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Branch: Biological Sciences

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Study of the effect of fluorine and lead on the metabolism of lichens: case of the species *Xanthoria parietina* (L.) Th. Fr.

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List of content

List of abbreviations

ATCC: American Type Culture Collection

BSA: Bovine serum albumin

BHT: Butylated hydroxyl-toluene

C_a: Chlorophyll a

C_{a+b}: Total chlorophyll

C_b: Chlorophyll b

DTNB: 5.5'-dithiobis 2-nitrobenzoic acid

FW: Fresh wight

GAEPGDW: Gallic acid equivalent per g dry weight

QEPGDW: Quercetin equivalent per g dry weight

GSH: Reduced glutathione

HNO_3: Nitric acid

IZd: Size of inhibition zone diameter

MDA: Malondialdehyde

MEXTL: Methanol extract of *X. parietina* treated with lead

MEXTF: Methanol extract of *X. parietina* treated with fluoride.

NaF: Sodium fluoride

$Pb(NO_3)_2$: Lead nitrate

Ph_a: Pheophytin a

Ph_b: Pheophytin b

Ph_{a+b}: Total pheophytin

ROS: Reactive oxygen species

SD: Standard deviation

TCA: Trichloroacetic acid

TNB: Thionitro-benzoic acid

X. parietina: *Xanthoria parietina*

List of figures

List of tables

Abstract

Our work aims to investigate the toxicity of fluorine and lead on various stress parameters on lichen species of *Xanthoria parietina* (L.) Th. Fr., chlorophyll, proteins and antioxidant system, to explore the differences in polyphenol and flavonoid contents and to study the antibacterial activity of its methanol extract. For this purpose, lichen thalli have been treated by sodium fluoride (NaF) and lead nitrate ($Pb(NO_3)_2$) at concentrations of 0, 0.5, 1.0, 5.0 and 10 mM, for time scale 0, 24, 48 and 96 h.

The obtained results revealed that all the evaluated parameters showed significant variations compared to the controls, it was noted that chlorophyll a (C_a), chlorophyll b (C_b), total chlorophyll (C_{a+b}) and protein contents decreased correlating with exposure times and/or increasing concentrations of NaF and $Pb(NO_3)_2$, with a significant increase of $C_{a/b}$ ratio showing that C_b is more affected than C_a and that this degradation is coupled with pheophytin accumulation. However, contents of catalase, hydrogen peroxide (H_2O_2), Glutathione (GSH), proline, Malondialdehide (MDA), soluble sugars increased with increasing exposure time and/or increasing concentrations of NaF and $Pb(NO_3)_2$.

The results also showed that high concentration of NaF and $Pb(NO_3)_2$ solutions disturbed the detoxification system, resulting in total glutathione decomposition and reduction of polyphenol and flavonoid contents. Furthermore, results indicate a favorable relationship between differences in the diameter of the inhibitory zone and the polyphenol and flavonoid levels. In light of these results, Gram-positive bacteria were found to be more sensitive to extracts than Gram-negative bacteria.

Keywords: Antibacterial activity, Antioxidant system, Fluorine, Lead, Lichen, Stress.

ملخص

يهدف عملنا إلى التحقق من سمية الفلور والرصاص على العوامل المختلفة للإجهاد عند الأشنة *Xanthoria parietina* (L.) Th. Fr. ; الكلوروفيل, البروتينات ونظام مضادات الأكسدة، لاستكشاف الاختلافات في محتوى البوليفينول والفلافونويد ولدراسة النشاط المضاد للبكتيريا لمستخلص الميثانول. ولهذا الغرض، تمت معالجة الأشنة بفلوريد الصوديوم (NaF) ونترات الرصاص ($Pb(NO_3)_2$) بتركيزات 0، 0.5، 1.0، 5.0 و10 ميلي مول لمدة 0، 24، 48 و96 ساعة.

أوضحت النتائج المتحصل عليها أن جميع المتغيرات المقيمة أظهرت اختلافات معتبرة مقارنة بالشواهد، ولوحظ أن كميات الكلوروفيل أ (C_a)، الكلوروفيل ب (C_b)، الكلوروفيل الكلي (C_{a+b}) والبروتينات تناقصت مع أوقات التعرض و / أو زيادة تركيزات NaF و $Pb(NO_3)_2$، مع زيادة كبيرة في نسبة $C_{a/b}$ دالة أن C_b أكثر تأثراً من C_a وأن هذا التحلل يقترن بتراكم الفيوفيتين. بينما كميات الكتلاز، بيروكسيد الهيدروجين (H_2O_2)، الجلوتاثيون (GSH)، البرولين، الملونديالدهيد (MDA) و السكريات القابلة للذوبان زادت مع زيادة وقت التعرض و / أو زيادة تركيزات NaF و $Pb(NO_3)_2$. أظهرت النتائج أيضًا أن التركيز العالي لمحلول NaF و$Pb(NO_3)_2$ أدى إلى اضطراب نظام مضادات الأكسدة، مما أدى إلى تحلل كلي للجلوتاثيون وتقليل محتويات البوليفينول والفلافونويد. علاوة على ذلك، أظهرت النتائج وجود علاقة إيجابية بين محتويات البوليفينول والفلافونويد والاختلافات في حجم قطر منطقة التثبيط. في ضوء هذه النتائج ، وجد أن البكتيريا إيجابية الجرام أكثر حساسية للمستخلصات من البكتيريا سالبة الجرام.

الكلمات المفتاح: الفلور، الرصاص، الإجهاد، الأشنة، نظام مضادات الأكسدة، النشاط المضاد للبكتيريا.

Résumé

Notre travail vise à étudier la toxicité du fluor et du plomb sur les différents paramètres du stress chez le lichen *Xanthoria parietina* (L.) Th. Fr.; chlorophylle, protéines et système antioxydant, pour explorer les variations de la teneur en polyphénols et flavonoïdes et d'étudier l'activité antibactérienne de son extrait méthanolique, à cette fin, les thalles de lichens ont été traités au fluorure de sodium (NaF) et au nitrate de plomb ($Pb(NO_3)_2$) à des concentrations de 0, 0,5, 1,0, 5,0 et 10 mM, à des échelles du temps 0, 24, 48 et 96 h.

Les résultats obtenus ont révélé que tous les paramètres évalués présentaient des variations significatives par rapport aux témoins, il a été noté que les contenus en chlorophylle a (C_a), chlorophylle b (C_b), chlorophylle totale (C_{a+b}) et protéines diminuaient en corrélation avec les temps d'exposition au NaF et au $Pb(NO_3)_2$, avec une augmentation significative du rapport $C_{a/b}$ montrant que C_b est plus affecté que C_a et que cette dégradation est couplée à une accumulation de phéophytine. Cependant, les contenus en catalase, peroxyde d'hydrogène (H_2O_2), glutathion (GSH), proline, malondialdéhide (MDA) et sucres solubles ont augmenté avec l'augmentation du temps d'exposition et/ou l'augmentation des concentrations de NaF et de $Pb(NO_3)_2$. Les résultats ont également montré qu'une concentration élevée de solutions de NaF et de $Pb(NO_3)_2$ perturbait le système de détoxification, entraînant une décomposition totale du glutathion et une réduction des teneurs en polyphénols et en flavonoïdes. De plus, les résultats montrent une corrélation positive entre les teneurs en polyphénols, en flavonoïdes et les variations de la taille du diamètre de la zone d'inhibition. A la lumière de ces résultats, les bactéries à Gram positif se sont révélées plus sensibles aux extraits que les bactéries à Gram négatif.

Mots-clés: Activité antibactérienne, Fluor, Lichen, Plomb, Stress, Système antioxydant.

Introduction

One of the major problems of current time is air pollution, the assessment of air quality through the use of lichens as bioindicators is a major concern at the moment. Lichens present a very important model of symbiotic organisms that include a fungus termed mycobiont, that capture fixed carbon from green algae and/or cyanobacteria called photobionts (Honegger, 1991; Nash III, 2008; Mitrović et *al.*, 2011; Calcott et *al.*, 2018). They are used in the bio indication of air quality (Bosch-Roig et *al.*, 2013; Kar et *al.*, 2014; Loppi, 2014; Kuldeep and Prodyut, 2015; Pescott et *al.*, 2015; Sulaiman et *al.*, 2018; Benítez et *al.*, 2019; Mohamed et *al.*, 2020; Quijano-Abril et *al.*, 2021). Lichens are devoid of roots, and mineral nutrition takes place from atmospheric inputs and wet deposits (Garty *et al.*, 2008; Gauslaa et *al.*, 2021), this property allows us to use them as one of the excellent bio-indicators of air pollution. Lichens are also characterized by the presence of many original compounds, especially bioactive secondary metabolites (Mukemre et *al.*, 2021), where, they can be used for their antimicrobial (Maciąg et *al.*, 2014; Sargsyan et *al.*, 2021; Gandhi et *al.*, 2022) and antioxidant activity (Gessner et *al.*, 2017; Rodríguez et *al.*, 2016; Kandelinskaya et *al.*, 2021), the most important of which are polyphenols thanks to their pharmacological activity (Stromsnes et *al.*, 2021). Polyphenols have an important role in a plant's ability to withstand various stresses (Kiani et *al.*, 2021; Tuladhar et *al.*, 2021; Kołton et *al.*, 2022). They are necessary for plant's growth, nutrition, survival, and defenses (Singh et *al.*, 2021). Curentlly, lichens are being used for a variety of purposes, including the therapy of many human illnesses, especially as an anti-cancer activity (Nugraha et *al.*, 2019; Solárová et *al.*, 2020; Chae et *al.*, 2021; Šeklić et *al.*, 2022).

Lichens are capable of absorbing large amounts of trace elements from the atmosphere (Caggiano et *al.*, 2015; Darnajoux et *al.*, 2015). Additionally, they serve as biomonitors (Kularatne and de Freitas, 2013; Ite et *al.*, 2014; Conti and Tudino, 2016; Demková et *al.*, 2017; Abas, 2021; Tarawneh et *al.*, 2021) and bioaccumulators of heavy metals (Węgrzyn et *al.*, 2016; Winkler et *al.*, 2019; Rola, 2020; Vannini et *al.*, 2021). They can develop on challenging supports, like coastal rocks (Dévéhat et *al.*, 2014), and distinct from the majority of other eukaryotic species in terms of their physiology, anatomy, and capacity to tolerate extreme stresses (Expósito et *al.*, 2022). Despite their distribution and diversity are influenced by climate, soil chemistry and geography (Škvorová et *al.*, 2022), lichens can resist various stressful conditions such as extreme drought and temperatures (Beckett et *al.*, 2021), salinity (Chowaniec and Rola, 2022), heat (Kraft *et al.*, 2022), nutrient deficiency (Hauck et *al.*, 2009) heavy metals (Rola, 2020) and fluoride (Roberts and Thompson, 2011). These stresses are the source of reactive oxygen species (ROS) and the most

important adaptation mechanism used by lichens for tolerance to stressful conditions is the scavenging of these ROS (Kranner et *al*., 2009). Lichens can also exhibit the typical stress-tolerant characteristics, such as reduced growth rates, significant longevity, low nutrient requirements, the presence of specific morphological and physiological adaptation, and changes in ecological behavior for surviving in the most hostile environments in the world (Armstrong, 2017).

Fluoride is found in a variety of environmental matrices and even at low quantities and is one of the most phytotoxic chemical elements for plants (Banerjee and Roychoudhury, 2019). It affects the metabolic activity of plants by decreasing nutrient uptake, germination, photosynthesis, growth, and productivity (Sharma and Kaur, 2018). Fluoride toxicity causes the generation of ROS, it has also a negative impact on enzyme activity, protein synthesis, and gene expression patterns (Choudhary *et al*., 2019). Heavy metals cause inhibition of chlorophyll synthesis in lichens (Rola et *al*., 2019). In green plants, abiotic stress seriously affects photosynthesis in all its stages (Ashraf and Harris, 2013). Lead causes a disturbance of the prooxidant/antioxidant equilibrium, which induces various cell and tissue damage that evolve towards damage at the subcellular level (Nareshkumar et *al.*, 2015).

Lichens react with atmospheric pollutants often having physiological, morphological and structural changes (Matos et *al.,* 2015). The most commonly used parameters to study the toxicity of atmospheric pollutants on lichens are chlorophyll degradation (Šujetovienė and Sliumpaitė, 2013; Balarinová et *al.*, 2014; Karakoti et *al*., 2014; Šujetovienė, 2015) and lipid peroxidation (Paoli et *al*., 2015; Šujetovienė et *al.*, 2019). Like all other plant components, including chlorophyll, polyphenols also respond differently to pollutants in the air. Plants use phenolic compounds to defend themselves from oxidative stress brought on by a variety of airborne pollutants (Nobile et *al.*, 2021). Plant polyphenols have the property of naturally acting as antioxidants and being only mildly harmful. Moreover, they can chelate lead because of their unique chemical molecular structure, which helps them withstand lead toxicity (Li et *al.*, 2021). Exposure to abiotic stress triggers the accumulation of amino acids and amines in different plant species. Soluble sugars play a crucial role in a variety of metabolic processes, acting as a signal to control gene expression in photosynthesis, osmolyte production, and sucrose metabolism (Khan et *al.*, 2020). Proline, on the other hand, is crucial for plants; it protects them from various stresses and helps in their faster recovery from stress (Mundada et *al.*, 2021).

To prevent ROS-induced damage, plants synthesize many enzymatic components like catalase (Lei et *al*., 2022) and non-enzymatic components like glutathione (Hasanuzzaman et *al*., 2020), and change their protein composition (Amnan et *al*., 2022). In response to abiotic stress, plants also produce H_2O_2 as one of the ROS (Zhang et *al.*, 2022b). Under stressful conditions, GSH levels rise in plants (Nahar et *al*., 2017) and it is one of the plant's adaptive methods for combating and tolerating stress (Gong et *al*., 2018). Catalase activity also increases in plant under fluoride stress correlating with high concentration (Sharma and Kaur, 2019) and with exposure time (Sharma et *al*., 2019).

Xanthoria parietina (L.) Th. Fr is among the lichen species that are most used in bioindication and biomonitoring programs (Nimis et *al.*, 2001; Pisani et *al.*, 2009). Although various species, such as *Hypogymnia physodes* and *Parmelia sulcata*, are utilized in bioindication. *X. parietina* is capable of accumulating considerable levels of heavy metals due to its wide surface in contact with contaminants (Cuny et *al.*, 2004).

Our work which relates to the study of the toxic effect induced by fluorine and lead on *X. parietina*, is the first to be carried out in Jijel (North-East of Algeria). Furthermore, the work is original as there is no information about investigation of the effects of fluorine on this lichen. For this reason, we tried to study the toxic effect of this halogen on the metabolism of this lichen, based on several stress indicators, and to compare its toxicity with that of lead.

The purpose of this study is to:

• Investigate the toxic effect of fluorine which is in the form of sodium fluoride (NaF) and lead which is in the form of lead nitrate ($Pb(NO_3)_2$) on the lichen *X. parietina* by measuring chlorophyll, protein content, catalase, hydrogen peroxide (H_2O_2), and reduced glutathione (GSH) as stress biomarkers.

• Explore the toxic effect of fluorine and lead on lipid peroxidation and chlorophyll integrity in *X. parietina* and to check for the accumulation of proline and soluble sugars as indicators of responses used for adaptation to induced stress.

• Examine the variations of polyphenol and flavonoid contents in *X. parietina* and to test in vitro the antibacterial activity of its methanol extracts under fluorine and lead stress.

Literature revue

I. General data on lichens

I.1. History and definition

Lichenology is a branch of biology; it is interested in the study of lichens. It is a discipline covering several aspects of these organisms: taxonomy, morphology, physiology, the algae-fungus relationship, ecology and bio-indication (Kranner et *al.*, 2009). The term lichen is of Greek origin (hence its pronunciation likèn), it was found for the first time in the writings of THEOPHRASTE (IV century before our era) who also designates the plants growing on the trunks of trees (Ozenda and Glauzade, 1970). The use of lichens as bioindicators and bioaccumulators has been studied for several years, the first study was carried out by William Nylander (1822-1899) who published an article in 1866 (Les lichens du Jardins du Luxembourg) in which he studied the relationship between environmental pollution and the disappearance of several lichen species (Vitikainen, 2009).

Lichens have long been thought of as composite organisms made up of algae and/or cyanobacteria (phycosymbiont) hosted in a mutualistic relationship by a fungus (mycosymbiont): **(1)** Phycosymbiont (algae): it ensures the carbon supply of the whole by its assimilating pigments, **(2)** Mycosymbiont (The mushroom): thanks to the felting of its filaments, it provides support to the algae and prevents its dehydration. It represents more than 90 % of the lichen biomass. Within the lichen thalli, several algae species, yeasts, and even viruses have all been gradually found (Morillas et *al.*, 2022).

I.2. Lichen morphology

Lichens are thallophytes, their vegetative apparatus is a thallus representing neither leaves, nor stems, nor conductive apparatus and carrying the reproductive organs, the thallus of lichens have very varied forms among which we distinguish several major morphological types (Ozenda, 2000). The fungus partner is principally responsible for determining the three main growth types of thallus structures: foliose, fruticose or crustose. Foliose and fruticose lichens are only partially attached to the substrate through anchor-like structures like rhizines and hapters, whereas, crustose lichens, which lack the lower cortex, are entirely attached (Büdel and Scheidegger, 2008). The phytobiont and its close physical contact with the mycobiont have a significant impact on the shape of the lichenized thallus. There are five different types of lichen thalli (Figure 1).

1.2.1. Principal type of lichen thalli

Lichens are in the form of: **(1)** Crustose thalli: Phytobiont in a distinct layer below an upper mycrobiont cortical layer with no lower cortex, forming a crust closely adherent to the support (bark, rocks or earth), it is by far the most numerous (more than 4/5 of the lichens). The thallus is generally inseparable from the support. Example: *Lecanora, Pertusaria, Ochrolechia*; **(2)** Leprous thalli: Groups of phycobiont surrounded by mycobiont, result from a coherent association of granules (0.1-0.2 mm) each consisting of a bunch of hyphae associated with a few algal cells. Example: *Leparia*. **(3)** Foliose thalli: Phycobiont in a layer under an upper cortex with a discrete cortex underneath, in the form of lobes easily separable from the substrate to which they are sometimes attached by rhizines. Example: *Parmelia, Physia, Xanthoria*; **(4)** Filamentous thalli: Phycobiont surrounded by a sheath of mycobiont possessing the appearance of pads, they are in the form of very fine filaments, tangled and spread on the support. Example: some species of *Usnea*; **(5)** Fruticose thalli: Shrub; erect, vertical or dragging; radial structure, often attached to the base, with the phycobiont in a layer inside the outer cortex, either tufts hanging from the trunk or branches of trees, or tufts of stems. Example: *Usnea,* and *Ramalina* (Elkhateeb et *al.*, 2022).

I.2.2. Other thalli

Lichen thalli can be presented in three other forms (Figure 2): **(1)** Gelatinous thalli: These are the ones that contain cyanobacteria. The thallus is in the form of a simple structure with little differentiation. Example: *Collema, Ephebe, Lichina, Placodium, Leptogium*; **(2)** Squamulose thalli: They are intermediate thalli between crustose and foliose thalli, are found in the form of more or less close together or overlapping scales, on board not adhering to the support. Example: *Psoradecipien, Hypocenomycescalaris, Normaninapulchella*; **(3)** Composite thalli: The composite thallus is a component of two thalli: the first is crustose, squamulose thallus or foliose, more or less spread out on the substrate. The second of a fructicose nature, growing perpendicular to the substrate. Example: *Cladonia* (Büdel and Scheidegger, 1996).

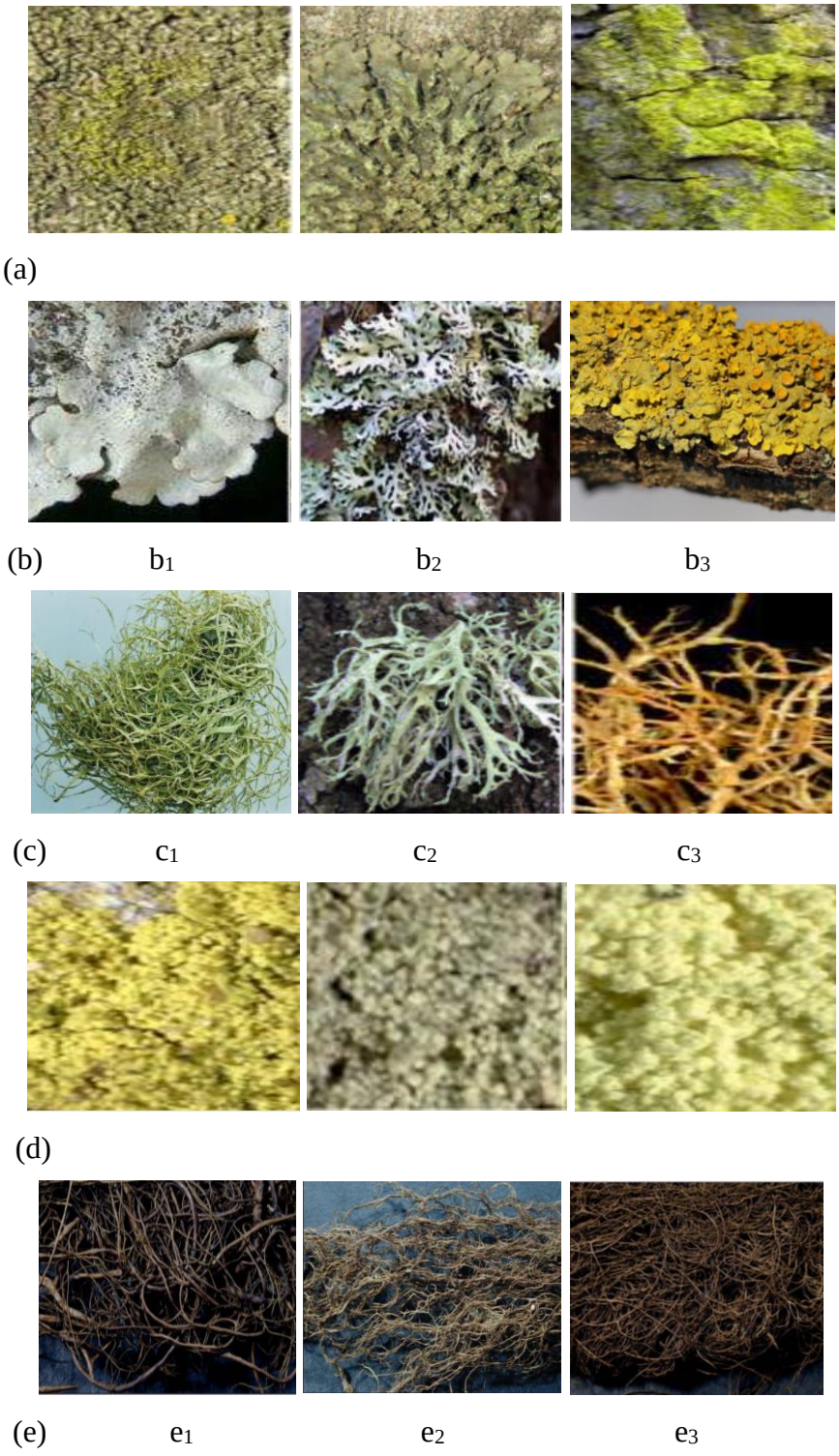

Figure 1. Growth-forms and general appearance of principal type of lichen thalli; (a): Crustose lichens, (b): Folisose lichens (b_1: *Parmelia*, b_2: *Hypogymnia*, b_3: *Xanthoria*), (c): Fructicose lichens (c_1: *Usnea*, c_2: *Ramalina*, c_3: *Teloschistes*), (d): Leprose lichens, e: Filamentous lichens (e_1: *Bryoria fremontii*, e_2: *Bryoria fuscescens*, e_3: *Bryoria pseudofuscescens*) (Alison, 2006; Nimis et *al.*, 2017)

(a)

(b)

Figure 2. Growth-forms and general appearance of squamulose and gelatinous lichens; (a): Squamulose lichens (*Leptogium species*), (b): Gelatinous lichens (Nimis et *al.*, 2017)

I.3. Lichen ecology

Lichens can develop on a variety of substrates and in a variety of weather situations: **(1)** Corticolous lichens are those that grow on tree trunk and bark; **(2)** Ramicolous lichens live on twigs; **(3)** Legnicolous lichens live on wood; **(4)** Saxicolous lichens live on rocks and boulders; **(5)** Muscicolous lichens live on moss; **(6)** Terricolous lichens live on soil; **(7)** Foliicolous lichens live on evergreen leaves (epiphyllous). Any lichen that grows on another plant is generally referred to as epiphytic (Nayaka, 2005).

I.4. Lichen reproduction

Lichens frequently combine sexual and vegetative reproduction. Vegetative means can simply involve thallus fragmentation or specialized organs like soredia or isidia. The fungal partner is often the sole one involved in sexual reproduction, and the variety of fruiting bodies or ascomata (sing. ascoma) is astounding. Not only that, but they also differ considerably among species and higher taxonomic levels like genus and family in terms of internal architecture such as tissue arrangement, spore sacs (known as asci), and the spores themselves (Perlmutter, 2009).

I.4.1. Vegetative Reproduction

The lichen complex can spread globally, either in the form of thallus fragments, or through the set of special organs, soralia and isidia (Figure 3). A soralia is a crack in the bark (formed by the fungus) at the level of the medulla of the lichen which resolves into a floury dust of soredia which are glomeruli formed of a few cells of algae surrounded by a bunch of hyphae of very small size and easy dispersal. While the isidia are small buds carried on the surface of the thalli, these isidia, which are heavier than the soralia, cannot be transported as far, they rather ensure colonization of the substrate, they are generally considered as organs of multiplication (Ozenda, 2000).

I.4.2. Sexual reproduction

It is carried out by the production of spores of the fungus, which, by germinating then give hyphae which capture the algae. Indeed, two sexually differentiated fungal hyphae merge and give the surface of the thallus structures in the form of buttons (apothecia), or more or less closed cups (perithecia) (Figure 3), in which specific cells (asci) will develop ascospores (generally 8 spores per ascus but the number can vary, and post-miotic mitoses make it possible to obtain in certain cases 32, 64, … ascospores, or much less) (Honegger, 1998).

I.5. Composition of lichen substances

Primary and secondary lichen compounds can be separated into two categories: Primary lichen compounds play structural and metabolic roles in biological processes. The majority of these are found in other plants. Lichens create a wide variety of peculiar secondary products that are not present in other plants. The functions of secondary lichen compounds are still not fully understood. They most likely function as antibiotics (acids), photosynthesis-related molecules (atranorin), light

a b c d

Figure 3. Organs for vegetative and sexual reproduction; a: soredia, b: insidia, c: apothecia, d: perithecia (Nimis et *al*., 2017)

filters (parietin), or agents that speed up the transfer of carbohydrates from the photobiont to the mycobiont or have roles in degrading the mineral substrates (Podterob, 2008). Lichen metabolites act as major factor in metal homeostasis and pollution tolerance of lichen (Bhattacharyya et *al.*, 2016).

I.5.1. Primary metabolites

Primary metabolites include proteins, amino acids, lipids, carbohydrates, polyols, carotenoids, vitamins, and other organic compounds vital to lichen metabolism and structure (Elix and Stocker-Wörgötter, 2008; Mitrović et *al.*, 2011).

I.5.2. Secondary metabolites

A wide variety of chemical groups are represented in lichen secondary metabolites (Nayaka and Haridas, 2020), such as aliphatic acids, sugar alcohols, quinines, chromones, xanthones, dibenzofurans, depsides, depsidones, depsones, terpenoids, steroids, carotenoids, and diphenyl ethers (Yousuf et *al.*, 2014). Secondary metabolites are produced by mycobionts, which then store them in the cortex or the medullary layer (Calcott et *al.*, 2018).

I.6. Uses of lichens

I.6.1. Medicinal uses of lichens

In traditional medicine, several lichens such ae *Usnea, Evernia, Cetraria Cladonia Ramalina Lobaria, Peltigera* and *Xanthoparmelia* are most frequently used by cultures across the world, particularly in temperate and arctic regions to treat wounds, skin problems, respiratory, and obstetrical difficulties. They have been used for both their carbohydrate and secondary metabolite contents (Crawford, 2015). Likewise, due to its bioactive compounds, lichens are used as antimicrobial and antioxidant agents (Elkhateeb et *al.*, 2021) also as antiviral agent (Bhattacharyya et *al.*, 2016). Currently, lichens are employed to treat a variety of human illnesses because of their anti-cancer, antigenotoxic, anti-inflammatory, analgesic and antipyretic activity (Ranković and Kosanić, 2019; Nugraha et *al.*, 2019; Šeklić et *al.*, 2022). In addition, the lichen's metabolites are being tested for their potential as hepatoprotective, cardiovascular protective, gastrointestinal protective, antidiabetic, and probiotic agents (Nayaka and Haridas, 2020).

I.6.2. Uses of lichens as food

Because of its richness in polysaccharides, certain enzymes and some vitamins, some lichens serve as important source of food for animals (*Cladonia rangiferina, Cetraria islandica,*

species of *Stereocaulon*, *Evernia*, *Parmelia* and *Lecanora*) and humans during famine (*Parmelia* species, *Lecanora esculenta, Cetraria islandica, Lecanora esculenta, Umbilicaria esculenta*) (Elkhateeb et *al.*, 2022).

I.6.3. Uses of lichens for mineral production

Lichens living on rocks can pull oxalate and turn it into oxalic acid particularly calcium oxalate monohydrate and calcium oxalate dihydrate (Marques et *al.*, 2016).

I.6.4. Uses of lichens as bio indicators of air pollution

The possibility that lichens are sensitive to air pollution was suggested as early as 1866 from observations made around Paris, France. Since then, several studies have been carried out in large urban areas on several continents, whose gaseous substances most involved in the decline of lichens appear to be sulphur dioxide which mainly affects the algal component by disrupting the process of photosynthesis and various fluorine compounds (Weaver, 1975). Because they are sensitive to changes in temperature, air pollution, and water availability, lichens are excellent markers of planetary change (Bajpai et *al.*, 2018). Their biodiversity is substantially impacted by even minor environmental changes (Eldridge and Delgado-Baquerizo, 2018). According to the researchers, urbanization and environmental pollution appear to have an impact on every functional aspect of lichens (Koch et *al.*, 2019).

Lichens have the ability to capture mineral nutrients from wet deposits (Gauslaa et *al.*, 2021), and to absorb significant amounts of trace elements and heavy metals from the atmosphere (Caggiano et *al.*, 2015; Darnajoux et *al.*, 2015; Węgrzyn et *al.*, 2016; Winkler et *al.*, 2019; Rola, 2020; Vannini et *al.*, 2021). Because of their physiological, morphological, and structural changes when exposed to atmospheric contaminants, lichens are one of the best bio-indicators and biomonitors of air pollution (Kularatne and de Freitas, 2013; Ite et *al.*, 2014; (Matos et *al.*, 2015; Conti and Tudino, 2016; Demková et *al.*, 2017; Abas, 2021; Tarawneh et *al.*, 2021).

I.7. Brief description of *X. parietina*

I.7.1. Morphology and taxonomy

X. parietina was first scientifically described by Carl Linnaeus in 1753, as *Lichen parietinus* (Linnaeus, 1753). It is a foliaceous thallus lichen with a color almost always yellow; and tends towards green when it is in the shade and towards orange when exposed in full sun (Figure 4). Orange parietin is the most important anthraquinone pigment produced by *X. parietina*, this

pigment is deposited as tiny crystals in the upper layer of the upper cortex (Solhaug et *al.*, 2003). *X. parietina* also produces the metabolite 2-methoxy-4,5,7-trihydroxy-anthraquinone (Ivanova et *al.*, 2000).

Thallus of *X. parietina* is composed of large, slightly wrinkled lobes that overlap. The underside of the thallus is whitish and has some rhizines (false rootlets, from the welding of hairs, fixing the thallus to its support). *X. parietina* is widespread in Europe, Africa, Asia, Australia and America (Lindblom, 1997; Brodo et *al.*, 2007) on different phorophytes especially woody plants in areas with high levels of nitrogen deposition (Fraser et *al.*, 2016).

Figure 4. The image represents the lichen *Xanthoria parietina* (L.) Th. Fr (Tsurykau et *al*., 2020)

X. parietina is a symbiotic association between a filamentous fungus of the genus *Ascomycotina,* and a green algae genus *Trebouxia* (Itten and Honegger, 2010). The green algae genus *Trebouxia* is home to the photosynthetic symbionts, or photobionts, associated with *X. parietina*. *Trebouxia* is represented by *T. arboricola* and *T. irregularis* species. Given that they have been discovered on both *X. parietina*–colonized bark and bark that hasn't been colonized by lichens, it is known that both of these photobionts occur freely in nature (Bubrick et *al.*, 2006).

Molecular analysis of *X. polessica* and the recently described species *X. coomae,* in addition of morphological characteristics (size of the thallus and lobe, their color, position apothecia, the shape of the ascospores and width of ascospore septum) clearly indicate that both taxa should be considered as synonyms of *X. parietina* (Table 1) (Tsurykau et *al*., 2020).

Table 1: Taxonomy of *X. parietina* (Tsurykau et *al.*, 2020)

Scientific classification	
Kingdom	Fungi
Division	Ascomycota
Class	Lecanoromycetes
Order	Telochistales
Family	Telochistaceae
Genus	*Xanthoria*
Species	*X. parietina*
Binomial name	
Xanthoria parietina (L.) Th. Fr (1860)	
Synonyms	
Xanthoria coomae	
Xanthoria polessica	

I.7.2. Reproduction of *X. parietina*

By use of symbiotic vegetative propagules such soredia, isidia, and blastidia, a large number of lichens disperse very effectively. However, *X. parietina* produces neither isidia nor soredia. It is capable of producing vegetatively through fragmentation, however, the frequency with which this species' thalli are discovered in apothecia suggests that sexual reproduction is highly valued in this species (Meier et *al.*, 2002).

I.7.3. Use of *X. parietina*

Thanks to its intermediate sensitivity to atmospheric pollutants (Agnan et *al.*, 2017), *X. parietina* can tolerate damage caused by air pollutants by certain types of defense mechanisms such as increased polyphenol and GSH contents, soluble sugars and proline accumulation and detoxification of active oxygen forms, in addition to the antioxidant role endowed by parietin

(Silberstein et *al.*, 1996). These properties allow us to qualify it as a broad-spectrum species of tolerance to air pollutants (Lorenz et *al.*, 2022). *X. parietina* can also be regarded as a source of unique chemicals compounds, particularly bioactive secondary metabolites, which can be employed for antibacterial activity (Karagöz et *al.*, 2009). In the past, due to its yellow color it was used as a remedy for jaundice (Vartia, 1973).

II. General data on fluorine

II.1. History and general properties of fluorine

After the discovery of sodium and potassium by the English chemist Humphrey Davy (1778-1829), André-Marie Ampère (1775-1836) grasped the idea that chlorine and fluorine were both chemical elements but did not publish his hypotheses. He pointed to the analogies between hydrochloric acid and hydrofluoric acid and concluded that an element first called oxy-fluoric and then fluorine must exist. On 1st November 1810, André-Marie Ampère even suggested the possibility of isolating the element fluorine by electrolysis of anhydrous hydrofluoric acid. In 1813, he announced that a new element had been discovered (Moissan, 1886).

Fluorine, the most electro-negative element of the halogen group, its electronegativity in the Panling scale is 4 against 3.5 for O_2 and 3 for Chlorine. Fluorine is a chemical element with an atomic number 9 and is denoted by F. While Fluorine gas is an elemental form of the element fluorine at standard temperature and pressure. The fluorine gas formula is F_2. Fluorine gas doesn't exist freely in nature due to its high reactivity. Fluorine combines directly with all elements other than oxygen and nitrogen, reacts vigorously with most oxidizable substances and organic compounds, which explains its toxicity (Chappuis, 1991).

II.2. Sources of fluoride

Parent rock is the main natural source of inorganic fluorides in the soil. Fluoride is naturally released into the environment by mineral weathering, volcanic emissions, and marine aerosols (Fuge, 2018). Different industrial uses exist for inorganic fluorine compounds. Fluoride is a chemical that is used in the manufacturing of aluminum, as a flux in the production of steel and glass fiber, and as a waste product that is released into the air, water, and land. Additionally, it may be discharged into the environment during manufacturing phosphatic fertilizers, bricks, tiles, and ceramics (Bonvicini et *al.*, 2006; Walna et *al.*, 2007).

II.3. Fluorine and main derivatives

II.3.1. Gaseous mineral compounds

Gaseous forms of fluorine include: **(1)** Fluorine: a yellow-green gas at ordinary temperature, (fluorine 18) prepared in nuclear reactors and used in animal experiments (Chappuis, 1991); **(2)** Hydrogen fluoride: also called hydrofluoric acid, it is the fluorinated compound whose industrial production is the most important, it is a strong corrosive chemical that can cause intense irritation, severe burns and necrosis (eye, lung, digestive tract) (Kim and Su, 1999); **(3)** Silicilated fluorine derivatives: also called silicon tetrafluoride (SiF4): is a very toxic gas, industrial pollutant of many industries (coal combustion, aluminum industry, superphosphate plant, brickworks, tile works, glassworks...), SiF_4 is also observed in terrestrial volcanic gases and is predicted to be the major F-bearing species in low-temperature (Schaefer and Fegley, 2005).

II.3.2. Fluorides

The main forms of fluorides are: **(1)** Alkaline fluorides such as calcium fluoride (CaF_2), strontium fluoride (SrF_2), Barium fluoride (BaF), magnesium fluoride (MgF_2), lithium fluoride (LiF) and sodium fluoride (NaF) (Song and Williams, 1993). Except LiF, all alkaline fluorides are soluble in water. The most common alkaline fluoride is NaF responsible for most accidental or voluntary poisoning by the fluoride ion (confusion with edible compounds, ingestion of insecticides, fungicides ...). In addition, NaF has a detrimental effect on immunity and humoral immunity by causing the reduction of the population of T and B lymphocytes (Kuang et *al.*, 2016); **(2)** Main mineral forms: such as fluorspar (CaF_2), Cryolite (3NaF, AlF_3) and Fluor-apatite [$3Ca_3(PO_4)_2$, CaF_2] constituting phosphate rocks and frequently responsible for endemic fluorosis (Choudhary et *al.*, 2019).

II.3.3. Organic fluorine compounds

The organic fluorine compounds have attracted the attention of scientists in recent decades. This is largely due to their widespread applications in pharmaceuticals, agrochemicals, and functional materials (Li et *al.*, 2018) like: **(1)** Chlorofluorocarbones (CFCs), commonly referred to as Freon, generally non-toxic. These are gases or more rarely liquids used as refrigerants, solvents and, until recent years, as aerosol propellants (Mishra et *al.*, 2022); **(2)** Fluorocarbones: characterized by a high gas-dissolving capacity (oxygen, carbon dioxide, inert gases), low viscosity, and chemical and biologic inertness. The reason why they are used therapeutically as

oxygen transporters instead of hemoglobin (van Hulst et *al.*, 2008); **(3)** Fluorinated anesthetics: widely used as an anesthetic agent, these are enflurane, isoflurane, sevoflurane, methoxyflurane, and fluorinated hydrocarbons such as halothane (Chen et *al.*, 2015).

II.3.4. Natural organic compounds

They are rare, including fluorooleic acid and fluoroacetic acid found in some plants. After ingestion, fluoroacetate is rapidly converted to the more toxic fluorocitrate (Talcott, 2018), while sodium fluoroacetate, very toxic, is used as a rat poison (Goncharov et *al.*, 2020).

II.4. Use of fluorine

Fluorine is a trace element since it represents only 0.0037 % of the human body, to do this, it is beneficial for human health in low concentrations, but is toxic in excess (Fordyce, 2011).

II.4.1. Caries and periodontal disease

The beneficial effect of fluoride derivatives in the prevention of tooth decay has been known since 1930, fluoridation of water or salt prevents tooth decay at the communal level as well as on an individual level through use at home and professional application (Pollick, 2018). While low levels of NaF exhibit a pharmacological role in the inflammatory response against the development and progression of periodontal disease (Kim et *al.*, 2019).

II.4.2. Therapeutic use of fluorine

Fluorouracil is used to treat actinic and solar keratosis, as well as carcinomas of the head and neck, colon, rectum, breast, stomach, bladder, and pancreas (Vardanyan and Hruby, 2006).

Due to its capacity to increase the lipophilicity of the molecule, fluorine greatly affects the hydrophobic interaction between the drug molecule and the receptor, for this reason the fluorine atom is used in the production of several therapeutically helpful medications combating numerous life-threatening disorders (Gupta, 2019).

Fluorinated molecules are widely used in bioengineering and nanotechnology due to its properties in forming C-F bonds, which can adapt membrane permeability and improve the pharmacokinetic properties of drugs (Zhang et *al.*, 2022a). The most common example of fluorination is that of fluorination of steroids, this one has been very remarkable especially in the anti-inflammatory field, this is the case of 9α- and 6α- fluorosteroids used in the treatment of rheumatoid arthritis and fluticasone propionate used as an anti-inflammatory in allergic rhinitis and asthma (Filler and Saha, 2009; Yamazaki et *al.*, 2009).

II.4.3. Prevention of osteoporosis and otosclerosis

Once bone has been destroyed, fluoride offers the greatest potential as an osteoporosis treatment, essentially common postmenopausal osteoporosis (Pak et *al.*, 2009). Rich and Ensinck first used sodium fluoride in the treatment of osteoporosis in 1961 (Kleerekoper, 1998). Low quality research suggests that sodium fluoride may be benefit to preserve hearing and reduce vestibular symptoms in patients with otosclerosis (Cruise et *al.*, 2010).

II.5. Toxicokinetic of fluorine

II.5.1. Absorption

Fluorine absorption can be done by: **(1)** Inhalation exposure: specific for hydrogen fluoride or mixtures of hydrogen fluoride and fluoride, this type of exposure is one of the industrial accidents that causes serious damage (Lee and Jeong, 2021); **(2)** Oral exposure: the gastrointestinal system easily absorbs soluble fluoride compounds such as sodium fluoride, hydrogen fluoride, and fluorosilic acid (Whitford, 1996), about 80 to 90 % of fluoride ingested through this route by passive diffusion (Zohoori and Duckworth, 2017); **(3)** Dermal exposure: the rapid entry of fluoride ions into the epidermis by hydrofluoric acid results in soft tissue necrosis, decalcification, and bone deterioration (Dennerlein et *al.*, 2016). Accidental cutaneous exposure to anhydrous hydrogen fluoride has been associated with systemic fluoride poisoning.

II.5.2. Distribution

After ingestion, fluorides pledge the plasma with maximum absorption within 60 minutes of exposure initiation (Buzalaf and Whitford, 2011). Once in the blood plasma, fluoride is distributed, to soft and mineralized tissues and reversibly incorporated mainly in bone, being released back to plasma during bone remodeling (Ekstrand, 1996; Whitford, 1996).

II.5.3. Excretion

About 60 % of fluoride that has been ingested by healthy adults is eliminated in urine via the kidneys, compared to about 45 % for children (Villa et *al.*, 2010; Buzalaf and Whitford, 2011). Most of the fluoride not absorbed from the stomach is absorbed from the small intestine and eliminated in feces (Buzalaf and Whitford, 2011). Fluoride is also excreted in sweat (Fawell et *al.*, 2006), and saliva (Ingram et *al.*, 2005).

II.6. Toxic effects of fluoride

Due to the excessive intake of fluoride through drinking water, fluoride toxicity adversely affects physiological and biochemical parameters in either agricultural crops, plants, animals, or human consumption (Tak, 2018).

II.6.1. Toxic effects on the environment

The use of chlorofluorocarbones (CFCs) as aerosol propellants expected to decrease and even disappear due to the action of freon components on atmospheric ozone (Mishra et *al.*, 2022). Fluorinated gases (F-gases) used as refrigerants, blowing agents and electrical insulators are powerful greenhouse gases and, therefore, their release into the environment creates a significant contribution to global warming (Sheldon and Crimmin, 2022).

II.6.2. Human toxicity

Fluoride has recently been grouped with hazardous metals (lead, methylmercury, arsenic) and polychlorinated biphenyls in epidemiological research, because of their adverse effects obtained in animal experiments, in particular on developmental toxicity, and the molecular mechanisms by which it can cause effects. Given the extensive fluoridation of drinking water and the ubiquitous use of fluoride in dental hygiene products like toothpaste, if this evaluation is accurate, it would be quite pertinent (Guth et *al.*, 2020). Acute Toxicity may be caused by accidental ingestion or, for suicidal purposes, of sodium fluoride, fluorosilicate, hydrofluoric acid or fluorosilic acid solutions, the symptoms of which are vomiting, diarrhea, salivation, respiratory arrest, cardiac depression, convulsions and leading coma (Norman and Arden, 1991).

Dental fluorosis is among the most well-researched long-term effects of fluoride in people (EFSA, 2013). Indeed, excessive incorporation of fluoride into tooth enamel before teeth eruption leads to hypomineralization of developing teeth. This problem is found when fluoride intake exceeds a concentration in drinking water of approximately 1 mg/L of fluoride, under conditions where drinking water is the only relevant source of fluoride. Another example of the long-term effects of fluoride is skeletal fluorosis (osteosclerosis and other bone abnormalities) caused by persistent fluoride toxicity, which results in excruciating pain and weakness, due to exposure to the environment or industry (Choubisa, 2021; Mohideen et *al.*, 2022).

Higher doses of fluoride (> 10 mg/L) can be correlated with debilitating fluorosis and carcinogenic risk. Confined studies suggest that fluoride may speed up the development of cells

that would eventually turn cancerous, but this claim is debatable because there is no conclusive evidence linking fluoride to the influence of carcinogenicity (Bajpai, 2013; Ali et *al.*, 2019). It should be mentioned that fluoride ions exhibit a number of genotoxic characteristics and may possess mutagenic effects when in prolonged contact with biological components, but there are only a few studies discussing the ability of fluoride ion to increase the level of genotoxic effects. The danger of genotoxicity associated with human exposure with fluoride compounds is still an open question (Ribeiro et *al.*, 2017; Mikheeva et *al.*, 2020)

II.6.3. Fluoride phytotoxicity

Fluoride is found in a variety of environmental matrices and even at low quantities, is one of the most phytotoxic chemical elements for plants (Banerjee and Roychoudhury, 2019). Fluoride can cause slower plant growth and browning of their leaves, generally poisoning begins with chloroses, followed by the destruction of tissues, color change and finally the appearance of necrosis. The most remarkable toxic effect of fluoride on plants is leaf damage (Figure 5); indeed, plant leaves are the site of fluorine accumulation which causes stomata closure and inhibition of photosynthesis, hence the appearance of necrosis. Fluorides also induce degradation of photosynthetic pigments, thereby inhibiting photosynthesis, interfere with active metabolism, decrease the rate of cell division and expansion which decreases the germination and development of seed (Ram et *al.*, 2014; Sharma and Kaur, 2018; Choudhary et *al.*, 2019; Pelc et *al.*, 2020; Sahariya et *al.*, 2021), length of root, length of shoot, plant height, number of leaves, size of leaf, number of flowers per plant and fruit-set percentage (Singh et *al.*, 2013; Sodani et *al.*, 2021). Fluoride toxicity also has an adverse effect on development, mineral nutrition, respiration, reproduction, and the activity of cellular enzymes (Sahariya et *al.*, 2021).

Figure 5. Leaf spots due to fluorine toxicity (Chatterjee et *al.*, 2020)

II.6.4. Toxic effects of fluoride on lichens

Fluorinated pollution can cause an almost total disappearance of some lichen species. A precise cartographic study makes it possible to establish the existence of a lichen desert in the immediate vicinity of the factories. As one moves away from it, the lichen flora gradually enriches (Perkins, 1992).

Numerous studies have shown a relationship between lichen damage and fluorine accumulation. This is because lichens are able to quickly accumulate large amounts of fluoride. This accumulation varies according to the lichen species, the time and the distance from the source of pollution (Deruelle and Lallement, 1983), climatic factors (Conti and Cecchetti, 2001), and fluoride concentration (Chakrabarti et *al.*, 2014)

In *X. parietina* under fluoride-induced stress, the color change of the pinkish gray thalli suggests that it is accompanied by a destruction of lichenic acids, this is due to the destruction of chlorophylls a and b, and transformation into phaeophytin, before the complete degradation of all pigments including carotene and xanthophyll (Deruelle and Lallement 1983; Zhang et *al.*, 2016; Yang et *al.*, 2019). Fluoride accumulation would also create favorable conditions for exosmosis causing permanent plasmolysis of lichen algal cells. This phenomenon of plasmolysis is explained by the inhibition of the metabolism of the cell wall compounds, there will be a slight dehydration resulting in the folding of the lichen upper cortex, including the necrosis formation (Figure 6). According to Nash III (1971) and Gilbert (1973), this necrosis is due to the destruction of lichenic acids.

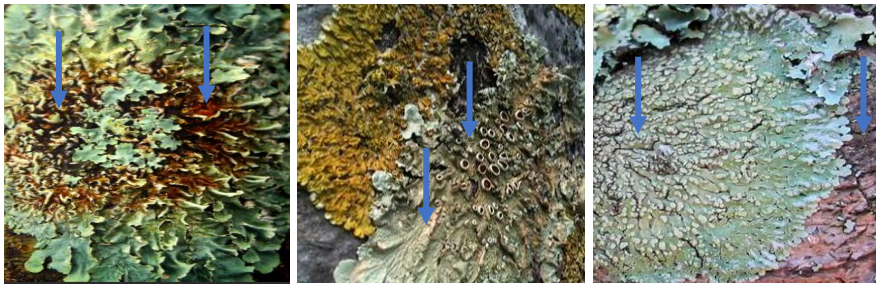

Figure 6. Fluoride injury symptoms (necrosis) in epiphytic lichens (LeBlanc et *al.*, 2011)

Fluoride also decreases germination of spore. Semadi (1989) studies have shown that low concentrations of NaF cause spore germination in *Physonia pulverulacea* lichen.

III. General data on lead

III.1. Definition and history

Lead (Pb) from the "Latin plumbum" with the atomic number 82 and atomic mass of 208, is a blue-gray non-radioactive metal which turns into gray once exposed to air, with a melting point of 327.5 °C and a boiling point at atmospheric pressure of 1740 °C. It is the most universally widespread element of heavy metals and the second most toxic heavy metal after arsenic. Lead and its oxides typically create covalent bonds when reacting with acids and bases (Boldyrev, 2018). It has been commonly used for nearly 6000 years in human activities (Papanikolaou et *al.*, 2005), and since at least 200 B.C. humans have been aware of its harmful effects, indeed Asia Minor saw the earliest known usage of lead between 6000 and 4000 B.C. (Hernberg, 2000). Meanwhile and with the huge development of population and economic growth, lead pollution has increased from an estimated 10 tons to 1 000 000 tons per year, with a global production about ten million tonnes in 2014 (Boldyrev, 2018).

III.2. Origin of lead

Lead occurs naturally in soil, water, plants and animals (Tiwari et *al.*, 2013). It is found naturally in the earth's crust at concentration of 15–20 mg/kg. In general, lead concentrations are around 5 to 25 mg/kg in soil, 1 to 60 µg in surface water, and around 1µg/m^3 in air (Lu, 1992). Lead can be emitted naturally either by erosion that carries it to soil, surface water and sediment, or by terrestrial or underwater volcanic eruptions (Popescu et *al.*, 1998). Industrial lead pollution originates from several industrial processes, especially mining and smelting, plastics, printing and phosphate industries, manufacturing batteries, metallurgy and lead refining complexes (Kapusta and Sobczyk, 2015; Zhang et *al.*, 2009; Kabir et *al.*, 2020) as well as car traffic and leaded aviation gasoline (Hashisho and El-Fadel, 2004; Lin et *al.*, 2011).

III.3. Lead, lead compounds and uses

(1) Metallic lead (Pb): lead is primarily used to create rechargeable storage batteries. Pb makes up the gray negative electrode in battery (Crompton, 2000); **(2)** Lead oxides: such as α-PbO (litharge) and β-PbO (cutter) used in the industries of glassware, enamels, drying oils of accumulators, α-PbO_2 (scrutinyite) and β-PbO_2 (plattnerite) used as energetic oxidants and make up the red color

positive electrode in battery (Crompton, 2000), and Pb_3O_4 (leas tetraoxide or minium) used in the composition of anti-rust paints (Pavlov, 2017); **(3)** Lead sulphides (PbS): formed by chemical reaction of lead acetate with thiourea at room temperature. It provides varnishes of yellow color, which can be colored brown by addition of manganese oxide or green by addition of copper oxide (Bhatt et *al.*, 2011); **(4)** Lead arsenate $PbAH_sO_4$ and lead stearate $Pb(C_{17}H_{35}COO)_2$: used in insecticides and herbicides (Gad and Pham, 2014); **(5)** Lead iodide PbI_2 and lead base acetate $Pb(CH_3\text{-}COO)_2$: used as a mordant in printing and dyeing textiles, as a lead coating for metals, as a dryer in paints, varnishes and pigment inks, and as a dye in hair dyes (Ghazi and Millette, 1964); **(6)** Alkyl derivatives of lead and organic lead compounds: such as tetraethyl lead and tetramethyl lead used as antiknock agents in gasoline (Filella and Bonet, 2017); **(7)** Lead nitrate $Pb(NO_3)_2$: used in the manufacture of matches and explosives, as a heat stabilizer in nylon, and as a coating on paper for photothermography (Nielsen, 2013); **(8)** Insoluble lead compounds: such us lead carbonate $Pb(CO_3)_2$ and lead sulfate ($PbSO_4$) used in paints synthesis (Nielsen, 2013), lead chromate ($PbCrO_4$) used as a yellow pigment in paints, rubber, plastics, and ceramic coatings (Gad and Pham, 2014), lead fluoride (Pb_3F_8) used as a pigment in ancient Rome and in anti-corrosion coatings (Bose et *al.*, 1983) with additional uses in the electronic and optical industries, lead naphthenate used as catalysts and as a varnish drier, lead phosphate and lead stearate used as stabilizers in the plastics industry and lead sulfate with zinc in galvanic batteries (Nielsen, 2013).

III.4. Toxicokinetic of lead

III.4.1. Absorption

We distinguish respiratory absorption specific to small Pb inorganic lead particles and aerosols that can be almost completely absorbed, while larger particles can be moved by mucociliary clearance to the oropharynx and swallowed (James et *al.*, 1994); gastrointestinal absorption influenced by age (40–50 % for children compared to 3–10 % for adults), diet, nutrition and physiological characteristics of Pb in the ingested medium (Ziegler et *al.*, 1978) and dermal absorption specific for inorganic forms of lead but less frequent than inhalation and oral exposure.

III.4.2. Distribution

Ingested lead undergoes the action of hydrochloric acid from gastric juice and that of bile secretions, which by determining partial solubilization and, consequently, increasing toxicity; absorption is maximum in the small intestine and duodenum. Once in the blood, lead mainly

attaches to red blood cells before being distributed to the bones (Barry,1981), lead can also be transferred from the mother to the fetus and also from the mother to infants via maternal milk (Gulson et *al.*, 1999; Li et *al.*, 2000; Papanikolaou et *al.*, 2005).

III.4.3. Metabolism

Inorganic forms of lead are not metabolized, so they form complexes with a variety of proteins and non-protein ligands, while organic compounds are actively metabolized in the liver by oxidative dealkylation by cytochrome P-450 enzymes (Philip and Gerson, 1994).

III.4.4. Excretion

About 50 - 60 % of eliminated lead is carried out mainly through the urinary and biliary excretion route (Ziegler et *al.*, 1978), lead elimination can be also done through sweat, saliva, hair, nails, breast milk and seminal fluid (Barbosa et *al.*, 2006).

III.5. Lead toxicity

III.5.1. Human toxicity

Lead poisoning originates from paints, water, food, dust, but in the majority of cases by oral ingestion and absorption through the gut. Acute lead-related toxicity is manifested by headaches, muscle tremors, abdominal cramps, kidney damage, loss of memory with encephalopathy (Papanikolaou et *al.*, 2005). Lead exposure has a negative impact on the hematological, renal, reproductive, neurological, digestive and respiratory systems. Lead also causes generation of ROS that results in critical damage to various biomolecules such as DNA, enzymes, proteins and membrane-based lipids, while simultaneously altering the antioxidant defense system (Flora et *al.*, 2012; Wani et *al.*, 2015). Once in the blood, lead can inhibit certain enzymes responsible for heme synthesis, particularly δ-aminolevulinic acid dehydratase (Sakai, 2000).

III.5.2. Phytotoxic effects of lead

In plants, lead is mainly absorbed by the root system and in minor amounts through the leaves. Once inside the plant, lead accumulates in the roots, but part of it is transferred to the aerial parts. At the cellular level, it accumulates in the wall, vacuoles and forms small deposits in the endoplasmic reticulum (Sharma and Dubey, 2005). Figure 7 shows an example of morphological distortion observed in the leaves of *Vicia faba* plants under lead stress.

Lead toxicity causes severe oxidative damage to plants, it limits the synthesis of photosynthetic pigments, proteins, and affects net assimilation rate, sweat rate, and stomatal

conductance (Arif et *al.*, 2019). Lead also reduces rate of seed germination, and plant growth, and causes retardation of carbon metabolism and blocks potassium in plants which causes membrane damage and stomatal closure (Zulfiqar et *al.*, 2019). Lead stress also inhibits activities of several enzymes especially that of the Calvin cycle (Sharma and Dubey, 2005), reduces gas exchange attributes and induces the oxidative stress markers and activities of antioxidant enzymes (Bamagoos et *al.*, 2022).

Figure 7. *Vicia faba* leaves grown on Hoagland nutrient solution under Pb stress, 0.0 mM (left) and 48 mM (right) (Kamel, 2008).

III.5.3. Toxic effects on lichens

Despite lichens retain high concentrations of lead extracellularly, mainly attached to cell walls and extracellular polymers, this metal has been shown to enter cells especially in fungal cortical compartments (Branquinho et *al.*, 1997), once inside the cell, the first important sign of its toxicity is the reduction of photosystem II (PSII) photochemical reactions, and consequently the reduction of integrity and chlorophyll content. Unlike fluoride which shows symptoms related to lesions in epiphytic lichens (Figure 6), the accumulation of heavy metals, with the exception of Cd, did not show significant morphological differences between stressed thalli and control (Šujetovienė et *al.*, 2019).

Lead causes membrane damages, especially through water loss and ion leakage (Expósito et *al.*, 2019), indeed, lipid peroxidation, which is one of the main consequences of prooxidant pollutants examined in thalli lichen, can cause these impairments (Álvarez et *al.*, 2015; Gurbanov and Unal, 2019). Indeed, membrane damage results in the degradation of its phospholipids which accumulate in the form of MDA residues (Zoungranan et *al.*, 2019).

Lead has the ability to stimulate the formation of ROS (Kováčik et *al.*, 2018; Wieners et *al.*, 2018) that react with several vital biomolecules of the species causing serious morphological, metabolic and physiological abnormalities (Zoungranan et *al.*, 2019). To neutralize and trap free radicals produced by lead, lichens accumulate polyphenols, usnic acide, cysteine and tripeptide glutathione, proline, soluble sugars, GSH and catalase (Kováčik et *al.*, 2017; Expósito et *al.*, 2019; Gurbanov and Unal, 2019).

Experimental part

I. Oxidative stress induced by fluorine and lead in *X. parietina*

I.1. Objective

The purpose of this study is to investigate the toxic effect of fluorine which is in the form of sodium fluoride (NaF) and of lead which is in the form of lead nitrates ($Pb(NO_3)_2$) on the lichen *X. parietina* by assaying different biomarkers of stress : chlorophyll, protein content, catalase, hydrogen peroxide (H_2O_2) and reduced glutathione (GSH).

I.2. Material and methods

I.2.1. Plant material

During February and March 2017, the sample thalli of *X. parietina* were collected in Djimar, Beni Metrane, and Bouaafroun regions located in Jijel North-East of Algeria (Figure 8), in an abandoned orange grove that spanned several hectares. The sites were chosen because they are in the rural areas far from any urban or industrial region. Collected thalli were transported to the laboratory in sterilized closed boxes and rinsed with distilled water to remove adherent particles. The thalli were then isolated from their supports and stored under laboratory conditions until analysis.

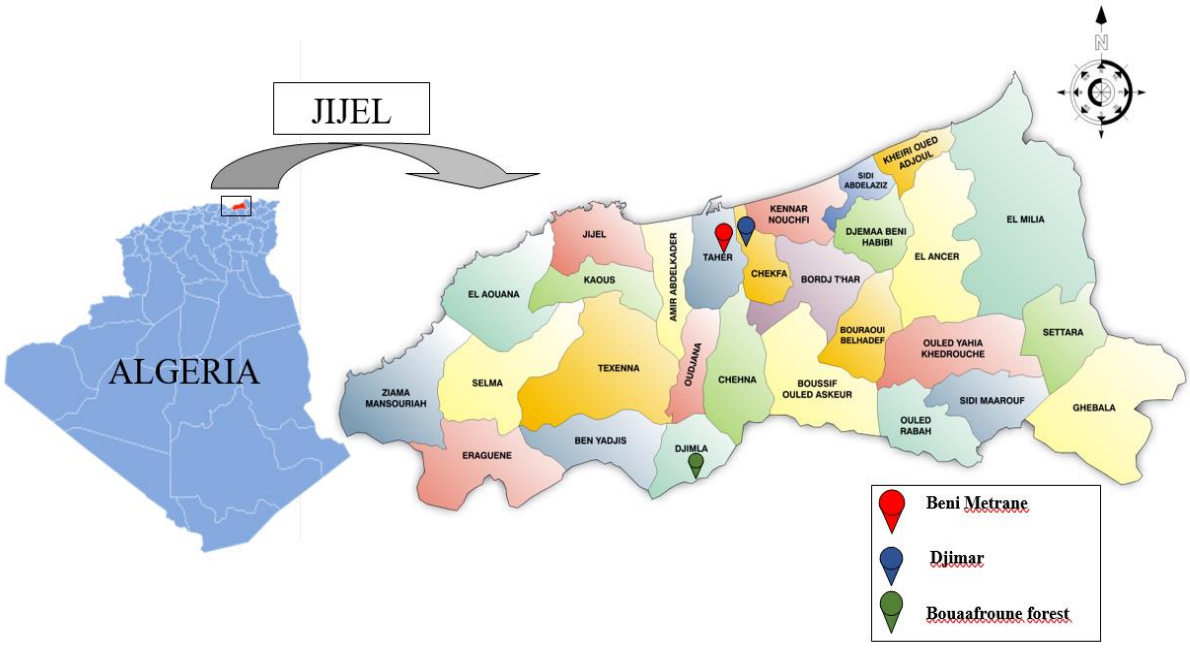

Figure 8. Localization of sampling sites

I.2.2. Fluoride and lead treatment

Under the binocular microscope, lichens were thoroughly cleaned. In each experimental vessel, 3 g of fresh thalli were soaked in 100 mL of 0.5, 1, 5, and 10 mM NaF and $Pb(NO_3)_2$ solutions and compared to control samples soaked in deionized water (0 mM). H_2SO_4 (9 M) or HNO_3 (11.19 M) were added to the solutions immediately before treatment to adjust pH to 3.5 (Carreras and Pignata, 2007). These solutions were then incubated in the dark for 0, 24, 48, and 96 hours at room temperature. They were then filtered and rinsed three times with deionized water for five seconds each time. Each experiment was carried out three times. At t0 and after 24, 48, and 96 hours, lichens were stored to perform the following varied dosages: chlorophyll, protein, catalase, H_2O_2, and GSH dosages.

I.2.3. Lead analysis

Flame atomic absorption spectrometry was used for the determination of lead. After drying at 90°C for 24 h, 3 mL of concentrated HNO_3 and H_2O_2 (2:1, v/v) was used to digest 100 mg of dry matter for 48 h (Dzubaj et *al.*, 2008). After filtration through a Whatman Filter paper N° 42, the mixture was completed to 10 mL with deionized water. Lead analysis was performed with the Shimadzu AA 6200 Flame Atomic Absorption Spectrophotometer. To quantify the accumulated lead, the internal stock was used to create a calibration curve. Results were expressed in $\mu g.g^{-1}$ (dry weight).

I.2.4. Chlorophyll and pheophytin analysis

To determine the content of chlorophyll a (C_a), chlorophyll b (C_b) and total chlorophyll (C_{a+b}), the method described by Lichtenthaler (1987) was used. The concentration of 80% acetone was used for the maceration of the fresh lichen sample; the maceration extract was then filtered and read by a spectrophotometer at A_{663} and A_{645}. Chlorophyll content was calculated according to the following equation:

$$C_a = 12.7 \times A_{663} - 2.69 \times A_{645}$$

$$C_b = 22.9 \times A_{645} - 4.68 \times A_{663}$$

$$C_{a+b} = 20.2 \times A_{645} - 8.02 \times A_{663}$$

Where A_{663}, A_{645} are absorbances at 663 and 645 nm, respectively. Results were expressed in $\mu g.\ g^{-1}$ dry wt.

Chlorophyll a/b ($c_{a/b}$) has become useful as an indicator for measuring the physiological activity of algal cells.

At 9.7 mL of chlorophyll extract above, the formation of pheophytin was favored by the addition of 0.3 mL of oxalic acid, the optical densities were read at the same two wavelengths (645 and 663 nm). Contents of pheophytin a (Ph_a), pheophytin b (Ph_b) and total pheophytin (Ph_{a+b}) were calculated according to the formula used for chlorophyll.

I.2.5. Proteins assay

The technique of Bradford (1976) was used to test the protein content. The Bradford assay is based on an absorbance shift of the dye Coomassie brilliant blue G-250. Homogenization of 100 mg of fresh weight of lichens in 2 mL of 0.05 M phosphate buffer pH 6.8 and centrifugation of the homogenate at 4 °C for 20 min at 12000 t / min were carried out. An amount of 2 mL of Bradford's solution was added to 50 µL of the supernatant. After 10 minutes, a reading was taken at 595 nm. The calibration curve was established by the BSA (bovine serum albumin) at different concentrations (0.01, 0.02, 0.03, 0.04, 0.05, 0.06, 0.07 and 0.08) mg/mL. Protein levels ($mg.g^{-1}$) were calculated using equation established by BSA ($y = 28.9$ x, $R^2 = 0.9911$).

I.2.6. Catalase activity assay

Using Chance and Maehly's method (1955), the activity of catalase was measured. Homogenization of 50 mg fresh weight of lichens in 2 ml of 0.05M phosphate buffer at pH 7 was made, followed by centrifugation at 15000 t / min at 5 °C for 20 minutes. 50 µL of the supernatant was added to 2.95 mL of 0.015 M H_2O_2 in the phosphate buffer. A first reading was performed immediately at 240 nm, then a second one was taken after 3 minutes. The enzymatic activity of catalase was calculated using the following formula:

$k = 2.303 / T \times \log (A_1/A_2)$

Of which:

K: the reaction rate constant**.**

T: Time interval in minutes.

A_1: Absorbance at t = 0.

A_2: Absorbance after 3 min.

1.2.7. H_2O_2 determination

Method of Sagisaka (1976) was used to determine the concentration of H_2O_2. Approximately 1g of lichen fresh material was homogenized with 2 mL of 5% trichloroacetic acid (TCA). The resulting mixture was centrifuged at 14000 g for 20 minutes at 0 °C. The amount of 1.6 mL of supernatant was added to 0.4 mL of TCA (50 %) and 0.4 mL of ferrous ammonium sulfate (1%) and 0.2 mL of potassium thiocyanate (1 %). The optical density at 480 nm was used to determine the amount of H_2O_2 in the supernatant. The H_2O_2 level (mmol. g^{-1} (dw)) was determined using an equation established by known concentrations of H_2O_2 standard ($y = 0.1864$ x + 0.2281, $R^2 = 0.09691$).

I.2.8. GSH assay

GSH assay was carried out by the colorimetric method described by Ellman (1959). In the presence of 5.5'-dithiobis 2-nitrobenzoic acid (DTNB), GSH is oxidized by releasing thionitro-benzoic acid (TNB) which shows absorbance at 412 nm. Homogenization in 50 mM phosphate buffer (pH 6.5) and centrifugation at 12000 g for 15 min at 4 °C of lichen thalli were carried out. For this assay, the absorbance at 412 nm of a mixture containing 100 µL of the supernatant and 1200 µL of DTNB solution was read. Results were expressed in mmol. g^{-1} using an equation established by known concentrations of GSH standard ($y = 0.2012x + 0.3852$, $R^2 = 0.9573$).

I.2.9. Statistical analysis

For the purpose of calculating the standard deviation (SD), three repetitions were carried out at each concentration. The ORIGIN 6.0 system's test univariate variance (one way ANOVA) was used to conduct the statistical analysis. Results were expressed as mean ± SD (standard deviation). The distinction was deemed insignificant when $p > 0.05$(NS), significant when $0.01 < p < 0.05$ (*), very significant when $0.001 < p < 0.01$ (**) and highly significant when $p < 0.001$ (***).

STATISTICA Version 10 software was used to analyze correlation matrices between NaF, $Pb(NO_3)_2$ and the different studied parameters.

I.3. Results

I.3.1. Lead accumulation

The accumulation of lead in treated lichens was increased with increasing exposure time. Figure 9 shows that treatment with rising $Pb(NO_3)_2$ concentrations resulted in a progressive buildup of lead.

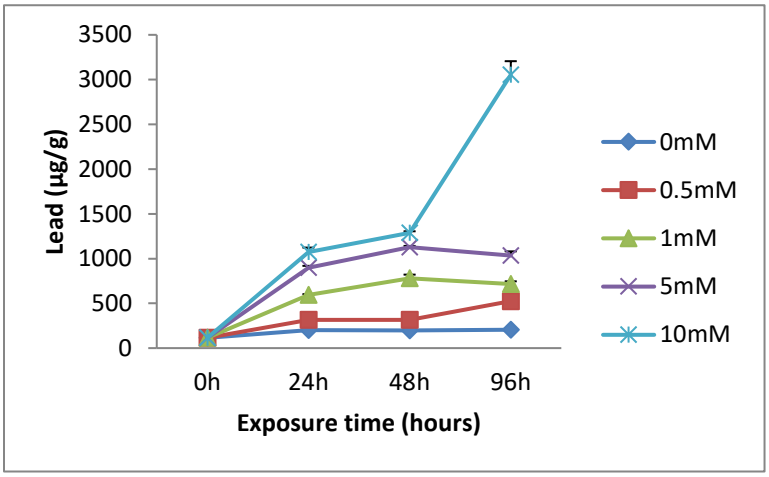

Figure 9. Variations of accumulated lead in thalli of *X. parietina* under $Pb(NO_3)_2$ stress

According to Figure 9, we found an important accumulation of lead in *X. parietina* from $Pb(NO_3)_2$ solutions, but this accumulation is not significant in thalli treated with the various concentrations after 24 and 48 h of exposure. However, significant increase in built-up lead in thalli treated with 10 mM $Pb(NO_3)_2$ was observed after 96 h of treatment (p = 0.018*), with a highest value of 3052 µg/g compared to 205.21 µg/g in the control test.

I.3.2. Chlorophyll content variations

Variations in C_a, C_b and C_{a+b} contents in *X. parietina* under NaF and $Pb(NO_3)_2$ stress are shown in Figures 10, 11 and 12, respectively, whereas, $C_{a/b}$ ratio variations in thalli treated by NaF and $Pb(NO_3)_2$ are presented in Tables 2 and 3, respectively.

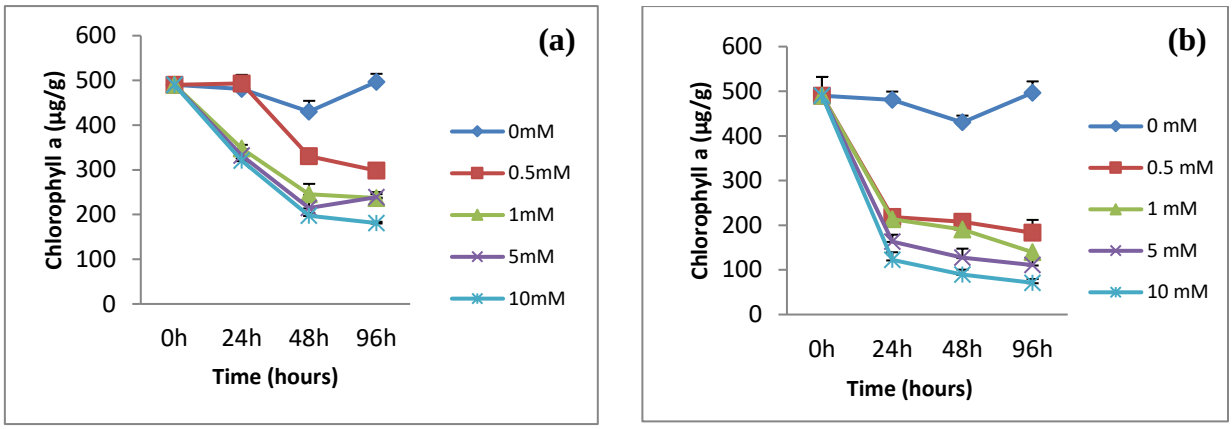

Figure 10. Chlorophyll a content variations in *X. parietina* after treatment of thalli by NaF (a), and $Pb(NO_3)_2$ (b) solutions

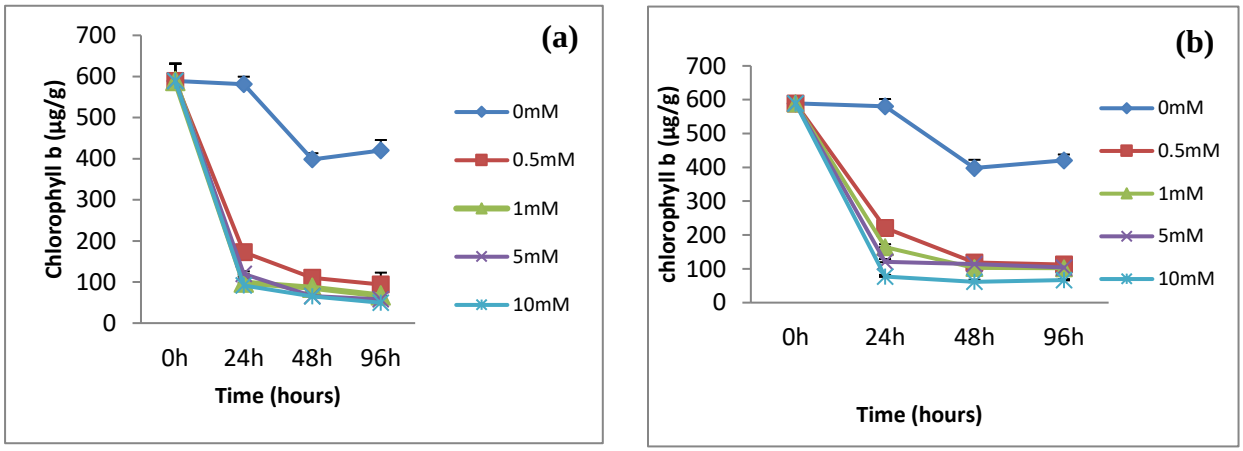

Figure 11. Chlorophyll b content variations in *X. parietina* after treatment of thalli by NaF (a), and $Pb(NO_3)_2$ (b) solutions

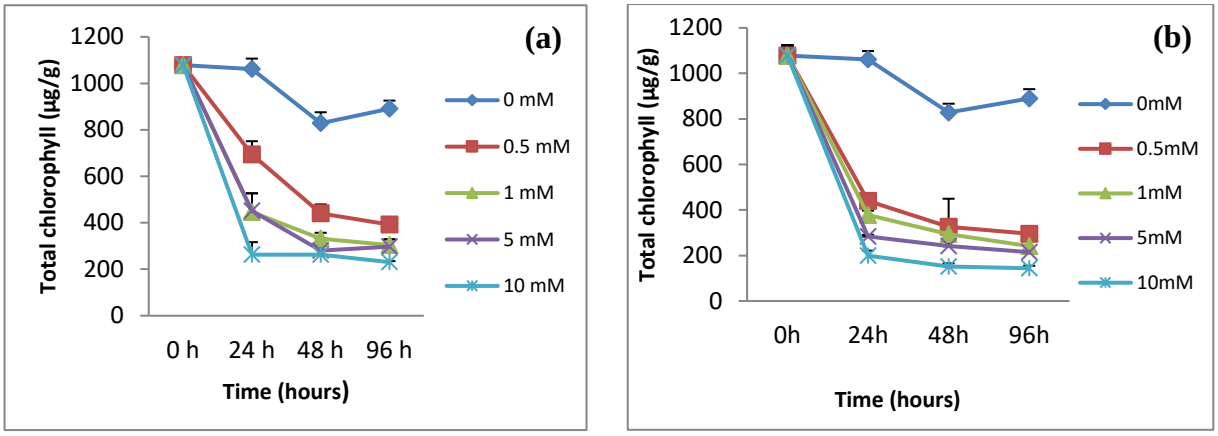

Figure 12. Total chlorophyll content variations in *X. parietina* after treatment of thalli by NaF (a), and $Pb_2(NO)_3$ (b) solutions

Lichens incubated in distilled water show the highest levels of C_a and C_b. Figure 10 (a) shows a significant decrease in C_a content as a function of different concentrations of NaF (p = 0.0052**) and as a function of exposure time (p = 0.0031**) within 48 hours of treatment. However, variations in C_a levels are not significant between 48 h and 96 h of exposure time (p > 0.05). Figure 10 (b), shows a significant decrease of C_a (p = 0.04*) after 24 h of treatment by $Pb(NO_3)_2$.

From the Figure 11 (a), it was noticed that all the concentrations of NaF exhibit the same effect on C_b content, where a very significant decrease was observed (p = 0.007**). According to the exposure time, a significant decrease in the content of C_b was also noted in the 24 h following the treatment (p = 0.0037**), between 48h and 96 h of treatment, C_b content variations were not significant ($p = 0.755^{NS}$). Under $Pb(NO_3)_2$ stress, Figure 11 (b) shows the same effect; a significant decrease of C_b content after 24 h of treatment (p = 0.02*) was noted, whereas, between 48 and 96 h of treatment, this decrease was not significant (p > 0.5)

Figure 12 (a) shows that the decrease in C_{a+b} content is significant as a function of exposure time (p = 0.0012**) as well as a function of NaF concentrations (p = 0.020*). According to Figure 12 (b), C_{a+b} decreases significantly (p = 0.0032**) in thalli treated with 10 mM of $Pb(NO_3)_2$ after 96 h of exposure, with a clear start-point from 24 h onward with all concentrations (0.5, 1, 5 and 10 mM).

The results presented in Figures (10, 11 and 12) allowed us to deduce that the variations in chlorophyll contents (C_a, C_b, C_{a+b}) in *X. parietina* under $Pb(NO_3)_2$ stress are much more influenced by the time factor. However, under NaF stress, these variations are significant as a function of exposure time as well as a function of NaF concentrations.

Table 2. Chlorophyll a/b ratio variations in *X. parietina* under NaF stress

	0 mM	**0.5 mM**	**1 mM**	**5 mM**	**10 mM**
0 h	0.83 ± 0.02	0.83 ± 0.02	0.83 ± 0.02	0.83 ± 0.02	0.83 ± 0.02
24 h	0.82 ± 0.02	2.86 ± 0.02	2.5 ± 0.02	2.77 ± 0.1	3.51 ± 0.02
48 h	0.9 ± 0.07	2.98 ± 0.08	2.84 ± 0.04	3.27 ± 0.02	3.03 ± 0.07
96 h	1.18 ± 0.05	3.17 ± 0.02	3.52 ± 0.03	4.13 ± 0.03	3.65 ± 0.05

The data in the table are represented as the mean ± SD.

From the results presented in Table 2, it was noted a significant increase of $C_{a/b}$ ratio (p = 0.00572**). This increase explains well that C_b is the most affected by fluorine compared to C_a.

Table 3. Chlorophyll a/b ratio variations in *X. parietina* under $Pb(NO_3)_2$ stress.

	0 mM	**0.5 mM**	**1 mM**	**5 mM**	**10 mM**
0h	0.83±0.07	0.83±0.07	0.83±0.07	0.83±0.07	0.83±0.07
24h	0.82±0.02	0.98±0.05	1.29±0.09	1.35±0.10	1.58±0.13
48h	1.08±0.03	1.74±0.20	1.85±0.23	1.11±0.08	1.45±0.11
96h	1.11±0.03	1.62±0.32	1.37±0.04	1.06±0.22	1.06±0.08

The data in the table are represented as the mean ± SD.

According to data presented in Table 3, the chlorophyll a/b ratio in thalli treated by $Pb(NO_3)_2$ solutions was not changed very dramatically, and we did not observe significant differences in this parameter between different exposure time of lead (p = 0.5198 ^{NS}).

I.3.3. Pheophytin accumulation

Accumulation of Ph_a, Ph_b and Ph_{a+b} in *X. parietina* under NaF and $Pb(NO_3)_2$ stress are shown in Figures 13, 14 and 15, respectively.

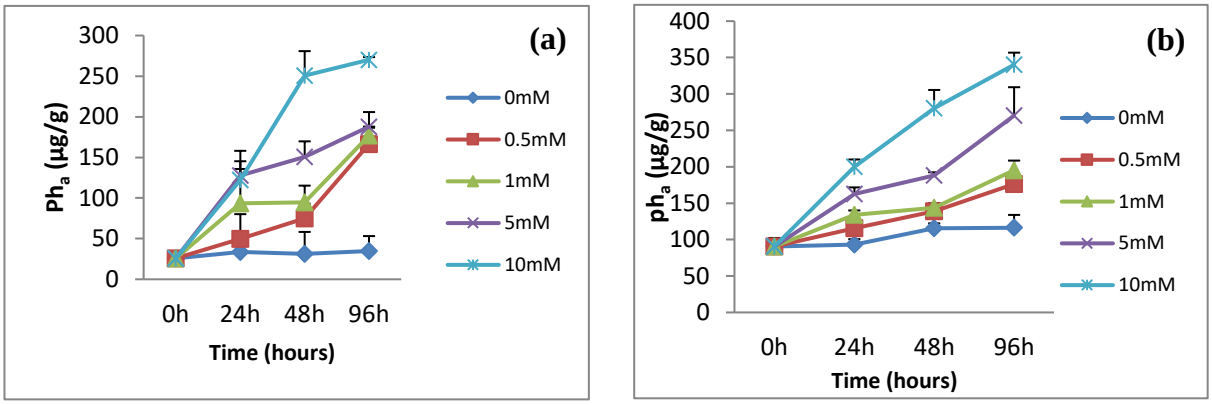

Figure 13. Ph_a accumulation in *X. parietina* after treatment of thalli by NaF (a), and $Pb(NO_3)_2$ (b) solutions

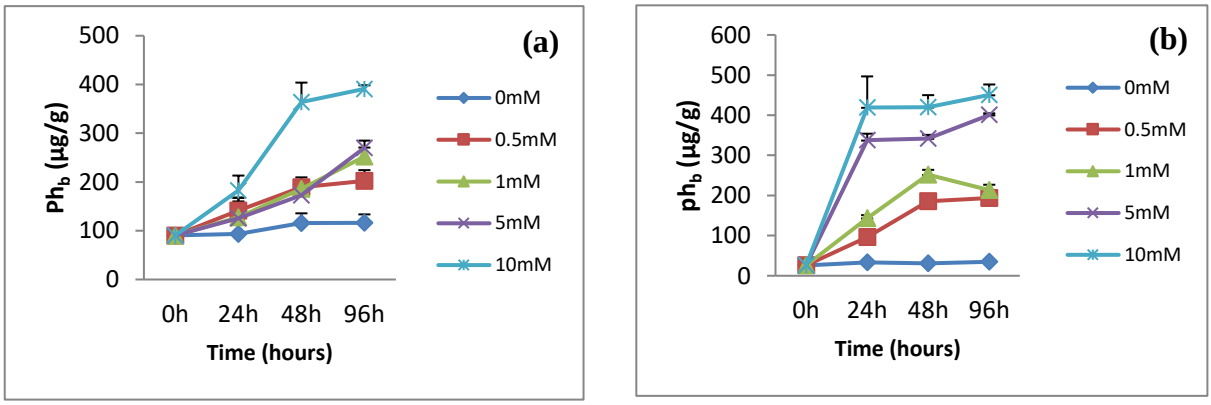

Figure 14. Ph_b accumulation in *X. parietina* after treatment of thalli by NaF (a), and $Pb(NO_3)_2$ (b) solutions

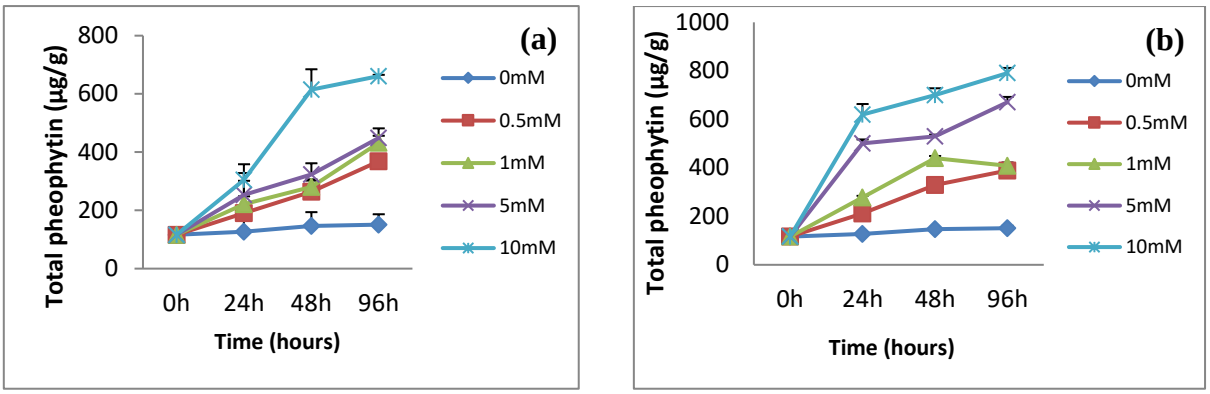

Figure 15. Ph_{a+b} accumulation in *X. parietina* after treatment of thalli by NaF (a), and $Pb(NO_3)_2$ (b) solutions

According to the data presented by Figure 13 (a), Figure 14 (a) and Figure 15 (a), it was found a non-significant increase in the level of Ph_a ($p = 0.251^{NS}$), Ph_b ($p = 0.39^{NS}$) and Ph_{a+b} ($p = 0.108^{NS}$) in thalli treated by 0.5 mM, 1mM and 5 mM of NaF. However, in those treated with the concentration 10 mM, a significant increase of Ph_a ($p = 0.03$*), Ph_b ($p = 0.017$*) and Ph_{a+b} ($p = 0.01$*) was noted. Similarly, Figures 13 (a), 14 (a) and 15 (a) show that Ph_a, Ph_b and Ph_{a+b} levels increase significantly with increasing exposure time to NaF ($p = 0.019$*, $p = 0.012$*, $p = 0.013$*, respectively). The same result is observed in thalli treated by $Pb(NO_3)_2$; Figure 13 (b) shows a non-significant increase of Ph_a with concentrations 0.5 mM, 1 mM and 5 mM ($p > 0.05$) and a significant increase ($p = 0.0032$**) in thalli treated by high concentration (10 mM). Whereas, from the results presented in Figure 14 (b) and Figure 15 (b), it was noted that the Ph_b and Ph_{a+b} levels increase significantly with increasing concentrations of $Pb(NO_3)_2$ ($p = 0.002$**, $p = 0.05$*, respecively). However, with increasing exposure time of $Pb(NO_3)_2$, the increase of Ph_b and Ph_{a+b} levels is not significant ($p > 0.05$).

I.3.4. Protein content variations

Protein content variations in *X. parietina* under NaF and $Pb(NO_3)_2$ stress are presented in Figure 16 (a) and Figure 16 (b), respectively.

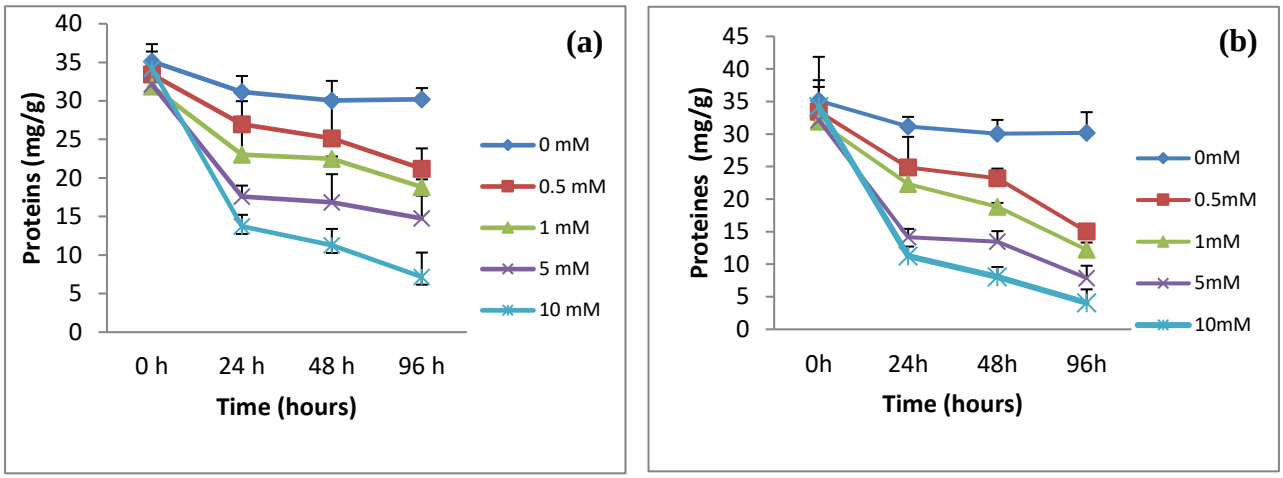

Figure 16. Protein content variations in *X. parietina* after treatment of thalli by NaF (a), and $Pb(NO_3)_2$ (b) solutions

Figure 16 (a) shows that protein levels are affected by NaF, with a significant decrease at all concentrations ($p = 0.0240$*), a significant decrease was also noted within 24 h of treatment ($p = 0.0062$**). However, between 24 h and 96 of exposure time, the decrease in protein contents is not significant ($p > 0.05^{NS}$).

Figure 16 (b) shows also a significant decrease of protein contents at all concentrations of $Pb(NO_3)_2$ after 24 h of exposure time ($p = 0.008$**) in comparison with the control. Between 24 h and 96 h of exposure time, this reduction is not significant ($p > 0.05^{NS}$).

I.3.5. Catalase activity variations

The effect of fluorine and lead on catalase activity is presented in Figure 17 (a) and Figure 17 (b), respectively.

According to figure 17 (a), it was found that the expression of the catalase increases with increasing concentration ($p = 0.047$*) and increasing exposure time of NaF ($p = 0.045$*).

Figure 17 (b) shows that the expression of catalase increases significantly in thalli with the increase of $Pb(NO_3)_2$ concentrations ($p = 0.014$*) and with increasing exposure time ($p = 0.036$*).

The greater amounts of catalase were recorded at concentrations of 10 mM of $Pb(NO_3)_2$ after 96 hours of the contact.

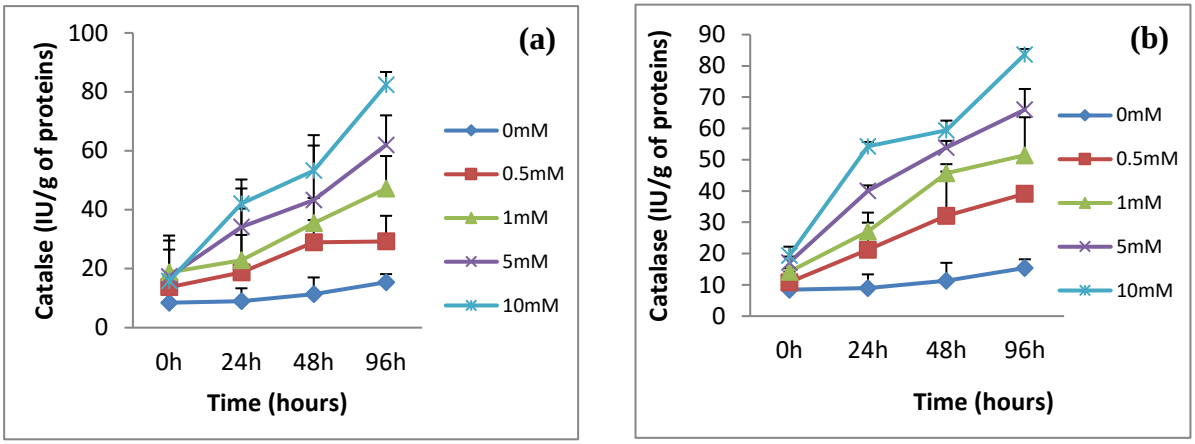

Figure 17. Catalase activity variations in *X. parietina* after treatment of thalli by NaF (a), and $Pb(NO_3)_2$ (b) solutions

I.3.6. H_2O_2 content variations

H_2O_2 content variations in *X. parietina* under NaF and $Pb(NO_3)_2$ stress are presented in Figure 18 (a) and Figure 18 (b), respectively.

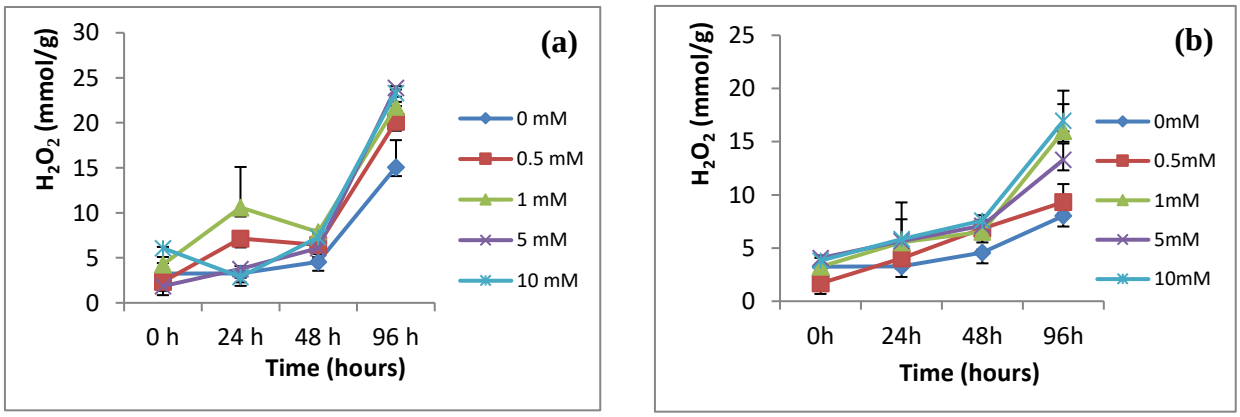

Figure 18. H_2O_2 content variations in *X. parietina* after treatment of thalli by NaF (a), and $Pb(NO_3)_2$ (b) solutions

According to the results presented in Figure 18 (a), it was noted that the variations of H_2O_2 concentrations are not significant either according to the different concentrations of NaF (p = 0.95^{NS}), or after the 48 h which follow the treatment (p = 0.16^{NS}). However, a significant increase was observed after 96 h of treatment (p = 0.017*). In thalli treated by $Pb(NO_3)_2$, the content of H_2O_2 increases with increasing exposure time, particularly after 96 h of treatment by concentrations of 1 mM, 5 mM and 10 mM (p = 0.0018**). We noted that low concentration (0.5 mM) has a negligible effect on the accumulation of H_2O_2 which is similar to the control. The increase in H_2O_2 concentrations was not significant in function of various concentrations of $Pb(NO_3)_2$.

I.3.7. GSH content variations

GSH content variations in *X. parietina* under NaF and $Pb(NO_3)_2$ stress are presented in Figure 19 (a) and Figure 19 (b), respectively.

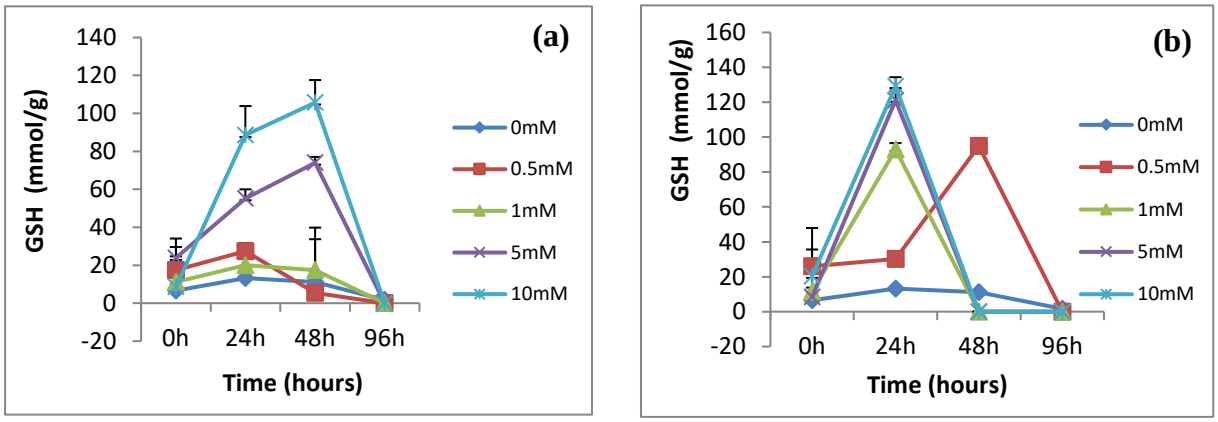

Figure 19. GSH content variations in *X. parietina* after treatment of thalli by NaF (a), and $Pb(NO_3)_2$ (b) solutions

From the data presented in figure 19 (a), it was noted that the variations in GSH content in thalli treated with low concentrations of NaF (0.5 mM and 1 mM) are negligible. Whereas, the high concentrations (5 mM and 10 mM) caused a significant increase after 24 h and a non-significant increase after 48 h of treatment (p = 0.64^{NS}). However, a significant decrease in the GSH content should be noted between 48 h and 96 h of exposure time (p = 0.02*) with complete degradation after 96 h of treatment.

At low concentration of $Pb(NO_3)_2$ (0.5 mM), Figure 19 (b) shows a significant increase in GSH contents after 48 h of treatment (p = 0.05*), followed by a significant decrease after 96 h of treatment (p = 0.05*). Conversely, the other concentrations of $Pb(NO_3)_2$ caused a significant increase (p = 0.0019**) in GSH contents just after 24 h of exposure time, these concentrations reached the maximum values after 24 h of treatment and then decreased significantly to zero after 48 h of exposure time (p = 0.0038**). Concerning the effect of concentrations, no significant variation of GSH content as a function of different concentrations were noted ($p > 0.05^{NS}$).

I.3.8. Correlation analysis

I.3.8.1. $Pb(NO_3)_2$ / lead correlation

Lead accumulation in *X. parietina* correlates with increased $Pb(NO_3)_2$ concentrations in solutions (Figure 20).

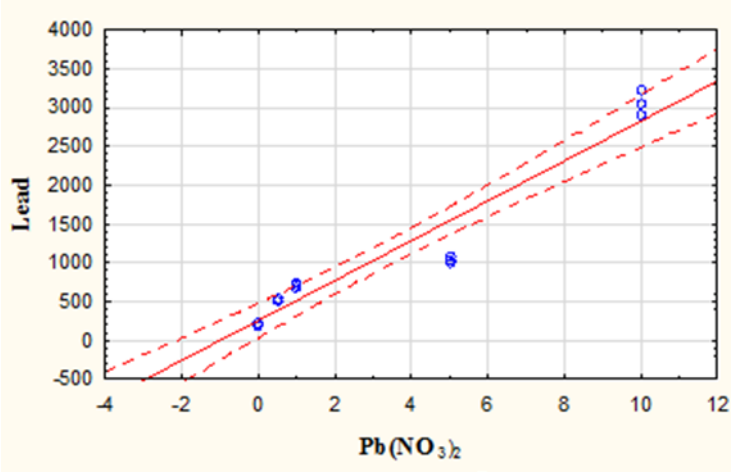

Figure 20. Correlation matrices between $Pb(NO_3)_2$ concentrations and lead buildup in *X. parietina* (r = 0.96122, $p < 0.001$***)

Figure 20 demonstrates a significant positive correlation between $Pb(NO_3)_2$ concentrations and lead buildup in thalli.

I.3.8.2. Correlation matrices between NaF, $Pb(NO_3)_2$ and different studied parameters

Correlation matrices between NaF, $Pb(NO_3)_2$ and different studied parameters are presented in Tables 4 and 5, respectively.

Table 4. Correlation matrices between NaF and C_a, C_b, C_{a+b}, $C_{a/b}$, proteins, catalase, H_2O_2 and GSH contents in thalli of *X. parietina*

Correlation matrices	**Correlation dependency**	***r***	***P***	**Significance**
NaF / C_a	Time (0 - 48 h)	-0.785	< 0.001	***
NaF / C_b	Time (0 - 24 h)	-0.955	< 0.001	***
NaF / C_{a+b}	Time (0 - 48 h)	-0.899	< 0.001	***
NaF / $C_{a/b}$	Time	0.818	< 0.001	***
NaF / proteins	Concentration	-0.872	< 0.001	***
NaF / catalase	Time and concentration	0.784	< 0.001	***
NaF / H_2O_2	Time (48 -96 h)	0.949	< 0.001	***
NaF / GSH	Concentration	0.969	< 0.001	***
	Time (48 - 96 h)	-0.6	0.06	NS

(NS): not significant, (*): significant, (**) very significant, (***): highly significant

Results of the statistical analysis presented in Table 4, show a significant negative correlation between C_a, C_b, C_{a+b} and exposure time of NaF and between proteins and increasing concentrations of NaF. A significant positive correlation was noted between $C_{a/b}$ ratio and exposure time of NaF, catalase and exposure time to increasing concentrations of NaF, H_2O_2 and exposure time of NaF, GSH and increasing concentrations of NaF. However, a non-significant negative correlation was noted between GSH and 48 h to 96 h of exposure of NaF.

Except for non-significant correlation between $C_{a/b}$ and exposure time of $Pb(NO_3)_2$, Table 5 shows a significant negative correlation between C_a, C_b, C_{a+b} and exposure time of $Pb(NO_3)_2$, and between proteins and increasing concentrations of $Pb(NO_3)_2$. Table 5 shows also a significant positive correlation between H_2O_2 and increasing concentration of $Pb(NO_3)_2$. While catalase and GSH variations are correlated with concentration and exposure time.

Table 5. Correlation matrices between $Pb(NO_3)_2$ and C_a, C_b, C_{a+b}, $C_{a/b}$, proteins, catalase, H_2O_2 and GSH contents in *X. parietina*

Correlation matrices	Correlation dependency	*r*	*P*	Significance
$Pb(NO_3)_2$/ C_a	Time (0- 24 h)	-0.816	0.004	**
$Pb(NO_3)_2$ / C_b	Time (0- 24 h)	-0.813	0.004	**
$Pb(NO_3)_2$/ C_{a+b}	Time (0- 24 h)	-0.814	0.004	**
$Pb(NO_3)_2$ / $C_{a/b}$	Time	0.420	0.06	NS
$Pb(NO_3)_2$ / proteins	Concentration	-0.790	0.0002	***
$Pb(NO_3)_2$/ catalase	Concentration	0.795	0.0002	***
	Time	0.797	0.0001	***
$Pb(NO_3)_2$ / H_2O_2	Concentration	0.866	0.00001	***
$Pb(NO_3)_2$ / GSH	Concentration	0.761	0.017	*
	Time (0- 24 h)	0.828	0.005	**
	Time (24- 48 h)	-0.982	0.0004	***

(NS): not significant, (*): significant, (**) very significant, (***): highly significant

I.3.8.3. Chlorophyll / pheophytin, H_2O_2 / proteins and H_2O_2 / GSH correlations

Correlation matrices between C_{a+b} / Ph_{a+b}, H_2O_2 / proteins and H_2O_2 / GSH are presented in Figures 21, 22 and 23, respectively.

The statistical analyses presented in Figure 21, Figure 22 and Figure 23 show a negative correlation between C_{a+b} and Ph_{a+b}, between H_2O_2 and proteins, and between H_2O_2 and GSH under NaF and $Pb(NO_3)_2$ stress, respectively. Similarly, the results show that C_{a+b} / Ph_{a+b} correlations under NaF and $Pb(NO_3)_2$ stress, and H_2O_2 / proteins correlation under $Pb(NO_3)_2$ stress were significant. However, H_2O_2 / proteins correlation under NaF stress and H_2O_2 / GSH correlations under NaF and $Pb(NO_3)_2$ stress were not significant,

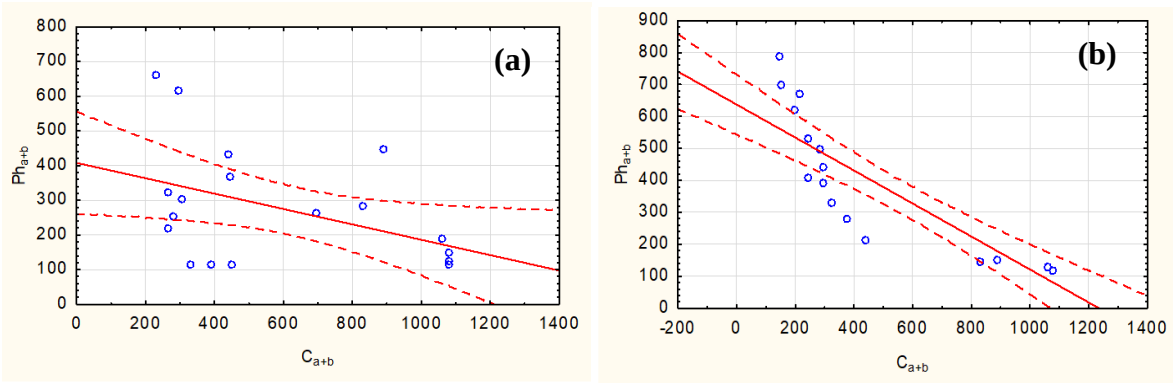

Figure 21. Correlation matrices between total pheophytin and total chlorophyll; (a): under NaF stress (r = -0.467, p = 0.37^{NS}), (b): under under $Pb(NO_3)_2$ stress (r = -0.881, p < 0.001***)

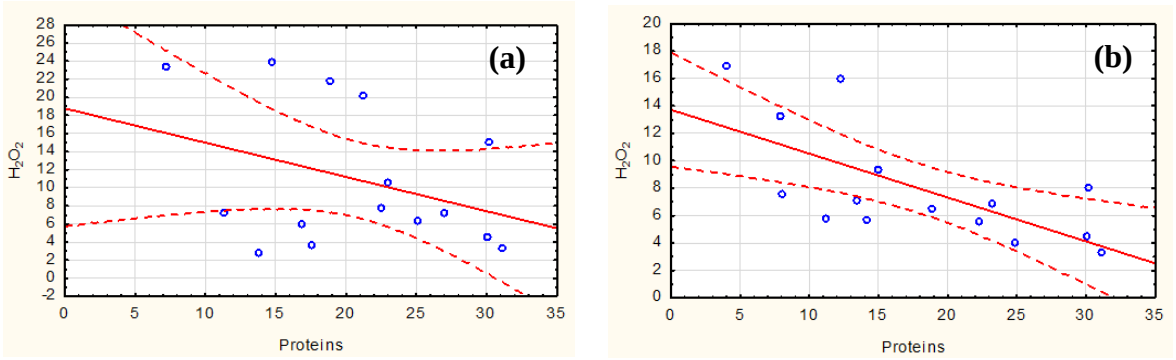

Figure 22. Correlation matrices between H_2O_2 and proteins; (a): under NaF stress (r = -0.3554, p = 0.19^{NS}), (b): under $Pb(NO_3)_2$ stress (r = -0.6716, p = 0.0061**)

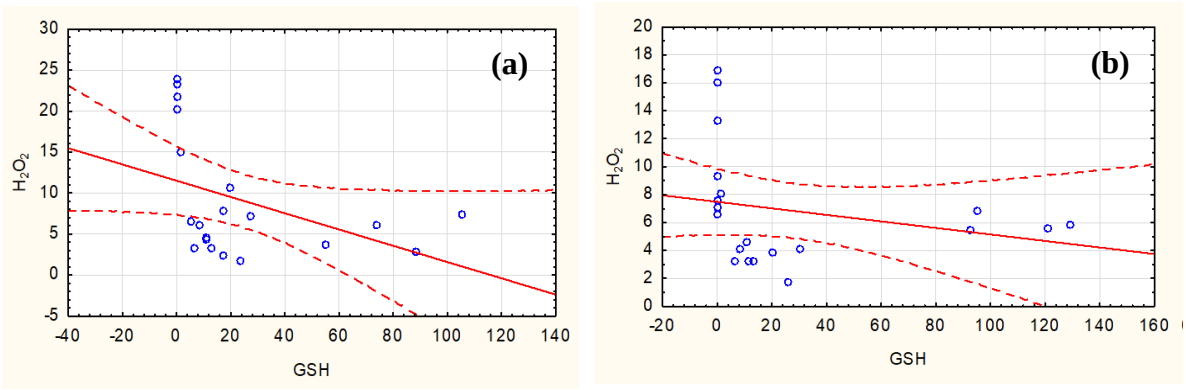

Figure 23. Correlation matrices between H_2O_2 and GSH; (a): under NaF stress (r = -0.416, p = 0.06^{NS}), (b): under $Pb(NO_3)_2$ stress (r = -0.24, p = 0.3^{NS})

Our results show that variations in chlorophyll, pheophytin, protein, catalase, H_2O_2 and GSH contents in *X. parietina* under $Pb(NO_3)_2$ stress are more intense than under NaF stress. Based on statistical analysis of these parameters, it was concluded that lead is significantly more toxic than fluoride (p < 0.001***).

I.4. Discussion

According to results obtained by Ma et *al.* (2016), it was found that vegetation is capable to accumulate heavy metals such as lead, and this accumulation induced oxidative stress (Mourato et *al.*, 2015). Because of their tendency to accumulate large levels of hazardous metals, foliose lichens are frequently used as biomonitors of metal pollution in the environment (Purvis, 2014). Our results confirmed the accumulation of lead in *X. parietina* thalli. According to Abas and Awang (2017), lichens are effectively used as powerful biological indicators for biomonitoring. *X. Parietina* is one of the most regularly employed lichens in metal accumulation research. According to Belguidoum et *al.* (2021), *X. parietina* can accumulate significant amounts of heavy metals in comparison with other fruticulous lichen such as *Ramalina farinacea*. Our results show that the accumulation of lead increases with increasing exposure time, the same result was obtained by Douibi et *al.* (2015), and Šujetovienė and Česynaitė (2021). Lichens have the ability to extract metals from their environment (Subhashini and Suganthi, 2014; Caggiano et *al.*, 2015; Darnajoux et *al.*, 2015). According to results obtained by Carreras and Pignata (2007), Hg, Cu, Cd, Ag, Pb, Zn are considered the most toxic heavy metals. Our results also demonstrate that *X. parietina* can accumulate lead correlating with increasing $Pb(NO_3)_2$ concentration (r = 0.96122, p < 0.001***).

Plants underwent many biochemical and physiological changes as a result of the stressful environment. Chlorophyll degradation is the most common metric used to investigate the toxicity of air contaminants on lichens (Šujetovienė, 2015). Photosynthesis and respiration are the processes most affected by fluoride (Sharma and Kaur, 2018). Compared with the control test, and depending on the increase of concentration and exposure time to NaF, our results show a significant decrease in C_a, C_b and C_{a+b} contents in *X. parietina*. According to the results of statistical analysis presented in Table 4, it was noted a significant decrease of C_a, C_b and C_{a+b} contents correlating with exposure time to NaF (r = -0.785, $p < 0.001$; r = -0.955, $p < 0.001$ and r = -0.899, $p < 0.001$, respectively). Our results are in the same line with those obtained by Chakrabarti et *al.* (2014) who reported that chlorophyll decreased in paddy (*Oryza sativa* L.) with increasing fluoride treatment. Mondal (2017) also found pigment degradation in four widely cultivated rice (*O. sativa*) varieties treated by 5, 10, and 20 mg dm^{-3} NaF. An other study carried out by Iram and Khan (2016) showed that C_a, C_b and C_{a+b} decreased in *Abelmoschus esculentus* (L.) Moench under NaF stress. Fan et *al.* (2022) also found a significant decrease in chlorophyll content after high concentration of NaF treatment in tall fescue (*Festuca arundinacea* Schreb).

According to the results presented in Figures 10 (b), 11 (b) and 12 (b), it was noted that lead exerts toxicity on lichen by the decrease of C_a, C_b and the total chlorophyll content, the same result was obtained by Emamverdian and Ding (2017) who indicate that lead is toxic for photosynthetic properties.The obtained results also show that the decrease in the chlorophyll content is important in thalli treated with high concentrations of lead. Zhao et *al*. (2021) found the same thing, indicating that high cadmium concentrations affect photosynthesis in *Sassafras* seedlings. Wang et *al.* (2021) also found that the content of C_a, C_b and C_{a+b}, decreases in tall fescue under lead stress. Significant decrease of total chorophyll content was also observed in *Lonicera japonica* Thunb. in response to 150 mg kg^{-1} or 200 mg kg^{-1} of cadmium (Li et *al*., 2022).

The accumulation of lead in the lichen thalli causes significant decrease in the content of C_a, C_b and total chlorophyll correlating with exposure time, especially after 24 h of exposure (r = -0.816, p = 0.004**; r = -0.813, p = 0.004**; r = -0.814, p = 0.004**, respectively).

Using treatment with $Pb(NO_3)_2$ concentrations for 24, 48 and 96 h of *X. parietina* thalli, in addition to control test, quantification of total chlorophyll content was done as a direct results of lead stress. Abu-Muriefah (2015), shows that the treatment with lead had a considerable impact on

the concentration of C_a. The levels of C_a decreased with increasing lead concentrations in solution, the lowest contents of this pigment were recorded in thalli treated with high concentrations of lead, implying that Pb had a negative impact on C_a content (Carreras and Pignata, 2007). According to a study conducted by Sędzik et *al.*, (2015), the application of lead nitrate causes a decrease in chlorophyll concentration in seedlings of diverse plant species.

Our results show a significant increase of $C_{a/b}$ ratio correlating with exposure time to NaF (r = 0.818, $p < 0.001$***). These results allowed us to conclude that C_b is more affected than C_a in thalli treated by NaF and the same results werw obtained by Purnama et *al.* (2015), who show a significant decrease in total chlorophyll ($p = 0.006$**) and C_b content in Seagrass under lead stress, even though, they also found that C_b was more affected than C_a as result of lead effect. Lichtenthaler and Babani (2022) also, found that C_b is destroyed far more quickly than C_a, resulting in steadily rising values for the $C_{a/b}$ ratio, up to values of 6 to 8 at a progressive C_{a+b} decrease in shade leaves and sun leaves. However, under $Pb(NO_3)_2$ stress $C_{a/b}$ ratio variations are not significant ($p = 0.420$, $r = 0.06^{NS}$), this allowed us to conclude that both types of pigments (C_a and C_b) have the same sensitivity to lead. This results is in agreement with those of Aboal et *al.* (2008) how found that the decrease of $C_{a/b}$ ratio is apparent marker of degradation by senescence or stress, also, the study of Bajpai et *al.* (2010) who were interested in the toxic effect of heavy metals accumulation in lichens and higher plants, showed that heavy metals (Zn, Cd and Cu) even at very low concentrations cause physiological changes and inhibit photosynthesis, while lead causes a decrease in total chlorophyll and $C_{a/b}$ ratio.

Pheophytin is the result of chlorophyll degradation under acidic conditions. This reaction is called pheophytinization, in other words, destruction of chlorophyll (C_a, C_b) and its transformation into pheophytin (Ph_a, Ph_b) (Dzubaj et *al.*, 2008 ; Pisani et *al.*, 2009). During pheophytinization, the magnesium ion found in the center of the porphyrin ring is replaced by two hydrogen ions, resulting in the color change of chlorophyll from bright green to olive- brown (Yilmaz and Gökmen, 2016). Our result show that pheophytin accumulation is associated with chlorophyll degradation, we found that pheophytin was negatively correlated with chlorophyll in *X. parietina* under NaF (r = -0.467, $p = 0.37^{NS}$) and $Pb(NO_3)_2$ (r = -0.881, $p < 0.001$***) stress. Similar result was achieved by Dissanayake et *al.* (2012) who showed that pheophytin is formed in considerable amount as an important derivative during the degradation of chlorophyll in bunching onion leaves. Also, Huang et *al.* (2022) found a significant decrease in chlorophyll content (from 6.57 to 1.28 mg/100 g)

accompanied by the formation of tawny pheophytin in the cotyledons and seed coats of mung beans during a heat treatment of 0 to 50 min.

According to Yang et *al.* (2019), pheophytin a is accumulated as a result of chlorophyll degradation but the amount was relatively less than that of degraded chlorophyll, while most of pheophytin a (Ph_a) was bound by forming aggregates with some other chlorophyll-protein (CP) complexes. Zhang et *al.* (2016) explain that the increase in the concentration of pheophytin is the result of the enzymatic activity of pheophorbide hydrolase which catabolizes the degradation of chlorophyll.

Plants vary their protein composition for rebuilding, tolerance, resistance, and responsiveness to stressful situations (Firuzeh et *al.*, 2015; Amnan et *al.*, 2022). Indeed, the plant changes its protein content to adapt or react against an abiotic stimulus (Nouri, et *al.*, 2015). Our results show a significant decrease in protein contents in *X. parietina* correlating with increasing concentrations of NaF (r = -0.872, $p < 0.001$) and $Pb(NO_3)_2$ (r = -0.790, p = 0.0002***). Similar result was achieved by Esposito et *al.* (2012) and by Chetia et *al*. (2021), who found a decrease in total protein contents to correlate with Pb, Cd, Zn, Cu, Co, Ni, and Cr in lichens growing in differently polluted areas. Khan et *al.* (2021) found a drop in total protein contents in cultivated rice grown in lead-contaminated soil. Sharma et *al.* (2019) also showed significant reduction ($p \leq 0.05$) in protein content in *Spirodela polyrhiza* (L.) Schleiden under treatment with fluoride at all the exposure periods (24, 72, 120 and 168 h). According to Szostek and Ciecko (2017), the decrease in total protein content caused by fluoride can be explained by channeling degraded products towards metabolic pathways for energy and stress management. On the other hand, treatment with 0.3 and 0.6 g/L lead acetate, resulted in an increase in total protein content in *Triticum durum* Desf. leaves and roots (Souahi et *al.*, 2021).

Plants increase the activities of antioxidant enzymes like catalase to trap ROS and detoxify their effects (Lei et *al.*, 2022). Ours results show that the expression of catalase in *X. parietina* increases correlating with increasing concentrations of NaF (r = 0.784, $p < 0.001$***). Mondal (2017) reported similar results, demonstrating that catalase activity increased with increasing fluorine concentrations in four widely cultivated rice (*O. sativa*). Elloumi et *al*. (2017) also showed that increased catalase activity is one of the indices of oxidative stress induced by fluoride air pollution in *Eriobotrya japonica.* Sharma and Kaur (2019) also found a significant increase of

catalase activity in *Spirodela polyrhiza* under fluoride stress at high concentration compared to the control at a very first exposure period of 24 h. In addition, Sharma et *al*. (2019) showed that fluoride treatment significantly increased catalase activity in exposed *S. polyrhiza* fronds when compared to control during all exposure periods (24, 72, 120 and 168 h). Ghosh et *al.* (2021) also found that during the ripening of chili fruits, treatment with chitosan and putrescine modulates reactive oxygen species metabolism, and causes an increase in catalase activity. However, Chakrabarti et *al.* (2014) found that catalase activity decreased with increasing fluoride treatment. Furthermore, Orabi et *al*. (2015) indicate that the excess of H_2O_2 caused a decrease in the activity of catalase.

Our results show that lead causes an increase of catalase activity correlating with increasing concentration (r = 0.795, p = 0.0002***) and increasing exposure time of $Pb(NO_3)_2$ (r = 0.797, p = 0.0001***), AL-Zurfi et *al.* (2021) found the same results, indicating that the plant *Hydrilla verticillata* reacts against cadmium stress by steadily increasing of the catalase enzyme concentration. Also, Abu-Muriefah (2015) and Khan et *al.* (2021) found that catalase increases significantly in plants exposed to lead. In addition, several other studies confirm that heavy metals cause oxidative stress in biomarkers (Taiwo et *al.*, 2014; Batool et *al*., 2018; Ullah et *al*., 2021). Emamverdian et *al.* (2015) report that some antioxidant components such as catalase are likely to act in an integrated manner at excessive levels of heavy metals and raise plant tolerance to heavy metals stress. Contrary to the results obtained by Orabi et *al.* (2015) which indicate that the excess of H_2O_2 causes decrease in the activity of catalase, our results show that the accumulation of H_2O_2 is accompanied with an increase in the catalase activity.

Plants create H_2O_2 as one of ROS in response to abiotic stress (Muneer et *al.*, 2014; Qi et *al.*, 2018; Zhang, 2022b). According to Sofo et *al.* (2015), H_2O_2 generation is regarded as a stress marker. The hydrogen peroxide performs a crucial role in the transfer of a signal all through abiotic stress in plants (Niu and Liao, 2016). The H_2O_2 is also vital for stressful tolerance conditions in plants (Černý et *al.*, 2018). According to Hung et *al*. (2005), plants have developed complex regulatory mechanisms to adapt to various environmental stresses, the most important of which is to convert the ROS formed into hydrogen peroxide. The statistical analyses results presented in Tables 4 and 5 show that H_2O_2 increases in correlation with the increase of the the exposure time to NaF (r = 0.949, p < 0.001***) and in correlation with the increase of $Pb(NO_3)_2$ concentrations (r = 0.866, p < 0.001***). These results are comparable with those of Panda (2007), who

investigated the effect of chromium on rice and discovered that this pollutant produces H_2O_2, which is proportional to exposure time and pollutant concentration. Furthermore, those obtained by Liu et *al*. (2010), who found that contents of H_2O_2 decrease in tomato seedlings when the concentration of Mn^{2+} reached 400-600 $\mu molL^{-1}$ under hypoxia stress. Our results are also similar to those obtained by Liu et *al.* (2021), which show that lead causes the increase of H_2O_2 in edible amaranth when lead from soils and atmospheric stresses are present, and Li et *al*. (2022), who discovered higher levels of H_2O_2 in response to high cadmium concentrations (150 mg kg^{-1} or 200 mg kg^{-1} Cd). According to Liu et *al.* (2020), trealose treatment of tomato under cold stress causes elevated H_2O_2 levels as a way of tolerance. Our results show that the accumulation of H_2O_2 is accompanied with the decrease in protein content, where we found a negative correlation between H_2O_2 and protein contents under NaF (r = -0.3554, $p = 0.19^{NS}$) and $Pb(NO_3)_2$ (r = -0.6716, p = 0.0061**) stress. Similar result was achieved by James et *al.* (2022) who showed a negative correlation between H_2O_2 and proteins in bleuet Northland under hypobaric storage.

The obtained results for glutathione assay are in agreement with those obtained by Sanità ditopi et *al.* (2008) who reported that lichens used glutathione in detoxification; the low levels of glutathione are observed in the controls. GSH increases in plants under stressful conditions (Nahar et *al*., 2017) and it is part of the adaptation strategies used by plants to combat and tolerate stressful conditions (Gong et *al*., 2018). The obtained results show that the GSH content increases correlating with increasing concentrations of NaF (r = 0.969, p < 0.001***) and $Pb(NO_3)_2$ (r = 0.761, p = 0.017*) and the same result was obtained by Li et *al*. (2022) who showed that in response to 150 mg kg^{-1} or 200 mg kg^{-1} of Cd, GSH increased in *Lonicera japonica* with increasing concentration of Cd.

Correlating with exposure time to NaF, our results show a decrease in GSH content between 48 h and 96 h of treatment (p = -0.6, $r = 0.06^{NS}$). However, in thalli treated by $Pb(NO_3)_2$, we found a significant increase of GSH correlating with exposure time (p = 0.828, r = 0.005**) between 0 and 24 h of treatment, followed by a significant decrease (p = -0.982, r < 0.001***) between 24 and 48 h, with a total destruction in thalli treated by highest concentrations (5 mM and 10 mM) after 96 h of treatment. According to Cempírková and Večeřová (2018), the long-term stress exposure of the individual species of green algal and cyanobacterial lichen had a significant impact on the antioxidant content resulting from high light stress. However, no light treatment did not change the total GSH content with various levels of irradiance for various time periods in

comparison to the control test. Our results are similar with those of Balarinová et *al*. (2014), who discovered that during the first 30-40 min of high light treatment, total GSH increased in two Antarctic lichens (*Usnea antarctica* and *Usnea aurantiaco-atra*), followed by a reduction at 60 min of treatment, and with those of Li et *al*. (2015) which reported that increased heavy metal concentrations resulted in a considerable reduction in GSH content in the roots and leaves of *Safflower* plants (*Carthamus tinctorius* L.). Sofo et *al.* (2015) also, indicate that elevated and non-metabolized cellular hydrogen peroxide due to stress conditions, can be possible to cause severe damage to biomolecules such glutathione. Our results are also similar to those obtained by Cao et *al.* (2004) and Freeman et *al.* (2004) who demonstrated that the level of GSH decreases with higher tolerance to pollutants at low concentrations, and by Pristupa et *al*. (2021) who found a decrease in GSH content in transgenic plants *Nicotiana tabacum* L. under abiotic stress conditions.

High concentrations of pollutants affect the detoxification system which explains the total degradation of glutathione. Chaabene et *al*. (2018) indicate that in plants exposed to copper, antioxidant enzyme activity increases as copper concentrations rise, while, at high concentrations, the defense system is disturbed and deregulated. Liu et *al.* (2021) also indicate that the antioxidant system can be significantly reduced by high levels of lead in plants. According to Shelly and Lu (2013), the availability of the sulfur amino acid precursor cysteine, as well as the activity of two enzymes glutamate cysteine ligase and GSH synthetase, is the primary determinants of GSH production, so we can say that the decrease in the GSH content is mostly due to an inactivation of enzymes involved in GSH biosynthesis.

According to our results (Figure 23), we found a negative correlation between H_2O_2 and GSH content under NaF (r = -0.416, $p = 0.06^{NS}$) and $Pb(NO_3)_2$ (r = -0.24, $p = 0.3^{NS}$) stress; these results are in the same line with those of James et *al*. (2022), who found that hydrogen peroxide was negatively correlated with GSH in bleuet Northland under hypobaric storage. Arianmehr et *al.* (2022) also investigated the role of GSH in reducing arsenic (As) toxicity in *Isatis cappadocica* DESV. and *Erysimum allionii* exposed to different concentrations (0, 400, and 800 M) of arsenic for 3 weeks, and discovered that application of GSH increased fresh weight and total chlorophyll while inhibiting H_2O_2 accumulation.

NB: Results of this part are published in *Acta Scientiarum Biological Sciences* and *International Journal of Secondary Metabolite.*

II. Other signs of damages and adaptation of *X. parietina* to stress induced by fluoride and lead

II.1. Objective

The aim of this work is to study the toxic effect of fluoride and lead on lipid peroxidation and chlorophyll integrity in *X. parietina*, and to check for proline and soluble sugars accumulation as indicators of responses employed for adaptation to produced stress.

II.2. Material and methods

II.2.1. Lichen material

Samples of *X. parietina*'s lichen thalli were collected in the Djimar and Beni Metrane regions, located in Jijel Nort-Est of Algeria in February and March 2018 (Figure 8). Collected sample thalli were transported to the laboratory in sterilized closed containers, then cleaned of impurities and rinsed in distilled water to get rid of adhering and surface dust. The fresh weights of the thalli were separated from their supports and acclimatized to laboratory conditions in each experimental vessel until examination. Lichens then, were incubated in various concentrations of NaF and $Pb(NO_3)_2$ solutions (see section Experimental part I.2.2) and stored to perform the following analyses: MDA, integrity of chlorophyll, proline and soluble sugars assay.

II.2.2. MDA assay

For the MDA assay, the Heath and Packer (1968) method was used. About 200 mg of the lichen thalli were homogenized in 2 mL of 0.1 % trichloroacetic acid (TCA) and centrifuged at 10000 g for 20 min. To 1 mL of supernatant, 1 mL of 20 % TCA containing 0.5 mL of thiobarbituric acid and 0.001 mL of butylated hydroxyl-toluene (BHT) at 4 % solution in ethanol were added. The mixture was heated at 95 °C for 30 min and centrifuged at 10000 g for 15 min, the supernatant absorbance was read at the wavelength 532 nm and corrected at 600 nm. MDA was calculated by multiplying by the coefficient of 155 $mM^{-1}cm^{-1}$; the results were expressed in µmol g^{-1} FW.

II.2.3. Integrity of chlorophyll assay

According to Ronen and Galun (1984), the integrity of chlorophyll was calculated. About 20 mg of lichen thalli were extracted in 3 mL of dimethylsulfoxide (DMSO) in dark at 65 °C for 40 min. The optical densities at 435 nm and 415 nm for the extract were read, and the ratio OD_{435}/OD_{415} was calculated to assess the degree of chlorophyll degradation. Ronen and Galun

(1984) estimate that a ratio between 1.4 and 1.45 was calculated in lichens in the case of minimal chlorophyll degradation into phaeopigments.

II.2.4. Proline assay

The method used for the determination of proline is that of Troll and Linsley (1955). About 100 mg of fresh lichen thalli were extracted in 2 mL of 40 % methanol at 85 °C for 60 min. After cooling, 1 mL of acetic acid and 1 mL of a mixture containing (120 mL of distilled water, 300 mL of acetic acid and 80 mL of acid orthophosphoric acid and 25 mg of ninhydrin) were added to 1 mL of the extract. The solution was brought to the boil for 30 min, it gradually turns red. After cooling, 5 mL of toluene was added; the upper phase which contains the proline was recovered and dehydrated by the addition of disodium sulphate. The optical density was determined by a spectrophotometer at a wavelength of 528 nm and the calibration curve was established by different concentrations of proline from a stock solution of 2 mg/100 mL of 40 % methanol. Proline contents were determined using equation established by known concentrations of proline (y = 28.0 x, R^2 = 0.9911); the results were expressed in µg g^{-1} FW.

II.2.5. Soluble sugars assay

The soluble sugar contents were quantified by the method of Dubois et *al.* (1956). 100 mg of fresh lichen material were extracted in 3 mL of 85 % ethanol for 48 h in the dark, then filtered and recovered with 20 mL of distilled water, 1 mL of 5 % phenol and 5 mL of 1.8 N sulfonic acid were added to 1 mL of the filtrate. After incubation for 15-20 min in a water bath adjusted to 30 °C, the optical densities were determined at the wavelength 490 nm. The calibration curve was established by glucose at different concentrations prepared from a stock solution of 250 mg/L. Soluble sugar contents (µg.g^{-1} FW) were calculated using equation established by known concentrations of glucose (y = 0.731x + 0.001, R^2 = 0.999).

II.2.6. Statistical analysis

For the purpose of calculating the standard deviation (SD), three repetitions were carried out at each concentration. The ORIGIN 6.0 system's test univariate variance (one way ANOVA) was used to conduct the statistical analysis. Results were expressed as mean ± SD. The distinction was deemed insignificant when $p > 0.05^{(NS)}$, significant when $0.01 < p < 0.05$ (*), highly significant when $0.001 < p < 0.01$ (**) and very highly significant when $p < 0.001$ (***).

STATISTICA Version 10 software was used to analyze correlation matrices between NaF, $Pb(NO_3)_2$ and different studied parameters.

II.3. Results

II.3.1. MDA accumulation

Results of the MDA content variations in the treated thalli of *X. parietina* by various NaF and $Pb(NO_3)_2$ concentrations for 0, 24, 48 and 96 h are presented in Figure 24. Correlation matrices between NaF / MDA, $Pb(NO_3)_2$ / MDA are presented in Figure 25.

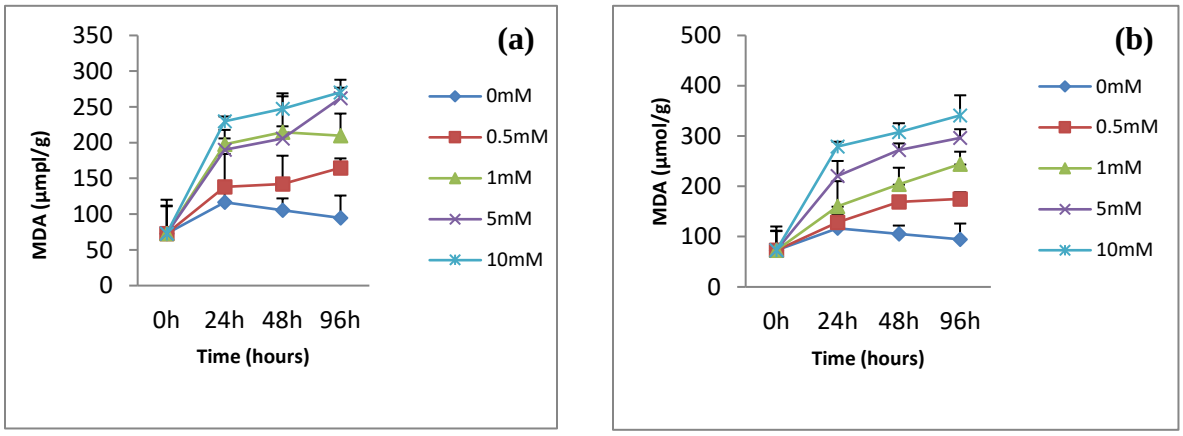

Figure 24. MDA accumulation in thalli of *X. parietina* under NaF (a) and $Pb(NO_3)_2$ (b) stress

According to Figure 24, no significant increase in the MDA content was noted as a function of the concentrations of NaF ($p = 0.179^{NS}$) and $Pb(NO_3)_2$ ($p = 0.109^{NS}$). However, depending on exposure time, a significant increase of MDA content was noted with treatment by NaF ($p = 0.005^{**}$) and $Pb(NO_3)_2$ ($p = 0.014^{*}$).

Results of the statistical analysis presented in Figure 25, show a significant positive correlation between MDA (Figure 25 a) and NaF concentrations, and between MDA and $Pb(NO_3)_2$ concentrations (Figure 25 b).

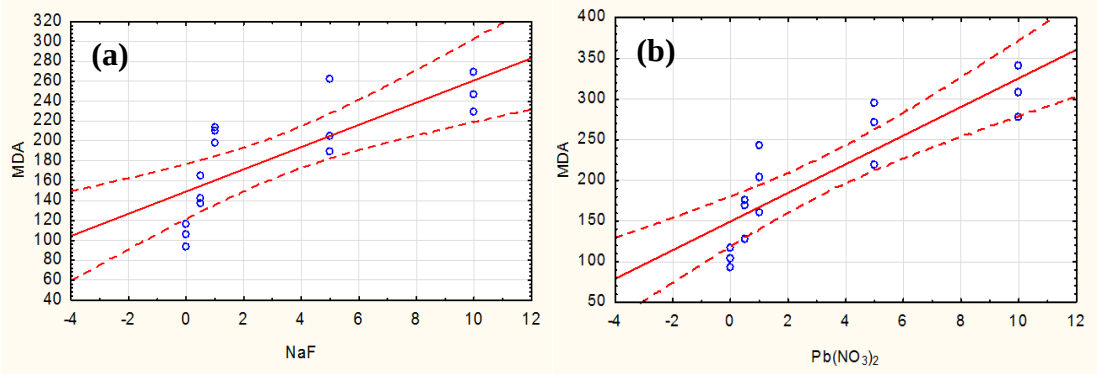

Figure 25. Correlation matrices between NaF / MDA (a), $Pb(NO_3)_2$ / MDA (b). (a)*: r* = 0.773, *p* = 0.000712***, (b): *r* = 0.865, *p* = 0.000031***

II.3.2. Chlorophyll integrity variations

Variations of the OD_{435}/OD_{415} ratio in *X. parietina* are presented in Figure 26. Correlation matrices between NaF / OD_{435}/OD_{415} ratio and $Pb(NO_3)_2$ / OD_{435}/OD_{415} ratio are presented in Figure 27.

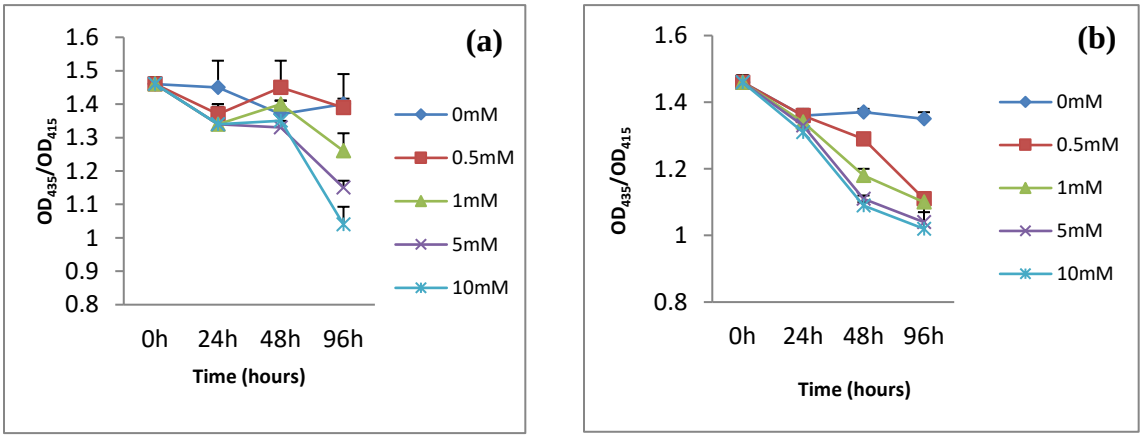

Figure 26. Variations of the OD_{435}/OD_{415} ratio in thalli of *X. parietina* under NaF (a) and $Pb(NO_3)_2$ (b) stress

Depending on exposure time, Figure 26 (a) shows that the variations of OD_{435}/OD_{415} ratio in thalli under NaF stress are significant (*p* = 0.009**), but not significant (*p* = 0.422^{NS}) depending of concentrations.

In thalli under different $Pb(NO_3)_2$ concentrations, a slight decrease in OD_{435}/OD_{415} ratio was recorded after 24 h of treatment ($p > 0.05^{NS}$). While, between 24 h and 96 of treatment, a significant decrease in this ratio was noticed ($p = 0.036$*).

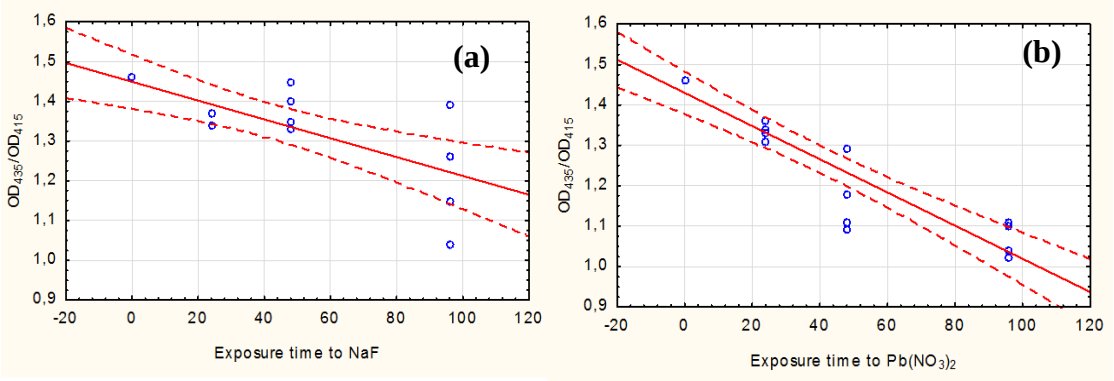

Figure 27. Correlation matrices between NaF / OD_{435}/OD_{415} ratio (a), $Pb(NO_3)_2$ / OD_{435}/OD_{415} ratio (b). (a): $r = - 0.737$, $p = 0.0011$**, (b): $r = - 0.926$, $p < 0.0001$***

Results presented in Figure 27, show a significant negative correlation between OD_{435}/OD_{415} ratio and exposure time of NaF (Figure 27 a) and between OD_{435}/OD_{415} ratio and exposure time of $Pb(NO_3)_2$ (Figure 27 b).

II.3.3. Proline accumulation

A general rising buildup of proline was observed in *X. parietina* (Figure 28) following treatment with increasing exposure time to increasing doses of NaF and $Pb(NO_3)_2$. Matrix correlation between NaF / proline and $Pb(NO_3)_2$ / proline are shown in Figure 29.

According to the Figure 28 (a), it was noticed that the accumulation of proline in thalli increases significantly with increasing NaF concentrations ($p = 0.0052$**) but not significantly with increasing exposure time ($p = 0.177^{NS}$).

According to Figure 28 (b), no significant accumulation of proline in the thalli treated with the 0.5, 1 and 5 mM concentrations of $Pb(NO_3)_2$ was noted ($p = 0.08^{NS}$). However, with the high concentration of $Pb(NO_3)_2$ (10 mM), proline accumulation was significant ($p = 0.016$*).

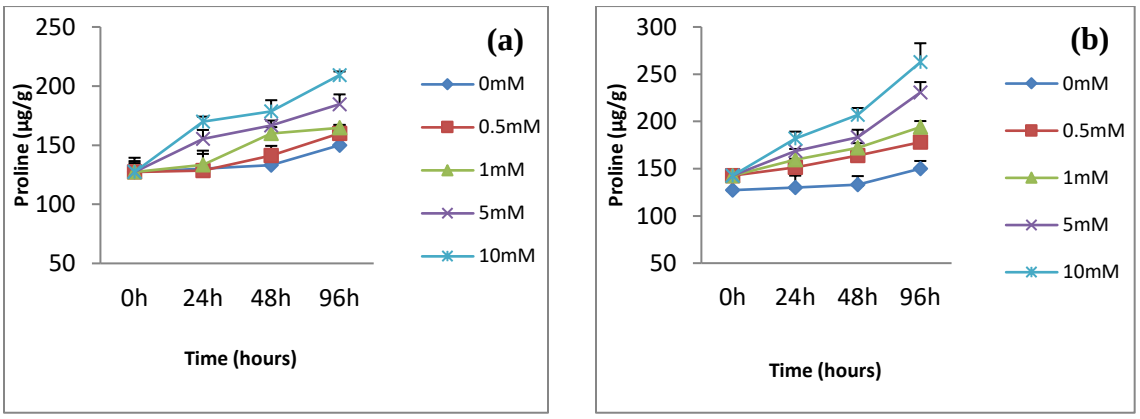

Figure 28. Accumulation of proline in thalli of *X. parietina* under NaF (a) and $Pb(NO_3)_2$ (b) stress

Depending on exposure time, significant accumulation of proline (p = 0.013*) after 96 h of treatment with all concentrations of $Pb(NO_3)_2$ was noted. Therefore, it can be concluded that the accumulation of proline varies much more with time than with concentration.

Figure 29 shows a significant positive correlation between proline and exposure time to NaF (Figure 29 a), and between proline (Figure 29 b) and exposure time to $Pb(NO_3)_2$.

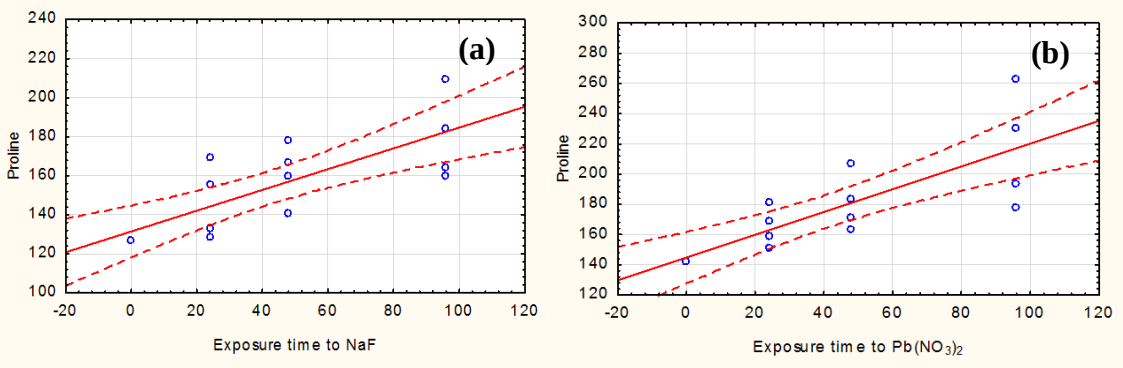

Figure 29. Correlation matrices between NaF / proline (a), $Pb(NO_3)_2$ / proline (b). (a): r = 0.783, p = 0.00032***, (b): r = 0.811, p = 0.00013***

II.3.4. Soluble sugars accumulation

The effect of different concentrations of NaF and $Pb(NO_3)_2$ on the content of soluble sugars in *X. parietina* are shown in Figure 30. Correlation matrices between NaF/soluble sugars and $Pb(NO_3)_2$ / soluble sugars are presented in Figure 31.

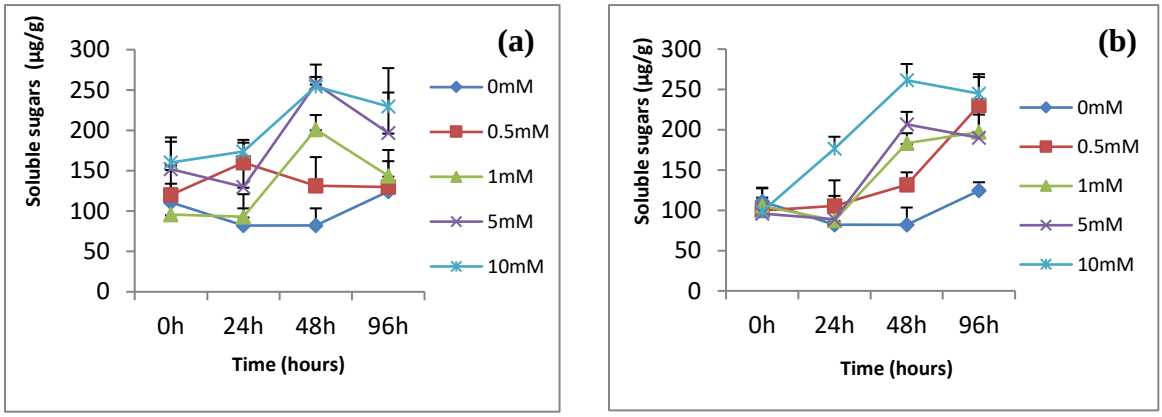

Figure 30. Variations of soluble sugar contents in thalli of *X. parietina* under NaF (a) and $Pb(NO_3)_2$ (b) stress

According to the results presented in Figure 30, the soluble sugar contents generally increase in thalli under various NaF and $Pb(NO_3)_2$ concentrations, but these contents decrease slightly after 96 h of $Pb(NO_3)_2$ treatment with 5 mM and 10 mM concentrations and with all concentrations of NaF (0.5 mM, 1 mM, 5 mM, and 10 mM). Figure 30 (a) shows a significant increase in soluble sugar contents in thalli treated with all concentrations of NaF (p = 0.01*), but depending of exposure time, the variations in soluble sugar contents were not significant ($p = 0.23^{NS}$). Unlike NaF, Figure 30 (b) shows no significant increase in soluble sugar contents depending of concentrations ($p = 0.238^{NS}$), and a significant increase depending of exposure time (p = 0.010*).

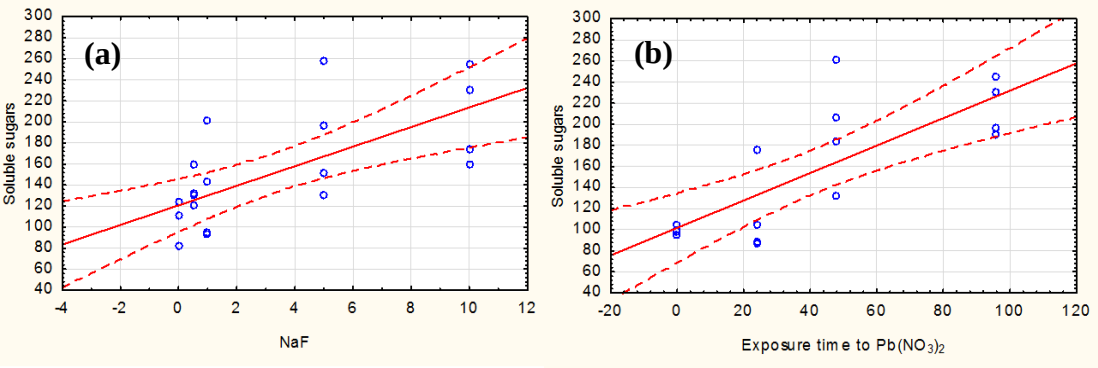

Figure 31. Correlation matrices between NaF / soluble sugars (a), $Pb(NO_3)_2$ / soluble sugars (b). (a): $r = 0.678$, $p = 0.0010$***, (b): $r = 0.780$, $p = 0.00036$***

Results of the statistical analysis show a significant positive correlation between soluble sugar contents and NaF concentrations (Figure 31 a), and between soluble sugars contents and exposure time to $Pb(NO_3)_2$ (Figure 31 b).

Our results show that chlorosis, lipid peroxidation, accumulation of proline and soluble sugars in *X. parietina* under $Pb(NO_3)_2$ treatment are more intense than under NaF treatment. Based on our statistical analysis, it was concluded that lead is significantly more toxic than fluoride ($p = 0.02$*).

II.4. Discussion

During lipid peroxidation, a wide variety of aldehydes are formed, among these aldehydes is MDA. MDA is used as an interesting biomarker and diagnostic for lipid oxidative damage under drought stress (Amine-Khodja et *al.*, 2022; Toto et *al.*, 2022). MDA is also accumulated in plants under heavy metal stress such us mercury (Singh et *al.*, 2020).

Compared to the control test, our results show a significant increase in MDA contents ($p < 0.05$*) in exposed *X. parietina* to NaF and $Pb(NO_3)_2$ solutions, where we found a positive correlation between MDA contents in *X. parietina* and increasing concentrations of NaF and $Pb(NO_3)_2$ ($r = 0.773$, $p = 0.000712$*** and $r = 0.865$, $p = 0.000031$***, respectively). Similar results were archived by Dzubaj et *al.* (2008) and Pisani et *al.* (2009) who showed that *X. parietina* reacts against fluorine, boron and lead-induced stress by increasing of the MDA content.

Our results are in agreement with those of El-Shora et *al.* (2021) which showed that lead stress increased MDA contents in the treated plants, and those of Alsherif et *al.* (2022) which

indicate that heavy metal contamination resulted in significant increases in MDA in plants. Also, our results are in the same line with those obtained by Fan et *al.* (2022) who show that MDA content increased in *Festuca arundinacea* Schreb after high concentration of fluorine treatment. Kacienė et *al.* (2015) showed that oxidative stress induced by stress factors of different origin-ozone, ultraviolet (UV)-B radiation, drought, cadmium and copper, causes the increase in the content of MDA in barley. Likewise, Gutiérrez-Martínez et *al.* (2020) noticed that the MDA content increases with increasing concentrations of cadmium accumulated in the leaves and roots of *Phaseolus vulgaris* plants under cadmium stress.

The most commonly used metric to quantify chlorophyll degradation is the ratio of optical density of chlorophyll samples read at 435 and 415nm. A ratio of 1.4 informs about chlorophyll integrity, any reduction in this value indicates the degradation of chlorophyll to provide stress to the organism (Munzi et *al.*, 2009; Bajpai et *al.*, 2010). Our results show that a ratio of 1.4 was obtained in treated lichen thalli with distilled water (control), but for those stored in different NaF and $Pb(NO_3)_2$ concentrations, a decrease in this ratio was noted. According to this results, we found that the decrease of the OD_{435} / OD_{415} ratio is in correlation with increasing exposure time of *X. parietina* to NaF and $Pb(NO_3)_2$ (r = - 0.737, p = 0.0011** and r = -0.926, p < 0.0001***, respectively). Our results are in agreement with those obtained by Shukla and Upreti (2008), who reported that OD_{435} / OD_{415} ratio values decreased with the increase in the amount of Cu, Pb and Zn in the lichen P*yxine subcinerea Stirton* and of Bajpai et *al.* (2015); Sharma and Singh (2016) and Chetia et *al.* (2021) who indicate that chlorosis increases in lichens under heavy metals stress, and with those obtained by Panda (2015), who reported that chlorosis is one of the symptoms of fluoride toxicity in plants.

Proline is part of a general adaptive syndrome to adverse environmental conditions (Liang et *al.*, 2013; Ghosh et *al.*, 2022). The accumulation of proline can be considered as a biomarker of stress (Amine-Khodja et *al.*, 2022) which varies depending on the plant species. Plants react against stress by accumulating proline to protect the structure of its macromolecules (Alhasnawi, 2019). Amri and Layachi (2018) reported that exogenous application of proline on a Faba bean (*Vicia faba*) plant cultived under cadmium stress helped the plant recover from the cadmium stress-induced physiological changes. The determination of proline in lichens is a detective method of the various possible stress phenomena. Our results show a significant increase in proline content in *X. parietina* correlating with increasing exposure time to NaF and $Pb(NO_3)_2$ (r = 0.783, p =

0.00032*** and r = 0.811, p = 0.00013***, respectively). Similar result was noted by Li et *al.* (2022) who reported that proline contents increased in maize varieties under Cd stress. Likewise, our results are similar to those obtained by Koleva et *al.* (2022), which were noted that *Phaseolus vulgaris* seedlings under cadmium-induced stress exhibited an increased level of proline. Several other studies indicate also that proline increases under the action of other types of stress: salt stress (Alhasnawi, 2019) water limitation (Bhaskara et *al.*, 2015), changing climate conditions Ghosh et *al.*, 2022), UV radiation (Metwally et *al.*, 2019), heat Stress tolerance (Iqbal et *al.*, 2019) and nutrient deficiency (Mundada et *al.*, 2021).

Like proline, soluble sugars are part of the adaptation strategies used by plants to combat and tolerate stressful conditions (Gangola and Ramadoss, 2018; Khan et *al.*, 2020; Amine-Khodja et *al.*, 2022). The obtained results (Figure 31) show a significant increase in the content of soluble sugars correlating with increasing concentration of NaF (r = 0.678, p = 0.0010***) and correlating with increasing exposure time of *X. parietina* to $Pb(NO_3)_2$ (r = 0.780, p = 0.00036***); these results are in agreement with the results obtained by Gandonou et *al.* (2011) who showed that soluble sugars accumulate in two sugarcane cultivars under salt stress. According to Abbaspour et *al.* (2012), the increase of the salinity stress resulted in the increasing concentration of soluble sugars in three pistachio cultivars. Also, several other studies showed that drought stress increased the contents of soluble sugars in the leaves of *Soybean* seedlings (Du et *al.*, 2020) and in *Sophora davidii* (Franch.) (Zhao et *al.*, 2022). Our results are also concomitant with those obtained by Aldoobie and Beltagi (2013), who reported that contents of total soluble sugars increased in common bean plants (*Phaseolus vulgaris L*. cv. Nebraska) in response to lead, cadmium and nickel stress.

According to Ahmad et *al.* (2020), plants accumulate soluble sugars as a defense mechanism against stressful conditions caused by drought and water scarcity, varying temperature from minimal to maximum level, and accumulation of salt and heavy metals.

NB: Results of this part are published in *Journal of Applied Biological Sciences.*

III. Polyphenols and flavonoids extraction quantification and study of the antibacterial activity of extracted methanol from *X. parietina* under fluoride and lead induced-stress

III. 1. Objective

Our work aims to study the toxic effects of fluoride and lead on contents of flavonoids and polyphenols in *X. parietina* lichen species, and to investigate the biological activity of its methanol extracts against several strains.

III.2. Material and methods

III.2.1. Lichen material

Samples of *X. parietina*'s lichen thalli were collected in the Beni Metrane and Djimar regions, located in Jijel, Nort-Est of Algeria (Figure 8) during the spring season 2019, and then transported in sterilized closed containers to the laboratory. Contaminants were taken out, and thalli were rinsed repeatedly in distilled water to get rid of adhering and surface dust. Fresh weights of thalli were separated from their supports and acclimatized to laboratory conditions in each experimental vessel until examination.

III.2.2. Fluoride and lead treatment

In comparison to control test, the samples of *X. parietina* were stored in solutions of sodium fluoride and lead nitrates (0.5, 1, 5, and 10 mM). Before treatment, the solutions were immediately acidified to adjust pH to 3.5 by adding H_2SO_4 or HNO_3. These solutions were then stored in the dark for 96 h at room temperature. After 96 h of treatment and before each analysis, the treated thalli were rinsed with distilled water and then dried at room temperature (Carreras and Pignata, 2007).

III.2.3. Extraction of phenolic compounds

Phenolic compounds can be extracted by several organic solvents such as ethanol, methanol, and acetone (Ajila et *al.*, 2011). But methanol is the most important solvent because it offers a good extraction yield and has the advantage of being easily removed (Owen and Johns, 1999) and when diluted with water (80%), it provides important extraction of polyphenols (Qasim et *al.*, 2016; Nakilcioglu and Otles, 2021).

The test consists of extracting 15 g of lichen thalli in 60 mL of methanol (80%). The resulting mixture is then continuously stirred for 48 h at room temperature without the presence of light to prevent oxidation phenomena. After maceration, filter paper was then used to filter the solutions.

Each preparation's methanol extracts were evaporated and then dried at 40 °C using a Heidolph type rotavapor until complete evaporation of the methanol. After evaporation, crude methanol was used to extract the mass of the obtained residue. The obtained extracts were then frozen at -20 ° C until they have been analyzed (Qasim et *al.*, 2016; Nakilcioglu and Otles, 2021).

III.2.4. Total soluble phenolic compounds determination

Method described by Slinkard and Singleton (1977) was used to determine total phenolic compounds in methanol extracts of *X. parietina* using gallic acid as standard. Essay was carried out by adding 1 mL of the Folin-Ciocâlteu reagent to 1 mL of the methanol extract, and 1 mL of Na_2CO_3 (20 g/L) after 5 min. After incubation of the mixture at room temperature for 2 h, the absorbance was measured at 760 nm. Applying the equation derived from the gallic acid calibration curve (y = 6.574 x, R^2 = 0.99), the results are reported in mg gallic acid equivalent per 1 g dry weight (mg GAEPGDW) of the extract.

III.2.5. Total flavonoid compounds determination

According to the method of Meda et *al.* (2005), total flavonoids were determined. To 2 mL of the extract 2 mL of aluminum trichloride ($AlCl_3$) was added, the mixture was then incubated at room temperature for 10 min, and the absorbance was measured at 415 nm using a spectrophotometer against a blank sample. Total flavonoid contents were expressed as Quercetin equivalents per g extract dry weight (QEPGDW) by referring to the equation established from the standard curve reference (y = 31.68 x, R^2 = 0.99).

III.2.6. Antibacterial activity of methanol extracts

III.2.6.1. Tested strains

All of the tested strains were provided by the American Type Culture Collection (ATCC). Two bacterial groups are represented by the examined strains, positive Gram bacteria (*Bacillus cereus* ATCC 10876, *Staphylococcus aureus* ATCC 25923, *Listeria monocytogenes* ATCC 15313) and negative Gram bacteria (*Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 700603, *Salmonella typhimurium* ATCC 25842, *Pseudomonas aeruginosa* ATCC 27853, and *Enterobacter spp.* ATCC 25639). First, these tested strains are cultured for 18 h at 37 °C in a nutritional broth, and then seeded using the method of the quadrant on agar culture medium to create bacterial culture that are well isolated (Nigussie et *al.*, 2021).

III.2.6.2. Inoculum preparation

Each bacterial suspension was made by dilution of a colony of the relevant strain from a fresh culture in 9 mL of saline water (NaCl 0.9%). The inoculum should be used within 15 min of preparation, and the suspension should have an opacity equivalent to 0.5 by the conventional McFarland method with around 10^8 CFU/mL (CFU = colony-forming unit) (Kassim et *al.*, 2016).

III.2.6.3. Antibacterial activity test

Using Mueller Hinton agar, the method of adapted disc diffusion was applied to investigate the bactericidal activity of methanol extracts of *X. parietina* treated fluoride (MEXTF) and lead (MEXTL) in vitro (Kassim et *al.*, 2016). In Petri plates, the agar culture medium is added. Following solidification, each inoculum is inoculated under strict aseptic circumstances by wiping the medium's surface with sterile swabs dipped in the bacterial suspensions. The culture medium was then covered with sterile disks of Whatman No. 1 paper, and each disk was then moistened with 20 µL of methanol extract (Wayne, 2018).

In control test, disks were moistened with distilled water, methanol, and different concentrations of NaF and $Pb(NO_3)_2$ solutions. For the purpose of calculating an average and a standard deviation, each test is conducted three times. After the diffusion of the methanol extract, the boxes were incubated at 37 ° C for 18 to 24 h (Devi et *al.*, 2011).

After incubation, the absence of the bacterial growth results in the appearance of a translucent halo (inhibition zone) around each disc, similar to that of sterile agar. Results were expressed as the (mm) of the inhibition zone diameter (IZd).

III.2.7. Statistical analysis

For the purpose of calculating the standard deviation (SD), three repetitions were carried out at each concentration. The ORIGIN 6.0 system's test univariate variance (one way ANOVA) was used to conduct the statistical analysis. Results were expressed as mean ± SD. The distinction was deemed insignificant when $p > 0.05$(NS), significant when $0.01 < p < 0.05$ (*), very significant when $0.001 < p < 0.01$ (**) and highly significant when $p < 0.001$ (***).

STATISTICA Version 10 software was used to analyze correlation matrices between NaF, $Pb(NO_3)_2$ and the different studied parameters.

III.3. Results

III.3.1. Variations of polyphenol and flavonoid contents

Variations of polyphenol and flavonoid contents in treated thalli of *X. parietina* for 96 h with different NaF and $Pb(NO_3)_2$ concentrations are shown in Figure 32 and Figure 33, respectively. Polyphenols / NaF, polyphenols / $Pb(NO_3)_2$, flavonoids / NaF, and flavonoids / $Pb(NO_3)_2$ correlation matrices are presented in Figure 36, Figure 37, Figure 39 and Figure 40, respectively. However, matrix correlation between polyphenols/lead and flavonoids/lead are shown in Figure 38 and Figure 41, respectively.

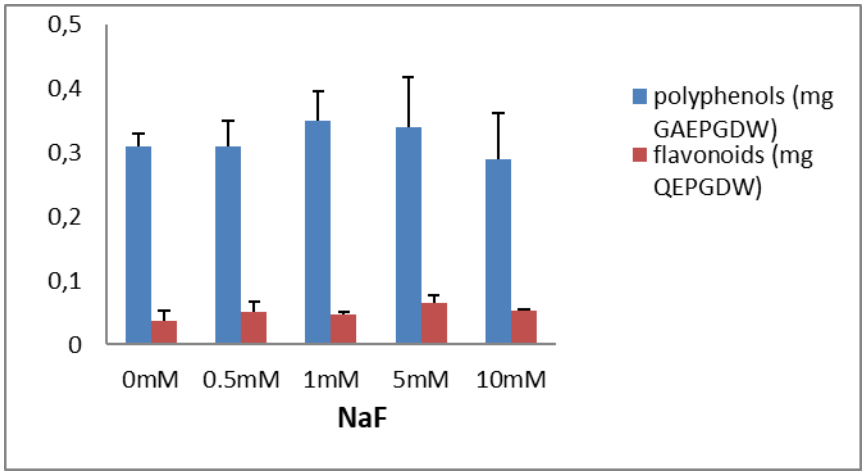

Figure 32. Variations of polyphenol and flavonoid contents in thalli of *X. parietina* under NaF stress, mg GAEPGDW : gallic acid equivalent per g dry weight, mg QEPGDW : quercetin equivalent per g dry weight.

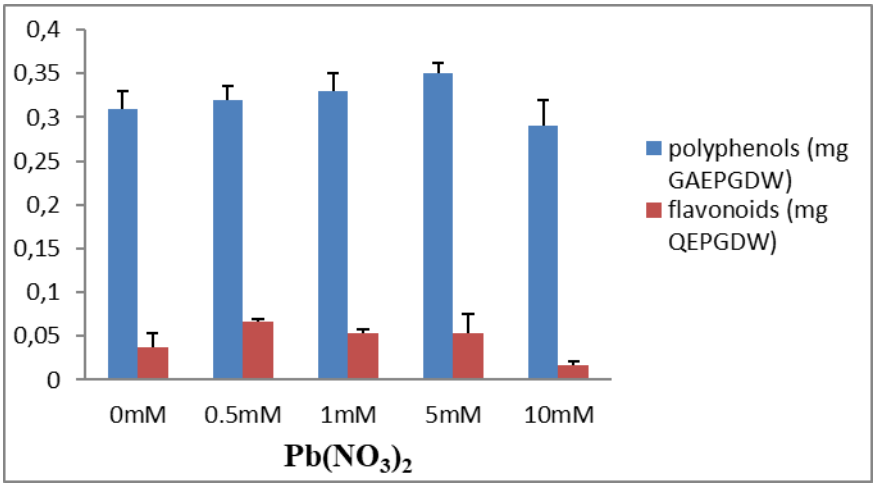

Figure 33. Variations of polyphenol and flavonoid contents in thalli of *X. parietina* under $Pb(NO_3)_2$ stress, mg GAEPGDW : gallic acid equivalent per g dry weight, mg QEPGDW : quercetin equivalent per g dry weight.

From the results shown in Figure 32 and Figure 33, it was found that the polyphenol contents in *X. parietina* was 0.31 mg, this value is registered in thalli of the control test, and in thalli treated by the concentration 0.5 mM of NaF at T (96 h). However, after 96 h, this value changes in the thalli treated with other concentrations of NaF and various concentrations of $Pb(NO_3)_2$, where a significant increase was observed in the thalli treated with the concentrations of 1 mM of NaF (p = 0.004**) and with concentrations of 0.5, 1 and 5 mM of $Pb(NO_3)_2$ (p = 0.04081*). In contrast significant decrease of total phenolic compounds was noted in thalli treated with the concentrations 5 mM and 10 mM of NaF (p = 0.00143**). With the concentrations 5 mM and 10 mM of $Pb(NO_3)_2$, the decrease in the polyphenol contents was not significant ($p > 0.05^{NS}$).

Significant variation in flavonoid contents was noted in thalli treated with different concentrations of NaF (p = 0.00334**) and $Pb(NO_3)_2$ (p = 0.00329**), the results show a remarkable increase in thalli treated with the concentration 0.5 mM of $Pb(NO_3)_2$. Whereas, results show a significant decrease in flavonoid contents in the thalli treated with the concentration 10 mM of NaF (p = 0.03821*) and the other concentrations (1, 5 and 10 mM) of $Pb(NO_3)_2$ (p = 0.0018**).

III.3.2. Antibacterial activity results

Antibacterial activity results of MEXTF (Methanol Extract of *X. parietina* Treated with Fluoride) and MEXTL (Methanol Extract of *X. parietina* Treated with Lead) on different studied strains are presented in Figure 34 and Figure 35, respectively.

From the results presented in Figure 34 (a) and Figure 34 (b), no effect of MEXTF 0.5 and 1 mM against *E. coli* and *P. aeruginosa* was noted. However, a significant increase ($p = 0.0183$*) of the the inhibition zone diameter (IZd = 13 mm) was noted with MEXTF 5 mM, followed by a slight reduction with MEXTF 10 mM ($p = 0.15387^{NS}$) against *E. coli.* A slight increase of IZd against *P. aeruginosa* with MEXTF 5 and 10 mM ($p = 0.07139^{NS}$) was noted.

According to the data presented in Figure 34 (c), there was a significant increase ($p = 0.00758$**) in the diameter of the inhibition zone from 11 mm to 13.33 mm with the MEXTF 5 mM against *Enterobacter spp.* From this concentration and with the MEXTF 10 mM, this diameter decreases significantly ($p = 0.02895$*) to reach 11mm, equivalent to that recorded in the thalli treated with distilled water. Compared to the control test (IZd = 10.33 mm), Figure 34 (d) shows a significant increase ($p = 0.04513$*) in antibacterial activity of MEXTF 1 mM against *K. pneumoniae* (IZd = 13 mm), followed by significant decrease with MEXTF 5 and 10 mM ($p = 0.004$**). Against *S. typhimurium* (Figure 34 (e)), no variation was observed between the different concentrations of the MEXTF ($p = 0.18636^{NS}$).

From the data presented in Figure 34 (f), it was noted a significant increase in antibacterial activity against *B. cereus* ($p = 0.01378$*) of MEXTF 1Mm. However, a significant decrease in the diameter of the inhibition zone ($p = 0.0110$*) was noted with MEXTF between 1mM and 10 mM.

Similar effect of methanol extract was noticed against *L. monocytogenes* and *S. aureus* (Figure 34 (g, h)), we found that the antibacterial activity increases significantly with increasing MEXTF concentrations (from MEXTF 0.5 mM to MEXTF 5 mM) when compared to the control test ($p = 0.02795$* and $p = 0.049$*, respectively). However, by the MEXTF 10 mM, a significant decrease in the inhibition zone diameter was noted with *L. monocytogenes* and *S. aureus* ($p = 0.04295$* and $p = 0.02895$*, respectively).

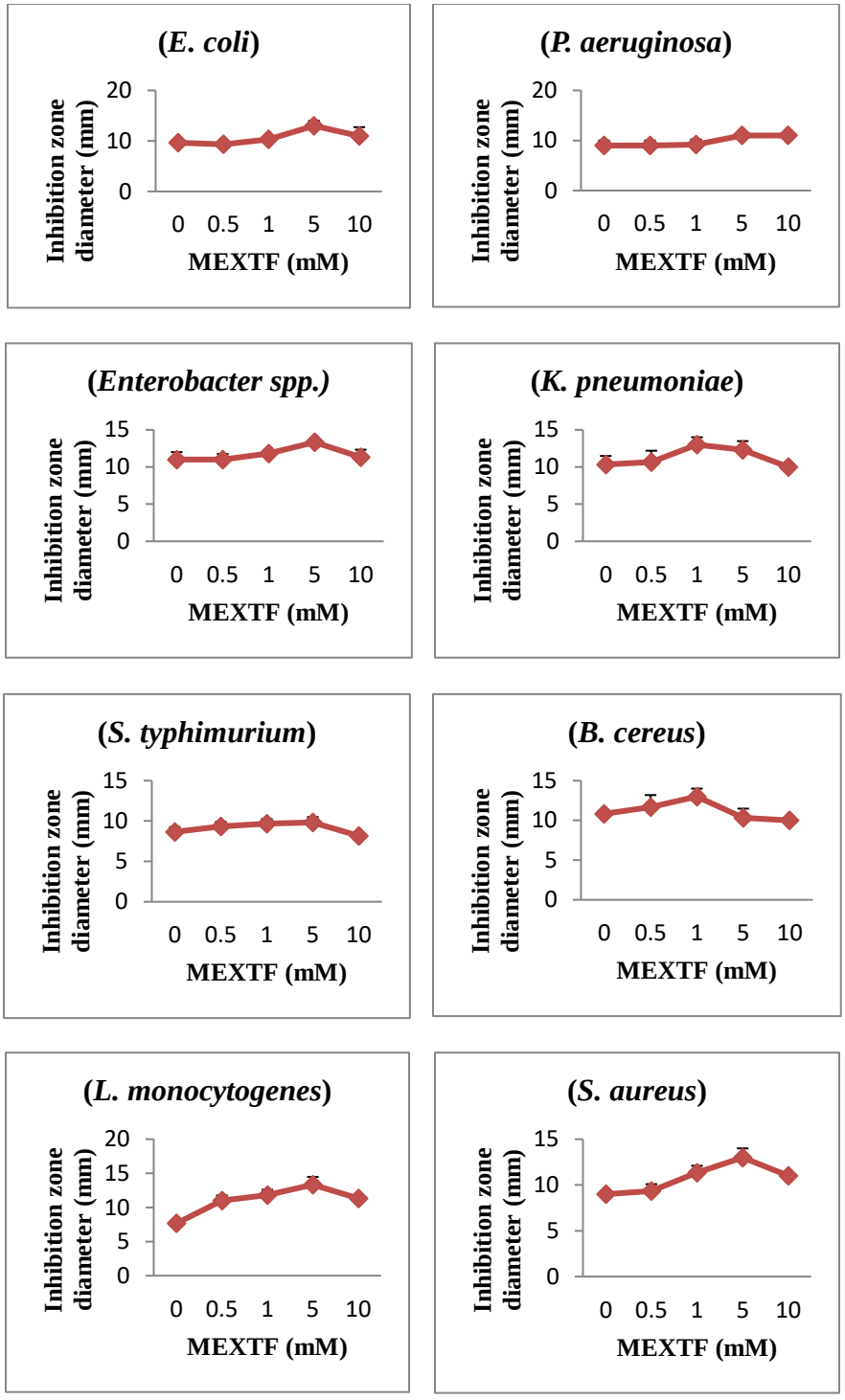

Figure 34: Methanol extracts' antibacterial effect of *X. parietina* thalli under NaF stress. MEXTF: methanol extract of *X. parietina* treated with fluoride.

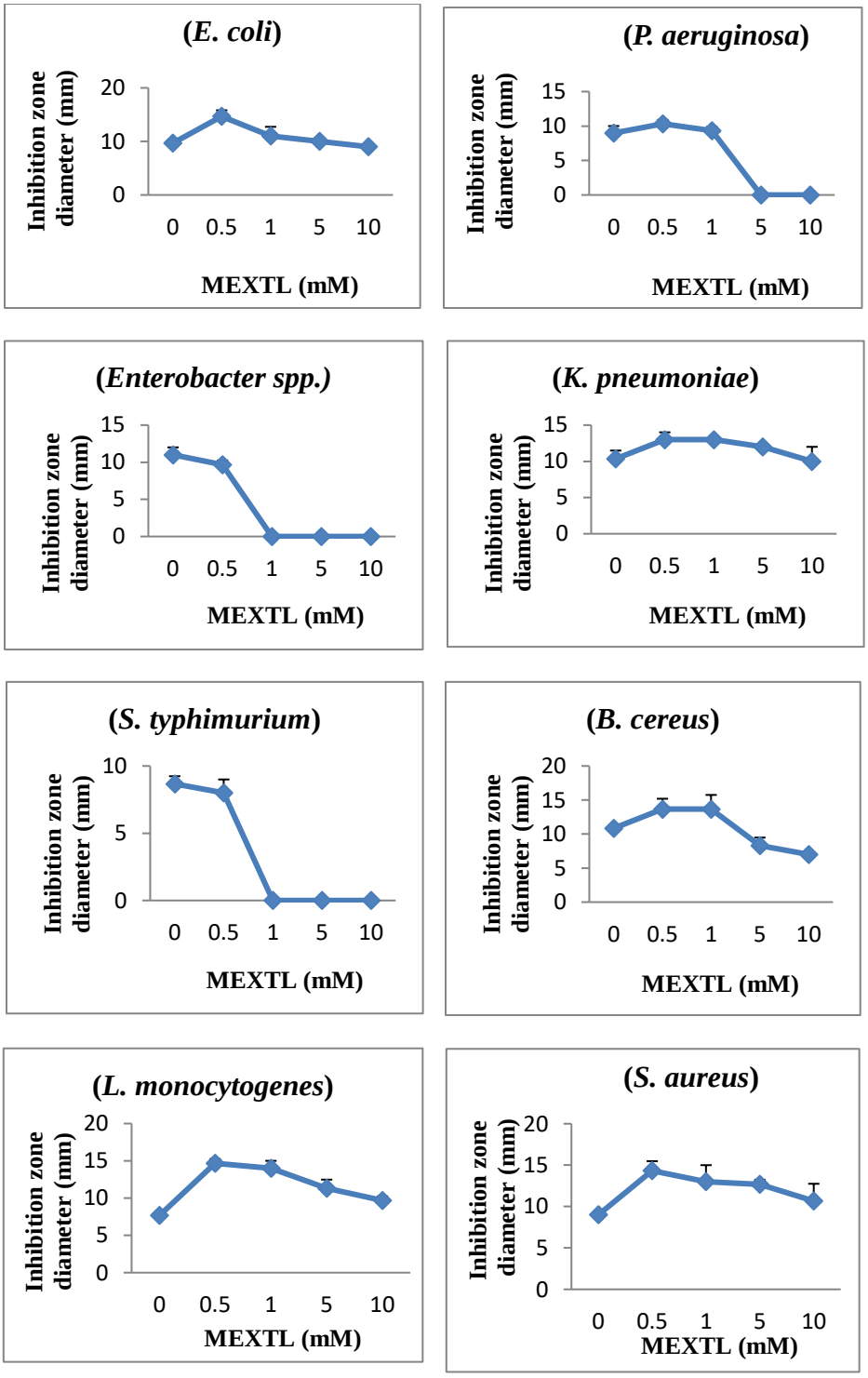

Figure 35. Methanol extracts' antibacterial effect of *X. parietina* thalli under $Pb(NO_3)_2$ stress. MEXTL: methanol extract of *X. parietina* treated with lead.

From the results presented in Figure 35, the extract (MEXTL) was found to have the same antibacterial action against *E. coli, B. cereus, L. monocytogenes,* and *S. aureus* bacteria. Only MEXTL 5 mM and 10 mM have no antibacterial action on *P. aeruginosa*. However, similar result was noted against *Enterobacter spp.* and *S. typhimurium* bacteria, only the MEXTL 0.5 mM has identical antibacterial activity to that obtained by the control. Whereas, against *K. pneumoniae* bacteria variations in the antibacterial activity of MEXTL are negligible.

From the Figure 35 (a), it can be noticed that the MEXTL 0.5 mM has an important effect against *E. coli* bacteria, with a highly significant increase (p = 0.003**) of the inhibition zone diameter (IZd = 14.67 mm). However, in thalli treated with MEXTL 1, 5 and 10 mM, a significant decrease of the antibacterial activity was noted (p = 0.019*).

Figure 35 (b) shows similar antibacterial activity of MEXTL 0 and 0.5 mM against *P. aeruginosa*, while, with MEXTL 0.5 mM a significant decrease (p = 0.02791*) was noted.

According to the results presented in Figure 35 (c) it can be noted a significant decrease of the antibacterial activity against *Enterobacter spp.* (p = 0.00211**) with increasing concentrations of MEXTL. No activity was noted with MEXTL 1 mM, 5 mM and 10 mM.

Figure 35 (d) demonstrates a significant increase (p = 0.0007***) in the antibacterial activity of MEXTL 0.5 mM against *K. pneumoniae* (IZd = 13 mm) when compared to the control (IZd = 10.33 mm); the same antibacterial effect was also seen with MEXTL 1 mM, with a slight reduction by MEXTL 5 mM; and a significant decrease (p = 0.01566*) of the antibacterial activity with MEXTL 10 mM.

With rising MEXTL concentrations Figure 35 (e) shows a significant decrease of the antibacterial activity against *S. typhimurium* bacteria (p = 0.00251**). This species was found to be completely resistant to MEXTL at concentrations of 1 mM, 5 mM, and 10 mM.

Figure 35 (f) demonstrates that MEXTL's antibacterial activity tends to rise significantly (p = 0.00001***) against *B. cereus* to achieve the maximum with MEXTL 0.5 mM and 1 mM, followed by significant decrease (p = 0.02144*) with MEXTL 5 mM and 10 mM.

From the Figure 35 (g, h), similar result was noted against *L. monocytogenes* and *S. aureus*, we found a significant increase in the inhibition zone diameters with MEXTL 0.5 mM (p = 0.02*, p = 0.004**, respectively) when compared to the control test. However, with the other MEXTL

concentrations (1 mM, 5 mM, and 10 mM) significant decrease in the inhibition zone diameters was noted against *L. monocytogenes* and *S. aureus* (p = 0.007**, p = 0.0089**, respectively).

No antibacterial effect on any strain (IZd = 0 mm) was observed with the control test (distilled water, methanol, different NaF and $Pb(NO_3)_2$) concentrations.

III.3.3. Correlation analysis

III.3.3.1. Polyphenols /NaF, polyphenols / $Pb(NO_3)_2$ and polyphenols / lead correlations

Polyphenols /NaF, polyphenols / $Pb(NO_3)_2$ and polyphenols / lead correlations are presented in Figure 36, Figure 37 and Figure 38, respectively.

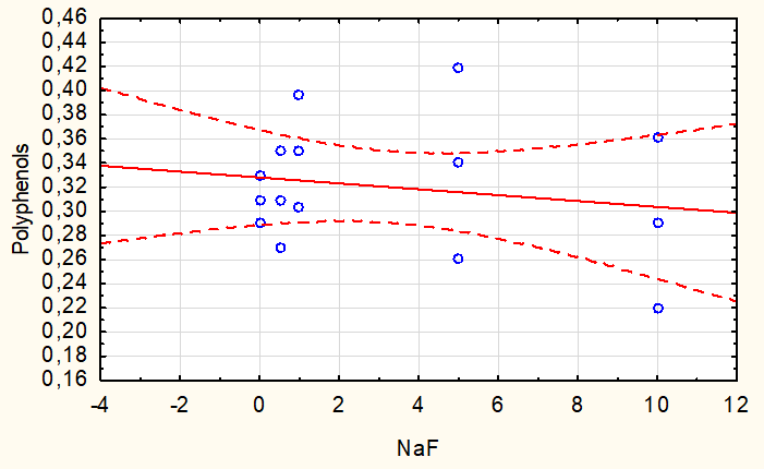

Figure 36. Matrix correlation between NaF and polyphenol contents in thalli of *X. parietina*, r = -0.18351, p = 0.51267, Significance: ^{NS}

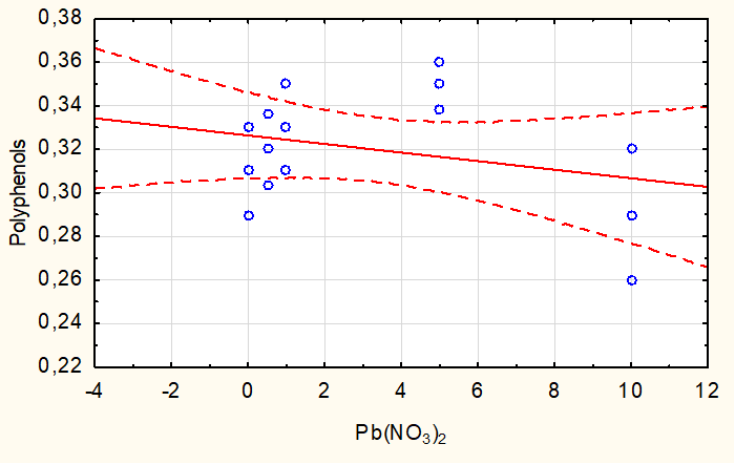

Figure 37. Matrix correlation between $Pb(NO_3)_2$ and total phenolic compounds in thalli of *X. parietina*, r = -0.28797, p = 0.29795, Significance: [NS]

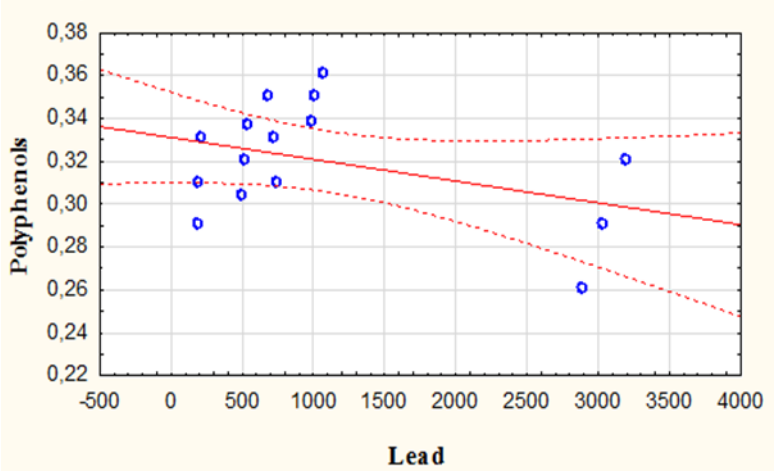

Figure 38. Matrix correlation between polyphenol compounds and accumulated lead in thalli of *X. parietina*, r = - 0.39744, p = 0 .142374, Significance: [NS]

Figure 36, Figure 37 and Figure 38 show negative correlations between polyphenols and NaF, polyphenols and $Pb(NO_3)_2$, polyphenols and lead accumulated in *X. parietina*, respectively. Similarly Figures (36, 37, and 38) show that polyphenols /NaF, polyphenols / $Pb(NO_3)_2$ and polyphenols / lead correlations are not significant.

III.3.3.2. Flavonoids /NaF, flavonoids / $Pb(NO_3)_2$ and flavonoids / lead correlations

Flavonoids /NaF, flavonoids / $Pb(NO_3)_2$ and flavonoids / lead correlations are presented in Figure 39, Figure 40 and Figure 41, respectively.

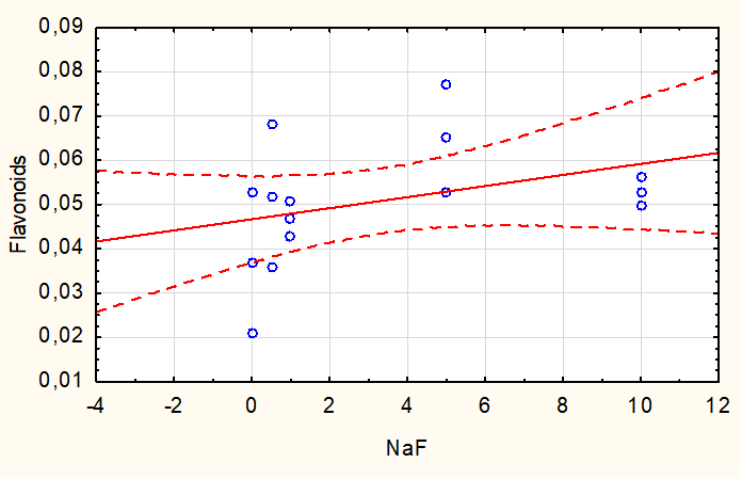

Figure 39. Matrix correlation between NaF and total flavonoid compounds in thalli of *X. parietina*, r = 0.35996, p = 0.18754, Significance: ^{NS}

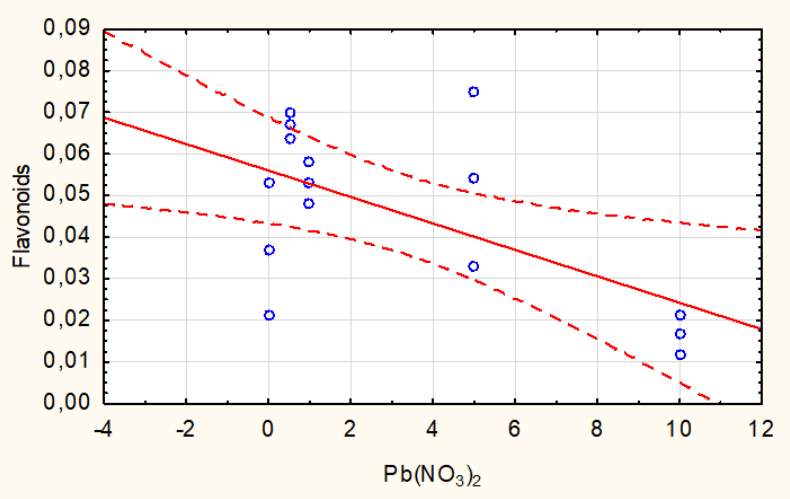

Figure 40. Matrix correlation between $Pb(NO_3)_2$ and total flavonoid compounds in thalli of *X. parietina*, r = -0.60273, p = 0.01739, Significance: *

Figure 39, Figure 40 and Figure 41 show positive correlation between flavonoids and NaF, and negative correlations between polyphenols and $Pb(NO_3)_2$, polyphenols and lead accumulated in *X. parietina*, respectively. From these results, we found a non-significant correlation between flavonoids and NaF, and a significant correlation between flavonoids and $Pb(NO_3)_2$, and between flavonoid contents and accumulated lead in *X. parietina*.

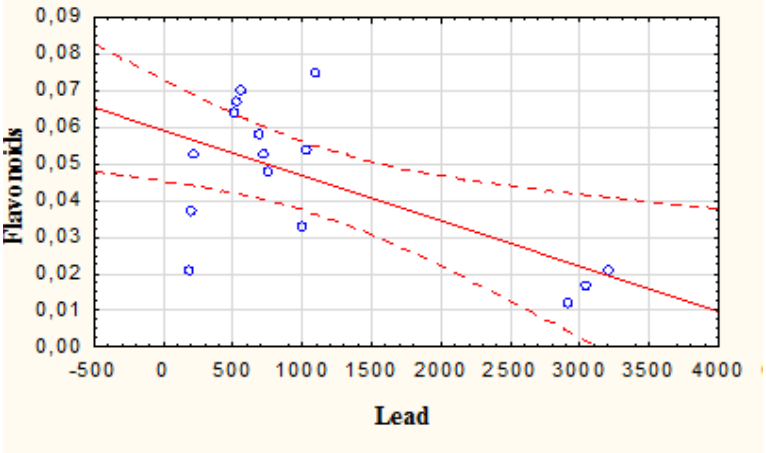

Figure 41. Matrix correlation between accumulated lead and content of flavonoids in thalli of *X. parietina*, r = - 0.62677, p = 0 .012402, Significance: *

III.3.3.3. Matrix correlations between polyphenols/antibacterial activity and between flavonoids/antibacterial activity

Polyphenols/antibacterial activity and flavonoids/antibacterial activity correlation matrices under NaF and $Pb(NO_3)_2$ stress are presented in Tables 6, 7, 8 and 9, respectively.

According to Tabe 6, we found a significant positive correlation between the polyphenol contents and the antibacterial activity against *E. coli, Enterobacter spp., K. pneumoniae, S. typhimurium, B. cereus* and *S. aureus*. However, against *P. aeruginosa and L. monocytogenes* no significant positive correlation was noted.

Table 6. Matrix correlation between total phenolic contents in thalli of *X. parietina* and antibacterial activity under NaF stress

Correlation	***r***	***P***	**Significance**
Polyphenols / *E. coli* IZd	0.52353	0.04518	*
Polyphenols / *P. aeruginosa* IZd	0.28321	0.30637	NS
Polyphenols / *Enterobacter spp.* IZd	0.69567	0.00397	**
Polyphenols / *K. pneumoniae* IZd	0.61949	0.01377	*
Polyphenols / *S. typhimurium* IZd	0.77640	0.00066	***
Polyphenols / *B. cereus* IZd	0.72742	0.00211	**
Polyphenols / *L. monocytogenes* IZd	0.42242	0.11674	NS
Polyphenols / *S. aureus* IZd	0.84156	0.00008	***

(NS): not significant, (*): significant, (**) very significant, (***): highly significant

Table 7. Matrix correlation between flavonoid contents in thalli of *X. parietina* and antibacterial activity under NaF stress

Correlation	***r***	***p***	**Significance**
Flavonoids / *E. coli* IZd	0.59349	0.01968	*
Flavonoids / *P. aeruginosa* IZd	0.78624	0.0005	***
Flavonoids / *Enterobacter spp.* IZd	0.85342	0.00005	***
Flavonoids /*K. pneumoniae* IZd	0.32903	0.23111	NS
Flavonoids / *S. typhimurium* IZd	0.62896	0.012	*
Flavonoids / *B. cereus* IZd	0.52811	0.04301	*
Flavonoids / *L. monocytogenes* IZd	0.54495	0.03566	*
Flavonoids / *S. aureus* IZd	0.41239	0.12662	NS

(NS): not significant, (*): significant, (**) very significant, (***): highly significant

From the results shown in Table 7, we note a significant positive correlation between the flavonoid contents and the antibacterial activity against all studied species with the exception seen in *K. pneumoniae* IZd and *S. aureus* IZd, where we found a not significant correlation.

Table 8. Matrix correlation between polyphenol contents in thalli of *X. parietina* and antibacterial activity under $Pb(NO_3)_2$ stress

Correlation matrices	***r***	***p***	**Significance**
Polyphenols / *E. coli* IZd	0.31132	0.25868	NS
Polyphenols / *P. aeruginosa* IZd	0.01132	0.96805	NS
Polyphenols / *Enterobacter spp.* IZd	- 0.13514	0.63108	NS
Polyphenols *K. pneumoniae* IZd	0.36287	0.18373	NS
Polyphenols / *S. typhimurium* IZd	- 0.16468	0.55753	NS
Polyphenols / *B. cereus* IZd	0.33484	0 .22248	NS
Polyphenols / *L. monocytogenes* IZd	0.41141	0.12761	NS
Polyphenols / *S. aureus* IZd	0.65596	0.00792	**
(NS): not significant, (*): significant, (**) very significant, (***): highly significant			

From the results of the statistical analysis presented in Table 8, positive correlation between polyphenol contents and the antibacterial activity against *E. coli, P. aeruginosa, K. pneumoniae, B. cereus, L. monocytogenes,* and *S. aureus* was noted. However, a negative correlation was noted against *Enterobacter spp.* and *S. typhimurium*. Against *S. aureus,* results also show a significant correlation between polyphenols and antibacterial activity

Results presented in Table 9 indicate a positive correlation between flavonoid levels and antibacterial activity against all strains bacteria. With regard to *E. coli*, *B. cereus*, *L. monocytogenes,* and *S. aureus*, a significant correlation between flavonoids and antibacterial activity was also discovered.

Table 9. Matrix correlation between flavonoid contents in thalli of *X. parietina* and antibacterial activity under $Pb(NO_3)_2$ stress

Correlation	*r*	*p*	Significance
Flavonoids / *E. coli* IZd	0.71298	0.00284	**
Flavonoids / *P. aeruginosa* IZd	0.41610	0.12290	NS
Flavonoids / *Enterobacter spp.* IZd	0.25209	0.36472	NS
Flavonoids *K. pneumoniae* IZd	0.51179	0.05115	NS
Flavonoids / *S. typhimurium* IZd	0.24803	0.37274	NS
Flavonoids / *B. cereus* IZd	0.69038	0.00438	**
Flavonoids / *L. monocytogenes* IZd	0.69477	0.004	**
Flavonoids / *S. aureus* IZd	0.67523	0.00574	**

(NS): not significant, (*): significant, (**) very significant, (***): highly significant

III.4. Discussion

Lichen extract is generally known for its richness in phenolic compounds. Based on our results, we were able to verify that these chemical compounds were present in these samples, and to determine a concentration of polyphenol compounds equivalent to 0.31 mg/g of dry weight of extract, expressed in equivalents of gallic acid.

In treated thalli of *X. parietina* with fluoride and lead solutions, our results show a significant increase in polyphenol and flavonoid contents with increasing NaF and $Pb(NO_3)_2$ concentrations, these results are similar with those obtained by Khedim et *al.* (2020) which found an increase in the total phenolic and flavonoid contents in *Atriplex canescens*, depending on the increasing concentration of heavy metals (zinc, lead and cadmium). The same results were also obtained by Ren et *al.* (2021) and Kiani et *al.* (2021) who show that plants accumulate phenolic compounds as mechanism of defense against stressful situations. In another study carried out by Kısa et *al.* (2016), it was noted that total phenolic compounds increased in all treatments of *Zea mays* by Cd, Cu, and Pb when compared to control test. Harangozo et *al.* (2014) also show that

polyphenol compounds in flax seeds increased with increasing concentrations of lead. Likewise, Benhabiles et *al.* (2020) show an increase in polyphenol levels under cadmium stress. Yet another investigation by Mamat et *al.* (2015) showed that the activity of cytochrome C oxidase in cells increases under high concentrations of copper which induces more synthesis of phenolic compounds. Our results are also in agreement with those obtained by Procházková et *al.* (2011), who indicate that the accumulation of total phenolic and flavonoid compounds in lichens under stressful conditions are employed as mechanism of biodegradation and detoxification of xenobiotics.

According to our results, we found a significant increase in polyphenol contents in thalli treated with the concentration 1 mM of NaF (p = 0.004**) and a significant decrease in those treated with the other concentrations (p = 0.00143**), also a significant decrease in flavonoid contents in the thalli treated with the concentration 10 mM of NaF (p = 0.03821*) was noted, these results are confirmed by the statistical analysis presented in Figure 36 and Figure 39, where we found negative correlation between NaF and polyphenol contents (r = -0.18351, p = 0.51267 ^{NS}) and positive correlation between NaF and total flavonoid compounds (r = 0.35996, p = 0.18754 ^{NS}). However, a non-significant decrease in polyphenol contents in thalli stressed by high concentrations of $Pb(NO_3)_2$ (10 mM) is noted ($p > 0.05^{NS}$), while flavonoid contents decrease significantly in thalli treated with all $Pb(NO_3)_2$ concentrations except 0.5 mM.

Results of the statistical analysis presented in Figure 37 and Figure 40 show no significant decrease in polyphenol contents and significant decrease in flavonoid contents in correlation with the various concentrations of $Pb(NO_3)_2$ (r = -0.28797, p = 0.29795^{NS}, r = -0.60273, p = 0.01739*, respectively). According to the statistical analysis presented in Figure 38 and Figure 41, the same results were noted; no significant decrease in contents of polyphenol and significant decrease in contents of flavonoid were obtained correlating with accumulated lead in *X. parietina* (r = - 0.39744, p = 0 .14237^{NS}; r = - 0.62677, p = 0 .0124*, respectively). The imbalance between the production of ROS and the drop in contents of polyphenol and flavonoid in stressed thalli causes oxidative stress in the biological system. Sharma et *al.* (2014) and Pizzino et *al*. (2017) state that these amounts decrease as a result of the secondary metabolism disruption and the breakdown of the antioxidant defense system. A considerable increase in the production of ROS caused by heavy metals creates an imbalance that damages cells and tissues. According to the results obtained by Kısa (2018), ascorbate peroxidase (APX), peroxidase (POD), and superoxide dismutase (SOD)

activities are said to be reduced in tomato grown under heavy metal-induced stress as a result of an excessive accumulation of heavy metals. High lead concentrations resulted in a decrease in the polyphenol content of *X. parietina*, which can be attributed to lead-induced free radical overproduction.

Our findings suggest that *X. parietina* may be used as a natural source of antibiotics; these results concur with those of Basile et *al*. (2015), Alqahtani et *al*. (2020), and Rankovi et *al*. (2014) with *Hypogymnia physodes* lichen. From the obtained results, we found an increase in the inhibition zone diameter of the studied strains under effect of the methanol extracts of fluoride and lead-treated lichen; the rise in the polyphenol and flavonoid contents can be used to explain this increase. Our findings are consistent with those of Coppo and Marchese (2014), who show that polyphenols are a potential source of antimicrobial agents, as well as those of Akpinar et *al*. (2009), Devi et *al*. (2011), Alqahtani et *al*. (2020), and Popovici et *al*. (2022), who show that lichen methanol extracts have antibacterial activity against several bacteria.

Our results show that methanol extract of thalli treated with high concentrations of fluoride and lead produce a reduction in the inhibition zone diameter in the examined strains. This drop can be attributed to a decrease in polyphenol and flavonoid contents under fluoride and lead stress, where we discovered a positive correlation between antibacterial activity of methanol extract and contents of polyphenol and flavonoid.

According to the results shown in Tables 6 - 10, in most instances the variations in the inhibition zone diameter are in correlation positive with polyphenol and flavonoid contents, we found a significant correlation between polyphenols / *E. coli* IZd (r = 0.52353, p = 0.04518*), polyphenols / *Enterobacter spp* IZd (r = 0.69567, p = 0.00397**), polyphenols / *K. pneumoniae* IZd (r = 0.61949, p = 0.01377*), polyphenols / *S. typhimurium* IZd (r = 0.77640, p = 0.00066***), polyphenols / *B. cereus* IZd (r = 0.72742, p = 0.00211**), polyphenols / *S. aureus* IZd (r = 0.84156, p = 0.00008***), flavonoids / *E. coli* IZd (r = 0.59349, p = 0.01968*), flavonoids / *P. aeruginosa* IZd (r = 0.78624, p = 0.0005***), flavonoids / *Enterobacter spp.* IZd (r = 0.85342, p = 0.00005***), flavonoids / *S. typhimurium* IZd (r = 0.62896, p = 0.012*), flavonoids / *B. cereus* IZd (r = 0.52811, p = 0.04301*) and flavonoids / *L. monocytogenes* IZd (r = 0.54495, p = 0.03566*) under NaF stress. However, under $Pb(NO_3)_2$ stress, only significant correlations between

polyphenols / *S. aureus* IZd (r = 0.65596, p = 0.00792**), flavonoids / *E.coli* IZd (r = 0.71298, p = 0.00284**) and flavonoids / *S. aureus* (r = 0.65596, p = 0.00792**) were noted.

Based on our results, MEXTF exerts the same effect against Gram-positive and Gram-negative bacteria. IZd = 13.33 mm was recorded with MEXTF 5 mM against *Enterobacter spp*. and *L. monocytogenes* followed by IZd = 13 mm recorded in *E. coli* and *S. aureus* with MEXTF 5 mM, and in *K. pneumoniae* and *B. cereus* with MEXTF 1 mM. Whereas, the most resistant strains are *P. aeruginosa* and *S. typhimurium*. However, the Gram-positive bacteria (*B. cereus, L. monocytogenes, S. aureus*) were found to be more susceptible to MEXTL than the Gram-negative bacteria (*S. typhimurium, P. aeruginosa, and Enterobacter spp.*). *B. cereus* (IZd = 10.83 - 13.67 mm), *L. monocytogenes* (IZd = 7.67 - 14.67 mm), and *S. aureus* (IZd = 9 - 14.33 mm) are the most sensitive strains, whereas, *S. typhimurium* and *Enterobacter spp.* (IZd = 0 mm) are the most resistant against MEXTL 1, 5 and 10 mM.

Our results demonstrate that methanol extract has a considerably greater antibacterial effect (p = 0.001***) against Gram-positive bacteria as compared to Gram-negative bacteria. Identical conclusion was reached by b Alghazeer et *al.* (2013) who report that both Gram-positive (*S. aureus, B. subtilis, Bacillus spp*., and *S. epidermidis*) and Gram-negative bacteria (*E. coli, S. typhi, Klebsiella spp*., and *P. aeruginosa*) were significantly inhibited by the methanol extract of marine green, and by Chen et *al*. (2022) who investigated the antibacterial activity of polyphenol extract from fresh sweet sorghum stems against *S. aureus, E. coli, Listeria spp.,* and *Salmonella spp*. and who came to the conclusion that the effect of the extract is significantly greater against Gram-positive bacteria than it is against Gram-negative bacteria. The existence of the outer membrane in Gram-negative bacteria, which is completely missing in Gram-positive bacteria, may be the cause of this discrepancy (Vollmer et *al.*, 2008).

Among Gram-negative bacteria, our results show that *E. coli* and *Enterobacter spp.* offer significant sensitivity to MEXTF 5 mM (IZd = 9.67 mm – 13 mm and IZd = 11 mm – 13.33 mm, respectively), whereas, *K. pneumoniae* offer significant sensitivity to MEXTF 1 mM (IZd = 10.33 mm – 13 mm). Under lead stress *E. coli* and *K. pneumoniae* offer significant sensitivity to MEXTL 0.5 mM (IZd = 9.67 mm – 14.67 mm and IZd = 10.33 mm – 13 mm, respectively). Similar result was attained by Aghraz et *al.* (2020), who show that *Cladanthus arabicus* and *Bubonium imbricatum* polyphenol extracts have potent antibacterial activity in vitro, particularly

against *E. coli*. Our result allowed us to propose the following hypothesis: ``Generating stress in plants increases polyphenol content and decreases resistance of Gram-negative bacteria``.

NB: Results of this part are published in *Journal of Applied Biological Sciences*.

Conclusion

The results of the current study revealed that *X. parietina* is able to accumulate lead correlating with increasing concentration of $Pb(NO_3)_2$. Treating *X. parietina* thalli with NaF and $Pb(NO_3)_2$ solutions caused a significant decrease in chlorophyll and protein contents, and a significant increase of H_2O_2, catalase, GSH, MDA, OD_{435}/OD_{415} ratio, pheophytin, proline, soluble sugar, polyphenol and flavonoid contents correlating with increasing exposure time and/or increasing concentrations of NaF and $Pb(NO_3)_2$. Results also showed that C_b is more affected than C_a under fluoride and lead induced-stress. Furthermore, the obtained results showed a negative correlation between total chlorophyll and total pheophytin contents, and between H_2O_2 and GSH contents.

We may deduce that the increase of OD_{435}/OD_{415} ratio and MDA accumulation provide information on the damage produced by fluoride and lead-induced stress, and that lichen used catalase, GSH, polyphenols, proline and soluble sugars as detoxification mechanisms in response to fluoride and lead-induced stress. Exposing thalli of the lichen *X. parietina* to high concentration of NaF and $Pb(NO_3)_2$ solutions disturbed the detoxification system, resulting in total glutathione decomposition and reduction of polyphenol and flavonoid contents.

Our results show also that methanol extracts of fluoride and lead-stressed *X. parietina* lichen were found to be more active against Gram-positive bacteria (*B. cereus*, *L. monocytogenes* and *S. aureus*) than compared to other Gram-negative bacteria (*S. typhimurium*, *P. aeruginosa*, and *Enterobacter spp.*). Among Gram-negative bacteria, our results show that *E. coli* and *Enterobacter spp.* offer significant sensitivity to MEXTF 5 mM (IZd = 9.67 mm – 13 mm and IZd = 11 mm – 13.33 mm, respectively), whereas, *K. pneumoniae* offer significant sensitivity to MEXTF 1 mM (IZ = 10.33 mm – 13 mm). Under lead stress *E. coli* and *K. pneumoniae* offer significant sensitivity to MEXTL 0.5 mM (IZd = 9.67 mm – 14.67 mm and IZd = 10.33 mm –13 mm, respectively)

Despite the fact that lead was more toxic than fluoride, *X. parietina* has a very high sensitivity to fluoride, we were able to draw the conclusion that fluoride must be classified among the most toxic air pollutants, and therefore to open the field to other works to study and compare the toxicity of fluorine with that of heavy metals on the various other ecosystems.

Further studies will be very effective in order to specify the site of action of fluorine and lead at the cellular, intracellular and molecular level, as well as to look for other molecules developed by this lichen to combat stressful conditions. In addition, further research will be crucial

to understand how polyphenols accumulate in lichens under fluorine and heavy metal stress and to pinpoint the ideal concentrations at which the polyphenol content is most significant, and therefore the exploitation of polyphenols as antibacterial agents.

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