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Potenciais benefícios para a saúde de extratos de *Artemisia gorgonum*

Potential health benefits of *Artemisia gorgonum* extracts

Dissertação
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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Molecular e Celular, realizada sob a orientação científica da Doutora Maria de Lourdes Pereira, Professora Associada c/Agregação do Departamento de Biologia da Universidade de Aveiro e co-orientação da Professora Doutora Helena Silva, Professora Auxiliar do Departamento de Biologia da Universidade de Aveiro.

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O Júri

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Palavras-chaves: *Artemisia gorgonum*, extratos de plantas, atividade antimicrobiana, atividade antioxidante, hepatoproteção.

Resumo

Relevância etnofarmacológica: *Artemisia gorgonum* (Asteraceae), conhecida como "losna ou lorna", é usada em Cabo Verde na medicina tradicional para o tratamento de inflamações, febre e gastroenterites. Estudos recentes sugerem que artimetina, isolada a partir de *Artemisa gorgonum*, poderia ser usada para o tratamento da malária devido à sua atividade antiplasmodial.

Objetivo do estudo: Avaliação *in vitro* da atividade anti-microbiana e sinergética dos extratos hidroetanol (70%) e metanol de *A. gorgonum* (EHAG e EMAG) em bactérias do trato urinário e uma espécie de fungo. A atividade antioxidante dos extratos de hidroetanol (70%), metanol, clorofórmio e clorofórmio-metanol (1:2), e o efeito protetor de EHAG contra lesões hepáticas em ratos induzidos com CCl_4 também foram analisados. *Material e métodos*: A atividade antimicrobiana dos extratos de *A. gorgonum* foi testada *in vitro* contra sete estirpes de microrganismos, incluindo bactérias Gram-positivas, Gram-negativas e uma espécie de fungo. O método DAA (*Decimal assay for additivity*) foi determinado para atividade antibacteriana do EHAG contra *Pseudomonas aeruginosa*. O efeito antioxidante *in vitro* de vários extratos de *A. gorgonum* foi analisado pelo método DPPH. A lesão hepática foi induzida por injeção intrapeitoral do CCl_4. Seguidamente, os ratos foram administrados oralmente com EHAG, diariamente, por um período de 7 dias. Resultados e Discussão: Foi observada atividade antibacteriana dos extratos de EHAG e EMAG contra todos os microrganismos usados neste estudo. O crescimento das estirpes de *Escherichia coli* e *Pseudomonas aeruginosa* foi o mais inibido por ambos os extratos, apresentando valores significativos, enquanto o crescimento das estirpes *S. aureus* e *Klebsiella spp*. foi o menos afetado. *Candida albicans* foi inibida fortemente pelo EMAG. As combinações de extrato hidroetanólico com antibióticos demonstraram atividade antibacteriana sinergética contra todos os patogénicos testados. Em contrapartida, a combinação de extrato metanólico com antibióticos permitiu observar efeitos antagónicos contra todas as bactérias, exceto *Klebsiella* spp. que apresentou atividade sinérgica. O EHAG e EMAG mostraram efeito significativo na eliminação do radical DPPH. A atividade hepatoprotetora foi observada em ratos previamente administrados com CCl_4. Estes estudos evidenciam os potenciais benefícios de *A. gorgonum.*

Keywords: *Artemisia gorgonum*, plant extracts, antimicrobial activity, antioxidant activity, hepatoprotection.

Abstract

Ethnopharmacological relevance: Artemisia gorgonum (Asteraceae) known as "losna" or "lorna" is used in Cape Verde as traditional medicine to treat inflammation, fever and gastroenteritis. Recent studies have suggested that artemetin isolated from *A. gorgonum* may be used to treat malaria, due to its anti-plasmodium proprieties.

Aim of the study: Evaluate the *in vitro* antimicrobial and synergistic activity of hydroethanol (70%) and methanolic extracts of *A. gorgonum* (HEAG and MEAG*)* against urinary bacteria and one species of yeast. Antioxidant activity of the hydroethanol (70%), methanol, chloroform and chloroform-methanol (1:2) extracts and the protective effect of HEAG against liver injury on rat model were also evaluated.

Material and methods: Antimicrobial *in vitro* activity of *A. gorgonum* leaves extracts were tested against seven microorganism species, which frequently cause urinary infections, including Gram-positive bacteria, Gram-negative bacteria and one yeast. The synergistic effect of the HEAG extracts against *Pseudomonas aeruginosa* was evaluated by decimal assay for additivity (DAA) method. The *in vitro* antioxidant activity of several extracts from *A. gorgonum* was evaluated by 2, 2-diphenyl-1-picryl hydrazyl (DPPH) methods. Liver injury was induced by intraperitoneal CCl_4. Rats were orally administrated with HEAG daily for one week. *Results and Discussion:* The antimicrobial activity of hydroethanolic (70%) and methanolic extracts from *A. gorgonum* was showed against all microorganisms used in this study. *Escherichia coli* and *Pseudomonas aeruginosa* growth was significantly inhibited by both extracts whereas *S. aureus and Klebsiella* spp. growth was less inhibited. The *Candida albicans* was strongly inhibited by the methanolic extract. The combinations of hydroethanolic extract with antibiotics demonstrated the synergistic antibacterial activity against all pathogens tested. In contrast, the combination of methanolic extract with antibiotics revealed antagonism effects against all bacteria, except *Klebsiella* spp. showed synergistic activity. The MEAG and HEAG showed significant radical scavenging effect in the DPPH assay. The hepatoprotective effect was observed in rats previously administered with CCl_4. These studies highlight the potential benefits of *A. gorgonum*.

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Chapter I: Antibacterial and antioxidant effects

1. Introduction

Traditional medicine is practiced for many centuries by a substantial proportion of the world population, particularly by inhabitants from rural areas, in order to provide essential physical and psychological health care. Medicinal plants are Nature's gift to human, helping them to pursue a disease-free healthy life, and thus can play an important role in preserving health.

In an extensive review of new drugs between 1981 and 2006, 48% of the 1184 new chemical entities approved as drugs by the US Food and Drug Administration, include 5% natural products, 23% natural-product derivatives and another 20% natural mimic compounds (Newman et al., 2009).Therefore, natural products have been perceived as a highly significant source to develop promising new drugs (Appendino and Banfi, 2011).

Phytomedicines derived from several plants demonstrated great promise in the treatment of intractable infectious diseases including viral infections (Cowan, 1999). The use of medicinal plants and their extracts have grown in health care due to its easy acceptability, availability and low cost. Numerous research studies have been carried out to screen the antimicrobial activity of several natural products. However attention has not been focused intensively on the study of the combinations of plant-drug for their antimicrobial activity (Abu-Shanab et al., 2004).

Several studies reported the antioxidant activity of some medicinal plants (Liu et al., 2004; Cavar et al., 2012; Rashid et al., 2013). Antioxidants retard oxidation to prevent or slow oxidative degradation of tissues. Antioxidant agents are effective due to different mechanisms such as free radical scavenging, chelating of pro-oxidant metal ions or quenching singlet-oxygen formation (Lopes-Lutz 2008) which are associated with several diseases. Due to their accessibility, much attention has been paid to natural antioxidants for preventing radical-induce damage and disease. Various medicinal plants could be used as natural antioxidants and antimicrobials (Lopes-Lutz, 2008; Kershaw, 2000).

2. Ethnopharmacological study of the *Artemisia* genus

Artemisia gorgonum Webb (Asteraceae) is an endemic small aromatic shrub from Cape Verde and this species is included in the ecosystem proposed by the "protected areas" project which revels its importance for the flora of this country (Cesarini and Furtado, 2006).

Artemisia species showed anti-coagulant, anti-inflammatory and immunosuppressive effect, antimicrobial, antioxidant, antimalarial, immunotherapeutic, neuroprotective, antifungal, and hepatoprotective activity (Shahriyary and Yazdanparast; 2007; Yin et al., 2008; Annan and Houghton., 2008; Ortet et al., 2008; Xie et al., 2008; Bora and Sharma, 2010; Amat et al., 2010 ; Ćavar et al., 2012; Wang et al., 2012.). Due to high immunosuppressive activity of *A. abrotanum*, its use in the treatment of autoimmune diseases was suggested (Remberg et al. 2004). *A. frigida* and *A. dracunculus* (tarragon) are used as spice and to preserve meat (Kershaw, 2000). These species could be a source of natural antioxidants and antimicrobials (Paulke et al., 2008). Artemisinin (sesquiterpene lactone) and derivates isolated from *A. anuua* showed effective action against protozoa, some viruses, tropical parasites and human cancer cells (Weathers et al., 2011).

The volatile oil from *A. gorgonum* displays several biological properties including outstanding antioxidant activity, showing efficacy against diseases caused by excess production of radicals (Ortet et al., 2010). These authors isolated previously several sesquiterpene lactones, furofurans lignans and a flavonoid (Figure 1) (Ortet et al., 2008, 2011).

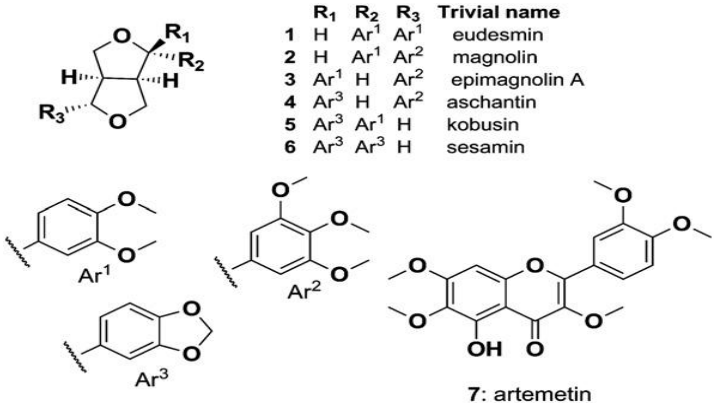

Figure 1: Representative six lignans furfurans compounds of *Artemisia gorgonum* extracts (Ortet et al., 2008, 2011).

The compounds ridentin, sesamin and artemetin isolated from *A. gorgonum* showed anti-plasmodium *in vitro* activity (Ortet et al., 2008, 2011).

Recently, seco-guaianolides isolated from the same species, showed higher phytotoxic activity, and the authors proposed them for development of a natural herbicide model (Figure 2) (Macías et al., 2012).

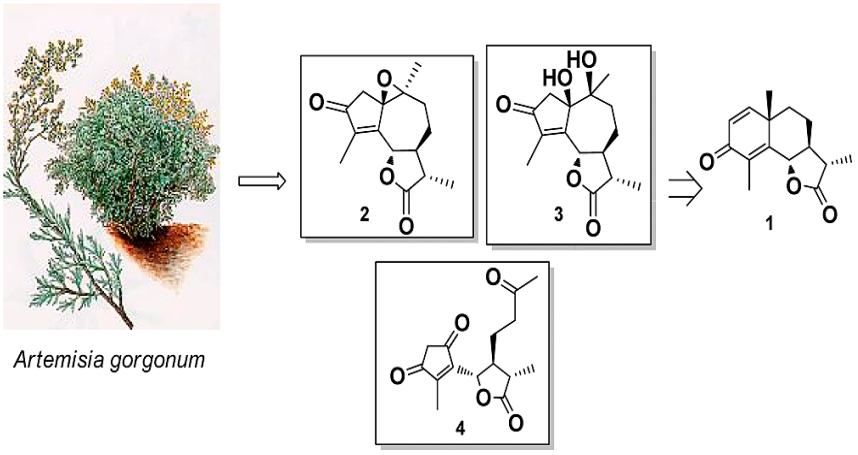

Figure 2: Seco-guaianoloides structure isolated from *Artemisia gorgonum* (Macías et al., 2012).

The aim of the present study was to evaluate the, antimicrobial and antioxidant potential of *A. gorgonum* leaves extracts.

3. Material and methods

3.1 Selection of bacterial and yeast strains

Representative microorganisms were kindly obtained from Medical Laboratory of Clinical Analysis (AVELAB): Gram-negative bacteria (*Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella* spp., and *Klebsiella spp*.), Gram-positive bacteria (*Staphylococcus aureus* and *Enterococcus faecalis*) and yeast (*Candida albicans*), categorized as frequent cause of urinary infection. Microorganisms were isolated from urine samples except *Salmonella* (isolated from faeces) and inoculums were prepared in Mueller Hinton Broth and maintained for 24h at 37ºC.

3.2 Sampling of A. gorgonum and preparations of plant extracts

Leaves of *A. gorgonum* were collected in Serra Malagueta Natural Park, Cape Verde, Santiago Island, in January 2012. Taxonomical identification was performed by Helena Silva (Department of Biology, University of Aveiro, Portugal). A voucher specimen was deposited in the Herbarium of the referred University.

The extracts were obtained from dried and powdered leaves of *A. gorgonum*. Four portions of vegetal material (500 mg) were extracted with one of the following extractants: hydroethanolic (70%), chloroform, chloroform-methanol (1:2) and methanol (20 mL each), during 30 minutes at 70 ºC and then maintained at room temperature for 24 hours (Smith et al., 2007). All extracts were filtrated using a 0.45 μm membrane Whatman filter paper.

3.3 Bacteria inoculums preparation and disc diffusion method

The sensitivity determination of microorganism on plant extract was performed according to the disc diffusion method, described by Kirby-Bauer (1966), following the recommendations of National Committee for Clinical Laboratory Standards - NCCLS (2002). The Mueller Hinton agar media was used for determination of extracts antibacterial activities (Rashid et al., 2013). To standardize, the inoculums were made

with bacterial suspension with turbidity of 0.5 McFarland scale. Sterile filter disc (6 mm in diameter) was impregnated with 30 µL of plant extract (625 µg/mL) and delivered into the well (Meléndez and Capriles, 2006; Sakunpak and Panichayupakaranant, 2012). After incubation at 37 °C for 24 h, the discs were examined for any zones of inhibition. Petri dishes (86 mm internal diameter) were filled with this system.

The antimicrobial activity was determinate by measuring the diameters of zones of inhibition (mm) produced after incubation at 37ºC for 24 hours.

The diameter of the inhibition zones (mm) were measured by ImageJ software. The standard antibiotics for positive control were Ciprofloxacin (CIP) (5µg) for *P. aeruginosa* and amoxicillin (875 mg) + clavulanic acid (125 mg) (AMC) (5µg) for other microorganisms. The negative control was used the solvents without extracts.

3.4 Antifungal activity test

The Sabouraud agar medium was used for determination of antifungal susceptibility of extracts. The starting inoculum was 1.0×10^6 CFU/mL and then was incubated at 37 ºC, the diameters of inhibition zone (mm) were recorded after 24h of incubation. The susceptibility endpoints were defined as the lowest concentration of antifungal agent that resulted in total inhibition of diameter growth measured by ImageJ software. Nystatin (Sigma Aldrich) was used as the positive control.

3.5 Determination of Decimal Assay for Additivity (DAA) methods

The interaction between hydroethanolic (70%) and methanolic extract of *A. gorgonum* (HEAG and MEAG) with antibiotics (CIP and AMC) was investigated, in vitro assay (Sanders et al., 1993), for their antimicrobial activity against microorganisms, using combinations of AMC+ HEAG, AMC +MEAG, CIP+ HEAG and CIP+ MEAG (Table 1). After bacteria were inoculated and grown overnight at 37º C, the sterile filter disc (6mm) was impregnated with different proportion of plant extract and antibiotics, placed on the agar plates. The final antibiotics and plant extract concentration was 500 µg/mL per disc. After an incubation period of 24 h at 37º C, inhibition zones were measured in millimetres. The experiment was repeated in triplicate.

Table 1: Decimal assay for additivity of hydroethanolic (70%) and methanolic extract of *Artemisia gorgonum* combined with Amoxicillin+ clavulanic acid and Ciprofloxacin against bacteria.

Decimal mixture	**Ratio (A:B)**	**Antibiotic combination**	
		A	B
0	10:0	**1.0**	**0.0**
1	9:1	**0.9**	**0.1**
2	8:2	**0.8**	**0.2**
3	7:3	**0.7**	**0.3**
4	6:4	**0.6**	**0.4**
5	5:5	**0.5**	**0.5**
6	4:6	**0.4**	**0.6**
7	3:7	**0.3**	**0.7**
8	2:8	**0.2**	**0.8**
9	1:1	**0.1**	**0.9**
10	0:10	**0.0**	**1.0**

A: Antibiotics; B: Plant extracts;

3.6 Determination of antioxidant activity

Antioxidant activity was assayed by the DPPH (2, 2-diphenyl-1-picryl hydrazyl) radical scavenging method (Blois, 1958) modified by Barreto et al. (2012). Briefly, to different concentrations of ethanolic solutions of each extracts (hydroethanolic 70%, methanolic, chloroform-methanol (1:2) and chloroform) were added fixed volumes of DPPH ethanolic solution and solvent (ethanol) to obtain in each case a fixed total volume. In each assay, a control was prepared, in which the sample or standard (quercetin and BHT) was substituted by the same amount of solvent.

The absorbance of each solution was measured at 517 nm against a corresponding blank (ethanol solution) after 30 min. in dark at room temperature. The percentage of DPPH inhibition was calculated as follows:

$$\%\, DPPH\ Inhibition\ = \frac{A\ control - A\ sample}{A\ control}\ \text{X}\ 100$$

Where Acontrol is the absorbance of the assay control and Asample is the absorbance of assay with extract or standard. The extract concentration needed to have 50 % of DPPH inhibition (EC_{50}) was obtained by interpolation from % DPPH inhibition versus extract concentration curve. The test was carried out in triplicate and the results were expressed as EC_{50} media with standard deviation.

3.7 Statistical analysis

All data were represented as a mean of three independent measurements. Data were analyzed by one-way ANOVA. Results are expressed as mean±S.D. (standard deviation) data, using the Statistic 7 Software. Significance was set at $P < 0.001$ for all tests.

4. Results and Discussion

4.1 Anti-microbial effects of plant extracts

The diffusion method is generally used as a preliminary screening for antimicrobial activity prior to more detailed studies (Hammer et al., 1999). The results of antimicrobial activity showed that methanolic and hydroethanolic (70%) extracts from *A. gorgonum* inhibited significantly the microorganisms growth. Both plant extracts were active against the microorganisms assayed, however *P. aeruginosa* and *E. coli* presented the highest inhibition whereas *S. aureus* and *Klebsiella* spp., displayed the lowest growth inhibition (Table. 2). The fungus, Candida albicans, was less susceptible to the hydroethanolic (70%) extract compared with methanolic extract, but both extracts inhibit it significantly. The hydroethanolic (70%) extract was more active against all bacteria than methanolic extract. The Gram-positive bacterium *S. aureus* was sensible to hydroethanolic (70%) and methanolic extracts at the highest concentration (1250 µg/mL). Antimicrobial activity evaluation of the hydroethanolic (70%) and methanolic extracts of *A. gorgonum* suggests the need for further studies considering the isolation of substances responsible for the antibacterial activity and study of the mechanism involved in the modulation of multidrug-resistance, if necessary for development of new drugs.

Table 2: Antibacterial effects of hydroethanolic (70%) and methanolic extracts from *Artemisia gorgonum*. The results expressed as the mean and standard deviation calculated. Data are referred to two separated experiments (ANOVA followed by Dennett's test), p-value < 0.001 as compared to control group (C-).

Microorganism		**Zone of inhibition (mm)**					
		Plant extracts				Positive control	Negative control
		HEAG		MEAG		Antibiotic reference #	Only solvents*
		mean	S.D	Mean	S.D		
Gram – negative bacteria	*Escherichia coli*	20***	0,11	18***	1,69	28	*6*
	Salmonella spp.	15***	1,08	15***	1,92	-	*6*
	Pseudomonas aeruginosa	23***	1,19	17***	1,55	25	6
	Klebsiella spp.	13**	0,77	11**	0,80	11	6
Gram- Positive bacteria	*Enterococcus faecalis*	14**	1,31	11**	0,99	27	6
	Staphylococcus aureus	10	0,98	9	1, 27	26	6
Fungi	*Candida albicans*	17***	2,95	19***	0,77	40	*6*

*** Significance P < 0.001, compare with negative control group.
** Significance P < 0.01, compare with negative control group.
#Antibiotic reference (0, 05mg.ml^{-1}): AMC for Gram –negative bacteria except pseudomonas were used ciprofloxacin; Nystin for fungi -without reference antibiotic

2. DDA analysis

The interactions between plant extract and antibiotics have demonstrated anti-bacterial activity against all bacteria used in this study (Table 3). These pathogens are very important and have the ability to cause infections in various kinds of diseases. The increasing number of multidrug resistant strains of *Klebsiella pneumonia* and *P. aeruginosa* has become a serious clinical and epidemiological problem (Rashid et al., 2013).

The highest zone inhibition resulted by the combination plant-drug was showed on the proportion 5:5 in both extracts (Table 3). HEAG with antibiotics combinations showed increased inhibition zone diameter when compared with antibiotics and HEAG separated in all bacteria tested. The strongest combination effect was observed against *P. aeruginosa* that was inhibited by combinations of HEAG with CIP, followed by *S. aureus*, *E. coli*, *E. faecalis* and *Klebsiella* spp. using combinations of HEAG with AMC.

Combinations between MEAG and antibiotics showed decreased antibacterial activity against all bacteria excepted *Klebsiella* spp.. The *E. faecalis*, *E. coli* and *S. aureus* demonstrated less susceptibility in MEAG with AMC combined. The antagonism effect was observed in combination of AMC and HEAG against *P. aeruginosa*. Currently, several scientific data support the antibacterial potentiality of herbal extracts or their isolated compounds (Mahady et al., 2008). A potential interaction between plant-drug can be demonstrated from pure plant extracts and antibiotics, which may or not, be beneficial. Advantageous interactions can result from additivity or synergy and the contrary from antagonism or toxic effects (Hemaiswarya, 2008). In addition, according to these results the decimal assay for additivity identified the component ratio at which drug interaction was maximum, and it deserve further studies to identify which component was affecting the other.

Table 3: Additivity assay against Gram –positive and Gram-negative, using HEAG and MEAG with CIP (for only *Pseudomonas aeruginosa*) and AMC (others bacteria) combinations.

Microorganisms	Gram	Inhibition zone (mm)				Antibiotics references
		Combined HEAG with Antibiotics		Combined MEAG with Antibiotics		
		Mean	S.D	Mean	S.D	
Escherichia coli	-	30,1**	1,5	26,3	3,5	28
Pseudomonas aeruginosa	-	32,7***	2,08	19	2	25
Klebsiella spp*	-	19,7**	1,5	13,7	0,6	11
Enterococcus faecalis	+	29,7	5,5	26,5	0,6	27
Staphylococcus aureus	+	32,1***	5,5	25,7	3,2	26

*** Significance P < 0.001, compare with negative control group.
** Significance P < 0.01, compare with negative control group.
#Antibiotic reference (0, 05mg.ml^{-1}): AMC for Gram –negative bacteria except pseudomonas were used ciprofloxacin;

4.3 Antioxidant analysis

The antioxidant activity of the extracts was evaluated using the DPPH assay; quercetin and BHT (butylated hydroxytoluene) were used as positive controls. The plant extracts exhibit very different EC_{50} values but in all cases higher than the standard compounds (Table 4). The methanolic extract was the most active followed by hydroethanolic (70%) and chloroform-methanol (1:2) extracts. The radical scavenging activity of the first two extracts, the most polar ones, was similar to the obtained for BHT, a synthetic antioxidant widely used in food industry. These results suggest that

phenolic compounds, which usually are more polar compounds, can be regarded as the responsible for the antioxidant activity displayed. The EC_{50} value of the chloroform extract was not obtained because, for higher concentration, the extract is not soluble in ethanol (at 0.783 mg/mL the DPPH inhibition was 22.4%).

Table 4 : Radical scavenging activities of *Artemisia gorgonum* extracts determined by DPPH method.

Extracts	**EC_{50} media (mg/mL)**	**Standard deviation**
Hydroethanol (70%)	0.157	0.0130
Methanol	0.139	0.00703
Chloroform-methanol (1:2)	0.235	0.0130
Chloroform	>783	
Quercetin	0.00274	0.00041
BHT	0.0771	0.00598

The tested extracts, except chloroform, showed higher radical scavenging activity than essential oils from the same species (Ortet et al., 2010) (EC_{50} = 0.48±0.02 mg/mL), higher than essential oils from *A. annua* (Juteau et al., 2002) (antioxidant activity equivalent to 18% of the α-tocopherol) and also than *A. absinthium* ethanolic extracts (Craciunescu et al., 2012) (EC_{50} = 0.57±0.05 mg/mL). However, the aqueous extract of *A. vulgaris* exhibited an EC_{50} lower than the tested extracts of *A. gorgonum* (EC_{50}= 11.4 μg/mL) (Temraz and El-Tantawy, 2008).

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Chapter II: Hepatoprotective effects of *A. gorgonum* extracts on rats

1 Introduction

The plants extracts had been frequently investigated for their hepatoprotective and antifibrotic effects in animal models (Amat et al., 2010; Wang et al., 2012). Hepatic cirrhosis is one disease where necrosis, inflammation, fibrosis, nodular regeneration and formation of vascular anastomosis develop normally at the same time. It is generally caused by long-term action of noxious factors, especially alcohol abuse, which is 50% of the cases in the world (Silbernagl, 2006).

Artemisia tripartita stimulated macrophage production by both pro-inflammatory and anti-inflammatory cytokines. The production of inflammatory chemokine, monocyte chemotactic protein 1 (MCP-1), also was induced by polysaccharides (Xie et al. 2008).

In a study by Amat and other authors (2010), oral administration of aqueous extracts of *Artemisia absinthium* in mice at all doses tested showed significantly reduced levels of TNF-α, IL- 1 and hepatic enzymes AST and ALT, compared to related model group animals.

Yin and co-workers (2008) isolated 9 flavones from *Artemisia vestita* which were evaluated per se for their inhibitory activity on the proliferation and activation of T cells in vitro . Among these flavones, cirsilineol, 6-metoxitricin, and apigenin, significantly inhibited the proliferation and activation of T cells.

The aim of the present study was to investigate the hepatoprotective properties of hydroethanolic (80%) extracts of *Artemisia gorgonum* against liver injury induced by CCl_4 on rats.

1.1 Carbon tetrachloride (CCl_4)-induced chemical liver injury in rats

The CCl_4 has been widely used to induce hepatic injury on experimental animals, (Manibusan et al. 2007). Carbon tetrachloride is a chemical hepatotoxicant that causes hepatocellular damage mediated by free radicals (Weber and Stampfl 2003; Upur et al., 2009).

1.2 Liver

The liver is located inferiorly to the diaphragm in the right upper quadrant abdominal and is the largest internal organ in the body. The liver performs a variety of functions, including detoxification of chemical substances, phagocytosis of old red blood cells, synthesis of proteins and lipoproteins, glucose metabolism (glycogen storage and gluconeogenesis), storage of certain vitamins and bile production (Leboffe, 2005). Most functions are performed by hepatocytes, and many of them require an intimate contact with the blood. The anatomical location, as well as their role in the biotransformation, makes the liver a body susceptible to injury induced by various chemical agents (Williams et al., 2000). It is the primary target of toxicity and carcinogenesis, resulting in fatty degeneration, cell necrosis, fibrosis and cirrhosis (Manibusam, et al., 2007).

Liver damage is usually caused by long-term action of harmful factors, pathogens or virulence factor, which results in increased formation of highly reactive metabolites of oxygen (O^-_2, HO_2, H_2O_2) with concomitant deficiency of antioxidants (eg. glutathione peroxidase) and / or damage of protective enzymes, glutathione peroxidase (GPx) and superoxide dismutase (SOD) (Williams, 2000). Low levels of antioxidants, or inhibition of the antioxidant enzymes, cause oxidative stress and may damage or kill cells.

2 Materials and methods

2.1 Extract preparation

The leaves of *A. gorgonum* were extracted in 80% ethanol during 30 min. at the boiled temperature and then kept for two days at room temperatures in the dark. After filtration, the ethanol was evaporated to dryness in rotary evaporator and the residue, lyophilized.

2.2 Animals and experimental design

Male wistar rats purchased from Harlan (Spain) were kept under standard laboratory conditions (temperature 22±2 ºC and relative humidity (40-60%) on vivarium on light-dark 12h/12h cycle).Water and food was provided *ad libitum*. Rats were allowed to acclimate for one week before experimental use. Daily monitoring was carried out to evaluate the behavior and survival rate. Guidelines for ethics on animal experimentation were followed.

2.3 Treatment groups

Rats were divided randomly into four groups (n=5): I group (normal control) were given orally 0.5% CMC (extract suspended in 0.5% sodium carboxymethyl cellulose) for 7 days and then intraperioneally injected with 10 mL/ kg/body weight olive oil. The II group (hepatotoxicity control) was orally given 0.5% of CMC for seven days and then i.p. injected with CCl_4 (0.1% in olive oil, 10mL/ kg). The III group was pretreated with HEAG at only dose (200mg/kg per day) during the same period and then hepatotoxicity was induced, by CCl_4 (0.1% in olive oil, 10mL/ kg) i.p. injected (Amat et al., 2010). The IV group was pretreated with one dose of Silymarin (positive control) (25 mg/kg per day) during seven days with subsequent administration of CCl_4 (0.1% in olive oil, 10mL/ kg). After this period, all animals were weighed and sacrificed. Blood samples were collected for biochemical analysis. Organs (liver, thymus and spleen) were collected and weighed.

2.3 Histopathology

The liver tissues fixed in Bouin's solution were embedded in paraffin and sectioned into 5μml sections for histomorphological analysis. Sections were stained with hematoxylin and eosin (H&E) and Masson's trichrome. Photographs were done using a digital camera (Olympus Camedia C-5060) coupled to a microscope (Olympus BX41 Olympus, Tokyo, Japan).

2.4 Serum biochemical analysis

Blood of rats was collected from the abdominal aorta. The serum levels of aspartate transaminase (AST), and alanine transaminase (ALT), were determined using an Auto Chemistry Analyzer (AU600; Olympus).

2.5 Statistical analysis

All data were represented as a mean of three independent measurements. Data were analyzed by one-way ANOVA. Results were expressed as mean±S.D. (standard deviation) data, using the Statistic 7 Software. Significance was set at $P < 0.001$ for all tests.

3 Results and discussion

A survival rate of 100% was observed in the present study. The behaviour was normal in all groups, excepted for the group II. The results showed several changes on hepatic sections from group II, administrated with CCl_4 only (hepatotoxicity control). Histopathological analysis indicated hepatocellular necrosis, ballooned hepatocytes, vacuolation, hemorrhage at portal vein, and bridging collagen accumulation in the CCl_4 group, whereas HEAG (80%) treatment evidently alleviated these features (Figure 1) . Our results demonstrated that hydroethanolic (80%) extract of *A. gorgonum* strongly indicate the hepato-protective against liver injury induced by CCl_4 which may be attributed to its immunomodulatory or antioxidative activity, and thereby scientifically supports its traditional use.

The analysis of organ weights showed a raise in body, liver and spleen mass, whereas the thymus mass demonstrated no differences between groups compared with CCl_4 groups and with normal groups. The groups treated with *A. gorgonum* after CCl_4 chemically induced showed reduction clearly in all weights organs except thymus.

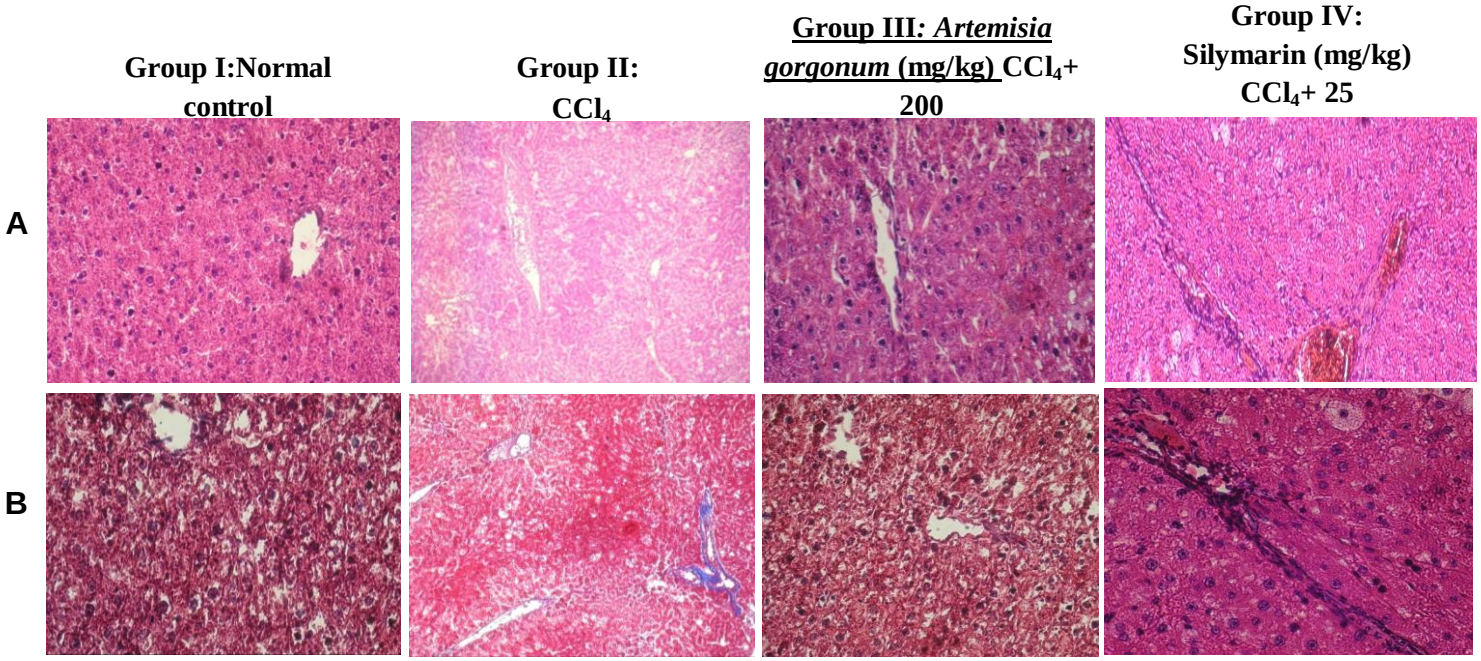

Figure 3: Histomorphological observation of liver. The effect of HEAG (80%) on liver of CCl_4 hepatotoxicity induced rats. The liver tissues were stained with hematoxylin and eosin (A) and Masson's trichrome (B); x200.

There was an increase in the level of serum, alanine transaminase (ALT) and aspartate transaminase (AST) in the CCl_4 treated rats when compared with the normal (control) rats (Table 4). The intraperitoneal administration of CCl_4 to experimental animals resulted in increased serum aminotransferase activities (used for assessing liver function). In rats treated with CCl_4, and with one dose of *A. gorgonum* extract (200mg/kg), the activity of these enzymes was lower.

Treatment with HEAG (80%) 200mg/kg showed activity almost comparable to the group treated with silymarin, a potent hepatoprotective drug used as positive control.

Table 1: The organ weights and serum biochemistry parameters.

Groups	**Normal**	**silymarin**	**CCL_4 only**	***A. gorgonum* (mg/kg)**
Body mass (g)	234±8,6	320±17	258±17	243±2,5**
Liver mass (g)	7,78±0,54	10,9±0,23	12,02±3,2	9,8±0,799***
Spleen mass (g)	0,42±0,079	0,47±0,07	0,55±0,11	0,51±0,78**
Thymus (g)	0,263±0,0296	0,296±0,05	0,298±0,002	0,299±0,07
AST (IU/L)	102±1,76	123,5±6,4	539±55,86	141±53,7***
ALT (IU/L)	70±41	87,5±13,4	446±62,2	77±39,6***

*** Significance $P < 0.001$, compare with hepatotoxicity control (group II).
** Significance $P < 0.01$, compare with hepatotoxicity control (group II).

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Chaper III: Conclusions and Future perspectives

To our knowledge, this is the first study providing data on antibacterial, antifungal, hepatoprotective, and antioxidant activities of the extracts of *A. gorgonum* from Cape Verde (Annexes I, II, and III).

The chemical composition of hydroethanolic (70%) and methanolic extracts responsible for antibacterial and antifungal activities will be also explored in detail in future studies. The extract investigated in the present work is quite interesting from a pharmaceutical standpoint due to its antimicrobial properties.

Our results showed the antioxidant potential of *A. gorgonum* more polar extracts, which are more active than the extracts of other *Artemisia* species and point out that the methanolic and hydroethanolic extracts should be, in a near future, subjected to a phytochemical study in order to isolate the secondary metabolites responsible for the radical scavenging activity displayed and herein reported.

The in vivo studies revealed protective effect of hydroethanolic (80%) extract of *A. gorgonum* against CCL_4 administered group. Future research will be conducted to understand the possible mechanisms of activity.

The antioxidant, antibacterial, synergistic antibacterial and hepatoprotective activities of hydroethanolic (70%) and methanolic extracts of *A. gorgonum* suggests that this species is a potential source of natural antibacterial and antioxidant compounds and justify its future use in traditional medicine.

ANNEX

Annex I: Journal of Ethnopharmacology (submitted)

Antimicrobial and antioxidant potential of *Artemisia gorgonum* leaves extracts

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Abstract

Ethnopharmacological relevance: *Artemisia gorgonum* (Asteraceae) known as "losna" or "lorna" is used in Cape Verde as traditional medicine to treat inflammation, fever and gastroenteritis. Recent studies have suggested that artemetin isolated from *A. gorgonum* can be used to treat malaria, due to its anti-plasmodium properties.

Aim of the study: Evaluate the antimicrobial and synergistic activity of hydroethanolic (70%) and methanolic extracts from aerial parts of *A. gorgonum* against urinary bacteria and one species of yeast. Antioxidant activity of the hydroethanolic (70%), methanol, chloroform and chloroform-methanol (1:2) extracts was also evaluated.

Material and methods: Antimicrobial *in vitro* activity of *A. gorgonum* leaves extracts were tested against seven microorganism species, including Gram-positive bacteria (*Staphylococcus aureus and Enterococcus faecalis*), Gram-negative bacteria (*Escherichia coli*, *Salmonella* spp., *Pseudomonas aeruginosa*, and *Klebsiella* spp.) and one yeast species (*Candida albicans*). The synergistic effect of the *A. gorgonum* extracts against bacteria was evaluated by decimal assay for additivity (DAA) method. The *in vitro* antioxidant activity of several extracts from *A. gorgonum* was evaluated by 2, 2-diphenyl-1-picryl hydrazyl (DPPH) method.

Results and Conclusions: The antimicrobial activity of hydroethanolic (70%) and methanolic extracts from *A. gorgonum* was showed against all bacteria used in this study. *E. coli* and *P. aeruginosa* growth was significantly inhibited by both extracts whereas *S. aureus* and *Klebsiella* spp. growth was less inhibited. *C. albicans growth* was strongly inhibited by the methanolic extract. The combination of hydroethanolic extract with antibiotics was demonstrated the synergistic antibacterial activity against all pathogens tested. In contrast the combination of methanolic extract with antibiotics was revealed antagonism effects against all bacteria, except *klebsiella* spp. was showed synergistic activity.
The methanolic and hydroethanolic extracts showed significant radical scavenging effect in the DPPH assay.

Keywords: *Artemisia gorgonum*, plant extracts, antimicrobial activity, antioxidant activity, microbial infection, drug interaction.

Annex II: Abstract of **Poster** presented on Congress: XXIII National Meeting of the Portuguese Society of Chemistry, University of Aveiro, June 2013

XXIII Encontro Nacional da SPQ

Aveiro 12 a 14 de Junho de 2013

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universidade de aveiro

Antioxidant activity evaluation from *Artemisia gorgonum* extracts

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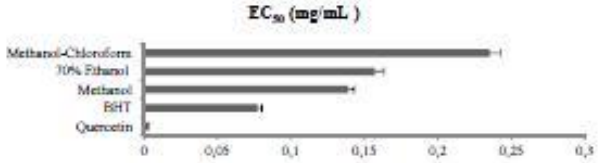
Artemisia gorgonum (Asteraceae) known as "losna or lorna" is used in Cape Verde in traditional medicine to treat inflammation, fever and gastroenteritis.[1] The sesquiterpene lactone ridentin, furofuranlignansesamin and the flavonoid artemetin isolated from *A. gorgonum*showed anti-plasmodium *in vitro* activity.[2,3] Recently, sesquiterpene lactones (*seco*-guaianolides) isolated from this plant, showed higher phytotoxic activity, and the authors suggested that they can be used as inspiration to develop new herbicides.[4]
A few years ago was established that *A. gorgonum* volatile oil displays several biological properties including outstanding antioxidant activity.[5] However, to our best knowledge, no study on the antioxidant potential of other *A. gorgonum* extracts has been published.
Thus, the antioxidant activity of methanol, 70% ethanol, chloroform-methanol and chloroform extracts from *A. gorgonum* leaves was evaluated using the DPPH (2,2-diphenyl-1-picrylhydrazyl) assay.[6] The radical scavenging effect of these extracts willbe presented, discussed and compared with the radical scavenging effects of quercetin and BHT (butylatedhydroxytoluene), used as positive controls.



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Annex III: Poster will be presented in Congress 3rd Joint Congress of the Portuguese and Spanish Microscopy Societies and Israel Society, September 2013.

microscopy AT THE FRONTIERS OF SCIENCE 2013
17-20 September 2013, Tarragona, Spain
3rd Joint Congress of the Portuguese and Spanish Microscopy Societies and Israel Society for Microscopy as invited guest

POTENCIAL ANTI-INFLAMATORY EFFECTS OF *ARTEMISIA GORGONUM* ON RAT LIVER INJURY INDUCED BY CCL_4

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Introduction

Artemisia gorgonum (Asteraceae) is a common traditional Cape Verde medicinal plant which has been used intensively to treat several inflammatory diseases. However, the most recent studies reported the anti-malaria activity [1], antiviral, anti-tumoral, anti-pyretic, anti-hemorrhagic, anti-coagulant, anti-fungal, anti-microbial, anti-oxidant, anti-malarial, anti-ulcerogenic and antispasmodic and hepatoprotective activities have been reported for the *Artemisia genus* [1-5]. Therefore, our aim is to investigate the protective effect of aqueous extract of *A. gorgonum* (AEAG) against liver injury on rat model.

Material and methods

The HEAG were extracted in 80% ethanol during 30 min at the boiled temperature and then two days at room temperature in dark. After filtration, the ethanol was evaporated to dryness in rotary evaporator and residue, lyophilized.
Male wistar rats were divided randomly into three groups (n=5): First group (normal control) given orally 0.5%CMC (extract suspended in 0.5% sodium carboxymethyl cellulose) for 7 days and then intraperioneally injected with 10 ml/ kg/body weight olive oil. The second group (hepatotoxicity control) was orally given 0.5% of CMC for seven days and then i.p. injected with CCl_4 (0.1% in olive oil, 10ml/ kg,i.v). The third group was pretreated with AEAG at only dose (200mg/kg per day p.o) during the same period and then hepatotoxicity was induced, by CCl_4 (0.1% in olive oil, 10ml/ kg,i.v) i.p. injected [1]. On the final experimental day, after 24 h all the animals were weighed and sacrificed. The liver tissues fixed in Bouin's solution were embedded in paraffin and sectioned into 5µml sections for histomorphological analysis. Section slides were stained with hematoxylin and eosin (H&E) and Masson's trichrome.

Results:

The results showed several changes on hepatic sections from second group who were administrated only CCl_4 (hapatotoxicity control). Histopathological analysis indicated hepatocellular necrosis, ballooned hepatocytes, vacuolation, hemorrhage at portal vein, and bridging collagen accumulation in the CCl_4 group, whereas AEAG treatment evidently alleviated these features (Fig. 1A and B).

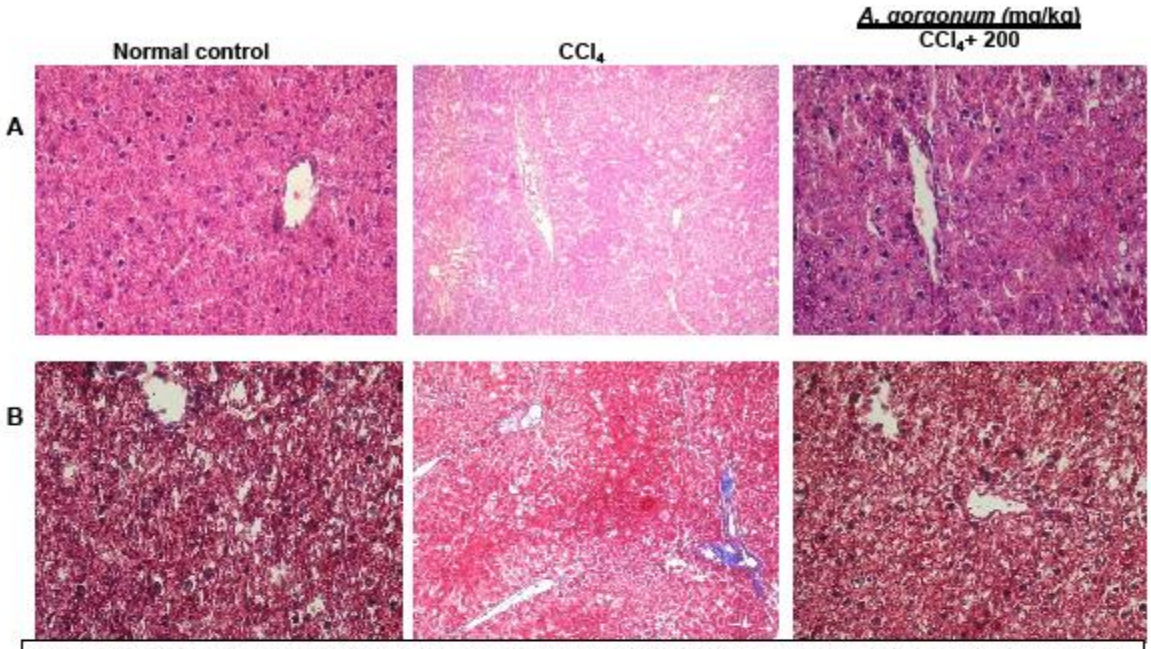

Figure 1: Histomorphological examination. The effect of AEAG on liver of CCl_4 hepatotoxicity induced rats. The liver tissues were stained with hematoxylin and eosin (A) and Masson's trichrome (B) and then pathophysiologic analysis was performed under light microscopy at 200 × magnification.

Conclusions:

Our results demonstrated that aqueous extract of *A. gorgonum* strongly indicate the hepato-protective against liver injury induced by CCl_4 which may be attributed to its immunomodulatory or antioxidative activity, and thereby scientifically supports traditional use.

Acknowledgement

Funding for this work was provided by the Research Centre on Ceramic and Composite Materials (CICECO) from the Aveiro University (Portugal), Pest-C/CTML/LA0011/2011-FCT. Thanks are due to Fundação para a Ciência e a Tecnologia (FCT, Portugal), European Union, QREN, FEDER and COMPETE for funding the QOPNA research unit (project PEst-C/QUI/UI0062/2011).

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