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Table of Contents: Volume 12 Number 1 8 January, 2018

## ARTICLES

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African Journal of Pharmacy and Pharmacology

*Review*

# Absorption, distribution, metabolism and elimination (ADME) and toxicity profile of marine sulfated polysaccharides used in bionanotechnology

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**Sulfated polysaccharides extracted from marine algae and bacteria constitute an important class of biomacromolecules as they are characterized by biocompatibility, biodegradability and low immunogenicity. Recent advances in bionanotechnology are attributed to identification of marine sulfated polysaccharides of unique composition and functional properties. Promising results obtained so far justify the need for additional research in the study of absorption, distribution, metabolism and elimination (ADME) of these novel biopolymer-based nanomaterials in human body after administration by oral or parenteral route for therapeutic or diagnostic purpose. *In vitro* enzymatic degradation pathways should be investigated in order to yield commercially valuable oligomers. The goal of the present review is to enlighten on the ADME, cytotoxicity and *in vitro* enzymatic degradation of three marine sulfated polysaccharides, fucoidan, ulvan and mauran, obtained from brown seaweeds or macroalgae in the class of Phaeophyceae, members of *Ulvales* (green algae) and halophilic bacteria, respectively. They are presently being exploited in fabrication of nanoplatforms with novel applications in the field of controlled drug delivery, tissue regeneration scaffolds, cancer therapy, and bioimaging. However, significant research still needs to be carried out to characterize ADME of mauran and to improve production of the biopolymers on a large scale in order to find out clinically relevant solutions to establish these sulfated polysachharide-based nanotools as novel bionanotechnology strategies in future.**

**Key words:** Cytotoxicity, fucoidan, fucoidanase, mauran, sulfated polysaccharides, ulvan, ulvan lyase.

## INTRODUCTION

Marine environment is a comparatively underexploited and renewable reservoir for discovery of new natural biopolymers. Consumption of alga-derived foods for their dietary fibres and as a source of prebiotics, essential amino acids, polyunsaturated fatty acids, vitamins and minerals is steadily increasing day-by-day owing to evidences of potential nutritive value and health promoting effects. Marine heteropolysaccharides of algal

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origin represent a highly promising platform for controlled release novel drug delivery systems, cancer nano-theranostics, hydrogels for biomedical applications as in development of tissue engineering and tissue regeneration scaffolds, wound dressings, etc. (Silva et al., 2013; Popa et al., 2014; Posocco et al., 2015; Manivasagan et al., 2017; Majee et al., 2017a; Wells et al., 2017). Green algae have also attracted attention globally due to their pivotal role in marine and terrestrial ecosystem (Collen et al., 2011). In addition to marine algal polysaccharides, exopolysaccharides (EPS) secreted by marine bacteria constitute an important group of renewable biopolymers with considerable importance and demand for high-value applications. Some of the well-known EPS already in use commercially or being investigated for novel applications include xanthan, curdlan, levan, pullulan, dextran, sphingans, succinoglycans, mauran, etc. (Majee et al., 2017b). Bacterial EPS which is comparatively a new entrant is mauran, a sulfated anionic EPS which reveals itself with immense potential (Ates, 2015).

Nanobiotechnology or bionanotechnology is an emerging research arena which focuses on nanostructures and nanodevices fabricated from biomaterials for medical application and cell biology research (NSTC website; Raveendran et al., 2017). Nanotechnological endeavor has taken a great stride with identification of marine biopolymers of unique composition and functional attributes.

The advent of nanomaterials in the form of nanoparticles, nanocrystals, nanocapsules, nanospheres, nanogels, nanorods, and nanotubes has revolutionized the field of pharmaceutical technology, therapeutics, cancer therapy, oral vaccine delivery, imaging, biosensors, veterinary medicine, biotechnology, regenerative medicine, etc. Nanoparticles offer unique structural features owing to their nano-dimensions, shape and surface area rendering them capable of protecting the entrapped molecules, sustaining their release, improving bioavailability and enhancing intestinal epithelial absorption or cellular internalization. Selection of suitable natural polymers for development of nanoparticles provides additional benefits of enhanced stability, biocompatibility, biodegradability, non-immunogenicity and non-toxicity. New generation of nanoparticles can be developed with newer natural polymers with remarkable biopharmaceutical properties such as health-promoting effects, inherent pharmacological activities, haemocompatibility, responsiveness to specific internal stimuli and tunable chemical properties (Bahrami et al., 2015; Fernández Diaz et al., 2017). Quantum dots are specialised nanocrystals or nanofactories used as fluorescent markers for live cellular imaging, especially in cancer therapeutics because of their superior size control effect through compartmentalization in the periplasmic space and vesicles. But, techniques currently available involve harmful chemicals and also deleterious processing conditions thereby ultimately yielding hydrophobic and water-insoluble quantum dots with doubtful safety profile. Thus, suitable stabilization techniques should be employed for capping of quantum dots with biocompatible polymers to enhance their biocompatibility and improve their cellular uptake (Raveendran et al., 2014; Srivastava et al., 2015). Therefore, potential of nanomaterials can be expanded and flexibility in applications can be enhanced by exploring new natural polymers which can be used alone or can be combined with different synthetic polymers to alter absorption, biodistribution, biodegradation, elimination, cytotoxicity, biocompatibility, stability, and site-specificity (Raveendran et al., 2017). The multiple applications of marine sulfated polysaccharides are schematically given in Figure 1. The objective of the review article is to reveal the current status in the area of evaluation and interpretation of interactions of nanoparticles fabricated from selected marine polysaccharides of algal and bacterial origin, viz., fucoidan, ulvan and mauran, at cellular level with special emphasis on absorption pattern, biodistribution, metabolism, route and kinetics of elimination, that is, Absorption, Distribution, Metabolism and Elimination (ADME). An attempt has also been made to assimilate the results of cytotoxicity assays of these polysaccharides. Understanding the overall *in vivo* profile or ADME profile of the biopolymers and various *in vitro* enzymatic degradation pathways will strengthen the knowledge base for risk assessment, better management in exploitation of the natural resources for design of novel nano-platforms in various fields and ultimately establishing the applicability of the biopolymers as consumer-oriented product.

## FUCOIDAN, ULVAN AND MAURAN: MARINE SULFATED POLYSACCHARIDES OF CHOICE IN NANOTECHNOLOGY

### Occurrence and chemistry

#### *Fucoidan*

Fucoidan or sulfated fucose containing homo- or hetero-polysaccharide has been obtained from cell wall matrix and intercellular spaces of various edible species of brown seaweeds or macroalgae in the class of Phaeophyceae, such as *Cladosiphono kamuranus*, *Saccharina japonica* (as *Laminaria japonica*), and *Undaria pinnatifida* and also marine invertebrates like sea cucumber and sea urchin (Atashrazm et al., 2015; Wells et al., 2017). They are distributed among algae of several orders such as Fucales and Laminariales but also in Chordariales, Dictyotales, Dictyosiphonales, Ectocarpales, and Scytosiphonales. However, it is typically absent in green, red and golden algae and also


**Figure 1.** Applications of marine sulfated polysaccharides.

freshwater algae (Berteau and Mulloy, 2003). Fucoidan is a linear or an irregularly branched, high molecular weight polysaccharide and is primarily composed of α(1-3)- or alternating α(1-3)- and α(1-4)-linked L-fucopyranosyl residues as in *Fucus serratus*, *Fucus evanescens*, and *Fucus distichus*, with acetyl groups, sulfates or various side chains attached at different locations of the polymer backbone. Small amounts of other monosaccharides such as galactose, glucose, xylose, mannose and uronic acid are also present. Some fucoidans obtained from species belonging to the order of Fucales may have equal proportions of fucose and galactose and are thus termed as sulfated galactofucans. These polysaccharides consist of (1→6)-β-D-galactose and/or (1→2)-β-D-mannose residues. Chemical composition, physicochemical and functional properties of fucoidans thus vary greatly with geographical region, season, source, species of algae, method of harvesting, storage, method of extraction and processing techniques (Qianqian et al., 2011; Kimura et al., 2013; Cunha and Grenha, 2016; Felisilda et al., 2017). Sulfation occurs usually at C4, C2 or both C2 and C4 positions with rare instances of the sulfate group at C3 position. In some cases, sulfation and acetylation may occur simultaneously. Short fucoside side chains (fuco-oligosaccharide) may occur as O-linked to the α L-fucopyranosyl backbone residues as in *C. kamuranus*, *Chorda filum* (Ale et al., 2011; Silchenko et al., 2013).

### *Ulvan*

Ulvan is a complex, water-soluble sulfated anionic polysaccharide obtained from cell wall matrix of green algae, members of *Ulvales* and its structure is similar to those of L-rhamnose specific lectins in humans and animal glycosaminoglycan regulators such as dermatan sulfate, heparin and heparin sulfates. The species from which ulvan extraction has been reported include *Ulva pertusa*, *Ulva lactuca*, *Ulva clathrata*, *Ulva compressa*, *Ulva conglobata*, and *Enteromorpha prolifera*. 3-Sulfated rhamnose (Rha3S) is the principal monosaccharide present in ulvan, constituting 16.8 to 45% by weight, the rest being composed of sugars such as xylose (Xyl), glucose, mannose, galactose, arabinose, glucuronic acid (GlcA) and its C5 epimer, iduronic acid (IduA). The molar ratio of different monosaccharides is Rha: Gal: Xyl: Man: Glu = 1: 0.1: 0.11: 0.01: 0.21. The repeating units have been identified as disaccharides composed of Rha3S, linked either to GlcA, IduA, or Xyl, giving Rha3S-GlcA (ulvanobiouronicacid A), Rha3S-IduA (ulvanobiouronic acid B), and Rha3S-Xyl, respectively. These repeating units are α- or β- (1,4) linked to sulfated rhamnose. Xylose residues may also be sulfated at C2. The sulfate

content is 14.3 wt% and uronic acid is present at 19.3 wt%. Variations in molecular weight and rheological properties have been observed in different ulvans depending primarily on extraction conditions (Silva et al., 2013; Yoshimura et al., 2014; Cunha and Grenha, 2016; Collen et al., 2017; He et al., 2017; Melcher et al., 2017).

### *Mauran*

Mauran is an acidic sulfated polysaccharide with high uronic acid content and is extracted from moderately halophilic bacterium, *Halomonas maura*. The monosaccharides present in mauran include mannose, galactose, glucose and glucuronic acid in addition to sulfate and phosphate groups (Raveendran et al., 2013). In contrast to other anionic polysaccharides, it possesses unique property of being resistant to high salt concentrations and its conformation or its viscosity remains unaffected in the presence of cations (Rehm, 2009).

## Therapeutic benefits

Global demand for fucoidan is growing at a constant pace because of several studies indicating its potential anticoagulant, antiviral, anti-inflammatory, immune-modulatory, anti-oxidant and anti-cancer activity. It is consumed as dietary component by the people of Japan, Korea and China. Investigations have also proven its efficacy for nutraceutical and cosmetic applications (Kimura et al., 2013; Atashrazm et al., 2015; Felisilda et al., 2017; Majee et al., 2017a). It is also reported to facilitate neovascularization and angiogenesis through mobilization of endothelial progenitor cells, sequestration and enhancement of the activity of vascular endothelial growth factor (VEGF) (Rujitanaroj et al., 2014). Owing to its characteristic molecular weight, sugar composition and unique charge distribution, ulvan shows great promise with respect to bioactivities and nutritional value (Fernández Diaz et al., 2017). Mauran has demonstrated immunomodulatory and anti-proliferative effects in cancer cells as well as antioxidant, antihemolytic and anti-thrombogenic activities (Raveendran et al., 2014; Majee et al., 2017a).

## Marine sulfated polysaccharides as bionanotechnology platforms

Natural polysaccharides obtained from marine microbial world have been employed in fabrication of nano-particulate drug delivery systems. Factors governing their utilization are their easy availability, well-characterised physical, chemical and rheological behavior, low immunogenicity, usefulness in design of stimuli-responsive or ‘smart’ drug delivery systems, ability of being conjugated with proteins and bioactives and ability of being modified to achieve tailor-made functionalities. They include cellulose, agar, alginate, carrageenan, chitin, chitosan, heparin, hyaluronic acid, chondroitin sulfate, pectin, pullulan, dextran and many more. Modified natural polysaccharides can also be used to develop interpenetrating polymeric networks (IPN) and semi-IPN. Since, most of these polysaccharides carry specific charges, they can be exploited in designing of mucoadhesive drug delivery system involving interactions with mucin. Among seaweed polysaccharides, alginates and carrageenans are extensively studied biopolymers for diverse applications in nanotechnology with positive outcomes with respect to particle size distribution, high drug incorporation efficiency, controlled drug release profile, easy penetration into target sites and biocompatibility paving the way for safe and effective drug delivery (Venkatesan et al., 2016; Rydahl et al., 2017). In the recent years, several studies have been reported on the use of fucoidan, ulvan and mauran as nanotechnology platforms, some of which are being discussed in very brief.

Fucoidan encapsulated in nanoparticles exhibited cytotoxicity and induced apoptosis both *in vitro* and *in vivo* in xenograft osteosarcoma model. Permeability and hence bioavailability of the nanoparticles across Caco-2 cells was also higher than native fucoidan. Similar enhanced anticancer effect and inhibition of tumor growth and metastasis was also observed with nanoparticle fucoidan in the lungs of LM8 mice tumor xenografts (Kimura et al., 2013). Gold nanorods have been employed in photothermal, photodynamic therapy and also in photoacoustic imaging. In order to reduce the toxicity of cetyltrimethylammonium bromide-coated gold nanorods to normal cells, fucoidan coating has been employed with better results during photothermal cancer therapy (Manivasagan et al., 2017).

Nanoparticles prepared with unaltered ulvan extract from *Ulva ohnoi* macroalgae demonstrated successful stimulation of *Solea senegalensis* macrophages as indicated by intracellular and extracellular production of reactive oxygen species (ROS) in *in vitro* oxidative burst assay (Fernández Diaz et al., 2017). Ulvan-based intercalated and exfoliated nanoclays and nanofibres have also been developed (Robic et al., 2008; Melcher et al., 2017).

Another polyelectrolyte marine polysaccharide, mauran has been considered an interesting candidate for design of nanotools such as mauran quantum dots for bioimaging purposes and also for novel platforms for drug delivery in cancer therapy. Fabrication and active targeting of these nanocrystals has significantly improved binding to and uptake by cancer cells and tumor masses, promoting site-specific delivery and imaging. It has also attracted attention in the repertoire of tissue engineering for development of porous nanoscaffolds for cellular

regeneration due to its enhanced cell adhesion and proliferative activities observed during *in vitro* cell culture studies (Raveendran et al., 2014; Raveendran et al.,2015)

## ADME OF MARINE SULFATED POLYSACCHARIDES

For a molecule to be pharmacologically or nutritionally active without eliciting any toxicity or adverse effects, it must be released from drug delivery system or food matrix, undergo conversion to the simplest building block during digestion for a nutrient, must permeate across gastrointestinal mucosal epithelial barriers, must be absorbed into target cells or tissues for entering into systemic circulation, and finally must be metabolized and excreted out of the system. Algal polysaccharides are characterized by β(1→4) linkages and hence cannot be digested by humans. They constitute what is known as indigestible dietary fibres which pass along the small intestine without being metabolized and are fermented partially by resident colonic bacteria into short chain fatty acids (SCFA). They prove to be beneficial for humans because of their immunostimulant effect and their ability to alter the human gut microbiome or microbiota. Some of them have also been found to possess lower glycemic index than normal carbohydrate-rich vegetables. Fucoidans and ulvans belong to the class of soluble dietary fibres. Owing to their high intrinsic viscosity in aqueous media, they are capable of retarding the process of digestion, reduce the bioavailability of minerals and other vital nutrients by chelating them and may also enhance the count of Bifidobacteria and Lactobacillus in cecum and large intestine, respectively (Toboada et al., 2009; Raposo et al., 2016; Hemsworth et al., 2016).

Human digestive enzymes lack the ability to degrade fucoidan because of its high molecular weight. Low gastric pH also has very limited effects on fucoidan breakdown *in vivo*. In animal studies, jejunal epithelial cells, mononuclear cells in the jejunal lamina propria and sinusoidal non-parenchymal cells in the liver showed positive results for fucoidan accumulation. Trace amounts of fucoidan could be seen in ileum of rats. It has been reported in a study with human volunteers that fucoidan in its native form can be transported across the intestinal wall in very small amounts followed by internalization in intestinal macrophages. Macrophage scavenger receptors in Kupffer cells may be involved in fucoidan uptake. The apparent permeability coefficient of fucoidan is comparatively smaller than that of heparin. Transcellular route of fucoidan transport via pinocytosis or transporter protein has been envisaged. Inter-personal variability in rate of absorption could be noted. Rate of permeation across the intestinal wall has been increased by nitrosamine. Unchanged fucoidan could be detected in nanogram levels in serum. In urine, the amount excreted was ten times higher than that in serum but the presence of lower molecular weight fractions suggest possible metabolism by kidney. Following ingestion of *Undaria* dried biomass, low molecular weight fucoidan derivative, S-galactofucan has been identified in human blood. The degree of permeation of native fucoidan was significantly low when investigated across human colon adenocarcinoma Caco-2 cell monolayer. Similarity in characteristics between fucoidan and heparin indicate that fucoidan may follow first-order elimination kinetics upon oral administration. Biodistribution studies for 20 days after injection of anti-epidermal growth factor receptor (anti-EGFR) encapsulated in fucoidan-coated gold nanorods revealed the highest accumulation of gold in liver and spleen followed by heart, lungs, kidney and tumors. Fucoidan can thus be said to exhibit preferential accumulation in liver and low levels in systemic circulation (Atashrazm et al., 2015; Nagamine et al., 2015; Manivasagan et al., 2017). Rapid clearance of ultrasmall superparamagnetic iron oxide nanoparticles coated with $^{99m}$Tc labeled fucoidan (USPIO-FUCO) was recorded with half-life of 9 min for rapid component when administered intravenously by a single injection in male Wistar rats. Clearance could be explained by a two-compartment model where this rapid component accounted for observed 70% of the activity. Only 4.4% of the injected dose could be traced an hour after injection. Excretion of USPIO-FUCO is suggested to occur via reticuloendothelial system (Suzuki et al., 2015). Radiolabelled fucoidan with $^{99m}$Tc demonstrated excellent selectivity and sensitivity with respect to tissue uptake and retention. From biodistribution study, very low levels of radioactivity could be detected in the brain indicating negligible transport of fucoidan across blood brain barrier. Though fucoidan can act as an excellent P-selectin targeting agent in nuclear imaging of platelet-rich arterial thrombus and endothelial activation after an acute ischemic effect, chances of detection of early stages of inflammatory disease of brain are rare (Rouzet et al., 2011). It did not exert any influence on CYP450 enzymes or COMT pathways and therefore can be regarded as safe for administration either singly or as an adjuvant in cancer chemotherapy (Mathew et al., 2016). Pullulan-dextran electrospun fibres endowed with fucoidan were developed for delivery of vascular endothelial growth factor for eliciting angiogenic response. They were characterized by complete biodegradation within a week after subcutaneous implantation in mice (Rujitanaroj et al., 2014).

As in case of fucoidan, very low degree of ulvan fermentation by colonic microflora was noted when tested in human volunteers and production of SCFA in colon was also significantly less (Wells et al., 2017). Similar results were obtained in *in vitro* incubation study performed for 3 weeks with human faecal flora in a semi-continuous fermentor. However, its constituent mono- and disaccharides are rapidly metabolized. Ulvan in its native form demonstrated excellent biodegradation which

was reduced by introducing cross-linking (Bobin-Dubigeon et al., 1997; Yoshimura et al., 2016). Since the sulfate content in ulvan is considerably high, it should be consumed with caution on a long-term basis. Free sulfate can be readily transformed by resident gut microbiota such as sulfate-reducing bacteria into sulfide with potential health risks due to over-utilization of hydrogen. Released hydrogen sulfide is harmful for humans as it may actually potentiate the occurrence of inflammatory bowel disease, ulcerative colitis, etc. (Durand et al., 1997; Taboada et al., 2010).

## *IN VITRO* ENZYMATIC DEGRADATION OF MARINE SULFATED POLYSACCHARIDES BY MICROBIAL ENZYMES

Microbial enzymes responsible for hydrolytic degradation of marine polysaccharides include a diverse group of extracellular enzymes having substrate-specificity. These enzymes are collectively referred to as hydrolases and are distributed across huge microbial population (Steen et al., 2012). Glycosyl hydrolases, detailed information which are available in Carbohydrate-Active Enzymes (CAZy) Database, utilize the linkage between anomeric carbon and the bridging oxygen of glycosidic bond. Polysaccharides containing uronic acid residues are depolymerised through O-C4 bond cleavage via an elimination reaction to produce an α,β-unsaturated residue at the newly created non-reducing end of the sugar (Garron and Cygler, 2014; Ulaganathan et al., 2017).

Enzymatic hydrolysis of fucoidan into its constituent oligomers can be brought about by a class of enzymes, known as fucoidanase, found in brown algae themselves and also in other microbial species such as marine bacteria and fungi and mollusks. Fucoidanases or fucoidan hydrolases have been isolated from *Flavobacterium* species F-31, *Formosa algae* strain KMM 3553, which are induced by fucoidan itself and not by any of its degradation products. They can be classified as endo- and exo-fucoidanases. The end-products of fucoidan breakdown by endo-fucoidanases are oligosaccharides with varying, reduced molecular weights, produced by hydrolysis of glycosidic bond. L-fucanase is an example of endofucoidanase obtained from *F. algae*, which can produce fuco-oligosaccharides with immunomodulatory activity. Exofucoidanases act on the non-reducing end of sulfated fucose residues to produce oligomers and reduce the molecular weight at a relatively slower rate (Berteau and Mulloy, 2003; Ohshiro et al., 2010; Silchenko et al., 2013).

Ulvan-degrading enzymes or ulvan lyases have been identified in *Alteromonas* species from the faeces of small marine animals, uncharacterized Gram-negative marine bacterium found in decomposing algae of soil, marine Bacteroidetes, *Nonlabens ulvanivorans*, isolated from the feces of sea hares (*Aplysia punctata*) fed with green algae, Proteobacteria species, *Ochrobactrum tritici*, found in soil. These enzymes cleave glycosidic linkage between sulfated rhamnose and GlcA or IduA and the end-products of lyase-mediated β-elimination reaction are unsaturated oligosaccharides with degree of polymerization greater than 2, terminal positions being occupied by uronyl residues at the non-reducing end e.g. Δ, 4-deoxy-L-threohex-4-enopyranosiduronic acid (Collen et al., 2014; He et al., 2017; Ulaganathan et al., 2017). Complex nature of ulvanolytic degradation into monosaccharides indicates participation of other lyases such as 6S-rhamnosidases, xylanases, and sulfatases (Collen et al., 2011). Enzymes acting on ulvan oligomers have also been identified such as β-glucuronidase, a glucuronanlyase, and an ulvan hydrolase (Melcher et al., 2017).

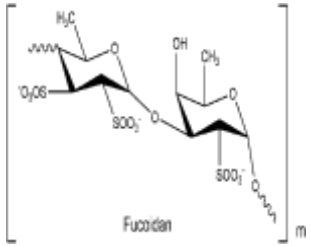
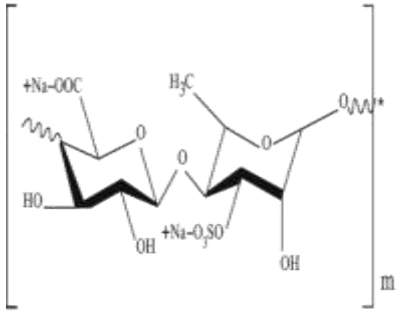
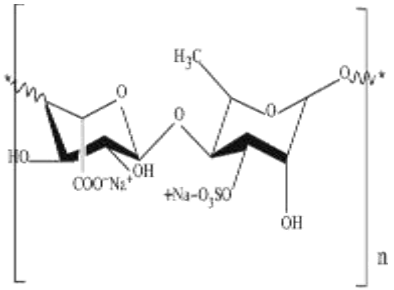
*In vitro* enzymatic degradation routes of fucoidan and ulvan by microbial enzymes, as cited in literature are summarized in a tabular form in Table 1.

## CYTOTOXICITY OF MARINE SULFATED POLYSACCHARIDES

Unearthing the non-cytotoxic nature of marine sulfated polysaccharides in normal cells is an essential step when biological applications are envisaged. Cytotoxicity of the sulfated polysaccharides has been tested by standard toxicological procedures based on evaluation of biochemical parameters such as double stranded DNA quantification, protein content through estimation of total protein, cellular metabolic viability through MTS/MTT assay and checking of membrane integrity through lactate dehydrogenase assay. For the purpose of comparison, a non-cytotoxic control sulfated polysaccharide such as hyaluronic acid is taken (Alves et al., 2013). Ulvan and fucoidan have been proved to be less cytotoxic compared to marketed antiviral drugs, at concentrations up to 100 µg/ml when studied in Vero cells. The 50% cytotoxic concentrations ($CC_{50}$) for ulvan and fucoidan have been reported as 810 and 1336 µg/ml, respectively (Aguilar-Briseno et al., 2015).

Fucoidan has demonstrated cytotoxicity towards specific cancers such as hematopoietic, lung, breast and colon cancers, at low dose such as 20 µg/ml and also at higher dose like, 3 mg/ml. It is reported to induce apoptosis in cancer cells through activation of caspases 3 and 7 in human colon cancer cells and also is capable of activating caspases 8 and 9. Mitochondrial function is also altered. From *in vitro* studies conducted on normal fibroblasts, fucoidan is considered safe at dose effective for cancer therapy. Fucoidan, extracted from *U. pinnatifida* and *L. japonica*, did not exhibit any toxicity in mice or Wister rats, when administered orally at dose up 300 mg/kg/body weight daily over a period of 6 months. No toxicity was observed in rabbits even after

**Table 1.** *In vitro* enzymatic degradation of marine sulfated polysaccharides by microbial enzymes.

| S/N | Marine sulfated polysaccharides | Sources | Enzymes for *in vitro* degradation | End products | Reference |
|---|---|---|---|---|---|
| 1 | Fucoidan  | *C. kamuranus*, *S. japonica*, and *U. pinnatifida* | Endo- and Exo-fucoidanases | Fuco-oligosac-charides | Berteau and Mulloy (2003); Oshiro et al. (2010); Silchenko et al. (2013). |
| 2 | Ulvan  Ulvanobiouronic acid A  Ulvanobiouronic acid B | *Ulva pertusa*, *U. lactuca*, *U. clathrata*, *U. compressa*, *U. onglobate*, and *E. prolifera* | Ulvan lyases, 6S-rhamnosidase, Xylanases, and Sulfatases | Δ, 4-deoxy-L-threohex-4-enopyranosidur-onic acid | Collen et al. (2011, 2014); He et al. (2017); Ulaganathan et al. (2017). |
| 3 | Mauran | *Halomonas maura* | NA | NA | - |

NA: Not available in literature.

administration for 2 weeks intramuscularly at a considerably high dose (Suzuki et al., 2015). Fucoidan-coated gold nanorods loaded with anti-EGFR did not exhibit any cytotoxicity or any histological changes even after 48 h incubation when investigated in MDA-MB-231 cells, although slight lowering of cell viability was noted in a time- and dose-dependent manner (Manivasagan et al., 2017). Viability of peripheral blood mononuclear cells is diminished by fucoidan at dose of 3 mg/ml and coagulopathic evidence with higher clotting time has been recorded at dose of 900 to 2500 mg/ml. Anti-thrombotic activity of fucoidan is responsible for observed defects in control of bleeding process. Deviations in potassium level within clinical reference range has been noted after 28 days in volunteers to whom a mixture of three extracts [*Fucus vesiculosis* (85% w/w), *Macrocysti spyrifera* (10% w/w), and *L. japonica* (5% w/w)] in capsules containing up to 187.5 mg fucoidan were administered daily (Atashrazm et al., 2015). During assessment of fucoidan activity *in vivo* as P-selectin inhibitor in experimental ischemia-reperfusion model, administration of fucoidan decreased neutrophil infiltration, platelet deposition in reperfused mycocardial tissue and also decreased the extent of necrosis (Rouzet et al., 2011). Therapeutic intervention for advanced or recurrent colorectal cancer involves administration of 5-fluorouracil/leucovorin or irinotecan plus 5-fluorouracil/leucovorin combination with serious side effects as consequence. Co-administration of oral liquid preparation of fucoidan daily to patients for a period of 6 months suggested reduction in toxicity of the chemotherapeutic agents as evidenced by lowering in incidences of peripheral neuropathy, thrombocytopenia and liver dysfunction. Fucoidan also suppressed development of fatigue in the patients. Although there was a marginal increase in occurrence of leucocytopenia, neutropenia, anemia and stomatitis, the survival rate and quality of patients improved with concurrent fucoidan therapy (Ikeguchi et al., 2011).

Dose-dependent cytotoxic effect for ulvan was responsible for management of hepatocellular carcinoma, human breast cancer and cervical cancer. Negative effect of ulvan on cellular metabolic activity did not correlate with any negative effect on cell viability. These results suggest the potential of ulvan to be used as a non-toxic compound in therapy, diagnosis and as nutraceutical (Alves et al., 2012; Thuy et al., 2016).

Mauran exhibited dose-dependent cytotoxicity in L929 cells when given in the form of autoclaved maura reduced graphene oxide (MRGO). Toxicity was also evident in white blood cells as manifested by reduced proliferation, generation of reactive oxygen species (ROS) and slight apoptosis at significantly low dose. These toxic effects can be attributed to the damage of exopolysaccharide layer on MRGO as a result of autoclaving (Cherian et al., 2014). In another study, mauran-chitosan composite nanoparticles did not induce ROS generation and thus did not affect the polyunsaturated fatty acids of fatty acid membrane or initiate chain reaction posing threat to the tissue system (Raveendran et al., 2015). Mauran-chitosan nanoparticles loaded with 5-fluorouracil did not elicit any toxicity in mouse connective tissue fibroblast cells (L929) (Posocco et al., 2015). Stabilization by mauran is also reported to reduce the toxicity, augment biocompatibility and improve cellular uptake of quantum dots without any compromise in efficacy when employed as fluorescent marker in *in vitro* imaging studies (Raveendran et al., 2014).

## FUTURE DIRECTION

Marine environment is undoubtedly a rich source of novel biopolymers with potential biotechnological applications and industrial prospects, the macromolecules being extracted from marine algae, bacteria, other micro-and macroflora as well as marine micro-and macro fauna. However, expected commercialization cannot be achieved primarily due to heterogeneity in composition and functionality with variation in environmental conditions, harvesting, fermentation, and extraction procedures. Therefore, sophisticated screening and isolation techniques such as bioassay-guided fractionation need to be adopted for separation of these novel sulfated polysaccharides with fixed chemical composition and defined functional properties. Recent advances in the field of genetic engineering and fermentation technology have proved successful in overcoming these problems resulting in generation of over-producing strains. Production of microbial exopolysaccharides involves huge expenditure which can be reduced and productivity can be optimized through deeper understanding of interrelations between metabolic pathways and EPS biosynthesis mechanism and by application of omics technologies and systems biology tools. Acquired knowledge will also enable in improving product quality and characteristics and also design of novel strains capable of yielding new molecules with modified composition and chain length (Ates, 2015). Alternatively, a list of structural characteristics essential to their optimal performances can be prepared for each of the polymer, against which matching can be done, when isolated from a new source or same source but under different conditions (Rouzet et al., 2011; Rujitanaroj et al., 2014; Salek and Gutierrez, 2016).

Fucoidan has demonstrated high affinity for P-selectin and also it is a natural mimic of ligand of P-selectin, sialyl Lewis X ($SLe^x$). Thus, it shows great potential for being developed on a commercial scale as a contrast agent in detection of active intraluminal thrombi occurring during acute coronary syndrome and stroke (Suzuki et al., 2015).

Application of novel hydrogels prepared from sulfated

polysaccharides of marine algal origin in tissue engineering for repair of cartilage defects have shown positive outcomes in *in vitro* studies as well as in animal models. Integration of these natural cell-carrying matrices with cells can be used in fabrication of *in situ* gelling system for parenteral administration, which can provide shape and can lead to regeneration of any type of defect *in vivo*. Significant research needs to be carried out to find out clinically relevant solutions and to establish these sulfated polysachharide-based hydrogels as novel tissue engineering scaffolds in humans (Popa et al., 2015).

## CONCLUSION

Sulfated polysaccharides of marine origin have gained popularity across the scientific community for diverse applications as they are biocompatible, less cytotoxic and possess low immunogenicity. Fucoidan and ulvan, obtained from seaweeds and mauran, obtained from extremophilic marine bacteria are examples of sulfated polysaccharides which are recently being investigated for fabrication of nanomaterials with novel applications in drug delivery, tissue regeneration and cancer theranostics. Comprehensive review of published data reveals that fucoidan and ulvan are not degraded in human digestive system but they are selectively and preferentially absorbed in certain organs and tissues with no evident signs of toxicity to normal cells. Literature lacks any data on ADME of mauran but reports on cytotoxicity studies are available. Several carbohydrate active enzymes have been identified which can effectively hydrolyse or degrade fucoidan and ulvan to specific well-defined oligomers. With all these promising findings on ADME of specialized marine sulfated polysaccharides, it is evident that strong efforts should be taken to improve the yield of these renewable biopolymers in a cost-effective manner.

## CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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*Full Length Research Paper*

# Antidiarrhoeal and antispasmodic activity of leaves of *Syzygium cumini* L. (Myrtaceae) mediated through calcium channel blockage

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***Syzygium cumini* L. Skeels (Myrtaceae) commonly known as jambolan is used as traditional medicine to treat gastrointestinal disorders in children in Brazil. This work is one of the first to evaluate the antidiarrhoeal and antispasmodic activity of the standardized extract of *S. cumini* leaves (HESc) in experimental models *in vitro* and *in vivo* rodents. Mice pre-treated with HESc (100, 250 and 1000 mg/kg) and atropine (1.0 mg/kg) had reduced intestinal transit velocity of 11.0; 23.2 and19.1%, respectively compared to saline control (46.6±0.9). In isolated rats jejunum, HESc (50, 150 and 300 µg/mL) shifted to the right cumulative concentration-response curves to ACh with changing maximum effect ($E_{max}$), which is characteristic of non-competitive antagonism to ACh. HESc also promoted relaxation ($E_{max}$ 90.2±5.8%) in preparations pre-contacted with KCl (75 mM). Additionally, it reduced the maximal $CaCl_2$-induced response in 15.4; 56.3 and 92.1% in a concentration-dependent manner. The study results show that HESc has an antidiarrhoeal and spasmolytic potential that can be partly explained by the reduction of intestinal transit velocity and blockage of the voltage-dependent calcium channels in the smooth intestinal muscle.**

**Key words:** *Syzygium cumini*, antidiarrhoeal activity, antispasmodic effect, leaves, rat jejunum.

## INTRODUCTION

Diarrhoea can be defined as a symptom of the gastrointestinal disorder; it is characterized by increase in stool frequency and alteration in consistency. It results from the imbalance between the absorptive and secretory mechanisms in the intestinal tract accompanied by hypermotility, bringing about excess loss of body fluids and electrolytes in feces (Sharma et al., 2015; Fernández-Bañares et al., 2015; Nemeth and Pfleghaar, 2017).

Treatment of gastrointestinal disorders and the search for new therapeutic agents are still a challenge. A potential antidiarrhoeal agent may exhibit its effect by inhibiting the gut motility (spasmolytic) and/or electrolyte outflux in the form of wet droppings, for example. The World Health Organization (WHO) has approved the use of traditional (folklore) medicines for treating many

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diseases (Hasler, 2003; Achem, 2004; Bellini et al., 2014; Qi and Kelley, 2014; Megbo et al., 2017; Schiller, 2017).

Natural products continue to play a highly significant role in drug discovery and development process. When analyzing drugs approved by Food and Drug Administration (FDA) between 1981 and 2014, approximately half of the drugs were based on natural products or derivatives thereof (Newman, 2013; Lahlou, 2013; Harvey et al., 2015; Newman et al., 2016).

Medicinal plants till date are quite used to treat various diseases. For example, *Syzygium cumini* is extensively used for the treatment of different diseases, such as inflammation, constipation, diarrhoea, obesity, urinary disorders, diabetes and hypertension. Despite this extensive use in folk medicine, most of the time there are no scientific studies that prove people use them (Zahoor et al., 2017; Mukherjee et al., 2017; Baliga et al., 2011; Ayyanar and Subash-Babu 2012).

*Syzygium cumini* L. Skeels*, of* the Myrtaceae family, popularly known as jamun, is included in the List of Medicinal Plants of Interest to the Public Health System (Renisus), issued by the Ministry of Health, which includes plants with potential use for medicines (Souza, 2005; Brasil, 2009; Swami, 2012; Subash-Babu and Ayyanar et al., 2013). All parts of *S. cumini* are widely used as traditional medicine, for example, juice of tender leaves is given in goat milk to treat diarrhoea in children (Morton, 1963; Corrêa, 1974; Lainetti and Brito, 1979; Nadkarni, 1976).

Pharmacological studies show that *S. cumini* species have several functions, among them, antioxidants, antibacterial, antifungal, anti-allergic, anti-inflammatory, anti-hyperlipidemic, gastroprotective, cardioprotective, hepatoprotective, anticancer, radioprotective and antidiarrhoeal (Jagetia and Baliga, 2002; Barh and Viswanathan, 2008; Chaturvedi et al., 2009; Schoenfelder et al., 2010; Patel et al., 2010).

The antidiarrhoeal activity was investigated using extracts obtained from the stem bark and seeds of *S. cumini* (Mukherjee et al., 1998; Mazumder et al., 2006; Shamkuwar et al., 2012; Chandra, 2013). However, there is no report available on the hydro-alcoholic extract of the leaves of *S. cumini* on its anti-diarrhoeal or antispasmodic activity despite its medicinal use in diarrhoea.

The research group previously showed that a standardized hydroalcoholic extract prepared from the leaves of *S. cumini* (HESc) is safe, with no evidence of toxicity in rodents (Silva et al., 2012). We subsequently demonstrated the hypotensive and antihypertensive activity of HESc, causing a reduction in vascular reactivity associated with the inhibition of extracellular calcium influx, whose mechanism was attributed to the marked presence of flavonoids (Mahmoud et al., 2001; Ribeiro et al., 2014). This work is the first to evaluate HESc in experimental models *in vitro* and *in vivo* animals, analyzing the intestinal transit velocity in Swiss albino mice, and the contractile activity in isolated rat jejunum, respectively.

## MATERIALS AND METHODS

### Plant material

Leaves of *S. cumini* were collected from the Campus of the Federal University of Maranhão (2°33'11.7"S 44°18'22.7"W), São Luís, Brazil, in January 2014. A voucher specimen was identified and deposited in the herbarium of the “Profa. Dra Berta Lange de Morretes” Medicinal Plant Garden, UFMA (No. 01079/1079).

### Preparation of crude extract

The leaves were mechanically ground to give 920 mg powder. This was added to 1 L of ethanol (70%) and mixed at 8 h each for 72 h. After this period the hydroalcoholic extract was filtered using a cotton funnel. After this process, the extract was concentrated using a rotatory evaporator under reduced pressure and filtered again. We obtained a concentrate of 150 mg/ml and a yield of 16.3% proportional to the 920 mg initially obtained. Such concentrate was denominated in a hydroalcoholic extract of *S. cumini* (HESc). Finally, the extract was lyophilized to obtain a powder and the dry residue obtained was solubilized in distilled water to a concentration of 10 mg/ml. It was re-diluted in distilled water as needed for each experimental protocol.

### Experimental animals

Swiss albino mice (25 to 30 g) and Wistar albino rats (250 to 300 g) of either sex from the Universidade Federal de São Luis, Brazil were used. Animals were housed under controlled temperature (25±1°C) and lighting (lights on 06:00 to 18:00 h); they had free access to food and potable water. All procedures described in the present study were approved by the Animal Research Ethics Committee of the State University of Maranhão, Brazil (Protocol number 003584/2014- 97).

### *In vivo* experiments

#### *Small intestinal transit*

Swiss albino mice were fasted for 6 h prior to the experiments, but were allowed free access to water. The animals were treated with HESc (100, 250 or 1000 mg/kg, *p.o*., respectively), atropine sulfate (1.0 mg/kg, *p.o*.) or saline (10 mL/kg, *p.o*. n=6), 60 min prior to the administration of a 5% charcoal suspension in 1% guar gum (0.1 mL/10 g body weight, *p.o.*). After 30 min, the animals were euthanized and their small intestines were removed. The distance traveled by the charcoal plug from the pylorus to the cecum was measured and expressed as a percentage of the total intestinal length (adapted from Freire et al., 2011).

### *In vitro* experiments

#### *Effect of HESc on ACh-induced cumulative dose-response curves in isolated rat jejunum*

All rats were euthanized by decapitation with guillotine following the principles of laboratory animal care based on the guidelines of the bioethics committee. Segments of jejunum (1.5 cm long) were suspended in a 10 mL organ bath containing Tyrode’s solution

(composition in mmol/L: NaCl, 137; KCl, 2.7, $MgCl_2 \cdot 6H_2O$, 0.5; $CaCl_2 \cdot 2H_2O$, 1.8; $NaH_2PO_4$, 0.4; $NaHCO_3$, 12; glucose, 5.5), aerated with 95% $O_2$, 5% $CO_2$ (pH 7.4) and maintained at 37°C. The preparations were set up under a tension of 1 g and responses were recorded on a smoked Kymograph paper through an isotonic frontal writing lever (magnification x 6). After 30 min equilibration period, cumulative concentration-response curves for ACh ($10^{-9}$ to $10^{-4}$ M) were recorded in the absence and presence of HESc (50, 150 and 300 µg/mL). This curve was compared with those obtained in the absence of HESc and the results were expressed as percentages of the maximal response to ACh alone (Van Rossum, 1963).

***Effect of HESc on KCl-induced tonic contractions in isolated rat jejunum***

Isolated rat jejunum was obtained as described earlier. Segments of jejunum (1.0 cm long) were suspended in a 5 mL organ bath containing Tyrode's solution aerated with 95% $O_2$, 5% $CO_2$ (pH 7.4) and maintained at 37°C. The preparations were set up under a tension of 1 g. In addition, for the recording of the isometric tension, the thread from the muscle strips was attached to an isometric force transducer that was connected to a bridge amplifier (ADInstruments Ltd, Grove House, Hastings, U.K.). Isometric tension changes were digitized using either PowerLab/4SP (ADInstruments Ltd, Grove House, Hastings, U.K.) and stored on a personal computer for later analysis. After 30 min equilibration period, segments of jejunum were contracted with KCl (75 mM) and when a stable contraction was attained (15-20 min), HESc (9; 27; 81; 243 and 729 µg/mL) was cumulatively added in an attempt to obtain dose-relaxation curves. The relaxant effect induced by HESc was expressed as the reverse percentage of the initial contraction force elicited by KCl.

***Effect of HESc on $CaCl_2$-induced cumulative dose-response curves in calcium-free solution in isolated rat jejunum***

The jejunum was mounted as described earlier. The preparations were set up under a tension of 1 g, and responses were recorded on a smoked Kymograph paper through an isotonic frontal writing lever (magnification x 6). After the stabilization during 30 min in normal Tyrode's solution, the external calcium was eliminated with depolarizing Tyrode's solution (KCl, 70 mM; $Ca^{2+}$-free). Cumulative concentration-response curves of $Ca^{2+}$ were obtained by cumulatively adding $CaCl_2$ ($3 \times 10^{-5}$ to $10^{-1}$ M) in the absence and presence of HESc (27, 81 and 243 µg/mL), which were added to the bath 10 min before addition of $Ca^{2+}$. This curve was compared with those obtained in the absence of HESc and the results were expressed as percentages of the maximal response to $CaCl_2$ alone (Van Rossum, 1963).

**Statistical analysis**

Values were expressed as mean ± S.E.M. Statistical analysis was performed using GraphPad Prism 5.01 software (GraphPad Software Inc., San Diego, CA, USA). Differences between means were compared using t-test (non-paired) and one-way ANOVA followed by Bonferroni's test as appropriate and $p$ values < 0.05 were considered indicative of significance.

## RESULTS AND DISCUSSION

In this study, it is shown for the first time the investigation of the antidiarrhoeal and antispasmodic activities of the HESc, analyzing the intestinal transit velocity in Swiss albino mice, and the contractile activity in isolated rat jejunum, respectively. It is demonstrated that HESc has antidiarrhoeal and antispasmodic effect by decreasing the speed of intestinal transit and blocking the influx of calcium, respectively.

Charcoal meal test in mice is a method used to study the effect of drugs on the motility of intestine (Misar, 2000). The transit velocity of the small intestine compared to the effect produced by saline control (46.6±0.9%), was reduced to 11.0; 23.2; 19.1 and 38.5%, respectively, after pre-treatment of mice with HESc (100, 250 and 1000 mg/kg) and atropine (1.0 mg/kg), administered orally, 60 min before administration of coal meal. Although HESc was less efficient in decreasing intestinal transit compared to atropine (standard drug), it reduced intestinal transit significantly in all doses tested compared to the control as shown in Figure 1.

Anti-diarrhoeal activity has already been demonstrated in studies carried out with the seeds and bark of the stem of *S. cumini*. In both studies, it was possible to show a significant reduction in gastrointestinal motility in tests of coal meal in rats. The underlying mechanism of action of the plant extract appeared to be antispasmodic whereby the extract produced relief from diarrhea (Mukherjee et al., 1998; Shamkuwar et al., 2012; Srivastava and Chandra, 2013).

It is well known that aqueous herbal medicines are traditionally used for their antispasmodic and antidiarrhoeal activity in various countries (Hajhashemi et al., 2000; Mujumdar et al., 2000; Sadraei et al., 2003). Non-specific anti-diarrhoeal drugs involve actions on intestinal transit that results in symptomatic improvement in a variety of conditions. Furthermore, the study on antispasmodic effect might help to deduce the possible mechanism of action (Schiller, 1995).

Antispasmodics may be classified, for example, into antimuscarinics, smooth muscle relaxants (that is, drugs that directly inhibit smooth muscle contractility, for example, by increasing cyclic AMP levels or by interfering with the intracellular calcium pool), similar agents papaverine and $Ca^{2+}$ blocking channels (especially $Ca^{2+}$ L-channel blockers) (Christen, 1990; Singh et al., 2003).

Muscarinic receptors of the $M_3$ subtype are present in the intestinal smooth muscle. These receptors are responsible for initiating contraction in response to acetylcholine (ACh) binding. This neurotransmitter is released by parasympathetic postganglionic neurons that innervate the digestive tract (Weiser et al., 1997). Upon binding with ACh, the $M_3$ receptor initiates a cellular signaling cascade. Briefly, the alpha subunit of the Gq/11 protein activates the effector phospholipase C (PLC), which increases the inositol triphosphate ($IP_3$) secondary messenger responsible for releasing calcium from the sarcoplasmic reticulum. This $Ca^{2+}$ release activates voltage-gated $Ca^{2+}$ ($Ca_V$) channels indirectly that leads to influx of $Ca^{2+}$ from extracellular fluid (Caulfield, 1993;

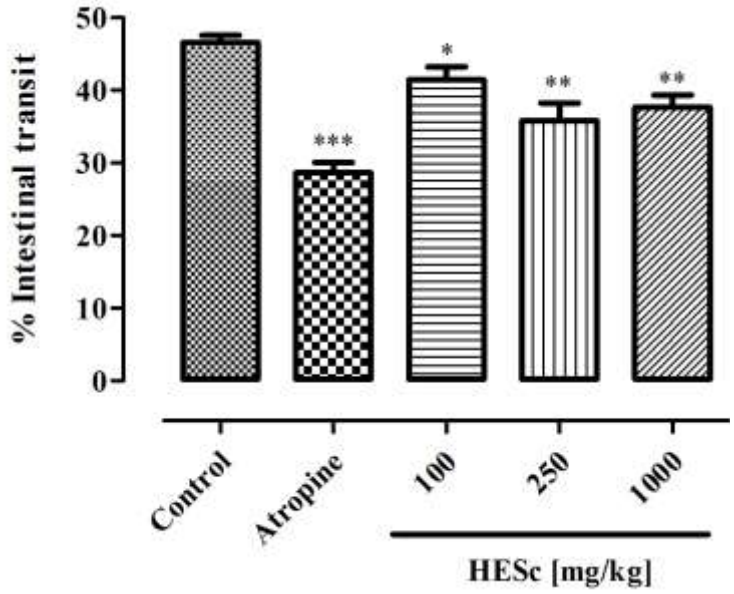


**Figure 1.** Effect of HESc on small intestinal transit in mice. Columns and bars represent means and S.E.M., t-test, *$p < 0.05$, **$p < 0.01$ (Control vs. HESc), ***$p < 0.001$ (Control vs. atropine) (n = 6).

Eglen et al., 1996; Honda et al.,1996; Catterall et al, 2005).

The isolated rat jejunum was used in this study to initially investigate whether the reduction of intestinal motility caused by HESc was mediated by competitive antagonism to the $M_3$ receptor, with consequent interference in the availability of intracellular $Ca^{2+}$. For this, the effect of HESc on cumulative concentration-response curves to the addition of ACh ($10^{-9}$ to $10^{-4}$ M) was evaluated. The $pD_2$ value ($pD_2$ = -log $EC_{50}$, negative logarithm of molar concentration of agonist that caused half-maximal response) was 6.4±0.05 M. In the presence of HESc (50, 150 and 300 µg/mL), the $pD_2$ value was altered to 5.8±0.2, 6.2±0.2 and 5.1±0.1 M, respectively, and $E_{max}$ to ACh was reduced in 17.8, 34.3 and 57.3% (Figure 2), suggesting a non-competitive type antagonism.

Spasm is characterized by a muscle contraction and in the smooth muscle this contraction occurs after the elevation of the intracellular calcium concentration ($[Ca^{2+}]_i$) due to the opening of the voltage-dependent calcium channels ($Ca_V$) present in the plasma membrane or due to its release of sarcoplasmic reticulum (RS) controlled by secondary messengers, for example, $IP_3$. The functional regulation of $[Ca^{2+}]_i$ to trigger a contractile response in smooth muscle is related to two stimuli that lead to two types of couplings: (1) electromechanical coupling, which is involved with the membrane potential change (Vm) and (2) drug-mechanical coupling when the contraction induced by an agonist is always greater than that observed only with the change of Vm (Al-Zuhair et al., 1996; Rembold, 1996; Bolton, 1979).

The contraction in the smooth muscle in response to several agents is often composed of two phases: a fast and unstained phasic component, followed by a slow and sustained tonic component (Breemen and Saida, 1989). The phasic component is due in part to the $Ca^{2+}$ of the sarcoplasmic reticulum and the tonic component is mainly due to $Ca^{2+}$ from the extracellular medium entering the cell through the $Ca_V$ (Abdellatif, 1989; Kobayashi et al., 1989; Takano and Kamiya, 1996).

In order to verify whether HESc would promote relaxation of the pre-contracted jejunum, which would be suggestive, at a functional level, of blocking $Ca^{2+}$ influx through the plasma membrane, its effects were evaluated on the tonic component of the contractile response induced by KCl 75 mM (electromechanical coupling). It was observed that HESc promotes relaxation of the jejunum (Figure 3B) in a concentration-dependent manner with a $E_{max}$ of 90.2±5.8% (Figure 4); we hypothesized that HESc would prevent $Ca^{2+}$ influx through $Ca_V$.

To confirm this hypothesis, the effect of HESc on the cumulative concentration-response curves to the $CaCl_2$ was evaluated. The jejunum was contracted with increasing concentrations of $CaCl_2$ (3 x $10^{-5}$M to $10^{-1}$ M), with a $pD_2$ value of 2.07±0.09 M. In the presence of HESc, at 27, 81 and 243 µg/mL, the $pD_2$ values were lowered to 1.59±0.12, 1.83± 0.14 and 1.29±0.06 M, respectively. In addition, HESc displaced the cumulative

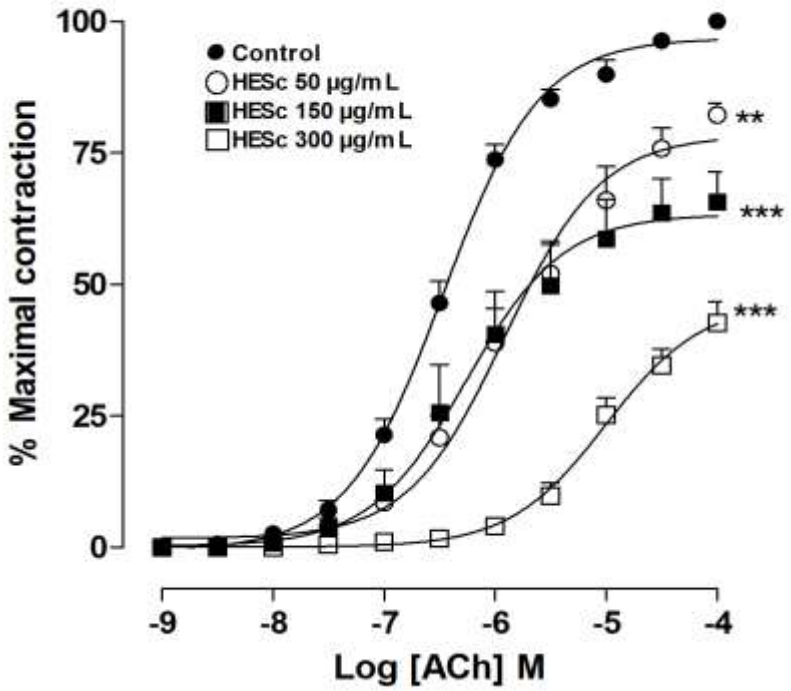


**Figure 2.** Effect of HESc on isolated rat jejunum contractile-response to ACh. Symbols and vertical lines indicate means ± SEM, respectively. One-way ANOVA followed by Bonferroni's test (Control vs HESc), ***p* < 0.01; ****p* < 0.001 (n = 4 – 6).

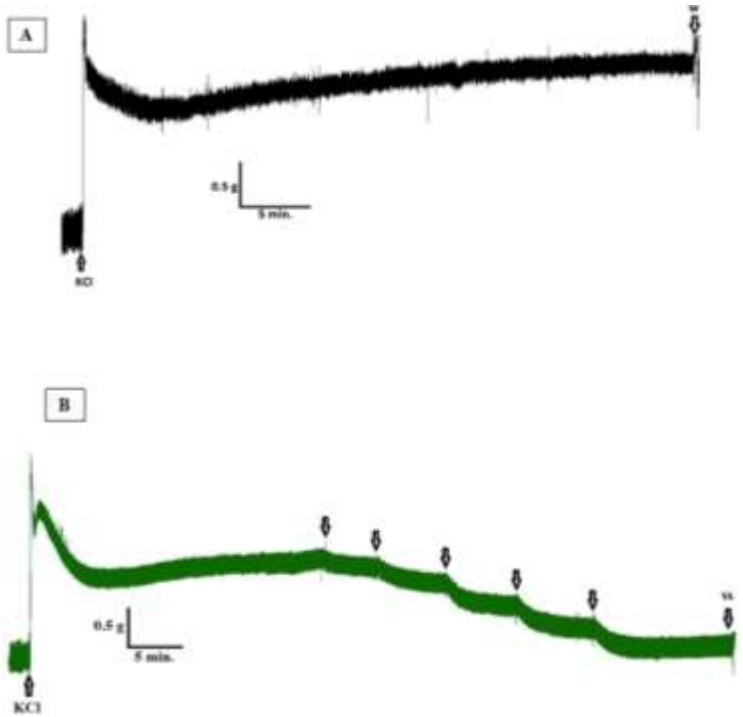


**Figure 3.** Representative originals records in the absence (A) and presence of HESc (B) on isolated rat jejunum contractile-response to KCl. Arrow represents concentration of HESc (9, 27, 81, 243, and 729 µg/mL). KCl: potassium chloride and W: washout.

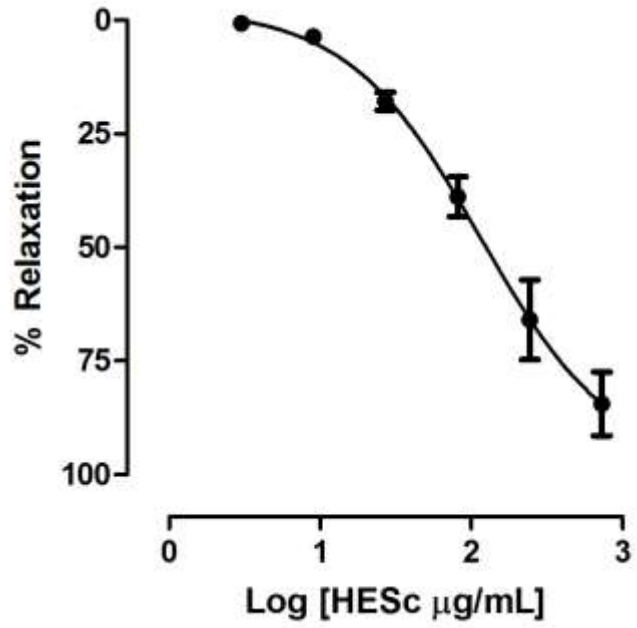


**Figure 4.** Effect of HESc on KCl-induced (75 mM) tonic contraction in isolated rat jejunum. Symbols and vertical lines indicate means ± SEM, respectively (n = 4).

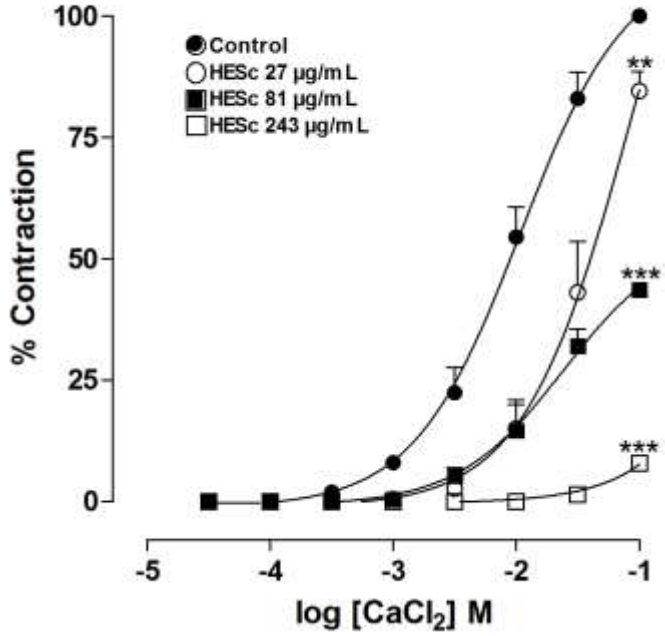


**Figure 5.** Effect of HESc on isolated rat jejunum contractile-response to $CaCl_2$. Symbols and vertical lines indicate means ± SEM, respectively. One-way ANOVA followed by Bonferroni's test (Control vs EHF-SC), $^{**}p < 0.01$; $^{***}p < 0.001$ (n = 3).

concentration-response curves of $CaCl_2$ to the right, in a non-parallel manner and the maximal response was reduced, in a concentration-dependent manner, by 15.4; 56.3 and 92.1%, respectively (Figure 5), suggesting that

the antispasmodic effect is possibly mediated through the inhibition of $Ca^{2+}$ influx probably through of $Ca_V$.

Other studies show that antispasmodic constituents present in various medicinal plants mediate their effect generally by blocking the calcium channel (Ghayur et al., 2006; Gilani et al., 2006; Shah et al., 2010). Similar results of inhibition of contractile responses to calcium were found in previous studies with the hydroalcoholic extract of *S. cumini* by our laboratory in preparation of vascular arteries rings isolated from normotensive and spontaneously hypertensive rats. The effects were attributed to the presence of flavonoids detected by phytochemical screening (Abreu et al., 2002; Ribeiro et al., 2014)

## Conclusion

In this study, it can be concluded that the antidiarrhoeal effect of HESc, observed by the reduction of the intestinal transit, can be explained by the blockage of calcium influx through $Ca_V$ responsible for the antispasmodic activity. These properties may explain the use of *S.* cumini as an antidiarrhoeal agent in traditional medicine and contribute to the future indication of *S. cumini* as a possible therapeutic alternative to treat gastrointestinal diseases. However, further studies are needed to explore the secondary metabolites responsible for the results obtained in this study.

## CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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