Universidade de Aveiro Departamento de Biologia
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Potencialidades terapêuticas de *Salicornia ramosissima* em ratinhos

The therapeutic potential of *Salicornia ramosissima* on mice

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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 Professora Doutora Maria de Lourdes Pereira, Professora Associada com Agregação do Departamento de Biologia da Universidade de Aveiro, e da Professora Doutora Helena Silva, Professora Auxiliar do Departamento de Biologia da Universidade de Aveiro.

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“La science? Après tout, qu’est-elle, sinon une longue et systématique curiosité?”

André Maurois

Aos meus pais.

o júri

presidente

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palavras-chave

Salicornia ramosissima, figado, rim, tetracloreto de carbono, toxicidade, histopatologia, proteção, regeneração

resumo

No seio da comunidade científica tem vindo a crescer o interesse pelas plantas de sapal, a partir do momento em que as halófitas se começaram a revelar como potenciais fontes de novos compostos com atividade terapêutica. O objectivo do presente estudo foi avaliar os efeitos de *Salicornia ramosissima* no metabolismo e excreção de ratinhos controlo e aquando da exposição a tetracloreto de carbono (CCl_4). Para tal, o fígado e os rins foram sujeitos a uma avaliação histopatológica. Foi preparado um extrato etanólico liofilizado (SREE) a partir das partes aéreas da planta e, juntamente com as suas sementes secas (SRS), foram administrados durante três semanas a ratinhos macho da estirpe ICR-CD1, divididos da seguinte forma: nos estudos da planta *per se*, dois grupos receberam SREE e SRS nas doses de 50 mg/kg e 2000 mg/kg, respetivamente, por via oral; nos estudos de proteção, outros grupos de ratinhos foram pré-tratados com SREE (50 mg/kg) ou SRS (2000 mg/kg), seguido de uma injeção subcutânea de 0.25 ml de CCl_4 (0.2ml/kg) dissolvido em azeite, no último dia da experiência; nos estudos de regeneração, outros grupos foram pré-tratados com CCl_4 nas condições acima descritas, seguido do tratamento com SREE (50 mg/kg) ou SRS (2000 mg/kg) por via oral, até ao fim da experiência. Foi considerado um controlo negativo para as três situações, o qual recebeu unicamente água. Nos estudos de proteção e regeneração foi incluído um controlo positivo o qual foi tratado com água no período anterior e posterior à administração de CCl_4, respetivamente. O SREE *per se* causou severas lesões no fígado e rim, sugerindo que possa existir uma fração tóxica no extrato, enquanto o SRS provocou apenas lesões renais. Não houve uma efectiva proteção do SREE e SRS contra a toxicidade do CCl_4; o SREE permitiu uma ligeira recuperação do fígado após a administração do tóxico, enquanto o SRS provocou um aumento da mortalidade e foram verificadas algumas lesões degenerativas persistentes em ambos os órgãos.
Embora ainda não sejam totalmente conhecidos os mecanismos biológicos da ação de *S. ramosissima*, as evidências sugerem a necessidade de futuros estudos de investigação de forma a elucidar os seus efeitos toxicológicos.

keywords

Salicornia ramosissima, liver, kidney, carbon tetrachloride, toxicity, histopathology, protection, regeneration

abstract

There has been a growing interest in salt marsh plants among scientific community since halophytes have been evidencing strong potential as a source of novel compounds with therapeutic activity. The aim of the present study was to assess the metabolic and excretory effects of *Salicornia ramosissima* on mice under normal and carbon tetrachloride (CCl_4) exposure conditions. Accordingly, a histopathological evaluation on liver and kidneys was performed. A lyophilised ethanolic extract (SREE) was prepared from the aerial portions of *S. ramosissima*, and its dried seeds (SRS) were reduced to powder. These extracts were given during 3 weeks to ICR-CD1 male mice divided as follows: for the plant effects *per se*, groups received 50 mg/kg of SREE or 2000 mg/kg of SRS suspended in the drinking water; for protection studies, mice received a pre-treatment of SREE (50 mg/kg) or SRS (2000 mg/kg), followed by a single 0.25 ml subcutaneous injection of CCl_4 in olive oil (0.2 ml/kg) on the last day; for regeneration studies, animals received a pre-treatment with CCl_4 in the conditions described above, followed by SREE (50 mg/kg) or SRS (2000 mg/kg) suspended in the drinking water until the end of the experiment. A negative control group was considered for the three settings, which received exclusively tap water. A positive control was included in protection and regeneration studies in which mice received tap water after and before CCl_4 administration, respectively. SREE *per se* caused severe hepatic and renal damage, suggesting that SREE may contain a toxic fraction, whereas SRS displayed only kidney injury. SREE and SRS evidenced no effective protection against CCl_4-exposed mice; SREE had a little positive influence in the outcome of liver recovery after CCl_4 administration, whereas SRS increased mortality and liver and kidney impairment was still noticed. Although the biological mechanisms of *S. ramosissima* are not fully understood, the evidences suggest the pertinence of developing further research on *S. ramosissima* extracts in order to elucidate their toxicological effects.

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Index of Abbreviations

ANOVA	Analysis of Variance
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytoluene
b.w.	body weight
$CCl_3^{\cdot}$	Trichloromethyl radical
$CCl_3OO^{\cdot}$	Trichloromethyl peroxy radical
CCl_4	Carbon tetrachloride
CYP2B1	Cytochrome P450 2B1
CYP2B2	Cytochrome P450 2B2
CYP2E1	Cytochrome P450 2E1
EtOH	Ethanol
GFR	Glomerular Filtration Rate
H&E	Hematoxylin & Eosin
PMA	Phorbol 12-myristate 13-acetate
ROS	Reactive Oxygen Species
SREE	*S. ramosissima* ethanolic extract
SRS	*S. ramosissima* seeds
WHO	World Health Organisation

I. Introduction

1. The importance of natural plant resources to human welfare

Since ancient times, the humankind has been trying to understand and make use of natural resources of the planet, especially plants (Ahmad *et al.* 2006). Of the 270 000 terrestrial plant species on earth (Tan *et al.* 2006), around 10 000 species of higher plants are currently used on a wide range of therapeutic purposes (McChesney *et al.* 2007). Despite the popularity of chemically synthesised drugs by pharmaceutical industry, 80% of the total human population still treat their health problems with traditional remedies based mainly made on plants, according to the World Health Organization (WHO) (Morales 1996). In fact, the population growth, inadequate supply of drugs, prohibitive cost of treatments, side effects and development of resistance to currently used drugs for infectious diseases have led to the increasing use of phytotherapy for a wide variety of human ailments (Ahmad *et al.* 2006).

The practice of traditional medicine is based on the folk knowledge, passed down from generation to generation by word of mouth while the documentation of medicinal plants is a developing field and the properties of them are still unknown (Ahmad *et al.* 2006; Neves *et al.* 2009). The Portuguese origins of folk medicine are scattered and have been, in the recent past, replaced by conventional medical practise (Neves *et al.* 2009). Having also the option for planting crops and consuming processed food, they no longer gather wild edible plants as before (Pardo-de-Santayana *et al.* 2007). However, it is now globally known that natural products, such as wild plant resources, represent a rich supply of biologically active compounds and are an example of molecular diversity, with significant potential in the discovery and development of new medicine (Harvey 2000). These bioactive compounds, also known as phytochemicals, are secondary metabolites, able to modulate molecular and cellular responses through interaction with many different molecular and cellular targets (Milner 2004). Hence these interactions can promote health, providing for instance, beneficial antioxidant and enzyme inhibitory or modulatory activities to the human body (Huang *et al.* 1994).

For this reason, it is necessary to conduct more research on herbal products' quality, safety and efficacy whose scientific recognition might lead them to a definitive

integration into conventional medical practise and thus a better alternative to improve human welfare (Mendonça-Filho 2006).

1.1. Herbal medicine in the treatment of liver diseases

The liver is continuously exposed to environmental toxicants, alcohol and pharmacological drugs, whose diseases pose currently a serious challenge to public health (Adewusi *et al.* 2010). Chronic liver diseases, such as viral hepatitis B and C, alcoholic liver disease, non-alcoholic fatty liver disease and hepatocellular carcinoma, are among the leading causes of death in Western countries (Kim *et al.* 2002). Also conventional and modern medicine fails to act in response due to the lack of drug availability and effectiveness, only displaying symptomatic relief, the existence of side-effects associated with prolonged usage and high probability of relapse if the therapy is discontinued. As a result, there has been an increasing interest in plant-derived hepatic therapies which are more accessible and apparently less hazardous (Stickel *et al.* 2007). Unlike conventional medications, which generally consist of a standardized formulation of a specific agent in a known concentration, herbal preparations often consist of mixtures of different ingredients, frequently in impure form and varying concentration (Stickel *et al.* 2005). In addition to different harvest seasons, sites and extract methods, herbal contamination with microorganisms, pesticides or heavy metals may also contribute to hepatotoxic reactions (De Smet 2002). Therefore, rigorous scientific testing and appropriate regulations are needed to evaluate the potential benefits and hazards of herbal preparations (Stickel *et al.* 2007). Despite these limitations, a number of herbals show promising effects in the treatment of acute and chronic liver diseases, including for instance silymarin, extracted from *Silybum marianum*, as a potential antifibrotic (Boigk *et al.* 1997), *Phyllantus amarus* as an antiviral in chronic hepatitis B (Ott *et al.* 1997) or glycyrrhizin, an extract of *Glycyrrhiza glabra*, as a hepatoprotectant in chronic hepatitis C (Kumada 2002).

2. Medicinal halophytes

Over the last decades, considerable scientific and commercial interest has focused on marine natural resources, which might constitute new sources for, among other applications, pharmaceuticals (Becker *et al.* 2008; Newman *et al.* 2006; Smit 2004), enzymes (Pennisi 1997) or functional foods (Zhang *et al.* 2012).

Salt marshes have been the subject for extensive biological research over many years. Located in estuaries, tidal inlets and in other coastal areas, they are a physical boundary between land and sea which provide an exclusive habitat for a large number of species that cannot survive in other environments. Salt marsh plants were recognized as one of the most important ecosystems communities in a tidal zone because they play vital roles in the tidal ecology, such as serving as buffers, protecting the shorelines from erosion caused by the force of waves, and filtering contaminants from the land (Townend *et al.* 2011; Vernberg 1993).

Halophytes (halo= salt + phyte= plant), which are plants capable of completing their life cycle under highly saline conditions, are found in many salt marshes around the world (Yensen 2006). There are more than 2500 halophyte species known and among them, several could be suitable candidates to be used as edible plants, fodder, biofuel, medicine, chemicals and ornamentals (Abdelly *et al.* 2006). Interestingly, numerous halophyte species have been used in folk medicine (Qasim *et al.* 2010) and, recently, extracts from halophytes have proven activity against human, animal, and plant pathogens and more investigations have been carried out to identify the metabolites responsible for their bioactivities (Falleh *et al.* 2011; Gnanadesigan *et al.* 2011; Oueslati *et al.* 2012).

Unfavourable environmental conditions, such as salt-induced stress, increase production and accumulation of reactive oxygen species (ROS) in plants, which lead to cellular damage, metabolic disorders, and aging processes (Menezes-Benavente *et al.* 2004). Due to its powerful antioxidant system, halophytes are able to tolerate and quench these toxic ROS (Ben Amor *et al.* 2007) by inhibiting the initiation or propagation of oxidative chain reactions and consequently, delaying the oxidation of lipids and other biomolecules (Tepe *et al.* 2006). Since the natural antioxidants contained in halophytes exhibit a strong biological activity, their identification is a promising, safer and economical

alternative for their use in preventive medicine to replace synthetic antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) (Tadhani *et al.* 2007). Although some phytochemical bioactive compounds are less effective than those in pharmaceutical drugs, their regular ingestion in significant levels through the diet may lead to long-term physiological effects (Espin *et al.* 2007).

In summary, halophytes have proven strong potential as a new source of novel compounds with beneficial proprieties, and are one step further of being a source of health products for food and pharmaceutical industries.

2.1. Salicornia *species as medicinal plants*

The highest percentage of halophytic genera (around 44%) and the largest number of species belong to the Chenopodiaceae family (Flowers *et al.* 1986). The genus *Salicornia*, (Chenopodiaceae), is world-widely distributed in saline environments and found on every continent with the exception of Antarctica (Shepherd *et al.* 2005). *Salicornia* is represented in Portugal by only one species, *Salicornia ramosissima* J. Woods (Castroviejo *et al.* 1990). This annual edible halophyte, included in the species aggregate *S. europaea* agg. is broadly distributed in the salt marshes and salt pans of Ria de Aveiro and also present in many salt marshes of the Iberian Peninsula (Castroviejo *et al.* 1990; Silva *et al.* 2007). This species is characterised by articulated photosynthetic succulent stems and distinctive reduced leaves in each segment of the stem. These inflorescences are spike-like with two opposite three-flowered cymules in each segment, which produce small seeds in the two lateral flowers and a larger seed in the central flower of the cymule (seed dimorphism) (Davy *et al.* 2001).

Figure 1 - *S. ramosissima* in vegetative stage (Aveiro, Portugal).

Little information is available about *S. ramosissima* constituents and biological activities. However, a previous study conducted on our laboratory has shown adverse hepatic and renal effects of a *S. ramosissima* ethanolic extract on mice, mainly severe cellular degeneration and haemorrhages (Ferreira *et al.* 2012).

Regarding medicinal properties, *S. europaea* is known in folk medicine for the treatment of numerous ailments, including hypertension, cephalalgia, and scurvy (State Administration of Traditional Chinese Medicine of PRC *in* Yin *et al.* 2012), and *S. herbacea* in Asia as a treatment for hepatitis, nephropathy, diarrhea or constipation (Rhee *et al.* 2009), obesity, diabetes, cancer, asthma or even arthritis (Lee *et al.* 2006). The biological mechanisms for the majority of these activities have not been totally clarified.

Currently, it is still used as a seasoned vegetable by some people living in coastal areas; its aerial parts are used as an ingredient of vinegar in Western Europe and, in Korea, consumed in diverse forms, such as a seasoned vegetable, salad and fermented food (Kim *et al.* 2011). Due to the recent discovery of new, functional and biological active compounds with beneficial effects, consumption of this plant has been extended into the functional foods and medicinal herb category (Rhee *et al.* 2009). Despite all of this, there is still a lack of public awareness and scientific literature regarding successfully established halophytes such as *Salicornia* (Böer 2006).

2.1.1. Bioactive chemical compounds

Several studies on the nutritional value of *Salicornia* have characterised the chemical composition of the whole plant, aerial parts, or seeds and their amount through assessment of a variety of solvent extracts. Indeed, many valuable primary and secondary compounds have been found and identified. On the whole, this genus is characterised by relatively high protein content, carbohydrates, fibres and considerable presence of aminoacids (aspartic acid, glutamic acid, arginine and essential aminoacids such as leucine, isoleucine or valine); minerals are represented by the presence of sodium, potassium, calcium, magnesium and chloride in considerable amounts (Durant *et al.* 2006; Lu *et al.* 2010; Rhee *et al.* 2009). Many phytochemicals were isolated and identified from *S. herbacea* aerial or whole plant, such as flavonol glucosides of quercitin and isorhamnetin, chlorogenic acid derivatives, triterpenoid saponins and some sterols (Kim *et al.* 2011; Kim *et al.* 2012; Lee *et al.* 2004). Triterpenoid saponins can also be found in fresh *S. europaea* (Yin *et al.* 2012). Fresh whole *S. bigelovii* contains beta-carotenes and ascorbic acid (vitamin C) (Lu *et al.* 2010), while nortriterpenoid saponins and triterpenoid glycosides were recently found in dry specimens (Wang *et al.* 2012). Lipid content is generally not abundant in the whole plant but *S. bigelovii* and *S. brachiata* have been cultivated for

animal fodder and their seeds exceptionally used for oil production in Middle East over the past years (Charnock *et al.* 1988; Eganathan *et al.* 2006). Detailed studies on seed oils from *S. bigelovii* and hybrid varieties SOS-7 and -10 have revealed a significant amount of proteins, natural antioxidants (tocopherols and flavonoids), sterols (7-stigmastenol, b-sitosterol, spinasterol) and polyunsaturated fatty acids, particularly linoleic acid, which is known to have a great value in human nutrition (Anwar *et al.* 2002; Attia *et al.* 1997; Elmallah *et al.* 1994; Elshami *et al.* 1993; Glenn *et al.* 1991). A *S. europaea* seed profile had been previously characterised revealing a fatty acid composition similar to the above described. Additionally, sucrose, nitrogen, mineral phosphate, potassium and magnesium were represented significantly (Austenfeld 1986).

2.1.2. Biological and pharmacological activities

Several studies reported the value of extracts and some active constituents of *Salicornia* species, each presenting considerable biological and pharmacological activities, such as antioxidative (Kim *et al.* 2011), anti-inflammatory (Kim *et al.* 2009), anti-hyperlipidemic (Hwang *et al.* 2007; Park *et al.* 2012), anti-hyperglycemic (Park *et al.* 2006), antithrombotic (Jang *et al.* 2007), antitumor (Kang *et al.* 2011; Kong *et al.* 2008; Wang *et al.* 2012), among others.

An aqueous extract of *S. herbacea* was shown to be capable of minimising ROS-induced cell damage and recovering liver injury in an ovariectomised rat model of oxidative stress, through induction of antioxidant enzyme levels (Ha *et al.* 2006); various fractions from a seed extract were recently reported to inhibit lipid peroxidation and exhibit significant cytotoxic effects on human colon cancer cells while being less toxic to a non-cancerous intestinal cell line (Kang *et al.* 2011). Another potential chemopreventive agent for cancer is isorhamnetin 3-O-β-D-glucoside, a flavonoid compound extracted also from *S. herbacea* which is able to block the proteolytic activities of metalloproteinase enzymes and enhances a metalloproteinase inhibitor molecule, important in impeding malignant tumour progression (Kong *et al.* 2008).

Isorhamnetin 3-O-β-D-glucopyranoside and quercetin 3-O-β-D-glucopyranoside, have also been confirmed to prevent and treat streptozotocin-induced diabetes in rats, by inhibiting an enzyme involved in the pathogenesis of diabetic complications (Lee *et al.* 2005). Tungtungmadic acid (3-caffeoyl-4-dihydrocaffeoylquinic acid), isolated as an

antioxidant from *S. herbacea*, showed a protective effect against tert-butyl hydroperoxide-induced hepatotoxicity in Hepa1c1c7 cells, increasing the cellular antioxidant defence capacity (Hwang *et al.* 2009) and was found to have anti-inflammatory properties on murine macrophage cells exposed to the tumour promoter phorbol 12-myristate 13-acetate (PMA) (Han *et al.* 2010).

Finally, polysaccharides originated from *S. herbacea* evidenced potent immunomodulatory activity on monocyte/macrophage lineage cells, which is an important step for the triggering of the immune response. This leads to the recruiting of other immune cells and upregulation of cytolytic molecules against a variety of tumours (Im *et al.* 2007; Lee *et al.* 2006). Moreover on another study, these compounds provoked cell cycle arrest and apoptosis on human colon cancer cells, evidencing an anti-proliferative activity. Therefore, polysaccharides might be one of the main reasons for *S. herbacea* anti-cancer effects (Ryu *et al.* 2009).

The literature thus confirms the importance of these naturally-occurring dietary compounds as interesting resources for health promotion through its various physiological actions on animals, cultured cells and chemical assay systems.

3. Toxicity studies

Several studies have been conducted to investigate the protective or regenerative potential of plant extracts, by inducing cell damage through toxic agents. A toxic agent most commonly used in laboratory experiments is carbon tetrachloride, whose histopathological effects are well documented.

3.1. Carbon tetrachloride

Carbon tetrachloride (CCl_4) is a colourless liquid halogenated alkane, synthetically produced and once widely used as a solvent, fumigant, cleaner and degreaser, both for industrial and home handling. Due to its severe toxic effects, most of these uses were discontinued in the past (Bergman 1979). Although CCl_4 is no longer employed for such purposes, it is the best characterised system of xenobiotic-induced hepatotoxicity and animal models are frequently used for the screening of anti-hepatotoxic and/or hepatoprotective activities of drugs and herbal extracts (Jain *et al.* 2012; Rubinstein 1962; Rusu *et al.* 2005).

CCl_4 poisoning can occur by inhalation, ingestion or dermal contact and, depending on the dose and period of exposure, it covers a variety of effects. The injuries of haloalkane exposure are even worsened by their inherent tendency to display synergism with certain solvents or chemicals, leading to potentiation of some aspects of toxicity (Plaa 2000).

Although hepatic toxic effects are the predominant clinical features following exposure to CCl_4, other systemic (renal, respiratory and cardiovascular), reproductive and central effects and nervous system depression have been reported (Ahmad *et al.* 1987; Weber *et al.* 2003).

3.2. The liver and its pathological changes

The liver is the largest gland in the human body, representing nearly 2% of an adult's body weight. Anatomically, it is characterised by two main lobes and two minor ones. It acts as an interface between the digestive system and the blood; the position of the liver in the circulatory system is optimal for gathering, transforming, and accumulating metabolites from blood and for neutralising and eliminating toxic substances, through the bile (Mescher 2009). It also plays an important role in the carbohydrate and lipid metabolism, for instance, glycogen storage, triglycerides and cholesterol synthesis, and in protein (albumin, carrier proteins and clotting factors) synthesis (Fox 2006).

The liver is surrounded by a collagenous capsule which becomes continuous with the internal stroma of the liver at the hilus, the region where the portal vein and hepatic artery enter the organ and the hepatic and lymphatic ducts exit (Mescher 2009; Rogers *et al.* 2012).

The structural and functional units of the liver, the hepatic lobules, are polyhedral structures which contain hepatocytes arranged into radial interconnected plates or cords. Each hepatic lobule displays several portal tracts at the periphery and a central vein. The portal zones are located at the corners of the lobules and consist of a portal triad structure embedded in collagenous connective tissue: a venule branch from the portal vein, an arteriole from the hepatic artery and a duct of cuboidal epithelium from the bile duct system. Blood flows from the periphery to the centre of each hepatic lobule, first supplying periportal hepatocytes with oxygen and metabolites, which are more active in protein synthesis, while centrilobular ones remain less exposed to nutrients and are more involved with detoxification and glycogen metabolism. Mono or binucleated hepatocytes secrete bile into minuscule tubular spaces between them, the bile canaliculi, which anastomose into the common bile duct, where bile is excreted into the duodenum (Mescher 2009).

The spaces among hepatic cords are the liver sinusoids, formed by a discontinuous layer of fenestrated endothelial cells and supported by reticular fibres of type III collagen. In sinusoids reside stellate Ito cells, which store vitamin A in lipid droplets, macrophage Küpffer cells, which filter foreign particulate matter entering the liver and break down aged erythrocytes, and other cells involved in immune surveillance. Between the sinusoid and the hepatocyte is the perisinusoidal space of Disse, where free exchange of macromolecules between plasma and hepatocytes occurs (Rogers *et al.* 2012).

The liver is the main target organ for CCl_4 deleterious effects. Briefly, the main CCl_4-induced changes in the liver are the impairment of membrane permeability, activation of lipid peroxidation, impairment of triglycerides secretion, resulting in steatosis (fatty degeneration), and inhibition of protein synthesis, which contribute to the breakdown of membrane structure and loss of organelle and cell functions. When the injury is relatively mild, it may be reversible; however, in severe cases and prolonged exposure, it leads to a complete destruction of hepatocytes and a subsequent decrease in liver function, culminating in liver fibrosis, cirrhosis or hepatocellular carcinomas (Weber *et al.* 2003).

3.3. The kidney and its pathological changes

The kidney is a bean-shaped organ from the urinary system, lying in the upper retroperitoneal area of the body. Its main functions include the maintenance of water, electrolyte and acid-base homeostasis, renin and erythropoietin synthesis, and the excretion

of many toxic metabolic waste products in the urine, particularly urea and creatinine molecules (Treuting *et al.* 2012; Young *et al.* 2006).

The kidney is divided into two zones, an outer cortex and an inner medulla. In humans, the renal medulla consists of several conical structures called renal pyramids, which are separated by medullary extensions of the cortex. Each medullary pyramid, along with the cortical tissue at its base and along its sides, constitutes a renal lobe (Mescher 2009).

The functional and structural unit of the kidney is the nephron, which performs osmoregulation and excretion by the processes of filtration, selective absorption and secretion (Young *et al.* 2006). Histologically, it consists of a renal corpuscle, responsible for plasma ultrafiltration in a globular network of anastomosing capillaries, called the glomerulus; this structure is surrounded by a double-walled epithelial capsule, the Bowman's capsule. It also contains a renal tubule of a convoluted shape and lined by a single layer of epithelial cells. It has four distinct zones, each having a different histological appearance and roles in tubular function, related to the selective reabsorption of water, inorganic ions and other molecules from the ultrafiltrate: the proximal convoluted tubule, the loop of Henle (with descending and ascending limbs), the distal convoluted tubule and the collecting tubule (Mescher 2009; Young *et al.* 2006).

Briefly, nephrons arise in the cortex, descend into the medulla and return to the cortex. The collecting ducts receive the fluid and descend again into the medulla to discharge urine from renal papilla, subsequently funnelled through the renal pelvis and out of the kidney in the ureter (Young *et al.* 2006).

Because of its high rate of perfusion, active transport capabilities and concentrating functions, the kidney often is exposed to higher concentrations of chemicals and toxicants than other organs. The kidney is capable of metabolically altering a wide variety of endogenous and exogenous substances, and chemicals such as CCl_4 may produce a direct renal cytotoxicity through the conversion of its toxic products via cytochrome P450 enzyme metabolism, or indirectly by altering renal haemodynamics, or by a combination of both. The site along the injured nephron is regularly the site of cellular accumulation of the chemical or its metabolites, whose effects are predictable and dose-related (Kluwe *et al.* 1980; Lock 2009).

4. Framing and purpose of the experimental study

Overall, the importance of *Salicornia* species is being recently widespread, through the recognition of its nutritional value and effects of its chemical compounds. Currently many traditional remedies from plant sources are tested for its potential antioxidant effects, which are important in the recovery processes of the intoxicated liver (Giri *et al.* 2011; Rusu *et al.* 2005). About the medicinal properties of *S. ramosissima*, little is known so far. However, other *Salicornia* species were reported to have beneficial health effects, so it is likely that *S. ramosissima* would be included in that frame as well.

The general goal of this study was to assess the influence of *S. ramosissima* on organism metabolic and excretory systems, under toxicological conditions and focusing on liver and kidney impairment and recovery. The chosen animal model was the mouse since it is often used as a laboratory research animal due to easy maintenance, short life cycle and a well-known physiology (Ng 2008). Moreover, bioactivation of CCl_4 is similar between rodents and humans (Thrall *et al.* 2000). Therefore, mice were subjected to CCl_4, already described as a potent hepatotoxic substance. The prospective hepatoprotection and hepatoregeneration of *S. ramosissima*, along with the effects in the kidneys, were evaluated by histological analysis.

II. Material and Methods

1. Plant treatment and extract preparation

Fresh specimens of *S. ramosissima* were collected on 28th October 2011 at Troncalhada salt pan, Ria de Aveiro. Samples were identified by Professor Helena Silva from University of Aveiro, Portugal. After rinsed with distilled water, samples were stored at -20°C until further use.

The fresh aerial parts (1.2 kg) of *S. ramosissima* were chopped into small pieces and extracted with ethanol p.a. (EtOH, 5 L, three times) at room temperature for 24h using an overhead stirrer (Yellow line OST basic, *IKA®-Werke* GmbH & Co. KG, Germany). The mixture was filtered and the ethanolic extract was obtained. The solvent was removed using a rotary vacuum evaporator (Laborota 4000, Heidolph, Germany) at 50°C. The resulting extract was then freeze-dried at - 40°C (Flexi-Dry MP, FD-3-85A-MP model, FTS Systems, Stone Ridge, USA) for 5 days, yielding 34.7 g of lyophilised product.

Additionally, dried specimens of *S. ramosissima* were collected on 11th November 2011 at Troncalhada salt pan, Ria de Aveiro, and further air-dried at room temperature for 1 week.

In order to retrieve seeds, the dried plants were shaken vigorously into a plastic tray and the remains were passed through 2 and 1 mm sieves, and then through 500 and 355 μm sieves to clean the sample from residues. The resultant amount of seeds (115 g) was homogenised to a fine powder with a blender and stored at room temperature.

2. Animal handling

All animal experiments followed the guidelines for the care and handling of laboratory animals. Five-week-old male ICR(CD-1®) mice (*Mus musculus* L.), supplied by Harlan laboratories (Harlan, Barcelona, Spain) and weighing 34,71 ± 3,83g, were housed in polycarbonate cages placed in an acclimatised chamber suitable for small rodents under standard conditions: constant temperature of 22 ± 2°C, relative humidity of 40-60%, and 12h light/dark photoperiod. Mice were fed with regular rodent chow (SAFE-Scientific Animal Food & Engineering, Augy, France) and tap water *ad libitum*. The acclimatisation period to laboratory conditions lasted for 5 days.

Animals were randomly divided into 9 experimental groups of 4-5 mice each:

→ **Group I**: negative control, treated with tap water *ad libitum*;

→ **Group II**: treated orally with ethanolic extract of *S. ramosissima*, suspended in the drinking-trough water, at a dose of 50 mg/kg b.w;

→ **Group III**: treated orally with powdered dried seeds of *S. ramosissima*, suspended in the drinking-trough water, at a dose of 2000 mg/kg b.w;

→ **Group IV**: positive control, treated with tap water *ad libitum*, followed by 0.25 mL of a CCl_4 solution (olive oil as a vehicle) at a single dose of 0.2 mL/kg b.w. on the 22^{th} day;

→ **Group V**: treated orally with ethanolic extract of *S. ramosissima*, suspended in the drinking-trough water, at a dose of 50 mg/kg b.w, followed by 0.25 mL of a CCl_4 solution (olive oil as a vehicle) at a single dose of 0.2 mL/kg b.w. on the 22^{th} day.

→ **Group VI**: treated orally with powdered dried seeds of *S. ramosissima*, suspended in the drinking-trough water, at a dose of 2000 mg/kg b.w, followed by 0.25 mL of a CCl_4 solution (olive oil as a vehicle) at a single dose of 0.2 mL/kg b.w. on the 22^{th} day.

→ **Group VII**: positive control, treated with 0.25 mL of a CCl_4 solution (olive oil as a vehicle) at a single dose of 0.2 mL/kg b.w., followed by tap water *ad libitum*;

→ **Group VIII:** treated with a with 0.25 mL of a CCl_4 solution (olive oil as a vehicle) at a single dose of 0.2 mL/kg b.w, followed by oral administration of the ethanolic extract of *S. ramosissima*, suspended in the drinking-trough water, at a dose of 50 mg/kg b.w.

→ **Group IX**: treated with 0.25 mL of a CCl_4 solution (olive oil as a vehicle) at a single dose of 0.2 mL/kg b.w, followed by oral administration of the powdered dried seeds of *S. ramosissima*, suspended in the drinking-trough water at a dose of 2000 mg/kg b.w.

The study was carried out for 3 weeks (22 days) and mice were weighed at the start of the experiment, once a week and at the sacrifice moment, with an accuracy of 0,1g. Animals were sacrificed by cervical dislocation 24h after the last treatment on each group. CCl_4 solution was administered by subcutaneous injection and its dosage was retrieved

from Irie and co-workers (2010). The ethanolic extract dosage was adapted from Pinto (2011) while the seed dosage was based on Meneguetti and co-workers (2011).

The experimental study is summarised below:

Table 1 – Structure of the experimental design.

Group	***Study type***	***Treatment given***	***Plant dosage***	***CCl_4 dosage***
Group I	Negative (normal) control	Tap water	N/A	N/A
Group II	Aerial plant extract experimental group	*S. ramosissima* EtOH extract	50 mg/kg	N/A
Group III	Dried seed experimental group	*S. ramossisima* dried seeds	2000 mg/kg	N/A
Group IV	Positive control for protection group	Tap water + CCl_4	N/A	0.2 mg/kg
Group V	Protection experimental group	*S. ramosissima* EtOH extract + CCl_4	50 mg/kg.	0.2 mg/kg
Group VI	Protection experimental group	*S. ramossisima* dried seeds + CCl_4	2000 mg/kg	0.2 mg/kg
Group VII	Positive control for regeneration group	CCl_4 + tap water	N/A	0.2 mg/kg.
Group VIII	Regeneration experimental group	CCl_4 + *S. ramosissima* EtOH extract	50 mg/kg	0.2 mg/kg
Group IX	Regeneration experimental group	CCl_4 + *S. ramossisima* dried seeds	2000 mg/kg	0.2 mg/kg

3. Organ collection and histological techniques

After opening the abdominal cavity, the liver and kidneys were excised from mice and weighed (AND model HR-120). Portions and left sides of the aforementioned organs were used for histological studies. Firstly, they were immersed in Bouin's solution for fixation purposes. Subsequently, dehydration was performed through graded ethanol series, followed by infiltration and embedding in paraffin (melting point 52-58°C). Sections of 4-8 µm thickness were prepared from the tissues using a rotary microtome (Leitz 1512, Wetzlar, Germany). The samples were dried in a stove (40°C) then deparaffinised and rehydrated. Sections were stained with hematoxylin and eosin dyes and received further dehydration and clearing with xylene. The preparations were mounted in the Eukitt® synthetic medium (EMS, Hatfield, USA) and examined microscopically for cell

abnormalities (Olympus microscope model BX41TF, coupled with photographic system, Japan).

4. Histological semi-quantitative analysis

Histological lesions of liver and kidney were graded according to a previously described semi-quantitative score (Shackelford *et al.* 2002). One slide section of each organ for each group was considered. The lesion's score was evaluated as follows: 0: Not present, 1: Minimal (< 10%), 2: Mild (10–39%), 3: Moderate (40–79%), 4: Marked (80–100%).

5. Statistical analysis

All values are presented as mean ± standard error (SE). The results were computed statistically (IBM SPSS Statistics, version 20.0.0, IBM Corp.) using two-way ANOVA without interactions (incomplete factorial design) to analyse differences between mean values of relative organ weights, among groups. Since relative weight is a more sensitive toxicity indicator than absolute weight (Uemitsu *et al.* 1986), the latter was deliberately excluded from statistical testing. A p-value <0.05 was considered significant.

III. Results

The results herein described attempted to elucidate the effects of *S. ramosissima* ethanolic extract (SREE) and seeds (SRS) on the liver and kidney structure and function under normal and toxicological conditions.

1. Mice survival and behaviour

All mice have survived during the present study, except in the groups V and IX where 25% and 75% of mortality was noted, respectively. Deaths occurred between 24h and 7 days after CCl_4 administration. No significant changes in mice behaviour and physical appearance from groups I to III were observed; however, all mice exposed to CCl_4 presented, 24h after the injection, pilo-erection, less activity and weak response to handling. Mice from groups VII, VIII and the remaining from group IX recovered gradually until the end of the experiment.

2. Organ weight

Table 2 represents the liver and kidneys absolute weights from all experimental and control groups.

Table 2 – Values of the absolute organ weights from all groups.

Group	*Absolute organ weight (g)*			*n*
	Liver	**Right kidney**	**Left kidney**	
Group I (control)	2,2963±0,0876	0,2762±0,0116	0,2683±0,0183	4
Group II (SREE)	2,3725±0,1564	0,2559±0,0205	0,2599±0,0245	4
Group III (SRS)	1,5844±0,0760	0,2242±0,0053	0,2154±0,0065	4
Group IV (water + CCl_4)	2,1693±0,1500	0,2888±0,0123	0,2807±0,0120	5
Group V (SREE + CCl_4)	2,0689±0,1727	0,2475±0,0327	0,2344±0,0259	3
Group VI (SRS + CCl_4)	1,9372±0,0600	0,3082±0,0186	0,3055±0,0255	4
Group VII (CCl_4 + water)	2,0719±0,0757	0,2954±0,0168	0,2897±0,0104	4
Group VIII (CCl_4 + SREE)	1,0700±0,1151	0,1795±0,0152	0,1754±0,0101	4
Group IX (CCl_4 + SRS)	1,7397±ND	0,2739±ND	0,2696±ND	1

Data are mean ± SE values.
n, number of animals; ND, not determined.

Table 2 shows a stable kidney weight in all the groups. On the other hand, there was more variability in the liver weight among groups. However, only statistical significant differences among the different groups were measured in the organ-to-body-weight ratio due to the reasons described earlier.

Table 3 shows the mean values of the relative organ weight (in percentage) from the several experimental and control groups, as well as the results of the statistical test.

Table 3 - Comparison of the relative organ weights of experimental and control groups.

Group	Final body weight (g)	Relative organ weight (g/g of the body weight, %)		
		Liver	Right kidney	Left kidney
Group I (control)	38,325±1,114	5,9898±0,1326	0,7217±0,0306	0,6998±0,0436
Group II (SREE)	36,775±1,344	6,4404±0,2756	0,6973±0,0546	0,7055±0,0545
Group II (SRS)	32,018±0,842	4,9505±0,2275	0,7024±0,0321	0,6749±0,0335
Group IV (water + CCl_4)	37,960±1,003	5,6979±0,3036	0,7615±0,0305	0,7390±0,0222
Group V (SREE + CCl_4)	38,000±1,436	5,4577±0,4901	0,6490±0,0701	0,6152±0,0535
Group VI (SRS + CCl_4)	42,375±1,967	4,5848±0,1272	0,7267±0,0181	0,7192±0,0368
Group VII (CCl_4 + water)	41,050±1,201	5,0497±0,1429	0,7200±0,0363	0,7060±0,0191
Group VIII (CCl_4 + SREE)	27,173±2,310	3,9166±0,1140	0,6658±0,0476	0,6529±0,0406
Group IX (CCl_4 + SRS)	33,923± ND	5,1319± ND	0,8080± ND	0,7953± ND

Data are mean ± SE values (n=4). Group IV (n=5), Group V (n=3), Group IX (n=1).
ND, not determined.

No statistical significant differences were observed in the relative kidney weight among the presented groups (plant treatment *per se*, protection studies and regeneration studies). Although the relative liver weights, mainly from group II, VI and VIII, were lower than their respective controls, these differences had no statistical significance. Due to the low survival rate, Group IX was excluded from statistical analysis.

3. Macroscopic aspect of the organs

The liver and kidneys of all groups exhibited normal morphology, except for CCl_4-treated livers, which displayed pale colour and were slightly mottled, 24h after the administration of the toxicant.

4. Histopathological findings

The histopathological effects of *S. ramosissima* ethanolic extract and whole seeds were evaluated on the liver and kidney. Additionally, protective and regenerative effects, of both ethanolic extract and seeds, after the CCl_4 administration were assessed.

4.1. Hepatic evaluation

4.1.1. Effects of *S. ramosissima* ethanolic extract and seeds

Table 4 summarises the hepatic changes on mice treated with SREE and SRS during 3 weeks. Figure 2 shows the main histopathological findings.

Table 4 - Frequency of hepatic histopathological features in groups treated with SREE and SRS *per se*.

	Group treatment		
Histopathological findings	Control group	Group II	Group III
Hydropic degeneration/vacuolar change	0	4	0
Disorganisation of hepatic parenchyma	0	4	2
Inflammatory foci	0	1	1
Haemorrhages	0	3	1
Focal necrosis	0	0	0

Lesions were rated as 0: not present, 1: minimal, 2: mild, 3: moderate and 4: marked.

Liver sections from negative control group revealed normal architecture of the parenchyma (Fig. 2A). On the other hand, the groups treated with 50 mg/kg of SREE *per se* exhibited significant changes, mainly severe and diffuse hydropic degeneration of hepatocytes, with subsequent loss of their radial arrangement (Fig. 2B). Animals treated with 2000 mg/kg of SRS *per se* showed no hepatocyte degeneration, although some loss of parenchymal structure and prominence of microvesicules along the focally widened sinusoids between the hepatocyte plaques could be observed (Fig. 2C).

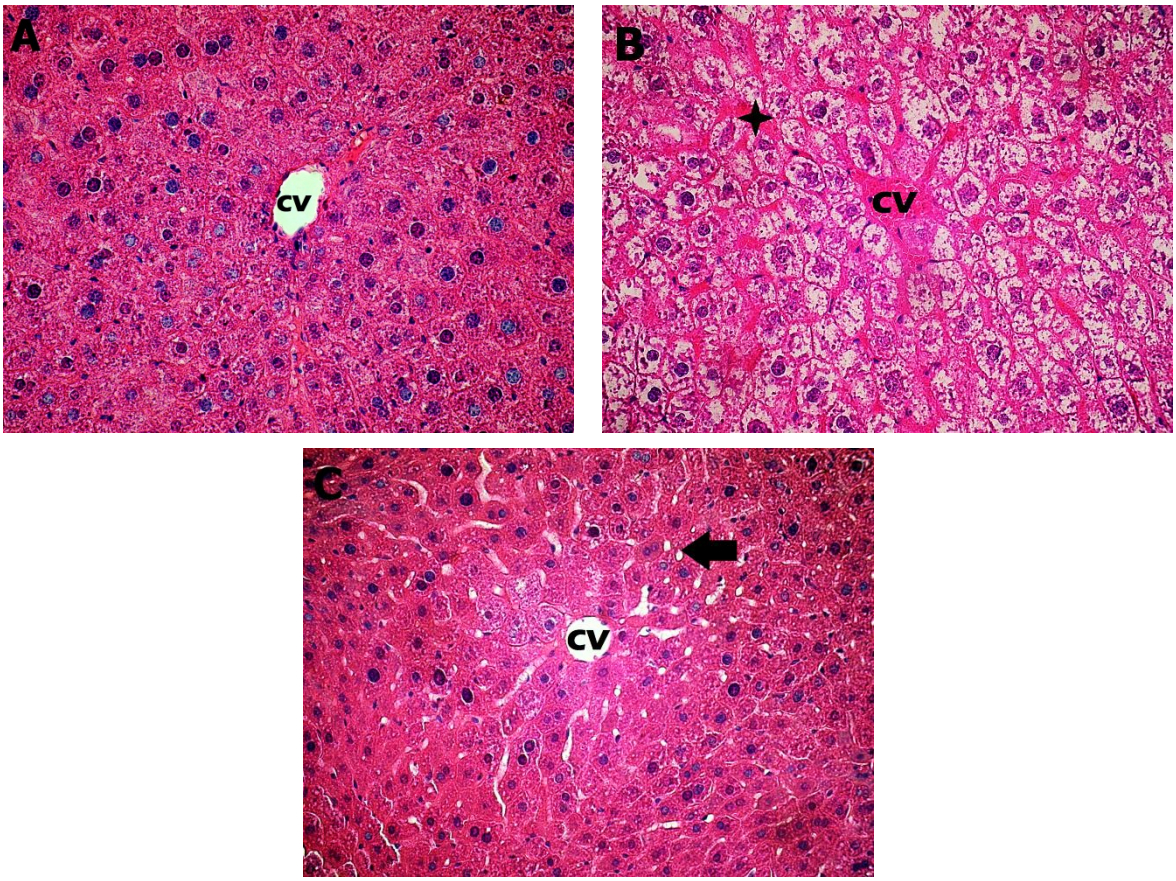

Figure 2 - Light microphotographs of liver sections (H&E stain, 400× original magnification): A) control group; B) SREE-treated group; C) SRS-treated group. (CV: central vein, ✦: haemorrhage, ➡ : microvesicules).

4.1.2. Protection studies

Table 5 summarises the hepatic changes on mice treated with SREE or SRS during 3 weeks followed by CCl_4 administration. Figure 3 shows the main histopathological findings.

Table 5 - Frequency of hepatic histopathological features in groups of protection studies.

	Group treatment			
Histopathological findings	Negative Control	Pos. Control (Group IV)	Group V	Group VI
Hydropic degeneration/ vacuolar change	0	3	4	3
Disorganisation of hepatic parenchyma	0	3	4	3
Inflammatory foci	0	1	1	1
Haemorrhages	0	2	4	2
Focal necrosis	0	3	4	3

Lesions were rated as 0: not present, 1: minimal, 2: mild, 3: moderate and 4: marked.

The CCl_4-affected organs displayed hepatocyte necrosis within the centrilobular zone as the predominant histopathologic lesion, together with parenchymal disorganisation and hepatocellular degeneration, as evidenced by histological changes (Figs. 3B). [SRS + CCl_4]-treated group (Group VI) revealed similar histological pattern as the positive control group, with a more pronounced microvesicular fatty change (Fig. 3D), whereas the livers from [SREE + CCl_4]-treated mice (Group V) showed a multiple lesion profile, within the group: animals either exhibited centrilobular necrosis plus reasonably degenerated periportal zone, exhibited a nearly intact centrilobular zone plus severe periportal hepatocyte degeneration (Fig. 3C), or no necrosis but only hepatocyte degeneration.

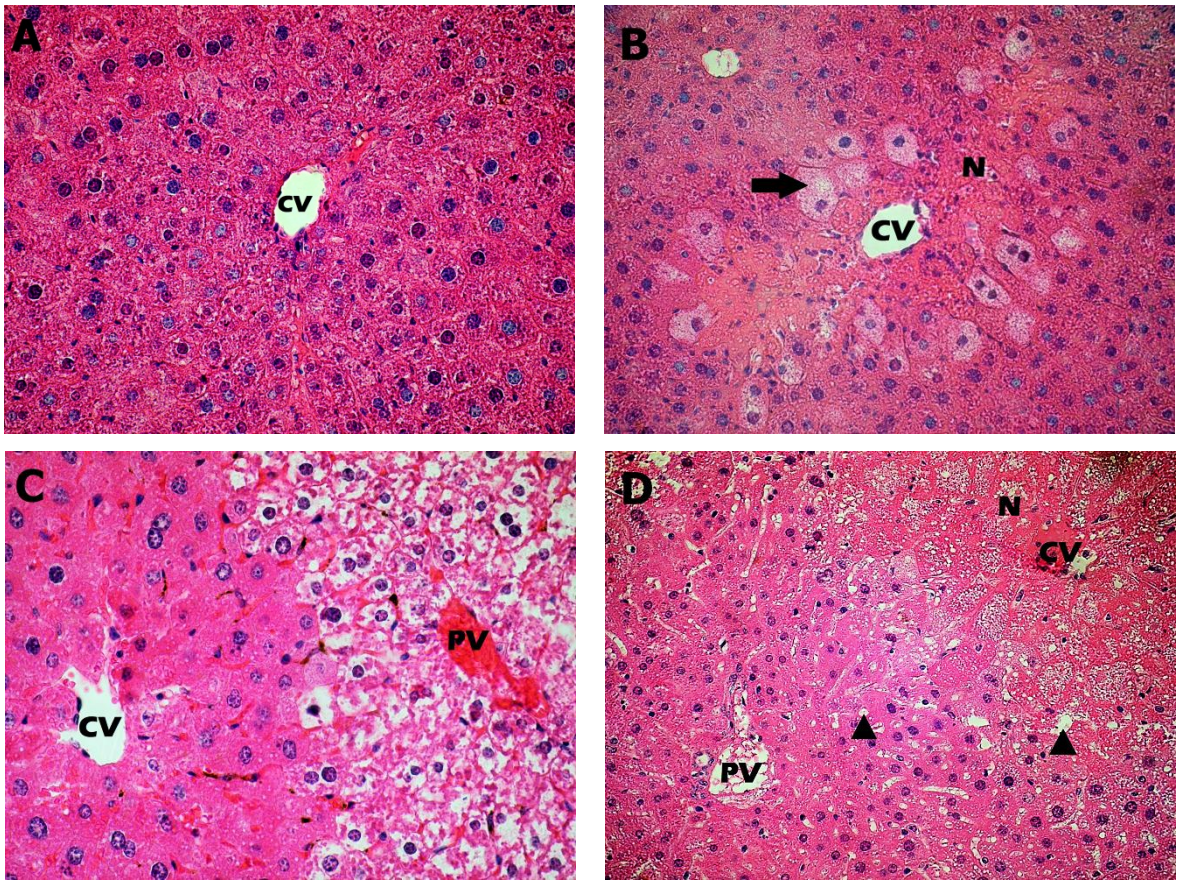

Figure 3 - Microphotographs of hepatic sections (H&E stain, 400× original magnification): A) negative control group; B) [Water + CCl_4] control group; C) [SREE + CCl_4]-treated group; D) [SRS + CCl_4]-treated group. (CV: central vein, PV: portal vein, N: necrosis, ➡: hydropic degeneration, ►: microvesicules).

4.1.3. Regeneration studies

Table 6 summarises the hepatic changes on mice treated with CCl_4 followed by SREE or SRS administration during 3 weeks. Figure 4 shows the main histopathological findings.

Table 6 - Frequency of hepatic histopathological features in groups of regeneration studies.

Histopathological findings	***Group treatment***			
	Negative Control	Positive Control (Group VII)	Group VIII	Group IX
Hydropic degeneration/vacuolar change	0	2	1	2
Disorganisation of hepatic parenchyma	0	2	1	1
Inflammatory foci	0	1	1	2
Haemorrhages	0	0	0	0
Focal necrosis	0	0	0	0

Severity was rated as 0: not present, 1: minimal, 2: mild, 3: moderate and 4: marked.

Concerning the positive control group, mild cellular degeneration and an irregular structure of hepatocyte cords were evidenced (Fig. 4B), when compared with the negative control. The experimental groups revealed a similar hepatic profile, though slightly ameliorated in Group VI, whereas liver from Group VII was mainly characterised by microgranulomas and pigmented cells randomly distributed throughout the parenchyma (Fig. 4C).

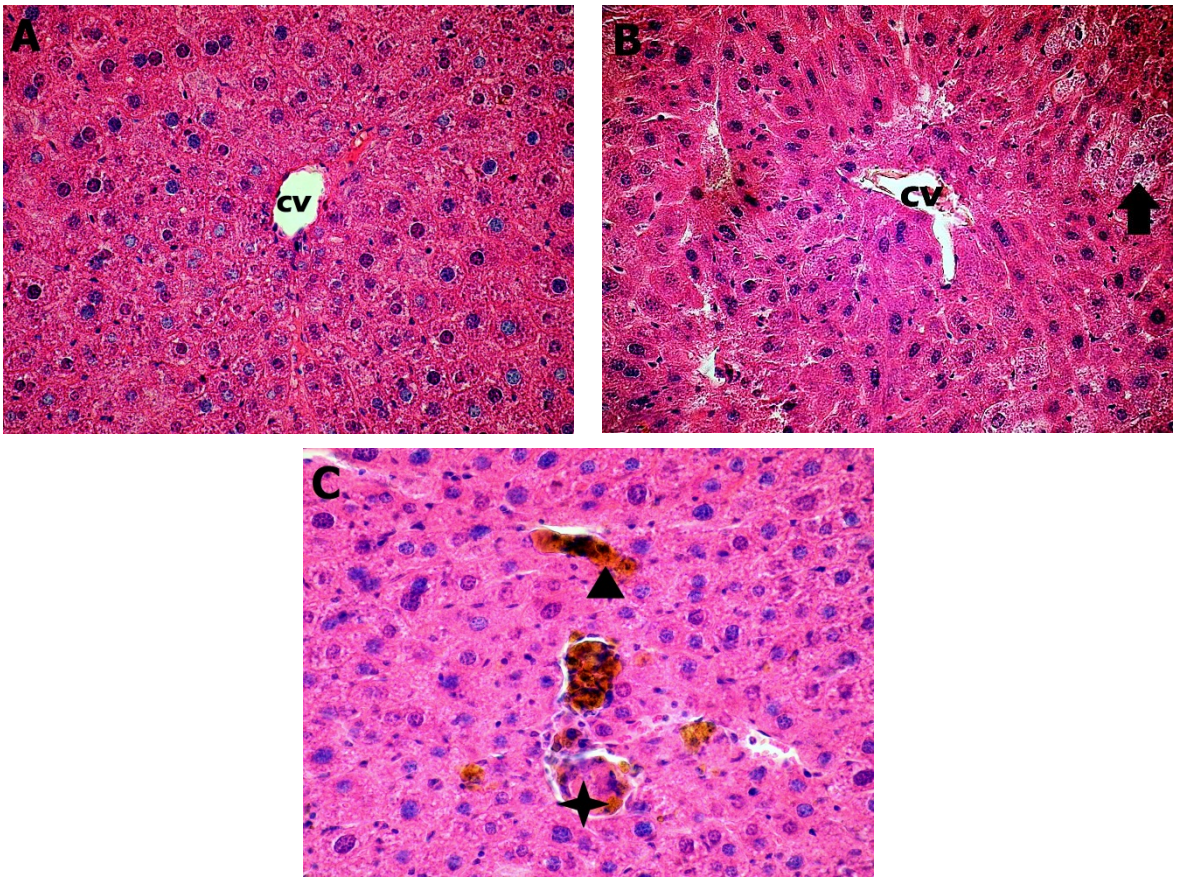

Figure 4 - Histological sections of liver (H&E stain, 400× original magnification): A) negative control group; B) [CCl_4 + water] control group; C) [CCl_4 + SRS]-treated group. (CV: central vein, ➡: hydropic degeneration, ✦:granulomatous mass, ►: pigmented cells).

4.2. Renal evaluation

4.2.1. Effects of *S. ramosissima* ethanolic extract and seeds

Table 7 summarises the renal changes on mice treated with SREE and SRS during 3 weeks. Figure 5 shows the main histopathological findings.

Table 7 - Frequency of renal histopathological features in groups treated with SREE and SRS *per se*.

	Group treatment		
Histopathological findings	Negative Control	Group II	Group III
Cellular debris within cortical tubular lumen	0	0	0
Glomerular structural changes	0	2	2
Inflammatory foci	1	3	3
Haemorrhages	0	3	3

Lesions were rated as 0: not present, 1: minimal, 2: mild, 3: moderate and 4: marked.

Renal profile of negative control group evidenced normal features (Fig. 5A). However, significant and similar histological alterations were found in both SREE and SRS exposed groups: cortical interstitial haemorrhages and inflammatory cell infiltration (Fig. 5B), associated with glomerular surrounding zones particularly in SREE-treated mice (Group II). Glomerular integrity was slightly compromised in both groups.

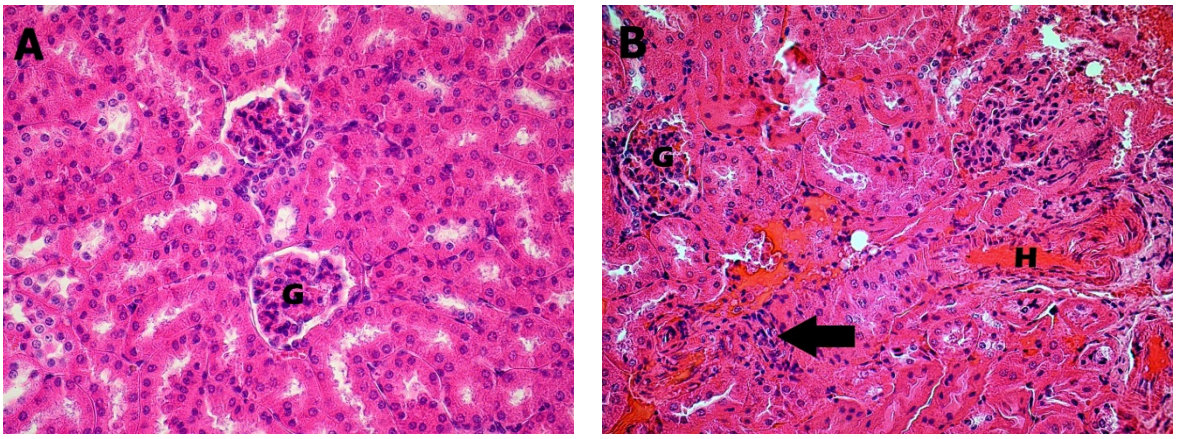

Figure 5 - Light micrographs of kidney sections (H&E stain, 400× original magnification): A) negative control group; B) SRS-treated group. (G: glomerulus, H: haemorrhage, ➡: inflammatory focus)

4.2.2. Protection studies

Table 8 summarises the renal changes on mice treated with SREE or SRS during 3 weeks followed by CCl_4 administration. Figure 6 shows the main histopathological findings.

Table 8 - Frequency of renal histopathological features in groups of protection studies.

	Group treatment			
Histopathological findings	Negative Control	Pos. Control (Group IV)	Group V	Group VI
Cellular debris within cortical tubular lumen	0	4	4	4
Glomerular structural changes	0	4	2	3
Inflammatory foci	1	2	2	2
Haemorrhages	0	1	3	2

Lesions were rated as 0: not present, 1: minimal, 2: mild, 3: moderate and 4: marked.

Kidneys exhibited significant CCl_4-induced alterations compared with the negative control, mainly severe glomerular structural changes with different grades of degeneration, and cell breakdown products and debris within the lumen of the tubules (Fig. 6B). Pre-treatment with SREE (Group V) revealed the same renal pattern, with the exception of significant fewer glomerular changes and increased haemorrhages (Fig. 6C). Pre-treatment with SRS (Group VI) differed from the CCl_4 control in a slight decrease of glomerular changes and in the luminal content, where the latter revealed increased exfoliated cells (Fig. 6D).

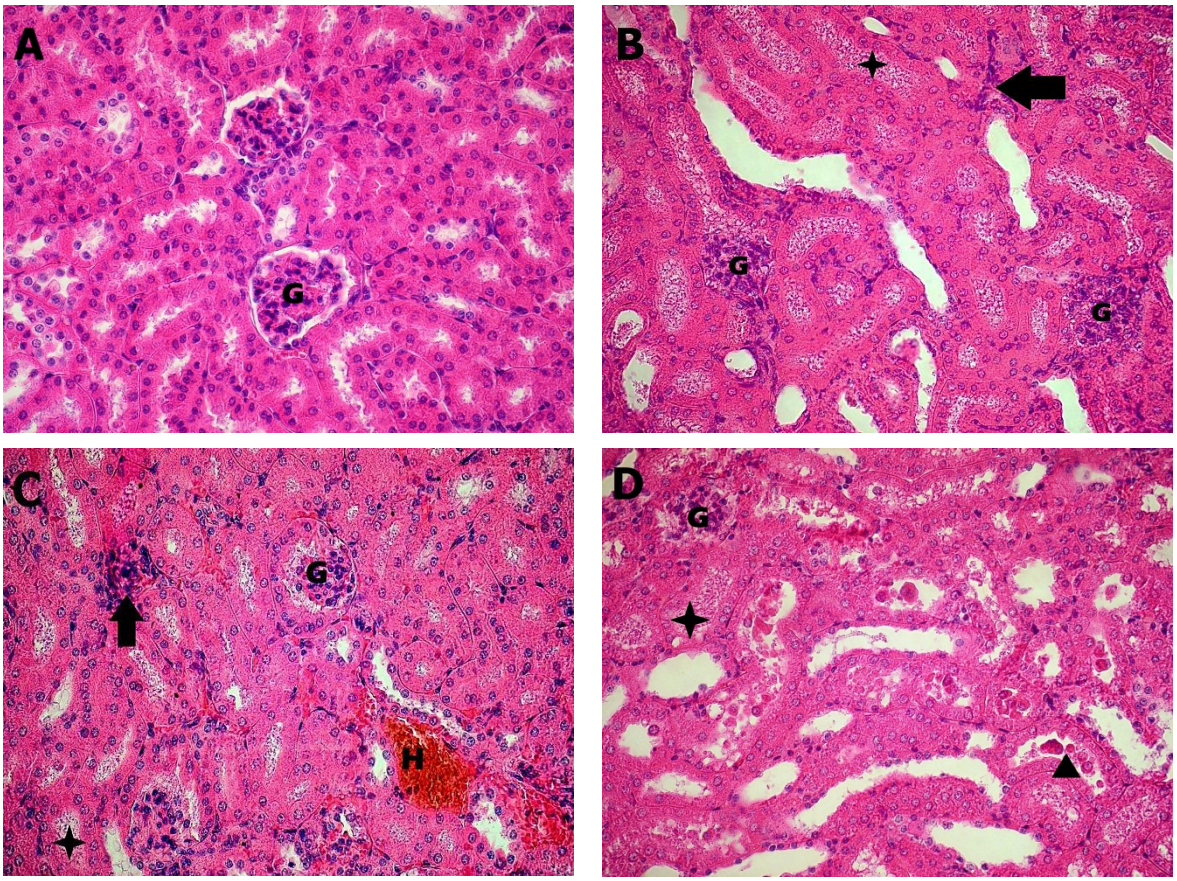

Figure 6 - Renal histological sections (H&E stain, 400× original magnification): A) negative control group; B) [Water + CCl_4] control group; C) [SREE + CCl_4]-treated group; D) [SRS + CCl_4]-treated group. (G: glomerulus, H: haemorrhage, ►: intact cells, ✦: cellular debris, ➡: inflammatory focus).

4.2.3. Regeneration studies

Table 9 summarises the renal changes found on mice treated with CCl_4 followed by SREE or SRS administration during 3 weeks. Figure 7 shows the main histopathological findings.

Table 9 - Frequency of renal histopathological features in groups of regeneration studies.

Histopathological findings	*Group treatment*			
	Negative Control	Positive Control (Group VII)	Group VIII	Group IX
Cellular debris within cortical tubular lumen	0	0	0	1
Glomerular structural changes	0	2	2	2
Inflammatory foci	1	3	3	3
Haemorrhages	0	3	3	3

Lesions were rated as 0: not present, 1: minimal, 2: mild, 3: moderate and 4: marked.

Kidney sections treated with CCl_4 + SREE revealed no histopathological findings in the cortical tubular lumen, nearly as seen in positive control group (Fig. 7B). All treated groups were characterised by extensive haemorrhages and leukocyte infiltration foci, and Group IX particularly revealed several foci of tubular basophilia (Fig. 7C).

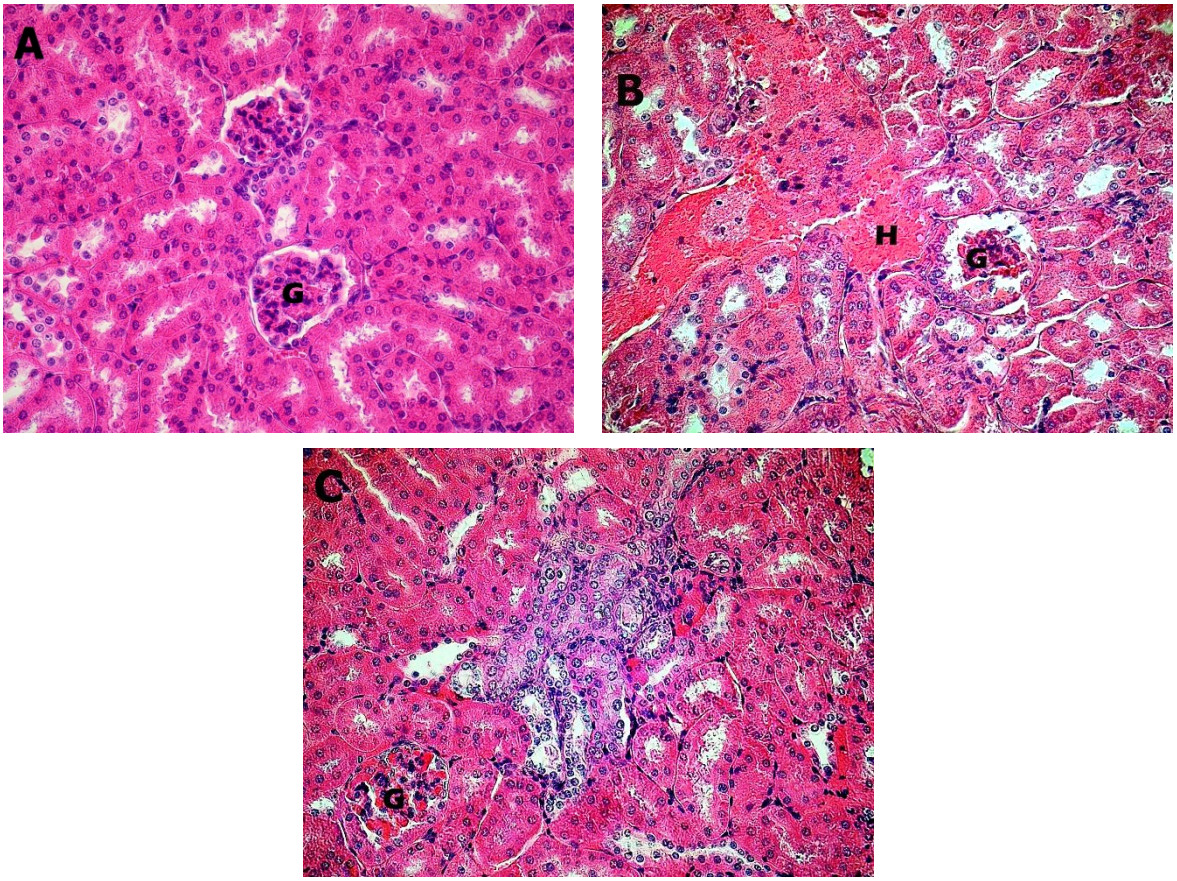

Figure 7 - Microphotographs of renal histological sections (H&E stain, 400× original magnification): A) negative control group; B) [CCl_4 + water] control group; C) [CCl_4 + SRS]-treated group. (G: glomerulus, H: haemorrhage).

IV. Discussion

The aim of the present experimental study was to explore the potentialities of *Salicornia ramosissima* as a therapeutic agent. The hepatic and renal effects of *S. ramosissima* ethanolic extract and dried seeds were characterised in three different settings: their influence *per se* in normal control animals, their protective and regenerative actions under toxic insult (CCl_4 exposure).

Both liver and kidneys were selected in this work since they are intimately related organs, as they participate in several metabolic and excretion events. Therefore, any abnormality in the liver may also lead to kidney function impairment.

1. Effects of *S. ramosissima* ethanolic extract and seeds

The uptake of 50 mg/kg of SREE by mice during 3 weeks (Group II) revealed severe hepatotoxicity, and inflammation and haemorrhages in the kidney. However, neither significant liver weight nor survival changes were noticed compared with the control. Haemorrhage often accompanies acute injury, inflammatory processes or vascular injury (Frazier *et al.* 2012). In this case, both organs confirm the presence of significant damage induced by the extract. Consistently, the same histological findings were obtained previously (Ferreira *et al.* 2012). Moreover, a previous study demonstrated high activity of *Salicornia europaea* on *A. salina* (brine shrimp) and *D. magna* tests for cytotoxicity. *S. europaea* ethanolic extract caused more than 50% of toxicity in almost all testing (Lellau *et al.* 2003). These tests are frequently used for the screening of toxic compounds from plant extracts and consequently to assess biological responses of lethality (Adema 1978; Meyer *et al.* 1982). The biological toxicity verified by SREE is in agreement with the aforementioned study. According to these results, the extraction methodology used on *S. ramosissima* possibly retained and concentrated toxic compounds and contaminants.

The uptake of 2000 mg/kg of SRS by mice during 3 weeks (Group III) was based on the dosage of a previous study about the effects of *Chenopodium quinoa* seed extract on rats (Meneguetti *et al.* 2011), a plant from Chenopodiaceae, as well as *S. ramosissima*. Histologically, the hepatic parenchyma exhibited a nearly normal structure and organisation, apart from the fact that some sinusoidal spaces revealed to be widened and

microvesicular, and this was perceptible only at a higher magnification. An early sign of lipidosis could be hypothesised.

The renal profile of this group is similar to the previously described Group II but there was no clear relationship between the minor hepatic changes and the noticeable kidney compromise. Contrary to these results, extracts of *S. herbacea* seeds were reported to have antioxidant activity and cytotoxicity against carcinoma cells *in vitro* (Kang *et al.* 2011). Moreover, no renal or liver toxicity was attained with 2000 mg/kg of Hydrolysed Quinoa extract (Meneguetti *et al.* 2011). Therefore, we hypothesise that SRS may contain noxious compounds or contaminants, since no purification and extraction method was employed in the seeds. The kidney was the most affected organ probably due its physiological functions. Despite comprising only a small portion of total body weight, the kidney receives approximately 25% of the circulating blood and has an extensive reabsorption capacity which ensures that high levels of toxicants are delivered and uptaken by renal cells, compared with other organs (Bhadauria *et al.* 2012).

2. Protection studies

The main goal of protection studies is to evaluate the preventive action of a substance towards an organism stressor. Animal models for hepatic injury are important tools for the study of liver physiopathology and extrapolation of related therapeutics into human diseases. The chemical chosen for the induction of hepatotoxic (and nephrotoxic) action was the CCl_4 due to its well-known toxicological specificity and broadened usage in laboratory experiments.

For the first positive control group (Group IV), a single low dose of CCl_4 was administered, and its histopathological effects were noticed 24h after the exposure as expected. No significant organ weight or survival changes were noticed compared with the negative control. CCl_4 is known to cause an increase in liver weight (Uemitsu *et al.* 1984) but the low dose administrated may have not been appropriate to induce such significant increase. However, the administration of CCl_4 caused significant morphological damage to the liver and kidneys, especially to the renal cortex. These findings are consistent with those described previously (Bhadauria 2012; Irie *et al.* 2010; Khan *et al.* 2009). Indeed,

CCl_4 bioactivation occurs in the centrilobular zone, the selected site of detoxification. CCl_4 metabolism begins with the formation of the trichloromethyl free radical ($CCl_3^{\cdot}$) through the action of the cytochrome P450 oxygenase system of the endoplasmic reticulum. The major cytochrome isozyme to execute biotransformation of CCl_4 is CYP2E1, but CYP2B1 and CYP2B2 may also be involved. The resulting $CCl_3^{\cdot}$ radical is reactive enough to bind covalently to CYP2E1, thus causing its own inactivation. The $CCl_3^{\cdot}$ radical in the presence of oxygen is transformed into a more reactive radical, the trichloromethyl peroxy ($CCl_3OO^{\cdot}$) (Weber *et al.* 2003). Both radicals react with several important biological substances (proteins, lipids, nucleic acids and amino acids) having preference for fatty acids from membrane phospholipids. This leads to sequential free radical reactions until the complete disintegration of the membrane and leakage of microsomal enzymes, in a process called lipid peroxidation (Manibusan *et al.* 2007).

Meantime, the susceptibility of the kidney to toxicant-induced injury is mediated by physiological and biochemical factors. Physiologically, the large luminal surface area, high renal reabsorption capacity and the ability to concentrate solutes can result in extraordinarily increased amount of toxicant exposure and its metabolites (Frazier *et al.* 2012). Biochemical factors include the presence of several biotransformation enzymes which mediate the formation of toxic metabolites and reactive intermediates. The proximal tubule is the main site of xenobiotic transformation and is particularly sensitive to chemical insult since it contains cytochrome P450 enzymes, mainly the CCl_4-metabolising enzyme CYP2E1 (Hu *et al.* 1990). CCl_4-induced tubular necrosis leads to the detachment of tubular epithelial cells from the basement membrane (Khan *et al.* 2009), which form aggregates that can obstruct filtrate flow and increase intraluminal pressure and decrease glomerular filtration rate (GFR), inducing the back leak of filtrate into the interstitium, which further decreases GFR (Goligorsky *et al.* 1993). Another factor is the renal sensibility to vasoactive substances. The vasoconstriction induced by CCl_4 produces an ischemic local environment, which leads to cellular injury by deteriorating the membrane integrity (Khan *et al.* 2010). This fact, added to low GFR, may ultimately cause glomerular dysfunction. Accordingly, our results show that the glomerular function and the capacity of tubular absorption may have been altered with CCl_4 exposure, leading to functional overloading of nephrons with subsequent renal failure as also reported (Khan *et al.* 2009).

Concerning experimental groups, pre-treatment with SREE (Group V) provided different hepatic responses within the group, 24h after CCl_4 administration. The centrilobular hepatocytes either (a) appeared nearly normal with SREE-typical pattern on adjacent periportal zone, (b) revealed CCl_4-necrosis with moderately disorganised architecture of periportal zone, and (c) only SREE-pattern was attained.

Oppositely, a previous study on the halophyte *Sarcocornia perennis* subsp. *perennis*, which is taxonomically close related to *S. ramosissima*, demonstrated a protective effect against CCl_4-treated mice, mainly on liver and spleen, although the plant *per se* revealed testicular toxicity (Pinto 2011). Moreover, another member of the Chenopodiaceae, the halophyte *Suaeda maritima*, exhibited significant hepatoprotection against concanavalin-A-exposed rats (Ravikumar *et al.* 2011).

The most interesting fact about the hepatic results of the present work is that these complete unexpected interactions between SREE and CCl_4 occurred in the same experimental conditions; therefore these differences can only be explained with molecular mechanisms, where specific compound interactions occur. Consequently, these findings were probably due to the multitude of different chemical compounds present in the extract, since they can act synergistically, enhancing the hepatic pathological features, or antagonistically to CCl_4 (Venkataramanan *et al.* 2006). Through the results attained from the surprising histological finding (a), we also hypothesise that CCl_4 nature can be even modified when its hepatic site of action is previously injured by SREE toxic compounds, reversing CCl_4 toxicological action into a repairing one. Alternatively, and in agreement with the histological finding (b), a previous report demonstrated an increased CCl_4-induced hepatotoxicity by *Salvia officinalis* tea and, unlike SREE, it does not trigger any toxicity *per se*. Pre-treatment with the *S. officinalis* tea for 14 days before CCl_4 administration exacerbated centrilobular necrosis *versus* water drinking mice, and increased the expression and activity of CYP2E1 (Lima *et al.* 2007). An induction or an over-expression of CYP2E1 is correlated with higher CCl_4 toxicity as CCl_4 becomes toxic upon activation primarily through this cytochrome (Weber *et al.* 2003).

Nevertheless, each different response may depend on the herb compound concentration, their sufficient amount intake at a given time period and the genetic susceptibility of the animals, so that the compounds can take beneficial and/or adverse effect properly (Yang *et al.* 2006). Furthermore, the liver handles compounds through a

complex network of feedback mechanisms, which are interconnected by similar regulatory elements and substrates (Venkataramanan *et al.* 2006).

On the other hand, the kidneys exhibited uniform morphological changes among the group, and differed from the positive control in the increased haemorrhage extent and decreased number of degenerated glomeruli. Although SREE have failed to protect the CCl_4-affected cortical tubules, at least it partially inhibited the extension of the lesions into the glomerulus. As mentioned earlier, SREE *per se* caused mild glomerular changes. Altogether, these evidences may support the idea of SREE-induced damage in normal state of the organism, whereas under extreme cellular stress, the extract shows an opposite effect and protects discriminately some renal areas, probably by preventing excessive CCl_4 vasoconstriction and reabsorption.

Regarding SRS + CCl_4-treated animals (Group VI), prior seed administration did not positively influence the histological outcome in the liver, as it failed to reduce the extent of CCl_4-induced injury. Furthermore, SRS enhanced CCl_4-induced fatty vacuolar changes around all the parenchyma, suggesting an increased lipidosis. In contradiction to these results, a previous study on a seed extract of *Spinacia oleracea*, also a Chenopodiaceae, demonstrated hepatoprotective effect against CCl_4-induced injury. Through acute toxicity *in vitro* studies, this extract was also found to be safe up to 2000 mg/kg (Jain *et al.* 2012).

In the renal tissue, similar CCl_4-features occurred in Group VI, with the exception of the additional cellular content inside the tubular lumen, suggesting exfoliated tubule cells. Viable and non-viable renal tubular epithelial cells may also be sloughed into the tubular lumen, which promotes the loss of epithelial integrity (Racusen 1998). In addition to not conferring protection to tubular epithelium and yet somewhat intensifying the lesions, SRS did not significantly reduce the number of degenerated glomeruli, compared with SREE+CCl_4-treated group. All these findings show the SRS incapacity to prevent a toxicological insult and the inherent oxidative responses.

3. Regeneration studies

The main goal of the following regeneration studies is to simulate human diseases in animal models and to evaluate the potential curative and therapeutic action of the tested substances.

For the second positive control group (Group VII), the same single dose of CCl_4 was administered and its histopathological effects were evaluated after 3 weeks. Histologically, a certain degree of recovery was observed in hepatocytes and liver lobular arrangement, although some cell degeneration still persists. Indeed, the liver has a strong capacity for regeneration and the loss of hepatic tissue from the action of toxic compounds initiates a mechanism by which the surviving hepatocytes undergo promitogenic activity, until the original mass of tissue and its functions are restored. (Mescher 2009).

CCl_4 elicits two opposing responses, tissue repair and tissue damage. Normally, with lower acute doses of CCl_4, hepatocellular recovery will begin as soon as centrilobular necrosis starts to become evident. To accomplish this, cells must have sufficient energy supplies and receive no further injury from the same level of toxic exposure. Since the single dose of the present study was low, CCl_4 did not deplete cellular ATP levels completely, as earlier reported for higher dosages (Soni *et al.* 1994), and enabled the regenerative process take its course.

Concerning the kidneys, necrotic tubular injury was clearly decreased, although significant haemorrhage and inflammatory foci were still sustained after this time period. Normally after an acute chemical insult, the proximal tubular epithelium can be recovered within 5 to 7 days (Frazier *et al.* 2012), through the sequential process of dedifferentiation, migration, proliferation and redifferentiation of surviving cells which restores the tubular cell layer and function (Nony *et al.* 2003). After 21 to 28 days the tubules show a fully mature epithelium, functionally normal (Frazier *et al.* 2012). Our findings on Group VII are consistent with this outcome, and by this time CCl_4 may be already completely excreted from the organism. However, inflammatory processes remains and obviously more time would be needed for a complete recovery.

With regard to experimental groups, post-treatment with SREE (Group VIII) provided similar but ameliorated histological hepatic features.

It would be expected a hepatic histological profile similar to the Group II, though this has not happened. Previous reports indicated that pre-treatment with low CCl_4 doses before a lethal dose from the same toxicant, evidences an autoprotective effect (Pound *et al.* 1975). Moreover, heteroprotection can be equally achieved, preventing the action of the second-administered compound with higher dose (Chanda *et al.* 1995). This may be due to the fact that while hepatocytes undergo regenerative processes caused by the first toxicant, the CCl_4, all other mechanisms of injury progression induced by SREE toxic compounds, in this case, seem to be inhibited. On the other hand, and assuming that the extract contains a variety of beneficial compounds, the action of antioxidants will probably be more emphasised, since toxic compounds may be supressed. This hypothesis may explain the faster hepatic recovery from the combined CCl_4 and SREE-induced hepatotoxicity in this group, compared with the respective positive control.

While hepatic improvements were evident, the renal structure of this group was similar to the positive control. It is possible that beneficial compounds may have not reached the kidney within this time frame.

Post-treatment with SRS (Group IX) caused high mortality rate within the group. The histopathological evaluation of the remaining animals revealed a slightly aggravated morphology of the liver compared with the positive control and featured no additional recovery. In fact, some small granulomatous-like cell masses were observed, associated with yellow-brown pigmented cells. Granulomatous changes are most probably caused by precipitated poor water-soluble metabolites (Greaves 2007). Similar findings were described previously (Sobol *et al.* 2006). Pigmentation can result from cellular and erythryoid breakdown products, lipid peroxidation of cell membranes or altered heme metabolism (Thoolen *et al.* 2010). Circulatory disorders and xenobiotic treatment and may induce lipofuscin, hemosiderin or porphyrin accumulation in hepatic tissue (Greaves 2007). A previous study showed microgranulomas and lipofuscin pigments concomitantly present in the liver from beagle dogs after a chronic drug treatment, suggesting that basal maintenance and turnover of membranes were disturbed, with a subsequent increased accumulation of degradation products (Walsh *et al.* 1999). The pigmentation nature in the

present study is still unknown, although it is believed to result from the long-term and high dose exposure of SRS, which deprived hepatocytes of its full recovery and enhanced liver impairment, previously under stress conditions.

Concerning the kidneys, they presented several foci of tubular basophilia, suggesting an earlier sign of tubular regeneration. These basophilic cells are characterised by high mitotic activity and are normally present in the reconstructive tissue after 5 to 7 days of tubular cell necrosis, where the basement membrane remained intact (Frazier *et al.* 2012). However, in this experimental group, these cells persisted in the renal tissue during 22 days, indicating that, although the epithelial lining is complete, epithelial cell turnover persisted for a considerable time (Haagsma *et al.* 1980).

This evidence demonstrates that SRS treatment for 22 days after CCl_4 administration delayed the normal regenerative process and caused cell abnormalities, mainly in the liver. These facts may be some of the reasons behind the low survival rate associated with this group.

V. Conclusions and Future perspectives

Based on the study of *S. ramosissima* potential as a therapeutic agent, the current approach demonstrated some adverse histopathological effects. The histological data of the present work has shown that 50 mg/kg of *S. ramosissima* ethanolic extract revealed to be hepatotoxic and nephro-inflammatory; the seeds of this species at a dose of 2000 mg/kg caused significant renal inflammation and a slight hepatic changes.

Through histological analysis of CCl_4-subjected mice, it can be also inferred that neither *S. ramosissima* ethanolic extract nor their seeds were able to effectively protect CCl_4-induced injury on liver and kidney, on considered experimental conditions.

S. ramosissima ethanolic extract was able to promote mild regeneration on CCl_4-induced liver injury, but not in the kidneys. The extent of recovery achieved in this study exclusively happened through kidney's own regenerative processes.

The seeds of the studied species were unable to successfully promote regeneration on CCl_4-induced injury on liver and kidney, on considered experimental conditions. Contrariwise, it had a deleterious reverse effect, causing death possibly through impairment of normal function.

The findings of the present work raised numerous questions regarding the efficacy of *S. ramosissima* as a protective and regenerative agent of hepatorenal toxicity.

For this reason, further research is required in order to elucidate the effects of extracts of this plant. The following strategies should be addressed:

- New methodological approaches evidencing the precise biological mechanisms which are responsible for the plant-induced effects, such as histochemistry, immunohistochemistry and oxidative stress enzyme assays.
- Dose-dependent toxicological assays with the *S. ramosissima* extract and seeds, along with several times of exposure;
- The fractioning of the extract and isolation of its phytochemical compounds or potential contaminants, in order to establish a relationship between their chemical structure and the biological activities tested;
- Preparation of a seed extract and compound isolation for the same reasons above.

Although the biological mechanisms of *S. ramosissima* are not fully understood, the evidences suggest the pertinence of developing further research on this spontaneous halophyte that inhabits salt marshes and salt pans of Ria the Aveiro and is accessible to the general population.

V. Conclusions and Future Perpectives

VI. References

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VII. Appendix

Salicornia ramosissima ethanolic extract on mice: a light microscopy approach on liver and kidney

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Salicornia ramosissima J. Woods (Chenopodiaceae), included in the species aggregate *S. europaea* agg., is an annual succulent halophyte and one of the most salt tolerant plants, broadly distributed in the salt marshes and salt pans of Ria de Aveiro and in many others of the Iberian Peninsula [1]. *Salicornia* L. has been used not only as a seasoned vegetable, salad and fermented food in coastal areas of Europe and Asia [2], but also as folk medicine for disorders such as constipation, obesity and diabetes [3]. To corroborate this, the literature reports immunomodulatory, antioxidative, anti-inflammatory, anti-hyperlipidemic and antidiabetic effects [4]. Moreover, some bioactive compounds from its aerial parts were recently isolated and identified, exhibiting also antioxidant and cytotoxic activities [2, 5].
Factors such as the proper botanical identification, season and harvest site, extraction and purification methods, characterisation of the active constituents and the extract effects in a dose-and time-dependent manner are crucial to assess the therapeutic potential of herbal extracts [6]. The aim of this study was to investigate the possible hepatic and renal effects of an ethanolic extract of *S. ramosissima* on mice.

Aerial portions of *S. ramosissima* were collected from a salt pan in Ria de Aveiro (Portugal). The ethanolic extract (50 mg/kg b.w.) was orally administered, during 3 weeks, to male ICR-CD1 mice, purchased from Harlan (Spain). Control group was also considered. Liver and kidney were collected and prepared for histology. Animal procedures were followed according to guidelines for ethics and animal care.
Central and portal vein congestion and severe hydropic changes of the hepatocytes were noted in the liver of exposed group, compared with controls (Figs. 1A, 1B). Renal profile of control group evidenced normal features (Figs.1C, 1E). However, significant histological alterations were found in the exposed group: cortical interstitial haemorrhages (Fig. 1D), inflammatory cell infiltration and tubular cell degeneration within medulla and cortico-medullary junction (Fig. 1F).
In conclusion, this pilot study has demonstrated considerable effects in the mouse metabolism of *S. ramosissima* ethanolic extract, with significant hepatic and renal lesions. Further studies are needed to correlate these data with isolated active constituents for a more reliable evaluation of the potential of this species.

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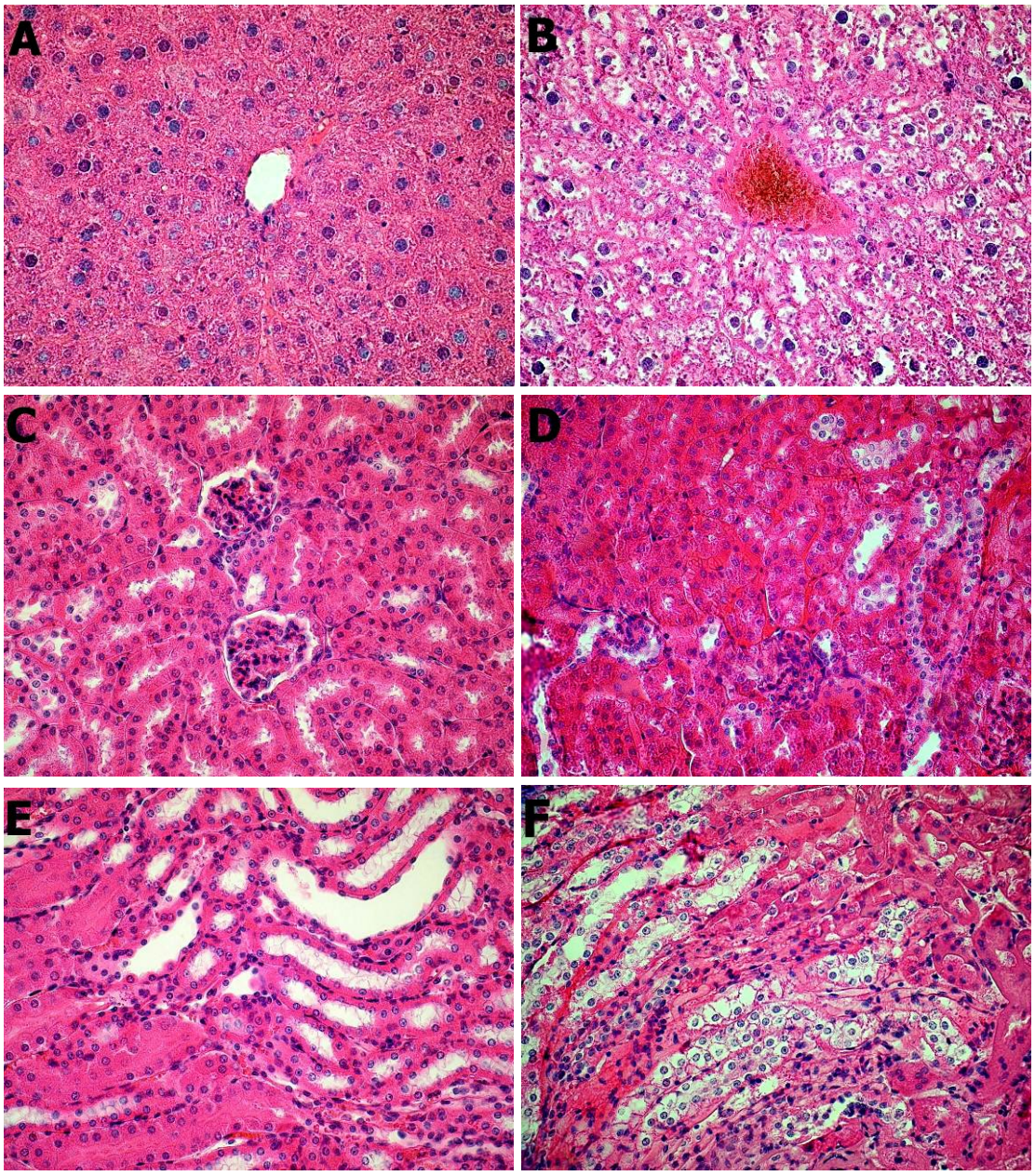

Fig 1. Light microphotographs of liver (A, B) and kidney sections (C-F) (H&E stain, 400× original magnification): A, C and E) Control group; B, D and F) *S. ramosissima* ethanolic extract-treated group.

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