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Describtive study to Foliose Lichen Xanthoria parietina From Misurata, Libya

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دراسة وصفية للأشن الورقي زانثوريا بارنتينا من مدينة مصراتة ، ليبيا

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Abstract:
Lichens are a stable lasting symbiotic relationship between mycobiont (fungus) and a photobiont (algae or cyanobacteria). Lichens have great potential in biotechnological applications due to their wide biodiversity, ability to produce bioactive secondary metabolites, and effective capacity to accumulate air depositions. This study aimed to study the foliose lichen associated with the Pinus sp. tree in Misurata. According to the results, Xanthoria parientina was the only and common foliose lichen found on the trunk of the Pinus tree in the studied areas. Collected Xanthoria parientina revealed as a phenotypic variant with a small thallus size (1-1.5 cm). Total soluble protein and chlorophyll contents in the control sample were 1.5 mg/ml and 0.8 µg/mg dry mass, respectively. This preliminary study provides baseline information about this unstudied field to draw the attention of researchers to start local lichenological studies in the east and west of Libya.

Keywords: Lichens, Xanthoria parientina, Fungi , Algae, Libya.

الملخص
الأشنات هي كائنات تتكون من علاقة تبادل منفعة طويلة و دائمة بين شريك فطري (فطر) و شريط ضوئي (طحلب). نظرًا لتنوعها البيولوجي الواسع وقدرتها على إنتاج المواد الأيضية الثانوية ذات الفعالية الحيوية، بالإضافة إلى قدرتها الفعالة على تراكم الرواسب الجوية؛ فإن للأشنات تطبيقات حيوية كبيرة. استهدفت هذه الدراسة دراسة الأشنات الورقية المرتبطة بشجرة الصنوبر في مصراتة. وفقًا للنتائج، كانت زانثوريا بارنتينا Xanthoria parientina الأشنات الورقية الوحيدة والشائعة الموجودة على جذع شجرة الصنوبر في المناطق المدروسة . كما ظهرت بسمات مظهرية كنوع متغير ذو حجم ثالوس صغير (1-1.5 سم). كانت كمية البروتين القابل للذوبان والكلوروفيل في العينة الضابطة 1.5 ملغ/مل و0.8 ميكروغرام/ملغ من الكتلة الجافة على التوالي. توفر هذه الدراسة الأولية معلومات أساسية حول هذا المجال غير المدروس لجذب انتباه الباحثين لبدء دراسات الأشنات المحلية في شرق وغرب ليبيا.

الكلمات المفتاحية: الأشنات، زانثوريا بارنتينا، فطريات، طحالب، ليبيا.

Introduction

Lichens are long-lived symbiotic associations between a fungus, which is called a mycobiont, and an alga or cyanobacterium, which is referred to as a photobiont. In general, about 90% of the lichen biomass consists of fungal filaments that form a protective layer for the algae partner [1]. Taxonomically, lichensbelong to the kingdom of fungi and are referred to as lichenized fungi. The lichen species is named after the name of the fungal species that form it [2]. Fungi incorporated into lichens are largely (98%) ascomycetes, and 42% of all

ascomycetes are reported as lichen-forming fungi. There are also a few basidiomycetes and deuteromycetes that form lichens [3]. Lichens can contain numerous distinctive photobionts, which can be different types of algae, cyanobacteria, or a combination of the two [1]. Nearly 40 genera of cyanobacteria and algae have been identified as photobionts, and the three most prevalent genera of photobionts are Trebouxia, Trentepohlia, and Nostoc. The green algal genera Trebouxia and Trentepohlia are eukaryotic, while the genus Nostoc is an oxygenic photosynthetic bacterium (cyanobacteria). The large majority of the approximately 20.000 described lichen species are formed with green algae, which are also known as "phycobionts," whereas cyanobacterial photobionts, which are occasionally referred to as "cyanobionts," represent 10% of lichen photobionts [2][4].

Notably, lichens are more than the sum of two organisms, because they create substances that are typically lacking when the fungus or the photobiont are grown individually and possess structures that were not formed by either of the partners. In support of this hypothesis, investigations proved that 11% and 12% of the genes from algae and 16% and 28% of the genes from fungi, respectively, were overexpressed during the "pre-contact" and "initial contact stage," proving that the potential symbionts begin communicating before physical contact [2]. In fact, lichens create over 800 different compounds, many of which are unique to nature [3][5]. The lichens are divided into crustose, foliose, and fruticose lichens based on the structure and shape of the thallus [6].

Xanthoria parietina, the type species of Xanthoria, is a lichen forming a scomycete; with the photobiont partner, it forms foliose lichens recognized by their yellow or green, gray leafy thallus [7]. The size of the thallus varies from a few centimeters (cm) in diameter to 15 cm, loosely attached to the substrates by rhizines [8]; the lobes of the thallus are imbricated and wrinkled with slight marginal borders [7]; the upper surface appears yellow to orange when it has access to sunlight and can be green in fully shaded sites [9]. Neither idisia nor soridia are found in the thallus of *X. parietina*, but its central thallus area is covered by apothecia. The presence of abundant and apothecia in the majority of its stages distinguishes this species from other Xanthoria species [8].

Xanthoria parietina belongs to the Teloschistales on order and to Teloschistaceae on family level [10]. Treboxia sp. is the ideal photobiont. It is coccoidal unicellular green algae that belongs to the Treboxiophyceae family. Three species are found specially associated with *X. parietina*: *T. arboricola*, *T. decolorans*, and *T. crenulata* [11].

X. parietina is globally distributed. It is one of the most common and widespread lichen species on the European continent; it has been reported from America, Africa, and Asia and is also regarded as an introduced species in Australia and New Zealand [11]. In addition to being one of the common air pollution bioindicator organisms, *X. parietina* has been chosen as a model organism to represent lichen-forming fungi due to many features: it is widespread, has recognized morphology, has a high capacity for regeneration, and is docile to laboratory cultivation [12]. *X. parietina* produces many secondary metabolites as deposited crystals on the surface of the hyphae of mycobionts, such as anthraquinones (parietin), steroids, terpenes, and carotenoids. Biological activities of various secondary metabolites were confirmed in several studies, such as anti-tumoral, anti-inflammatory, and bactericidal effects [13] [14].

Despite the importance of lichens, there are still unexplored areas in Africa where interest in lichens has been very faint in deserts and semi-deserts; the literature about the Libyan lichen biota is very scarce. Its description and classification are problematic since all the previous work was done by European scientists, and most of the collections were deposited in different herbaria outside the country [15].A checklist of 151 taxa was constructed from literature published up until 1950; an additional 49 taxa were added by Thor and Nascimbene (2010) [15]. There is a study conducted in 2021 in the Libya-El-Jabal El-Akhdar region about lichen; in this study 15 different species of lichen were collected from J. phoenicea trees belonging to six families and eight genera. Five of the lichens could not be fully identified to the species level. Seven lichens belong to the family Teloschistaceae, three to Lecanoraceae, two to Pertusariaceae, and the Cladoniaceae, Physciaceae, and Ramalinaceae are each represented by only one taxon; and the most important species, respectively, are *Xanthoria parietina* (L.) Beltr., *Lecanora gr. subfusca* (L.) Ach., *Pertusaria sp*., *Cladonia foliacea* subsp. *endiviifolia* (Dicks.) Boistel, *Tornabea scutellifera* (With.) J.R. *Laundon, and Ramalina maciformis* (Delile) [16]. This study was conducted to identify and characterize an abundant lichen type in Pinus sp. trees in Misurata through studying the morphology and anatomy of the lichen thallus in addition to chlorophyll and protein contents. Briefly, this study provides baseline information about the predominant lichen associated with the Pinus tree to open the way for initiating the local lichenological studies.

Material and methods

Sample collection

The lichen samples were collected from Pinus spp trees in the AL- fallaga region in Misurata city. Carefully, the lichen specimens collected a long with their substratum above the ground at least 50cm from trunk of the trees. The collected lichen sample are transferred to the laboratory of genetics department in a paper bags.

Morphology description

In the laboratory , the lichen samples were first cleaned from dust and washed by distilled water to be examined by naked eye , and stereomicroscope . Morphologocal features was recorded especially growth form , color and shape of thallus , type of mycoboint fruting bodies, presence of finger like projection (isidia), granular powder (soredia) and rhizines. Then the lichen specimens were identified primarily according to two sources [9][17].

Direct section:

Cleaned air dried thallus and apothesia were undergone to direct sectioning by a razor blade . The cuts were performed by freehand and a mounted on a surface of slide with drop of distilled water , then examined under compounded microscope with magnification of 40X to observe the major characters like arrargment of the algal and fungal layers and presence of ascospores in Ascus [18].

Lichen spot assay:

Chemotaxonamic test were carried out to investigate the presence of a certain secondary metabolite in the lichen thallus in which differentiate the genus of lichen [18] .The tests were done by applying few drops of the K, P and C reagents on the top of lichen thallus and color reaction was recorded.

Thallus and apothesia anatomy examination

In order to examine the mycobiont and photobiont layers in the lichen samples, paraffin slide techniques were used as described by Lesniewska et al. [19]. Cleaned thallus fragments containing apothecia were put in cassettes and softened by soaking in lichen softening solution for 24 h. Then the water content of the thallus was removed by soaking the thallus in an ascending series of ethanol solutions (30% to 96%) for 30 min in each solution. After that, the filtrated ethanol was replaced by xylene through soaking in a mixture of absolute ethanol and xylene at the ratio of (2:1), (1:1), and (1:2) for 30 mins and then in pure xylene three times. Finally, the thallus was saturated with 58°C melted paraffin and covered with excess wax to make paraffin blocks. Three micrometer sections were obtained by cutting the blocks by microtome. Slides of sections were stained by Ehrlich hematoxylin and examined under a light microscope.

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Isolation of Lichen mycobionts:

The fungus was isolated using ascospore discharge techniques briefly.The surface of the thullas was cleaned with a paintbrush to remove any soil and debris. The apothecia were dissected from the thallus and placed into dishes containing distilled water, washed several times with distilled water, and soaked for 30 minutes in water until fully rehydrated. These were then attached with Canada balsam glue to the middle inside of the cover of a petri dish containing Modified Bold's Basal (MBB) agar. The petri dish was placed upside down in the incubator in the dark at 25°C. The plate surface was examined and replaced with a new MBB plate every three days. Fungal growth was examined every 2 to 3 days, and contamination was cut out [20].

Isolation of Lichen photobionts:

The thallus method was used to isolate algae from the lichen. The lichen thallus was cut about 1 cm from the apical regions and washed for 5-10 min with sterilized water with brushing to clean the surface of the water. Then the upper surface of the thallus (cortex) was shaved away by using a small scalpel, one drop of sterilized water was added on the slide, and it was covered with the other slide and squeezed using light pressure to separate algal cells from the hyphae of mycobionts. The drop containing free photobionts was transferred from the slide into Bold's Basal Media (BBM) agar plates using pipettes. The algae were cultivated in light at 20-22°C. The plates were investigated 2 times a week [20].

Determination of total soluble protein content

To remove extracellular phenolic compounds, the lichen thalli were washed several times with acetone and then air-dried. A weight of 2 mg of frozen lichen thallus was homogenized with 2 ml of cold 0.2 M phosphate buffer saline (pH 7) by a cold mortar and pestle. The homogenized mixture was transferred into 1.5 ml microcentrifuge tubes and centrifuged at 13000 rpm for 20 minutes [21]. Subsequently, supernatant was transferred to a new clean microcentrifuge tube. The total soluble protein concentration was measured using a Thermo Scientific NanoDrop.

Determination of total chlorophyll contents:

Chlorophyll contents of lichen thallus was extracted according to Liu *et al*. method [22]. Thallus of lichen was washed by distilled water several times to remove the soil or any contaminant and then air dried . The weight of 20mg from clean thallus was washed five times (5min per wash) with 3ml of 100% CACO3 saturated acetone solution to dissolve any lichenic acids which may degrade chlorophyll during extraction . Then the thallus was deposed on clean filter paper and leaved to air dried to evaporate all the acetone contents. After that, sample were cut into very small pieces and placed in a darkened bottle containing 10ml of DMSO. After 72h the pieces of

lichen thallus was removed and the volume was adjusted to be exactly 10ml with the DMSO solvent . Absorbance of the extract was measured using Jen way 6305 UV/VIS Spectrophotometer at 665 , 649 and 480 nm wavelengths after checking that the absorbance of the extract at 750 nm did not exceed (0.01) .
To calculate the concentration (µg/mg) of chlorophyll a , chlorophyll b and total carotenoids, the following equations were used [23]:

"Chlorophyll a = 12.19 A665 – 3.45 A649"

"Chlorophyll b = 21.99 A649 – 5.32 A665"

"Total carotenoids = (1000 A480 – 2.14 Ch a – 70.16 Cl b) / 220"

Results and discussion

Although lichens have been the subject of renewed interest for many countries worldwide, the lichen biota in Libya remains poorly known despite the presence of a considerable number of published floristic papers. Coastal areas are likely to harbor diverse lichen biota like other neighboring countries (Tunisia, south of Italy) [24]. The Mediterranean climate and higher precipitation and humidity, in addition to the presence of mountains with green cover in some regions, provide suitable habitat for lichen growth. The comprehensive published checklistof lichen in Libya by Thor and Nascimbene (2010) summonsed a wide range of published sources and sporadic collection field works, mainly by Italian authors listed. 151 taxes are found in Libya [15]. Recent fieldwork was performed during 2020 by Saed and his colleagues to identify lichens that are associated with Juniper sp. trees in El Jabal. EL-Akdar region revealed the presenceThe EL-Akdar of 15 taxa belonging to eight genera, which were previously reported in Libya except for one taxon, Pertusaria hymenea. 16.In the present study, Pinus sp. trees in Alfellaga showed little to moderate dense growth of foliose lichens. Fruticose lichen could not be observed. Notably, lichen growth was found on the trunks of large, mature trees and more significant growth on declining ones. While young pine trees do not have lichen growth. This is consistent with the findings of Saaed et al. in Juniperus trees in Al Jaball EL-Akhdar [16].This may be due to two factors: The young trees have strong and compact bark, which cannot hold or retain water for a long period sufficient to support the growth of lichen. Also, the lichens grow very slowly annually, which needs many years to be visible in young trees [25].

Species Identification

The morphology and anatomy of the predominant foliose lichen were studied by thallus description and performing sections. The handmade section of fruiting bodies and lichen thalli showed the presence of the two main components of lichen: fungus and algae arranged in the layers of upper cortex, then photosynthetic layer, then medulla, and lower cortex, as shown in figure (1B). The presence of a lower cortex indicates that the current lichen sample belongs to foliose lichens, which is the only form that is discriminated by having a lower cortex [26].

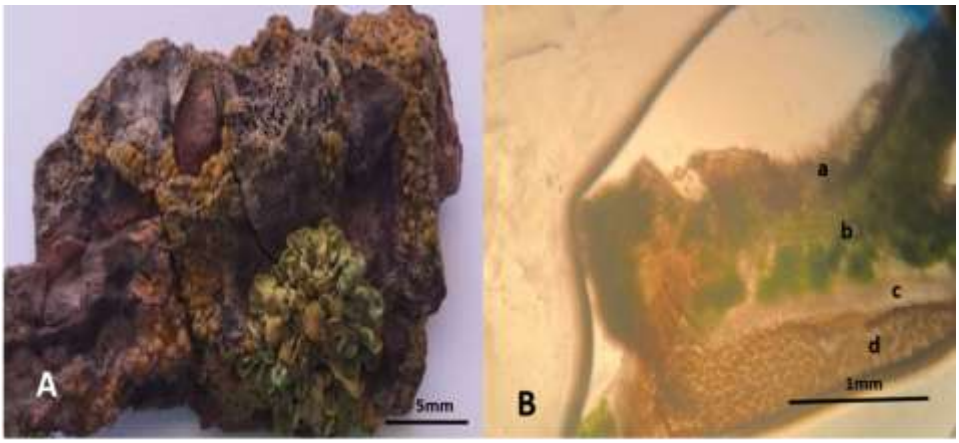

Figure (1): Primary identification of collected lichen sample. A: leafy growth form. B: Handmade vertical section under compound microscope (100X). a: upper cortex (fungi), b: algae layer, c: medulla (fungi), d: lower cortex (fungi).

Regarding the morphological features, The upper surface of the thallus has yellow to green gray color with size varies from 0.5cm in diameters to 2cm with mean of 1.3cm ± 0.3. It is loosely attached to the substrate by root similar structures called rihizines.

The leafy thallus composed of wrinkled and imbricated lobes and bears bright yellow or yellow orange cup – shaped fruiting body (apothesia) which gathered in the center of thallus . The apothesia size ranges from 0.5 to 2mm in diameter. Neither finger like Structure (isidia) or granular powder like structure (soridia) are found on the examines thalli (figure 2). The morphological and anatomical characters given above perfectly match the characters of foliose lichen *Xanthoria parietina* [26] .

Despite recently described *X. parietina* differs in size comparing with well-known and previously described *X. parietina* worldwide, which have thallus can reach to 10 to 15 cm in diameter, phenotypic variant with small thallus size (1-1.5 cm) was reported in Australia, Russia and UK in agreement with our Libyan samples [27] .

The identifications was more confirmed by chemical spot assay that depends on reaction between specific chemical reagent with accumulated secondary metabolites that produced by mycobionts in the surface cortex or medulla of thallus. This assay adds discriminative feature helps in more identification of Lichen genera. Our sample revealed positive reaction (purple colour) with KOH in K test indicating to presence of anthraquinone (paretin) ; paretin is secondary metabolites that is abundant in Xanthoria Species [28] .

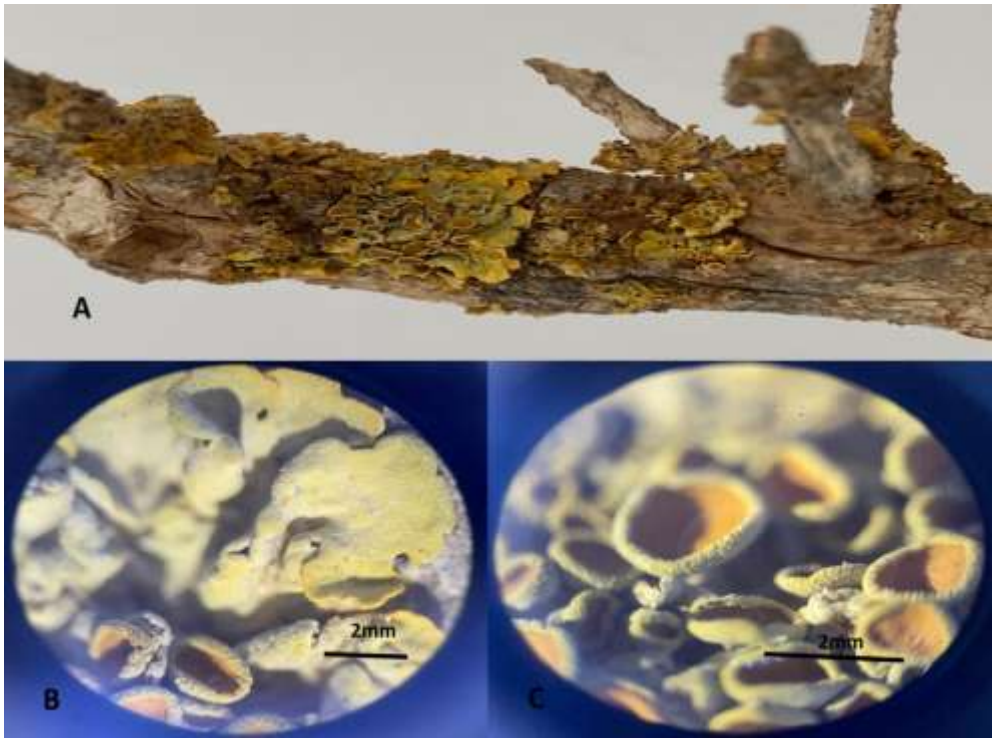

Figure (2): *X. parietina* thallus morphology. A: leafy growth on a vertical branch of Pinus sp. B: detail of thallus surface with wrinkled lobes under compound microscope (40X). C: cup shaped apothesia with orange color.

Anatomy of *X. parietina* thallus

Permanent paraffine slide was prepared to study the structure of thallus and the apothesia in identified *X. parietina*. A thallus cross section figure (3) showed the presents of the typical foliose lichen layer. Notably, coccoidal algal cell of *Treboxia. sp*. stained heavily by hometoxyline and appeared under the fungal cortex to be access to sunlight. Apothesia cross section figure (4) revealed the present of hymenium layer as upper tissue of *X. parietina* apothesia containing ascus and strile hyphae called paraphyses. The hymeruim is spported with hypothecium tissue which give the orange color to apothesia of the *X. parietina* .

Isolation of lichen mycobionts and photobionts

In this study, mycobiont was isolated by ascospore discharge technique which reported to be the most successful method with low contamination rates [20] .When this method was applied in the current study the ascospores was discharge at least after two days of incubation at 20±3C° in the dark. One out of four cultures success to form white colonies after 20 days with diameter 4 mm (figure E). The remaining cultured plates either showed contaminated growth or did not have any growth (sterile). The maximum diameter of *X. parietina* fungi colonig was 5cm after two months. Noteworthy, the colonies achieved coloration from whitish to pale salmon to dark brown, probably because of accumulation of related carotenoid that produced by the *X. praientina* fungus in the media. The production of pigmented secondary metabolites was reported previously in the literature [29].

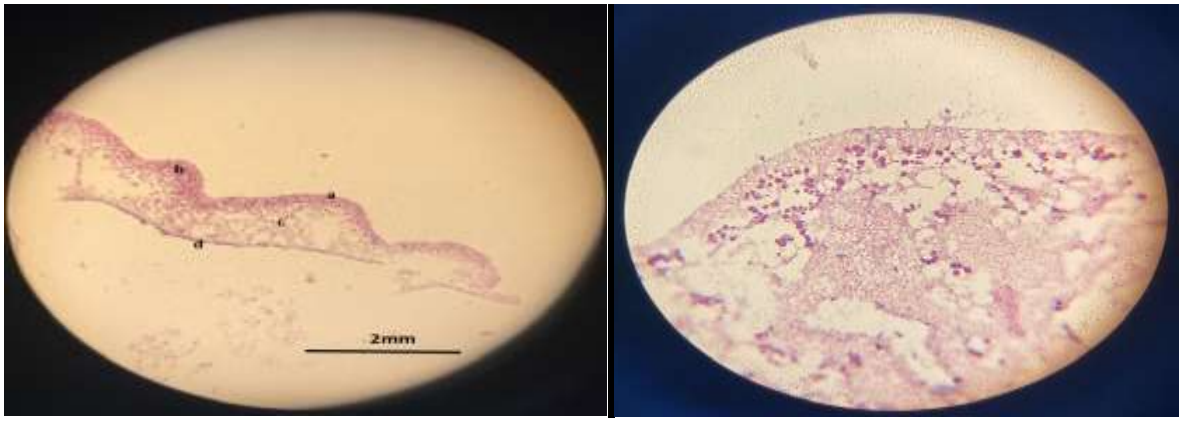

Figure (3): Transverse section of thallus stained with hematoxylin (x100). a: upper cortex (fungi), b: algae layer, c: medulla (fungi), d: lower cortex (fungi).

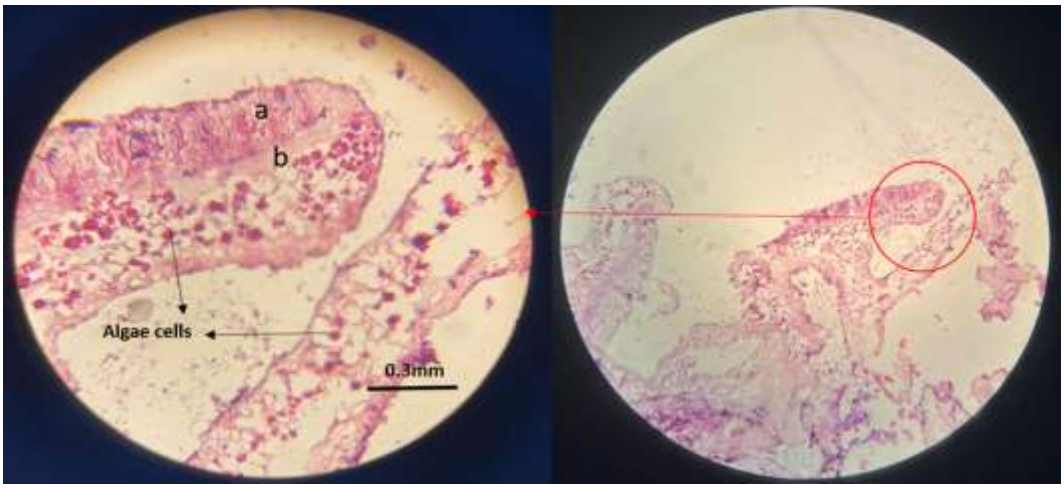

Figure (4): An apothecial cross section from *X. parietina*. a: hymenium, b: hypothecium.

In the other side, photobiont from *X. parietina* lichen could not be isolated successfully by enrolled thallus method; weak algae growth on the Petri dishes of BBM was visible after about a month of incubation on light but was contaminated with fungal growth. For obtaining true axenic algae culture additional reported regimes depend on separation of algae from fungi by serial washing and density gradient centrifugation steps and inoculation of separated algal cells into BBM broth instead of solid media revealed [30].

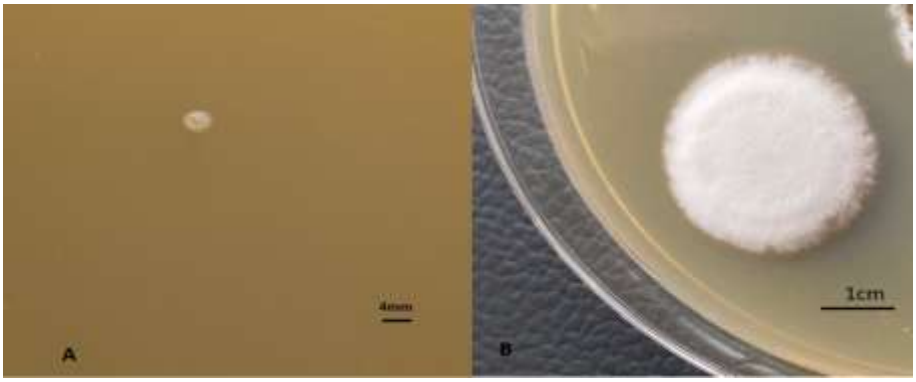

Figure (5): Isolation and cultivation of *X. parietina* fungi by ascospore discharge method in MBB medium. A: colony of fungal mycelium after 20 days of cultivation. B: colony after 1 months.

chlorophyll and protein contents

Lichen are very sensitive to climate change and environmental pollution because of their physiological characteristics. Chlorophyll degradation, respiration rate and photosynthesis in lichen are particular indicator of air pollutions and other environmental changes. Among these indicators, chlorophyll contents in the photobiont is one of the most evident sign of accruing of damage to the sensitive lichen [22].

In this study chlorophyll contents was extracted by DMSO method chlorophyll was more than chlorophyll b (0.5µg/mg) and (0.39µg\mg) respectively. While the total carotenoids was about 0.2µg/mg/dry mass of lichen.

There has been a renewed and growing interest in lichen as a sourse of novel, biologically active molecules including proteins compounds. Lichen extracted proteins with antiprion, UVB protection, antifreeze protein and oxidative enzymes has been reported recently [31]. Low protein contents (1.5mg/ml) was extracted by 0.2 M potassium phosphate buffer solution from acetone washed thallus (20mg) of *X. parietina* in the our study. However. Studies on the lichen proteome are impeded by number of factors; in particular, lichens contain a tiny amount of cytoplasm, little protein contents, presence of significant amount of phenolic compounds and the high activity of their proteolytic enzymes which result in decrease protein yield and changes in the property and activity of extracted proteins [32].

Conclusion

In the present study, one foliose lichen species (*X. parietina*) was observed associated with Pinus sp tree in the Al-Fellaga regions in Misurata City .The identified *X. parietina* represents a phenotypic variant with small thallus size (1-1.5 cm) comparing with well-known and typical *X. parietina* reported samples. Average chlorophyll a and chlorophyll b contents were (0.5µg/mg) and (0.39µg\mg) respectively. While the total carotenoids was about 0.2µg/mg/dry mass of *X. parietina* thallus. Low protein contents (1.5mg/ml) was extracted by 0.2 M potassium phosphate buffer solution method . Mycobiont could be isolated in pure culture successfully by ascospore discharge method. Axenic culture of algae could not be obtained by thallus method. This study opens interesting perspectives regarding the investigation lichens have not received attention from researches for many years in Libya. Furthermore, expanding this preliminary study to filed lichenological study in east and west regions will contribute to a broader knowledge about lichen biota in Libya.

Compliance with ethical standards

Disclosure of conflict of interest

The authors declare that they have no conflict of interest.

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