New Record of *Pseudodistoma arborescens* Millar, 1967a (Tunicata, Ascidiacea) in the Red Sea with Some Notes on the Cytotoxic Activity of the Alkaloid Content on MCF-7 Cell Lines

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Abstract. The ascidian, *Pseudodistoma arborescens*, is reported for the first time in the Saudi Arabian water of the Red Sea. It is found to be an important component of the coral reefs at a water depth of 6-13 meter of Janabiyat Island, Doga area (19° 46' 09.14" N, 40° 34' 16.33" E) in December 2014. The species is redescribed and briefly its cytotoxic activity was tested. The anti-proliferative activity of the alkaloid content of *Pseudodistoma arborescens* has been evaluated against the MCF-7 cell line; showed IC_{50} value of 0.70 ± 0.01 mg/L.

Keywords: Ascidian; coral reefs; *Pseudodistoma arborescens*; new record; cytotoxicity; Red Sea.

Introduction

Ascidians or sea squirts (Phylum: Chordata, Class: Ascidiacea), are the largest and most diverse class of the subphylum Tunicata. They include nearly 3,000 accepted species recorded from marine habitats (Cameron *et al.*, 2000; Kott, 2005; Monniot *et al.*, 1991; Shenkar *et al.*, 2012). All ascidians are sessile distributed from shallow coastal seas to deep waters of all world oceans (Millar, 1971, 1988; Monniot *et al.*, 1991; Monniot, 1993, 1998; Young and Vázquez, 1997; Sanamyan and Sanamyan, 1998, 2002; Lambert, 2005; Kott, 2008a, b; Primo and Vázquez, 2007; Monniot *et al.*, 2011). Most of ascidians are filter feeders (feed on bacteria, phytoplankton and particulate matter) using a mucous net lining inside the branchial sac (Bone *et al.*, 2003). While some deep-water species however are carnivorous (Monniot and Monniot, 1990; Lescano *et al.*, 2011; Tatián *et al.*, 2011;

Mecho *et al.*, 2014). All ascidians are hermaphrodites and they generally avoid self-fertilization by developing only eggs or only sperm at any point of time (Newlon *et al.*, 2003).

Class Ascidiacea is divided into three orders based on the structure of the adult branchial sac (Lahille, 1886): Aplousobranchia (with simple slits), Phlebobranchia (with vessels) and Stolidobranchia (with folds). In the Red Sea, 73 ascidian species belonging to 13 families were recorded (Shenkar, 2012). The majority of these Red Sea species are distributes in the Indo-Pacific, Mediterranean Sea and Atlantic Ocean (Shenkar, 2012). Only 16 % of these species are endemic to the Red Sea which is considerably low compared to other regions. The percentage of endemism were: 43 % in New Zealand, 45 % South Africa, 44 % in the Antarctic region (Primo and Vazquez 2007, 2009), and 40.9 % in the

Eastern Mediterranean (Koukouras *et al.*, 1995). In the Red Sea, the family Styelidae are the most diversified group which is the second most diverse family of the class Ascidiacea (Shenkar and Swalla, 2011). Moreover, the genus *Polycarpa*, which is well known for its diversity (Monniot *et al.*, 1991) is represented with the highest number of species. During fieldwork assessing the coral reef status in Doga area, Saudi Arabian waters of the central Red Sea, an unrecorded species of *Pseudodistoma* was found. The general morphological characteristic of this species were close to those of *P. arborescens* originally described from Madagascar by Millar (1967).

The marine biota is characterized by ramification of the living organisms which live in the harsh perimeter (Blunt *et al.*, 2011). Marine invertebrates, suffering from the absence of natural defenses (e.g. spines) led to the production of defense metabolites (Grabowski and Schneider, 2007). Cancer is a dreadful disease that is the direct cause of almost 14.5% of all death cases all over the world, which is found to be increased with the aging of the population (Avendaño and Menéndez, 2015; Al-Alwan *et al.*, 2011). This paper records the first occurrence and redescription of *P. arborescens* from the Red Sea with some notes on its cytotoxic activity.

Materials and Methods

Specimens were collected from Janabiyat Island, Doga area, central Red Sea (19° 46' 09.14" N, 40° 34' 16.33"E) in December 29, 2014 by SCUBA diving from 6 to 13 m depth. The samples were submerged in fresh seawater for one hour after collection and then a small menthol crystal (*ca.* 2 mg) was placed on the specimen, and kept in a fridge (4°C) for 3 to 5 hours. Subsequently, sufficient concentrated formaldehyde (37%) was added to give an approximately 10% formalin solution as final

concentration. Preserved ascidian specimen was then washed with fresh water to remove formalin before dissection. Organs were stained with Mayer's hemalum stain (Humason, 1979). Specimen was dehydrated with ethyl and butyl alcohols and then mounted on a slide using canada balsam. Specimen was then examined using bright-field and differential interference microscopes (Nikon DM 6000). Drawings and measurements were made with a camera lucida attached to the microscope and an ocular micrometer respectively.

Extraction, isolation and cytotoxicity assays

The air-dried *P. arborescens* (150 g) was extracted with MeOH for 24 hours (2.5 L X 3 batches, room temperature). The extract was then concentrated in vacuo (100 ml). After that, H₂O (300 ml) was added, and the mixture was extracted with CHCl₃. The organic layer was concentrated to dryness. The residue (2.1 g) was dissolved in CHCl₃ and extracted with 10% HCl until a negative Mayer test was obtained. Removal of CHCl₃ from the pooled organic fraction gave an inactive extract (1.65 g). The acidic medium was basified with NH₄OH and partitioned with CHCl₃. Concentration of the pooled CHCl₃ fractions gave the active extract (crude alkaloids, 0.3 g). The alkaloid content was tested against MCF-7 cells. Sulforhodamine assay was performed as formerly described by Tolba *et al.* (2013). By using 0.25% Trypsin-EDTA, exponentially growing cells were collected and coated in 96-well plates at 1000-2000 cells/well. The cells were laid bare for 48 hours to the compound under investigation, thereafter, under dark incubation for 4 hours with SRB solution, which are then dissolved in DMSO. Measurement of color intensity was done at 750 nm. The equation % Cell viability = $\frac{E_{max} \times (1-M) + R}{E_{max}}$ is applied to estimate the dose response model of each metabolite, where R is

the resistance fraction (unaffected portion) and $M = [D]^m / [K_d]^m + [D]^m \cdot K_d$: compound concentration that causes a one-half depress of the maximum inhibition rate, D: drug concentration used, and $E_{max} = 100 - R$.

Results and Discussion

Pseudodistoma arborescens Millar, 1967

Synonyms *Pseudodistoma arborescens* Millar, 1967, p. 359-361, fig. 1. Gaill, 1972, p. 45, fig. 3. Monniot, F., 1987, p. 508-511, fig. 4. Monniot, C. *et al.*, 1991, p. 1-246. Kott, 1992, p. 424.

Material Examined

Janabiyat Island, Doga (19° 46' 09.14" N, 40° 34' 16.33" E), central Red Sea. Saudi Arabia, 29 December 2014. Depth 6-13 m.

Description

Colonies, up to 5-7.5 cm high (Figs. 1 and 2), consists of numerous lobes fused together at their lower ends with a diameter of 2 mm, gradually increased to about 3.7 mm below the heads. Living specimen dark violet (or deeply colored blue) and pigment slowly dissolve in preserved materials. These lobes usually divided into 2 to 4 branches, each branch with one, two or three terminal heads. The head up to 10 mm in length and less than 10 mm in width. Heads are mostly soft compared to the stalks and a little bit cloudy or opaque. On these heads, there are superficial round apertures that mark the contracted zooid position (Figure 2). This superficial layer becomes damaged in time after preservation. Zooids are long and narrow divided into thorax, abdomen and post-abdomen with lengths ranged between 0.7-1 mm, 1-2 mm and 4-6 mm respectively (Figure 3A). The atrial siphon located short distance behind the oral siphon. Both apertures (siphons) have six evenly rounded lobes (Figure 3 B). In

contracted individuals, the oral tentacles could not be clearly observed and their number was not possible to determine. Seven to 10 narrow longitudinal muscular bands are along each side of the thorax, and condense into a diffuse band that turns along each side of the abdomen to its posterior end (Fig. 3 B). There are three rows of branchial stigmata, each with about 11-13 stigmata. In less contracted zooids the oesophagus is moderately long and narrow leads to a small ovoid, smooth-walled stomach. The wall of stomach is indistinctly divided into four shallow folds. A short duodenal region opens into a roundish posterior stomach in the pole of the gut loop. The lower end of the ascending intestine has two small pouches. Gonads are restricted to the posterior half of the post-abdomen (Fig. 3 C). The ovary of zooids in the female phase is anterior to the position of the testis of zooids in the male phase. Ovary is represented by a compact group of oocytes. Testis consists of a longitudinal series of globular mass of follicles arranged in two rows along the posterior abdomen. The long straight sperm duct passes forward to the base of the thorax (Fig. 3 C). Embryo were in various developmental stages and are located in the anterior part of the oviduct within the abdomen. The larval trunk is 0.6-0.8 long, and has a rudimentary siphon, branchial sac with three rows of stigmata, sensory vesicle containing ocellus and otolith and rudimentary duct (Fig. 3 D). The genus *Pseudodistoma* Michaelsen, 1924 is represented by two species previously located in the family Polyclinidae Milne Edwards, 1842 (in the subfamily Euherdmaniinae) as defined by Kott (1992). The genus *Pseudodistoma* Michaelsen, 1924, *Citoclinum* Monniot, F. and Millar, 1988 and *Anadistoma* Kott, 1992 represent those species in which the zooids open separately to the surface of the colony and zooids have three rows of stigmata.

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The genus *Citoclinum* is represented by a single species *C. laboutei* Monniot, F. and Millar 1998 from New Caledonia. The genus *Anadistomais* represented by a single species *A. attenuatum*, Kott, 1992 from Australia (Kott, 1992). The zooids of the species in the genus *Pseudodistoma* are very similar. The zooids have strong musculature and zooids are often contracted to such an extent that characteristics are not visible.

Fig. 1. Underwater photo of *Pseudodistoma arborescens* from the Red Sea.

Fig. 2. Close up for the *Pseudodistoma arborescens* from the Red Sea.

Fig. 3. *Pseudodistoma arborescens* from the Red Sea: a) zooid, in female phase, b) thorax, c) end of the post-abdomen of zooid in male phase showing testis, d) larva ready for settlement.

As a result of the similarity of the zooids of the species in this genus and the contractibility of the zooids (Kott 1992) noted that the number of ampullae present in the larvae, the position of the gonads as well as the shape of the colony constitute the principle characteristics

that distinguish a species in this genus. Characters generally used in the literature to distinguish between the species in the genus *Pseudodistoma* are compared for the thirty-one species known worldwide are: Shape of the colony, number of stigmata per half row,

caeca, position of gonads, position embryo's are incubated, larval tail, number of larvae incubated and ampullae. The genus *Pseudodistoma* is represented by thirty-one species worldwide. Four species have been described for the Mediterranean Sea: *P. crucigaster*, *P. cyrnusense*, *P. obscurum* and *P. arnbacki* (Gaill, 1972; Pérès, 1952; Pérès, 1959). A large number of species have been described for Australia and the Pacific Ocean with eleven species occurring in Australian waters, two from the Federated States of Micronesia, one from and shared by Fiji and French Polynesia, one from Papua New Guinea and the Philippines. Three species occur off the Japanese coast, and one off Korea. The Indian Ocean is well represented by the genus *Pseudodistoma*. A single species, *P. arborescens*, is known from the coastal waters of Madagascar (Millar, 1967), and one species, *P. mauritiana*, for Mauritius (Vasseur, 1967). Two species were described from the South African coast, *P. africanum* Millar, 1954b (Millar, 1954b, 1962; Monniot, C. *et al.*, 2001) and *P. delicatum* Monniot, F., 2001 (Monniot, *et al.*, 2001). Eight species are characterized by the absence of sand particles within the test or on the colony surface. These colonies are generally cushion-shaped, of which the surface may be produced into lobes. Colonies that have a distinctive stalk and heads may have sand particles distributed throughout the interior test, as is the case for *P. africanum* Millar, 1954b (Millar, 1954b) or the sand may be restricted to the basal stalk of the colony. The *Pseudodistoma* species with a small number of stigmata per half row are *Pseudodistoma acutum* Kott, 1992, *P. antioja* Tokioka, 1949b and *P. opacum* (Brewin, 1950) which have less than ten branchial stigmata per half row while *P. kanoko* Tokioka and Nishikawa, 1975 and *P. poculum* Monniot, F. and Monniot, C. 1996 have fifteen or less stigmata per half row. *Pseudodistoma arborescens*, Millar, 1967, *P.*

arnback Pérès, 1959, *P. brieni* Pérès, 1949, *P. cyrnusense* Pérès, 1952, *P. mauritiana* Vasseur, 1967 and *P. obscurum* Pérès, 1959 have ten to fifteen stigmata per half row. The number of ampullae present around the anteriorly located adhesive organs varies from species to species. *Pseudodistoma delicatum* Monniot, F. 2001 (Monniot, C. *et al.*, 2001) described from South Africa has no ampullae present as is the case in some *P. oriens* larvae from Australia (Kott, 1992). *Pseudodistoma saxicavum* Gaill, 1972 has four ampullae, *P. crucigaster* Gaill, 1972 has four to six ampullae, *P. gracilum* Kott, 1992 and *P. fragile* Tokioka, 1958 has six ampullae present in the anterior part of the larvae. The remaining species, of which the larval structure is known, have eight ampullae which are generally arranged in four pairs on each side along the lateral line.

Ecology and Distribution

The percentage of coral cover was estimated using line transect method. The mean ($\pm$ SD) of total hard cover at 2.5 meters was 72.74% $\pm$ 23.18% (massive = 8.97%; arborescent = 61.97%). While at 7.5 meters the mean total hard coral was 18.95% $\pm$ 17.95% (massive = 8.57%; arborescent = 9.62%). Regarding distribution, this species is first described from Madagascar (Millar, 1967). Subsequently, it was recorded from New Caledonia (Monniot, F., 1987) and Philippines (Kott, 1992). This is the first record of this species in the Red Sea.

Cytotoxicity Assays and Viability Analysis

The anti-proliferative activity of the alkaloid extract has been evaluated against the MCF-7 cell line and it showed the effect with IC₅₀ value 0.70 $\pm$ 0.01 mg.

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تسجيل نوع جديد من الغلاليات (*Pseudodistoma arborescens*) في البحر الأحمر مع بعض الملاحظات عن تأثير المستخلص القلويدي تجاه خلايا سرطان الثدي

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المستخلص. من خلال هذا البحث يتم تسجيل نوع لأول مره في البحر الأحمر في المياه السعودية بمنطقة دوقه حيث إن هذا النوع تواجد بصورة كثيفة في عمق ما بين ٦ و ١٣ متر في جزيرة الجانيبات (١٤, ٩" ٤٥' ١٩° شمالاً و ٣٣, ١٦" ٣٤' ٤٠° شرقاً) خلال شهر ديسمبر ٢٠١٤م. تم وصف الشكل الظاهري لهذا النوع ووصف البرقة الخاصة به. وكذلك تم اختبار النشاط السام للمستخلص القلويدى لهذا النوع، حيث تم تقييم النشاط على الخلايا MCF-7 وقد أوضحت النتائج أن التأثير السام (LC_{50}) كان $0.07 \pm$ ٠,٠١ ميللجرام لكل لتر.

الكلمات الدالة: الغلاليات - الحيد المرجاني - *Pseudodistom arborescens* - تسجيل جديد - قياس السمية - سرطان الثدي - البحر الأحمر.