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First record of *Leptoxyphium madagascariense* (Dothideomycetes, Capnodiales) from sugarcane juice

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Abstract

During surveys of mycobiota of fresh juices in Assiut area, Egypt, a synnematous dark fungus was isolated from sugarcane juice on malt extract yeast extract 50% glucose agar, MY50G. Critical phenotypic and ITS sequencing revealed its relatedness to *Leptoxyphium madagascariense* (99.42% sequence similarity with Genbank accession nos. NR_137731 & GQ303277 of the type strain CBS124766^T). The strain was deposited at Assiut University Mycological Centre and its ITS sequence was deposited at the National Centre for Biotechnology Information, NCBI. This species is recorded for only the second time after its original description from trees in a tropical rain forest of Madagascar.

Key words – Egypt – ITS sequencing – phenotypic features – sooty moulds – synnemata

Introduction

The family Capnodiaceae was introduced by Höhnel (1910) with the generic type *Capnodium* Montagne (1849) and presently includes 14 genera and 117 species (Kirk et al. 2008, Lumbsch & Huhndorf 2010). Capnodiaceae are sooty moulds with bitunicate asci borne in ostiolate ascomata; the family however is based mostly on ecological characters (von Arx & Müller 1975). The first complete monographic review of capnodiaceous sooty moulds recognized both sexual and asexual species in Eucapnodiaceae (Fraser 1935). Batista & Ciferri (1963a, b) later provided a monograph of Capnodiaceae in the order Capnodiales. Hughes (1972) reviewed and re-classified Capnodiaceae, which is characterized by the structure of the hyphae, presence or absence of pseudoparaphyses and by deviating conidial states. Members of this family also have superficial ascomata with ovoid asci in fascicles and hyaline to dark, one to multiseptate ascospores (Hughes 1976), superficial mycelium of interwoven, mucilaginous, brown, cylindrical or tape ring hyphae, mostly constricted at the septa, and occur as leaf epiphytes associated with the honeydew of insects (Hughes 1976, Blakeman & Fokkema 1982, Andrews 1992). These fungi tend to live in complex communities, often with multiple fungal parasites (Faull et al. 2002, Hughes 2003). They produce darkly pigmented hyphae, often of very characteristic morphology (Hughes 1976, Reynolds 1998). Asexual state reported in Capnodiaceae are *Acanthorus*, *Apiosporium*, *Conidiocarpus*,

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Conidioxyphium, *Fumagospora*, *Fumiglobus*, *Leptoxyphium*, *Mycogelidium*, *Phaeoxyphiella*, *Polychaetella*, *Polychaeton*, *Scolecoxyphium*, and *Tripospermum* (Hyde et al. 2011).

Members of Capnodiaceae and Chaetothyriaceae are referred to as sooty moulds since they share the same ecological niche and are similar in appearance. The main distinguishing features between these two families are found in the ascomata having single locules in Capnodiaceae, and ascostromata, often with more than one locule in Chaetothyriaceae. In addition, the ascomata on leaf surfaces are subglobose to globose, with or without setae in Capnodiaceae (von Arx & Müller 1975), while ascostromata are surrounded by a pellicle of superficial mycelium in Chaetothyriaceae (Chomnunti et al. 2012). Phylogenetic analyses have shown them to be unrelated and they were placed in two separate classes, Dothideomycetes and Eurotiomycetes, respectively (Geiser et al. 2006, Schoch et al. 2006, 2009, Chomnunti et al. 2012).

The sooty mould genus *Leptoxyphium* Speg. was introduced for *L. graminum* (Pat.) Speg. (≡*Capnodium graminum* Pat.) (Spegazzini 1918). *Leptoxyphium* is a relatively poorly known genus of sooty moulds in Capnodiaceae (Hyde et al. 2013, Yang et al. 2014). The genus is saprobic on sugary exudates from insects growing on the surface of living leaves. Mycelia are superficial, grey-brown to brown, branched, septate, constricted at the septa, forming an irregular network. *Leptoxyphium* is mainly characterized by the synnemata arising from helically twisting hyphae or ropes of repent hyphae, expanded to become funnel-shaped with a terminal conidiogenous zone (Hughes 1976), and conidia ellipsoidal, hyaline, 1-celled, guttulate (Woronichin 1926, Hughes 1976). In a molecular study (Chomnunti et al. 2011) *Leptoxyphium* clustered in Capnodiaceae.

Leptoxyphium species were thought to be restricted to the glandular trichomes of leaves. The genus occurs worldwide in form of sooty mould and frequently in pure colonies (Hughes 1976). Interestingly, *L. kurandea* Crous & R.G. Shivas described from leaves of *Eucalyptus* sp. (Myrtaceae), in Queensland, Australia (Crous et al. 2011), was later recovered on a living leaf of *Psidium guajava* L. (Myrtaceae) in Yunnan Province, China (Yang et al. 2014), and from an insect gut (dusky cotton bug) from Western Ghats, India (Kajale et al. 2015). This genus includes 17 species (Index Fungorum, http://www.indexfungorum.org/names/Names.asp).

Leptoxyphium madagascariense Cheewangkoon & Crous was described from Madagascar Island, on leaves of *Eucalyptus camaldulensis* (Cheewangkoon et al. 2009). The purpose of this study is to describe a new record of the genus *Leptoxyphium* (family Capnodiaceae) isolated from fresh sugarcane juice marketed in Assiut city, Egypt.

Materials & Methods

Five juice samples of freshly squeezed sugarcane (*Saccharum officinarum* L.) were collected from different shops in Assiut city during the period from February to April 2013. The pH of the samples was determined and fungi were isolated onto Dichloran rose bengal chloramphenicol agar, DRBC (King et al. 1979), dichloran 18% glycerol agar base, DG18 (Hocking & Pitt 1980) and malt extract yeast extract 50% glucose agar, MY50G (Pitt & Hocking 1985).

Filamentous fungi were identified based on their macro- and microscopical features following the keys of Ellis (1971) and Seifert et al. (2011). A *Leptoxyphium* sp. was thereafter grown on Czapek yeast agar (CYA) and potato sucrose agar (PSA) at 25° C in the dark for 14 days. Colony measurements and microscopic features were determined.

For sequencing, the fungus was grown on CYA agar at 25° C for 7 days. A small amount of fungal biomass was scraped off, suspended in 100 µl of distilled water and boiled at 100 °C for 15 minutes following the manufacturer's protocol (SolGent Company, Daejeon, South Korea). The samples were directly sent for extraction and sequencing. Fungal DNA was extracted and isolated using SolGent purification beads at this company. Internal transcribed spacer (ITS) sequences of nuclear ribosomal DNA were amplified using the universal primers ITS1 (5'- TCC GTA GGT GAA CCT GCG G -3'), and ITS4 (5'- TCC TCC GCT TAT TGA TAT GC -3'). Amplification was performed using the polymerase chain reaction (PCR) (The GeneAmp® PCR System 9700 thermal cycler, Applied Biosystems, Foster City, California, USA). The PCR reaction mixture was prepared using SolGent EF-Taq. The PCR product was purified with the SolGent PCR Purification Kit-Ultra

Table 1 Closest matches in GenBank database and sequence similarity as inferred from Blastn searches of ITS sequences with the newly recorded species *Leptoxyphium madagascariense* strain AUMC 11702 isolated from fresh sugarcane juice in Egypt (accession GenBank number MG323879, length of base pairs = 545).

Genbank match # ITS		Sequencing similarity (%)	gaps	Species	Source (leaves)	Reference
Culture Collection code	Accession no.					
CBS 124766^{T}	NR_137731	515/518(99.42%)	1/518(0%)	*Leptoxyphium madagascariense*	*Eucalyptus camaldulensis*	Cheewangkoon et al. 2009
CBS 124766^{T}	GQ303277	515/518(99.42%)	1/518(0%)	*Leptoxyphium madagascariense*	*Eucalyptus camaldulensis*	Cheewangkoon et al. 2009
IFRD 9043^{T}	KF982307	518/531(97.55%)	1/531(0%)	*Leptoxyphium glochidion*	*Glochidion wrightii*	Yang et al. 2014
DSM1256	KX289331	530/545(97.25%)	1/545(0%)	*Leptoxyphium fumago*		Kellner et al. 2016
CPC 17274^{T}	JF951150	527/541(97.41%)	1/541(0%)	*Leptoxyphium kurandae*	*Eucalyptus* sp.	Crous et al. 2011
IFRDCC2650	KF982310	515/529(97.35%)	1/529(0%)	*Leptoxyphium kurandae*	*Psidium guajava*	Yang et al. 2014
MCC1085	KF826942	507/520(97.50%)	1/520(0%)	*Leptoxyphium kurandae*	Insect gut	Kajale et al. 2015
KUS-F27721	KM226890	492/504(97.62%)	0/504(0%)	*Leptoxyphium kurandae*	*Hibiscus rosa-sinensi*	Park et al. 2015
KUS-F27692	KP992873	491/504(97.42%)	1/504(0%)	*Leptoxyphium kurandae*	*Hibiscus cannabinus*	Choi et al. 2015
OP193	JN604454	451/463(97.41%)	0/463(0%)	*Leptoxyphium kurandae*	--	Taiwan, NCBI website
UBC F23788^{T}	KF263961	484/553(87.52%)	23/553(4%)	*Fumiglobus pieridicola*	*Pieris japonica*	Bose et al. 2014
CBS 124785^{T}	NR_132831	456/514(88.72%)	15/514(2%)	*Antennariella placitae*	*Eucalyptus placita*	Cheewangkoon et al. 2009
MFLUCC10-0064^{T}	KU358926	381/409(93.15%)	11/409(2%)	*Conidiocarpus siamensis*	*Mangifera* sp.	Hongsanan et al. 2015
MFLU15-3564^{T}	KU358919	378/409(92.42%)	10/409(2%)	*Conidiocarpus plumeriae*	*Plumeria* sp	Hongsanan et al. 2015
MFLU15-3565^{T}	KU358921	377/410(91.95%)	12/410(2%)	*Capnodium coffeicola*	*Coffea* sp.	Hongsanan et al. 2015

(SolGent, Daejeon, South Korea) and was sequenced in sense and antisense direction (Moubasher et al. 2017). Contig was created from the sequence data using the CLCBio Main Workbench program. The sequence was further analysed using BLAST from the National Center of Biotechnology Information (NCBI) website. Nucleotide sequence of the target strain together with those retrieved from the GenBank database were subjected to Clustal W analysis using MegAlign software version 5.05 (DNASTAR, Madison, Wisconsin, USA) for the phylogenetic analysis (Thompson et al. 1994). Sequence data were deposited in GenBank and accession numbers are given for them.

Results and Discussion

Sugarcane juice showed a mean pH of 6.45. The fungus (*Leptoxyphium madagascariense*) was isolated once from one sugarcane sample (out of 5 investigated) on only MY50G, and not on DRBC and DG18 agars. Therefore, this species could be considered a xerotolerant fungus since it was isolated on MY50G and was able to grow on CYA and PSA.

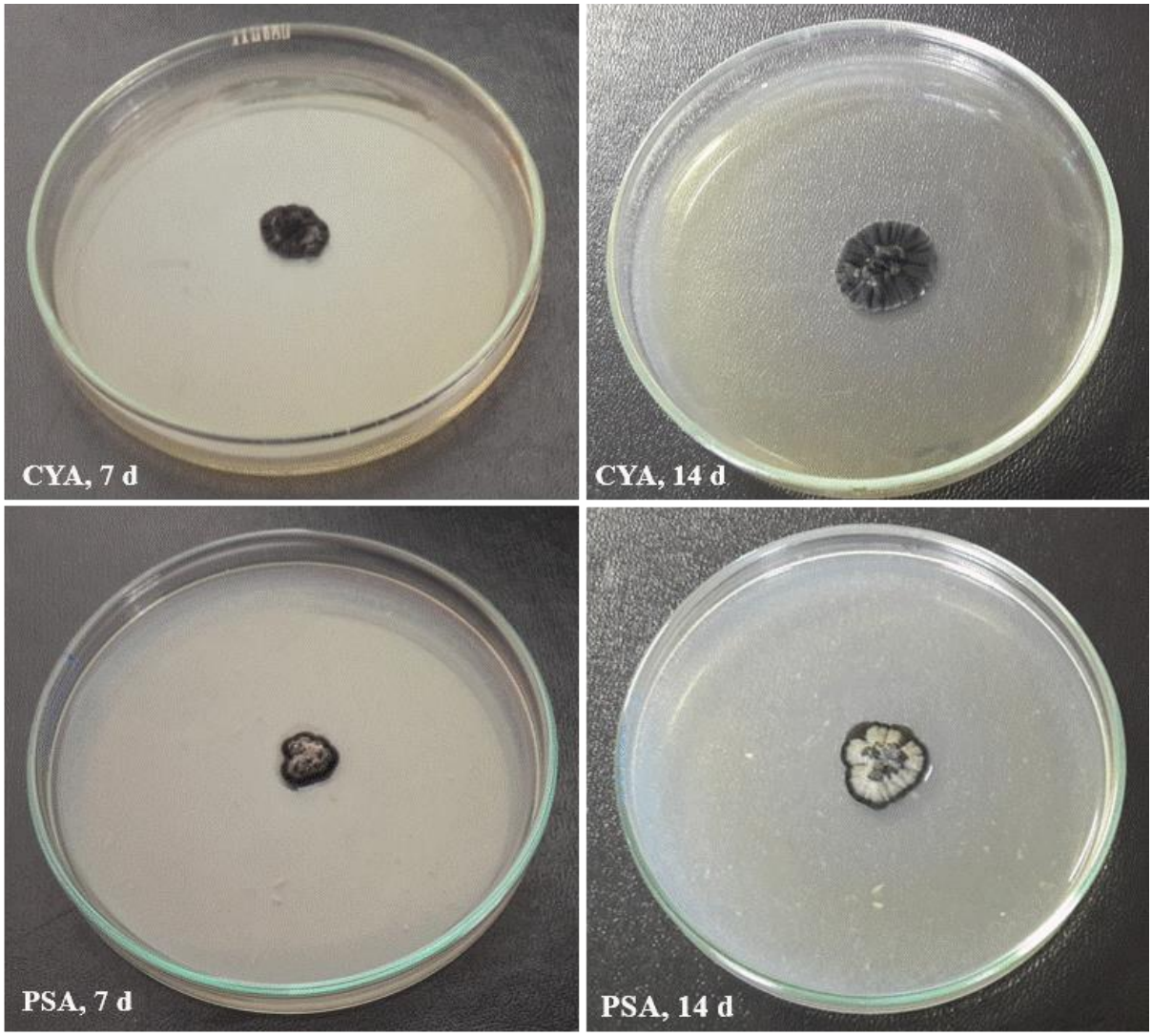

Fig 1 – *Leptoxyphium madagascariense* AUMC 11702 colony growth on Czapek yeast agar and potato sucrose agar after 7 and 14 days of incubation in the dark at 25°C.

Leptoxyphium madagascariense Cheewangkoon & Crous

Culture characteristics – Colonies were incubated in the dark at 25 °C; on CYA colonies attained 1 cm diam. after 7 days and 1.8 cm after 14 days; on PSA 0.9 cm after 7 days and 1.5 cm after 14 days. Colonies were slightly raised and obviously folded especially after 14 days, medium to dark brownish grey aerial mycelium. Especially on PSA, numerous, superficial, dark synnemata with whitish conidial masses were produced (Fig 1).

Microscopic characeristics (Fig 2) – Mycelium septate, frequently septate and wider in hyphae around conidiomata, dark grey-brown. Conidiomata determinate synnematal, cylindrical, subulate, superficial, arising from hyphal ropes; stipe composed of unbranched, parallel synnematous hyphae, sometimes with a helical twist, not enclosed in mucilage, occasionally producing 2–3 synnemata on a single hyphal rope; cylindrical part up to 300 µm high and 15 µm wide, expanding to a funnel-shaped hyphal apex up to 50 µm high and 60 µm wide, consisting of

several aggregated, synnematous hyphae that diverge close to the apex; hyphae 3–4.5 μm wide, septate, slightly thick-walled, flaring in apical part, appearing like a terminal hyphal fringe, terminating in rounded apices. Conidiogenous cells integrated, formed from the inner cell surface, intercalary, never terminal, monophialidic, denticle-like, with a truncate apex. Conidia rod-shape, with rounded ends, 1-celled, 4.5–5 × 3–3.5 μm, in a slimy mass at the apex of synnemata, conidia hyaline (not pigmented). Sexual stage not observed.

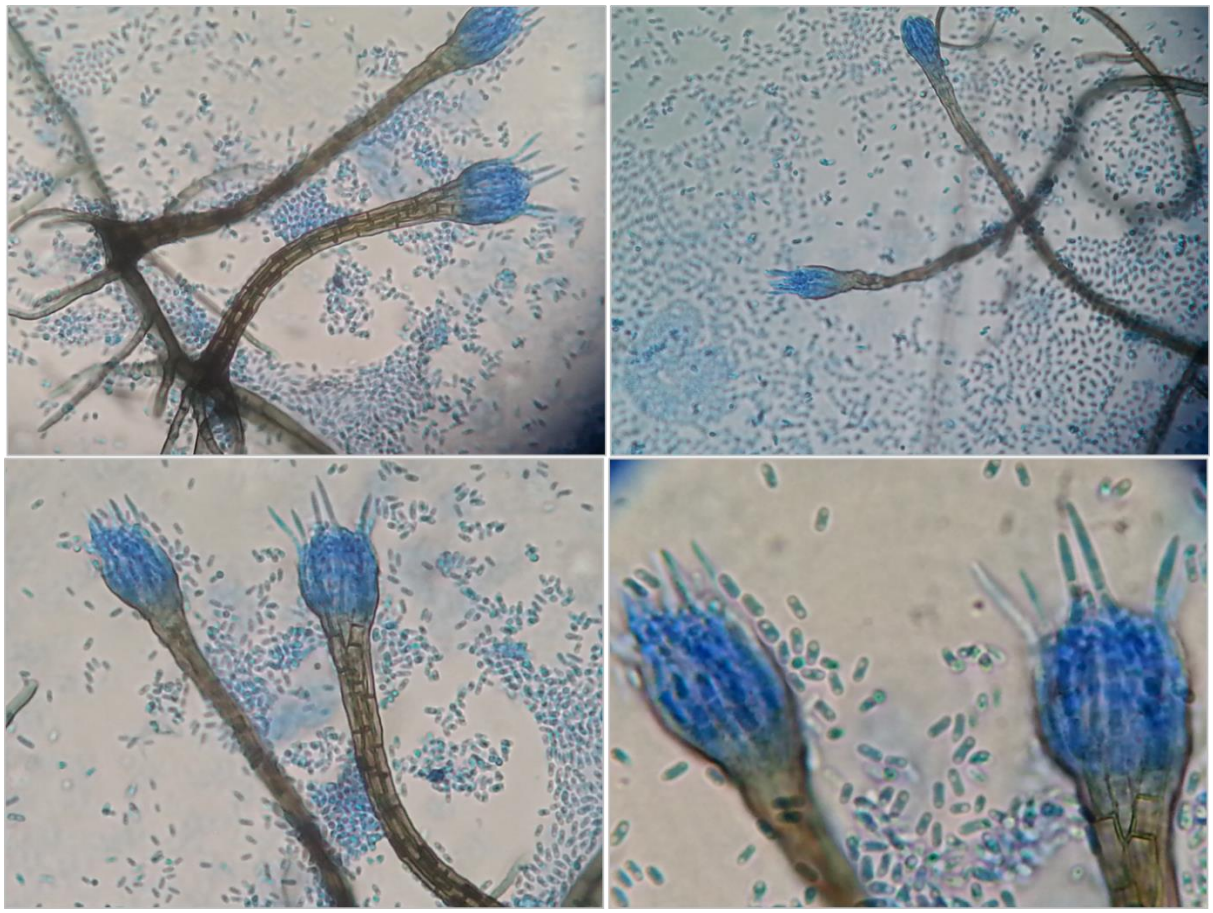

Fig 2 – *Leptoxyphium madagascariense* AUMC 11702; Synnematous conidiophores, conidiogenous cells and conidia.

Leptoxyphium madagascariense has the typical characterisics of the genus (Hughes 1976). It has elongated synnemata with a stout base, a long, narrow neck and a terminal conidiogenous zone. It produces conidia from monophialidic conidiogenous cells. *L. madagascariense* can be distinguished from other *Leptoxyphium* species by its conidial dimensions (Batista & Ciferri 1963b, Cheewangkoon et al. 2009). Unlike many other *Leptoxyphium* species, its conidia are not pigmented and remain non-septate during maturation (Batista & Ciferri 1963b, Hughes 1976).

Molecular identification – Phylogenetically the current strain clusters in the Capnodiales with the type strains of other sooty mould species such as *Leptoxyphium madagascariense* (99.42 % sequence similarity), *L. glochidion* (97.55%), *L. fumago* (97.25%), *L. kurandae* (97.35–97.62%), *Fumiglobus pieridicola* (87.52%), *Antennariella placitae* (88.72%), *Conidiocarpus siamensis* (93.15%), *C. plumeriae* (92.42%), *Capnodium coffeicola* (91.95%) with none or only one gap in case of *Leptoxyphium* species, and from 10 to 23 gaps in species of other genera (Table 1, Fig 3).

In the study of Schoch et al. (2006), *L. madagascariense* also clustered in the Capnodiales with *Microxyphium citri* (98% identical), *Leptoxyphium fumago* (98% identical), *Capnodium coffeae* (96% identical) and *Fumagospora capnodioides* (93% identical).

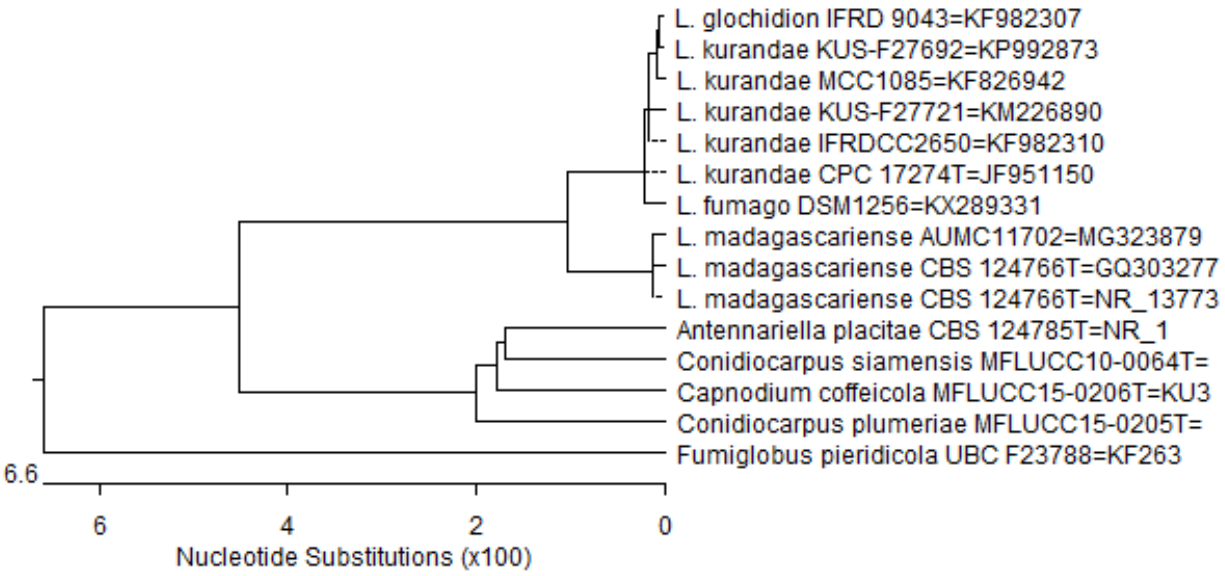

Fig 3 – Phylogenetic relationship of *Leptoxyphium madagascariense* strain AUMC 11702 with the related strains of *Leptoxyphium* and other genera.

This species was first collected from Madagascar Island, Morondavo, on leaves of *Eucalyptus camaldulensis*, in August 2007 and described as a new species (CBS 124766). To the best of our knowledge, this is the second addition worldwide of *L. madagascariense* after its original description (Cheewangkoon et al. 2009). Its isolation from sugarcane juice may be due to its probable presence on the sugarcane plant. This is also the first report of the genus *Leptoxyphium* in Egypt.

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