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Ecology and bioactivity of the sea hare *Notarchus indicus* schweigger 1820, from Lake Timsah, Suez Canal, Egypt

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ABSTRACT

Molluscs (bivalves, gastropods, cephalopods) along Lake Timsah have received much scientific concern during past decades; however, opithobranchs (sea hares) did not receive any attention yet. This investigation represents the first study, so far dealing with the ecology of the sea hare *Notarchus indicus* along the lake. Sea hare usually moves in depressions or ripples marks (wave marks) of sandy bottom of Lake Timsah forming chains of 3 to 5 individuals. Their densities ranged from 15 to 20 individuals/ transect (1mx10m). The size frequency distribution reveals that its individuals grow up to 7.25cm length with a model size of 2.75 – 3.50cm, representing 37% of its population. It usually feeds on organic matter and blue greens accumulated in depressions of sandy substrates and represent up to 5.2% of sediment dry weight. The water is typically saline (43‰), well aerated with normal alkaline pH. The sea hare usually secretes pink ink when attacked or disturbed. Investigation of the biological activity of the sea hare *Notarchus indicus*; and its ink showed that methanol extracts of large, medium and small organisms and the ink have a moderate bioactivity against three pathogenic bacterial strains, namely the Gram-positive bacterium *Staphylococcus aureus* NRRL B-767, and the Gram-negative bacteria *Klebsiella pneumoniae* NRRL B-14232 and *Escherichia coli* NRRL B-3704. Moreover, they have moderate bioactivity against *Candida albicans* NRRL Y-12983. Thus, such ink shows a most prominent antimicrobial activity. Moreover, the aqueous extract of large organisms as well as the ink showed strong cytotoxic activity against the nauplii of the brine shrimp *Artemia salina*. Ecologically, sea hares usually use the ink and other bioactive substances to protect itself from predation.

Keywords: Ecology, bioactivity, sea hare, *Notarchus indicus*, Lake Timsah, Suez Canal.

INTRODUCTION

The ecology and biology of molluscs (bivalves, gastropods and cephalopods) along Lake Timsah have received much attention during the past decades (Fouda and Abou Zaid, 1990; Abou Zied, 1991, Mohammed, 1992;

Gabr *et al.*, 1998, 1999a, 1999b) however, sea hares which belong to subclass Opisthobranchia were so far ignored. Unlike most molluscs, sea hares lack a shell for protection from attacks by predators. Instead, they rely on a variety of other anti-predation strategies such as cryptic coloration (Thompson, 1960), distasteful skin or body wall (Ambrose and Givers, 1979; Kinnel *et al.*, 1979), and the secretion of ink and opaiine (Hohnson and Willows, 1999). When a sea hare is disturbed or attacked by a predator, it usually releases ink and opaline independently from ink glands (Tritt and Byrne, 1980; Walters and Erickson, 1986; Prince *et al.*, 1998), mixed in the mantle cavity and directed toward the site of attack (Walters and Erickson, 1986; Walters *et al.*, 1993; Johnson and Willows, 1999).

Several studies have demonstrated that ink and opaline produced by sea hares can act as anti-feedant to predators such as birds, fishes, crustaceans and sea anemones (Dimattes, 1981, 1982, Walters *et al.*, 1993; Penning, 1994; Nolen *et al.*, 1995; Kicklighter *et al.*, 2005), suggesting that ink and opaline may serve as chemical defenses helping the survival of sea hares when attacked by predators. Tobach *et al.* (1965) proposed other functions for inking behavior of sea hares which included communication of information or reproductive status.

The present study aimed to highlight the ecology of the sea hare *Notarchus indicus* from Lake Timsah at different stations including temperature, salinity, oxygen content, pH of water, type of sediment, and organic content. Also, it aimed to detect the biological activity of methanol and aqueous extracts of small, medium and large organisms and the ink in order to figure out any pharmacological potential of this species.

MATERIALS AND METHODS

1. Ecological study

1.1. Ecological survey

A pilot survey was carried out early in summer 2007, and showed that the sea hare *Notarchus indicus* (Fig. 1) usually lives in shallow waters of Lake Timsah over sandy substrates. Late in summer 2007, another ecological survey was performed at seven stations along Lake Timsah (Fig. 2). Their GPS position and ecological status were mentioned in Table (1). Meanwhile, an ecological investigation was carried out in order to determine *in situ* water temperature (°C), salinity (‰), pH and oxygen concentration (mg/l) by electrodes. Samples of sediments were collected from investigated stations then dried, sieved and analyzed for particle size. Also, a sediment portion was analyzed for total organic carbon (TOC) according to Byers *et al.* (1984).

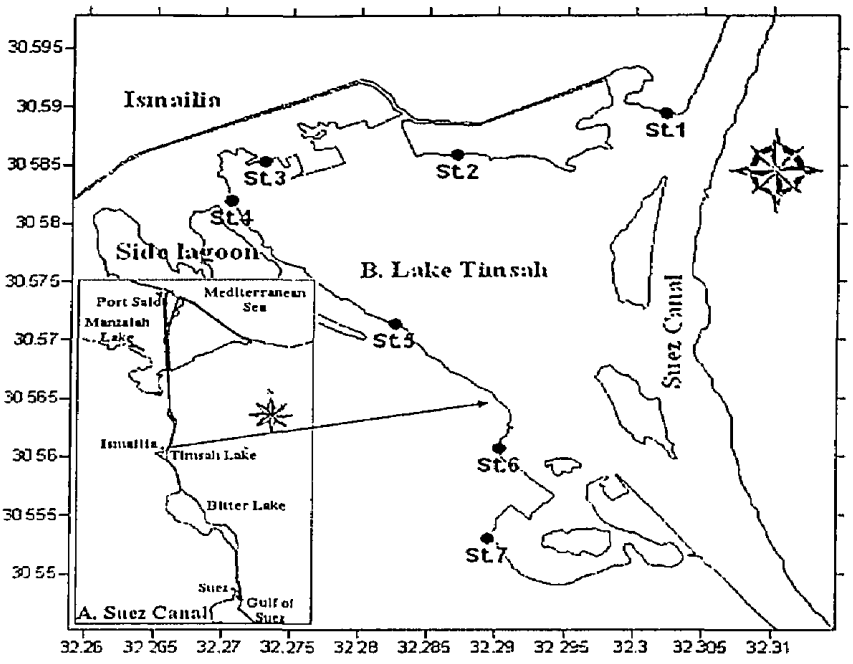

Fig. 2. Map of Suez Canal showing the surveyed stations along Lake Timsah.

Table 1. Sampling stations, ecological status and GPS position

Stations No.	Ecological status	GPS position
1	Sandy, shallow, turbid	30°35'14.31"N 32°18'16.46"E
2	Sandy, shallow, turbid	30°35'5.62"N 32°17'16.20"E
3	Sandy, shallow, turbid	30°35'6.93"N 32°16'22.59"E
4	Sandy, shallow, turbid	30°34'54.98"N 32°16'14.19"E
5	Sandy, shallow, turbid	30°34'17.00"N 32°16'57.53"E
6	Sandy, shallow, turbid	30°33'38.50"N 32°17'23.85"E
7	Sandy, shallow, turbid	30°33'10.84"N 32°17'22.14"E

To investigate the density of *Notarchus indicus* at the above stations, three belt transects (1m x 10 m) were established parallel to the shore along sand depressions or ripples marks at each site. Snorkeling was carried out along transects. Quantitative data were taken to assess the density of sea hare along various transects at each site.

1.2. Data analysis

Means and standard deviations were calculated for three replicate determinations of various parameters investigated. Analysis of variance (one-way ANOVA) test was used to evaluate the significance of differences between groups of measured parameters at different stations with a level of significance set at $p \leq 0.05$. Data were analyzed to determine size frequency distribution, and length-weight relationship of the sea hare under investigation.

2. Study of Bioactivity

2.1. Collection of the ink

Specimens of *Notarchus indicus* were collected from the Lake. The ink was collected by careful lifting individuals of the sea hare out of water and allowing excess water to drain, then gently massaging the surface of the mantle in the vicinity of the ink glands, rudimentary shell and gills. This procedure usually stimulates animals to release ink, which was drained in a small beaker. Several animals of the same size were usually de-inked at one time to provide sufficient ink for bioactivity assays. The ink was frozen at – 20°C until used.

2.2. Antimicrobial activity

Fresh tissues of small, medium or large sea hares were extracted with methanol (10 ml/ g fresh weight) for 5 min in a homogenizer, left to stand overnight, then filtered. The filtrate was evaporated to dryness in a rotary evaporator under reduced pressure at 40°C. The residue was dissolved in 3 ml methanol and tested for antimicrobial activity immediately.

Five bacterial strains were used for assaying the antimicrobial activity of sea hare. These bacteria are capable of human pathogenicity (Table 2). A *Candida* strain was also used for investigation of the anti-candidal activity (Table 1). The test strains were obtained from the United States Department of Agriculture, Northern Regional Research Laboratory (NRRL), Peoria, Illinois, U.S.A. An inoculum of each bacterial strain was suspended in 5 ml of Nutrient Broth and incubated overnight at 37°C. Yeast culture was suspended in 5 ml Sabouraud Dextrose Broth and incubated for 48-72 h at 30°C. The cultures were diluted to 1/10 with broth before use.

The disc diffusion method (Ericsson and Sherris, 1971) was used in screening crude extracts for antimicrobial activity. Bacterial strains were cultured on Nutrient Agar, while the yeast was cultured on Sabouraud Dextrose Agar. The Petri dishes were pre-seeded with 20 ml of Agar medium and 1ml of microbial culture. Paper discs (6mm) were impregnated with 20 µl of crude methanol extract and the residual methanol was evaporated at room temperature. Paper discs dipped in methanol were used as controls in each assay.

The hole-plate diffusion method was used for testing the antimicrobial activity of the ink of the sea hare. The seeded agar was poured into pre-sterilized Petri dishes and allowed to set. Wells were then punched out with sterile cork porer No. 5. Fifty µl of the ink was added to each well (6 mm diameter). Three replicates were tested for each extract and the experiment was done three times. The Petri plates were pre-incubated for 2 h at 5°C to permit maximum diffusion of the extracts into the medium. Plates for the antibacterial activity test were incubated at 30°C for 18-24 h and those for the anti-candidal activity incubated for 48-72 h at the same temperature. The diameter of inhibition zone (cm) was determined for each extract against the Gram-positive and Gram-negative bacteria or the yeast strain. Means of zones of inhibition and standard error of the means were recorded for each sample.

Table (2) Test microorganisms used for screening the antimicrobial activity of the sea hare *Notrachus indicus*.

Test organism	NRRL[a] strain	Classification
Staphylococcus aureus	NRRL B-767	Gram-positive bacteria
Klebsiella pneumoniae	NRRL B-14232	Gram- negative bacteria
Escherichia coli	NRRL B-3704	Gram- negative bacteria
Proteus vulgaris	NRRL B-123	Gram- negative bacteria
Pseudomonas aeroginosa	NRRL B-23	Gram- negative bacteria
Candida albicans	NRRL Y-12983	Yeast

[a] NRRL= Northern Regional Research Laboratory, U.S. Department of Agriculture, Peoria, Illinois, USA.

2.3. Cytotoxicity assay

Brine shrimp larvae (*Artemia salina*) are commonly used for cytotoxicity assays. These larvae are sensitive to toxic substances. The ratio between dead larvae and living larvae in comparison to the control is used to estimate the toxicity of the test solutions. The test is not only predicting cytotoxicity, but also used as a predictor of antitumor and pesticidal activity (Sanchez *et al.*, 1993).

A micro-well cytotoxicity assay using brine shrimp *Artemia salina* was used (Solis *et al.*, 1993). Brine shrimp eggs (obtained from Interpet Ltd. Dorking, England) were hatched in sea water supplemented with 6 mg/l dried yeasts, oxygenated with an aquarium pump and incubated in a warm room at 22-29 °C for 48 hours. Aqueous extracts were made by distilled water. Serial dilutions in 100 µl sea water were made into the 96 wells of microplates. Control wells with either sea water or distilled water were included in each experiment. The test was done three times. About 100 µl of the nauplii (containing 10-15 organisms) was added to each well and the covered plate incubated at 22-29 °C for 24 hours. Plates were then examined under a binocular microscope and the numbers of dead (non-motile) nauplii in each well were counted. Finally, all shrimps were sacrificed by adding 100 µl methanol to each well. After 15 minutes, the total numbers of shrimps/ well were counted. Probit analysis (Finney, 1971) was used for calculating the LC_{50} values.

RESULTS

1. Ecology of Sea hare

1.1. Physico-chemical parameters

No extreme values of temperature, salinity, pH and oxygen concentration were recorded along the different investigated stations. The parameters were more or less similar to each other at various stations.

Table 3. Distribution of temperature, salinity, pH and dissolved oxygen at the sampling stations.

Station No.	Temperature (°C)	Salinity (‰)	pH	Dissolved oxygen (mg/l)
1	28 ± 1	42 ± 1	8.1 ± 0.2	7.2 ± 0.2
2	29 ± 1	42 ± 1	8.2 ± 0.2	7.5 ± 0.3
3	29 ± 1	42 ± 2	8.3 ± 0.2	7.3 ± 0.2
4	28 ± 1	43 ± 2	7.9 ± 0.1	7.1 ± 0.2
5	28 ± 1	41 ± 2	8.0 ± 0.1	7.2 ± 0.1
6	29 ± 2	41 ± 1	7.9 ± 0.1	7.3 ± 0.1
7	29 ± 2	43 ± 2	7.9 ± 0.1	7.1 ± 0.1

Table 4. One way analysis of variance for different ecological parameters between different stations.

Source of variation	df	Sum of squares	Mean square	F. value	p. value
Temperature	4	12.833	3.208	0.721	0.590
Salinity	5	16.917	3.383	0.757	0.595
pH	7	36.283	5.183	1.412	0.280
O_2	6	7.750	1.292	0.237	0.957
Density of individuals	9	19.500	2.167	0.370	0.927
Organic matter in sediment	13	74.250	5.712	4.101	0.035

1.1.1. Temperature

Records of temperature are given in Table (3). Water temperature ranged from 28±1 °C at stations 1, 4, and 5 to 29±2 at stations 6, and 7. No significant differences (p = 0.590) were recorded between stations (Table 4).

1.1.2. Salinity, pH and dissolved oxygen

Records of salinity, pH and dissolved oxygen are given in Table (3). Measured salinity ranged from 41±2‰ at stations 1, and 5 to 43±2‰ at stations 4, and 7, with no significant difference (p= 0.595) between stations. The pH ranged from 7.9±1 at stations 4, 6, and 7 to 8.3±0.2 at station 3, with no significant difference (p= 0.280) between stations. Dissolved oxygen concentration ranged from 7.1±0.1 mgl^{-1} at station 7 to 7.3±0.3 mgl^{-1}at station 2, with no significant difference (p= 0.975) between stations (Table 4).

1.2. Sediment and total organic matter content

The shore of the studied stations was sedimentary and gently sloping. According to Went Worth scale, the sediments of the investigated stations were sandy, ranging from fine to very fine sand (Table 5). Total organic matter ranged from 3.9% at station 3 to 5.1% of sediment dry weight at station 7 (Table 5). This is due to accumulation of organic matter from blue green algae and particulate organic matter. There was low significant difference (Table 4) in organic matter content between stations (p= 0.03).

Table 5. Mechanical sediment analysis and organic matter content of different stations investigated.

Station No.	Sediment fractions						Sediment type	Organic matter
	VCS > 1000	CS 100- 500	MS 500-250	FS 250-125	VFS 125-63	S < 63		
1	3.0	7.0	20.0	63.7	6.0	0.3	FS	3.5
2	3.6	4.7	5.7	61.0	22	3.0	FS	4.5
3	0.5	1.3	4.5	40.7	48.8	4.2	FS - VFS	3.9
4	1.0	2.3	10.0	71.2	11.0	4.5	FS	4.0
5	2.0	13.0	12.0	23.0	46	4.0	VFS	5.2
6	3.0	6.0	5.7	10.3	70	5.0	VFS	4.9
7	4.0	8.0	7.0	12.0	59	10.0	VFS	5.1

VCS = Very coarse sand; CS = coarse sand; MS = median sand; FS = fine sand; VFS = very fine sand; S = silt.

1.3. Density

The sea hare recorded usually creeps in depressions at sandy sites in shallow water of Lake Timsah, beginning with 20 cm depth until 150 cm. It moves in chains of individuals ranging in number from 3 to 5 individuals each, with a mean density of 15±3 individuals/ transect at station 5 to 20±5 individuals/ transect at station 1 (Fig. 3). They feed on detritus and blue greens (which represent the main food item) accumulated by the wave action in depressions and ripples marks. No significant difference (Table 4) was recorded between density of *Notrachus indicus* at different stations (p= 0.927).

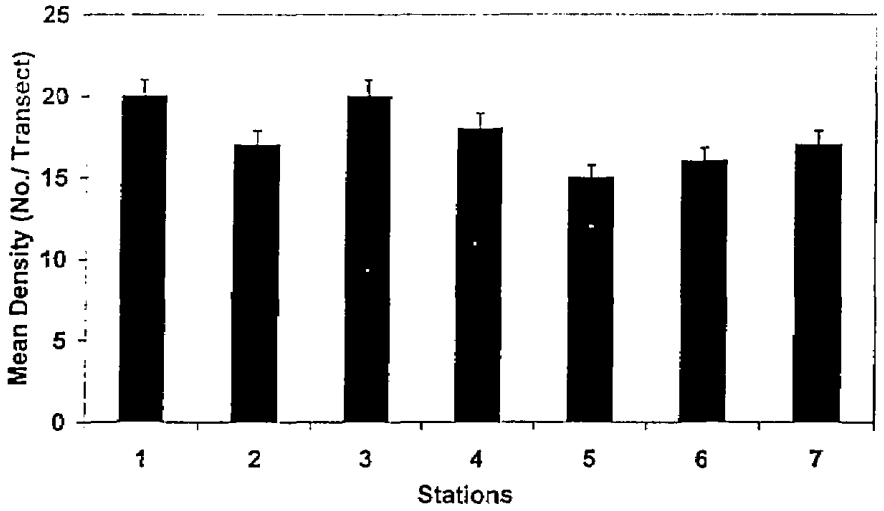

Fig. 3. Mean density (No./ 1mX10m transect) of the sea hare *Notrachus indicus* at different stations.

1.4. Size frequency distribution of *Notrachus indicus*

The individuals of *Notrachus indicus* at Lake Timsah ranged in size from 2cm to 7.25cm, with a model size of 2.75 – 3.50cm (Fig. 4) which represents about 37% of its population. The weight of recorded species ranged from 1.5g to 22.93g. The relationship between length and wet weight for *Notarchus indicus* is illustrated in figure 5. The regression formula was $Y = 0.2258X^{2.1497}$, where Y represents its weight and X

represents its length. The regression equation indicates that the body weight has a negative allometric function of length as the value of b (2.1497) was lower than 3.

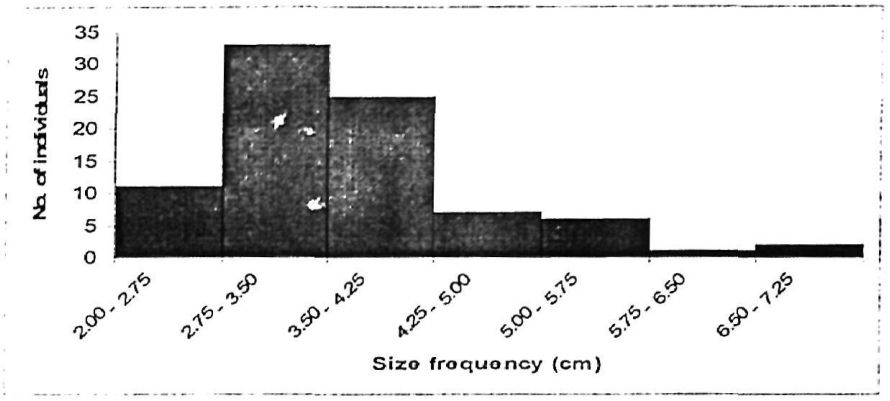

Fig. 4. Size frequency distribution of *Notarchus indicus*

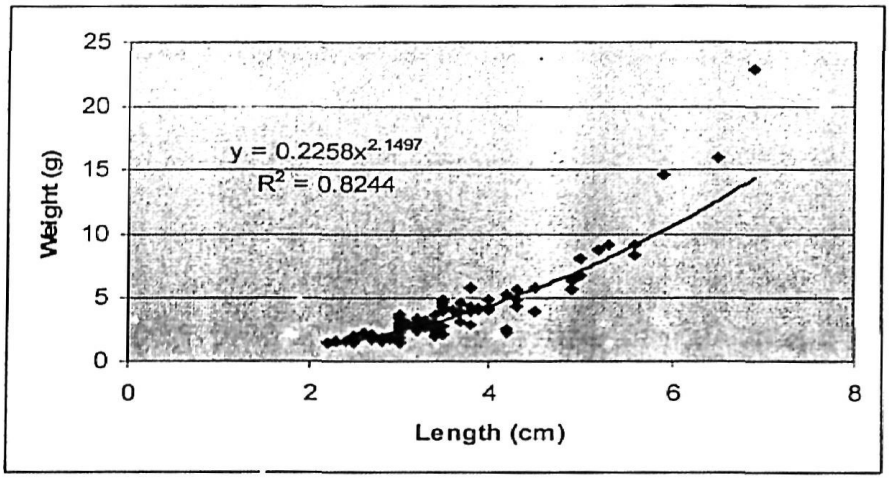

Fig. 5. The length-weight relationship of sea hare *Notarchus indicus*.

2. Bioactivity of sea hare

2.1. Antimicrobial activity

Methanol extracts of large, medium and small individuals of *Notrachus indicus* and the ink showed moderate antibacterial activity against three bacterial test strains including the Gram-positive bacteria *Staphylococcus aureus* NRRL B-767 and the two Gram-negative bacteria *Klebsiella pneumoniae* NRRL B-14232 and *Escherichia coli* NRRL B-3704 (Table 6). Moreover, moderate anti-candidal activity was produced from methanol extracts of large, medium and small organisms and the ink of *Notrachus indicus* against *Candida albicans*

NRRL Y-12983. Antimicrobial activity of the ink was most prominent against test strains. However, no antibacterial activity was reported against *Proteus vulgaris* NRRL B-123 or *Pseudomonas aeroginosa* NRRL B-23 (Table 6).

Table 6. Antimicrobial and cytotoxic activity of extracts of the sea hare *Notrachus indicus* and its ink.

Test organisms	Extracts of the sea hare			Ink
	Large individual	Medium individual	Small individual	
Staphylococcus aureus	1.54 ± 0.042	1.46 ± 0.16	1.24 ± 0.13	2.23 ± 0.13
Klebsiella pneumoniae	1.98 ± 0.013	1.65 ± 0.06	1.13 ± 0.07	2.98 ± 0.13
Escherichia coli	1.75 ± 0.07	1.49 ± 0.09	1.08 ± 0.15	2.55 ± 0.43
Proteus vulgaris	-	-	-	-
Pseudomonas aeroginosa	-	-	-	-
Candida albicans	1.77 ± 0.25	1.36 ± 0.06	1.26 ± 0.08	2.65± 0.17
Artemia salina nauplii LC_{50} (µl/ ml)	6.8 ± 0.48	24.4 ± 0.15	56.9 ± 0.09	5.4 ± 0.36

Numbers are the means of the diameter of inhibition zones (cm) ± standard errors of the mean
- = No antimicrobial activity against test organism
LC_{50} is estimated by Probit test using the brine shrimp microwell assay.

2.2. Cytotoxic activity

Table (6) also displays the LC_{50} (µl/ ml) of aqueous extracts of the sea hare and the ink. Data indicate that LC_{50} of the ink is 5.4 µl/ ml, which represents a high cytotoxic activity. Aqueous extracts of large organisms also exhibit strong cytotoxicity against brine shrimp nauplii (6.8 µl/ ml). On the other hand, aqueous extracts of medium and small organisms showed moderate 24.4 and 56.9 µl/ ml toxicity, respectively. These levels usually cause the nauplii of *Artemia salina* to shrivel and retract at first, accumulating in the middle of the micro- wells, loosing their activity and then die.

DISCUSSION

The present study represents the first work so far dealing with the ecology of sea hare *Notarchus indicus* along Lake Timsah. Most of the malacological studies of Suez Canal were conducted on bivalves (Fouda and Abou Zaid, 1990; Abou Zied, 1991, Mohammed *et al.*, 1992), gastropods (Mohammed *et al.*, 1992) or cephalopods (Gabr *et al.*, 1998, 1999a, 1999b) while sea hares were mostly ignored despite their presence in shallow water, with very slow movement to be easly observed.

It is a Red Sea species with an Indo-Pacific origin that had migrated to the Eastern Mediterranean Sea through Suez Canal (Gruvel, 1939; Barash and Danin, 1972). During the present investigation, it was recorded widely distributed in Lake Timsah, over sandy substrates of shallow water of Lake

Timsah, forming chains of individuals, creeping over fine sand and feeding on organic matter, which are mainly mats of blue greens.

Observing the species usually draws the attention, due to the pink secretion of ink, when its individuals are attacked or disturbed. Investigation of the biological activity of the sea hare and its ink showed that methanol extracts of large, medium and small individuals and the ink itself have moderate antibacterial activity against three pathogenic bacterial test strains including the Gram-positive bacterium *Staphylococcus aureus* NRRL B-767 and the Gram-negative bacteria *Klebsiella pneumoniae* NRRL B-14232 and *Escherichia coli* NRRL B-3704. Also, it produced moderate anti-candidal activity against *Candida albicans* NRRL Y-12983. The highest antibacterial and anti-candidal activity was produced from the ink. On the other hand, aqueous extracts of large individuals and the ink showed strong cytotoxicity against the nauplii of the brine shrimp *Artemia salina*. Related research studies carried out allover the world showed that the ink could be used by sea hares as defensive screen, serving to obscure the animal from predators (Eales, 1921), or repel them (Dimatteo, 1981) by chemoreception, where the ink has some types of distasteful and/ or noxious components (Dimatteo, 1982), since these animals are sluggish and do not possess the ability to make a rapid retreat (as in the case with cephalopods) so it would be more beneficial to have the ink that acts to repel potential predators (Dimatteo, 1982) due to complex repertoire of secreted chemicals inside the ink (Barsby, 2006). These chemicals in the ink or others secreted by glands (opaline gland) could be a source of bioactivity for these animals, as recorded during the present study. This work could draw the attention to study the chemistry of this species which is abundant in Lake Timsah.

Yang *et al.* (2005) showed that the ink of the sea hare *Aplysia californica* usually contains certain protein which has antitumor and antibacterial actions. This bioactive protein is known as escapin. Kicklighter *et al.* (2005) and Johnson *et al.* (2006) proved that opaline is the major source for escapin. Also, Kicklighter and Derby (2006) demonstrated that the ink but not opaline is aversive and that escapin is not responsible for ink's major aversive effects. They attempted to identify the aversive components in the ink and showed that multiple components in ink cause anemone tentacles to shrivel and retract. Also, they concluded that the aversive ink components are produced de novo or modified sequestered compounds.

Sea hares possess broad spectrum algal derived toxins in their skin and digestive glands (Carefoot, 1987) all of which could be defensive in function and play a role in warning to predators. Kinnel *et al.* (1979) proved that the skin of sea hares usually has defensive chemicals with bioactivity nature. Tsukamoto *et al.* (2005) showed that the extracts of *Aplysia kurodai* have cytotoxic and antibacterial activity. This activity is mainly related to the content of sea hare digestive glands which is derived from the diet that the animal usually ingests. It

could be hypothesized that ink might act to protect the sea hares at distance (i.e. without direct contact and taste) by irritating potential predators enough that would have the vicinity of sea hares.

Finally, it could be concluded that the sea hare *Notarchus indicus* is a successful colonizer in Suez Canal, which has migrated to Eastern Mediterranean. It is usually abundant in Lake Timash in shallow waters of sandy shores. Screening of its bioactivity showed antimicrobial and cytotoxic activity of the organism and its ink.

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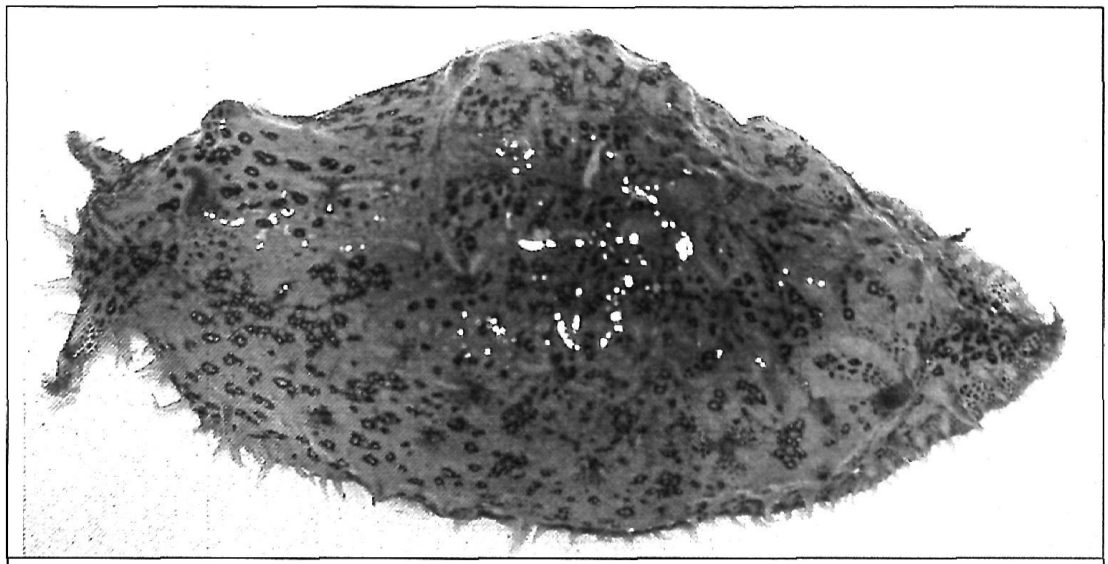
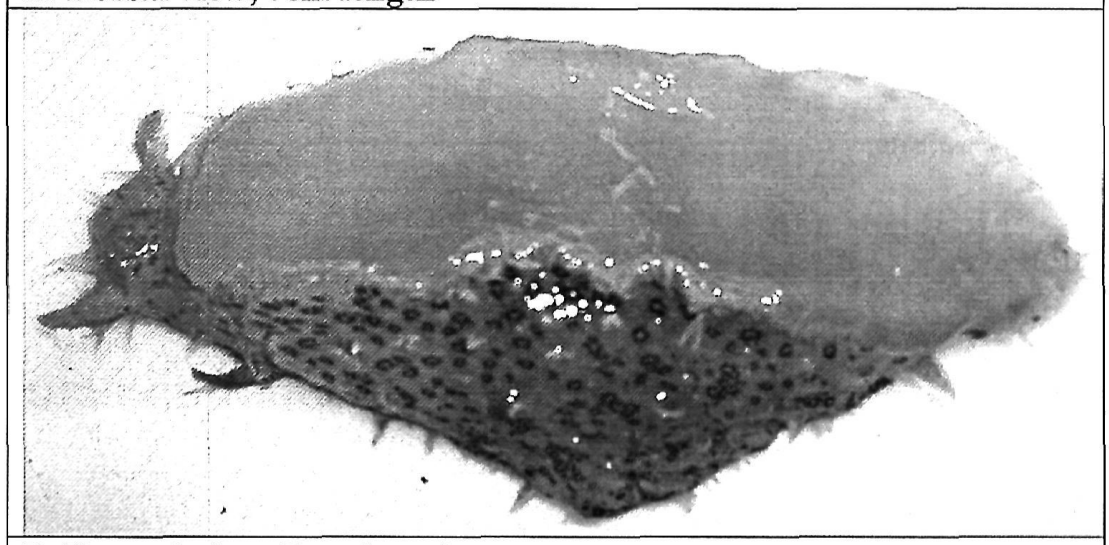


A. Dorsal view, 7cm length



B. Ventral view, 7 cm length

Fig. 1. The sea hare *Notarchus indicus*, Schweiger 1820, collected from Lake Timsah in summer 2007.