sodium Cacodylate Buffer pH 7.2 and stored at room temperature until the analysis were performed (Arruda et al., 2008). Glasses containing the larvae were prepared and photographed, using a digital camera (Sony), connected to a Zeiss inverted microscope (magnification of 40×).

Preparation of larvae homogenate

A. aegypti larvae homogenates, submitted to the extract and stearic acid by 6 h, were prepared according to Macedo et al. (1993), with some modifications. The gut of each larva was removed using a needle (8 mm length; 0.3 mm caliber) and immediately homogenized in tissue grinder with 1.0 ml of Tris/HCl Buffer 0.05 M (pH 8.0), and centrifuged in 17000 rpm during 20 min, 4°C. The supernatants were collected, and an additional 1.0 ml of Tris/HCl Buffer was added.

Protein concentration

The protein content in the larvae which were submitted to extracts and fractions was determined according to Lowry et al. (1951), using serum bovine albumin (31.25 to 500 µg/ml) as standard.

Acetylcholinesterase activity

For the acetylcholinesterase evaluation, 10 µl of the *A. aegypti* homogenates were incubated with 20 µl of acetylcholine, 0.062 µl of 0.25 mM DTNB for 3 min, at 25°C (Ellman et al., 1991). The increase in the absorbance was read at 405 nm (ε, 13.6 mM^{-1} cm^{-1}).

Determination of trypsin and chymotrypsin content

The total trypsin activity was determined using the N-benzoyl-D, L-arginine-ρ-nitroanilide (BAPNA) as substrate. About 500 µl of the larvae homogenate were incubated in 0.05 M Tris-HCl buffer (pH 8.0), to a final volume of 500 µl for 10 min before the addition of 1.0 ml BAPNA substrate. The reaction was allowed to proceed at 37°C for 20 min then stopped by adding 30% acetic acid (v/v). The trypsin activity was read in a microplate reader, at 410 nm (ε, 10.0 mM^{-1} cm^{-1}) in accordance with Silva et al. (2009)

Chymotrypsin activity was determined using N-succinyl-Ala-Ala-Pro-Phe-p Nitranilide (SAAP) as substrate. About 50 µl of the homogenates were incubated in 0.1 M Tris-HCl buffer (pH 8.0), to a final volume of 500 µl for 10 min before the addition of 1.0 ml SAAP substrate dissolved in pure dimethylsufoxide (DMSO). The reaction was allowed to proceed at 37°C for 20 min, then stopped by adding 30% acetic acid (v/v). The absorbance was read at 405 nm (ε, 8.8 mM^{-1} cm^{-1}) (Silva et al., 2009).

Statistical analysis

The values for lethal concentration (CL_{50}) in µg/ml were determined using the Probit analysis method (Fynnei, 1971). For each evaluated sample, triplicates were used, and data were submitted to analysis of variance and when a difference was detected, the averages were compared by Dunnet test, with 5% of probability.

RESULTS

The oil obtained by hydrodistillation had a yield of 0.84%; it has a yellow color and a characteristic odor. The solid-phase micro-extraction (SMPE) method was more efficient when considering the amount of identified substances and the time for chromatogram attainment (30 min), and may simplify considerably the sample preparing procedure by the hydrodistillation method. Furthermore, when combined with gas chromatography–mass spectrometry (GC/MS), it provides the adequate conditions of the optimization and identification of a greater number of volatile substances.

Eleven substances were identified: 1.2.4-trithiolane (31.8%), 1.3.5-trithiane (8.5%), 1.2.5-trithiepane (0.2%), 1.2.4.5-tetrathiane (7.0%), 1.2.3.4-tetrathiane (0.3%), 1.2.4.6-tetrathiepane (12.3%), 1.3.5.7.9-pentathiecane (1.2%), 1.2.5.6-tetrathiocane (0.4%), lenthionine (10.2%), hexathiepane (1.1%) and sulfur cyclic octaatomic (3.4%) (Table 1).

Based on this composition, these oils may be responsible for the poignant onion aroma, that is, exhaled from the plant, and that explains its popular name (garlic stick). Cyclic polysulfides have a restricted occurrence in nature, with few reports in plants. The presence of sulfur-containing substances in the oil composition turns it into quite a peculiar material regarding chemical composition. Antineoplastic activity of sulfur compounds has already been reported in the literature (Gmelin et al., 1981) and the presence of cyclic polysulfides such as 1.2.4-trithiolane and 2.4.6-tetrathiepane, have already been related in the brute extracts of *Parkia* species, exhibiting allelopathic activity.

Table 1. Chemical composition of the volatile sulfur compounds of *Microlobius foetidus* obtained by SPME (solid phase micro extraction).

Compounds[a,b]	MF	RI[c]	SPME
1,2,4-trithiolane	$C_2H_4S_3$	1095	31.8±5.6
1,3,5-trithiane	$C_3H_6S_3$	1249	8.5±1.2
1,2,5-trithiepane	$C_4H_8S_3$	1300	0.2±0.0
1,2,4,5-tetrathiane	$C_2H_4S_4$	1318	7.0±0.5
1,2,3,4-tetrathiane	$C_3H_6S_4$	1354	0.3±0.2
1,2,4,6-tetrathiepane	$C_3H_6S_4$	1488	12.3±2.8
1,3,5,7,9-pentathiecane	$C_5H_{10}S_5$	1545	1.2±0.4
1,2,5,6-tetrathiocane	$C_4H_8S_4$	1551	0.4±0.1
Lenthionine	$C_2H_4S_5$	1597	10.2±2.3
Hexathiepane	CH_2S_6	1685	1.1±0.5
Cyclic octaatomic sulfur	S_8	2006	3.4±0.3
Total			76.4%

MF: molecular formula; [a]Compounds listed in order of elution from a ZB-5 column; [b]Identification: RI, retention indeces, GC-MS, gas chromatography-mass spectroscopy; [c]Programmed temperature retention indices determined on apolar ZB-5 column (50 to 250°C; 3°C min^{-1}).

Table 2. Effect of different concentrations of the volatile oils of *M. foetidus* in the mortality and LD_{50} on *A. aegypti* larvae, in all the development stages.

Stage	Number of dead				LD_{50} (µg/ml)	Confidence interval 95%
	control	25µg/ml	50 µg/ml	100 µg/ml		
1°	2	36*	51*	60*	>45.68	36.72-25.96
2°	1	33*	48*	60*	>51.69	41.31-28.57
3°	3	31*	43*	60*	>58.42	42.002-28.78
4°	2	30*	58*	60*	>33.02	44.31-29.88

Table 3. Effect of different concentrations of the volatile oils of *M. foetidus* in the acute toxic unit (ATU), chronic toxic unit (CTU) and toxic load (TL) on *A. aegypti* larvae, in all the development stages.

Stage	ATU (µg/ml)	CTU (µg/ml)	TL (µg/ml)
1°	3.1	36.69	0.67
2°	2.9	35.42	0.65
3°	2.8	34.02	0.61
4°	2.7	32.68	0.56

The different concentrations of the volatile oils led to *A. aegypti* larvae mortality in the different stages. The 25 µg/ml concentration affected larvae survival by >50% (30 dead individuals). Larvae in the 1st (51 dead individuals) and 4th (58 dead individuals) stages were more affected by the volatile oils activity, with LD_{50} of >45.68 and >33.02 µg/ml, respectively. The greater concentration led to 100% larvae mortality in the different tested stages (Table 2).

The used concentrations were toxic to all larvae stages. Although all concentrations were effective, larvae in the 4th stage were more sensible, and presented ATU, CTU and TL by 2.7, 32.68 and 0.56 µg/ml respectively, demonstrating high insecticide potential (Table 3). Alterations in the shifting of the larvae stages were also observed. There was a decrease in the shifting of the different stages as a function of the used concentrations.

Living larvae of the 4th stage submitted to volatile oil concentrations and water control were evaluated after 8 h with an inverted optical microscope. Larvae treated with the 25 µg/ml concentration presented a thickening of the peritrophic membrane and a less thickened subperitrophic epithelium. The endoperitrophic space also appeared leaner and clearer when compared to control, and the elongation of the region between the thorax and the cephalic capsule was observed (Figure 1B and C). In a similar way, the 50 µg/ml concentrations led to thickening and darkening of the peritrophic membrane, and thickening of the subperitrophic epithelium. Endoperitrophic space showed discontinuations in the tissue segments

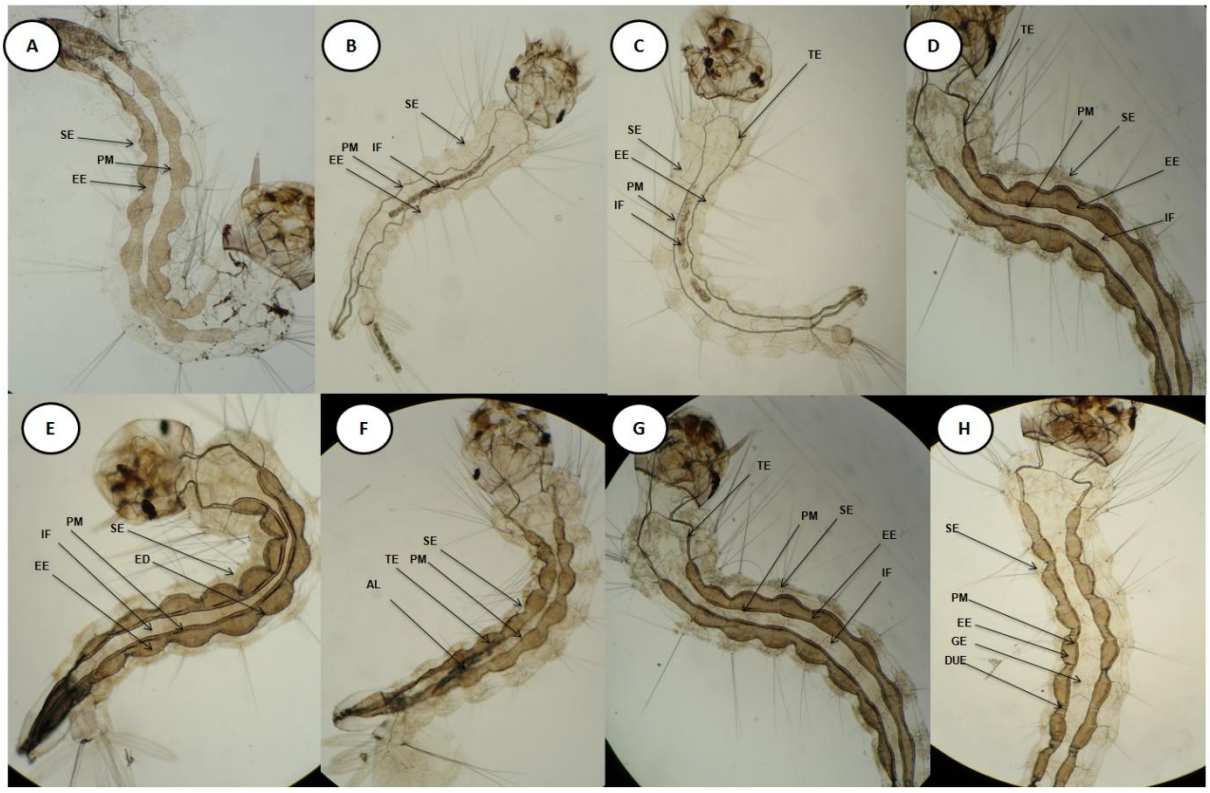

Figure 1. Photomicrography (40x, 900 µm) of the *A. aegypti* larvae on the 4th stage of development. A- control live, B and C- 25 µg/ml, D and E- 50 µg/ml, F G and H- 100 µg/ml. *Note = SE: subperitrophic epithelium, EE: endoperithrophic epithelium, PM: perithrophic membrane, IF: ingestion of food, TE: thorax extended.

and the region between the cephalic capsule and the thorax was also more elongated (Figure 1D and E). The same alterations were observed in the 100 µg/ml concentration (Figure 1F and G). The endoperitrophic space was also less thick, and an enlargement of the digestive tube in some larvae was also observed, and the non-continuous epithelium was also observed (Figure 1H).

The digestive enzyme activity decreased in larvae treated with different concentrations of the volatile oils of *M. foetidus* (Figure 2). Regarding trypsin activity, it was decreased in all development stages, and a lesser synthesis was verified in larvae of the 3rd (178 µmol/min) and 4th (176 µmol/min) stages that had been treated with the 100 µg/ml concentration (Figure 2A). Chymotrypsin activity was also decreased, and the least activity was observed with the 100 µg/ml concentration in larvae of the 4th development stage (0.11 µmol/min) (Figure 2B). The least trypsin and chymotrypsin synthesis observed in 4th stage larvae may be related to volatile oil ingestion, leading to intoxication and death.

Larvae treated with various concentrations of volatile oils showed a decrease in the acetylcholinesterase synthesis. Inhibitory effects were observed in all stages, with the lowest enzyme synthesis on the 100 µg/ml (0.256 µmol/min) concentration in the 4th larvae stage (0.172 µmol/min) (Figure 3). The differences between effects of 3 concentrations were not significant (subgroup b).

DISCUSSION

The increase of mosquito resistance to chemical insecticides led to an increase in research on natural resources and biodegradable insecticides, aiming to minimize the environmental impact and find new substances that promote mortality, preventing the proliferation of resistant larvae due to rotation of insecticides such as organophosphates and pyrethroids in Brazil.

Although many conventional synergists have relatively low acute toxicities to mammals, there is evidence that pyrethroids show adverse health effects with prolonged exposure to humans (Horton et al., 2011).

Some plant extracts have already demonstrated consistent insecticide activity, and these potential phytoinsecticides could become safer alternatives for mosquito control, as a high degree of degradation in the environment is expected (Lima et al., 2013; Rajasekaran and Duraikannan, 2012). Although many conventional

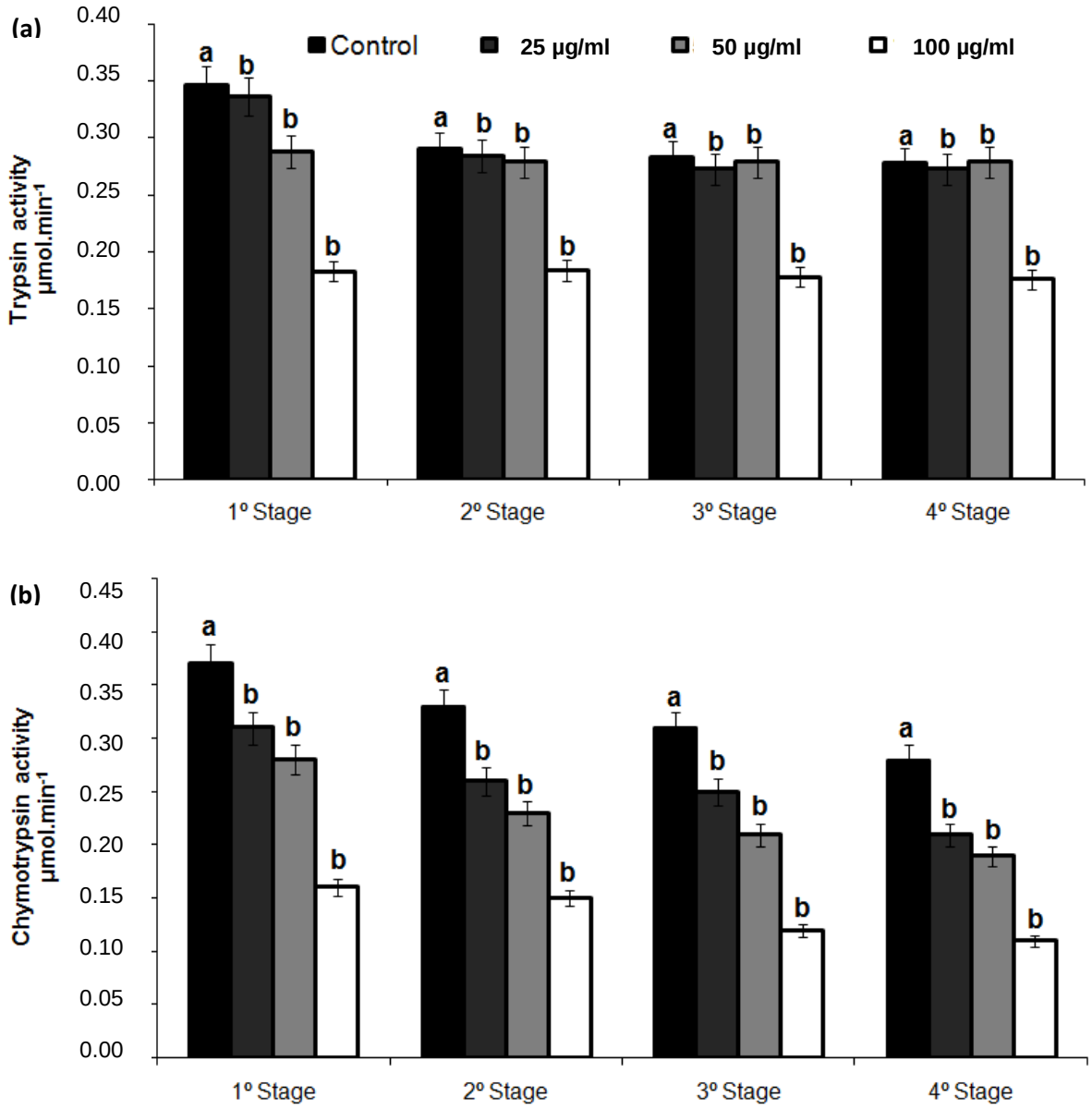

Figure 2. Effect of different concentrations of the volatile oils of *M. foetidus* on the trypsin and chymotrypsin activities in *A. aegypti* larvae(Mean ± Standard deviation or Standard error).
Means followed by the same letter do not differ statistically from the control group by Dunnet's test ($p < 0.05$).

synergists have relatively low acute toxicities to mammals, there is evidence that these chemicals show adverse health effects with prolonged exposure to humans (Horton et al., 2011).

The high larvae mortality in different stages exposed to the volatile oils of *M. foetidus* indicate its use as a natural insecticide. The alterations in internal morphology explain the high mortality rate arising from the intoxication and inhibition of trypsin and chymotrypsin.

The results showed significant repellent effect on larvae posture and general growth retardation on *A. aegypti* by medium containing aqueous of volatile oils. Few works have reported larvicidal activities of plant extracts against *A. aegypti*, describing the changes in the PM (Vieira et al., 2012), although potent larvicidal extracts have been described, with IC_{50} values less than 50 µg/ml (Lima et al., 2013).

The main biological functions of PM include the spatial organization of digestion, protection from ingested toxins, and serves as a physical barrier to pathogens (Zhong et al., 2012).

The PM not only plays important roles in facilitating food digestion and providing protection to the gut epithelium, but can also be a significant structural target

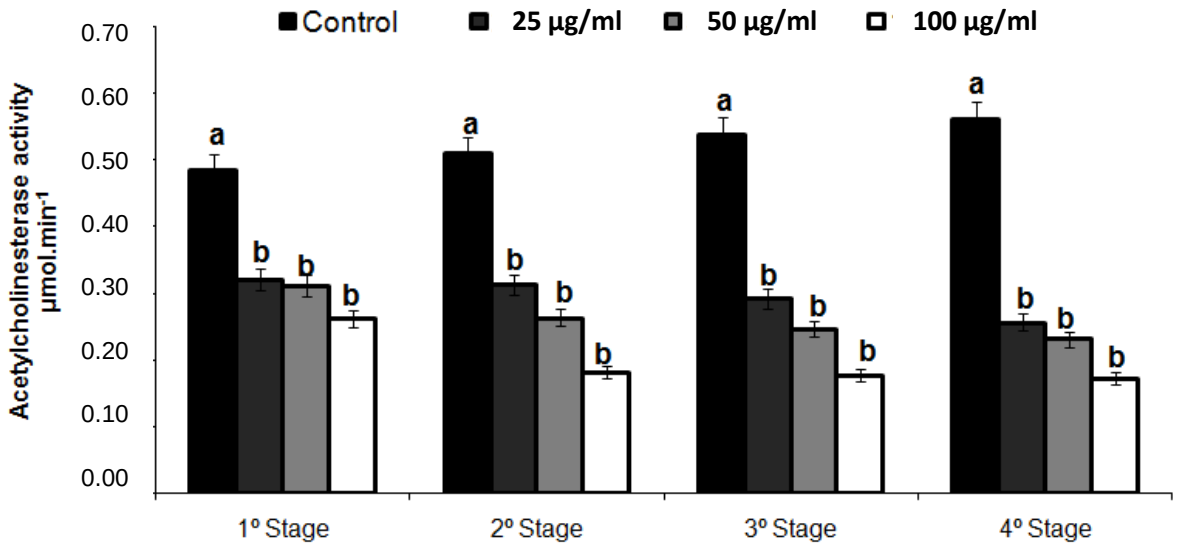

Figure 3. Effect of different concentrations of the volatile oils of *M. foetidus* on the acetylcholinesterase activity in *A. aegypti* larvae(Mean ± Standard deviation or Standard error?) . Means followed by the same letter do not differ statistically from the control group by Dunnet's test ($p < 0.05$).

for insect control (Zhong et al., 2012). Trypsin and chymotrypsin may play various important roles in food digestion, immune defense and zymogen activation in insects (Ge et al., 2012). Despite several studies concerning adult *A. aegypti* digestive biochemistry and molecular biology, very few studies have been performed to elucidate the digestion in *A. aegypti* larvae. Trypsin-like and chymotrypsin-like activities are known in *A. aegypti* larvae (Mesquita-Rodrigues et al., 2011).

Enzymatic inhibitors, when added to the diet of insects, interfere with their digestive process by decreasing the assimilation of nutrients, leading to delayed development and mortality (Napoleão et al., 2012).

Zhang et al. (2010) reported what enzymes involved in the food digestion, it has to be secreted into the interspaces between the epithelium and peritrophic membrane or the lumen of the gut, where it digests the ingested food proteins, and the results showed that digestive enzymes were present not only in the epithelium of the anterior, middle and posterior midgut, but also in the lumen food residues of the anterior, middle and posterior midgut, as well as the feces of the larvae, suggesting that the protein was secreted into the lumen of the gut.

Current strategies based on the elimination of breeding sites and applications of chemical insecticides for larval and adult mosquito control have resulted in development of resistance without eliminating the constant risk of dengue epidemics (Lima et al., 2011). Thus new approaches are urgently needed. Interest on possible use of environment friendly natural products such as oils of plants or plant parts increased for vector control. Plant derived products have received increased attention from scientists and more than 2000 plant species are already known to have insecticide properties (Pankaj and Anita, 2010; Kamaraj et al., 2011).

Vieira et al. (2012) reported that larvicidal activity of *Indigofera suffruticosa* had as main molecular targets: apoptosis, caspase 3 activation, DNA degeneration and mitotic catastrophe. Due to these actions, *I. suffruticosa* can impinge upon different conditions. Plausible infer that volatile oils of *M. foetidus* cause the same effects on larvae of *A. aegypti*, and these effects are associated with inhibition of gut enzymes.

Thus, the use of volatile oils for the control of insects is an alternative way to minimize the harmful effects of pesticides used to control the mosquito. Further, samples of locals where there has already been intensive use of pesticides by the control programs demonstrate that the larvae and mosquitoes became resistant, by mechanisms such as increase in the synthesis of acetylcholinesterase (Pinheiro and Tadei, 2002).

Volatile oils from the plants could be used in stagnant water bodies which are known to be the breeding grounds for mosquitoes. However, further studies on the active principals involved and their mode of action and field trials are usually needed to recommend any of these plant materials as an anti-larvicidal product used to combat and protect from mosquitoes in a control program.

Plant could be an alternative source for mosquito larvicides, because they constitute a potential source of bioactive chemicals and generally free from harmful effects. Use of these botanical derivatives in mosquito control instead of synthetic insecticides could reduce the cost and environmental pollution.

Taking this into consideration, the volatile oils of *M. foetidus* represent an alternative to the larvae control, as they acted not only as inhibitors of the synthesis of acetylcholine, but also as inhibitors of digestive enzymes such as trypsin and chymotrypsin, demonstrating its potential as physiologic pesticide.

The isolation of substances present in the volatile oils is underway, and new research on the isolated activity of these substances could improve our understanding on the popular indication of this plant as pesticide by the Pantanal population, and evolve to the development of formulations that could be used for the control of larvae and mosquitoes.

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Full Length Research Paper

Evaluation of activity exerted by a steroid derivative on injury by ischaemia/reperfusion

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There are reports which indicate that some steroid derivatives have activity atcardiovascular level; nevertheless, there is scarce information about the effects exerted by the progesterone derivatives on cardiac injury caused by ischemia/reperfusion. In this study, a new steroid (progesterone derivative) was synthetized with the objective of evaluating its activity on ischemia/reperfusion injury. The Langendorff technique was used to evaluate the effect of progesterone derivative on ischemia/reperfusion injury. Additionally, molecular mechanism involved in the activity exerted by the progesterone derivative on perfusion pressure and coronary resistance was evaluated by measuring left ventricular pressure in absence or presence of following compounds; mifepristone, prazosin, metoprolol, indomethacin and nifedipine. The results showed that the progesterone derivative reduces infarct size compared with control. Other results showed that the progesterone derivative significantly increase (p = 0.05) the perfusion pressure and coronary resistance in isolated heart. Other data indicate that the progesterone derivative increase left ventricular pressure in a dose-dependent manner (0.001 to 100 nM); however, this phenomenon was significantly inhibited by nifedipine at a dose of 1 nM (p = 0.05). In conclusion, these data suggest that progesterone derivative exert acardioprotective effect via the calcium channels activation and consequently induces changes in the left ventricular pressure levels. This phenomenon results in decrease of myocardial necrosis after ischemia and reperfusion.

Key words: Steroid, progesterone, left ventricular pressure, indomethacin.

INTRODUCTION

There are several reports which indicate that myocardial infarction is a major cause of death and disability worldwide (Yusuf et al., 2005; Thygesen et al., 2007), this cardiovascular disease is due to cell death of cardiac-myocytes caused by prolonged myocardial ischaemia. Acute myocardial infarction can produce alterations in the topography of both the infarcted and noninfarcted regions of the ventricle (Pfeffer, 1995).

Some reports showed that the most effective method of limiting necrosis is restoration of blood flow; however, the

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effects of reperfusion itself may also be associated with tissue injury (Klone at al., 1989). In search of therapeutic alternatives to reduce myocardial necrosis, some steroid derivatives has been used; for example, a study showed that 17β-estradiol reduced injury by ischemia/reperfusion via production of nitric oxide in an animalmodel (Node et al., 1997). Other studies showed that a progestin (norethindrone acetate) can also reduce ischemia-reperfusion injury in ovariectomized monkeys receiving estrogen therapy previously (Suparto et al., 2005).

Recently, a study indicated that a progesterone derivative induce acardioprotective effect on injury by ischaemia/reperfusion via M2 muscarinic receptor and activation of nitric oxide synthase in an animal model (Figueroa-Valverde et al., 2012). Nevertheless, there are reports which indicate that the administration of medroxyprogesterone acetate can inhibit the effects of estradiol that lead to protection of the myocardium from reperfusion injury and that this involves both neutrophil-dependent and neutrophil-independent mechanisms (Jeanes et al., 2006). Apart from these experiments, which also do not show clearly the cellular site and actual molecular mechanisms of progesterone and its derivatives, data are needed for characterizing the activity induced by this steroid on ischemia-reperfusion injury.

To test this aspect, the present study was designed to investigate the effects induced by a new steroid (progesterone derivative) in a myocardial infarction/ reperfusion model. In addition, molecular mechanism involved in the activity exerted by the progesterone derivative on left ventricular pressure was evaluated using some pharmacological tools for blocking various biological systems such as; mifepristone (progesterone receptor antagonist) (Couzintet et al., 1986), metoprolol (selective β1 receptor blocker) (Bengtsson et al., 1975), prazosin (selective α1 receptor blocker) (Graham et al., 1977), indomethacin (prostanglandin synthesis blocker) (Owen et al., 1975), nifedipine (antagonist of calcium channel) (Henry, 1980).

METHODOLOGY

Chemical synthesis

The compound 1 (1-[(2-Amino-ethylamino)-phenyl-methyl]-naphthalen-2-ol) was prepared according to a previously reported method (Figueroa-Valverde et al., 2013a). The other compounds evaluated in this study were purchased from Sigma-Aldrich Co., Ltd. The melting point for the progesterone derivative was determined on an Electrothermal (900 model). Infrared spectra (IR) were recorded using KBr pellets on a Perkin Elmer Lambda 40 spectrometer. ^{1}H and ^{13}C NMR (nuclear magnetic resonance) spectra were recorded on a Varian VXR-300/5 FT NMR spectrometer at 300 and 75.4 MHz (megahertz) in $CDCl_3$ (deuterated chloform) using tetramethylsilane (TMS) as internal standard. Electron impact mass spectroscopy (EIMS) spectra were obtained with a Finnigan trace gas chromatography polaris Q spectrometer. Elementary analysis data were acquired from a Perkin Elmer Ser. II CHNS/0 2400 elemental analyzer.

Synthesis of N-[2-(1-{3-[2-(2,4-Dinitro-benzenecarbonylamino)-ethylimino]-10,13-dime-thyl-2,3,6,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-17-yl}-ethylideneamino)-ethyl]-2,4-dinitro-benzamide **(compound 3)**

A solution of compound 1 (83 mg, 0.32 mmol), progesterone (100 mg, 0.32 mmol) and boric acid (40 mg, 0.65 mmol) in 10 ml of methanol was stirred for 48 h at room temperature. The reaction mixture was evaporated to dryness under reduced pressure, the residue washed 3 times with water. Then the precipitate was separated and dried at room temperature.

Synthesis of N-{2-[[1-(3-(N-(3-Chloro-2-oxo-cyclobutyl)-N-{2-[(3-chloro-2-oxo-cyclo-butyl)-amino]-ethyl}-2,4-dinitro-benzencarbonilamino])-10,13-dimethyl-2,3,6,7,8,9, 10,11, 12,13,14,15, 16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-17-yl)-ethyl]-(3-Chloro-2-oxo-cyclo-butyl)-amino]ethyl}-N-(3-Chloro-2-oxo-cyclobutyl)-2,4-dinitro-benzamide (compound 5)

A solution of compound 3 (100 mg, 0.13 mmol), chloroacetyl chloride (30 µl, 0.40 mmol) and triethylamine (55 µl, 0.39 mmol) in 10 ml of methanol was stirred for 48 h at room temperature. The reaction mixture was evaporated to dryness under reduced pressure, and the residue washed 3 times with water. Then the precipitate was separated and dried at room temperature.

Biological method

All experimental procedures and protocols used in this investigation were reviewed and approved by the Animal care and use Committee of University Autonomous of Campeche (No. PI-420/12) and were in accordance with the guide for the care and use of laboratory animals (Bayne, 1996). Male Wistar rats, weighing 200 to 250 g were obtained from University Autonomous of Campeche.

Reagents

All drugs were dissolved in methanol and different dilutions were obtained using Krebs-Henseleit solution (≤ 0.01%, v/v).

Experimental design

Briefly, the male rat (200 to 250 g) was anesthetized by injecting them with pentobarbital at a dose rate of 50 mg/kg body weight. Then the chest was opened, and a loose ligature passed through the ascending aorta. The heart was then rapidly removed and immersed in ice cold physiologic saline solution. The heart was trimmed of non-cardiac tissue and retrograde perfused via a non-circulating perfusion system at a constant flow rate. The perfusion medium was the Krebs-Henseleit solution (pH = 7.4, 37°C) composed of (mmol); 117.8 NaCl; 6 KCl; 1.75 $CaCl_2$; 1.2 NaH_2PO_4; 1.2 $MgSO_4$; 24.2 $NaHCO_3$; 5 glucose and 5 sodium pyruvate. The solution was actively bubbled with a mixture of O_2/CO_2 (95:5/5%). The coronary flow was adjusted with a variable speed peristaltic pump. An initial perfusion rate of 15 ml/min for 5 min was followed by a 15 min equilibration period at a perfusion rate of 10 ml/min. All experimental measurements were done after this equilibration period.

Perfusion pressure

Evaluation of measurements of perfusion pressure changes induced

by drugs administration in this study were assessed using a pressure transducer connected to the chamber where the hearts were mounted and the results entered into a computerized data capture system (Biopac).

Inotropic activity

Contractile function was assessed by measuring left ventricular developed pressure (LV/dP), using a saline-filled latex balloon (0.01 mm, diameter) inserted into the left ventricle via the left atrium (Figueroa-Valverde et al., 2011a). The latex balloon was bound to cannula which was linked to pressure transducer that was connected with the MP100 data acquisition system.

First stage

Ischemia/reperfusion model

After 15 min of equilibration time, the hearts were subjected to ischemia for 30 min by turning off the perfusion system (Booth et al., 2005). After this period, the system was restarted and the hearts were reperfused by 30 min with Krebs-Henseleit solution. The hearts were randomly divided into 2 major treatment groups with n = 9:

Group I: Hearts were subjected to ischemia/reperfusion but received vehicle only (Krebs-Henseleit solution).
Group II: Hearts were subjected to ischemia/reperfusion and treated with the progesterone derivative (compound 5) at a dose of 0.001 nM before ischemia period (for 10 min) and during the entire period of reperfusion.

At the end of each experiment, the perfusion pump was stopped, and 0.5 ml of fluoresce in solution (0.10%) was injected slowly through a side arm port connected to the aortic cannula. The dye was passed through the heart for 10 s to ensure its uniform tissue distribution. The presence of fluorescein was used to demarcate the tissue that was not subjected to regional ischemia, as opposed to the risk region. The heart was removed from the perfusion apparatus and cut into two transverse sections at right angles to the vertical axis. The right ventricle, apex and atrial tissue were discarded. The areas of the normal left ventricle non risk region, area at risk, and infarct region were determined using the technique reported by Boot and coworkers (2005). Total area at risk was expressed as the percentage of the left ventricle.

Second stage

Effect induced by the progesterone derivatives (compound 3 and 5) on perfusion pressure

Changes in perfusion pressure as a consequence of increases in time (3 to 18 min) in absence (control) or presence of the progesterone derivatives at a concentration of 0.001 nM were determined. The effects were obtained in isolated hearts perfused at a constant flow rate of 10 ml/min.

Evaluation of effects exerted by the progesterone derivatives (compound 3 and 5) on coronary resistance

The coronary resistance in absence (control) or presence of the progesterone derivatives at a concentration of 0.001 nM was evaluated. The effects were obtained in isolated hearts perfused at a constant flow rate of 10 ml/min. Since a constant flow was used, changes in coronary pressure reflected the changes in coronary resistance (Figueroa-Valverde et al., 2012).

Third stage

Effects induced by the progesterone derivative (compound 5) on left ventricular pressure through progestagen receptors

Intracoronary boluses (50 µl) of the compound 5 (0.001 to 100 nM) were administered and the corresponding effect on the left ventricular pressure was determined. The dose-response curve (control) was repeated in the presence of mifeprestone at a concentration of 1 nM (duration of preincubation with mifeprestone was by a 10 min equilibration period).

Effects induced by the progesterone derivative (compound 5) on left ventricular pressurethrough β1-adrenergic receptor

Intracoronary boluses (50 µl) of the compound 5 (0.001 to 100 nM) were administered and the corresponding effect on the left ventricular pressure was determined. The dose-response curve (control) was repeated in the presence of metoprolol at a concentration of 1 nM (duration of preincubation with metoprolol was by a 10 min equilibration period).

Effect exerted by the progesterone derivative (compound 5) on left ventricular pressure inthe presence of indomethacin

The boluses (50 µl) of the compound 5 (0.001 to100 nM) were administered and the corresponding effect on the left ventricular pressure was evaluated. The bolus injection administered was done in the point of cannulation. The dose response curve (control) was repeated in the presence of indomethacin at a concentration of 1 nM (duration of the pre-incubation with indomethacin was for a period of 10 min).

Effects of the progesterone derivative (compound 5) on left ventricular pressure throughthe calcium channel

Intracoronary boluses (50 µl) of the compound 5 (0.001 to100 nM) were administered and the corresponding effect on the left ventricular pressure was evaluated. The dose-response curve (control) was repeated in the presence of nifedipine at a concentration of 1 nM (duration of the pre-incubation with nifedipine wasfor a period of 10 min).

Statistical analysis

The obtained values are expressed as average ± SE, using each heart (n = 9) as its own control. The data obtained were put under analysis of variance (ANOVA) with the Bonferroni correction factor using the SPSS 12.0 program (Hocht et al., 1999). The differences were considered significant when *p* was equal or smaller than 0.05.

RESULTS

Chemical synthesis

The yield of the reaction product (compound 3) (Figure 1) was 64% with melting point of 172 to 174°C. In addition, the spectroscopic analyses show signals for IR (V_{max}, cm^{-1})

Table 1. 1H NMR (300 MHz, CDCl3) data for the compound 3.

Parameter
0.85 (s, 3H), 0.98-1.02 (m, 2H), 1.19 (s, 3H), 1.28-1.56 (m, 5H), 1.58 (s, 3H), 1.66-2.39 (m, 12H), 3.47 (t, 2H, *J* = 6.54), 3.67 (t, 2H, *J* = 6.54), 3.97 (t, 2H, *J* = 6.54), 3.99 (t, 2H, *J* = 6.54), 5.74 (m, 1H), 7.70 (t, 2H), 8.10-8.80 (m, 6H) ppm

Table 2. 13C NMR (300 MHz, CDCl3) data for the compound 3.

Parameter
13.20 (**C**-18), 17.12 (**C**-32), 21.16 (**C**-5), 22.52 (**C**-33), 25.28 (**C**-9), 26.32 (**C**-8), 30.40 (**C**-15), 31.13 (**C**-16), 31.31 (**C**-11), 31.60 (**C**-10), 35.26 (**C**-17), 35.61 (**C**-3), 35.84 (**C**-28), 36.42 (**C**-22), 38.04 (**C**-6), 42.80 (**C**-1), 51.97 (**C**-4), 54.20 (**C**-21), 54.38 (**C**-27), 56.52(**C**-2), 56.63 (**C**-7), 115.50 (**C**-13), 120.66 (**C**-36, **C**-42), 128.04 (**C**-38, **C**-44), 128.18 (**C**-39, **C**-45), 141.48 (**C**-34, **C**-40), 143.43 (**C**-35, **C**-41), 147.50 (**C**-37, **C**-43), 158.02 (**C**-12), 162.60 (**C**-19), 163.34 (**C**-24, **C**-30), 165.90 (**C**-14) ppm

Table 3. ^{1}H NMR (300 MHz, CDCl3) data for the compound 5.

Parameter
0.67 (s, 3H), 0.69 (m, 1H), 0.83 (m, 1H), 0.84 m, 1H), 0.93 (s, 3H), 1.07-1.67 (m, 11H), 1.66-1.70 (m, 2H), 1.71-1.94 (m, 4H), 2.00 (m, 1H), 2.07-2.22 (m, 3H), 2.37 (m, 2H), 2.64 (m, 1H), 2.65 (m, 2H), 2.67-2.75 (m, 4H), 3.49 (m, 1H), 3.60-3.62 (m, 2H), 3.64 (m, 1H), 3.65 (t, 2H,*J* = 6.25 Hz), 3.67-4.66 (m, 8H),4.86 (m, 1H), 8.30-8.80 (m, 6H) ppm

Table 4. ^{13}C NMR (300 MHz, CDCl3) data for the compound 5.

Parameter
14.30 (**C**-33), 15.20 (**C**-18), 19.00 (**C**-32), 21.12 (**C**-5), 28.25 (**C**-15), 28.36(**C**-8), 31.45 (**C**-11), 31.50 (**C**-10), 31.95 (**C**-39, **C**-53), 32.40(**C**-49), 32.66 (**C**-35), 34.40 (**C**-16), 36.16 (**C**-22), 38.24 (**C**-17), 39.10 (**C**-6), 42.80 (**C**-28), 43.05 (**C**-1), 50.70 (**C**-21), 50.76 (**C**-27), 54.58 (**C**-19), 54.70 (**C**-2), 55.70 (**C**-4), 56.22(**C**-7), 59.00 (**C**-14), 60.50 (**C**-40, **C**-54), 62.88 (**C**-36, **C**-50), 69.50 (**C**-38, **C**-52), 69.70 (**C**-48), 70.00 (**C**-34), 120.70 (**C**-44,**C**-58), 121.40 (**C**-13), 127.68 (**C**-47,**C**-61), 127.90 (**C**-46, **C**-60), 138.97 (**C**-42, **C**-56), 143.10 (**C**-57), 146.76 (**C**-12), 147.44 (**C**-45,**C**-59), 164.90 (**C**-24, **C**-30), 199.80 (**C**-41, **C**-55), 20.56(**C**-51), 202.12 (**C**-37) ppm

at 3338, 1648 and 1350. In addition, the chemical shifts of the spectroscopic analyses of ^{1}H NMR and^{13}C NMR for the compound 3 are shown in Tables 1 and 2. Finally, the results of mass spectroscopy (MS) (70 eV) was shown as m/z 786.30. Additionally, the elementary analysis data for the compound 3 ($C_{39}H_{46}Cl_4N_6O_{10}$) were calculated (C, 59.53; H, 5.99; N, 14.24; O, 20.33) and found (C, 59.50; H, 5.96). On the other hand, the yield obtained for the compound 5 (Figure 2) was 74% with melting point of 144 to 146°C. The spectroscopic analyses show signals for IR (V_{max}, cm^{-1}) at 3338, 1712, 1654, 1352 and 1200. In addition, the chemical shifts of the spectroscopic analyses of ^{1}H NMR and^{13}C NMR for the compound 5 are shown in Tables 3 and 4. Finally, the results of mass spectroscopy (MS) (70 eV) was shown as m/z 1198.30. Additionally, the elementary analysis data for the compound 3 ($C_{55}H_{62}Cl_4N_8O_{14}$) were calculated (C, 55.01; H, 5.20; Cl, 11.81; N, 9.33; O, 18.55) and found (C, 55.00; H, 5.18).

Biological activity

First stage

Effect of progesterone derivative (compound 5) on ischemia/reperfusion injury: The results (Figure 3) showed that the benzamide derivative reduces infarct size expressed as a percentage of the area at risk compared with vehicle-treated hearts (control).

Second stage

The activity exerted by the progesterone and its derivatives (compound 3 and 5) on perfusion pressure and coronary resistance in the isolated rat hearts was evaluated. The results obtained from changes in perfusion pressure as a consequence of increases in the time (3 to18 min) in absence (control) or in presence of the progesterone derivatives (Figure 4), showed that compound 5 (0.001 nM) significantly increase the perfusion pressure (p = 0.05) in comparison with the control conditions, progesterone and the compound 3 at a dose of 0.001 nM. In addition, another result (Figure 5) showed that coronary resistance calculated as the ratio of perfusion pressure at coronary flow assayed (10 ml/min) was higher in the presence of the compound 5 (p = 0.05) in comparison with the control conditions and progesterone and the compound 3 at a dose of 0.001 nM.

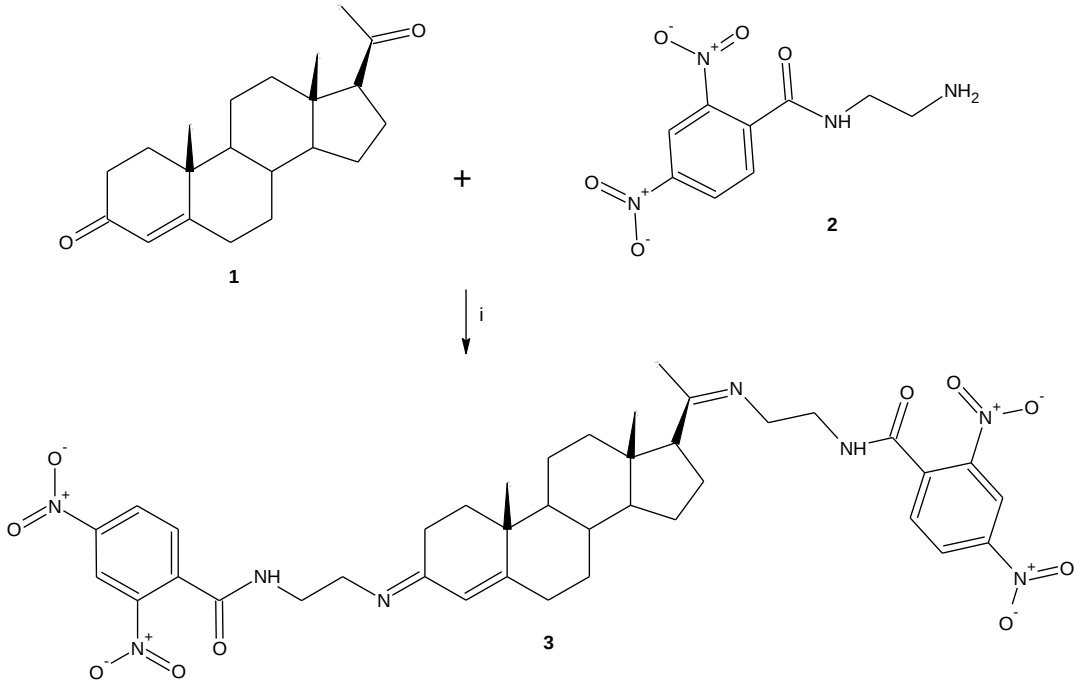

Figure 1. Synthesis of *N*-[2-(1-{3-[2-(2,4-Dinitro-benzenecarbonylamino)-ethylimino]-10,13-dimethyl-2,3,6,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1*H*-cyclopenta[*a*]phe-nanthren-17-yl}-ethylideneamino)-ethyl]-2,4-dinitro-benzamide (3). Reaction ofprogesterone (1) with 1-[(2-Amino-ethylamino)-phenyl-methyl]-naphthalen-2-ol (2) to form the compound (3) i = boric acid

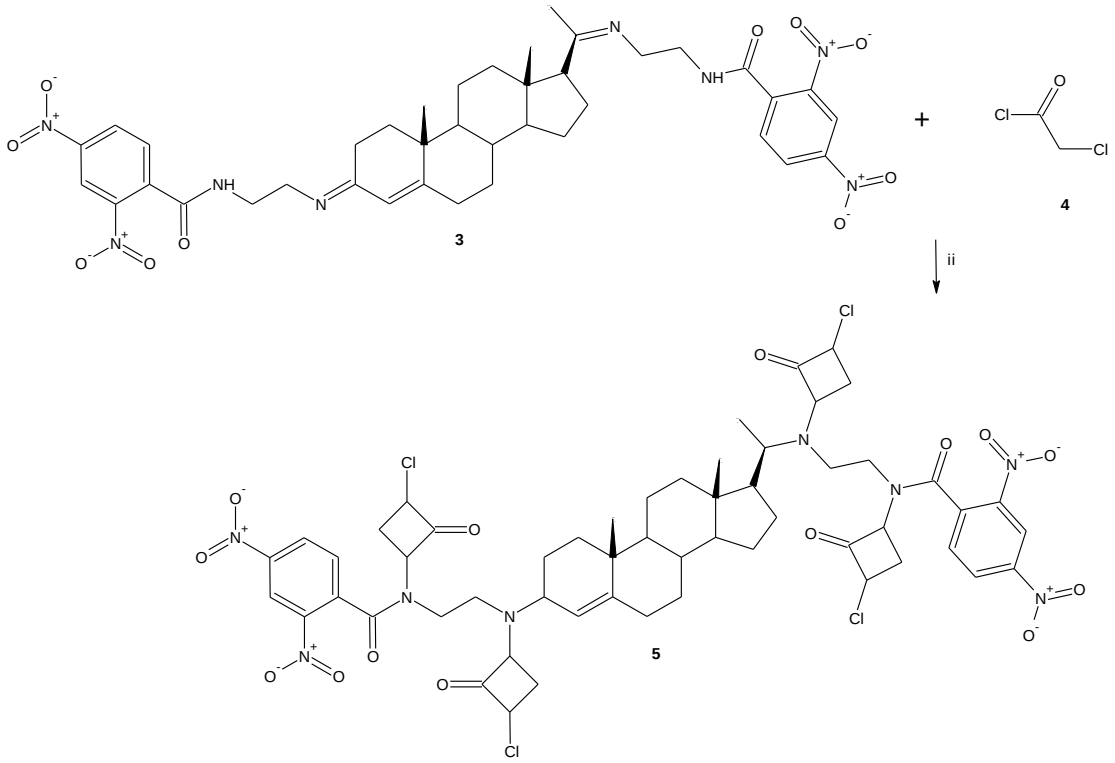

Figure 2. Synthesis of *N*-{2-[[1-(3-(*N*-(3-Chloro-2-oxo-cyclobutyl)-*N*-{2-[(3-chloro-2-oxo-cyclobutyl)-amino]-ethyl}-2,4-dinitro-benzencarbonilamino])-10,13-dimethyl-2,3,6,7,8,9,10,11,12,13,14,15, 16,17-tetradecahydro-1*H*-cyclopenta[*a*]phenanthren-17-yl)-ethyl]-
(3-Chloro-2-oxo-cyclo-butyl)-amino]ethyl}-*N*-(3-Chloro-2-oxo-cyclobutyl)-2,4-dinitro- benzamide(5). Reaction of 3 with chloroacetyl chloride to form 5.ii = triethylamine.

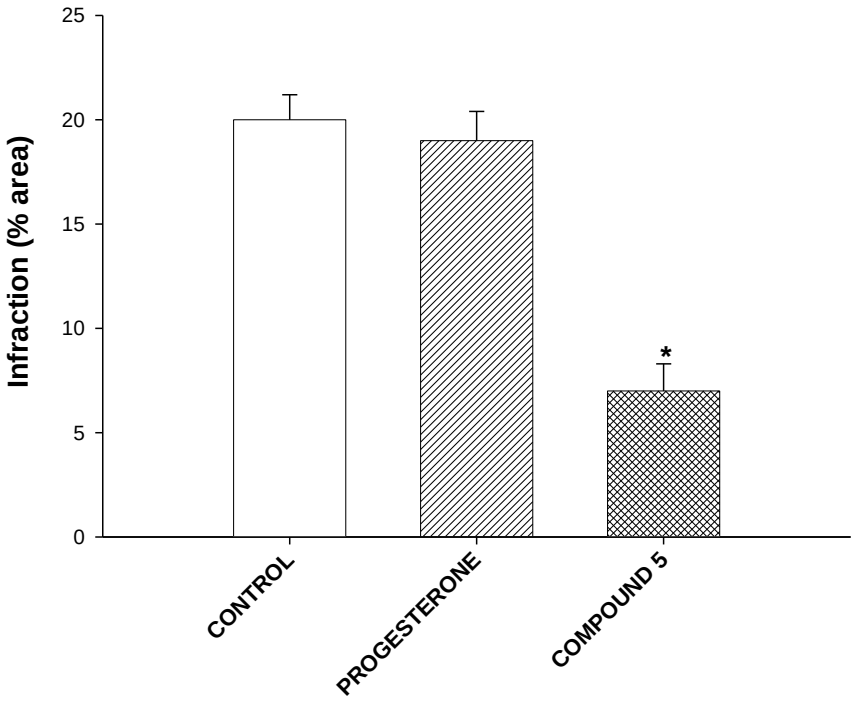

Figure 3. Effect exerted by the progesterone derivative (compound 5) on cardiac ischemia/reperfusion.The results showed that the progesterone derivative (0.001 nM) significantly reduced (p = 0.05) infarct size expressed as a percentage of the area at risk compared with progesterone (0.001 nM) and the vehicle-treated hearts. Each bar represents the mean ± SE of 9 experiments.

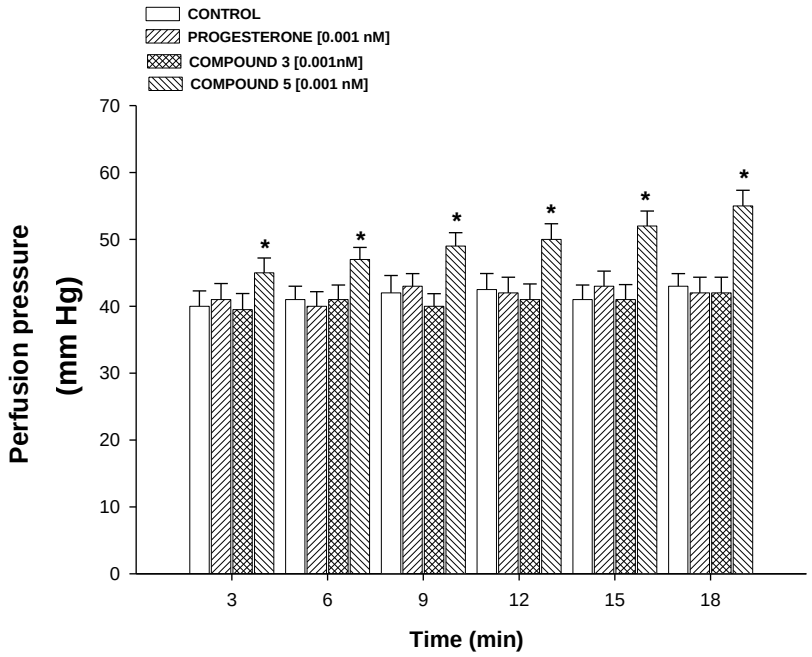

Figure 4. Effect induced by the progesterone derivatives (compound 3 and 5) on perfusion pressure. The results showed that the progesterone derivative significantly increase perfusion pressure (p = 0.05) through time in comparison with progesterone, the compound 3 and the control conditions. Each bar represents the mean ± SE of 9 experiments.

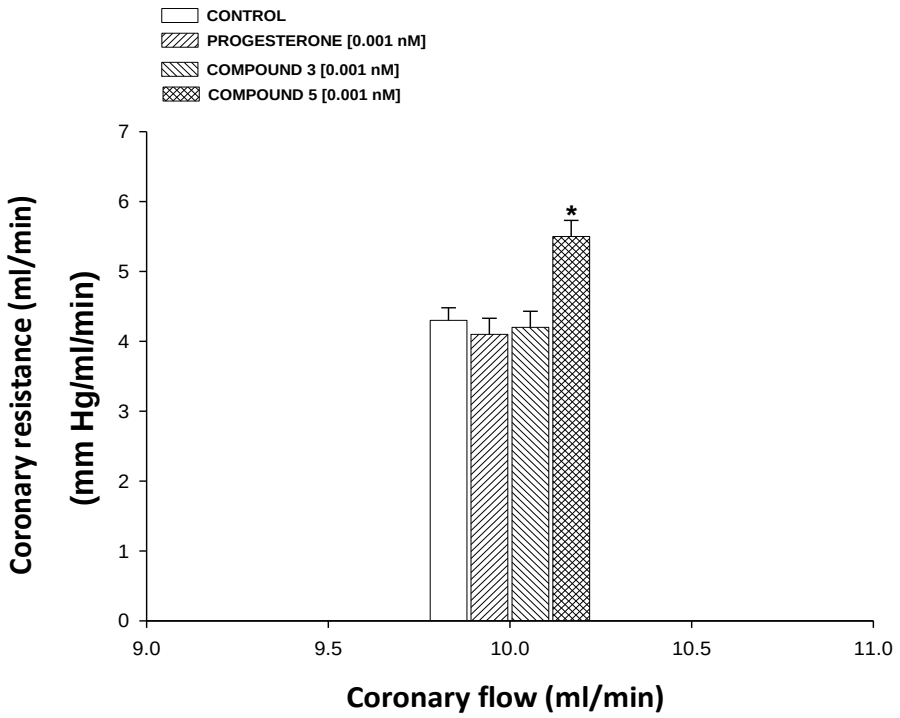

Figure 5.Activity exerted by the progesterone derivatives (compounds 3 and 5) on coronary resistance. The results show that coronary resistance was higher (p = 0.05) in the presence of the compound 5 in comparison with the control conditions, progesterone and the compound 3. Each bar represents the mean ± SE of 9 experiments.

Third stage

Other results showed that activity exerted by the compound 5 (0.001 to 100 nM) increased the left ventricular pressure and this effect was not inhibited in presence of mifepristone (Figure 6), metoprolol, prazosin (Figure 7) or indomethacin (Figure 8) drugs at a concentration of 1 nM. Finally, other data obtained (Figure 9) indicate that the progesterone derivative (compound 5) induces an increase in left ventricular pressure in a dose dependent manner (0.001 to 100 nM) and this effect was significantly inhibited by nifedipine (p = 0.05) at a dose of 1 nM.

DISCUSSION

Chemical synthesis

In this work we report some straight forward routes for the preparation of compound 5 (*N*-{2-[[1-(3-(*N*-(3-Chloro-2-oxo-cyclobutyl)-*N*-{2-[(3-chloro-2-oxo-cyclobutyl)-amino]-ethyl}-2,4-dinitro benzencarbonilamino])-10,13-dimethyl-2,3,6,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1*H*-cyclopenta[*a*]phenanthren-17-yl)-ethyl]-(3-Chloro-2-oxo-cyclo-butyl)-amino] ethyl}-*N*-(3-Chloro-2-oxo-cyclobutyl)-2,4-dinitro-benzamide). The first stage involve the synthesis of the compound 3 (*N*-[2-(1-{3-[2-(2,4-Dinitrobenzenecarbonylamino)-ethylimino]-10,13-dimethyl 2,3,6,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1*H*-cyclo- penta[*a*]phenanthren-17-yl}-ethylideneamino)-ethyl]-2,4-dinitrobenzamide) which have an imino group (Schiff base) involved in their chemical structure (Figure 1). There are several procedures for the synthesis of imino groups which are described in the literature (Shirayev et al., 2005; Uppiah et al., 2009; Figueroa-Valverde et al., 2013b).

In this study, the synthesis of the compound 3 was developed by the reaction of compound 1 (1-[(2-Amino-ethylamino)-phenyl-methyl]-naphthalen-2-ol) with progesterone using boric acid as catalyst to form the compound 3. The structure of 3 was confirmed using IR and NMR spectroscopy (Tables 1 and 2). The IR spectra contained characteristic vibrations at 3338 for imino group; at 1648 for amide group and 1350 for nitro groups. The ^{1}H NMR spectrum of the compound 3 shows signals at 0.85 and 1.19 ppm for methyl groups bound to steroid nucleus; at 1.58 ppm for methyl group bound to methanimine group; at 0.98 to 1.02, 1.28 to 1.56, 1.66 to 2.39 and 5.74 ppm for steroidnucleus; at 3.47 to 3.99 ppm for methylene groups bound to both imino and amide groups. Finally, other signals at 7.70 ppm for both amide groups; at 8.10 to 8.80 ppm for phenyl groups were found.

On the other hand, the ^{13}C NMR spectra displays chemical shifts at 13.20 and 17.12 ppm for both methyl groups bound to steroid nucleus; at 22.52 ppm for methyl group bound to imino group; at 21.16, 22.28 to 35.61,

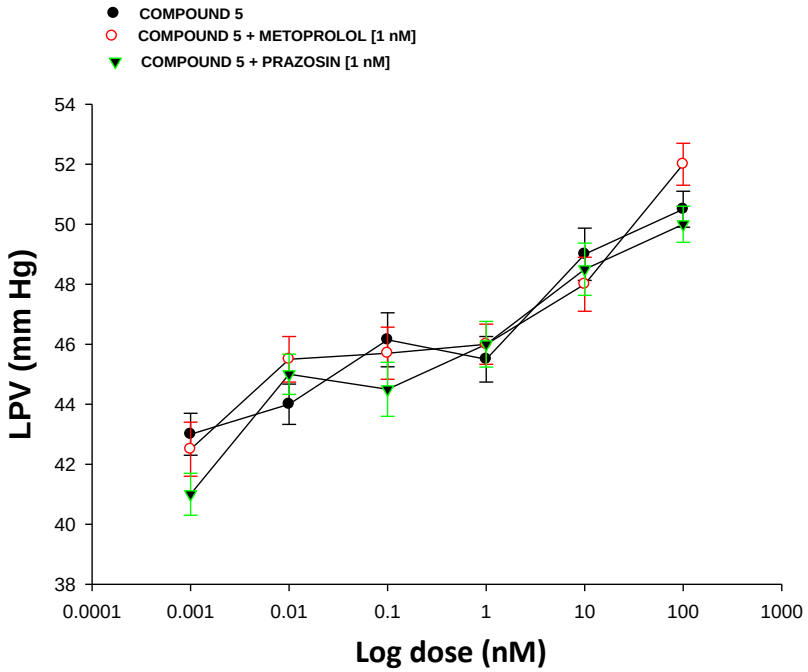

Figure 7. Activity exerted by the progesterone derivative (compound 5) on LVP through of adrenergic receptors. Progesterone derivative [0.001 to 100 nM] was administered (intracoronary boluses, 50 µl) and the corresponding effect on the LVP was evaluated in the absence and presence of prazosin or metoprolol. The results showed that activity induced by the progesterone derivative on LVP was not inhibited in the presence of prazosin ormetoprolol. Each bar represents the mean ± SE of 9 experiments. LVP = left ventricular pressure.

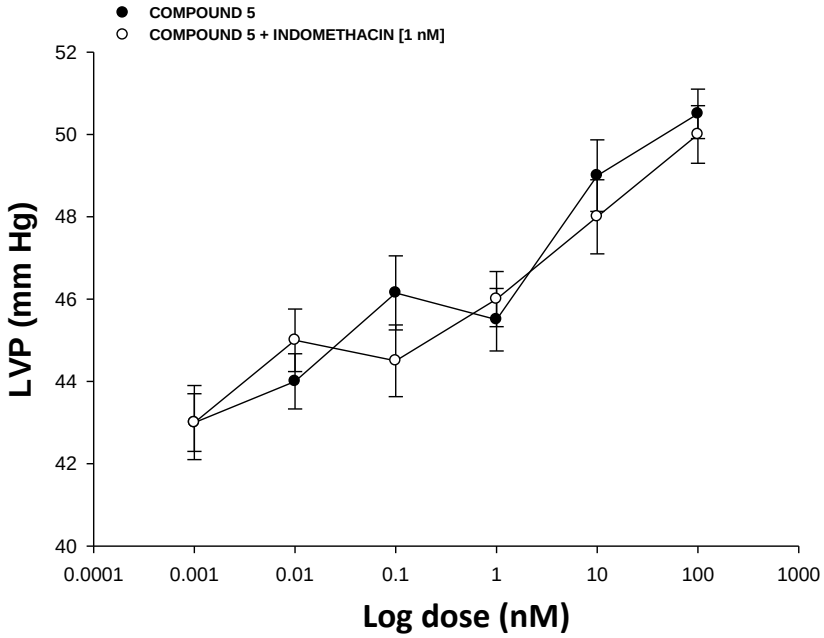

Figure 8. Effects induced by the progesterone derivative (compound 5) on LVP through prostaglandins synthesis. Intracoronary boluses (50 µl) of the progesterone derivative [0.001 to 100 nM] were administered and the corresponding effect on the LVP was determined in the absence and presence of indomethacin. The results showed that progesterone derivative increase the LVP in a dependent dose manner and this effect was not inhibited in the presence of indomethacin.
Each bar presents the mean ± SE of 9 experiments. LVP = left ventricular pressure.

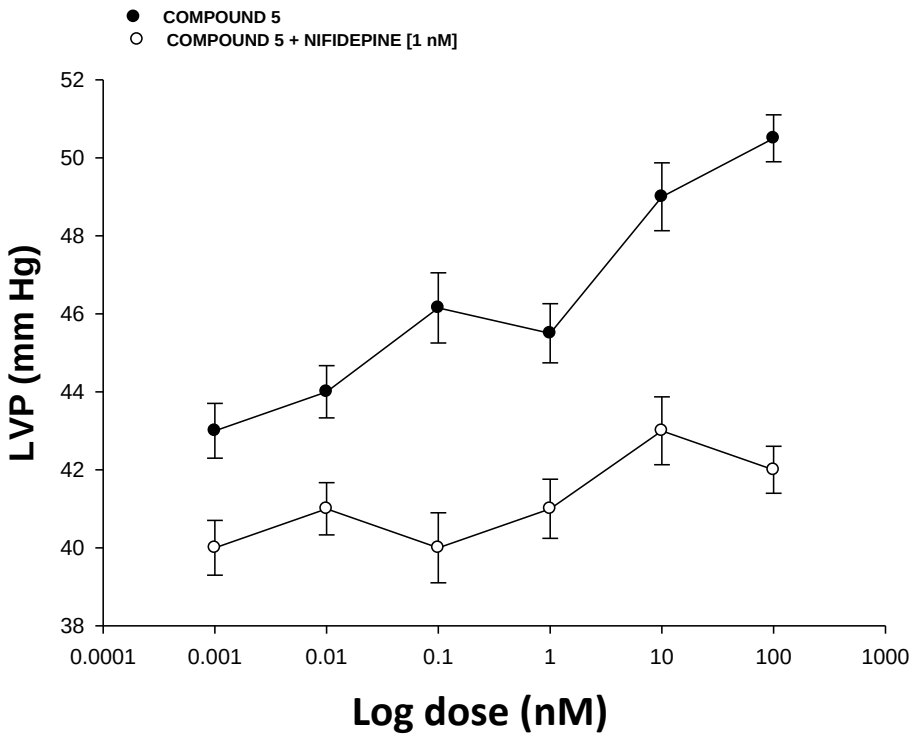

Figure 9. Effects induced by the progesterone derivative (compound 5) on LVP through calcium channel activation. Intracoronary boluses (50 µl) of the progesterone derivative (0.001 to 100 nM) were administered and the corresponding effect on the LVP was determined in the absence and presence of nifedepine. The results showed that the progesterone derivative increase the LVP in a dependent dose manner and this effect was significantly inhibited (p = 0.05) in the presence of nifedipine.
Each bar represents the mean ± SE of 9 experiments. LVP = left ventricular pressure.

38.04 to 51.97, 56.52 to 115.50, 158.02 and 165.90 ppm for steroid nucleus; at 36.42 to 54.20 for methylene groups bound to both imino and amide groups; at 120.66 to 147.50 ppm for both phenyl groups; at 162.60 ppm for carbon bound to both imino and methyl groups; at 163.34 ppm for both amide groups. Finally, the presence of benzamide derivative was further confirmed from mass spectrum which showed a molecular ion at m/z 786.30.

The second stage was achieved by the reaction of the compound 3 with chloroacetylchloride in presence of triethylamine to form the compound 5; this compound had characteristic of a cyclobutanone group in its chemical structure (Figure 2). The IR spectra contained characteristic vibrations at 1200 for amino group; at 1654 for amide group; at 1712 for carbonyl groups and 1352 for nitro groups. The ^{1}H NMR spectrum of the compound 5 (Tables 3 and 4) shows signals at 0.67 and 0.95 ppm for both methyl groups bound to steroid nucleus; at 0.83 for methyl group bound to methanoamino group; at 0.69, 0.83, 1.07 to 1.67, 1.77 to 1.94, 2.07 to 2.22, 2.64, 3.64 and 4.86 ppm for steroid nucleus; at 1.66 to 2.00, 2.37, 2.65, 3.67 to 4.66 ppm for cyclobutanone groups; at 2.67 to 2.75 and 3.60 to 3.62 and 3.65 to 3.67 ppm for methylene groups involved in the arms bound to both amide and amine groups; at 3.48 ppm for methylene group bound to both methyl and amino group; at 8.30 to 8.80 ppm for phenyl groups.

In addition, the ^{13}C NMR spectra displays chemical shifts at 14.30 ppm for methyl bound to methanoamino group; at 15.20 to 19.00 ppm for methyl groups bound to steroid nucleus; at 21.12 to 31.50, 34.40 to 39.10. 43.70, 54.70 to 59.00, 121.40 and 146.70 ppm for steroid nucleus; at 31.95 to 32.66 and 60.50 to 70.00 ppm for cyclobutanone groups; at 42.80 to 50.76 ppm for methylene groups involved in the arms bound to both amide and amine groups; at 54.58 ppm for carbon bound to both methyl and amino groups; at 164.90 ppm for both amide groups; at 127.68 to 143.10, and 147.44 for phenyl groups; at 164.90 for both amide groups; at 199.80 to 202.12 ppm for ketone groups. Finally, the presence of compound 5 was further confirmed from mass spectrum which showed a molecular ion at m/z 1198.30.

Biological evaluation

In this study, the activity of progesterone and its derivative (compound 5) on injury byischaemia/reperfusion in an isolated heart model was evaluated. The results showed that this progesterone derivative reduced

infarct size (expressed as a percentage of the area at risk) compared with both progesterone and vehicle-treated hearts. This effect can be conditioned by structured chemical difference between the progesterone and the compound 5 which may consequently bring activation of some biological structure (p.e. ionic channels or specific receptors) involved in the endothelium of coronary artery (Bouïs et al., 2000) or by the influence exerted by the progesterone derivative on blood pressure which consequently bring reduction in the infarct size, and decrease the myocardial injury after ischemia/reperfusion similar to other reports for other compounds such as estrogens (Beer et al., 2002).

In order to evaluate this hypothesis, the activity induced by the compound 5 on blood vessel capacity and coronary resistance translated as changes in perfusion pressure was evaluated in an isolated rat heart model. The results showed that the compound 5 significantly increase the perfusion pressure over time (3 to 18 min) compared with progesterone and the control conditions. Analyzing this data and evaluating the possibility of that cyclobutanone group involved in the chemical structure of compound 5 could be by itself responsible for their activity; in this study, the compound 3 was used such pharmacological tool for evaluate its effects exerted on perfusion pressure.

The results obtained indicate that compound 3 did not exert significant effects on perfusion pressure in comparison with the compound 5. These data suggest that activity induced by the compound 5 on perfusion pressure could modify vascular tone and coronary resistance of heart. Therefore, in this study the activity exerted by the compound 5 on coronary resistance was evaluated. The results indicate that coronary resistance was increased in presence of this compound in comparison with progesterone and the compound 3. These data suggest that the compound 5 exerts effect on vascular tone through generation or activation of vasoactive substances such as intracellular nitric oxide, calcium and others, and other such happening with other type of compounds such as the carbamazepine-alkyne derivative (Figueroa-Valverde et al., 2011a).

In order to characterize the molecular mechanism of this phenomenon we analyzed the reports of some investigations which indicate that progesterone induces its effect on blood pressure via activation of the progesterone receptor (Bayliss et al., 1987; Williams and Sigler, 1998). In this study, mifepristone (a progesterone receptor blocker) was used to determine if the effects of progesterone derivative on perfusion pressure were via the progesterone receptor. It is important to mention that interaction of progesterone-derivative with the progesterone receptor may be a key requirement for the biological activity as in the case of other progesterone derivatives (Melcangi et al., 1999). Our results showed that the effect of progesterone derivative was not inhibited by mifepristone, suggesting that the molecular mechanism is not via the progesterone receptor.

Therefore, in search of molecular mechanism involved in the activity of the progesterone derivative (compound 5), another study was analyzed which indicates that progesterone may stimulate synthesis or release of catecholamines (Tollan et al., 1993) which has an important role in the development or maintenance of elevated blood pressure (Lilley et al., 1976). In this sense, the effect exerted by the progesterone derivative on left ventricular pressure was evaluated in the absence or presence of prazosin or metoprolol. The results showed that the effect induced by the compound 5 was not inhibited in the presence of prazosin or metoprolol. These data suggest that the molecular mechanism involved in the activity of progesterone derivative is not via adrenergic system. Therefore, in the search of the molecular mechanism involved in activity induced by the progesterone-derivative on left ventricular pressure and analyzing previous reports, which indicate that some steroid derivatives exert effects on perfusion pressure via prostaglandins synthesis (Sheillan et al., 1983) and to evaluate the possibility that the activities induced by the compound 5 involve stimulation and secretion of prostaglandins.

In this experimental study, the activity exerted by the progesterone derivative on left ventricular pressure in the absence or presence of indomethacin was evaluated. The results showed that effect induced by the progesterone derivative on left ventricular pressure was not blocked by indomethacin. These data indicate that the molecular mechanism involved in the effect exerted by the progesterone derivative was not via prostaglandins.

Analyzing these experimental data and other reports which indicate that some steroid derivatives can induce changes to blood pressure by increasing calcium levels (Figueroa-Valverde et al., 2011b) and analyzing some reports which indicates that some positive cardiotonic agents act by an increase in intracellular Ca^{2+} and consequently induce an increase in the sensitivity of contractile proteins to Ca^{2+} ions or by combinations of the two mechanisms (Bowman et al., 1999). Therefore, in this study, we also considered validating the effect induced by the progesterone-derivative (compound 5) on left ventricular pressure via the calcium channels activation using it as pharmacological tool to nifedipine. The results showed that the effect induced by the progesterone derivative was significantly inhibited in the presence of nifedipine. All these data suggest that the molecular mechanism involved in the activity of the progesterone derivative is via the calcium channels activation. This phenomenon is similar to activity exerted by other drugs on left ventricular pressure (Thiemermann et al., 1997) which may contribute to decrease cell death caused by ischemia/reperfusion in men.

Conclusion

The progesterone derivative is a particularly interesting

drug, because the activity induced for this compound on injury by ischemia/reperfusion involves a molecular mechanism different in comparison with other drugs. This phenomenon may constitute a novel therapy for ischemia/ reperfusion injury.

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Full Length Research Paper

Evaluation of clarity and consistency in dosing directions and measuring devices for pediatric over the counter liquid medications used in United Arab Emirates

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The aim of this study was to evaluate the clarity and consistency prevalent in dosing directions and measuring devices used for over the counter (OTC) liquid pediatric medications available in United Arab Emirates (UAE). 130 pediatric oral liquid OTC medications with dosing information for children younger than 12 years were studied. The study specifically focused on issues like the inclusion of a measuring device, child resistance packaging, within product inconsistency between dosing directions on the bottle's label and dose markings on enclosed measuring device, across-product use of non standard units of measurements and abbreviations, use of numeric text according to the Food and Drug Administration recommendations (FDA) and the presence of definitions for abbreviations used. Out of 130 preparations studied, a measuring device was absent only in 16% (21 products, n=130). 31.5% (41 products, n=130) of the preparations studied did not have the child resistant cap. Dosing directions on the label/leaflet and markings on the measuring device were the same in 53.6% (52 products, n=97) and different in 46% (45 products, n=97) of preparations. Superfluous markings (marking in the dosing device that is not referred to in the product labeled dosage instructions) were present in only 21.5% (23 products, n=107) of the cases. Inconsistent text for units (milliliters) was observed in 41.2% (40 products, n=97) products. Inconsistency in expressing teaspoon and tablespoon (inconsistency in expressing teaspoons as tsps or TSP and tablespoons as tbsp. or TBS) was 18.8% (13 products, n=69) and 28.5% (20 products, n=70), respectively. The use of non standard units, tablespoons and teaspoons needs to be quickly reviewed and prevented in the best interest of pediatric population.

Key words: Over the counter drugs, United Arab Emirates, pharmaceutical preparations, oral drug administration, self administration.

INTRODUCTION

Challenges to pediatric drug therapy include excessive and unnecessary drug use, inappropriate dosage form, inaccurate dosing and improper drug administration (Lawrence, 2009). In November 2009, the Food and

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Table 1. Inclusion and exclusion criteria of OTC medications.

No.	Inclusion criteria	Exclusion criteria
1	Liquid pediatric medications to be taken orally	Liquid medication not indicated for oral use
2	Dosing directions given for a child younger than 12 years	No dosing instructions given for child younger than 12 years
3	Product selection: OTC liquids used for inflammation, cough/cold (respiratory disorders), allergic or gastrointestinal disturbances	Liquid medications containing vitamins and minerals for oral use

Drug Administration (FDA) released a new set of voluntary guidelines for the industries responsible for producing, selling and distributing the over-the-counter (OTC) medications (FDA, 2009). Several casualties resulting from unintentional overdoses of OTCs in children, which were likely due to the use of these OTC products with confusing labels, inconsistent dosage leaflets and inconsistent measuring devices with ambiguous dosing information, caused the impetus for these new guidelines.

In May 2011, the FDA issued revised guidelines for the production, marketing and distribution of liquid OTC drug products that are measured and dispensed with provided devices such as spoons, cups and droppers (FDA, 2011). The FDA notes that better measuring devices for OTC liquid drug products will help patients in self administration. Further, parents and other caregivers can administer the right amount of these medications, especially to children. Yin et al. (2010) in New York, Atlanta and Chicago set out to determine the prevalence of inconsistent dosing directions and measuring devices among popular pediatric OTC's at the time the United States (US) FDA released new voluntary guidelines to industry groups responsible for manufacturing, marketing, or distributing OTC liquid medications, particularly those intended for children. The study concluded that at the time, the FDA released its new guideline, top-selling pediatric OTC liquid medications contained highly variable and inconsistent dosing directions and measuring devices (Yin, 2010).

In March 2009, the United Arab Emirates (UAE) Ministry of Health (Medical Practice and License, 2009; Sharief and Elghandour, 2012) prohibited the use of OTC cough and cold products for children under six-years-old and stated that these medicines should only be prescribed by the physicians. In addition, these products should be dispensed for children of six years and above only by a pharmacist (MOH-RDCD-Policy, 2011).

In October 2011, the Ministry of Health (MOH) reviewed the mode of dispensing of all medications, and listed the majority of cough and cold products as Pharmacist Only Medications (Ph-OM), where a medicine may be supplied without prescription, but must be dispensed by a licensed pharmacist and placed behind the counter (Sharif and Ghandour, 2012).

If there is inconsistency between the measuring device and the dosing instructions, even correct information given by the pharmacist to the consumer may not be sufficient and still a child can suffer from overdose.

The objective of this study was to evaluate the clarity and consistency in dosing directions and measuring devices used for OTC liquid pediatric medications available in UAE. Secondarily, we looked at these medications to see if the revised guidelines recommended by the FDA were followed. In addition, the presence of child resistant caps as a means of preventing accidental administration and use of dosing device with only associated product will be evaluated. The findings of this study were compared with those of Yin et al. (2010)..

METHODS

Settings and preparations

130 pediatric oral liquid OTC medications were studied after obtaining permission from the Ethics and Research Committee of Ras Al Khaimah Medical and Health Sciences University. This study was conducted in various pharmacies in the UAE after obtaining the permission from each head of the pharmacy (both out patient and hospital pharmacies).

Criteria for analysis

Pediatric OTC medications analyzed in this study were chosen on the basis of the criteria listed in Table 1 and covered medications for allergy, gastrointestinal disorders, analgesics and respiratory disorders (Figure 1).

Study design

A descriptive comparative study was conducted using a checklist form which was filled for each OTC preparation, while comparing the dosage device and the leaflet given with it.

The features, inclusion of measuring device, presence of child resistance packaging, size of the measuring device in comparison to the largest dose prescribed, within product inconsistency

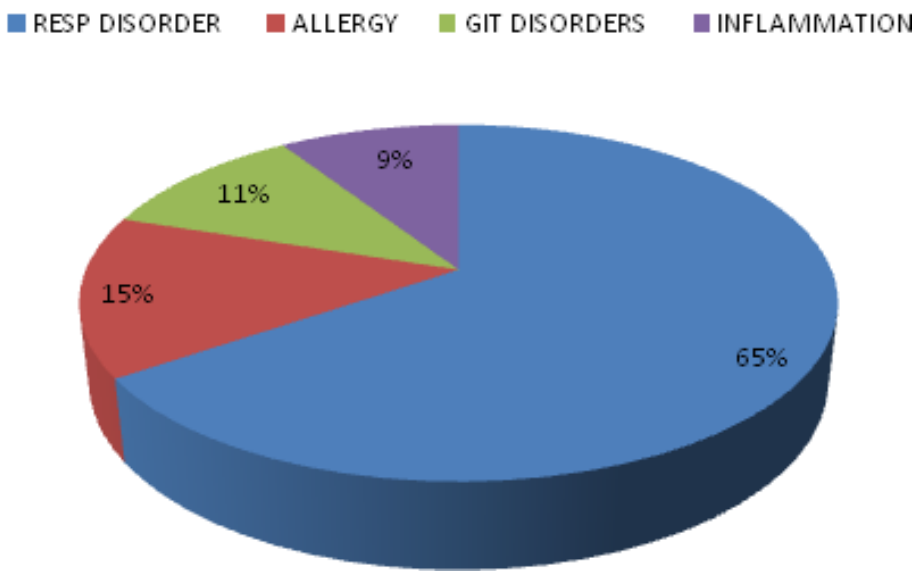

Figure 1. Pediatric OTC medications surveyed.

between dosing directions on the bottle's label/leaflet and dose markings on enclosed measuring device, across-product use of nonstandard units of measurements and abbreviations, use of numeric text (that is, leading zero before a decimal point, use of small numeral font size offset text format {e.g. ½} recommended by the FDA) and presence of abbreviation definitions, presence of elements designed to guide consumers on appropriate use, including: (1) a strategy to ensure that the measuring device is used only with the associated product (e.g. Inclusion of written statement or presence of a mechanism to secure the device to its product, such as a dropper that also serves as a cap for the bottle) and (2) a statement warning about appropriate use of the measuring device if the physician's recommended dose does not match doses marked on the device, were observed and compared between the OTC medications. The features in the form were marked as "present", "absent" or "non-applicable" in the checklist.

The parameters were observed and compared with findings of the study by Yin et al. (2010) on the basis of guidelines issued by the FDA in 2009 and 2011 for the production, marketing and distribution of liquid OTC drug products that are measured and dispensed with provided devices such as spoons, cups, droppers and the leaflets.

Data analysis

Descriptive analyses were performed for entire data using Microsoft excel. To compare the significance of the difference in the means of two groups, the Student "t" test was performed, $p<0.05$ was considered significant.

RESULTS

16.2% (21 products, n=130) of the products lacked dosing device and in 83.8% [(109 products, n=130) (95% CI: 77.8-89.8)] of the products, dosing device was included (Figure 2). Directions should clearly state that the dosing device is meant to be used only with the product with which it is packed. The probability of dosing errors increase when one dosing device is used for another product of different strength. Directions for using the dosing device with only the associated product were provided in 15.4% [(20 products, n=130) (95% CI: 9.1-21.7; $p<0.001$)] of the products. In 12.3% [(16 products, n=130) (95% CI: 6.6-18)] of the products, the dosing device was larger than the largest dose prescribed which may lead to overdosing. Child resistant packaging was absent in 41 products, (n=130) of the products studied ($p<0.001$) (Figure 2).

Physicians may tailor the dose as per the body weight or disease condition which occasionally may be different than the usual recommended dose. The dosing device may not have the markings for these revised doses. The packaging should bear warnings for the patient and the caregivers to use appropriate devices in such circumstances. Only 6.2% [(8 products, n=130) (95% CI: 2.0-10.4)] of the bottles and 37.7% [(49 products, n=130) (95% CI: 29.3-46.1)] of the leaflets studied in our survey carried such warnings.

In this present survey, 46.4% (95% CI: 43.5-63.7) of the products in our study showed inconsistency in dosing directions between bottle label and the dose markings on measuring device. Superfluous markings were also observed in 21.5% [(23 products, n= 107) (95% CI: 13.6-29.4; $p<0.001$)] of the products.

In our study, 41.2% [(40 products, n=97) (95% CI: 31.3 – 51.1)] of the products showed inconsistency in text for units which were expressed as milliliter and in 18.8% [(13 products, n=69) (95% CI: 9.4-28.2)] products, there was inconsistency in expressing teaspoons as tsps or TSP. Similarly, in 28.5% [(20 products, n=70) (95% CI: 17.1-39.2)] of the products tablespoons was expressed as tbsp or TBS (Figure 3).

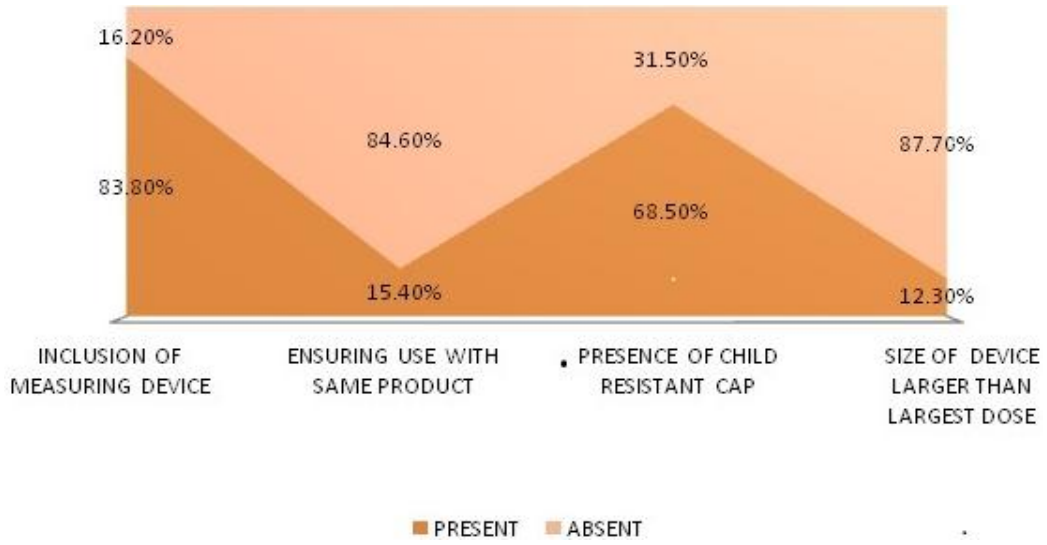

Figure 2. Area plot shown presence or absence of measuring device, directions and size of the dosing device. Key on X axis: 1: Inclusion of the dosing device; 2: Directions to ensure the device is used for only associated product; 3: Size of the dose device larger than the largest dose prescribed; 4: Child resistant packaging.

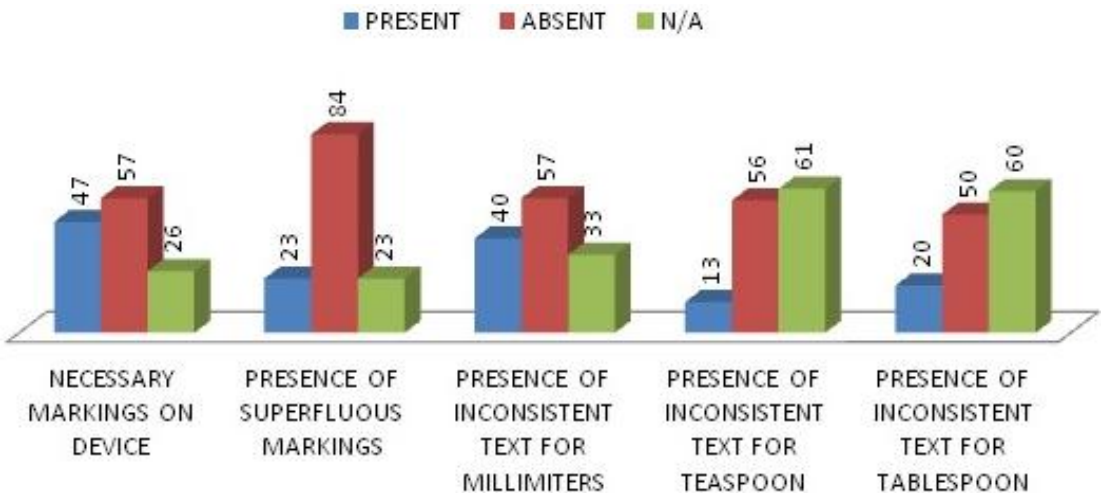

Figure 3. Inconsistency in the text for measuring units.

However in our survey, two products with a dose smaller than one omitted the leading zero, and in one product, the trailing zero was used. International standard units were used in 34.9% [(37 products, n=106) (95%CI: 25.7 – 44.1)] of the products and no product contained atypical units of measurement such as drams, fluid ounces, etc. In 40% (12 products, n=30) of the products in our survey, the tsp abbreviation was defined (Figure 4).

DISCUSSION

The developmental differences in gastrointestinal conditions and physiology in the pediatric population may manifest in differences in absorption, distribution, metabolism and excretion of drugs (Ali et al., 2013). Hence, overdose can prove fatal in this already vulnerable population group. Overdosing of OTC medications is estimated to result in emergency hospitalization of 5700 children in USA alone (Yin, 2010). Among many other factors, Yin et al. (2010) in their survey attributed inadequate and inconsistent dosing instructions and measuring device as one of the main causes for these emergencies. Stringent regulatory oversight is necessary to ensure these inconsistencies are minimized. Even though dosing device may be present, yet their use may not be easy or the instructions may not be clear enough

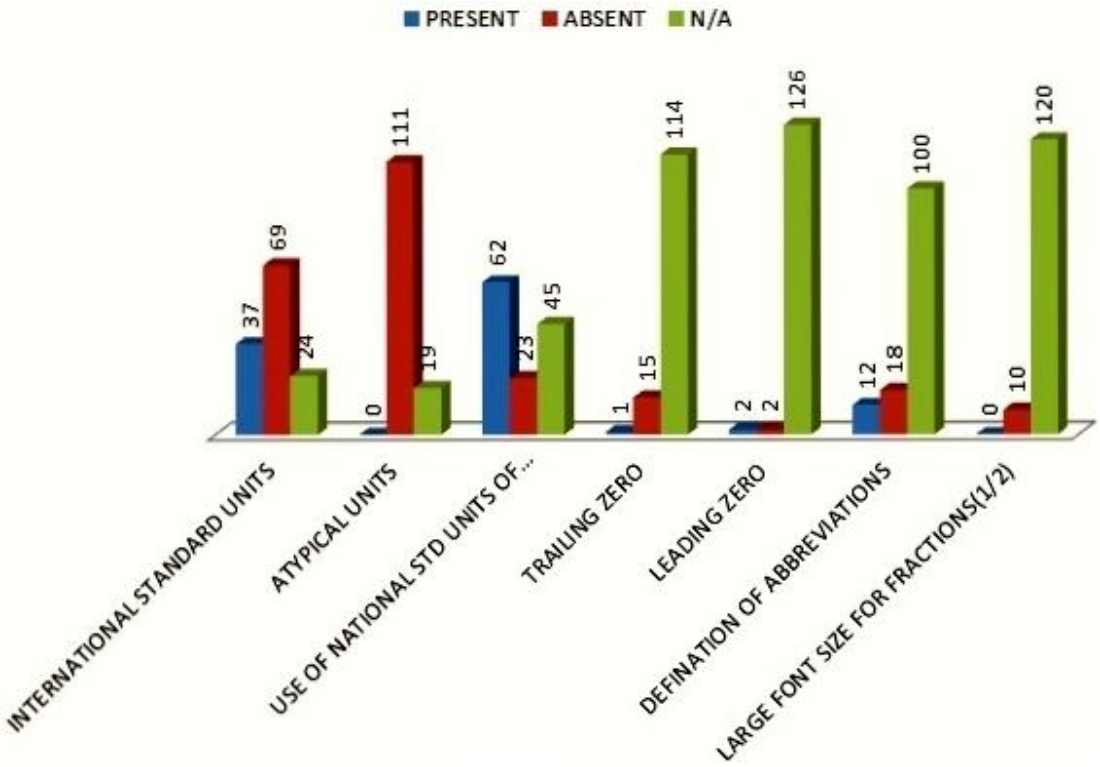

Figure 4. Units of measurement, use of zeros, abbreviations and fonts.

for caregivers to understand.

Dosing devices are provided in the form of cups, syringes, spoons and droppers. A person's preference for dosing devices (syringes and dosing cups) may vary. In one study, oral dosing syringe was found to have high dosing accuracy (92%) as compared to 85% accuracy when participants used dosing cups (Kay, 2000). Sobhani et al. (2008) in their survey on 96 subjects found that dosing cups were the most commonly used devices and oral syringe was preferred by the 80% of subjects for accurate dose measurement.

No common standard is followed in measurement of pediatric doses. The commonly used units are teaspoons, tablespoons, ounces, milliliters, cubic centimeters, etc. Confusion due to these markings often creates doubt in parents and caregivers mind which may result in overdose or under dose administration. It is estimated that 40 to 60% of parents and caregivers make errors during drug administration to children (Yin, 2010).

To address these concerns, various recommendations are proposed by the FDA guidance document for OTC liquid products (FDA, 2009). The FDA (2009) recommends that all OTC drug products should be accompanied by a measuring device and units of measurements and abbreviations used should be same on the device and in the dosing directions to avoid confusion and dosing errors (FDA, 2009). However, Perrigo (2010) in their response to FDA (2009) guidance document disagreed with the recommendation that device be unique to the associated packaged product. They argued that dosing errors will increase in cases of patients consuming multiple medications.

The guidance further says that no unnecessary markings should be used. Yin et al. (2010) reported 98.6% products with inconsistency between immediate packaging and the dosing device. Since the issuance of FDA guidance document, the inconsistencies have significantly gone down. Comparing the results of Yin et al. (2010) with our findings in UAE, 46% (95% CI: 34.5-53.9) of products had marking inconsistencies between label and the device, that is, the markings described in the label were not consistent with the dosing device. Our studies revealed that 31.5% of products lacked child resistant packaging. Besides being a regulatory requirement, child resistant packaging prevents self administration by children which can be insidious (FDA, 1999).

Muddle between "teaspoon" (tsp) and "Tablespoon" (Tbsp) can easily lead to over-or-under dosing of the medication. Further, these terms tend to give the impression that it is appropriate to use a kitchen spoon as a measurement device. Kitchen spoons are un-calibrated and are not recommended for dosing purpose. However, they are widely used, for example, in one survey Madlon-Kay and Mosch (2000) found that 73% of parents used a kitchen teaspoon to administer medication. As shown in

Table 2. Comparison of inconsistencies in dosing devices in USA and UAE markets.

Parameter	Present survey for UAE market	Survey by Yin et al. for USA market
Standard measuring device	83.8	74.0
Inconsistencies between the labeled directions and the dosing device	46.4	98.6
Superfluous markings	21.5	81.1
Nonstandard units of measurement	27	5.5
Use of milliliter as unit	73	71.5
Teaspoon	81.2	77.5
Tablespoon	71.4	18.5
All abbreviations defined	40	1.2
Leading zero absent	50	12.5
No statement mentioning that the measuring device should only be used with the product it is packed with	-	62.2
On the bottle	93.9	-
On the leaflet	62.3	-

Table 2, marked improvement (71.4%) has been made in the expression of tablespoon since Yin et al. (2010) study (18.5%). Smaller font for numernals in fractions as provided in FDA guidelines (2009) was used in all the applicable products (10 products, n=10) surveyed in this study. The supplemental materials accompanying a dosage form must be written at or below sixth-grade reading level and dosing devices should require only one measurement of medication to avoid dosing error (Wallace et al., 2010).

The use of standardized calibrated dosing devices needs to be advocated for reducing dosing errors arising from nonstandard dosing devices. Use of household spoon should be strongly discouraged. The American Academy of Pediatrics recommends using milliliter to enhance standardization and accuracy of dosing instructions (Yin, 2010). Well marked devices will further curtail the unintentional dosing errors. Use of metric units for dosage is also recommended by various other institutes (Official “do not use list” by the joint commission, 2012; United States Pharmacopoeial Convention, 2012; Lawrence and Isetts, 2009.). The standardization of dosing devices represents an area for further improvement (Table 2). Identification of dosage and corresponding measure on the label and on the dosing device, respectively, is of paramount importance in the prevention of dosing errors.

To further reduce ambiguity, the FDA recommends the use of one zero before the decimal point (FDA, 2009). The guidance further recommends that zeros should not be used after the decimal point (FDA, 2009).

Though the study by Yin et al. (2010) was conducted in different country with different socioeconomic measure and literacy rate. The comparison provides an insight into the areas which need further improvement. As shown in Table 2, since the inception of the FDA guidance document, significant improvements have taken place in dosing device consistency and uniformity. Further, MOH in UAE has made regulatory oversight stringent which has resulted in significant improvement in therapy.

Significant improvements have been made in terms of reducing the inconsistencies between the labeled directions and the dosing device, reducing superfluous markings, abbreviations, presence of leading zero and in avoiding the use of large font size. However, use of nonstandard units, tablespoons and teaspoons needs to be quickly reviewed and prevented in the best interest of pediatric population.

Conclusion

Absence of child–resistant caps in 31.5% of the syrup products subjects highly vulnerable children population to a high risk of overdose due to accidental ingestion. Different dosing directions were observed on the measuring device and the leaflets of almost half of the medications studied (46.4%). There is an urgent need to further streamline dosing directions and dosing devices to reduce the errors caused by confusion and ambiguities present in existing dosing devices. Replacement of teaspoons and tablespoons with standard units such as “ml” can mitigate the risk caused by inconsistencies in the expression of teaspoons and tablespoons.

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