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Assessment of DNA Damage in Lichens Caused by Air Pollution in Misurata Using Rapd-PCR

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ABSTRACT

Lichens are symbiotic organism consisting of fungi and algae grow together as a thallus that does not have a cuticle or roots. Lichens rely mainly on absorbing water and minerals from atmospheric inputs making them commonly utilized in biomonitoring research on air pollution, serving as bioindicators of air quality or as bioaccumulators of atmospheric deposits. In this lichen transplant study, foliose lichen *Xanthoria parietina* was used as bioindi cator to assess the genotoxicity of air pollutants in the central area of Misurata City; RAPD profiles revealed significant alteration in the band pattern and decreasing in genomic template stability (GTS) following four months of exposure to pollutants. These findings suggest that the lichen species *Xparietina* provides insights into the degree of potential genotoxic substances in the examined region. Nevertheless, this research must be expanded to cover larger regions for prolonged periods along with an ecological study carried out simultaneously to assess air quality in Misurata City.

تقييم الأضرار التي تلحق بالحمض النووي منقوص الأكسجين في الأشنات نتيجة لتلوث الهواء في مدينة مصراتة باستخدام تقنية تفاعل البلمرة المتسلسل للحمض النووي متعدد الاشكال المتضاعف (Rapd-PCR)

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الأشنات هي كائنات تكافلية تتكون من فطر وطحلب ينموان بشكل ثالوس لا يحتوي على غلاف أو جذور. تعتمد الأشنات على امتصاص الماء والمعادن من الهواء الجوي، مما يجعلها تُستخدم على نطاق واسع في أبحاث الرصد الحيوي إما كمؤشرات حيوية لمدى جودة الهواء أو كمتراكمات حيوية للمترسبات الجوية. في هذه الدراسة التي اعتمدت على زرع الأشنة زانثوريا باريتينا (Xanthoria parietina)واستخدامها كمؤشر حيوي لتقييم السمية الجينية للملوثات الهواء في مركز مدينة مصراتة، كشفت نتائج تحليل الحمض النووي متعدد الأشكال العشوائي المتضاعف (RAPD) عن تغييرات كبيرة في أنماط الحزم والخفاض في استقرار الجينوم (GTS) بعد فترة تعرض لمدة أربعة أشهر للملوثات. تشير النتائج إلى أن الأشنة زانثوريا باريتينا تقدم رؤى حول مستوى العوامل ذات التأثير السام المحتمل للجينات في المنطقة المفحوصة. ومع ذلك، يجب توسيع هذا البحث ليغطي مناطق أكبر ولفترة أطول مع إجراء دراسة بيئية في الوقت نفسه لتقييم جودة الهواء في مدينة مصراتة.

INTRODUCTION

A concentration of foreign particles in the air that negatively affects people's health and well-being is known as air pollution (Nassar *et al*., 2017). Air pollution is a major risk to human health causing over 4.2 million deaths according to the World Health Organisation report for 2019 (World Health Organisation, 2023). In response to this challenge, extensive studies have been conducted to find the most appropriate, sensitive, efficient, and cost-effective approaches for long time monitoring of the air quality. Equipment that has sensors are subjected to the number of disadvantages including the high purchase and maintenance costs, in addition to limitations in sampling and need to maintain fixed monitoring locations over long

periods to obtain a comprehensive picture of the concentration of air pollutants. Most importantly, the harmful effects of pollutants and their interaction with biological entities cannot be reflected by detector-based methods (Misra *et al*., 2020; Kang *et al*., 2022; Chaudhuri & Roy, 2024; Garcia *et al*., 2025).

The use of different groups of organisms as indicators, such as animals, vascular plants, fungi and lichens has been considered as a suitable alternative to evaluate the effects that may be caused by air pollution (Takano *et al*., 2024; Al-Alam *et al*., 2024; Petrova *et al*., 2024). Due to their anatomical, morphological, and physiological characteristics, lichens have been used as bio-indicators with successful results (Morillas, 2024). In other words, Lichens are a slow-growing associations of fungi and algae forming thallus that doesn't have a cuticle or roots. Therefore, lichens rely mainly on the absorbing water and atmospheric inputs of minerals and nutrients, including pollutants, easily which accumulate in fungal and algal cells. The presence of lichen species in specific regions may indicate the presence of a good air quality (Morillas, 2024; Dilrukshi *et al*., 2024).

Crustose lichens are the most pollution tolerant types, so they are more abundant and considered as bioaccumulation agents. In contrast, fruticose lichens are highly sensitive to pollution, making them disappear in polluted regions and therefore used as biomonitors. Foliose lichens act as both bioindicators and bioaccumulators (Abas *et al*., 2021; Augusto *et al*., 2013).

Various studies have consistently demonstrated that the foliose lichen *Xanthoria parietina* is an effective bioindicator for diverse pollutants. In a study conducted in Algeria in 2013, control *X. parietina* samples were transplanted in three oil stations to evaluate the effect of hydrocarbon pollutants. Transplanted lichens showed increasing levels of hexane-soluble compounds with a decrease in chlorophyll content in the three stations (Khelil *et al*., 2013). Belguidoum and her colleagues found that *X. parietina* was a common species in all studied sites, even polluted ones, so they recommended using *X. parietina* as a reliable pollutant-tolerant bio-monitor in urban systems (Belguidoum *et al*., 2022). Similarly, an in vitro study proved the bioaccumulation ability of *X. parietina*, as it revealed significant accumulation of lead, which was inversely proportional to chlorophyll content (Kouadria *et al*., 2020). *X. parietina* from various forest sites in North-eastern Morocco was used in a passive monitoring study to measure the background levels of five heavy metals (Fe, Cr, Zn, Pb, and Cu); there was a strong correlation between the concentration of metals and both urbanisation and vehicle activity. A recent study in Tunisia found that lichen diversity gradually declined near pollution sources, and that *X. parietina* had a higher capacity for bioaccumulation of the majority of heavy metals (Fe, Pb, Cr, Cu, and Ni) than the moss *Funaria hygrometrica* (Bousbih *et al.*, 2025).

Several air pollutants, particularly heavy metals, are genotoxic agents. They can directly or indirectly cause damage to the genomes of organisms such as addition, deletion, point mutation and rearrangement upon long term exposure. DNA damage can be detected using comet assay, micronucleus assay and chromosome aberration assay, compared with cytogenetic approaches. In PCR-based techniques any type of DNA damage can be scored even tiny mutational events. Studies have used random amplified polymorphic DNA PCR (RAPD-PCR) to evaluate the genotoxicity of air pollutants on lichens. RAPD profiles revealed significant alterations in the band pattern and a decrease in genomic template stability (GTS) in lichens exposed to pollutants (Cansaran-Duman *et al*., 2011; Cansaran-Duman *et al*., 2015; Hamutoolu *et al*., 2020).

The present study was conducted to assess the genotoxicity of air pollution in the centre of Misurata City by using lichens as bioindicators and random amplified polymorphic DNA (RAPD) technique as a DNA damage marker.

MATERIALS AND METHODS

Misurata situated on the Mediterranean Sea between 32°22′39.12″N and 15°05′31.26″E, an altitude of approximately 60 m above sea level, with a population of about 517 000 prior to the civil war. It is approximately 825 kilometres west of Benghazi and 187 kilometres east of Tripoli. Summer is hot and dry while winter is warm and rainy. The warmest month is August (30-5C°), the coldest month is February (13-8 C°), Misurata typically receives about 21.84 mm of precipitation unusually with humidity of 61.05%. Numerous industrial activities such as those of cement block industry, soap and detergent, iron and steel as well as active airport, seaport, and intercity highway are present in the city (Elsunousi *et al*., 2021).

The study was conducted in the city centre where there is heavy traffic due to the intense commercial, educational, and retail activity. Figure 1 shows where the zone under study is located.

Lichen sampling and identification

The lichen samples were collected from the trunk of Pinus tree at least 50cm above the ground in the AL- fellaga region, which is distant from major sources of air pollution and large urban and industrial settlements, thus far from important sources of air pollution. Morphological features were examined particularly growth form, colour of thallus, type of photobionts, presence of apothecia and rhizines. Direct sections of the thallus were performed to observe the anatomical arrangement of fungal and algal layers. Chemospot tests were conducted to investigate the presence of the parietin secondary metabolite in the lichen thallus in which characterizes *Xanthoria parietina*. The tests were performed by applying few drops of the K (potassium hydroxide 10%), C (commercial bleach), and P (alcohol solution of para-phenylenediamine) reagents on the top of lichen thallus and colour reactions were recorded (Zhang *et al*., 2021). *Xanthoria parietina* specimens were

identified according to several sources (Itten and Honegger, 2010; Thor and Nascimbene, 2010; Allen and Lendemer, 2021).

Figure (1): Lichen collection and transplantation regions. A: Al-fellaja region (collection region). B: Misurata City map: star indicates to region of control sample collection. Tringle indicates to city centre where the lichen samples were transplanted.

Lichen transplantation

In the laboratory, *Xanthoria parietina* samples with part of their substrate were cleaned of soil and washed several times with distilled water. Eight nylon mesh bags were assembled (3-4cm) in diameter containing 300-400mg lichen thalli (Gailey and LIoyd, 1986).

Three bags considered as controls and fixed on the Pinus trees in the original area while the remaining bags Three bags considered as controls and fixed on the Pinus trees in the original area while the remaining bags transplanted on tress in the city centre of Misurata at the beginning of August 2022. The lichen samples were examined twice: once at the time zero before transplantation and again after 4 months of exposure at the end of November 2022.

Genomic DNA extraction:

The Quick – DNA™ plant/seed mini prep kit (Zymo Research/ USA) was used for the lichen DNA extraction according to the manufacturer's instructions. The kit contains all the reagents required for DNA extraction and all steps were performed at room temperature. The quality and quantity of extracted DNA were assessed by measuring the absorbance at OD 260 and calculating the 260/280 nm absorbance ratios using a NanoDrop™ ND-1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA). The extracted genomic DNA was then electrophoresed on 1% agarose gel to check their integrity.

Random amplified of polymorphic DNA (RAPD) analysis of lichen DNA:

(RAPD) PCR was performed to generate fingerprints of lichen DNA with ten decamer oligonucleotide primers described by Honegger *et al* (Honegger *et al*., 2004). These primers (table 1) were selected because they were reported to amplify clear and reproducible bands from DNA of *Xanthoria Spp*. Polymerase chain reaction was carried out using 2x PCR MAXi master mix and the reaction were optimized in 20 µl volume containing:10µl master mix ,1µl primer, 2µl genomic DNA and 7µl deionized water. The reaction mixture was placed in the mini–Amp Plus thermoscycler. Each reaction consisted of 40 cycles, each including 30s at 94C°, 1min, for primer annealing at 33C°, and 1min for chain elongation at 72C°. The first cycle was proceeded by 5min of denaturation at 95C°, and the last cycle was extended by 5 min elongation step at 72C°. After the end of PCR, products were separated by electrophoresis on 1.5% agarose gel in 1x TAE buffer and the DNA bands pattern were visualized by GelDoc Go Imaging System. The reaction was conducted twice.

Table1: The primers for Random amplification of polymorphic DNA (RAPD)

Primers	Sequence 5' to 3'	Tm (C)
OPA – 09	GGGTAACGCC	34.00
OPA – 10	GTGATCGCAG	32.00
OPB – 10	CTGCTGGGAC	34.00
OPB – 17	AGGGAACGAG	32.00
OPC – 02	GTGAGGCGTC	34.00
OPC – 19	GTTGCCAGCC	34.00
OPD – 03	GTCGCCGTCA	34.00
OPD – 13	GGGGTGACGA	34.00
OPD – 16	AGGGCGTAAG	32.00
OPD – 20	ACCCGGTCAC	34.00

Tm: melting temperature

Genomic effect by RAPD analysis in the Cadmium Chloride treated *X. parietina* sample:

An in vitro experiment was conducted in parallel with transplantation study in order to confirm the concept that *X. parietina* could accumulate heavy metal contamination which can induce RAPD- PCR detectable DNA damage. Samples of *X. paritina* were treated with 30 mg/g Cadmium Chloride ($CdCl_2$) solution for 30 min, 3h and 6h incubating time (Vardar *et al*., 2014). Then the genomes of treated samples were extracted after each Cd incubation period. then RAPD analysis was performed by using four primer (OPB10 primer, OPB 17, OPD20 and OPC02) as same procedure mentioned above. Test samples exposed to cadmium were compared with control sample.

RAPD fingerprints result analysis:

The results of RAPD analysis were manually scored for disappearance of a control band and appearance of a new band in the transplanted lichen samples compared with RAPD profile of control samples. Changes in the thickness of the bands were ignored and bands of equal size were considered as homologous in this analysis.

The genomic template stability (GTS%) was estimated using the formula {1-(a/n)} × 100, where "a" stands for the RAPD polymorphic profiles of transplanted lichen sample that was transplanted in the central region of Misurata, and

"n" indicates the total number of bands in the control. A decrease in GTS was used to indicate changes in the RAPD patterns (Cansaran-Duman *et al*., 2011; Atienzar *et al*., 1999).

RESULTS AND DISCUSSION

Assessment of DNA Quality and Quantity

The concentration and quality of the extracted DNA were assessed using Nanodrop machine. The average recovery of DNA that could be obtained from 150 mg of lichen thallus using the Quick-DNA-plant/seed miniprep kit was 42ng/µl. The lowest DNA concentration obtained was 15.3ng/µl and the highest was 73ng/µl. The quality of DNA ranged from 1.7 to 2.2 ratio at (A260/A280). Both the concentration and quality were satisfactory for using in a conventional PCR reaction. The integrity of the extracted DNA samples also was assessed by electrophoresis in an ethidium bromide-stained agarose gel. All samples (figure 2) appeared as intact bands on the gel indicating a good DNA quality without any degradation.

Figure (2): Extracted genomic DNA from X. parietiena. The DNA samples were visualized in 1.5% agarose gel electrophoresis and stained with ethidium bromide

Evaluation of genotoxic effects by RAPD fingerprint in the Cd^{+2} treated *X. parietina* samples:

Heavy metals are the main toxic contaminants in air pollution. To prove that genotoxic effects may be induced by heavy metals on lichens, In vitro experiment was carried out in the laboratory; samples of *X. parietina* were treated with low concentration (30 mg/g) of CdCl2 solution for a specific period of times (0.5, 3, 6h). Cadmium is well established as carcinogen that may generate oxidative stress by interfering with metalloproteins involved in redox cycling in the cell (Apel & Hirt 2004). DNA was isolated from Cd treated samples and four different random primers were used in RAPD fingerprinting. Fingerprinting with the primer OPB10 revealed that new bands appeared after 30min of treatment (figure3). While in primer OPB17 one control band disappeared and additional band appeared upon exposure to Cd for 6h. In the OPD20 primer one control band disappeared after 3h of Cd exposure. At this stage of our study, we did not calculate the polymorphism percentages since our goal to confirm the genotoxicity of Cd and sensitivity of lichen sample to the environmental pollutants on one side and on the other side this experiment supports and confirms that contamination induced DNA damage can be observed by RAPD analysis clearly. Notably, elevated cadmium levels were found in soil and dust alongside the roads within Misurata City Centre and industrial areas (Elbagermi *et al*., 2013). Additionally, another study found that blood samples from cancer patients in the Misurata City had higher levels of cadmium than healthy control group (Alosta, 2018).

These results are in agreements with studies that reported *X. parietina* as a reliable pollutant tolerance bio-monitor since it had showed significant accumulation of heavy metal, Sulphur dioxide and hydrocarbons pollutants (Khelil *et al*., 2013; El Rhzaoui *et al*., 2020; Krjukovica *et al*., 2021; Bousbih *et al*., 2025).

The RAPD profile of the control and transplanted samples:

In the current study the genotoxic effects of expected environmental pollutants in the Misurata City Centre were tested with five *X. parietiena* samples collected from their natural habitats and transplanted in the city centre for four months. Ten different primers were used in RAPD analysis and eight of the primers yielded clear and reproducible bands.

The RAPD-PCR was tested for reproducibility by repeating the experiment twice for each primer, ignoring faint bands. Only repeatable bands from the repeated experiments were considered, and variations in band intensities were disregarded because they only showed up in trace amounts. The changes in bands can be observed in Figure 3. The number of new band appearances and disappearances for each primer and for each DNA sample obtained from the control and transplanted samples are shown in table 2. and the number of polymorphic bands / total bands×100 was used to compute the polymorphism ratio (Table 3).

Among the primers used, OPD20 exhibits the highest polymorphism, while OPD13 and OBP10 do not reveal any polymorphic band patterns. As RAPD primers scan almost the whole genome, it can be suggested that OPD20 and the rest of the primers except OPD13 and OBP10 found the DNA regions in which alterations have occurred upon 4 months exposure to air in Misurata City Center.

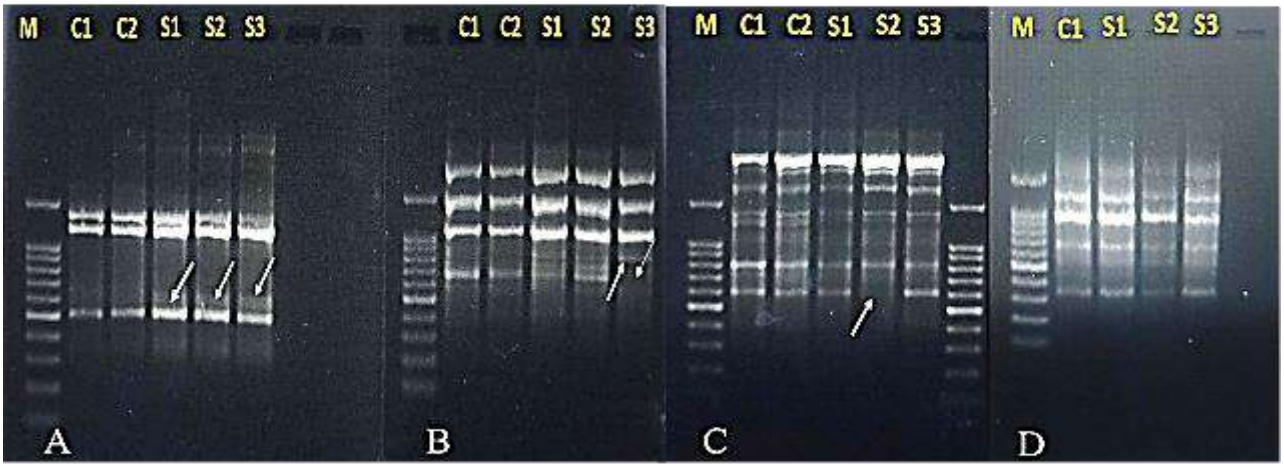

Figure (3): RAPD profile of genomic DNA from *X. parietiena* thallus exposed to (30mg/g) Cd^{+2} concentration. The DNA samples were visualized in 1.5% agarose gel electrophoresis and stained with ethidium bromide. M: ZR 50 bp DNA Marker. C: *X. parietiena* lichen control samples 1 and 2. S: Cd treated sample, 1: for 30minutes, 2: for 3hours, 3: for 6 hours. A: OPB10 primer. B: OPB 17. C: OPD20. Arrows indicate some of the band

The primary aim of biomonitoring is to provide data that supports effective practical ecological systems. Specifically, biomonitoring should function as an early warning system to assess the severity of pollution on living organisms through sensitive assays, even the pollutants are still at sub lethal concentrations. Genotoxicity of major air pollutants, such as halogenated aliphatic hydrocarbons, polycyclic aromatic hydrocarbons (PAHs) and elements oxides and heavy metals have been reported in many studies (Simmons *et al*., 2011; Verma *et al.*, 2012; Møller *et al*., 2013; Galeano-Páez *et al.*, 2024).

Table2: Changes of total bands in control and polymorphic bands after 4 months of exposure.

Primer	Control	After 4 months	
		a	b
OPD – 20	6	0	4
OPD – 13	2	0	0
OPB – 10	5	0	0
OPC – 19	5	1	2
OPC – 02	5	0	1
OPB – 17	5	1	0
DPA – 10	4	1	1
OPD -03	7	0	2
a+b	39	3	10

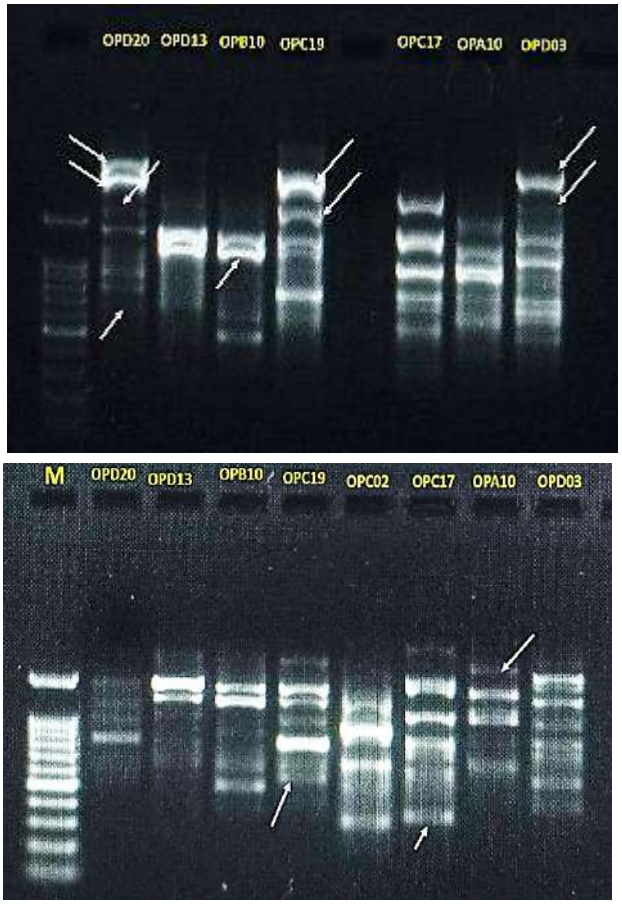

Figure (4): RAPD profile of genomic DNA from *X. parietiena* thallus transplanted in the Misurata centre after 4 months. The DNA samples were visualized in 1.5% agarose gel electrophoresis and stained with ethidium bromide. M: ZR 100 bp DNA Marker. A: RAPD profile of 4month sample (arrows indicate to appearance of new bands). B: RAPD profile control sample (arrows indicate to bands that disappeared in 4 months sample).

However, the coexistence of various gases in the atmosphere alongside with pollutants and radiation, in addition to subsequent reaction products presents significant challenges in accurate estimating of the genotoxic risk of air pollutants using traditional measurement methods. In this regard, the most effective approach to assess and quantify the genotoxicity of pollutants is through the direct observing of any DNA changes caused by genotoxic substances in living organisms; Several investigations have been conducted

to detect DNA damage using cytogenetic tests, comet assay, and other methods (Jayawardena *et al*., 2021; Musilova *et al*., 2023; Kaya *et al*. 2023). Cytogenetic approaches such as micronucleus assays don't detect damage at DNA level since they deal with nuclear DNA as one unit. The comet assay provides quantitative and qualitative data on DNA integrity including single-strand and double-strand breaks but cannot provide information about sequence changes in the DNA that occur as a result of mutagenic effects of pollutants (Šrut *et al*., 2013).

Recently, DNA – fingerprinting based molecular markers utilized to provide evidence for broader genomic alterations, such as sequence changes, mutations to large structural rearrangements (Atienzar *et al*., 2006). In this respect, DNA fingerprinting techniques like RAPD and amplified fragment length polymorphism (AFLP) have been employed to detect different types of DNA damage and mutations in lichens that are caused by either experimental exposure to pollutants or contaminated environmental conditions. In the current study, the *X. parietina* genome revealed high number of polymorphic bands in the samples exposed to atmospheric environment within the Misurata City Centre. Since the lichens lack cuticle and roots, they absorb aerosols and gases directly from the ambient air, therefore, pollutant concentration and its subsequent DNA damage often reflect the accumulated composition of the air. The appearance of new bands or absent of control bands means that changes in the sequence of the genomic DNA occurred and the random primers found new binding sites or lost priming sites respectively. Vardar *et al*. reported similar results in their investigations carried out with different species of folios lichens; lichens thallus close to or transplanted near pollution sources displayed high levels of heavy metal deposition and high polymorphic genome fingerprint comparing with distant samples (Vardar *et al*., 2014).

Additionally, the results of RAPD- PCR translated into semi- quantitative measurement called genomic template stability ratios (GTS), which indicates how much the genome altered upon the treatment or genotoxic exposure. The GTS of X. parietiena calculated and found to be 66.6% after 4 months transplantation in the centre of Misurata City. The GTS may be related to the level of DNA damage and efficiency of DNA repair and replication, so a high level of DNA damage that inhibits DNA repair may lead to a decreased GTS value (Abas *et al*., 2021). Misurata suffers from several air pollution sources. A key contributor is the Libyan Iron and Steel Company (LISCO), which emits pollutants like carbon dioxide, particulate matter, sulfur dioxide, and nitrogen oxides. Additionally, there's a soap and detergent factory in Misurata and other factories that add to the air emissions. The use of mobile electricity generators, anthropogenic emissions from road traffic also contribute to pollution. A study carried out in 2021 reported higher concentration of CO2 and particulate matter pollutants in city centre than other studied regions in Misurata City (Elsunousi *et al*., 2021).

The recorded GTS value in this study is less than that were recorded in studies in Turkey, which investigated the genotoxic effects of various contaminants including heavy metals in polluted areas on different types of foliose lichen: Evernia prunastri, and Pseudevernia furfuracea which ranged from 96% to 73% (Cansaran-Duman *et al*., 2011; Cansaran-Duman *et al*., 2015; Hamutoolu *et al*., 2020).

Previous studies have also indicated that that mutations, chromosomal alterations, and other DNA damage may reflected as differences in RAPD band patterns and reduced GTS (Atienzar *et al*., 2006).

Table3: polymorphic ratio of the primer

Primer	TB	PB	Ratio%
OPD20	6	4	66%
OPD13	2	0	0%
OPB10	5	0	0%
OPC19	5	3	60%
OPC02	5	1	20%
OPB17	5	1	20%
OPA10	4	2	50%
OPD03	7	2	28%

TB: Total bands, PB: Polymorphic bands

CONCLUSION

In the present study, lichen *X. parietina* that were collected from clean environment revealed changes in RAPD band patterns upon four months of transplantation in the centre of Misurata City. The genomic template stability (GTS) value was decreased to 66% comparing to the control sample. The presence of abnormalities in the bands profiles indicates to the genotoxic effect of air pollutants in the study area. DNA polymorphisms detected by means of RAPD analysis could be considered as useful biomarker assay that can be used as early warning system about the potential impact of pollution on the organisms, including human. This study should be extended to include more areas for a longer period with ecological study conducted in parallel to investigate the quality of the air in Misurata City.

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