

Screening for anticoagulant substances in some marine macroalgae

Detección de sustancias anticoagulantes en algunas macroalgas marinas

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ABSTRACT

The coagulation disorders have increased in the last decades and no new substances had been discovered that could regulate this illness. This fact makes the discovery of new anticoagulant substances a priority issue in research. Forty nine seaweed species collected off shore of several localities of Gulf of Mexico and Mexican Caribbean sea were screened in order to detect anticoagulant activity in both intrinsic and extrinsic pathways of clot formation. Standardized plasma coagulation tests (thrombin and prothrombin time) were used. Heparin, a sulphated glycosaminoglycan wide used in oral anticoagulant therapy, was used as reference in both proofs. The results showed that four species presented potent anticoagulant activity same as heparin in the two pathways: *Anadyomene stellata*, *Caulerpa cupressoides* (Chlorophyta), *Lobophora variegata* (Phaeophyta) and *Liagora farinosa* (Rhodophyta). *Caulerpa paspaloides* (Chlorophyta) was active only in the thrombin time test. Other seven species presented a slightly anticoagulant activity. We considered that algal extracts have substances capable of inhibit clot formation in the last steps of the coagulation cascade. The extracts could act preventing the conversion of prothrombin to thrombin, also they could act stopping the transformation of fibrinogen to convert it to fibrin o even in the polymerization of this last molecule. This is the first report of anticoagulant activity in *Anadyomene stellata*, *Lobophora variegata* and *Liagora farinosa*. We can concluded that these species can be considered as potentially alternative source of anticoagulant molecules.

Palabras clave: Anticoagulant activity, marine algae, Gulf of Mexico, heparin

RESUMEN

Los problemas de trombosis en el mundo se han agravado en las últimas décadas y no han sido descubiertas nuevas sustancias que regulen estos padecimientos, lo anterior, impone como una necesidad la búsqueda de sustancias anticoagulantes alternativas. Se analizaron 49 especies de macroalgas recolectadas en diversas localidades del Golfo de México y Caribe mexicano para identificar su actividad en la vía intrínseca y extrínseca de coagulación de la sangre por medio de pruebas estandarizadas de coagulación de plasma humano (tiempo de trombina y de protrombina). Se utilizó como anticoagulante de referencia heparina, glucosaminoglicano sulfatado utilizado en la terapia oral de la coagulación. Se detectaron cuatro especies con actividad semejante a la heparina, es decir, que impidieron la coagulación del plasma humano en las dos pruebas utilizadas: *Anadyomene stellata*, *Caulerpa cupressoides* (Chlorophyta), *Lobophora variegata* (Phaeophyta) y *Liagora farinosa* (Rhodophyta). *Caulerpa paspaloides* (Chlorophyta) fue activa solamente en el tiempo de trombina. Otras siete especies retardaron ligeramente el tiempo de coagulación. Se considera que los extractos algales poseen sustancias capaces de inhibir la coagulación en los últimos pasos de la cascada de coagulación de la sangre,

en la conversión de protrombina a trombina y pueden actuar deteniendo la transformación del fibrinógeno en fibrina o incluso en la polimerización de ésta última. Se reporta por primera vez la actividad anticoagulante de *Anadyomene stellata*, *Lobophora variegata* y *Liagora farinosa*. Se concluye que estas especies pueden ser consideradas como una fuente alternativa de nuevos anticoagulantes.

Key words: Actividad anticoagulante, algas marinas, Golfo de México, heparina

INTRODUCTION

Thrombosis is a health problem that affects many people in the world. The discovery of alternative anticoagulant molecules must be an important task for scientists. It has been done research in anticoagulant activity of polysaccharides and glycosaminoglycans of diverse sources such as: ascidians (Pavão *et al.*, 1998), sea cucumbers (Vieira *et al.*, 1991), echinoderms (Mourão *et al.*, 1996), tunicates (Cavalcante *et al.*, 2000). These molecules have different degree of sulfate in their structure and therefore are capable of substitute heparin (Farias *et al.*, 2000). This last molecule is a sulfated glycosaminoglycan used in oral therapy for anticoagulant disorders.

It is known that species of Chlorophyta synthesized polydisperse heteropolysaccharides with low sulfate content, glucuronoxilohamans, glucuronoxilohamagalactans and xyloarabinogalactans, some of them with a potent anticoagulant activity (Uehara *et al.*, 1992; Harada & Maeda 1998; Lee *et al.*, 1998; Hayakawa *et al.*, 2000). It has been reported also that brown algae have a potent anticoagulant activity in fucoidans (Chevolot *et al.*, 1999; Nishino & Nagumo 1992; Nishino *et al.*, 1995). In the other hand, a low anticoagulant potency has been reported for polysaccharides in species of Rhodophyta, as agar and carrageenans (Güven *et al.*, 1990 and 1991). Recently, a group of scientists had stand out the potentiality of some proteins and proteoglycans capable of interfere with blood clotting cascade, particularly from *Codium* (Matsubara *et al.*, 1998; 1999; 2000a and 2000b), this group had isolated and characterized proteins with strong anticoagulant activity. The research effort has been focused on one genus, forgotten the basic detection of different algae as a potential source of alternative anticoagulant molecules. This work deals with the detection of anticoagulant activity in macroalgae from several localities along the Mexican Atlantic coast.

Localities of collection. We collected algal material along the central coast of Veracruz, and some localities of Campeche, Yucatan and Quintana Roo States in order to have samples of the coast of the Atlantic Ocean in Mexico (Gulf of Mexico and Caribbean sea). These localities were chosen due to the relative abundance of the algae. The sites of collection of Veracruz state were: La Mancha, a rocky shore at 90°22'40" W, 19°36' N, where algae grow at great diversity and

abundance. Costa de Oro is a municipal shore in Veracruz harbor, is a shallow shore with pebble and boulders where algae grows attached to them, located at 96°02' W, 19°12' N, in front of Hotel Fiesta Americana in Boca del Rio district. In Campeche state we collected phycological material in: Champoton, located at 90°43'12"W, 19°24'00"N, this locality is an estuary with mixed sand and rocky bottom, the algae were collected near the mouth of this coastal lagoon. Tenabo a shore near the town of Tenabo, located at 90°12'34"W, 19°57'01" N, with marine seagrass *Thalassia testudinum*. In Yucatan state algae we collected in Celestun at 90° 27' W, 20° 48' 30" N, it is an open beach near the outlet of the coastal lagoon where seagrass and algae grow together. Near Chelmem town, this locality is located at 21°27'N, 89°7'W and is a typical tropical barrier island lagoon Class III-A according to Lankford's classification (Lankford, 1977). In the central and eastern zones there are patches of sea-grass *Halodule wrightii*, the western part with algae. Last locality was Puerto Morelos, which is in Quintana Roo state at 20°87'N, 86°87'W, in front of the Research Center of Instituto de Ciencias del Mar y Limnología, the collection of algal material was in the reef near the research facility.

MATERIALS AND METHODS

The samples for this study were manually collected from the seven localities described above, (from the Gulf of Mexico and Mexican Caribbean sea) during May 1996 to October 2001 (figure 1), were classified by genus and transported frozen to the laboratory using solid CO₂. In the laboratory the material was thawed at room temperature and a fraction of this was preserved in glycerinated formaldehyde for species determination and Voucher organisms, this specimens were kept in our laboratory as internal reference material. Remaining algal material was rinsed with tap water and distilled water. Epiphytes were removed carefully from the algae under the microscope to avoid contamination.

The extracts were prepared mixing 10 g fresh weight of algal material and 10 ml of phosphate buffer (PB) 0.1 M, pH 7.2. The mixture was homogenized in a Waring blender, centrifuged at 1000 x g for 15 minutes and supernatant filtered through 0.22 µm using a Millipore® system. Filtered solution was stored at -30 °C until used.

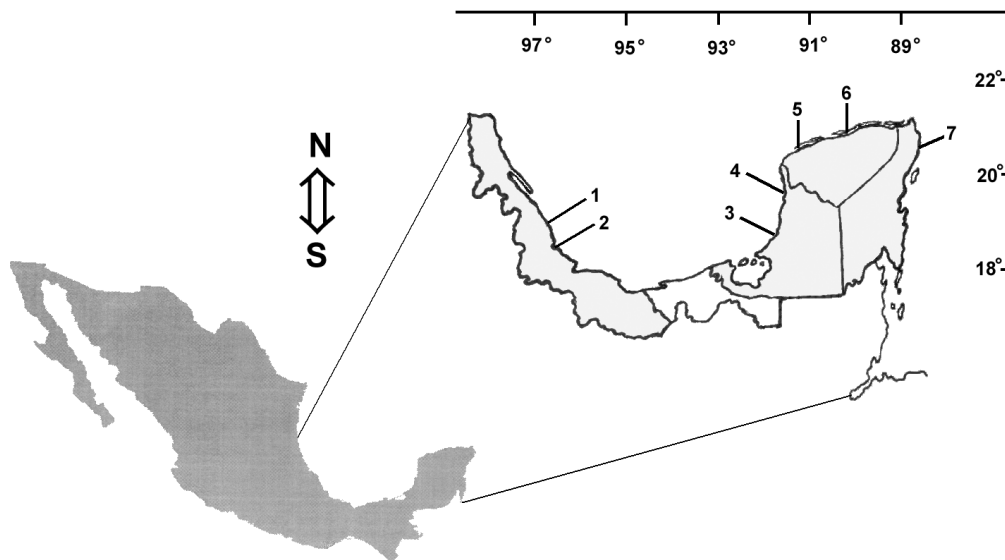


Figure 1. Localities of collection: 1. La Mancha (Veracruz), 2. Costa de Oro (Veracruz), 3. Champoton (Campeche), 4. Tenabo (Campeche) 5. Celestun (Yucatán), 6. Chelem (Yucatán), 7. Puerto Morelos Reef (Quintana Roo).

Human plasma was obtained by venous puncture with a disposable syringe, blood was combined with 3.0 % sodium citrate in PB (10:1 v/v). Blood was centrifuged immediately at 1000 x g and the plasma of all donors was pooled and stored at -50°C until used (Deacon-Smith *et al.*, 1985).

Standardized clotting tests were performed, thrombin time (Pitney & Dacie, 1953) using 4 U.I. ml^{-1} of thrombin and prothrombin time (Quick, 1945) with thromboplastin, both from Sigma Chemical Co., as described in Deacon-Smith *et al.* (1985). All tests were performed in duplicate and the average recorded (if a discrepancy $>5\%$ of times mean was obtained the test was repeated). As anticoagulant time reference 6.6 $\mu\text{g ml}^{-1}$ of heparin was used, we also included a control with PB. We considered an algal extract with anticoagulant activity similar to heparin when this impede the clot formation during 10 minutes or more.

RESULTS

Results for algal extracts in both anticoagulant tests are in table 1. Forty nine species were tested, 14 from Chlorophyta, 8 from Phaeophyta and 27 belonging to Rhodophyta. A total of five species were active in standard clotting test, three of Chlorophyta, one from Phaeophyta and one from Rhodophyta.

The extracts of *Anadyomene stellata* (Wulfen) C. Agardh, *Caulerpa cupressoides* (West in Vahl) C. Agardh, (Chlorophyta), *Lobophora variegata* (Lamouroux) Womersley

(Phaeophyta) and *Liagora farinosa* Lamouroux (Rhodophyta) were as potent as heparin impeding the clot formation in both thrombin and prothrombin time tests.

One of the extracts of *C. paspaloides* (Bory de Saint-Vincent) Greville (Chlorophyta) collected in July of 1997 was active only in thrombin time test, same results were obtained with the sample of *C. cupressoides* collected in June of 1995 and with *L. variegata* of April of 2000 all this species were collected in Puerto Morelos, Quintana Roo. The extract of *L. variegata* was only active in prothrombin coagulation test, this sample was obtained in November of 1996 in Puerto Morelos.

A delay in clot formation less than two minutes was observed in prothrombin time test for this species from Chlorophyta: *C. sertularioides* f. *farlowi*, *Dasycladus vermicularis* (Scopoli) Krasser, and *Galaxaura obtusata* (Ellis et Solander) Lamouroux (Rhodophyta). Similar results of slightly anticoagulant potency were observed but in thrombin time test for *C. cupressoides* collected in July of 1997, one extract of *C. paspaloides* from Puerto Morelos in May of 1996, *Cladophora prolifera* from Veracruz collected in July of 2001, *Bryocladia cuspidata* (J. Agardh) De Toni from Veracruz collected in October of 2001 and the sample of *G. obtusata* obtained in October 2001 from the same locality.

The extracts of *Halimeda incrasata* (Ellis) Lamouroux, *Penicillus capitatus* Lamouroux (Chlorophyta) and *Gratelopia filicina* (Lamouroux) C. Agardh (Rhodophyta) retarded the coagulation process of human plasma in both anticoagulant

Table 1. Time of clot formation in anticoagulant tests of macroalgae extracts.

	Date of Collection	Thrombin Time minutes	Prothrombin Time minutes
Chlorophyta			
<i>Anadyomene stellata</i> (Wulfen) C. Agardh	⁵ June 1998	>10	>10
<i>Avrainvillea longicaulis</i> (Kützinger) G. Murray et Boodle	⁷ July 1997	0.6	0.5
	⁷ May 1996	0.5	0.6
<i>Caulerpa cupressoides</i> (West in Vahl) C. Agardh	⁷ June 1995	>10	2.3
	⁷ May 1996	0.8	1.2
	⁶ May 1997	>10	>10
	⁷ July 1997	1.2	0.7
<i>Caulerpa paspaloides</i> (Bory de Saint-Vincent) Greville	⁷ May 1996	2.2	0.6
	⁷ July 1997	>10	0.7
<i>Caulerpa sertularioides</i> (S. G. Gmelin) M. Howe	² October 2001	0.5	0.5
<i>Caulerpa sertularioides</i> f. <i>farlowii</i> (Weber van bosee) Børgesen	¹ April 2001	0.3	1.0
<i>Cladophora prolifera</i> (Roth) Kützinger	¹ October 2000	0.3	0.3
	¹ July 2001	1.3	0.4
	² October 2001	0.6	0.3
<i>Codium simplex</i> Ined.	² April 2001	0.7	0.4
<i>Cymopolia barbata</i> (Linnaeus) Lamouroux	² July 2000	0.4	0.4
	² October 2000	0.4	0.2
	² April 2001	0.6	0.3
<i>Dasycladus vermicularis</i> (Scopoli) Krasser	⁷ July 1997	0.7	1.1
<i>Halimeda incrassata</i> (Ellis) Lamouroux	⁵ June 1998	1.2	1.3
<i>Penicillus capitatus</i> Lamouroux	⁷ May 1996	2.4	1.4
	⁷ July 1997	1.4	1.5
<i>Ulva fasciata</i> Delile	² October 2001	0.4	0.3
<i>Ulva lactuca</i> Linnaeus	¹ July 2000	0.6	0.2
Phaeophyta			
<i>Colpomenia sinuosa</i> (Mertens ex Roth) Derbès <i>et</i> Solier	¹ April 2001	0.4	0.3
	¹ July 2000	0.8	0.6
<i>Dictyota cervicornis</i> Kützinger	⁷ May 1996	0.4	0.3
<i>Dictyota linearis</i> (C. Agardh) Greville	⁴ May 1997	0.4	0.5
<i>Lobophora variegata</i> (Lamouroux) Womersley	⁷ May 1996	>10	>10
	⁷ November 1996	0.7	>10
	⁷ April 2000	>10	6.4
<i>Padina gymnospora</i> Kützinger	² April 2001	0.7	0.5
	April 2001	0.3	0.5
<i>Sargassum hystrix</i> var. <i>buxifolium</i> Chauvin in J. Agardh	¹ October 2000	0.4	0.3
<i>Sargassum vulgare</i> C. Agardh	¹ July 2000	0.4	0.4
<i>Spatoglossum schoederi</i> (C. Agardh) Kützinger	¹ April 2001	0.4	0.5

Table 1. Continuation

	Date of Collection	Thrombin Time minutes	Prothrombin Time minutes
Rhodophyta			
<i>Acanthophora spicifera</i> (Vahl) Børgesen	⁷ June 1995	0.6	0.4
	⁷ May 1996	0.4	0.6
	⁷ April 2001	0.6	0.5
	² October 2001	0.5	0.4
<i>Amansia multifida</i> Lamouroux	¹ July 2000	1.1	0.5
<i>Bryocladia cuspidata</i> (J. Agardh) De Toni	² October 2001	0.6	0.4
<i>Bryothamnion seaforthii</i> (Turner) Kützing	² April 2001	0.3	0.3
<i>Bryothamnion triquetrum</i> (S. G. Gmelin) M. Howe	⁵ May 1997	0.3	0.4
<i>Centroceras clavulatum</i> (C. Agardh in Kunth) Montagne in Durieu de Maisonneuve	² April 2001	0.2	0.2
	¹ October 2001	0.4	0.5
<i>Chondria cnicophylla</i> (Melville) De Toni	⁷ June 1995	0.5	0.3
<i>Chondria littoralis</i> Harvey	⁷ May 1996	0.4	0.3
	⁴ May 1997	0.4	0.5
<i>Chondrophycus gemmifeus</i> (Harvey) Garbay et Harper	³ July 1997	0.3	0.3
<i>Chondrophycus papillosus</i> (C. Agardh) Garbay et Harper	⁷ May 1996	0.3	0.4
<i>Chondrophycus poiteaui</i> (Lamouroux) Nam	¹ July 2000	0.3	0.3
	² April 2001	0.5	0.3
<i>Digenea simplex</i> (Wulfen) C. Agardh	⁷ May 1996	0.3	0.4
	⁷ July 1997	0.4	0.4
	¹ April 2001	0.3	0.2
<i>Galaxaura obtusata</i> (Ellis et Solander) Lamouroux	² April 2001	0.5	1.0
	² October 2001	1.2	0.4
<i>Gracilaria caudata</i> J. Agardh	¹ May 1999	0.5	0.3
	¹ October 2000	0.2	0.2
	² October 2000	0.3	0.2
	² April 2001	0.3	0.3
	¹ October 2001	0.3	0.3
	² October 2001	0.4	0.3
<i>Gracilaria cervicornis</i> (Turner) J. Agardh	² November 1994	0.3	0.3
	¹ July 2000	3.0	0.5
	¹ October 2000	0.6	0.4
	² April 2001	0.4	0.2
	¹ April 2001	0.3	0.3
	² October 2001	0.4	0.3
<i>Gracilaria mammillaris</i> (Montagne) M. Howe	² April 2001	0.3	0.2
	² October 2001	0.3	0.3
<i>Gracilariopsis lemaneiformis</i> (Bory de Saint-Vincent) E. Y. Dawson	² October 2000	0.3	0.2
	² October 2001	0.4	0.3

Table 1. Continuation

	Date of Collection	Thrombin Time minutes	Prothrombin Time minutes
Rhodophyta			
<i>Grateloupia filicina</i> (Lamouroux) C. Agardh	¹ July 2000	2.3	2.0
<i>Hydropuntia cornea</i> (J. Agardh) Wynne	⁷ June 1995	0.3	0.3
	⁷ May 1996	0.3	0.4
<i>Hypnea musciformis</i> (Wulfen in Jacquin)	¹ July 2000	0.5	0.3
	¹ April 2001	0.3	0.3
	² April 2001	0.2	0.3
<i>Hypnea spinella</i> (C. Agardh) Kützing	² October 2001	0.5	0.3
	¹ April 2001	0.2	0.3
<i>Laurencia intricata</i> Lamouroux	⁷ May 1996	0.3	0.4
<i>Laurencia obtusa</i> (Hudson) Lamouroux	⁷ July 1997	0.3	0.3
	³ July 1997	0.3	0.3
	² October 2001	0.5	0.3
<i>Liagora ceranoides</i> (Lamouroux) K. Fan	¹ April 2001	0.4	0.4
<i>Liagora dendroidea</i> (P. Crouan et H. Crouan in Mazé et Schramm)	⁷ May 1996	0.6	0.5
<i>Liagora farinosa</i> Lamouroux	⁷ July 1997	> 10	> 10
<i>Prionitis pterocladina</i> Wynne	¹ July 2000	0.8	0.6
Heparin		> 10	> 10

Superscripts numbers refers to the localities of collection: 1. La Mancha (Veracruz), 2. Costa de Oro (Veracruz), 3. Champoton (Campeche), 4. Tenabo (Campeche), 5. Celestun (Yucatán), 6. Chelem (Yucatán), 7. Puerto Morelos Reef (Quintana Roo).

tests for less than two minutes period and more than one minute.

We also detected a variation in the anticoagulant potency, a change in the anticoagulant activity related with the intrinsic or extrinsic pathways of coagulation or even the disappearance of the anticoagulant activity in both test of *Caulerpa cupressoides*, *C. paspaloides* and *Lobophora variegata* for the samples collected in different dates and localities.

DISCUSSION

Blood coagulation comprises a series of enzymatic reactions whereby an inactive proenzyme is converted to the active enzymatic form which, in turn, converts another proenzyme to its active form, next step is the conversion of fibrinogen to fibrin and its polymerization to cross-linked form and therefore the clot formation (Hougie & Baugh, 1980).

Extrinsic pathway of coagulation is considered when factors of plasma interact with tissular prothrombin and in-

trinsic pathway when factors of plasma interacts with phospholipids, both pathways converges in the conversion of fibrinogen to fibrin by thrombin (Girard & Broze, 1993).

We considered that extracts who acted on both pathways of clot formation, affect the cascade of coagulation in the point of convergence, that is in transformation of fibrinogen to fibrin. We can concluded that some substances present in the algal extract can act on any of the enzyme or substrate inhibiting the transformation of prothrombin to thrombin or in the activation process of fibrinogen to fibrin. Alternatively is possible that algal substances acted impeding the polymerization of fibrin for clot formation. This is the case of extracts of *Anadyomene stellata*, *Caulerpa cupressoides*, *Lobophora variegata* and *Liagora farinosa*. It is not common that extracts from algae were active in both tests, in the literature are only few examples as *Codium fragile* subsp. *tomentosoides* (Goor) Silva (Deacon-Smith *et al.*, 1985), *Halimeda discoidea* Decaisne (De Lara-Issasi & Alvarez-Hernández, 1995), the sulfated polysaccharides of some species from Chlorophyta, *Caulerpa* and *Monostroma* (Hayakawa *et*

al., 2000) and the fibrinolytic enzymes isolated from several species from *Codium*, also from Chlorophyta (Matsubara *et al.*, 1998, 1999, 2000a, 2000b). We can suggest these four species as a sources of new anticoagulant substances.

This work is the first one to report on the anticoagulant activity of *Anadyomene stellata*, *Lobophora variegata* and *Liagora farinosa*, however, these species can synthesized new and different anticoagulant molecules, for instance, *Caulerpa cupressoides* was reported previously only with activity in extrinsic coagulation pathway (De Lara-Isassi & Alvarez-Hernández, 1999) in contrast to present work where was active in both tests.

The reason for using a general extraction method of molecules (extracts from algae in saline or PB) as Deacon-Smith and Rogers (1982) and Deacon-Smith *et al.* (1985) did, is that we can obtained a wide pool of substances. Supporting the use of this method is the evidence that the results of Deacon *et al.* (1985) with this method of extraction guided Matsubara *et al.* (1998) and his group to do research related with the possibility of use of several species of *Codium* as a potential anticoagulant source and crystallized in the isolation and characterization of enzymes from this algae with application as alternative anticoagulant molecules. Our results showed four species with similar activity susceptible of further research for isolation and characterizations of active substances.

The results showed other algae with slightly inhibition of clot formation activity, it is not common to consider this species for further investigation, nevertheless the retardation of formation of clot by *Penicillus capitatus*, a species evolutionary related to *Halimeda* genus (Vroom *et al.*, 1998; Graham & Wilcox, 2000) which showed presence of molecules capable of impeding clot formation as heparin, did drive us to consider the *Penicillus* genus as capable of synthesized molecules with anticoagulant activity and therefore a candidate for further studies on this subject.

It is relevant to point out the dependence of anticoagulant potency due to the locality where algae grows, also the date of collection and thus, the climate season is other factor that could influence the synthesis of compounds, particularly in the brown alga *Lobophora variegata* we observed the double anticoagulant action in sample collected in May of 1996, but six months later (November 1996), same specie only was active in the prothrombin time test. Finally, sample collected in April of 2000 change the anticoagulant activity been active only in the thrombin time test. Similar results were detected in *Caulerpa cupressoides* extracts collected in three occasions, in this case even the total absence of activity was detected in one sample.

These variation has been suggested as been influenced by geographical factors such as climate and locality and has been probed with primary metabolites as lectins by Ingram (1985) and Fabregas *et al.* (1985). Also this variation has been recorded with secondary metabolites with antibacterial, anti-fungic and toxic activities (Fenical & Paul, 1984; Ballesteros *et al.*, 1992; Moreau *et al.*, 1988; Padmakumar & Ayyakkannu, 1997). However we consider more research has to be done, focused to this topic, in order to probe this hypothesis.

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