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2

SUMAR – CONTENT – SOMMAIRE – INHALT

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Original picture on front cover:
Kheloufi et al. *Sophora secundiflora* flowers, leaves, pods, and seeds.

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Assessment of circulating biomarkers in a rat model of doxorubicin-induced cardiotoxicity

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Abstract. The number of cancer survivors is increasing as cancer therapies become more and more effective. As a consequence, cardio-oncology is nowadays more shifted towards detecting and treating conditions such as CTOX, which refers to heart damage as a result of cancer treatment. Currently, a standardized way of evaluating and monitoring CTOX does not exist, and patients undergo nonspecific and lengthy tests, so they are often diagnosed when heart damage is irreversible. Thus, we assessed a panel of circulating biomarkers that can be used to monitor time-dependent changes in Wistar rats treated with conventional or liposomal DOX. After validation this panel might be applied in clinics to enhance accuracy of screening patients undergoing DOX-based therapy approaches.

Keywords: CTRCD, CTOX, DOX, doxorubicin, circulating biomarkers

Introduction

Heart disease and cancer are the top leading causes of death from non-communicable diseases worldwide (Dattani *et al.,* 2023). As the number of people affected by cancer increases, so are the efforts towards finding efficient treatments. This urges the need of finding solutions to boost the wellbeing of cancer survivors, especially as the global population is aging. In older cancer survivors, the effects of cancer and cancer treatment have a long-term negative impact (Schmidt *et al.*, 2022).

Cancer patients and survivors are undergoing many screenings aimed at monitoring the course of disease, effectiveness of treatment, or early spotting of relapses, and not enough attention is paid towards screening for potential health problems that arise from the cancer treatment itself. To address this issue, specific screenings for diseases that occur following cancer treatment can be conducted simultaneously with regular check-ups through biomarker panels.

Circulating biomarkers are an easily accessible, minimally invasive, and cost-effective way of patient screening (Ahmad *et al.*, 2023). One of the diseases that could benefit from this type of screening is cancer treatment-related cardiac dysfunction (CTRCD), that arises from various treatments, such as radiotherapy, cytotoxic chemotherapy, and targeted therapy (Bloom *et al.*, 2016). CTRCD manifests as cardiotoxicity (CTOX), which is one of the most important side effects of many cytotoxic anti-cancer agents. The most known type of therapy known to have this effect is anthracyclines (Cardinale *et al.*, 2020). CTOX presents as heart failure, hypertension, and decrease in left ventricle ejection fraction (LVEF), among other cardiovascular effects *(*Perez *et al.*, 2019).

Anthracyclines, a class of cytotoxic antibiotics extracted from *Streptomyces* bacteria, are used in the treatment of various solid tumors and hematologic malignancies (Morelli *et al.*, 2022). The most used anthracycline is doxorubicin (DOX), available in its conventional form or encapsulated in different types of liposomes (Rivankar, 2014).

Anthracyclines cause CTOX in a dose-dependent manner, and can occur at any point during treatment and up to several years post-treatment, when it could be exacerbated by other pre-existing cardiovascular conditions. Generally, CTOX arising during treatment or up to one year after the completion of treatment is considered acute, whereas CTOX diagnosed after this period is chronic. Acute CTOX can be reversed by cardioprotective strategies or dose reductions. Chronic, or late-onset CTOX is hard to diagnose, and is often irreversible (Cardinale *et al.*, 2020).

CTOX is currently diagnosed through imaging studies and by assessing heart disease-related blood biomarkers, and both means of diagnosis have some important downsides. Imaging tests require high expertise, are costly, often cannot be repeated due to risks associated with exposure to radiation, and in the case of pediatric patients, require sedation. Moreover, subclinical signs of CTOX cannot be detected through these procedures. The blood biomarkers used for the evaluation of CTOX, such as troponins and natriuretic peptides, were selected for their meaning in general cardiovascular disease assessments, using cut-off values that are not specific to CTOX, due to lack of standardization for this disease.

The present study aims at evaluating a panel of biomarkers in a rat model of DOX-induced CTOX, during and after treatment with conventional DOX, and only for the duration of treatment for a PEGylated liposomal formulation of DOX. The biomarkers were selected based on their association with oxidative stress produced by DOX administration and their specificity to heart-related conditions. Through the weekly assessments of our biomarker set, we focused on identifying periods at which biomarkers are more likely to show significant changes.

Ultimately, the overall goal of our research is to inform future human studies on the key time-points at which biomarkers should be evaluated in order to close the knowledge gap between which biomarkers work and what how we can use them to quicky diagnose patients and when cardioprotective strategies should be implemented.

Materials and methods

Animals, treatment and sample collection

The study was conducted in accordance with the requirements of the European Directive 2010/63/EU. The protocol was approved by the Scientific Council of the Babeș-Bolyai University of Cluj-Napoca under the reference number 14.172/02.11.2021. In total, 40 adult male Wistar rats were used, that were maintained in appropriate hygienic conditions with constant temperature and humidity, and were gently handled at the Laboratory Animal Facility (Zoobase) at the Babeș-Bolyai University in Cluj-Napoca. Rats were housed in standard cages, had an average weight of 250 g at the beginning of the experiment and were given free access to standard food and water, on a 12-hour light/dark cycle.

The DOX group (n=20) received weekly tail vein injections of 3.75 mg/kg body weight of doxorubicin hydrochloride (European Pharmacopeia Reference Standard, D2975000, Merck) dissolved in vehicle (0.9% sodium chloride, B.Braun) in order to reach a cumulative dose of 15 mg/kg over the course of 4 weeks. The same vehicle was administered to the Control group (n=20).

The LCL-DOX (n=5) group received DOX encapsulated in long-circulating liposomes, prepared using the method described by (Sesarman *et al.*, 2018), in the same cumulative dose and administration protocol as the DOX group. To serve as a control group, LCL-PBS rats (n=5) received PBS only, encapsulated in the same type of liposomes, using the same volume of the compound.

The i.v delivery of the therapeutic agents was chosen over intraperitoneal injection administration in order to limit pain and inflammation which could affect animal welfare.

Venous blood from the tail vein was collected weekly before treatment administration, in heparin-coated tubes. After centrifugation, plasma was stored at -20 degrees Celsius until analysis. At the end of the 4-week course of treatment, for the DOX and Control groups, half of each group (n=10) was sacrificed, and the remainder of the individuals were maintained without any treatment for an additional 4 weeks. The LCL-DOX and LCL-PBS groups were only kept in the experiment until the end of treatment. All rats were sacrificed by exsanguination under anesthesia.

Biochemical assays

Plasma Galectin-3 (Gal-3) and N-terminal prohormone of brain natriuretic peptide (NT-proBNP) levels were assayed using ELISA kits purchased from EIAab (Cat. no. E0498r and E0485r, respectively). Plasma calcium (1-413-0200), iron (1-418-0150), total cholesterol (1-023-0200) and triglyceride (1-053-0200) levels were evaluated using a BIOELAB ES-100C analyzer using reagent kits purchased from SwissFarm.

Statistical analysis

Student's t test was used for all analyses which were performed using GraphPad Prism 9.3.0 software (Boston, MA). Outliers were identified using the ROUT method (Q = 5%) and were removed from the analyses. Statistical significance was considered at p-values <0.05 such as follows: ns ($p>0.05$; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$, ****, $p<0.0001$).

Results

Calcium

In our experiment, plasma calcium concentration in the DOX group recorded an overall increasing trend compared to the Control group. The highest difference between these groups was recorded on week 2 (7.92% increase), after the first dose of treatment. At 4 weeks post-treatment, plasma calcium concentration was lower in the DOX group, and it was the only time-point comparison that

reached statistical significance (3.82% decrease, P-value of 0.0079). Compared to baseline, calcium concentration was 5.46% (P-value of 0.0173) lower at the end of the untreated period than at the beginning of the experiment (Fig. 1A.).

The liposome-treated groups had a similar trend, with a statistically significant increase of 26.68% (P-value of 26.68) after the first administered dose, followed by a slight decrease (4.39% and 2.34% at weeks 2 and 3, respectively). However, post-treatment, LCL-DOX plasma calcium values were 11.22 higher (P <0.0001) compared to LCL-PBS. Post-treatment, the LCL-DOX group calcium concentration was 4.77% higher than at the pre-treatment time-point, although not statistically significant (Fig. 1B.). The concentration of plasma calcium was 24.14% lower in the LCL-DOX group compared to DOX at the end of the treatment (P <0.0001) (Fig. 1C).

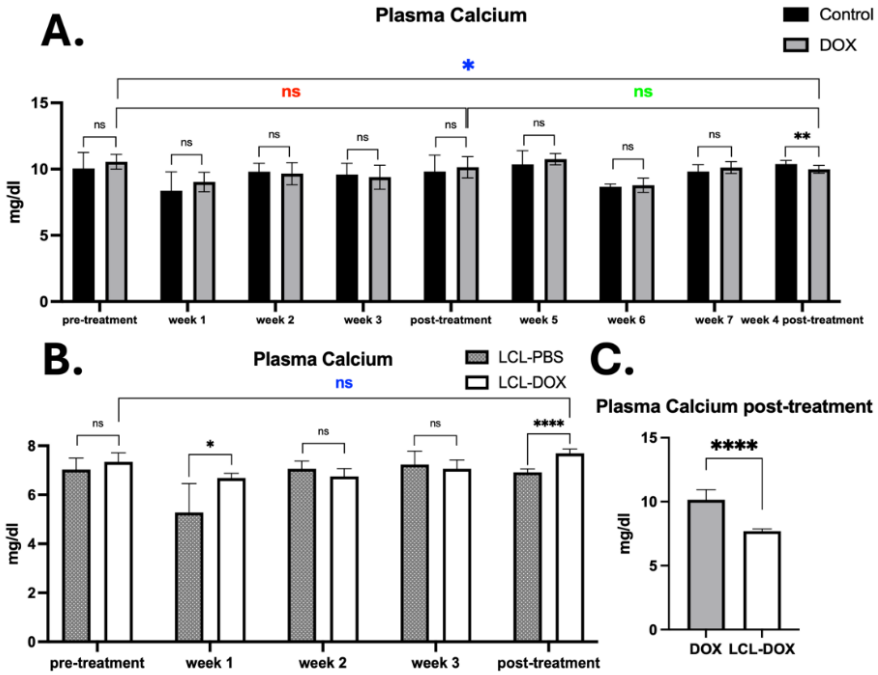

Figure 1. Plasma calcium concentration. A. weekly values recorded for the Control (vehicle treated) and DOX (DOX treated with a cumulative dose of 15 mg/kg divided into 4 weekly i.v injections) groups. B. weekly values recorded for LCL-PBS (vehicle treated) and LCL-DOX (liposomal DOX, with the same therapeutic protocol) groups. C. comparison between DOX and LCL-DOX treated groups at the end of the treatment. Data represent mean ± standard deviation (SD) for each group of animals in their corresponding week, pre-, during or post-treatment. Statistical significance was considered as follows: p>0.05; *, p<0.05; **, p<0.01; ***, p<0.001, ****, p<0.0001.

Cholesterol

Weekly recordings of the plasma cholesterol values showed a general upward trend that is highly visible in the post-treatment period (Fig. 3). Between the DOX and Control groups, the most statistically significant difference was recorded post-treatment, with a 27.43% increase (P-value <0.0001) for the DOX-treated group. Compared to baseline values, cholesterol concentration was 38.95% higher post-treatment (P <0.0001) and 103.66% higher at week 4 post-treatment (P-value of 0.0005, Fig. 2A.).

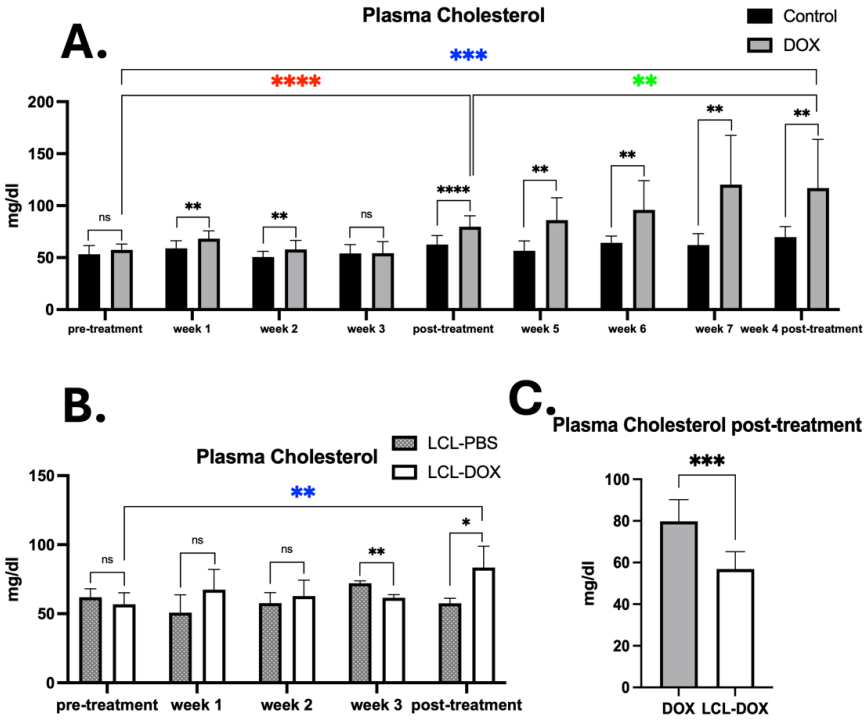

Figure 2. Plasma cholesterol concentration. A. weekly values recorded for the Control (vehicle treated) and DOX (DOX treated with a cumulative dose of 15 mg/kg divided into 4 weekly i.v injections) groups. B. weekly values recorded for LCL-PBS (vehicle treated) and LCL-DOX (liposomal DOX, with the same therapeutic protocol) groups. C. comparison between DOX and LCL-DOX treated groups at the end of the treatment. Data represent mean ± standard deviation (SD) for each group of animals in their corresponding week, pre-, during or post-treatment. Statistical significance was considered as follows: p>0.05; *, p<0.05; **, p<0.01; ***, p<0.001, ****, p<0.0001.

Regarding cholesterol values in the liposome-treated groups, the same increasing trend in concentration was observed for LCL-DOX, with a peak value achieved post-treatment (44.77%, P-value of 0.0146). However, at week 3, LCL-DOX cholesterol concentration was 14.51% lower compared to LCL-PBS (P value of 0.0146, Fig. 2B.).

On the comparison between the two formulations of DOX administered, the liposomal form of DOX decreased cholesterol values with 29.45% (P-value of 0.0003, Fig. 2C).

Triglycerides

For the DOX group, plasma triglyceride concentration was higher starting with week 3 compared to Control, and increased in the post-treatment period with a peak difference of 105.29% (P-value of 0.0013) 3 weeks after the last dose. Compared to pre-treatment, the concentration of triglycerides was 111.33% higher (P-value of 0.0072) at the end of the post-treatment period (Fig. 3A.).

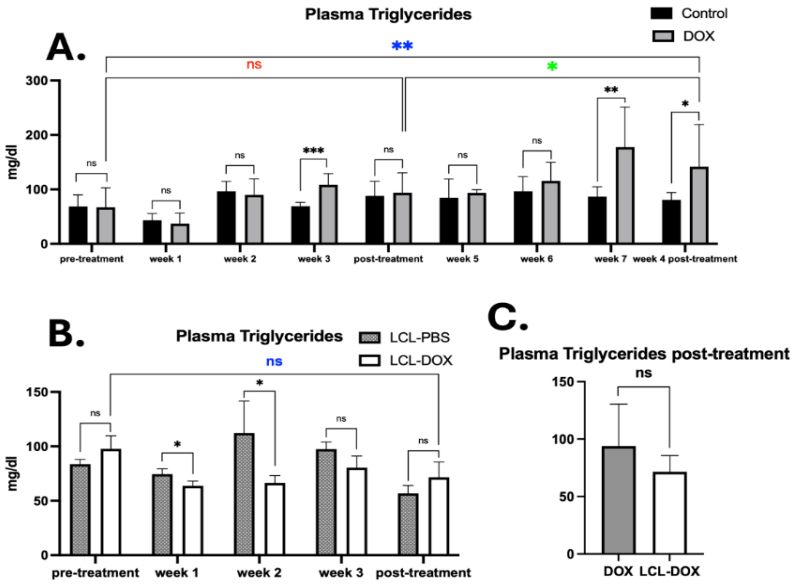

Figure 3. Plasma triglycerides concentration. A. weekly values recorded for the Control (vehicle treated) and DOX (DOX treated with a cumulative dose of 15 mg/kg divided into 4 weekly i.v injections) groups. B. weekly values recorded for LCL-PBS (vehicle treated) and LCL-DOX (liposomal DOX, with the same therapeutic protocol) groups. C. comparison between DOX and LCL-DOX treated groups at the end of the treatment. Data represent mean ± standard deviation (SD) for each group of animals in their corresponding week, pre-, during or post-treatment. Statistical significance was considered as follows: p>0.05; *, p<0.05; **, p<0.01; ***, p<0.001, ****, p<0.0001.

Liposome-treated groups followed the opposite trend, as LCL-DOX triglyceride values were lower than LCL-PBS during treatment and increased by 25.56% post-treatment. The most statistically significant difference was observed at week 2 (40.77% decrease and P-value of 0.0198, Fig. 3B.). Although not statistically significant, triglyceride values were 23.81% lower for LCL-DOX compared to the DOX group at the end of treatment (Fig. 3C.).

Iron

In the DOX and Control groups, plasma iron values showed a significant degree of variability over the course of the experiment. The most notable differences between these two groups were observed in the first week (65.82% higher values for DOX, P-value of 0.0047), followed by a sharp decline in week 3 (19.86% lower, P-value of 0.0404) during treatment. In the post-treatment week, peak values were identified where DOX group plasma iron concentration was 62.04% higher compared to Control (P-value of 0.0134). Compared to pre-treatment, iron concentration was lower at both post-treatment time-points (Fig. 4). This assay was not performed for the LCL-PBS and LCL-DOX groups.

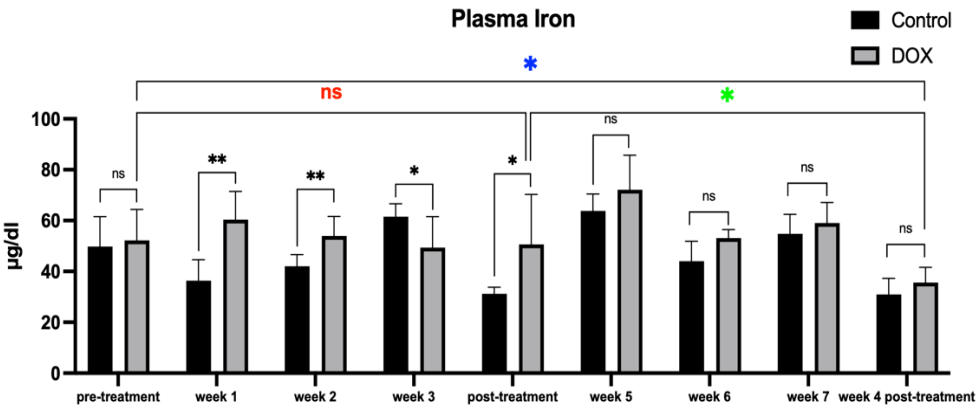

Figure 4. Plasma iron concentration. Weekly values recorded for the Control (vehicle treated) and DOX (DOX treated with a cumulative dose of 15 mg/kg divided into 4 weekly i.v injections) groups. Data represent mean ± standard deviation (SD) for each group of animals in their corresponding week, pre-, during or post-treatment. Statistical significance was considered as follows: p>0.05; *, p<0.05; **, p<0.01; ***, p<0.001, ****, p<0.0001.

Galectin-3

In our experiment, free DOX treatment did not generate any statistically significant differences regarding plasma Gal-3 concentration in the comparison between DOX and Control groups. However, following the first two weeks of treatment, an increase in Gal-3 levels of 31.13% and 21.81% were observed. Similarly, in the last two weeks of the post-treatment period, DOX Gal-3 values increased by 25.2 and 38.56%. Compared to baseline, week 4 post-treatment values for the DOX group were almost 38% higher (Fig. 5A.).

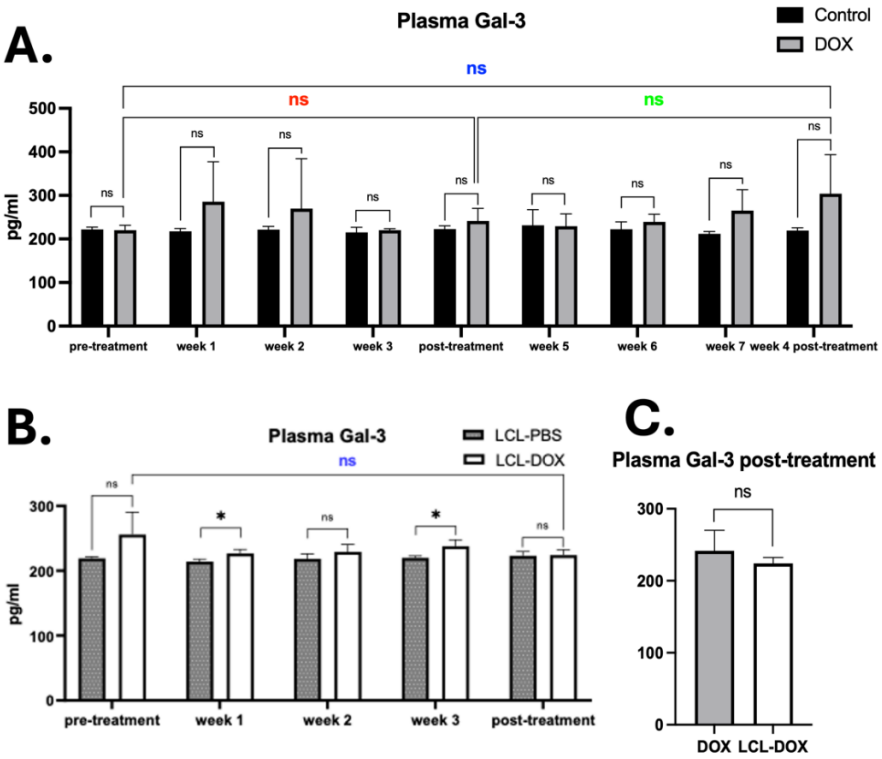

Figure 5. Plasma Gal-3 concentration. A. weekly values recorded for the Control (vehicle treated) and DOX (DOX treated with a cumulative dose of 15 mg/kg divided into 4 weekly i.v injections) groups. B. weekly values recorded for LCL-PBS (vehicle treated) and LCL-DOX (liposomal DOX, with the same therapeutic protocol) groups. C. comparison between DOX and LCL-DOX treated groups at the end of the treatment. Data represent mean ± standard deviation (SD) for each group of animals in their corresponding week, pre-, during or post-treatment. Statistical significance was considered as follows: p>0.05; *, p<0.05; **, p<0.01; ***, p<0.001, ****, p<0.0001.

As opposed to the trend seen for the DOX and Control groups, liposomal DOX treatment led to an increase in Gal-3 plasma concentration that is most noticeable at weeks 1 and 3 (5.84% and 8.19%, respectively), with a decrease of 12.47% post-treatment compared to baseline (Fig. 5B.). No significant difference was observed between the two forms of DOX treatment (Fig. 5C.).

N-terminal prohormone of brain natriuretic peptide

For the classic administration of DOX, NT-proBNP concentration was overall higher in the DOX group compared to Control. However, values for both groups slowly declined until the post-treatment week, and continued to increase in the following weeks. The only statistically significant difference between the DOX group is seen in the post-treatment week (48.36%, P-value of 0.0081) compared to baseline (Fig. 6A.).

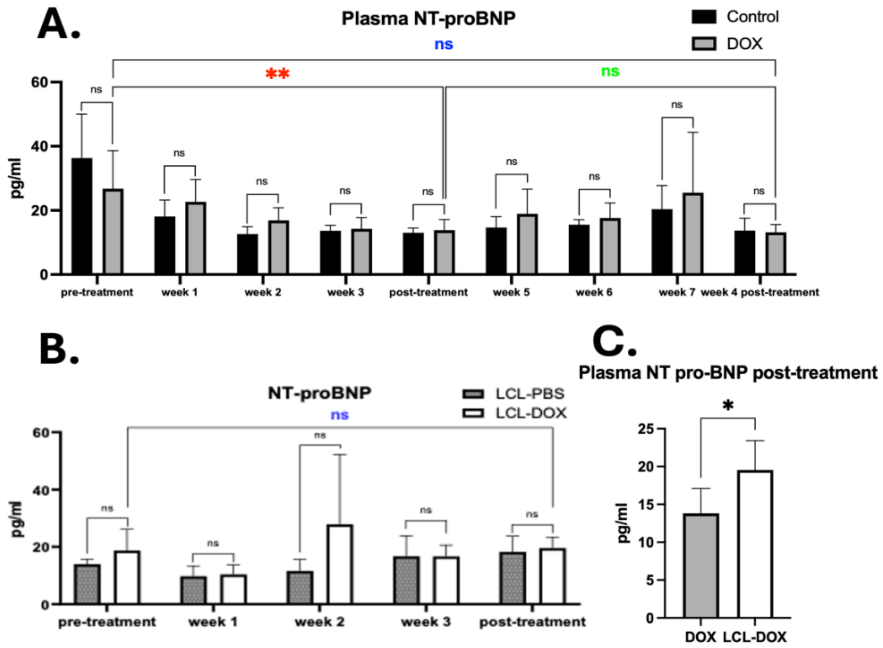

Figure 6. Plasma NT-proBNP concentration. A. weekly values recorded for the Control (vehicle treated) and DOX (DOX treated with a cumulative dose of 15 mg/kg divided into 4 weekly i.v injections) groups. B. weekly values recorded for LCL-PBS (vehicle treated) and LCL-DOX (liposomal DOX, with the same therapeutic protocol) groups. C. comparison between DOX and LCL-DOX treated groups at the end of the treatment. Data represent mean ± standard deviation (SD) for each group of animals in their corresponding week, pre-, during or post-treatment. Statistical significance was considered as follows: p>0.05; *, p<0.05; **, p<0.01; ***, p<0.001, ****, p<0.0001.

The liposomal form of DOX followed the same trend, with weekly values higher in the treated groups, apart from week 3, when values were similar for both groups. The most significant difference was recorded on week 2, when LCL-DOX NT-proBNP concentration was 139.76% higher than LCL-PBS (Fig. 6B.).

Liposomal administration of DOX led to a 41.43% increase in plasma NT-proBNP concentration compared to free DOX (Fig. 6C.).

Discussion

DOX is a highly potent chemotherapeutic agent, and along with its high efficacy in treating cancer, the long-term use of this compound should also be taken into account when choosing a DOX-based therapeutic regimen. This is especially important in the case of pediatric patients and in cancers with high survival rates, where post-treatment quality of life should also be considered.

One of the many ways DOX stimulates tumor cell apoptosis is through disruption of calcium homeostasis. This is mainly done by inhibiting mechanisms responsible for calcium reuptake, thus producing an increase in calcium concentration and the overproduction of ROS. This mechanism, although beneficial for treating cancer, is one of the contributing factors to the occurrence of CTOX (Shinlapawittayatorn *et al.*, 2022). Calcium homeostasis is of utmost importance for cardiac cells, due to its role in contractile function. Moreover, ROS are an important threat for these cells, due to their high vulnerability to oxidative stress (Pagan *et al.*, 2022).

In our experiment, free DOX administration did not lead to any significant changes in plasma calcium concentration, while liposomal DOX exhibited increases in calcium concentration during the first and last week of treatment, which shows that this formulation has a bigger impact on hypercalcemia even though weekly mean values were overall lower in both liposomal treatment groups. This result suggests a possible ameliorative effect of liposomal formulations on calcium dysregulation induced by DOX.

Cholesterol metabolism is severely impaired by both cancer and its therapeutic strategies, which often leads to other comorbidities besides CTRCD, thus increasing disease burden especially in older, individuals.

Belger *et al.* (2024) reviewed recent findings regarding risk factors for the development of DOX-induced CTOX and concluded that despite the limited data available regarding dyslipidemia as a risk factor for CTOX, current studies show that hyperlipidemia as a result of DOX treatment is strongly associated with cardiovascular events. Due to the lack of studies investigating long-term effects of anthracycline-based regimens on lipid metabolism, the duration of lipid dysregulation following treatment is unclear (Bhatnagar *et al.*, 2022).

Our results show that free DOX administration leads to hyperlipidemia that worsens in the post-treatment period. Total cholesterol values increased more post-treatment, and were doubled at the end of the experiment compared to baseline values and were 0.5-times higher than at the end of treatment.

Due to the small number of animals that were administered the liposomal formulations, the effect of this type of treatment was only investigated for the treatment period. Despite this limitation, the results in the LCL-DOX group at the post-treatment time-point are similar to those seen for the free DOX group. With these results, we can conclude that both therapies had the same effect on total cholesterol during treatment.

Triglyceride levels followed the same pattern as cholesterol in the post-treatment period, although differences between DOX and Control groups were actually lower. For LCL-DOX rats, a decrease in concentration was only observed at week 3.

The link between disrupted lipid metabolism and the development of heart disease is well-demonstrated, and DOX therapy has been proven as a contributing factor to hyperlipidemia in both cancer patients (Sharma *et al.*, 2016) and animal models (Abdulkareem Aljumaily *et al.*, 2021). In rats, one of the proposed mechanisms for the lipid metabolism alteration is through the inhibition of adipogenesis by the downregulation of the peroxisome proliferator activated receptor gamma (PPARγ) (Arunachalam *et al.*, 2013). Dyslipidemia is known to increase oxidative stress, especially in the heart, but due to the lack of long-term thorough studies focused on this side effect of DOX, its role in CTOX is inconclusive. In a study conducted by Simões *et al.* (2021), where breast cancer patients were followed for 1 year, it was shown that high triglycerides could offer a cardioprotection in some patients.

Another mechanism by which anthracyclines, and implicitly DOX contribute to CTOX development is by interfering with iron metabolism. This class of medications increase iron overload and contribute to ROS production (Huang *et al.*, 2022), which could exacerbate CTOX through oxidative stress (Kumfu *et al.*, 2022).

In our experiment, free DOX treated rats had increased plasma iron concentration, which seemed to slowly return to baseline, suggesting the contribution of excess iron on CTOX development could be temporary.

Gal-3 is a novel biomarker with prognostic value for heart failure and other cardiovascular diseases (Vucic *et al.*, 2023; Jiang *et al.*, 2021), but its purpose in CTOX prediction or monitoring is not yet clear. In the CECCY trial (NCT01724450), Gal-3 was not able to predict CTOX development (de Barros Wanderley *et al.*, 2022). In other human studies, this biomarker was successfully used for the prediction of treatment effectiveness (Shafiq *et al.*, 2020; Niang *et al.*, 2022). To our knowledge, this biomarker has not been evaluated

in animal models of CTOX thus, we sought to identify if Gal-3 could be included in a battery of biomarkers that could serve as a tool for the identification of CTOX.

In the free DOX group, Gal-3 spikes were observed at the start and before the end of treatment, and at the last analyzed time-point DOX Gal-3 levels were clearly higher than the ones for the Control group. For the liposomal DOX formulation, we observed a similar trend, except that the difference between LCL-DOX and LCL-PBS weekly values is smaller. This may be due to the less damaging side effects of liposomal DOX formulations (Franco *et al.*, 2018).

NT-proBNP is currently one of the few biomarkers recommended by the European Society of Cardiology for the screening of patients undergoing anthracycline-based therapies (McDonagh *et al.*, 2024). Multiple studies investigating its potential biomarker role have been conducted, but the results are inconclusive. NT-proBNP showed predictive power for the detection of subclinical CTOX in childhood cancer survivors (Demissei *et al.*, 2020), patients undergoing anthracycline-based therapies followed for 6 months post-treatment (Bisoc *et al.*, 2020), or followed during the course of treatment (Dong *et al.*, 2022). In smaller studies, an association between CTOX and NT-proBNP elevation could not be established (Posch *et al.*, 2022; Ruggiero *et al.*, 2013). Due to conflicting results between studies, as in the case of Gal-3, we chose to include NT-proBNP in our battery of biomarkers.

Similarly to Gal-3, NT-proBNP levels seemed to normalize after two weeks of treatment, and then gradually increased in the post-treatment period in the free DOX group. In the case of liposomal DOX, a similar trend was observed, but due to the absence of data in the post-treatment period for this group, we cannot compare the effects of the two formulations during the 4 weeks after the end of treatment.

Taken together, our results show that the most notable changes in biomarker levels occur at the beginning of treatment and close to the completion of the treatment. In order to correctly establish a set of biomarkers for prediction and diagnostic purposes, further studies should focus on establishing cut-off values for each biomarker that showed predicting/diagnosing power.

Conclusions

In this article, we sought to evaluate several biomarkers during the course of DOX treatment, and investigate whether or not a liposomal DOX formulation could influence these biomarkers differently. Our results showed that some of the CTOX-inducing effects of DOX administration are temporary, as iron levels slowly decrease after treatment. Free DOX administration induced changes in blood lipid levels that worsen post-treatment, so the search for cardioprotective strategies

based on lipid lowering compounds should focus on the post-treatment side effects of DOX administration. Lastly, a liposomal formulation of DOX could potentially alleviate sudden changes in these biomarkers, but close attention should be paid to the hypercalcemia produced by this formulation.

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Diversity of cereal pests (wheat and barley) grown in arid climate in Ziban region (provence of Biskra – southeastern Algeria)

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Abstract. This study investigated cereal pests (wheat and barley) grown in southeastern Algeria's arid climate. Using three sampling techniques (yellow pan traps and Pitfall traps, a comprehensive collection of insect specimens was obtained, comprising 2526 individuals taxonomically classified into 20 distinct species, 15 genera, ten families, and six orders. Homoptera and according to quantitative analysis, Thysanoptera was the most dominant taxa, with six and four occurrences, respectively. In contrast, the dominant species were Mayetiola destructor (Say 1817) (Diptera, Cecidomyiidae) with 412 specimens, *Rhopalosiphum maidis* (Fitch 1856) (Homoptera, Aphididae) with 404 specimens then *Oulema melanopa* (Linné 1758) (Coleoptera, Chrysomelidae) with 342 specimens. The observed and expected species richness diversity parameters were comparatively more significant in the cereal ecosystem cultivated in the El Outaya location than in the Sidi Okba location. (Kruskal–Wallis, df = 3, $P = 0.019$). The results obtained from the Generalised Linear Models (GLM) indicated a statistically significant variation in species richness across the different sites and crops (wheat and barley) (df = 3, Mean Square = 47.70, F = 3.58, $P = 0.020$).While there were very significant differences in the average number of individuals per species (N/S ratio) ($df = 3$, mean square = 22.08, F = 5.526, $P = 0.002$).

Species richness extrapolation revealed that diversity is anticipated as the number of individuals captured increases, however, at a slower rate as the sampled population increases. The species distribution showed that some insect species are found at all phenological stages and all sampling sites.

Keywords: insect, crop, biodiversity, dry climate, richness, extrapolation.

Introduction

Since antiquity, cereals have been the main staple food and are strategically important in human nutrition and animal feed. As well as cereal farming has played an essential role in the development of various civilizations (rice for Asian civilizations, maize for pre-Columbian civilizations, and wheat for the Mediterranean basin and the Near East) (Boulal *et al*., 2007).

Cereals are the staple diet in most southern Mediterranean countries. They are therefore considered strategic in the food security of populations (Lemeilleur *et al*., 2009). In Algeria, cereal growing plays a leading role in the national economy. It occupies the first place in strategic crops; most farmers practice it; according to statistics from the Ministry of Agriculture, the general census of agriculture (RGA) in 2013 gives us about 600.000 cereal farmers, or nearly 60% of all farms without taking fallow land into account. According to the FAOSTAT database (2021), the area occupied by cereals is 1.941.863 ha. This agricultural area is very narrow compared to the total area of Algeria which amounts to 238 million hectares of which 191 million are unproductive. Additionally, cereal production in Algeria is affected by various factors, including changes in weather patterns, which constitute the critical factor in determining the profitability of production. This strong correlation between climatic conditions and production causes a significant irregularity in cereal yields from year to year. Furthermore, according to figures from the Minister of Agriculture quoted by the official agency (APS, 2016), the national production of cereals (barley, oats, durum wheat and common wheat) decreased to 34.750 million quintals during the 2015/2016 campaign. The estimated yield rate was 16 quintals per hectare. It was 40 million quintals in 2014/2015, 35 million quintals in 2013/2014 and 49.1 million quintals in 2012/2013.

Despite the application of several programs to develop the agricultural sector in Algeria, in particular the national agricultural and rural development plan (PNDAR) in 2001/2002, cereal yields are still low and very irregular: 14.337 quintals/ha during the 2020-2021 agriculture campaign (FAOSTAT,

2021). Thus, in terms of productivity, Algeria is lagging behind Mediterranean countries, North Africa and Europe. This can be explained by various constraints such as environmental conditions (soil and climate), technical (seeds, cultivation practices) or human (organization and training of producers).

Besides, cereals in Algeria are frequently attacked by several pests that can damage the crop and reduce their yields. The most significant damages are due to insects; Indeed, these latter can cause severe crop losses by direct and indirect damage (some species are vectors of viruses and other diseases). Several authors have studied cereals entomofauna as well as the population dynamics of certain pests, such as Boujite (2007); Boughida & Dif (2010); Kellil (2019) and Bakroune (2021). Nevertheless, limited research was conducted in Algeria regarding pests affecting cereal crops, especially in arid regions under drought conditions and desertification. Thus, the present study presents a comprehensive inventory of pests that inhabit cereal ecosystems cultivated in the arid Ziban region (Biskra, southeastern Algeria). The aims of the investigation are: (i) assessment of species richness of insects pests in a cereal agrosystem in arid climat; (ii)determine the influence of varieties (wheat and/or barley) on insects diversity and (iii)determine the influence of phenological stages of crops on the diversity and the distribution of insects.

Materials and methods

Study area

This investigation was carried out from January to May 2016 in four sites across the Ziban region in Biskra province (Southeast Algeria), the study area is located at (34° 50' 13.326'' N, 5° 45' 3.7728'' E at an elevation: 97.623 m a.s.l.) (Fig. 1). Meteorological data for two decades (2000-2020) in Biskra indicated a dry climate in the region; with a maximum temperature of 41.76 °C in July (hottest month) and a minimum temperature of 17 °C h in December (coldest month). Rainfall has been scarce recently, with a maximum in winter (20.33 mm in January) and a minimum in summer (0.80 mm in July).All these factors led to a prolonged dry period; characteristic of North Africa's arid zones (Le Houérou, 1992). The ombrothermic diagram shows a six-month dry period, starting from May and ending in October (Fig.1). According to Emberger's climagram, the Biskra region is in an arid climatic stage (with a mild winter) characterized by low rainfall, high temperatures, high luminosity, and intense evaporation.

Insects sampling

Our work was carried out from January to May 2016. Samplings were done on four plots located in two different zones in Biskra region (southeastern Algeria). Two in El Outaya and two others in Sidi Okba. The plots for wheat and barley at every site are situated 5 kilometers apart from each other. During our experiment, three methods were used for trapping insects: (i) sight hunting (Colas, 1974), which involves random summation, (ii) yellow pan traps, which are commonly used in faunistic and entomological studies of agricultural environments due to their effectiveness, simplicity, and low cost. These traps can be used on a large scale, and (iii) pitfall traps. In this method, the traps are filled to a level of two-thirds with a solution of soapy water, which reduces the surface tension of the water and dissolves the layer of fat that envelops the bodies of the captured insects (Winchester, 1999). This layer of fat is known to hinder the escape of the insects from the trap. A total of 05 yellow pan traps and 05 Pitfall traps were placed at each field on a homogenous square parcel of land measuring 20 by 20 meters, with a total area of 400 square meters (Benkhelil, 1992), at least 50 meters away from the edges to prevent the edge effect. The experimental traps are filled with soapy water up to two-thirds of their capacity. Samples are collected every ten days throughout the experiment (Fig. 1).

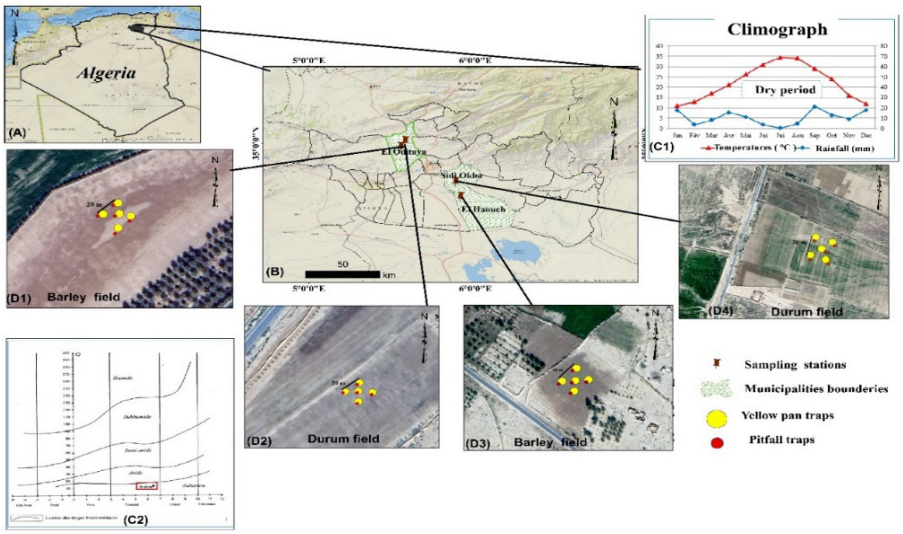

Figure 1. Geographical location of study sites (D_1, D_2, D_3, D_4) located in arid climate in the Ziban region (C_1, C_2) in the Province of Biskra (B) in southeastern Algeria (A).

Insects identification

Captured insects are retrieved using a filter that allows us to collect only insects. The filter designed specifically for collecting entomofauna. The insects are kept in pill boxes containing 70% alcohol and labeled with trap type; collection date, location, and species of cereal. After collecting the insect samples, they are taken to the laboratory for analysis under a binocular microscope. The insects are then sorted in a systematic order to help with identification and counting. To accurately identify the insects, we consulted with specialists from the Department of Agricultural and Forestry Zoology of El-Harrach and the C.R.S.T.R.A. of Biskra.

We collected insects from cereals during different growing stages are counted and examined them under a binocular microscope in the laboratory. To identify these insects, we referred to keys from Remaudiere and Seco Fernandez (1990); Leclant (1999); Leraut (2007).

Data analysis

Biodiversity analysis

- *Taxonomic diversity (species diversity).* Diversity of insect pests of cereals in each plot was evaluated by calculating the following ecological indices: (i) the relative frequency (RF) of each insect, which was determined by calculating the percentage of the number of individuals of a species in each station relative to the total number N; (ii) the Density/400m^2 (D), which was computed as $D = N/P$, where N represents the total number of individuals of a species collected on the surface considered, and P is the total number of samples; (iii) species richness *(S)*, which represents the overall number of determined species;(iv) Frequency of occurrence *(FO%),* according to Dajoz (1985), it shows the proportion of species appearances to all species for a specific species it is calculated by the following formula: *F.O % = ni1 / N2 x 100*. To determine the number of constancy classes (N.c.), we used the Sturge index (Scherrer, 1984). The formula is as follows: N.c. = 1 + (3.3 log 10 N3), where N3 represents the total number of captured individuals.;(v) The Shannon diversity index (H): $H = ((ni/N)\ log2(ni/N))$, where ni denotes the frequency of a species and N denotes the total number of individuals in a sample; (vi) evenness (E): $E = H/Hmax$, where $Hmax = log2S$; and (vii) the Simpson reciprocal index, $SRI = (1/D)$, with $D = \Sigma(ni(ni-1)/N(N-1))$. (viii) Menhinick's diversity index is calculated as the number of species (S) in the sample divided by the square root of the total number of individuals (N) in the sample written as IMn=S/√N. (ix)The Margalef richness index (RMg) estimates absolute species richness, regardless of sample size. RMg index used to assess diversity across various sites. It does not have a

defined threshold and allows for weighting of sample sizes. Despite being easy to calculate, this index can be greatly influenced by the amount of sampling effort put in (Margalef, 1969). The value of this index is obtained by the following formula: *RMg = S-1/ln (N)*. (x) the value of Fisher alpha index, a diversity index, defined implicitly by the formula S=a*ln (1+n/a), where S is number of taxa, n is number of individuals and a is the Fisher's alpha. (xi) Berger-Parker dominance, this index calculates the proportion of the community represented by the most abundant species. All other species are ignored (Berger, 1970). (xii) the value *1/S*. (xiii) the ration *N/S.*

- *Species accumulation curves.* The impact of the number of captured individuals on the cumulative specific richness observed (S_{obs}) by site and by cereal variety was evaluated. Using the Estimate S program (Colwell, 2013), the diversity index (Richness S, Shannon index H'and Simpson index), were utilized in order to carry out the species richness estimates (S_{est}).

Statistical analysis

The normality of the abundance data was assessed using the Shapiro-Wilk test, followed by the evaluation of species abundance variation using the nonparametric Kruskal-Wallis's test ($\chi 2$). For each sampling site in the study area, the calculated ecological diversity index (N, S, H, E, SRI, and the ratio N/S), and descriptive statistics were displayed in the form of boxplots for each insect family. The study employed generalized linear models (GLMs) to examine the diversity parameters of aphids across different seasons. A one-way analysis of variance (ANOVA) was performed, and a Tukey's HSD post hoc significance test (p 0.05) was used to determine if there was a significant difference in the means of N and S between and within the sampling locations. Statistical analyses was carried out using the free software R (R Core Team, 2019). Principal component analysis (PCA) was employed to investigate the impact of crops on the distribution pattern of insect pests across different locations. A visual representation of the frequency distribution of pest insect species across various phenological stages was presented through Venn diagram.

Results

Inventory

The plots fauna inventory comprised 20 cereal pest taxa that were categorized into six orders and ten distinct families, as presented in Table 1. The order with the highest representation was Homoptera (Fig. 2), which comprised six aphid species dependent on cereals. The aphid community at the

El Outaya location exhibits a higher level of attraction towards cereals than the Sidi Okba site, which only harbours four taxa. The Thysanoptera which ranked second in terms of species diversity exhibited a total of four species belonging to the Tripidae family. Both Lepidoptera and Diptera were comprised of three distinct species each. The Hemiptera taxonomic group comprises two species that rely on cereals as their primary source of sustenance, namely *Aelia germari* and *Aelia acuminata*. The Coleoptera taxa were observed to be comprised of two additional significant cereal pests, namely the Chrysomelidae *Oulema melanopa* and the Scarabaeidae *Geotrogus deserticola*. El Outaya and Sidi Okba sites harbor 18 and 17 species, respectively as determined by the qualitative analysis. Statistically significant difference in insect species diversity was observed among the two sites (Kruskal-Wallis, df = 3, $P = 0.019$).

The results of principal component analysis (PCA) shown that the ordination structure is divided into three clusters in wheat and four in barely (Fig. 3). The species *Mayetiola destructor*, *Oulema melanopa*, *Rhopalosiphum padi, Limothrips cerealium* and *Rhopalosiphum maidis*are are characterized by high values for the variables Occurence.%, Frequency, N Density Ind/400m^2 (variables are sorted from the strongest) (Tab. 2).

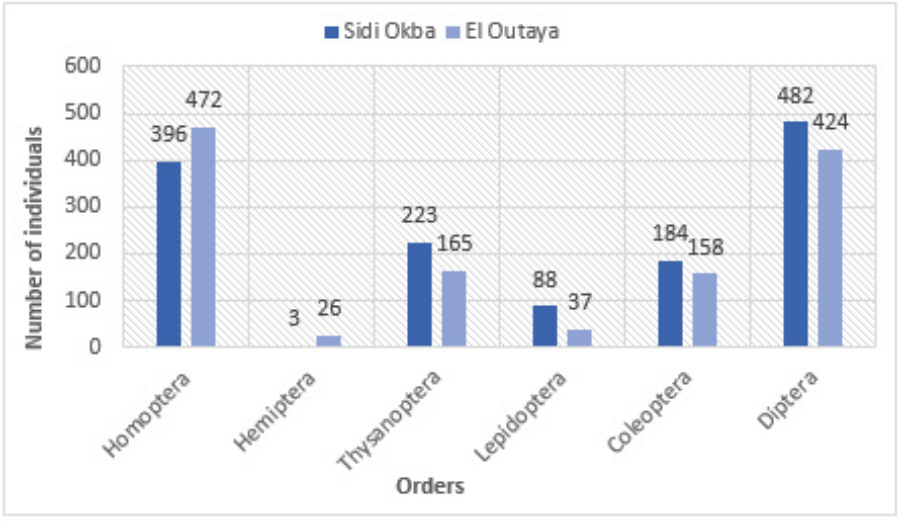

Figure 2. Abundance of the orders of the main cereal pests in the two study sites.

Table 1. Systematic list, abundances (N), frequency and occurrence (occ) of cereals pests (wheat and barley), recorded in an arid climate (El Outaya and Sidi Okba) in the Ziban region (Biskra, Algerian south-east).

Ordres	Families	Species	EPPO codes	Sidi Okba								El Outaya							
				Wheat				Barely				Wheat				Barely			
				N	Freq	Occu %	D Ind/ 400m²	N	Freq	Occu %	D Ind/ 400m²	N	Freq	Occu %	D Ind/ 400m²	N	Freq	Occu %	D Ind/ 400m²
Coleoptera	Chrysomelidae	*Oulema melanopa* (Linné, 1758)	*OULEME*	141.00	5.01	83.33	11.75	43.00	1.79	77.78	4.78	97.00	3.53	76.92	7.46	61.00	3.30	84.62	4.69
	Scarabaeidae	*Geotrogus deserticola* (Blanchard, 1851)	*GEOTDE*	7.00	0.25	41.67	0.58	8.00	0.33	66.67	0.88	15.00	0.55	61.54	1.15	0.00	0.00	0.00	0.00
Homoptera	Aphididae	*Metopolophium dirhodum* (Walker, 1849)	*METODI*	0.00	0.00	000	0.00	17.00	0.71	66.67	1.89	25.00	0.91	53.85	1.92	24.00	1.30	53.85	1.85
		Rhopalosiphum padi (Linné, 1758)	*RHOPPA*	71.00	2.52	75.00	11.08	68.00	2.82	100.00	9.11	66.00	2.40	84.62	5.08	55.00	2.98	76.92	4.23
		Rhopalosiphum maidis (Fitch, 1856)	*RHOPMA*	133.00	4.72	100.00	5.92	82.00	3.41	100.00	7.56	101.00	3.67	100.00	7.77	88.00	4.76	92.31	6.77
		Sitobionavenae(Fabricius, 1775)	*SITOAV*	34.00	1.21	75.00	2.83	24.00	1.00	88.89	2.67	58.00	2.11	76.92	4.46	20.00	1.08	61.54	1.54
		Sitobion fragariae (Fabricius, 1775)	*SITOFR*	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	10.00	0.36	53.85	0.77	17.00	0.92	53.85	1.31
		Schizaphis graminum (Rondani, 1852)	*SCHIGR*	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	5.00	0.18	30.77	0.38	3.00	0.16	23.08	0.23
Hemiptera	Pentatomidae	*Aelia germari* (Küster, 1852)	*AELIGE*	0.00	0.00	0.00	0.00	3.00	0.12	44.44	0.33	12.00	0.44	53.85	0.92	6.00	0.32	30.77	0.46
		Aelia acuminata (Linnaeus, 1758)	*AELIAC*	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	3.00	0.11	15.38	0.23	5.00	0.27	30.77	0.38
Thysanoptera	Tripidae	*Limothrips cerealium* (Haliday, 1836)	*LIMOCE*	0.00	0.00	0.00	6.17	50.00	2.08	100.00	5.56	58.00	2.11	100.00	4.46	44.00	2.38	100.00	3.38
		Thrips tabaci(Lindeman, 1889)	*THRITA*	48.00	1.70	83.33	4.00	31.00	1.29	66.67	3.44	44.00	1.60	69.23	3.38	11.00	0.60	38.46	0.85
		Melanthrips pallidior (Priesner, 1919)	*MELAPA*	12.00	0.43	58.33	1.00	0.00	0.00	0.00	0.00	6.00	0.22	30.77	0.46	2.00	0.11	15.38	0.15
		Aeolothrips fasciatus (Linnaeus, 1758)	*AEOLFA*	0.00	0.00	0.00	0.00	8.00	0.33	66.67	0.89	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Lepidoptera	Noctuiidae	*Helicoverpa armigera* (Hübner, 1808)	*HELIAR*	13.00	0.46	50.00	1.08	8.00	0.33	44.44	0.89	0.00	0.00	0.00	0.46	6.00	0.32	38.46	0.46
		Agrotis segetum(Denis &Schif., 1775)	*AGROSE*	0.00	0.00	0.00	0.00	9.00	0.37	55.56	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	Gelechiidae	*Sitotroga cerealella* (Olivier, 1789)	*SITOCE*	30.00	1.07	66.67	2.50	28.00	1.16	100.00	3.11	15.00	0.55	53.85	1.15	10.00	0.54	46.15	0.77
Diptera	Cecidomyiidae	*Mayetiola destructor (Say, 1817)*	*MAYEDE*	144.00	5.11	100.00	12.00	147.00	6.10	100.00	16.33	121.00	4.40	100.00	9.31	0.00	0.00	0.00	6.77
	Agromyzidae	*Lyriomiza trifolii* (Burgess, 1880)	*LYRITR*	73.00	2.59	91.67	6.08	78.00	3.24	100.00	8.67	124.00	4.51	92.31	9.54	35.00	1.89	76.92	2.69
	Ephydridae	*Hydrellia griseola* (Fallen, 1813)	*HYDRGR*	8.00	0.28	50.00	0.67	32.00	1.33	100.00	3.56	29.00	1.05	69.23	2.23	0.00	0.00	0.00	2.08

N: Number of individuals;Freq: frequency; Occu % : occurrence; D Ind/400m² : density.

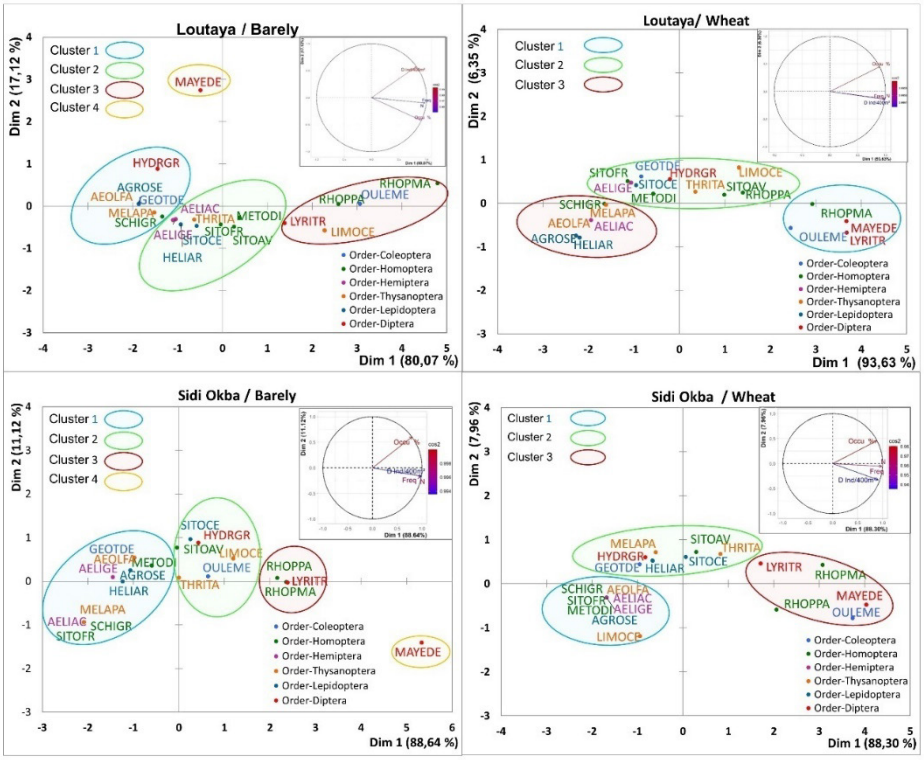

Figure 3. Plot of the results of the principal component analysis (PCA) of the pest/crop/locality ordination in the Ziban region (Biskra, south east Algeria).

Distribution of insects in the sites

The statistical analyses conducted using the Kolmogorov-Smirnova and Shapiro-Wilk tests revealed a significant correlation between species richness, insect abundances, and the N/s ratio in the ecosystems (of the two sites and the four plots), as presented in Table 2, with respective statistical values of S ($F = 3.582$, $P = 0.020$), N ($F = 3.569$, $P = 0.021$) and the ratio N/S ($F = 5.535$, $P = 0.002$) (Fig. 3). Nevertheless, the remaining diversity parameters exhibit no significant variations across the study sites (Shannon diversity index: F = 1.797, $P = 0.160$, Inverse of diversity index: F = 1.311, $P = 0.282$, Evenness: $X^2 = 19.961$, $P< 0.001$) (Fig. 4).

Following the Tukey's post-hoc test, and the Dunn's test (for Evenness), the identical letters linked with average values (circles) are not significantly different.

Table 2. Kolmogorov-Smirnov and Shapiro-Wilk normality tests for the ecological indices studied in overall (whole region).

	Normality tests					
Ecological indices	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Statistic	*df*	Sig.	Statistic	*df*	Sig.
Taxa_S	0.122	46	0.081	0.952	46	0.058
Individuals	0.111	46	0.197	0.940	46	0.019
Dominance_D	0.170	46	0.002	0.817	46	0.000
Shannon_H	0.126	46	0.063	0.927	46	0.006
Simpson_1D	0.170	46	0.002	0.817	46	0.000
Evenness_E	0.135	46	0.036	0.953	46	0.061
Menhinick	0.160	46	0.005	0.952	46	0.057
Margalef	0.093	46	.200*	0.966	46	0.200
Equitability_J	0.080	46	.200*	0.988	46	0.922
Fisher_alpha	0.139	46	0.026	0.918	46	0.003
Berger-Parker	0.146	46	0.015	0.846	46	0.000
1/S	0.128	46	0.055	0.886	46	0.000
N/S	0.114	46	0.167	0.936	46	0.014

df: degree of freedom, Sig.: significance

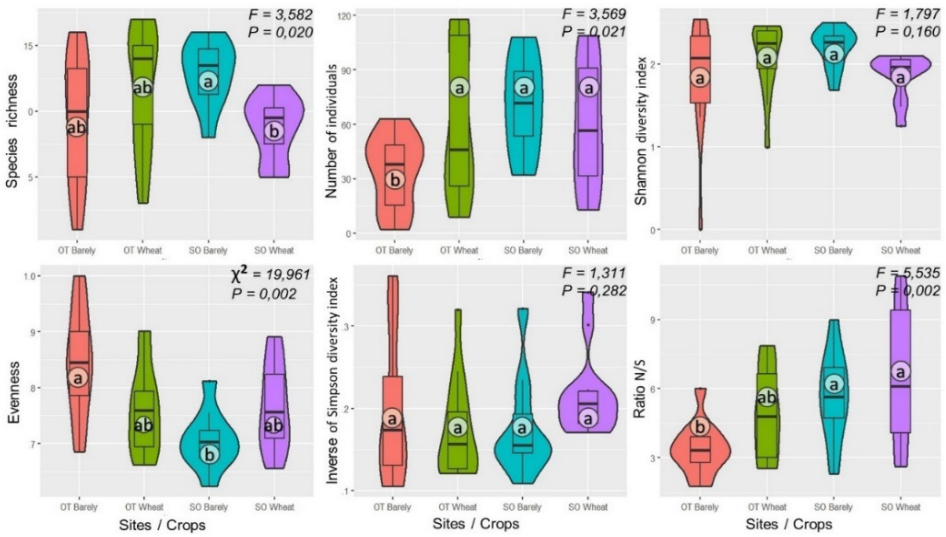

Figure 4. Boxplots representing the Ziban region's (Biskra, *SE* Algeria) various sites' insect diversity parameters.

Variation of insect diversity parameters

Overall, El Outaya site's cereal ecosystem reported higher diversity parameters than Sidi Okba site, with monthly variation values ranging from 4 to 18 in El Outaya and 8 to 15 in Sidi Okba. The abundance of cereal pests captured in Sidi Okba was higher (714 specimens in wheat with a range of 0.268 - 0.143 and 636 specimens captured in barley with a range of 0.243 - 0.105), than El Outaya (789 individuals in wheat with a range of 0,262 - 0,1001 per month and 387 specimens in barley with a range of 0,308 - 0,098) (Tab. 2). The study's findings indicate that there was a significant difference in species richness between the various sites and crops (wheat and barley) as determined by the generalized linear models (GLM) analysis (df = 3, Mean Square = 47.70, F = 3.58, *P* = 0.020). The N/S ratio exhibited a substantial degree of variation across species, as evidenced by a statistically significant result (df = 3, mean square = 22.08, F = 5.526, *P* = 0.002) (Tab. 3). The El Outaya site exhibited higher values of Shannon diversity index and evenness in both wheat (H′ = 2.494, E = 0.712) and barley (H′ = 2.473, E = 0.892) compared to the Sidi Okba site (H′ = 2.064, E = 0.877 in wheat and H′ = 2.477, E = 0.793 in barley) (Tab. 4). The Simpson reciprocal index (Simpson_1-D) exhibited a comparable pattern across the various sites, irrespective of the crop type.

In addition, the GLM revealed that the diversity parameters of Menhinick, Margalef, Fisher_alpha and Berger-Parker have no significant variation (*P* value > 0.05).

Table 3. Variation of diversity indices of pests of cereal sampled in arid lands of southeastern Algeria.

Index	**Sidi Okba**				**El Outaya**			
	Wheat		**Barely**		**Wheat**		**Barely**	
	Min	Max	Min	Max	Min	Max	Min	Max
Taxa_S	5.00	12.00	8.00	15.00	5.00	18.00	4.00	16.00
Individuals	13.00	303.00	37.00	199.00	21.00	197.00	9.00	144.00
Dominance_D	0.14	0.27	0.11	0.24	0.10	0.26	0.10	0.31
Shannon_H	1.48	2.06	1.68	2.48	1.23	2.49	1.27	2.47
Simpson_1-D	0.73	0.86	0.76	0.89	0.62	0.90	0.69	0.90
Evenness_e^H/S	0.66	0.88	0.65	0.79	0.65	0.71	0.70	0.89
Menhinick	0.63	1.39	1.32	1.63	0.98	1.30	0.88	1.37
Margalef	1.35	2.18	1.94	3.15	1.31	3.22	1.34	3.05
Equitability_J	0.83	0.92	0.81	0.91	0.76	0.89	0.87	0.92
Fisher_alpha	1.94	3.04	2.88	5.29	2.08	4.82	2.14	4.70
Berger-Parker	0.18	0.38	0.21	0.41	0.18	0.57	0.18	0.44

Estimation of species richness (rarefaction and extrapolation)

The rarefaction curves exhibited an upward trend as the number of captured individuals increased, eventually attaining a plateau (Fig. 5). As the sampled population grew, the number of species observed S grew, but at a slower rate. The estimated specific richness of the pests captured (S) reached specific stability from 810 and 250 individuals for wheat and barley respectively in El Outaya site, and 700 and 600 individuals for barley, and wheat respectively in Sidi Okba site. The use of three estimators (Richness S, Shannon index H', and Simpson index Simpson_1-D) showed that the estimated species richness curves had a striking resemblance in shape. Additionally, the populations of each site in the three estimators were combined when the total number of samples collected (the reference number) was smaller than the actual number of individuals.

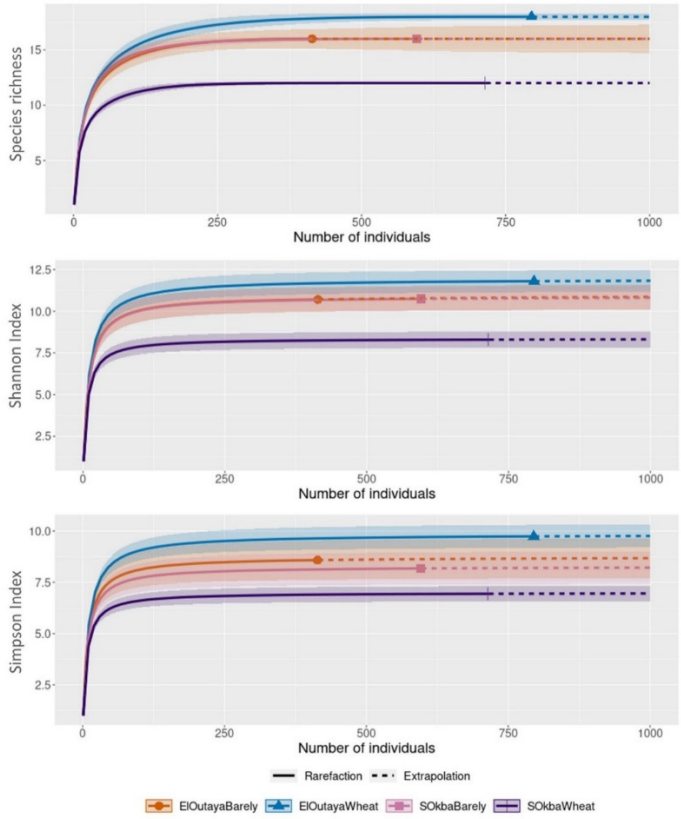

Figure 5. Rarefaction curves of expected species richness in a cereal ecosystem with a dry climate (Biskra, southeast Algeria). The lower and higher limits of the 95% confidence intervals for the number of species (S) are shown by the light-colored areas

Table 4. Evaluation of the fluctuation of insect diversity parameters among sites in Southeast Algeria using generalized linear models

Diversity index	Source	Type III sum of squares		Mean square	F	Sig.
S	Corr. model	143.120[a]	3	47.707	3.582	0.020
	Intercept	5913.547	1	5913.547	444.049	0.000
	site_crop	143.120	3	47.707	3.582	0.020
N	Corr. model	9587.450[a]	3	3195.817	3.570	0.021
	Intercept	165000.7	1	165000.7	184.313	0.000
	site_crop	9587.450	3	3195.817	3.570	0.021
Dominance_D	Corr. model	.049[a]	3	0.016	0.946	0.426
	Intercept	1.774	1	1.774	102.214	0.000
	site_crop	0.049	3	0.016	0.946	0.426
Shannon_H	Corr. model	1.025[a]	3	0.342	1.803	0.159
	Intercept	206.595	1	206.595	1090.104	0.000
	site_crop	1.025	3	0.342	1.803	0.159
Simpson_1-D	Corr. model	.049[a]	3	0.016	0.946	0.426
	Intercept	34.566	1	34.566	1991.904	0.000
	site_crop	0.049	3	0.016	0.946	0.426
Evenness_E	Corr. model	.125[a]	3	0.042	7.487	0.000
	Intercept	30.754	1	30.754	5507.929	0.000
	site_crop	0.125	3	0.042	7.487	0.000
Menhinick	Corr. model	1.051[a]	3	0.350	2.226	0.097
	Intercept	113.073	1	113.073	718.547	0.000
	site_crop	1.051	3	0.350	2.226	0.097
Margalef	Corr. model	4.522[a]	3	1.507	2.535	0.068
	Intercept	304.592	1	304.592	512.222	0.000
	site_crop	4.522	3	1.507	2.535	0.068
Fisher_alpha	Corr. model	21.704[a]	3	7.235	2.228	0.097
	Intercept	976.355	1	976.355	300.617	0.000
	site_crop	21.704	3	7.235	2.228	0.097
Berger-Parker	Corr. model	.015[a]	3	0.005	0.290	0.833
	Intercept	4.242	1	4.242	240.399	0.000
	site_crop	0.015	3	0.005	0.290	0.833
N/S	Corr. model	66.251[a]	3	22.084	5.526	0.002
	Intercept	1355.067	1	1355.067	339.098	0.000
	site_crop	66.251	3	22.084	5.526	0.002

Mean square values: are variance estimates.

Insect diversity depending on phenological stages

The Venn diagram (Fig. 6), indicates that Sidi Okba was host to seven pest species, namely *Hydrellia griseola, Limothrips cerealium, Lyriomiza trifolii, Mayetiola destructor, Rhopalosiphum maidis, Rhopalosiphum padi*, and *Sitotroga cerealella*, throughout the entire sampling period. While only two species shared among the months on wheat in the same site (*Lyriomiza trifolii*, *Mayetiola destructor*), and three species shared between all months of study under barely in El Outaya (*Limothrips cerealium*, *Oulema melanopa* and *Rhopalosiphum maidis*), However, four species were recorded in wheat at El Outaya (*Limothrips cerealium*, *Lyriomiza trifolii*, *Mayetiola destructor* and *Rhopalosiphum maidis*). According to Sturge's rule, all these species are considered omnipresent. Through our study, it was shown that February, March and April are the most diverse months, this coincided with the cereals phenological stages, boot, heading, flowering and grain fill.

Additionally, a notable disparity was observed in the frequency of the species identified during the phenological phases (Kruskal-Wallis, $df = 4$, $P = 0.045$).

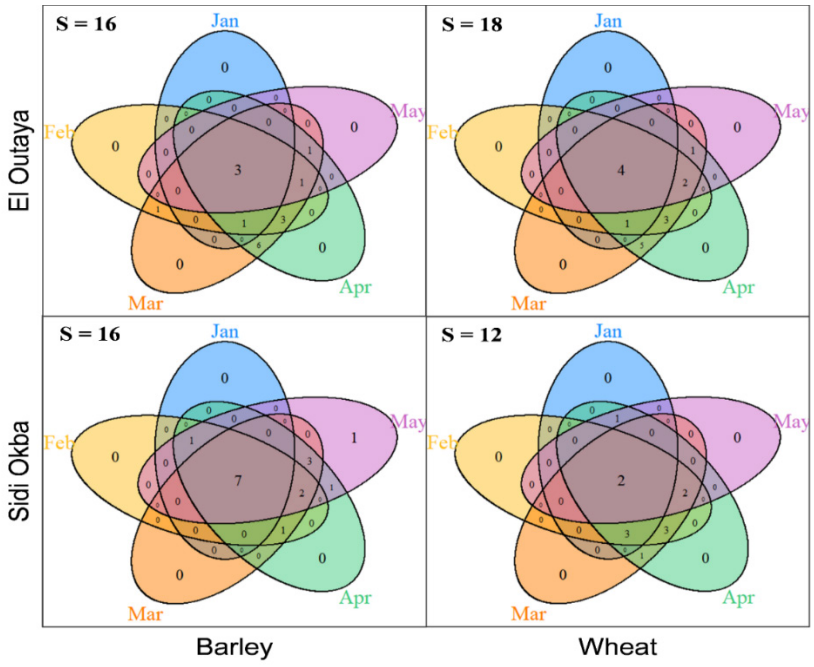

Figure 6. Venn diagram illustrating the distribution of the richness of pests found (S) over the five months of investigation at sample locations for the connected cereal ecosystem of the research area.

Discussion

The census of insect pests we conducted in a cereal agrosystem covered a wide range of bio-aggressor species in the Ziban (Biskra, Algeria) region, were dispersed among six orders, ten families, sixteen genera, and twenty species. Compared to Sidi Okba (17 species), El Outaya location has a higher diversity of 18 species. Homoptera significantly outnumbers the other orders in abundance in both locations, whether on wheat or on barely. Six Aphididae species that depend on cereals are included; they are among the most prevalent pests in Algeria that endanger cereal crops (Laamari, 2004; Assabah, 2011; Saharaoui 2017). Bakroune in 2012 had listed 26 species of aphids in the Biskra region, including 18 in El Outaya. For their part, Laamari *et al*. (2010) mentioned a richness of 30 species in several localities of the Biskra region. Tahar Chaouche (2019) inventoried 32 species in the wild in different localities in the Biskra region. In the region of Sidi Okba, Laamari *et al*. (2009), cited 11 aphid species belonging to the genera *Aphis*, *Brachycaudus* ,*Brevicoryne*, *Capitophorus*, *Dysaphis*, *Rhopalosiphum*and *Sitobion*. Hamidi *et al*. (2013), identified 10 species of aphids in Biskra city. In addition, in the high plain's region of Setif, Kellil in 2019 identified 24 taxa. Additionally, Ketfi (2018) noted the presence of *R. padi, R. maidis,* and *S. avevae* in Constantine region, with a combined total of 508 individuals on durum wheat and 531 individuals on soft wheat. In the sub-humid bioclimatic stage in Oued Smar, Assabah (2011), had inventoried four species of aphids dependent on cereals in a plot of durum wheat, where the species *R. padi* is quantitatively the most dominant with an abundance of 63.53%. Followed by *R. maidis* with 6.87%. The species *S. avenae* and *S. fragariae* represent very low proportions not exceeding 4%. According to Belkahla and Lapierre (1999), *R. padi*, *S. fragariae* and *S. avenae* are among the aphids considered potential vectors of the virus responsible for the incipient jaundice disease of barley in cereal-growing areas in Algeria.

The spatiotemporal evolution of each species shows that the species *R. maidis, R. padi* and *S avenae* are present in the two sites on both varieties (wheat and barley); they are constant species present during all the plant phenological phases. The activity of aphids is related to the biological cycle of the species and the phenological stage of the host plant, as well as the influence of climatic conditions. Hulle and d'Acier (2007) reported that the minimum temperature for the development of aphids is 4°C on average, below this threshold, they no longer multiply. According to Ortega (1988), *R. maidis* is a very popular pest worldwide. But in harsh climatic conditions such as winter, it does not survive (Blackman and Eastop, 2007). This species is probably the most important cereal aphid in hot, tropical and subtropical regions of Africa and Asia (So *et al*., 2010).

Four species of thrips were listed in the context of our study, among these taxa only the species *Limothrips cerealium* has the status of pest of cereals. The other taxa *Thrips tabaci*, *Melanthrips pallidior* and *Aeolothrips fasciatus*are much more stretched out by the spontaneous vegetation in or around the plot. The *L. cerealium* species has already been reported in the Biskra region by Rechid (2011) on *Echium parviflorum*, *Asphodelus refractus* and *Beta vulgaris* and by Razi (2017), on onions in several localities. On their part Benmessaoud *et al*. (2011), noted the presence of this species on *Triticum* and *Jasminus* in Algiers. It is also mentioned in North Africa, in Morocco by Zur Strassen (1968), in Tunisia Jenser (1982), in Egypt Preisner (1960). According to Elimen *et al*. (2014), it is a very popular pest in cereal fields in Europe. The appearance of thrips coincides with the first vegetative stages of the host plant (wheat and/or barley).

The infestation of cereal plots by thrips increases over time, especially by *L. cerealium* and *T. tabaci,* to reach the peak of pullulation in the spring (between March and April), it is matched with heading and flowering stages. Several conditions can play a determining role in diversity, abundance and dynamics of thrips such as plant phenology (Mehra and Singh, 2013), climatic conditions (Toapanta *et al*., 2001), the diversity of crops practiced within the study sites (Razi, 2017).

Diptera order occupies the third position with three cereal pests, where the *Mayetiola destructor* (Cecidomyiidae family) is the most dominant species in the two cereal varieties. This species was reported by the National Institute for Plant Protection services (NIPP, Algeria) in the Annaba and Guelma regions (Bakroune, 2021). Other researchers have reported this Diptera in several regions of Algeria (Berchiche, 2004; Saidouni, 2012), in the Mitidja region on soft wheat and barley. Today this pest has spread to all regions of Algeria. In Morocco, *M. destructor* has been observed in all cereal-growing regions (Nadjimi *et al*., 2002; Nsar Ellah and Lhaloui, 2006). According to Roy *et al.* (2008), this pest is recognized as being very harmful for the cultivation of wheat *Triticum aestivum* (Linné, 1753) and *T. turgidium* (Linné, 1753) where the damage can reach 100% of the yield. This species developed at least three generations during the 2015/2016 cereal campaign. Lhalouiet *et al*. (2004) mentioned that the number of generations varies according to climatic conditions. It varies between 2 to 6 generations per year (Elimen *et al.,* 2018). The results of monitoring the Hessian fly (*M. destructor*) in Morocco show that this species has three generations per year, two complete generations and a partial one which can only develop if the end of the growing season is rainy (Lhaloui, 1995). This confirms that the number of generations is related to climatic conditions. The Agromyzidae *Liriomyza trifolii* is the second cereal pest with intense activity in the two study sites. It is a very polyphagous species (Spencer, 1990), it has been observed in 29 taxonomic families (Mujica *et al.,* 2016). *L. trifolii,* is native to North America, Central America and South America. It spread to other parts of the world in the 1960s-1980s (Mujica *et al.,* 2016).

Hydrellia griseola is the third species that deserves to be reported as a critical cereal pest. It was captured in both cereal varieties. It is a Diptera, also called grain fly or grain miner, sometimes the tiny rice miner; it belongs to the Ephydridae family. Hydrellia is cosmopolitan, with more than 120 species identified. Hassan *et al.* (2019), noted that the Ephydridae family has a wealth of 2000 species identified across the world. The damage of this species is mainly due to the larvae (miners), which attack the blade and the sheath of the leaves as well as the roots.

Lepidoptera includes two families, namely the Noctuidae and the Gelechiidae. The Noctuidae predominate with two species, *Helicoverpa armigera* and *Agrotis segetum.* As an indication, more than 35000 Noctuiidae are described worldwide, and perhaps more than 100000 species in total, with more than 4200 genera (Murlis *et al.,* 2000). Gourari (2015) identified 12 species of Noctuidae, representing a present rate of 19% of all entomofauna captured in Séttif region. In addition, Barkou *et al.* (2017), in his study on the diversity of butterflies in the Algerian coastal regions, had cited the species *Agrotis segetum* and *Helicoverpa armigera* in his inventory. The Gelechiidae family is represented by *Sitotroga cerealella* which is considered as a cereal pest. This species showed intense activity in both study sites. According to Athanassiou *et al*. (2005), *S. cerealella* attacks all varieties of cereals (barley, wheat, rice, millet, sorghum); weight loss can reach 50% for wheat. Infestations begin during plant growth and continue during storage (Bushra and Aslan, 2014). This species one of the main pests of stored cereals in Morocco (Benayad, 2013).

According to Akter *et al.* (2013), adult longevity of *S. cerealella* is 2–4 weeks depending on developmental conditions, with 5–6 generations per year. Adjalian *et al*. (2014), indicated that the development of *S. cerealella* populations is maximal in a range of temperatures oscillating between 20 and 30° C.

Among beetles, *Oulema melanopa* is the only key bioaggressor of cereals that predominates. It is a species belonging to the Chrysomelidae family. Adults and larvae consume cereal leaves (Bai *et al*., 2002). In Algeria, Kellil (2019) indicated that studies on the cereal leaf beetle are insufficient, despite the significant damage that this species can cause to production. However, Rouag *et al.* (2014), reported the presence of two species of the genus *Oulema* in the Setif region (*O. melanopus* and *O. hoffmannseggi*).

Heavy infestations of *O. melanopa* occurred during boot, heading and Flowering stages, between the end of February and mid-April. These results are consistent with those obtained by Kellil in 2019 in his study of the population dynamics of *O. melanopa* on cereals in Setif. Kher *et al*. (2011) point out that the beetle's phenology and adaptability differ from region to other. According to Philips *et al*. (2012), many physical and biological factors intervene directly or

indirectly in the fluctuations of the beetle population, such as habitat, microclimate, food, quality of the host plant and the abundance of natural enemies or competing species.

Two species of hemipterans of the Pentatomidae family recognized as pests of cereals were identified. These are the bugs *Aelia germari* and *Aelia acuminata*. Our inventory results show that the two *Aelia* species are practically absent on durum wheat in Sidi okba. On the other hand, the species *A. germani* is present only on barley in the same site. In El Outaya the two species are present in the two cereal varieties.

Safavi (1968) in Zair (2016), reports that cereal crops in North Africa are frequently attacked by various species of Pentatomidae belonging mainly to the genus Aelia, and record considerable damage. According to the N.I.P. report (2016), the specie *A. germari*, constitutes a permanent danger in cereal-growing regions (High-Plains). In Constantine, Benaoun and Meziani (2015) captured 15% of the bug of the genus *Aelia*. In the region of Tlemcen, Zair (2016), reported the species *A. germari* in 4 localities of the Wilaya in a total area of 108 ha. The installation of the two species of *Aelia* in cereal plots should be on time. They intervene at the grain fill and maturation stages (mid-March to end of the cereal campaign). At the same time Boutheldja and Orlici (2014) indicated that this insect causes damage in the field, before grain maturity.

Conclusion

The present study allowed us to better understand the diversity and distribution of cereal bioagressors in an arid climate (the region of Ziban, Biskra, south-eastern Algeria). Throughout the cereal campaign, the cereal agrosystem has a varied richness of insect species. A distinct insect community accompanies each phenological stage. The diversity varies according to the cereal varieties (wheat, barley) and the collection sites. The results obtained from this study can potentially function as a benchmark for other ecological inquiries about the interdependent associations between insects and cereal agroecosystems.

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First report of *Planktothrix rubescens* bloom from the Algerian freshwater reservoir Hammam Debagh

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Abstract. Massive cyanobacteria blooms have become a worldwide concern problem due to the multiple nuisances they can cause. The impacts of climate change are distinct from other environmental constraints controlling its population dynamics. The current study was accomplished in the monomictic reservoir Hammam Debagh (North-Eastern Algeria). A sampling campaign was conducted during May and June 2012 in four surface sampling stations and the water column in the center of this reservoir. This study aims to investigate the surface bloom of *Planktothrix rubescens* (De Candolle ex Gomont 1892) Anagnostidis and Komarek, 1988; observed for the first time in an Algerian reservoir with a focus on the environmental variable changes in 2012.

During this thick epilimnetic bloom, *P. rubescens* spread in all water column layers, reaching 6.4 x 10^6 cells mL^{-1} at 2m from the surface recorded on 11.05.2012. Meanwhile, at the surface *P. rubescens* abundances ranged from 3 x 10^5 to 3.6 x 10^6 cells mL^{-1}. The biomass was so high that the signal from the phycocyanin probe became saturated attending a value of 200 µg L^{-1} PC in the top 1 m. In addition, the biogenic compounds especially P-PO_4 and NO_3 show a high concentration of 0.48 mg L^{-1} and 5 mg L^{-1} respectively. The red pigmented cyanobacterium bloom altered environmental conditions in

the reservoir Hammam Debagh, since primarily oxygen concentration shows a minimum of 2.63 mg L^{-1} and water transparency did not exceed 1m (min= 40cm). The vertical profile of temperature performed on 29.05.2012 over 33m; shows a stratified water column ranging from 22.6°C in the upper subsurface to 9.8°C in the bottom hypolimnion. Finally, suitable meteorological conditions were observed during 2012.

Keywords: cyanobacteria, *Planktothrix rubescens,* phycocyanin, microcystin, Reservoir Hammam Debagh

Introduction

In recent years, lakes hydrodynamics and thermal stratification period have been significantly altered due to the rise in water temperature and the extension of the period with suitable temperature for potential harmful algae growth (Komatsua *et al.*, 2007; De Stasio *et al.*, 1996). Cyanobacteria cells are equipped with the necessary physiological tools to thrive in changing environments (Neilan *et al.*, 2013). Thus, a global intensification of cyanobacteria bloom was expected (Chirico *et al.*, 2020; Paerl 2014; Wood *et al.*, 2011). During cyanobacteria bloom episodes, their concentration can maintain cells densities of more than 500 000 cells L^{-1} over several months, thus creating a significant restraint on the use of the water resource (Sulis *et al.*, 2014; Ernst *et al*., 2007). Hence, this increasing problem impacts the ecosystem integrity as well as human and animal health by drinking unfiltered water, fish consumption or swimming in it (Downing *et al.,* 2001; Carmichael *et al.*, 2001). Cyanotoxin production is one of the most dangerous consequences of cyanobacterial bloom, with microcystins, primarily associated with liver injuries, being the most widespread and likely to occur when certain cyanobacterial taxa are present (Bartram *et al*., 19999). Other toxins, such as neurotoxins, are less common.

Planktothrix rubescens (basionym *Oscillatoria rubescens,* De Candolle ex Gomont 1892) Anagnostidis and Komarek, 1988 is a filamentous bloom-forming cyanobacteria. This red pigmented species can tolerate low temperatures and prefers low light. Moreover, it exhibits high plasticity in the water column due to the presence of gas vesicles. Hence, it can form blooms in deeper, thermally stratified lakes with moderate nutrient pollution (Janse *et al.,* 2005). In fact, Reynolds *et al.* (2002) describe it as an R-strategist. When compared with *Planktothrix agardhii*; *P. rubescens* exerts a significant allelopathic effect on the diversity and biomass of phytoplankton (Lenard *et al.,* 2022).

Freshwater cyanobacteria bloom became a common problem in Algeria. During the last decade, several species have been reported including *Microcystis sp.* (Bouhaddada *et al.,* 2016; El-Herry *et al.*, 2009; Nasri *et al*., 2008), *Planktothrix agardhii* (Benayache *et al.*, 2022; Saoudi *et al.*, 2017), *Cylindrospermopsis raciborskii* (Charifi *et al.*, 2019; Bouaicha and Nasri, 2004) and *Aphanizomenon issatschenkoi* (Boussadia *et al.*, 2015). Blooms attributed to the species mentioned above occur commonly in the Mediterranean freshwater ecosystems. However, *P. rubescens* is a common bloom forming cyanobacterium in deep Northern and prealpine European oligotrophic to mesotrophic lakes (Vareli et al., 2009). The current study aims to describe a *P. rubescens* bloom observed in the reservoir Hammam Debagh (North of Algeria) in 2012, being the first record of this species blooming in Algeria. The focus was on environmental variations to better understand this "burgundy-blood phenomenon" observed in the surface of the reservoir.

Materials and methods

Study area

Hammam Debagh (36.4713889 N, 7.214166 E) is a warm monomictic reservoir in the north east of Algeria (Fig. 1), situated at 800m above sea level and the depth can vary from20m to 60m. The total volume of about 185 million cubic meters occupies a surface area of 6.5km^2. The main water source for the lake is the river Bouhamdene, formed by the confluence of the rivers Zenati and Sabath. This is a source of drinking water for 180000 inhabitants, irrigation (13.000 ha), and small fish farming activities (e.g. *Carassius carassius*, *Cyprinus carpio*). Meteorological data such as air temperature, precipitation, and water evaporation were provided by the weather station situated near to the Hammam Debagh reservoir.

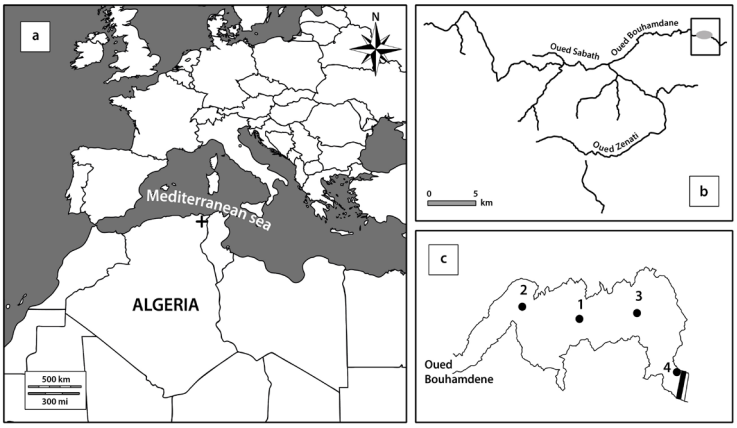

Figure 1. Geographical positions. a) Localization of the reservoir in Algeria; b) The Bouhamdane watershed; c) location of the four sampling stations in the reservoir

Sampling strategies

Surface water sampling was carried out along a west east transect, from May to July 2012, from four sampling stations named S1, S2, S3 and S4 for *P. rubescens* abundances and Microcystins analysis. Samples were collected at various depths in the water column using an integrated Ruttner sampler (Hydro-Bios, Germany). Samples were collected at 20cm interval in the first 1m and then at 2, 3, 5, 10, 18, 23, 33m in order to determine the abundances of *P. rubescens*.

Limnological parameters and phycocyanin measurements

Secchi depth (Zs) was used to estimate water transparency with a 25cm diameter disc. Zs is used for calculating the euphotic zone (i.e. the layer receiving sufficient light for photosynthesis occurring) as Zeu = 2.5 x Zs. Water temperature, pH, conductivity, and dissolved oxygen concentration were measured in *situ* using a 3420 IDS multi-parameter (WTW, Germany). In addition, phycocyanin (PC) concentration was measured with a TriOS microFlu-blue fluorescent prob with an accuracy of 0.02 µg/LPC. The sensor is equipped with ultra-bright red LEDs, of an excitation wavelength of 620 nm, detection wavelength of 655 nm, and band-width 10 nm. Finally, the nutrient (PO_4, NO_3) analysis was performed by the National Agency of Hydraulic Resources (ANRH).

P. rubescens identification and biomass estimation

Cyanobacteria taxonomic determination took place according to Komárek and Anagnostidis (1999, 2005) by microscopic observations of morphological characteristics. *P. rubescens* abundance was determined in a Nageotte chamber using a light Axiostar Plus microscope (Carl Zeiss, Germany) equipped with a UI-1240SE camera (IDS, Germany) as described in Guellati *et al.*, 2017. Cells quantification was estimated by dividing the length of the filament by the mean lengths of the cells. The number of cells per filament is the mean of 30 filaments.

Microcystins concentration

Samples for Microcystin analyses were filtrated through a GF/C microfiber glass filter (Whatman, Germany), and then frozen. Microcystin was extracted from a 1000 mL filtered water sample in 10mL of 75% methanol for 1h. Filters were crushed then the suspension was centrifuged (10 min at 4000 rpm) and the resulting supernatant kept at room temperature for the analysis. Enzyme-linked immunosorbent Assay technique (ELISA) with a 96-well Microcystin ADDA ELISA Kit (Abraxis LLC, Warminster PA) utilized for the microcystin analyses. The ELISA quantifiable toxin range was 0.15–5 mg/L. Finally, absorbance was read at a single wave length of 450nm using a microplate photometer (MindrayMR-96A). All samples were treated using the same approach.

Results

Task force and strategies to cope with the emergency

The observation of this cyanobacterium was first reported in March 2012 by a local inhabitant of the Hammam Debagh municipality. The spectacular bloom of *P. rubescens was* observed in June 2012, since which a task force was created to cope with the emergency and tackle jointly the contamination of this reservoir, which supplies drinking water to the Guelma willaya, and irrigates the Guelma-Bouchegouf valley. This group includes the scientific team of the EMMAL laboratory, the National Agency of Dams and transfers (ANBT), the Hammam Debagh reservoir water treatment agency (ADE), the National agency of Hydraulic Resources (ANRH), and the Environment Department of Guelma. Several actions conducted by the force task including i) Several actions to reduce human exposure and food consumption (banning fishing, alternative drinking, and irrigation sources. ii) A monitoring campaign started immediately to characterize the hazard within the reservoir and in the treated water: cyanobacterium counting and toxin analysis. iii)A bank filtration was conducted to reduce *P. rubescens* biomass (Fig. 2). This can also be highly effective in removing both cyanobacterial cells and dissolved toxins (Chorus and Bartram, 2021). iv) Additional water treatment using activated carbon for example.

Change in limnological parameters and nutrient

Physico-chemical variables recorded in the subsurface water during the peak of growth period of *P. rubescens* bloom summarized in table 1. As a consequence of the bloom special conditions were observed as attested by the low water transparency not exceeding 1m combined with low dissolved oxygen concentration (min=2.63 mg L^{-1}) during the sampling period. It is interesting to show that the highest values of $P\text{-}PO_4$ in 2012 noticed on May with 0.48±0.16 mg L^{-1}. However the highest values of NO_3 recorded in February and March with 5 mg. L^{-1} (Fig. 3). A vertical profile of temperature was performed on 29.05.2012 over 33m; it shows a stratified water column ranging from 22.6°C in the upper subsurface to 9.8°C in the bottom hypolimnion. The metalimnion defined as the layer with a temperature gradient ≥0.5°C extended from 5m to 18m (Fig. 1S).

a.

b.

c.

Figure 2. *Planktothrix rubescens* surface bloom in the reservoir Hammam Debagh. a) global view of the bloom. b), c) bank filtration to reduce the cyanobacteria biomass

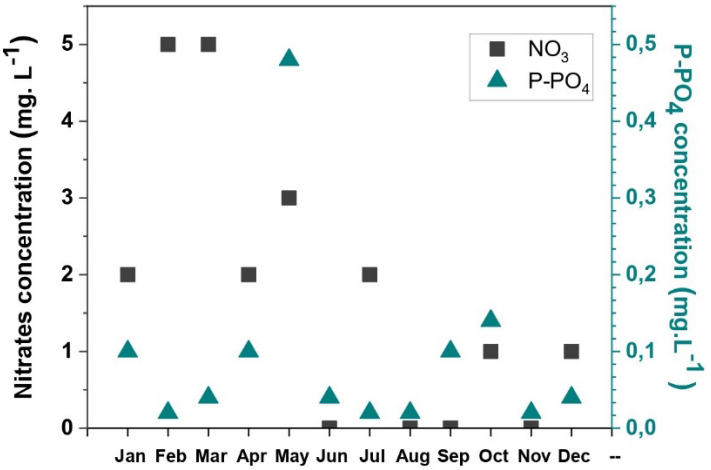

Figure 3. Concentration of biogenic compounds (NO_3, $P\text{-}PO_4$) in the water surface of the reservoir Hammam Debagh during 2012

Microscopic identification and population dynamics of P. rubescens

During the spectacular *P. rubescens* bloom a drastic reduction of green algae and diatoms diversity occurred. Maximum abundances reached 6.4 x 10^6 cells mL^{-1} in the water column as observed at 2m from the surface of the reservoir Hammam Debagh. The distribution pattern of *P. rubescens* included the entire water column being significantly lower in the hypolimnion layer with 5000-7500 cells mL^{-1} (Fig. 4 b). On the surface high abundances ranging from 3 x 10^5 to 3.6 x 10^6 cells mL^{-1} estimated respectively on the 06 June at St1 and on the 29 May in St4 (Fig. 4a). It is important to note that the senescence state of *P. rubescens* observed on 29 May and 06 June.

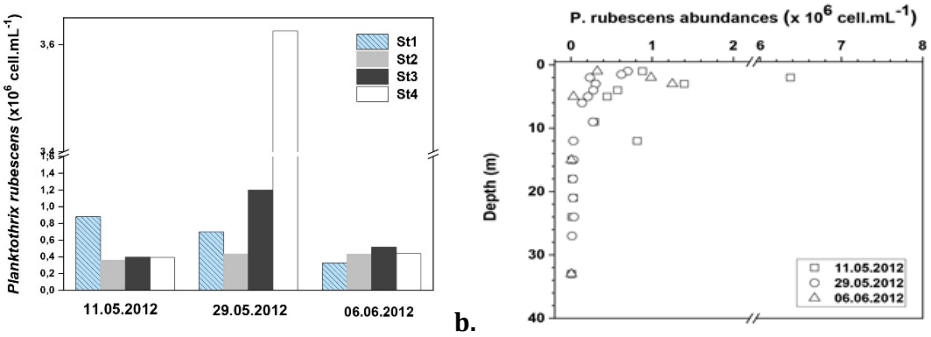

Figure 4. Variation of *P. rubescens* abundances in Hammam Debagh reservoir. a) Surface water in the four sampling stations, b) Water column in the center (St1)

Change in cyanobacterial related phycocyanin pigment

Fig. 5 demonstrates clearly the *P. rubescens* bloom dynamics in the four sampling stations over the euphotic zone. Globally, fluorescens measurements on 29.05.2012 show the highest concentration of the phycocyanin pigment. Moreover, the biomass was so high in St3 and St4 that the signal from the PC probe had become saturated in the top 1 m at a value of200 µg L^{-1} PC.

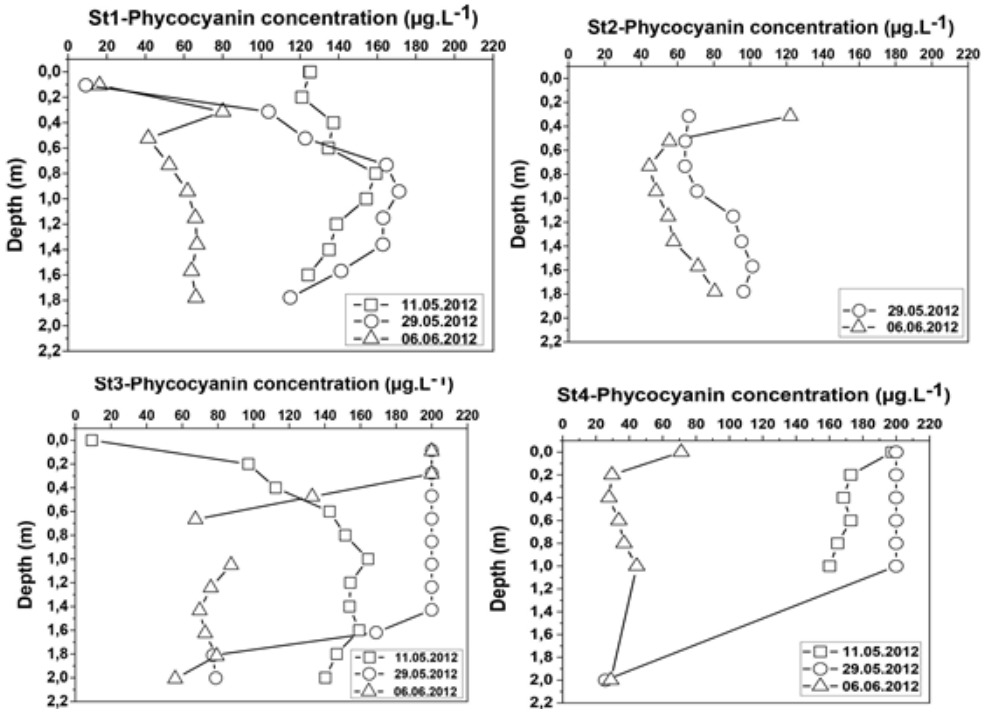

Figure 5. Phycocyanin concentration measured in the four sampling stations with fluorescent probe TRIOS Micro-Flue blue

Microcystin analysis

Intracellular Eq. MC LR concentrations detected at the water surface were below the WHO guidelines for drinking water (1 µg L^{-1}). They were only detected on 29 May with 0.45 µg L^{-1} Eq. MC-LR in St4 (data not shown). On the same date, samples taken at the water treatment agency (ADE) showed concentrations of 0.23 and 0.1 µg L^{-1} Eq. MC-LR before and after the treatment of water used for human consumption.

Discussion

The surface bloom of red colored cyanobacterium observed in the Hammam Debagh reservoir was an unexpected observation in our region. Generally, *P. rubescens* particularized as a cold water stenotherm species (Legnani *et al.*, 2005). It is known to form a metalimnetic blooms in deep-lakes located in Central and Northern Europe (Garneau *et al.*, 2013; Jann-Para *et al.*, 2004; Jacquet *et al.*, 2005; Ernst *et al.*, 2009; Fastner *et al.*, 1998; Micheletti *et al.*, 1998), and in shallow lakes in Scandinavia (Halstvedt et *al.*, 2007) and in Canada (Nürnberg *et al.*, 2003). Despite the importance of the reservoir Hammam Debagh, and in both terms of size and utilization (drinking water supply, irrigation and fisheries), there have been only a few studies of the phytoplankton. The lack of a long series of data, does not allow the identification of the process that causes the *P. rubescens* bloom. However, results from this study show strong variability in$P\text{-}PO_4$ on the water surface during 2012 with a maximum concentration of 0.48mg L^{-1} recorded in May. Similar concentrations observed in Vico Lake located in central Italy during a bloom of *P. rubescens* (Manganelli *et al.,* 2016). Hence, neither phosphorus nor nitrate (max= 5mg L^{-1} in February and March) were limited during the growth of *P. rubescents* in the reservoir Hammam Debagh*.* These findings are in accordance with previous studies which conclude that increased nutrient levels lead to *P. rubescens* surface blooms (Almodóvar *et al.* 2004; Sulis *et al.*, 2014; Trbojević *et al.*, 2019).

A study conducted from 2013 to 2015 in this monomictic reservoir showed that thermal stratification occurs from April to September. However, the overturn began in autumn (October-November) (Guellati *et al.*, 2017). It is interesting that 2012 characterized by a lowest rainfall and a snowywinter (Fig. 2S), enabling an increase of nutrients reaching the upper parts of the water column by the overturn. Our observation suggests that this cold stenotherm population resisted successfully to these conditions making an inoculum population for the following spring. Moreover, *P. rubescens* hardly grazed by herbivorous consumers, partly because of its filamentous morphology, formed by hundreds of single cells in up to 5 mm long fibers (Knapp *et al.*, 2021). In addition to good nutrient conditions and resistance to low water temperature in winter, spring 2012 was warmer; not to mention the role of "warm" conditions in spring and summer likely to enhance cell metabolism and lead to cyanobacterium bloom expansion (Moiron *et al.*, 2021; Posch *et al.*, 2012; Gallina *et al.*, 2011). Akçaalan *et al.* (2014) have demonstrated that air temperature indirectly affects cyanobacteria via the stabilization of the water column, favoring the buoyant cyanobacteria to produce more gas vesicles to float up to the surface leading to the Burgundy-blood phenomenon.

It is evident that the *P. rubescens* bloom altered environmental conditions in the reservoir Hammam Debagh, since the primary oxygen concentration shows a minimum of 2.63 mg L^{-1}. Several studies attested that during Cyanobacteria bloom, oxygen levels are low mainly due to the increased microbial decomposition of organic material (Grossart and Simon, 2007; Hoikkala *et al.*, 2016). Moreover, the surface bloom clearly affected concomitantly alkalinity and transparency (40cm in May 29) in the water column. However, despite the high abundance of *P. rubescens* attending several millions of cells per milliliter, very weak concentrations of Eq. MC-LR detected during the pic of the "Burgundy-blood phenomenon" observed in June. A study carried out in 2012 in Lake Alto Flumendosa Sardinia by Stefanelli *et al.* (2017) reported a concentration of 9.74 µg L^{-1} of total microcystins when *P. rubescens* was dominant on May 2012 with an abundance on the order of 1.3 $x10^6$ cells ml^{-1}. Most of the toxicity studies on this red filamentous cyanobacterium reported a high concentration of hepatotoxic microcystins (Humbert *et al.*, 2001; Jacquet *et al.,* 2005; Halstvedt *et al.*, 2007) with two to four variants especially MC-LR and MC-RR (Briand *et al.*, 2005; Blom *et al.,* 2001, Cerasino *et al.*, 2016; Moiron *et al.*, 2021). The absolute identification of microcystin variants among *P. rubescens* population in the reservoir Hammam Debagh would require further studies.

In addition, studies on the microcystin activated and inactivated gene (mcy) filament quotas could explain the low concentration of microcystin in this reservoir. A study performed by Kurmayer *et al.* (2004) reported that all the studied *P. rubescens* filaments have the MC synthetase genes, and that the co-occurrence of MC producers and non-MC producers were only due to the inactivation of the mcy genes. The same study reported a particular high percentage of inactive mcy genotypes found in a lake with a higher density of *P. rubescens*. Hence, the mcy genes might down regulated in these particular strains by environmental factors. Thus, the question of how environmental factors influence the toxicity of cyanobacteria remains an important challenge. In fact, several studies reported the role of nitrogen, phosphorous, trace metals, growth temperature, light and pH likely to influence the metabolic pathway of hepatotoxins production (Sivonen 1990; Neilan *et al.*, 2013). Nutrients indirectly influence the toxin production since they affect the growth rate of cyanobacteria as attested by a positive linear relationship between the microcystin content of cells and their specific growth rate. Moreover, low iron concentrations correlated with increased toxin production (Long *et al.*, 2001).

Conclusion

In this study we suggest that the low light and winter low temperature conditions favor the emergence of *Planktothrix rubescens* to constitute the inoculum population when environmental condition were better in spring (nutrient, temperature, stratified and stable water column). However, the questions on the low toxicity of *P. rubescens* despite the high abundances recorded in this reservoir need further studies particularly molecular analysis of the involvement of mcy gene in microcystin production. Finally, sampling for longer period is needed to build predictive models of water quality of the reservoir Hammam Debagh.

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Seed dormancy and germination in *Sophora secundiflora* (Fabaceae)

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Abstract. Given the ecological and horticultural significance of *Sophora secundiflora* (Ortega) DC., effective dormancy-breaking techniques are crucial for enhancing its cultivation and ensuring successful establishment in both natural and managed environments. Seeds have an extremely hard and water impermeable testa. This study evaluated the effects of mechanical scarification, sulphuric acid soaking for 30, 60 and 90 minutes and hydrogen peroxide soaking for 10 and 20 minutes on final germination percentage (FGP), mean germination time (MGT), time to 50% germination (T50) and coefficient of velocity of germination (CVG). Mechanical scarification and 60-minute sulphuric acid treatments were the most effective, achieving FGPs of 95% and 93%, respectively, and showing efficient germination processes as indicated by T50 and CVG metrics. Mechanical scarification resulted in the fastest and most consistent germination. Sulphuric acid treatments showed time-dependent efficacy, with the 60-minute treatment optimising both germination speed and percentage, whereas the 90-minute treatment caused potential seed damage, reflected in a poorer FGP. Hydrogen peroxide treatments were less effective overall, with a maximum FGP of 33% for the 20-minute soaking. Statistical analyses highlighted significant differences among

treatments, particularly for FGP ($p < 0.0001$), T50 ($p = 0.0020$) and CVG ($p = 0.0348$). These findings support the role of physical and chemical scarification in breaking dormancy in Fabaceae seeds, offering valuable insights to optimise germination protocols for *S. secundiflora* and similar species.

Keywords: coat dormancy, germination, mescal-bean, *Sophora secundiflora*, scarification.

Introduction

Dormancy and germination are crucial seed traits that are integral to the plant life cycle (Kildisheva *et al*., 2020; Nautiyal *et al*., 2023). Research indicates that 15 families of angiosperms display physical dormancy, notably within the three subfamilies of Fabaceae: Mimosoideae, Papilionoideae, and Caesalpinoideae (Baskin *et al*., 2000). Seed coat dormancy, or physical dormancy, is characterised by a hard, impermeable seed coat that inhibits the penetration of water and gases, thereby preventing the embryo from accessing the conditions necessary for germination (Bhatla and Lal, 2023).

Seed dormancy poses a considerable challenge in cultivating *Sophora secundiflora* (Ortega) DC. (Fabaceae), also known as *Dermatophyllum secundiflorum* or *Calia secundiflora*, commonly referred to as mescal-bean. This species is known for its ornamental and ecological benefits (Fu *et al*., 2016). *S. secundiflora* is an evergreen shrub or small tree native to western Texas, New Mexico, and northern Mexico (Aly *et al*., 2020). Mescal-bean typically reaches a height of up to 4 m. The plants flower in March or April, producing attractive light purple petals. The fruit is a woody pod containing several seeds. These seeds are bright orange to scarlet-red and possess very hard seed coats (Jordan, 2014). Although mescal bean can be a noxious plant on rangelands, it is frequently cultivated as an ornamental and is highly effective for rehabilitating degraded soils in arid and semi-arid regions due to its symbiotic relationship with *Rhizobium* bacteria (Correll and Johnston, 1970; Taylor and Ralphs, 2019; Oono *et al*., 2021). The genus *Sophora* (Papilionaceae) comprises 30 species with a worldwide distribution. *S. secundiflora* is considered as an excellent native plant for landscaping in Texas due to its tolerance to alkaline soils and moderate drought conditions (Niu *et al*., 2011). However, there is limited research on the seed biology of *S. secundiflora* during germination, and protocols for pretreating seeds to break seed coat dormancy are scarce. Previous studies have indicated that natural regeneration

of *S. secundiflora* is very poor, with several extrinsic and intrinsic constraints identified (Kildisheva *et al*., 2013; Ihtisham *et al*., 2021). Therefore, breaking this dormancy is essential for the successful propagation and cultivation of this species.

In the Fabaceae family, seed coat dormancy is commonly addressed through physical and chemical scarification techniques (Kildisheva *et al*., 2020; Jara-Peña and Marín-Bravo, 2023). Without pretreatment, germination can be erratic and prolonged, sometimes extending over many years (Rehmani *et al*., 2022). Therefore, artificial or natural dormancy-breaking treatments are employed to improve germination of such hard-coated seeds (Baskin and Baskin, 2020). Previous research has demonstrated the effectiveness of sulphuric acid treatments and mechanical scarification in enhancing germination by breaking down the hard seed coat. Sulphuric acid treatments chemically erode the seed coat, making it more permeable, while mechanical scarification physically disrupts the seed coat, allowing water and gases to penetrate more easily (Kheloufi *et al*., 2018; Kheloufi *et al*., 2019; Kheloufi *et al*., 2020; Mansouri and Kheloufi, 2021; Kheloufi, 2022; Mansouri and Kheloufi, 2023; Kheloufi, 2024). Another method involves the use of hydrogen peroxide to enhance the internal conditions of the seed, promoting the metabolic processes necessary for germination (Bhatla and Lal, 2023).

This study aims to evaluate the effectiveness of these pretreatment methods in breaking the dormancy of *S. secundiflora* seeds. By evaluating the relative effectiveness of these methods, We aim to provide practical recommendations to enhance the germination and propagation of *S. secundiflora*. The results from this study will contribute to the existing body of knowledge on seed dormancy in Fabaceae and offer insights into optimising germination protocols for *S. secundiflora*.

Materials and methods

Seed harvest and morphometry

Mature pods of *S. secundiflora* were collected on January 2024, from 9 shrubs located in the municipal park of Oran in North-West of Algeria (35°4’12” N, 0°38’42” W; 111.8 m a.s.l.) (Fig. 1). Seeds were stored in paper bags under standard laboratory conditions until they were used on March 2024. Seed morphological characteristics of *S. secundiflora* used in this study are presented in Table 1. The seed sample for this experiment was obtained by mixing all the collected seeds. The 1000 seeds weighed 785.7 g.

Figure 1. *Sophora secundiflora* flowers, leaves, pods, and seeds.

Table 1. Morphometric characteristics of *Sophora secundiflora* seeds (n=100).

Parameters	Mean ± SD	Minimum	Maximum
Length (cm)	1.33 ± 0.18	1.18	1.52
Width (cm)	1.11 ± 0.07	1.02	1.22
Thickness (cm)	0.96 ± 0.06	0.87	1.06
Weight (g)	0.74 ± 0.08	0.39	1.01

Experimental design

Different pretreatments were given to freshly collected seeds of *S. secundiflora* (4 replicates of 25 seeds) to break seed coat-imposed dormancy. The treatments were 1) control (untreated seeds), 2) chemical scarification of intact seeds with concentrated sulphuric acid (H_2SO_4) for 30, 60 and 90 minutes followed by washing in tap water for 5 minutes, 3) chemical scarification of intact seeds with hydrogen peroxide (H_2O_2) for 10 and 20 minutes followed by washing in tap water for 10 minutes, 4) mechanical scarification (making a small and shallow cut of the endocarp using the corner of nail clippers) (Kheloufi, 2024).

Seeds from each treatment were germinated in plastic container between two layers of moist filter paper in total darkness under 25 °C (±2 °C) for 17 days. Germination was defined as the emergence of radicle from the seed coat. As part of the experiment, it was essential to maintain a certain level of humidity for the seeds. A complete randomised design was used to conduct the germination test.

Germination Parameters

Final germination percentage (FGP): The FGP represents the total number of seeds germinated out of the total seeds. This germination parameter was calculated using the formula:

$$\text{FGP (\%)} = \frac{\sum \text{ni}}{\text{N}} \times 100 \text{ (1)}$$

where FGP is the final germination percentage, *ni* is the number of germinated seeds on the last day of the test, and *N* is the total number of seeds incubated per test (Côme, 1970).

Mean Germination Time (MGT): The MGT index showed that how fast the seeds emerged in a population. This was calculated using the following formula:

$$\text{MGT (days)} = \frac{\sum(ti.ni)}{\sum ni} \text{ (2)}$$

where MGT is the mean germination time, *ti* is the number of days since the beginning of the test, *ni* is the number of germinated seeds recorded at time *t(i)*, and *Σni* is the total number of germinated seeds (Orchard, 1977).

Time to 50% germination (T_{50}): The T_{50} was developed to find out the time required for 50% seed germination. This is reported through the following formula:

$$\text{T50 (days)} = \frac{\text{ti} + (\text{N}/2 - \text{ni})(\text{tj} - \text{ti})}{(\text{nj} - \text{ni})} \text{ (3)}$$

where N final number of seeds emerged, nj and ni are the cumulative numbers of seeds emerged after adjacent counts during tj and ti, when ni < N/2 > Nj (Coolbear *et al*., 1984).

Coefficient of the velocity of germination (CVG): The CVG represents the velocity of germination of seeds in an experiment, which will increase with an upsurge in the frequency of germinated seeds. The highest theoretical CVG value will be obtained when all sown seeds grow on the first day. This is calculated using the formula:

$$\text{CVG (\%)} = \frac{\text{N1} + \text{N2} + \text{N3} \ldots \text{Nx}}{100} \times \text{N1T1} \ldots \text{NxTx (4)}$$

in which *N* is the frequency of seeds germinating every day and *T* represents the time from sowing to germination of seed *N* (Khan *et al*., 2019).

Statistical analyses

The effects of different pretreatments on the four variables studied were tested by one-way analysis of variance (ANOVA). Differences between treatments following ANOVA were made by means comparison. Multiple comparisons of means were carried out using Tukey's test ($p < 0.05$). All statistical analyses were performed using SAS software Version 9.0 (Statistical Analysis System) (2002).

Results

Germination kinetics

Figure 2 presents an analysis of the daily cumulative germination percentages of *S. secundiflora* seeds subjected to various pretreatments aimed at breaking dormancy and enhancing germination. The treatments include different durations of soaking in sulphuric acid (SA), soaking in hydrogen peroxide (HP), and mechanical scarification (MS). The results show distinct patterns of germination responses over a 17-day period. The germination kinetics of *S. secundiflora* seeds can be interpreted by examining the cumulative germination curves over time for different pretreatments. These curves typically exhibit three distinct stages: latency, exponential growth, and plateau. Understanding these stages provides insights into the effectiveness of the dormancy-breaking treatments and the overall germination process.

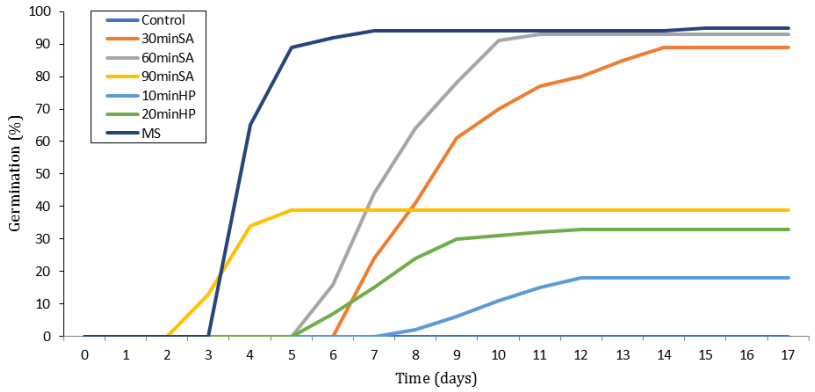

Figure 2. Cumulative germination percentages of *Sophora secundiflora* seeds after different pretreatments for 17 days. Control (untreated seeds); 30minSA (30 minutes soaking in sulphuric acid); 60minSA (60 minutes soaking in sulphuric acid); 90minSA (90 minutes soaking in sulphuric acid); 10minHP (10 minutes soaking in hydrogen peroxide); 20minHP (20 minutes soaking in hydrogen peroxide); MS (Mechanical scarification).

The initial latency stage is characterised by low to no germination. During this period, seeds are absorbing water and initiating internal biochemical processes necessary for germination but have not yet begun to sprout. The latency period varies depending on the pretreatment method. In untreated seeds (control), the latency period extends indefinitely, as no germination is observed throughout the experiment. In contrast, seeds treated with 30minSA and 60minSA show a latency period of about 6-7 days before germination begins. Mechanical scarification (MS) significantly reduces the latency period, with germination starting as early as day 4-5.

During the exponential stage, germination occurs rapidly. The seeds that have broken dormancy begin to germinate, and the cumulative germination percentage increases quickly. For the 60minSA and 30minSA treatments, this stage begins around day 7-8, with a rapid increase in germination observed until day 11-12. The MS treatment enters the exponential phase earlier and reaches near-complete germination by day 7-8, indicating a very effective dormancy-breaking process. The hydrogen peroxide treatments (10minHP and 20minHP) enter the exponential growth stage later and exhibit a slower rate of increase, reflecting their reduced efficacy in breaking seed dormancy.

The final plateau stage is characterised by a leveling off of the cumulative germination curve, where the rate of new germination decreases and the curve approaches an asymptote. This stage indicates that most viable seeds have germinated, and further increases in germination percentage are minimal. For the 60minSA treatment, the plateau of germination is reached around day 12-13 with an FGP of 93%. Germination under the 30minSA treatment reaches a plateau around day 15, with an FGP of 89%. On the other hand, germination under the MS treatment reaches its plateau the earliest, by days 9-10, with an FGP of 95%. However, germination under the 90minSA treatment reaches its plateau much earlier, around day 6, but at a reduced FGP of 39%, suggesting potential seed damage due to overexposure to sulfuric acid. Germination under the HP treatments plateaus at decreased FGPs, with 33% for the 20minHP treatment and 18% for the 10minHP treatment, by days 14-15.

Germination traits

The statistical analysis of the pretreatments for *S. secundiflora* seeds, indicated by F-values and p-values, reveals significant differences in their effectiveness. The final germination percentage (FGP) exhibited highly significant differences among treatments (F-value = 181.60, $p < 0.0001$), indicating that the pretreatments had a substantial impact on breaking seed dormancy and promoting germination. While the mean germination time (MGT) showed no significant differences (F-value = 2.46, $p = 0.0725$), the time to 50% germination

(T50) displayed significant variation (F-value = 6.00, p = 0.0020), suggesting differences in how quickly 50% of the seeds germinated across treatments. The coefficient of velocity of germination (CVG) also showed significant differences (F-value = 3.13, p = 0.0348), reflecting variability in the speed of germination among the treatments. These statistical results highlighted the importance of choosing the right pretreatment method to optimise germination, with mechanical scarification and sulphuric acid treatments being particularly effective (Tab. 2).

The control group, consisting of untreated seeds, showed no germination (FGP = 0%), highlighting that *S. secundiflora* seeds have intrinsic dormancy that requires intervention for germination to occur. Consequently, mean germination time (MGT), time to 50% germination (T50), and the coefficient of velocity of germination (CVG) were not calculated for this group (Tab. 2).

Seeds treated with 30minSA achieved a high FGP of 89%, which was statistically similar to other high-performing treatments. The mean germination time (MGT) was 6.14 days, with the time to 50% germination (T50) at 9.06 days. The coefficient of velocity of germination (CVG) was 16.3%, indicating a relatively efficient germination process (Tab. 2).

Table 2. Effects of different pretreatment on the final germination percentage (FGP), mean germination time (MGT), time to 50% germination (T_{50}) and coefficient of velocity of germination (CVG) of *Sophora secundiflora.*

Pretreatments	**FGP (%)**	**MGT (days)**	**T_{50} (days)**	**CVG (%)**
Control	0.00^{d}	NC	NC	NC
30minSA	89 ±4.83^{a}	6.14 ±0,53^{a}	9.06 ±0.46^{a}	16.3 ±1.46^{b}
60minSA	93 ±3.03^{a}	7.05 ±0.86^{a}	8.26 ±0.80^{ab}	14.3 ±1.82^{b}
90minSA	39 ±4.87^{b}	4.78 ±0.58^{a}	4.15 ±0.58^{c}	21.4 ±2.63^{ab}
10minHP	18 ±5.16^{c}	3.87 ±1.29^{a}	5.29 ±1.99^{abc}	27.7 ±6.75^{a}
20minHP	33 ±6.83^{b}	7.66 ±0.33^{a}	8.12 ±0.86^{ab}	13.1 ±0.66^{b}
MS	95 ±3.83^{a}	5.32 ±0.28^{a}	4.75 ±0.15^{bc}	18.8 ±1.03^{ab}
F-value	181.60	2.46	6.00	3.13
p-value	<0.0001	0.0725	0.0020	0.0348

The different letters in the same column indicate a significant difference at $p < 0.05$, as evaluated by Tukey's test. NC (not calculated); Control (untreated seeds); 30minSA (30 minutes soaking in sulphuric acid); 60minSA (60 minutes soaking in sulphuric acid); 90minSA (90 minutes soaking in sulphuric acid); 10minHP (10 minutes soaking in hydrogen peroxide); 20minHP (20 minutes soaking in hydrogen peroxide); MS (Mechanical scarification).

A similar pattern was observed for seeds treated with 60minSA, which had a slightly higher FGP of 93%. However, the MGT was slightly longer at 7.05 days, and the T_{50} was 8.26 days. The CVG for this treatment was 14.35%, slightly

decreased than the 30-minute treatment, suggesting a marginal decrease in germination speed despite the higher overall germination percentage. However, seeds treated with 90minSA showed a significantly poorer FGP of 39%. Despite this reduced final germination rate, these seeds had a faster germination process with an MGT of 4.78 days and a T_{50} of 4.15 days. The CVG was 21.43%, indicating a rapid initial germination phase, possibly due to a more aggressive scarification. However, the poorer FGP suggests potential seed damage from prolonged sulphuric acid exposure (Tab. 2).

Soaking seeds in 10minHP resulted in an FGP of 18%, with an MGT of 3.87 days and a T_{50} of 5.29 days. This treatment had the highest CVG of 27.7%, indicating a quick germination onset. However, the overall germination percentage remained low, signifying that while this treatment may accelerate the initiation of germination, it does not sufficiently break dormancy for a large proportion of seeds. Extending the soaking time in 20minHP improved the FGP to 33%, with an MGT of 7.66 days and a T_{50} of 8.12 days. The CVG was reduced to 13.1%, indicating a slower germination process compared to the 10-minute treatment (Tab. 2).

Mechanical scarification was the most effective treatment, achieving the highest FGP of 95%, which was statistically similar to the 60minSA treatment. The MGT was 5.32 days, and the T50 was 4.75 days. The CVG was 18.8%, indicating a relatively efficient germination process. Mechanical scarification effectively breaks seed dormancy by physically damaging the seed coat, allowing for rapid and high-percentage germination (Tab. 2).

Discussion

Seed surface morphology reveals that a hard and impermeable testa is the primary barrier to imbibition, consequently delaying germination (Lamont and Pausas, 2023). Studies on *S. secundiflora* seeds demonstrate the effectiveness of various pretreatments in breaking seed dormancy and improving germination. These findings are consistent with general observations in the Fabaceae family, where physical and chemical scarification are commonly used to enhance germination (Kheloufi, 2020; Jaganathan and Biddick, 2021). Pretreatments such as soaking in sulphuric acid, mechanical scarification, and soaking in hydrogen peroxide have shown varying degrees of success in breaking seed coat dormancy and promoting germination (Nautiyal *et al*., 2023).

Sulphuric acid treatments significantly improved germination rates, with the 60-minute treatment showing the highest final germination percentage at 93%. This method is effective because sulphuric acid breaks down the hard seed coat, allowing water to penetrate and initiate the germination process. The time-dependent nature of SA is evident, as the 90-minute treatment resulted in

a reduced FGP (39%), possibly due to overexposure causing seed damage. These results are consistent with other studies on Fabaceae species. Kheloufi (2022) showed similar improvements in germination with sulphuric acid treatments. Additionally, Baskin and Baskin (1998) noted that sulphuric acid is a commonly used method to overcome physical dormancy in seeds with hard seed coats. Kheloufi *et al.* (2018) reported that treating seeds with sulphuric acid removed some or all of the cuticular layer, resulting in rapid germination.

Mechanical scarification was the most effective overall, achieving an FGP of 95%. This method directly damages the seed coat, facilitating water uptake and germination. The rapid beginning and high percentage of germination align with findings in other Fabaceae species, where physical scarification has been shown to effectively break seed dormancy (Tang *et al*., 2022).

Treatments with hydrogen peroxide were less effective than SA and MS. The 10-minute soaking resulted in a low FGP of 18%, while extending the soaking to 20 minutes increased FGP to 33%. This method appears to be less efficient in breaking seed dormancy, likely due to insufficient physical or chemical disruption of the seed coat. Similar observations have been made in other Fabaceae species, where hydrogen peroxide treatments alone were less effective compared to chemical or mechanical scarification (Sirkeck and Singh, 2023). Moreover, Kheloufi (2022) found that hydrogen peroxide soaking did not significantly enhance germination in *Acacia* species, highlighting the necessity for more aggressive scarification methods.

According to Wang (1991), untreated fresh seeds of *S. secundiflora* required 10 weeks to achieve 50% germination, whereas untreated one-year-old seeds exhibited only 8% germination by the end of the experiment. Additionally, mechanical scarification did not significantly enhance the germination of fresh seeds, with a germination rate of only 42%.

The development of a very hard seed coat in its fully desiccated state is typically attributed to the substantial presence of heavily thickened galactomannan or mannan polymers lining the endosperm cell walls (Steinbrecher and Leubner-Metzger, 2017). Within the endosperm, the presence of hydrophilic galactomannan leads to a mucilaginous transformation upon imbuing and subsequent hydrolysis (Zandi *et al*., 2015). While impermeability of the seed coat and its mechanisms for delaying germination are common traits among legumes, they serve to delay seed germination under adverse environmental conditions (Naik and Deshpande, 2021). The strength of the seed coat and also the endocarp helps in safeguarding the seeds against mechanical harm, facilitating their survival in arid soils during droughts, or enabling natural dispersion and recolonization following fire events (Fenner, 2017; Shiferaw *et al*., 2018; Dalling *et al*., 2020; Kheloufi, 2024).

Conclusions

This study demonstrates that mechanical scarification (MS) is the most effective pretreatment for breaking seed dormancy and enhancing germination in *S. secundiflora*, achieving a final germination percentage (FGP) of 95%. This method outperformed all other treatments, including various durations of sulphuric acid soaking and hydrogen peroxide treatments, in both germination percentage and efficiency metrics. Sulphuric acid treatments, particularly the 60-minute soak, also showed high effectiveness with an FGP of 93%, but the mechanical scarification provided the fastest and most consistent results. These findings highlight the critical role of seed coat disruption in overcoming dormancy for *S. secundiflora*. The statistical significance of the results enhances the reliability of these methods, highlighting their practical applicability in improving germination outcomes. Researchers and horticulturists working with *S. secundiflora* or similar hard-seeded species in the Fabaceae family should consider mechanical scarification as the primary method for optimising germination.

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The effects of water stress and plant density on vegetative and reproductive characteristics of safflower in the semi-arid region

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Abstract. The limited availability of water for irrigation in semi-arid areas necessitates the use of deficit irrigation techniques. Deficit irrigation is an irrigation practice whereby water supply is reduced below maximum levels and a mild water-deficit stress affect the crop yield. Current field experiment was aimed to investigate different densities of safflower (*Carthamus tinctorius* L.) (33, 40, 50, 66 plants m^2 abbreviated as P_{33}, P_{40}, $P_{50,}$ and P_{66}, respectively) under different soil moisture content (irrigation up to 100%, 70%, and 50% field capacity showed as FC_{100}, $FC_{70,}$ and FC_{50}, respectively) on the growth characteristics of safflower in a semi-arid region in the northwest of Iran. Number of days to maturity decreased significantly under deficit irrigation (FC_{70} and FC_{50}). The longest growth period was recorded for plants grown under P_{66}-FC_{100} conditions which was 7% higher than P_{50}-FC_{100}. The decrease in plant density caused an increase in chlorophyll content and this trend was more evident under Fc_{100}. Increasing the density under FC_{100} conditions increased the plant height. However, this trend was not observed under deficit irrigation conditions. Decreasing the plant density per unit area induced the lateral growth, and increased the number of capitula per plant, the weight of capitulum, the number of achenes per capitulum and the 1000-achenes weight. This trend was more prominent under FC_{100} conditions. Mild

deficit-irrigation (irrigation up to FC_{70}) resulted approximately 20% reduction in achene yield. Thus, water management is very important to certify a conservational water supply in semi-arid regions. Irrigation up to FC_{70} under the studied area is a reasonable and good management method to save irrigation water.

Keywords: *Carthamus tinctorius* L., chlorophyll, deficit-irrigation, field capacity, phenology.

Introduction

Safflower (*Carthumus tinctorious* L.) is an annual xerophilous plant from the Asteraceae family and native to semi-arid Mediterranean regions. Ancient surveys show that safflower is one the oldest crops and its cultivation dates back to the 12th century (Emongor, 2010). Although safflower is equipped to some characteristics such as deep root system and osmolytes accumulation to adapt to semi-arid areas, this plant sometimes classified as the forgotten or underutilized crops due to the lack of sufficient information about agronomic practices and slow breeding trends (La Bella *et al*., 2018). Global warming and the occurrence of dry-spell in the growth season have been exacerbated by climate change during the recent decades, and this emphasizes the identification and use of drought-resistant plants in crop rotations (Janmohammadi and Sabaghnia, 2023a). Safflower has unique characteristics such as qualitative oil, forage, medicinal properties and it should be specifically considered in crop rotation in its original ecological zone (Kizil *et al*., 2008; Abou Chehade *et al*., 2022). Safflower is well adapted to crop rotation design, especially with cereals, and safflower insertion in rotation with cereals as dominant crops of semi-arid regions can break the lifecycle of cereal root diseases and improve ecological biodiversity (Hertel, 2016).

Adjusting the plant density is one of the most important factors influencing the achievement of the genetic potential of yield per unit area. However, the optimum plant density can be influenced by many other factors such as environmental conditions (soil type, depth of topsoil, readily available moisture, soil fertility, land slope, height above the sea level, air and soil temperatures, length of growth period), production systems and cultivar characteristics (Özaşık *et al*., 2019). The robust structure and special anatomy of safflower to produce fertile lateral branches under low plant densities are among the things that should be considered in planting decisions. However, there is a compensatory

state between the achene yield components, and under the high plant densities, some flowers may remain sterile or produce low or smaller achenes (Elfadl *et al*., 2009). However, there is still no comprehensive information about the response of safflower to plant densities and how to determine the optimal plant density.

On the other hand, the climate changes that happened during the last decades have aggravated the water shortage situation and the limitations of water resources prevailing in semi-arid regions (Janmohammadi and Sabaghnia 2023a). Therefore, the optimal use of available water resources and increasing the efficiency of water consumption are highly emphasized. Response of achene yield of safflower to plant densities is different in various soil moisture conditions. Deficit irrigation is a method of irrigation with predetermined less than usual volume of water, in which it is tried to improve the efficiency of water consumption (Pasandi *et al*., 2014). Abd El-Lattief (2013) reported that the l*ow*-density plantings under deficit irrigation could produce an acceptable yield of safflower in the semi-arid region. High water consumption efficiency can be achieved by using precise deficit irrigation methods with a slight drop in seed yield. Sefaoğlu and Özer (2022) indicated that in planting safflower at different inter-row distances (20, 40, and 60 cm) along with various amounts of seed used (20, 40, and 60 kg ha^{-1}) under fully irrigated conditions, the highest seed yield and oil content were obtained at high plant densities with low inter-row spacing. Also, increasing the density of safflower increased the leaf area duration (LAD), chlorophyll content, and increased the length of the development period (Moatshe *et al*., 2020). Therefore, assessment of safflower response to water shortage and different planting densities can be very advantageous for in semi-arid areas. The present experiment aimed to evaluate the effect of planting density and different irrigation levels on the growth, agronomic, phenological characteristics, and yield components of safflower in the semi-arid region in the northwest of Iran.

Materials and methods

Site description

The present experiment was carried out in the research farm of the Faculty of Agriculture, University of Maragheh, Maragheh, in North West of Iran (latitude 37°23' N, longitude 46°16' E and, height from sea level 1485 m) during the growing season of 2021-2022. Based on the Köppen-Geiger climate grouping, the region is cold and semi-arid in terms of climate and has predominant winter and spring rains (early and middle months). Some metrological characteristics during the safflower growing season in the Maragheh region in northwest Iran are shown in Tab. 1. Soil sampling was performed in the experimental field to determine

its physicochemical characteristics. The soil texture was clay loam consisting 41% clay, 37% silt and 22% sand. Some chemical soil characteristics were: pH= 7.51, electrical conductivity (EC) =1.14 dsm^{-1}, organic matter = 0.69 g kg^{-1}, nitrogen (N) = 0.082%, available phosphorus = 14.21 mg kg^{-1} and available potassium (K) = 320 mg kg^{-1}. The amount of annual potential evaporation and transpiration in the studied area was 1375 mm.

Soil preparation and application of farmyard manure

The primary tillage was done by a moldboard plow on November 2021, then 15 t $ha^{-1}$of rotted farmyard manure was integrated with the topsoil by a disk and a rotary harrow. At the time of planting, 90 kg ha^{-1} superphosphate and 50 of urea kg ha^{-1} were utilized. In early March, secondary plowing was done and finally, ridging operations was done using the Mini Tiller-furrower. In order to prevent the loss of moisture stored in the final plowed soil or to prepare the seed bed, the minimum amount of soil was turned over. The experiment was conducted as a complete factorial experiment in split-plots with three replications. In this experiment, the effects of two factors (plant density and irrigation regimes) were investigated. Plant densities in four levels were: 33, 40, 50 and 66 plant m^2 (abbreviated as P_{33}, P_{40}, P_{50}, and P_{60}, respectively) which was created through various inter-row planting distances (30, 40, 50 and 60 cm). Irrigation regimes in three levels were: water supply up to 100%, 70% and 50% field capacity (abbreviated as FC_{100}, FC_{70} and FC_{50}, respectively). The main plots were allocated to different plant densities (P_{33}, P_{40}, P_{50}, and P_{60}). Sub-plots were assigned to irrigation regimes (FC_{100}, FC_{70}, and FC_{50}). Each experimental unit (secondary plot) had an area of 16 m^2 (4×4m) with 6-13 planting rows according to the different inter-row spacing. After completing the preparation of seedbed safflower achenes (Cv. Saffeh) were manually planted on an intra-row spacing of 5 cm at the depth of 3 cm on March 9. Immediately after planting, all plots were fully irrigated uniformly up to the field capacity.

Irrigation treatments

The following formula was used to calculate the water required to reach the field capacity (Hasanuzzaman *et al.,* 2016). RI= (SFC × SM) ×BD × RD. Where RI is the required irrigation (mm), SFC: is the selected field capacity percentage, SM: is soil moisture content before irrigation (through gravimetric method), BD: is soil bulk density g cm^{-3}, RD: is the rooting depth (Average root development in the soil of the studied area). Soil water content was determined by a Time

Domain Reflectometry sensor (TDR 200, Campbell Scientific, Inc. USA) in three-day intervals. A pressure plate applied for measuring the soil moisture in suctions between 0.3 -15 bar and data were used for determine the irrigation schedules. A drip irrigation system with mainline polyethylene pipes was applied for irrigation. Irrigation conveyance and distribution efficiency was 95%. All treatments were irrigated primarily with an identical quantity of water (FC100) for three weeks to ensure ideal seed germination and seedling establishment. The irrigation was performed when half of the available water was depleted compared to the first days of the previous irrigation in each soil moisture condition. In order to prevent moisture leakage between the plots, one meter of margin was left uncultivated. Except for irrigation and plant density treatments, other agronomic managements were implemented uniformly on all experimental plots. Weed control was done manually at the same time as thinning and also during other growth stages.

Plant growth measurements

Monitoring the stages of plant growth was done regularly with daily inspection of the field. The stages of plant development were recorded based on reaching 70% of the plants in each plot to each stage of development (Flemmer *et al.*, 2015). The number of days to reach physiological maturity (BBCH: 87, 70% of the capitulum area yellow: fruits reach physiological maturity) was calculated by regularly visiting the field and counting the days from planting date. In the physiological maturity stage, the height of the ten plants was randomly measured using a meter from the soil surface to the tip of the main capitulum. Measurement of chlorophyll was measured through the portable chlorophyll meter (SPAD 502, Minolta, Japan) in the leaves above the canopy at the end stages of the development of the main capitulum at the top of the main shoot (BBCH scale= 61). The percentage of ground coverage by canopy was obtained visually by evaluating the amount of visible ground from the top of the canopy in each experimental unit. Ground cover was estimated during the final stages of reproductive growth when capitulum and fruits reach final size (BBCH scale= 79). Measurement of the diameter of the stem was performed in the early reproductive stages (capitulum emergence at the main stem) with a caliper. In the physiological maturity stage, after removing the marginal effects, 10 plants were randomly selected from the middle parts of the experimental plots, and the agronomic characteristics and yield components such as the number of capitula per plant, the mean diameter of the capitulum, achene number per main and secondary capitula, weight of capitula, thousand achene weight, achene number per secondary capitula, achene yield, and biological yield. Biological yield was determined by arbitrarily placing a 100 cm quadrat in central parts of each of the experimental units. After determining the harvesting area with quadrat, the plants

were harvested and dried in oven (72 °*C* for 48h), and the dry weight of the whole plant and the weight of the seeds were weighed. The harvest index was obtained through the ratio of achene yield to biological yield per unit area. The gathered data of evaluated traits were evaluated in terms of uniformity with Kolmogorov Smirnov and Bartlett's tests.

Statistical data analysis

Data statistically analyzed based on linear model procedure for a split-split plot design by SAS software (version 9.4). Means were separated using least significant differences test at 95% level of probability. Principal component analysis (PCA) and related statistic and graph provided by Minitab software (version 19.2). Box plots were drawn with Statistica software (version 13.0) to compare the average mutual effects of irrigation and plant density.

Results

The evaluation of meteorological data showed that with the progress of plant development stages and approaching the summer season, the amount of precipitation decreased significantly, and on the other hand, the amount of actual evapotranspiration increased (Tab. 1).

Table 1. Meteorological characteristics during the safflower growing season in Maragheh region in northwest Iran

Parameter	Jan	Feb	Mar	Apr	May	Jun	July	Aug
Maximum temperature (C°)	2.1	9.9	15.9	24.7	30.5	35.3	37.4	32.2
Minimum temperature (C°)	-5.7	-0.3	5.2	11.4	15.3	21.7	22.5	19.7
Air humidity (%)	68	63.4	46.3	41.5	42.8	27.5	32.4	26.4
Precipitation (mm)	21.6	9.1	34.9	10.7	1.2	0.5	0.2	%0.0
Actual evapotranspiration (mm)	27.41	35.24	56.72	78.86	98.49	108.51	127.41	134.65

Evaluation of growth characteristics

The evaluation of one of the phenological characteristics of the plant, i.e. the number of days to reach physiological maturity, showed that the mutual effects of irrigation regime and plant density on this component are significant ($p \leq 0.05$). The longest growth period for plants grown under favorable conditions of irrigation (FC_{100}) and high density (P_{66}) was recorded with about 151 days. However, reducing the density under optimal moisture conditions ($FC1_{00}$-P_{33})

reduced the number of days to maturity by 22 days. The shortest length of the growth period was recorded in FC_{50} conditions and no difference in days to maturity was observed between different planting densities. In FC_{70} irrigation conditions, reducing the density from P_{66} to P_{33} accelerated the number of days to maturity by about 14% (Tab. 2).

Table 2. Effect of different soil moisture regimes and plant density (inter-row spacing) on morphological and growth characteristics of safflower (*Carthamus tinctorius* L.) in the semi-arid area of Iran.

	Treatments	DTM	PH	TBN	SD	GCP	BBY
Soil moisture regimes	FC_{100}	137.66^{a}	72.66^{a}	30.75^{a}	11.59^{a}	52.20^{a}	4662.40^{a}
	FC_{70}	118.91^{b}	63.41^{b}	22.50^{b}	10.30^{b}	43.80^{b}	3613.92^{b}
	FC_{50}	102.34^{c}	49.44^{c}	16.66^{c}	10.29^{b}	34.40^{c}	2981.25^{c}
Plant density	P_{33}	127.17^{a}	58.44^{c}	18.88^{d}	8.36^{d}	38.66^{d}	3313.33^{d}
	P_{40}	121.73^{b}	59.72^{bc}	22.22^{c}	10.16^{c}	42.26^{c}	3525.04^{c}
	P_{50}	117.60^{c}	63.26^{ab}	24.77^{b}	11.32^{b}	44.80^{b}	3790.84^{b}
	P_{66}	112.04^{d}	65.93^{a}	27.33^{a}	13.06^{a}	48.12^{a}	4380.87^{a}
		Statistical significance					
	S	**	**	**	ns	*	**
	P	**	**	**	**	**	**
	S*P	**	*	ns	ns	ns	*
	CV	6.01	9.88	7.34	10.14	4.62	4.27

FC: Irrigation amount according to field capacity, P_{33}-P_{66}: Plant density per square meter with 30 cm to 60 cm inter-row spacing. DTM: day to maturity, PH: plant height (cm), TBN: total branch numbers, SD: stem dimeter (mm), GCP: ground cover percentage by plant canopy, BBY: biological weight (kg ha^{-1}). CV: coefficient of variation. Values in a column with the same letter (s) do not have a statistically significant difference, whereas values with dissimilar letters are statistically different. ns = not significant, * = significant at 5% level of probability, ** = significant at 1% level of probability.

The tallest plants were recorded in FC_{100} P_{66} conditions and reducing the density by half (P_{33}) caused a 24% decrease in plant height. The reducing effect of density on the height was clear only under favorable irrigation conditions, and under deficit irrigation conditions (FC_{50} and FC_{70}), the effect of density on the height was not significant. Only the adoption of high planting densities in the conditions of moderate irrigation ($FC_{70}P_{66}$) caused a slight increase in the

height of the plant. With the decrease in density, the height of the first capitulum on the plant from the ground level also decreased.

The lowest number of lateral branches was recorded in $FC_{50}P_{66}$ with 13 branches. Reduction of density in all irrigation regimes reduced the number of lateral branches. The highest number of side branches (35 branches) was recorded under $FC_{100}P_{33}$ conditions. The effect density on the number of side branches was more evident under favorable irrigation conditions (Tab. 2). Reducing density from P_{66} to P_{33} increased the number of lateral branches by 44%.

The evaluation of the stem diameter showed that this vegetative component was not significantly affected by the irrigation regime. However, the increase in plant density per unit area greatly reduced the stem diameter, the lowest stem diameter was under $P_{66}FC_{50}$ conditions and $P_{66}FC_{70}$ and the maximum stem diameter was recorded in $FC_{100}P_{33}$ conditions.

The evaluation of the chlorophyll content showed that the decreasing the soil moisture caused a significant decrease in this pigment. A 30% and 50% reduction in irrigation resulted in a 15% and 29% decrease in the chlorophyll content, respectively (Fig. 1). On the other hand, decreasing the density from P_{66} to P_{50}, P_{40} and P_{33} increased chlorophyll by 3%, 12% and 8%, respectively. The highest amount of chlorophyll was recorded under $FC1_{00}$ P_{40}, while the plants grown in FC_{50} P_{66} showed the lowest amount of chlorophyll.

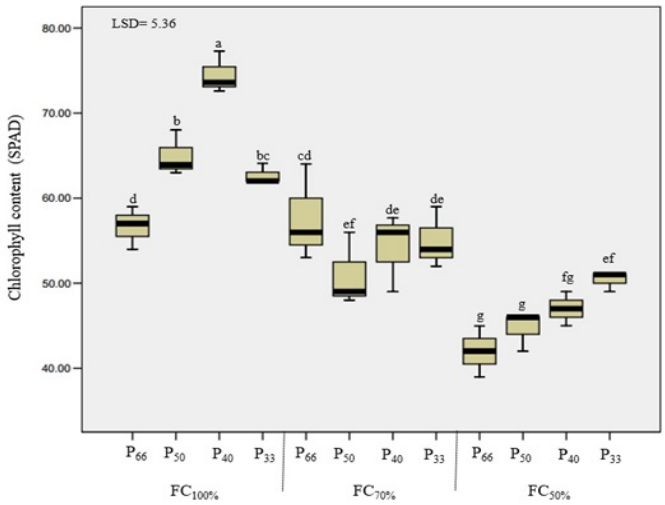

Figure 1. Mean comparison of chlorophyll content of the upper leaves of safflower plants cultivated in different densities and under different soil moisture regimes in the semi-arid region in the northwest of Iran. In each box, the horizontal dashed line represents the mean. FC: Irrigation amount according to field capacity, P_{33}-P_{66}: Plant density per square meter with 30 cm to 60 cm inter-row spacing. Boxes with different letters have statistically significant differences ($p<0.05$). *LSD:* least significant difference.

Estimation of ground canopy cover showed that the mutual effects of irrigation regime and density on this trait was significant at the statistical level of 5%. The highest ground canopy cover was recorded under FC_{100} and P_{66} conditions. The lowest amount of ground canopy cover was observed in densities P33 and P40 under FC_{50} (38%). Although the increase in the density increased the ground canopy cover, this trend was more noticeable under favorable irrigation conditions.

The evaluation of biological yield showed that a 30% and 50% reduction in irrigation resulted in a 23% and 36% decrease in the biological yield, respectively. The trend of decreasing biological yield with decreasing density was more evident in FC_{70} conditions. The biological yield under FC_{70} conditions decreased from 4216 kg ha^{-1} to 3221 kg ha^{-1} by decrease of plant density from P_{66} to P_{33}.

Achene yield components

The effects of soil mositure regimes and plant densities are summerized in Tab. 3.

Table 3. Impact of water stress and different inter-row spacing on achene yield component of safflower (*Carthamus tinctorius* L.) in northwest of Iran.

	Treatments	TCN	TCW	ANM	ANS	CMD	HI
Soil moisture regimes	FC_{100}	30.33^{a}	48.87^{a}	354.83^{a}	260.40^{a}	2.82^{a}	22.31^{a}
	FC_{70}	23.33^{b}	34.34^{b}	301.00^{b}	210.60^{b}	2.46^{b}	20.17^{b}
	FC_{50}	17.00^{c}	25.22^{c}	193.73^{c}	136.94^{c}	2.00^{c}	21.52^{a}
Plant density	P_{33}	29.44^{a}	42.03^{a}	314.66^{a}	237.77^{a}	2.67^{a}	20.18^{a}
	P_{40}	24.00^{b}	39.28^{a}	293.85^{b}	209.92^{b}	2.44^{b}	20.18^{a}
	P_{50}	22.11^{c}	34.37^{b}	275.90^{c}	200.97^{b}	2.40^{b}	21.67^{a}
	P_{66}	18.66^{d}	28.88^{c}	248.33^{d}	161.91^{c}	2.44^{b}	21.66^{a}
		Statistical significance					
	S	**	**	**	**	**	**
	P	**	**	**	**	**	Ns
	S*P	*	ns	ns	ns	**	**
	CV	6.43	8.67	5.51	6.92	3.77	5.44

FC: Irrigation amount according to field capacity, P_{33}-P_{66}: Plant density per square meter with 30 cm to 60 cm inter-row spacing. TCN: total number of capitula per plant, TCW: weight of capitula per plant (g), ANM: achene number per main capitula, ANS: achene number per secondary capitula, CMD: diameter of capitulum (cm), HI: harvest index (%). *CV:* coefficient of variation. Values in a column with the same letter (s) do not have a statistically significant difference, whereas values with dissimilar letters are statistically different. ns = not significant, * = significant at 5% level of probability, ** = significant at 1% level of probability.

The number of capitulum per plant (TNC) decreased with the decline in soil moisture, so that with a 30% and 50% decrease in water supply compared to FC_{100}, TNC decreased by 23% and 44%. Although the reduction of plant density per unit area in all irrigation regimes increased number of capitula per plant, this increase was significant in FC 100 conditions (Tab. 3). The highest number of capitula per plant was recorded under FC_{100} P_{33} (38.26) and the lowest number under FC_{50} P_{66} (12.60). A relatively similar trend was observed for the weight of the capitulum, and the decrease of 30% and 50% of irrigation compared to FC_{100} caused a decrease of 30% and 48% of the weight of the capitulum. Seed planting in rows with 60 cm intervals (P_{33}) increased the capitulum weight by 45% compared to dense planting at 30cm row spacing (P_{66}). Total achene number (TAN) in the plant was strongly affected by irrigation regimes and under FC_{70} and FC_{50} conditions it decreased by 17% and 46% compared to optimal irrigation conditions. On the other hand, with a decrease in plant density from P_{66} to P_{50}, P_{40} and P_{33} TAN showed an increase of 16%, 22.7% and 34% respectively. The highest TAN was recorded under FC_{100} P_{33} with 689 achenes and the lowest TAN was related to plants grown under FC_{50} P_{66} with 268.2 achenes (Fig. 2).

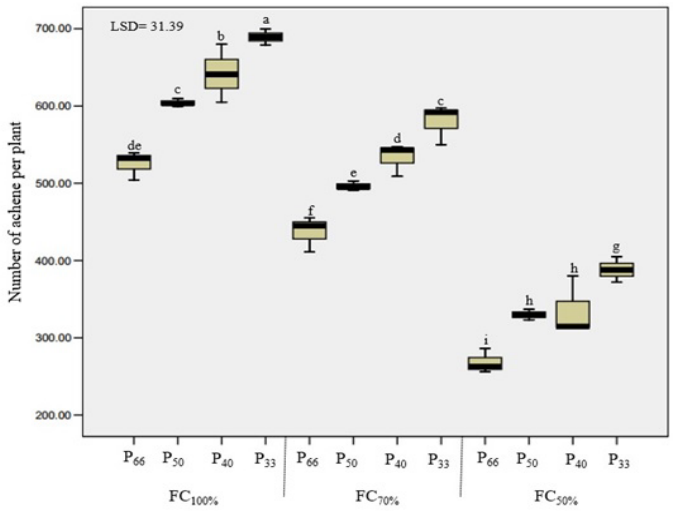

Figure 2. The effect of different safflower planting densities and different irrigation regimes on number of achenes per plant. FC: Irrigation amount according to field capacity, P_{33}-P_{66}: Plant density per square meter with 30 cm to 60 cm inter-row spacing. Boxes with different letters have statistically significant differences ($p<0.05$*). LSD:* least significant difference.

The mutual effects of irrigation and density were significant at the 1% level on the capitulum diameter. In all moisture regimes, planting in wide rows (P33) significantly increased the capitulum diameter. under FC_{100} conditions, the lowest capitulum diameter was recorded in P_{66} (2.41 cm) and reducing plant density to P_{50}, P_{40}, and P_{33} increased capitulum diameter by 19%, 23%, and 29%. However, plants grown under deficit irrigation conditions showed the

significant increase in capitulum diameter only at very low plant densities (P_{33}). The investigation of thousand achenes weight (TAW) indicated a significant effect of irrigation regime on this component. A decrease of 30% and 50% of irrigation compared to optimal irrigation conditions led to a decrease of 12% and 21% of TAW. The lowest amount of TAW was recorded under severe deficit irrigation (FC_{50}) and the highest amount of TAW was recorded under FC_{100} with densities of P_{33} and P_{40} (Fig. 3).

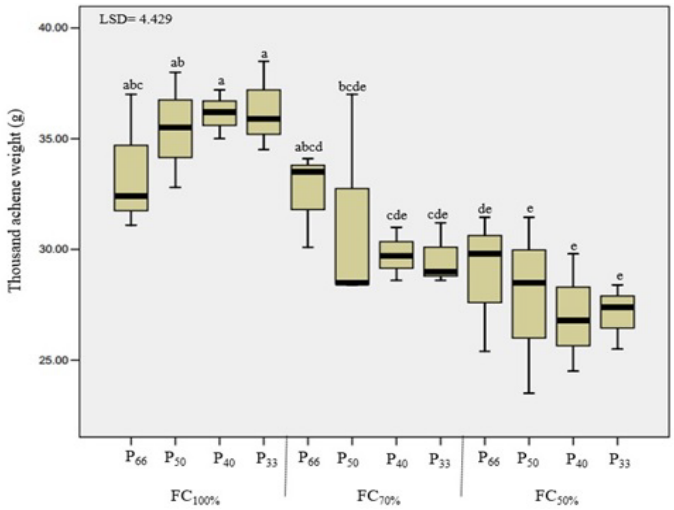

Figure 3. investigating the effect of different planting densities under different soil moisture conditions on the 1000- achene weight of safflower in the semi-arid region of northwestern Iran. FC: Irrigation amount according to field capacity, P_{33}-P_{66}: Plant density per square meter with 30 cm to 60 cm inter-row spacing. Boxes with different letters have statistically significant differences ($p<0.05$*). LSD:* least significant difference.

Achene yield per unit area was strongly influenced by the investigated treatments and the mutual effects of irrigation regime and plant density were significant on this trait. With a 30% and 50% decrease in irrigation water supply, achene yield decreased by 29% and 38%. However, the increase in plant density significantly increased achene yield only under FC_{100} and FC_{70} conditions. Reducing the plant density up to P_{33} under optimal irrigation conditions caused a 25% decrease in achene yield, while planting in low plant densities under FC_{70} decreased achene yield by 33%. However, the reduction of plant density under severe deficit irrigation reduced achene yield by 21%, but no difference between achene yield was observed between P_{33}, P_{40} and P_{50} densities (Fig. 4).

Principal component analysis (PCA) provided the possibility of summarizing the data and the first component was able to distinguish the irrigation regimes from each other and the best safflower performance was obtained under the

optimal moisture condition (FC_{100}) and under mild deficit irrigation (FC_{70}). On the other hand, the second component was able to distinguish the high plant densities (P_{66} and P_{50}) that produced the highest achene yield (Fig. 5). The results of PCA showed that high densities even under mild deficit irrigation conditions can be a suitable solution for acceptable safflower production in a semi-arid region.

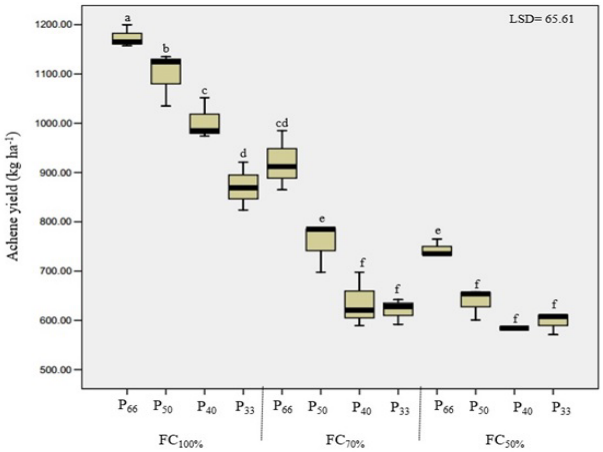

Figure 4. The effect of different planting densities and different irrigation regimes on safflower achene yield. FC: Irrigation amount according to field capacity, P_{33}-P_{66}: Plant density per square meter with 30 cm to 60 cm inter-row spacing. Boxes with different letters have statistically significant differences ($p<0.05$*). LSD:* least significant difference.

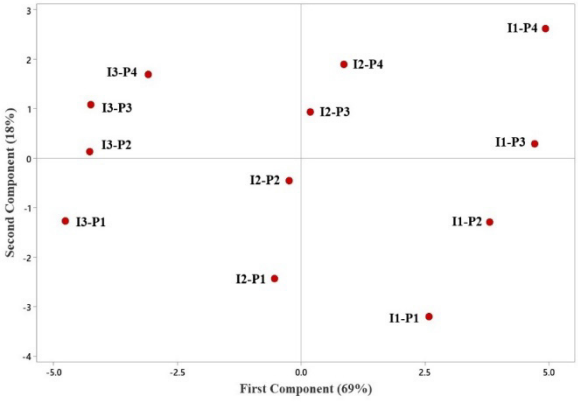

Figure 5. Plot of the first two principal component (PC) scores, PC1 vs. PC2 related to the distribution of different combined treatments (plant density and soil moisture regime) and their similarity in influencing the evaluated agronomic traits. FC: Irrigation amount according to a proportion of field capacity, P33-P66*:* Plant density per square meter with 30 cm to 60 cm inter-row spacing

Discussion

The trend of temperature changes and the amount of precipitation during the growing season in the studied area indicates that the amount of soil moisture loss through evaporation and transpiration is very high. In such a situation, it seems that rainfed farming cannot have high success and the use of irrigation is necessary to achieve acceptable yields. Although safflower is a plant with relatively good adaptability to water deficit conditions and can guarantee its survival and production by self-regulating its growth in different densities, the results obtained in this experiment showed that it was strongly affected by moisture conditions and densities. The low quality of the soil such as low permeability and low water holding capacity due to the low organic matter of the soil and the application of improper crop rotations and tillage systems as well as the intensification of climate change in semi-arid areas are among the possible reasons for strong response of safflower to moisture conditions and plant density in the mentioned area (Janmohammadi and Sabaghnia, 2023a).

Plant phenology was affected by irrigation regimes and densities. The length of the plant growth period can be strongly influenced by environmental conditions (temperature, humidity, soil condition, length of the growing season in the region, etc.) and plant density (Calıskan and Calıskan, 2018). Under favorable environmental conditions, increasing the density can accelerate flowering and maturity by increasing the speed of growth and development (Moatshe *et al*., 2020). However, it appears that the moisture content of the soil in mild and severe deficit irrigation conditions (FC_{70} and FC_{50}) due to the continuation of deficit irrigation conditions and lack of sufficient rainfall during the growth period has largely become yield-reducing drought stress. Response of yield components to water stress were somewhat different. Studies have shown that there is a tight correlation between the number of capitula and achene yield (Janmohammadi *et al*., 2016; Sefaoğlu and Özer, 2022; Janmohammadi and Sabaghnia, 2023; Fatthi *et al*., 2024). However, increasing plant density seems to have several pros and cons depending on environmental conditions. In the present experiment, the number of lateral branches and the number of capitula increased significantly by reducing the plant density per unit area. However, due to the presence of negative and compensatory relationships between yield components and the limited capacity of the plant to produce capitula, the increase of some of the plant components in the conditions of low densities could not compensate the low plant numbers and the yield did not show a significant increase. Contrary to the findings of Ehsanzadeh and Zareian Baghdad-Abadi (2003) we did not find any improving effects from low density. These researchers reported that the use of low densities of 16 and 22 plants m^{-2} through 50 cm inter-

row spacing and 9-12 cm intra-row spacing under well-irrigated conditions in the semi-arid region showed the best performance compared to high densities caused by the reduction of intra-row spacing. In any case, according to the humidity limits and soil conditions of the studied area, increasing the number of plants in the intra-row can increase intra-species competition. One of the important factors affecting the performance of achene is the leaf surface index. Usually, leaf area index of about 3-5 is necessary to produce maximum dry matter (Steberl *et al*., 2019). The obtained results showed that high planting densities in the FC_{100} and FC_{70} irrigation regimes could increase the ground cover by the canopy, and this can be attributed to the increase in the leaf area index. The increase in the leaf area increases the absorption of solar radiation and subsequently improves the production of dry matter by photosynthesis. The findings showed that the source-sink relationship in safflower plants impacted by both agronomic management and external environmental factors. As was anticipated, in very high densities, the amount of dry matter production in a distinct plant decreased probably due to shading and the reduction of the photosynthesis rate of the lower leaves of the canopy.

Under semi-arid region it is very important the quickly covering the soil surface by the canopy to prevent the loss of soil moisture through evaporation and also to reduce the invasion of weeds (El-Beltagi *et al*., 2022) therefore the planting in narrow rows with 30 cm inter-row spacing seems to be more justifiable. However, modification of the leaf area index through changing the plant density can affect the durability of the leaf area, chlorophyll content and the efficiency of photosynthesis per unit of leaf area. The results showed that under well-irrigated conditions (FC_{100}) the chlorophyll content of leaves increased with decreasing plant densities, still under deficit irrigation conditions, plant density did not have much effect on chlorophyll content. Probably, under deficit irrigation, the drought stress caused the destruction and inhibition of chlorophyll synthesis (de Almeida Silva *et al*., 2023). The study showed that the seed weight was less affected by the studied treatments. This trait is strongly influenced by genetic characteristics and is less influenced by environmental factors (Licata *et al*., 2023).

In this experiment, plant densities did not have a significant effect on seed weight. With the increase in density under favorable moisture conditions, probably due to the increase in inter-plant competition, the yield components decreased to some extent (Fasoula and Tollenaar, 2005). But the increase in the number of plants was able to compensate for the loss caused by the competition between plants and the total yield in unit of area increased (Zheng *et al*., 2021). However, increasing the density of plants per unit area can affect access to needed resources such as water, nutrients, and light (Chen *et al*., 2022), so conscious

and accurate fertilizer management, improving soil permeability through the use of animal manures and plant residues can be effective. There was 20% decrease in achene yield under mild deficit irrigation conditions (FC_{70}) compared to well-irrigated conditions. However, by considering the positive and negative attributes of arguments and due to the water scarcity in the studied area and also the coincidence of safflower growth with the cultivation of other cash crops, the use of this deficit irrigation technique is still recommended.

Conclusions

In total, the reduction of irrigation volume reduced the growth and all yield components. Reducing the irrigation amount by 50% accelerated the maturity of plants. Growth characteristics and yield components under deficit irrigation conditions did not significantly affect by planting densities. Applying low plant density under the well-irrigated or mild deficit irrigation (FC_{70}) conditions led to a considerable increase in lateral growth of plants. However, increased plant lateral growth at low planting densities could not compensate for the decrease in achene yield caused by the low plant and capitula number per unit area. Applying the 30 cm inter-row spacing with mild deficit irrigation produced an acceptable achene yield (900 kg ha^{-1}) and led to significant water saving (30%). By utilizing narrow row (P_{33}) and cover of the soil surface the evaporation reduced and most of the sun's radiation is received by the canopy. However, it is not recommended to use the deficit irrigation throughout the crop cycle. Because results showed that decrease in soil moisture during the reproductive period led to the reduction in the number of fertile branches, decreased length of the development period, and reduced the achene yield. Applying deficit irrigation during the vegetative growth period and providing an optimal level of water (FC_{100}) during the sensitive reproductive phase may improve the efficiency of water use.

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Direct and delayed effect of the plant *Cleome amblyocarpa* Barratte & Murb (*Capparidaceae*) on the two species of (Blattodea) *Blattella germanica* (Linnaeus, 1767) and *Shelfordella lateralis* (Walker, 1868)

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Abstract. Finding substantial substitution of chemical pesticides to control cockroaches, which were proved to link with multiple health issues, has become the major target of many researchers. In fact, subjecting this pest to the effects of toxic plants extracts is considered ideal to primarily avoid jeopardizing human health. This study is divided into two objectives. The first is to confirm the toxicity of the ethanolic extract from the plant *Cleome amblyocarpa* Barratte & Murb, on two species of urban cockroaches; which are *Blattella germanica* (Linnaeus, 1767) and *Shelfordella lateralis* (Walker, 1868), while the second is to verify the effect of the attractive odor of the extract on the feeding behavior of the two already mentioned species. Therefore, the findings demonstrated that the extract causes a

mortality rate of 60% of *B. germanica* after 30 days of treatment at a high concentration of (3g/l). The mortality rate in *S. lateralis* does not exceed 6.7%. Further, findings exhibited that the proportion of *B. germanica* attracted by the smell of the plant soaked in hexane at a specified time (15, 30 and 60 minutes) was estimated at 40%, whereas that of *S. lateralis* exceeded 60%. The results provide the presence of toxic elements within the examined extract, suggesting the potential for developing bio-insecticides derived from *Cleome amblyocarpa* for utilization in the pesticide industry.

Keywords: *Blattella germanica*, *Cleome amblyocarpa*, feeding behavior, *Shelfordella lateralis,* toxicity.

Introduction

Cockroaches are classified within the arthropod phylum, fall under the class of Insecta, Blattodea. Actually, more than 4500 species of cockroaches have been globally recognized (Hashemi-Aghdam and Oshaghi, 2015). So, a relatively small number, of cockroach species, have adapted to domestic habitats (Robinson, 2005). The majority of cockroach species are not household pests; in fact, the major pest species constitute less than 1.0% of all cockroach species (Rehn, 1945).

These pests are abundantly present in human environments, which is a result of the availability of appropriate conditions of humidity and temperature in some places such as food stores, restaurants, restrooms and culinary places (Nedelchev *et al.*, 2013). In fact, urban cockroaches carry diseases; as a consequence, they can contaminate foods consumed by humans with pathogenic organisms and cause asthma, especially for children (Hashemi-Aghdam and Oshaghi, 2015). Moreover, cockroaches excretions likes feces, molded skins and saliva can cause severe allergic symptoms (Sookrung and Chaicumpa, 2010).

Despite acknowledging their toxicity to human health, chemical compounds such as fipronil, imidacloprid and sulfonamide (Cutler *et al.,* 2017) have been used to control cockroaches; hence, to avoid the consequences of contracting diseases resulting from contact with this creature. However, the use of these products is being gradually avoided these years, this is due to the increasing resistance to common insecticides (Ko *et al.,* 2016). The previous facts contribute to the constant increase of the necessary of alternative methods such as biological struggle which replace environmentally harmful chemicals (Cutler *et al.,* 2017).

In effect, more than 2000 plant species with insecticidal activity have already been identified (Shilaluke and Moteetee, 2022). *Cleome amblyocarpa* is used in traditional medicine by the nomads of the Sahara as an analgesic for neuralgic pain (Khlifi *et al.,* 2021). In Saudi Arabia the whole plant is used to treat scabies, rheumatic fever and inflammation. While in Mauritania, roasted leaves are cooked into a food taken for kidney and back infections and as an aphrodisiac (Schmelzer and Grurib-Fakim, 2013).

Regarding Algeria, entomological investigations and studies related to plants extracts are scarce. Information is still fragmentary, incomplete or even non-existent (Aouissi *et al.*, 2021, Farhi *et el.,* 2022, Deghiche-Diab *et al.,* 2022). This study fills in an important gap in the knowledge of North African biodiversity in general.

The aim of the current study is first to test the toxicity of the ethanolic extract of *Cleome amblyocarpa* on two species of urban cockroaches, namely *Blattella germanica* and *Shelfordella lateralis*, in addition to examining the effect of this plant soaked in hexane in the food attractiveness of the two species, *Blattella germanica* and *Shelfordella lateralis.*

Materials and methods

1. The investigated target species of Blattodea

❖ *Blattella germanica* are cosmopolitan insects, closely associated with human accommodation and activities. They depend on the warm climate of homes and other habitat. Some abiotic factors such as food and water also affect their population (Gul *et al.,* 2017; Gul *et al.*, 2022).

❖ *Shelfordella lateralis,* the Turkestan cockroach, also known as the rusty red cockroach, is a lively mainly external cockroach, native to an area from North Africa to Central Asia (Kim and Michael, 2013). This species is commonly found in fields, houses and especially in steam tunnels (Gul *et al.,* 2017).

Breeding

The mass breeding of the individuals of the studied cockroaches (*B. germanica* and *S. lateralis*) was carried out in plastic boxes with a perforated lid so that the cockroaches did not escape from the boxes. The cockroaches were fed dog kibble, which was provided approximately weekly. To ensure the humidification of the environment, the boxes were equipped with tubes filled with water and plugged with cotton; the water was changed every four days.

2. The investigated target plant species

Cleome amblyocarpa: is a perennial green grass belonging to the Capparidaceae family (Ozenda, 1958). The plant used in this study was collected in the year of 2018 from Bousaâda region, located in the state of M'sila; Algeria.

Extraction of plant active ingredients

The powder of the plant *C. amblyocarpa* is macerated into 150 mL of distilled water and 350 mL of ethanol, then left to cool with stirring for 48 h. The obtained mixture is filtered using Whatman filter paper (3 mm). Then, in order to obtain an exact estimate of the quantity of plant matter dissolved in the aqueous extracts, the latter were concentrated by evaporation in an oven heated to 50°C for 48 hours, until a dry residue was obtained, of which the quantity is expressed in g.

3. Toxicity test

The larvae of *B. germanica* and *S. lateralis* are separated and arranged in groups within three repetitions contain 10 individuals in each box (13x11x5 cm), which contain dog food as well as water tube with added concentration from the extract of *C. amblyocarpa* (1g/l, 2g/l and 3g/l The control group is watered with pure water. Each experiment was repeated three times and followed for 30 days; the number of mortalities is recorded daily to determine lethal concentration and time (LC/ LT).

4. Food attractiveness test

In a flask containing 30 ml of hexane, 25 g of the leaves of *C. amblyocarpa* emerge; after 15, 30 and 60 min of incubation under laboratory conditions. A piece of filter paper emerges in each extract to perform the food attractiveness tests on the larvae of two species (*B. germanica* and *S. lateralis*) by the use of a tube of form (Y) for the tests in the various times 15 individuals were used.

5. Statistical analysis

Regarding the results acquired from the toxicological test done, calculations were conducted following the mathematical methods defined by Finney (1971). The lethal time (LT50% and LT90%) and lethal concentration (LC50% and LC90%) were determined for the extract used.

For the feeding behavior, we compared the results obtained from ethological tests conducted in an olfactometer using Monte-Carlo simulations, based on a Chi-2 (χ^2) test at the threshold p=0.05 (Vaillant and Derrij, 1992). The results

were also analyzed using the analysis of variance at a threshold variation criterion (ANOVA). Additionally, the View stat software on iMac was used for all these calculations.

Results

1.Effect of the ethanoic extract of C. amblyocarpa on the mortality of B. germanica and S. lateralis larvae

Mortality rate. Figure 1 provides the rates of the mortality of *B. germanica* and *S. lateralis* obtained after the 5th, 15th and 30th day of exposure to different concentrations of *C. amblyocarpa*.

B. germanica larvae: the use of a concentration of 1 g/l causes a mortality of 6.7% after 5 days of exposure and increases as a function of time to reach 30% after 30 days. A mortality of 20% to 46.7% was recorded after 5 and 30 days of exposure for the 2 g/l concentration. The 3 g/l concentration causes a mortality of 20% at the end of the 5th day to reach 60% at the 30th day. The analysis of variance indicates significant variations in mortality rates after 30 days of treatment among the different concentration. (Fobs = 2.06; P < 0.0001***) (Fig. 1).

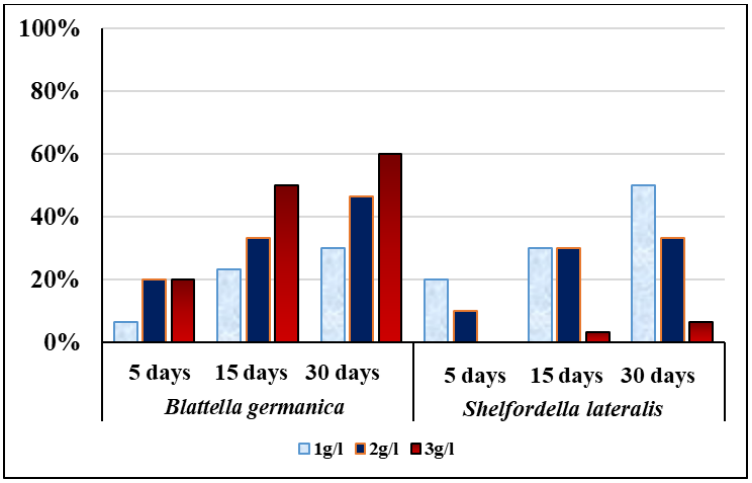

Figure 1. Mortality rate of *B. germanica* and *S. lateralis* larvae by different concentrations of ethanoic extracts of *C. amblyocarpa*.

S. lateralis larvae: the use of a concentration of 1 g/l causes a mortality of 20% after 5 days of exposure and increases as a function of time to reach 50% after 30 days. A mortality of 10% to 33.3% was recorded after 5 and 30 days of

exposure for the 2 g/l concentration. The 3 g/l concentration causes a mortality of 0% at the end of the 5th day to reach 6.7% at the 30th day. The analysis of variance demonstrates a significant variation between the mortality rates registered after 30 days of treatment with the different concentrations (F obs = 0.34; P = 0.72) (Fig. 1).

Toxicological parameters. Table 1 presents the toxicological parameters of the larvae of *B. germanica* and *S. lateralis* treated with different concentrations of the ethanolic extract of *C. amblyocarpa*.

Table 1. The toxicological parameters of different species treated with *C. amblyocarpa*.

B. germanica			
Lethal concentrations			
Exposure time	**5 days**	**15 days**	**30 days**
Regression	Y= 3.55+1.47X R= 0.93	Y=4.22+1.48X R=0.96	Y=4.45+1.63X R=0.99
LC50%(g/l)	0.98	0.52	0.33
LC90%(g/l)	1.85	1.39	1.12
Lethal times			
Concentrations	**1g/l**	**2g/l**	**3g/l**
Regression	Y=2.67+1.26X R=0.97	Y=3.55+0.85X R=0.99	Y=3.20+1.41X R=0.99
LT50%	6.35	5.50	3.58
LT90%	17.55	24.82	8.80
S. lateralis			
Lethal concentrations			
Exposure time	**5 days**	**15 days**	**30 days**
Regression	Y=4.66-7.98X R=0.83	Y=4.66-2.46 R=0.77	Y=5.12-2.99X R=0.92
LC50%(g/l)	0.958	0.879	1.040
LC90%(g/l)	0.816	0.517	0.678
Lethal times			
Concentrations	**1g/l**	**2g/l**	**3g/l**
Regression	Y=3.45+0.95X R=0.97	Y=3.77-0.19X R=0.96	Y=-2.94+4.62X R=0.95
LT50%	5.111	0.0015	5.576
LT90%	16.667	$0.00000018*10^{3}$	7.357

To achieve a 50% mortality rate of *B. germanica* larvae after 5 days, the concentration needs be equal to 0.98 g/l. On the other side, ensuring the mortality of 90% of the larvae in the same period necessitates the use of a concentration of (1.85g/l) of this insecticide. Further, 50% of these larvae can be eliminated after 15 days when a concentration of 0.52 g/l is applied, while 90% require the use of a concentration of (1.39g/l) in 15 days. For a mortality of the order of 50% of these larvae, the necessary concentration is (0.33 g/l), and for a mortality of 90% of the larvae a concentration of (1.12 g/l) is necessary, after 30 days (Tab. 1).

Concerning *S. lateralis* larvae, and in order to insure 50% mortality of these spices after 5 days, the concentration should be equal to (0.958 g/l). whereas (0.816 g/l) of this whereas (0.816 g/l) this insecticide ensures the mortality of 90% of the larvae in the same period. More, 50% of these larvae can be eliminated after 15 days when a concentration of (0.879 g/l) is applied, while 90% require the use of a concentration of (0.517g/l). For a mortality of the order of 50% of these larvae, the necessary concentration is (1,040g/l), and for a mortality of 90% of the larvae, a concentration of (0.678 g/l) is necessary after 30 days (Tab. 1). Thus, a positive correlation was observed between the mortality rates recorded and the exposure time and/or the concentration of the extract used against the larvae of *B. germanica* and *S. lateralis* (Tab. 1).

2. Effect of the hexanoic extract of C. amblyocarpa on the food attractiveness of B. germanica and S. lateralis

The percentage of *B. germanica* attracted to the odor of the foods tested at the specified time (15.30 and 60 minutes) is estimated at 40% (Fig. 2). However, the percentage of *S. lateralis* attracted by the smell of the foods tested at the specified time (15.30 and 60 minutes) is estimated at 60% (Fig. 2).

The detection time. Table 2 provides a summary of the statistical analysis conducted on the latency times of *B. germanica* and *S. lateralis* larvae in detecting the tested odor.

B. germanica larvae seem more attracted to the *C. amblyocarpa* extract with hexane for 15 min (2.454 ± 0.997), and they take longer with the *C. amblyocarpa* hexane extract 30 minutes (10.893 ± 4.757). The statistical analysis of the mean detection times reveals significant differences (F=2.290; P = 0.038*) (Tab. 2).

S. lateralis larvae seem more attracted to the *C. amblyocarpa* extract with hexane for 30 min (0.756 ± 0.342). The statistical analysis of these detection times reveals significant differences (F=1.891; P = 0.080*) (Tab. 2).

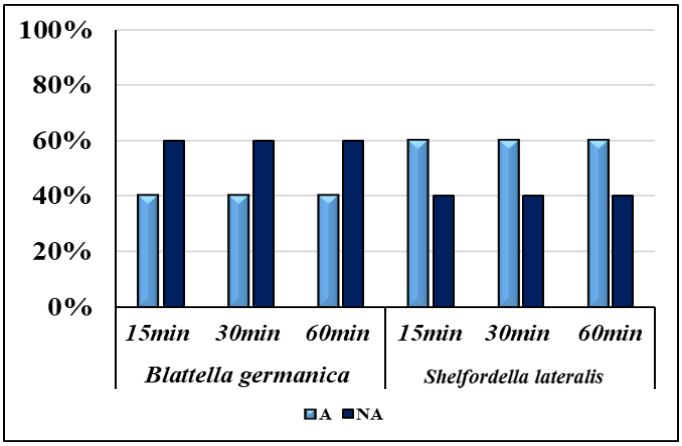

Figure 2. Food attractiveness rate of *B. germanica* and *S. lateralis* (NA: not attracted; A: attracted)

Table 2. Detection time of larvae to *C. amblyocarpa.*

***B. germanica* larvae**			
Time	**15min**	**30min**	**60mins**
Detection	2.454±0.997	10.893±4.757	8.933±4.417
F	2.461	2.290	2.023
P	0.027*	0.038*	0.063
***S. lateralis* larvae**			
Time	**15min**	**30min**	**60mins**
Detection	12.301±6.505	0.756±0.342	2.118±0.684
F	1.891	2.214	3.096
P	0.080	0.045*	0.008*

(*: Significant)

The arrival time. Table 3 illustrate the statistical analysis of the different latency times taken by larvae of *B. germanica* and *S. lateralis* to arrive at the tested odor. When the filter paper is soaked in the extract for 60 minutes, the majority of *B. germanica* larvae were noted to move towards a vacuum, i.e. they are not attracted by the smell of the *C. amblyocarpa* extract at an average of (49.040 ± 11.231). Statistical analysis of these mean latency times of indicates significant differences (F= 4.366; P = 0.001*) (Tab. 3).

Larvae of *S. lateralis* are more attracted to the smell of the hexanoic extraction of *C. amblyocarpa* at 60 min (4.782 ± 2.189). Statistical analysis suggests that there is a significant difference between the means arrival time (F = 2.185; P = 0.046*) (Tab3).

Table 3. Arrival time of larvae to *C. amblyocarpa* extract

***B. germanica* larvae**			
Time	**15min**	**30min**	**60mins**
Arrival	32.239±11.666	24.632±6.917	49.040±11.231
F	2.763	3.561	4.366
P	0.015*	0.003*	0.001*
***S. lateralis* larvae**			
Time	**15min**	**30min**	**60mins**
Arrival	6.800±2.134	11.255±7.852	4.782±2.189
F	3.186	1.433	2.185
P	0.007*	0.145	0.046*

(*: Significant)

Discussion

In his attempt to control harmful insects such as urban cockroaches, man deploys considerable efforts, and seeks new methods of physical, biological or chemical control in order to limit their proliferation (Kim *et al.,* 1995; Lyon, 1997).

The application of chemical methods to fight against harmful cockroaches cause harmful consequences on the environment and on man (Amin *et al.,* 2022). This is why the world is starting to look for another plant-based alternative to protect the globe with its different components. Plant products act on the sensory system and cause behavioral effects on insects, they include pheromones, extracts and oils of plants, plant growth regulators and insect growth regulators. Bio-insecticides work in harmony with integrated pest management programs (Veer and Gopalakrishnan, 2016). The effects of secondary plant metabolites responsible for insecticidal activity are described as suppression of calling behavior, growth retardation, toxicity, disruption of mating, deterrence of oviposition, inhibition of Diet and Reduced Fecundity and Fertility (Veer and Gopalakrishnan, 2016).

In Algeria, this type of research focuses on the use of plant extracts. It has gradually begun to develop in recent years with great concentration on aqueous plant extracts against urban pests, through several recent works (Benhissen *et al.,* 2018; Habbachi *et al.,* 2019; Boublata *et al.,* 2020; Saadane *et al.,* 2021; Hedjouli, 2022). This study, which uses ethanoic plant extracts as a means of control of the pests *B. germanica* and *S. lateralis*, focuses on the toxicological effect of different concentrations of ethanoic extracts from the leaves of the *C. amblyocarpa* plant, with the study of food attractiveness.

Based on the results obtained, we demonstrated that the leaves *C. amblyocarpa* affects the larval mortality of *B. germanica* and *S. lateralis*. The mortality rates of these larvae increase with the different exposure times and the concentrations used of the ethanolic extract of this plant. The results also indicated that the mortality rate of *B. germanica* larvae is greater than the mortality rate of *S. lateralis* larvae, so that *B. germanica* is more sensitive to this bio-insecticide.

These results are similar to a recent work by Hedjouli (2022). They demonstrated that the aqueous extract of *C. amblyocarpa* has an effect on the mortality of adults (male and female) of *B. germanica*. Further, he stated that the mortality rates in both sexes vary according to the duration of exposure and the concentrations used of *C. amblyocarpa*. They found that the values of LC 16%, LC50%, LC84, LC90%, LT16%, LT50%, LT84% and LT90% decrease with the increase of the treatment time and/or product concentration. The results also indicate that female mortality is greater than that of males. Therefore, females are more sensitive to this bio-insecticide. Habbachi *et al.* (2019) showed that the aqueous extract of *C. amblyocarpa* at different concentrations act on the time of mortality of *Drosophila melanogaster* larvae as a function of the concentration applied. More, the concentration of 35 µg/ml has a weak larvicidal activity. For the three concentrations (70, 100 and 200 µg/ml), 50% of the population is killed after 15 days of treatment. Kemassi *et al.* (2018) revealed that the aqueous extract from the leaves of *C. amblyocarpa*, acts on the mortality of adult males and females of *Schistocerca gregaria*. They added that a mortality percentage of 76.67% and 86.67% is noted in male and female larvae respectively. As for the signs of intoxication, they are observed in treated individuals, including decreased activity mobility, intense defecation, unusual water loss in the form of diarrhea, blockage and/or difficulties during moulting. Another study carried out by Korichi-Almi (2016) on larvae and adults of *Ectomyelois ceratoniae*, which were treated with different concentrations of the aqueous extract of *C. amblyocarpa*. The study Showed the existence of a positive correlation between the time and the mortality rate. Korichi noted that at the 7th day of the treatment, mortality rates reach 42.2%, 73.3% and 82.2% respectively for the 5%, 10 and 15% concentrations.

Cockroaches display advanced chemical communication capabilities, as described by Cornnette (1997). Their feeding behavior progresses through a series of distinct behavioral sequences triggered by the detection of odors. Initially, they engage in odor detection behavior, followed by moving towards the source of the scent, whether larvae or adults. Dajoz (1998) explains that a chemical composition enables cockroaches to locate food through olfactory attraction or repulsion. Within the genus, dietary preferences remain consistent, with cockroaches consistently being omnivorous, consuming both carbohydrates and proteins.

An extraction with hexane on *C. amblyocarpa* was carried out in order to obtain the apolar molecules. These extracts were tested on *B. germanica* and *S. lateralis* larvae. The latter are well attracted according to their concentration. The more the concentration of the extract increases, the more the attraction of the two cockroaches increases. *S. lateralis* larvae were noticed to be more attracted to the extract than *B. germanica* larvae.

These results show that *C. amblyocarpa* affect treated *S. lateralis* larvae. The number of *S. lateralis* attracted by the extracts is found much higher than that of *B. germanica*. Our results are comparable to those of Elbah (2017), who tested the effect of aqueous extracts of *Peganum harmala* and *Daphne gnidium* at sub-lethal concentrations on the gregarious behavior of *B. germanica*. The two products tested act on the perception of adult cockroaches; *B. germanica* treated and tested in an olfactometer did not significantly detect the odors tested.

The behavior of insects and all animals is controlled by interactions between neurons within their nervous systems. Insecticides have been selected, and sometimes designed, for their remarkable ability to kill insects (Souto *et al.,* 2021), and most of them attack specific sites in the insect's nervous system. It is therefore not surprising that insecticides can affect behavior at levels but that do not cause mortality. This study was important to fill a gap in Algerian biodiversity, specifically by bringing an entomological investigation related to plants extract. However, further studies are more than needed in order to better understand the underlying mechanisms related to animal behavior.

Conclusions

In this study, the purpose of the first experiment was to know the mortality rate of the larvae of these two cockroaches treated with the extracts of *Cleome amblyocarpa*. It was observed that the mortality rate on the 30th day at different concentrations (1 g/L, 2 g/l and 3 g/l) in *B. germanica* is 60%, whereas this rate is 6.7% in *S. lateralis*. The mortality is positively correlated with the different concentrations used, the duration of exposure and the different species. In the second experiment, the aim was to find out which of

these species is most attracted to *Cleome amblyocarpa* soaked in hexane for a duration of (15, 30 and 60 minutes). The results showed that *S. lateralis* is well attracted by the extracts because the ratio of reproduction to odor exceeds 60%, whereas it is just 40% in *B. germanica*.

The results of this work prove the presence of toxic substances in the studied extract which may lead to the development of bio-insecticides based on *Cleome amblyocarpa* to be used in the pesticide market.

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Conflict of interest: The authors declare that they have no conflict of interest.

Ethics approval: The conduct of this study was approved by the local ethics committee of the University of Mohamed Boudiaf-M'sila.

The ethics of research involving animals: All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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Reprotoxicity of zinc oxide nanoparticles synthesized with *Crataegus monogyna* leaves extract: testis and sperm function

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Abstract. This work examines the effect of three doses of zinc oxide nanoparticles (ZnONPs), synthetized with *Crataegus monogyna* leaves using zinc acetate, on the sperm quality of Wistar rats. Animals were divided into 4 groups; the control group maintained without treatment, while ZNP1, ZNP2, and ZNP3 received respectively 10 mg ZNP/kgbw, 50 mg ZNP/kgbw, and 100 mg ZNP/kgbw by gavage for 15 days. Epididymis sperm was collected for sperm parameters: concentration, live sperm, motility, velocity (VCL, VSL, and VAP), linearity (LIN), amplitude lateral head (ALH), and beat cross frequency (BCF). DNA fragmentation was measured in three samples selected from control, ZNP1, and ZNP2. Testicular and epididymis malondialdehyde (MDA), glutathione (GSH), and glutathione peroxidase (GPx) were evaluated. Compared to the control, ZNP1 has a significant reduction of testicular and epididymis weights, sperm concentration, live sperm, motility, VCL, VSL, VAP, LIN, and BCF, with a significant increase of MDA and a significant decrease of GSH levels. The ZNP2 group demonstrated a significant increase in epididymis weight, a raise in sperm parameters (concentration, motility, VCL VSL, VAP, LIN, ALH, and BCF), and an

augmentation in GSH and GPx levels. However, ZNP3 has a significant increase in VSL and ALH while ZNP1 and ZNP2 showed no effect on spermatozoa DNA. interestedly, we found that the lower dose of ZNP1 acted as toxic to testicular and epididymis parameters, while the higher ones may help to improve sperm quality and reduced oxidative stress.

Keywords: zinc oxide; nanoparticles; *Crataegus monogyna*; sperm quality.

Introduction

In recent decades, there has been a remarkable expansion in the prevalence of several diseases including diabetes, cardiovascular disorders, and fertility issues (Iavicoli and Bergamaschi, 2009; Bergman *et al.,* 2013). According to the World Health Organization (2001), approximately 90% of male infertility investigated cases are linked to sperm anomalies. Sperm concentration, vitality, and motility are considered as important markers of male fertility, along with DNA fragmentation, which has become a crucial tool in diagnosing genetic abnormalities. Reactive oxygen species (ROS), as stated by Iommiello *et al.,* (2015), can adversely affect sperm membrane and DNA integrity, leading to strand breaks and chromatin cross-linking (Agarwal and Sengupta, 2020).

The World Health Organization estimated that 80% of pharmaceutical drugs based on medicinal plants showed therapeutic efficacy (Prairna *et al.,* 2020). This opens up a new area of biomedical nanotechnology that focuses on the study of nanostructure properties derived from plants known for their therapeutic effects. By exploring and understanding these properties, researchers aim to detect and target molecules associated with various diseases (Logothetidis, 2012). Many recent investigations showed the uses of plants as nanomaterials' producers by different methods (Zong *et al.,* 2014; Ramesh and Viruthagiri, 2015). Biosynthesis of nanostructures is an eco-friendly and a non-toxic process used as antimicrobial agents, drug delivery, biosensors, imaging contrast agent, transfection vectors (Vishwakarma, 2013), cosmetics, environment protection (Hussein *et al.,* 2018; Rasmussen *et al.,* 2010), imaging and identification of cells, destruction of viruses and cancer cells, and repairing damaged cells (Abo Alhasan, 2014; Hartung and Mansoori, 2015).

Zinc is known as reparative element to DNA and has a protective effect against cellular necrosis (Ho, 2004). It stabilizes the suppression gene P53 activity as an apoptosis regulator (Ng *et al.,* 2011). In addition, it plays an important role in the formation of DNA and RNA synthesis (Chvapil, 1973).

Many previous findings showed its effect on male reproduction as an antioxidant, testosterone booster, and spermatogenesis activator (Roy *et al.,* 2013), in which there was a significant positive correlation between zinc and semen quality (Vásquez and Arango, 2003). The addition of zinc acetate to plants could produce zinc oxide nanoparticles (ZnONPs) that are considered as safe and biocompatible (Raghupathi and Manna, 2011; Rosi and Mirkin, 2005). Thus, humans can be exposed to this synthetic ZnONPs via skin, inhalation, oral, and intravenous routes (Elshama and Abdel-Karim, 2018), which easily enter through cell membrane and could be either cytoprotective or cytotoxic (Espanani *et al.,* 2013). Moreover, ZnONPs are considered as powerful antioxidants (Shen *et al.,* 2013); as well they could increase antioxidant enzymes, also it may decrease malondialdehyde (Elshama and Abdel-Karim, 2018).

Among 280 species of hawthorn (Arya and Thakur, 2012), *Crataegus monogyna* is known for its medicinal properties, because it was used to treat certain diseases as cardiovascular disorder (Chang *et al.,* 2002), heart failure, and some nervous system troubles (Bechkri *et al.,* 2017). *C. monogyna* was used also as food (Gürsoy and Yıldız 2019), as a source of vitamins and phenolic compounds (Bernatoniene *et al.,* 2009), and oils that make hawthorn as one of the powerful antioxidant plants (Bahorun *et al.,* 1994). In addition, *C. monogyna* was reported to have a protective activity on male fertility against the toxicity of cyclophosphamide (Shalizar and Malekinejad, 2011) and doxorubicin (Shalizar and Hasanzadeh, 2013).

Therefore, the objective of this study is to investigate the effect of different doses of ZnONPs, synthesized from *C. monogyna* leaves' extract, on the sperm quality, sperm DNA fragmentation, and testicular and epididymis oxidative stress in male Wistar rats.

Materials and methods

Plant preparation

C. monogyna was harvested in November from Annaba area, Northeast Algeria. Fresh leaves were washed, shade dried, and then 10 ml of distilled water was added to 10 g of the powdered leaves, mixed in a hitting magnetic stirrer for 1h at 60°C, and the filtered extract was obtained.

Nanoparticles extraction

5 g of zinc acetate was added to 50 ml of the filtered leaves' extract, and then the mixture was stirred for 2h at 95°C. Afterward, the mixture was incubated in an oven for 2h at 80°C in which a powder of (ZNP) was formed.

Experimental design

24 male Wistar rats were reared in the animal house at standard conditions and given water and standard diet *ad libitum*. Animals were divided into 4 groups; the control group maintained without treatment, the ZNP1 group received 10 mg ZNP/kgbw, the ZNP2 group administrated with 50 mg ZNP/kgbw, and the ZNP3 group given 100 mg ZNP/kgbw. After 15 days of treatment by gavage, animals were sacrificed by decapitation. The sperm was immediately collected from the epididymis for sperm quality test, while testis and epididymis were weighed and stored for further uses. Animals' treatments were authorized by the Ethical Committee of Animal Sciences at the University of Badji Mokhtar-Annaba, before starting the experimental work.

Semen analysis

Semen analysis was performed with the Computer-Assisted Sperm Analysis Method (CASA) using Sperm Class Analysis (SCA®, Microptic, Barcelona, Spain). The epididymal semen was obtained and then, a drop of semen (about 1 µl) was diluted with a physiological solution of NaCl 0.09%. Afterward, 5 µl of the mixture was placed in an empty chamber slide (GoldCyto model). The slide was then placed on a Nikon Eclipse (Nikon E200-LED) microscope using the phase objective (x4). The sperm markers of concentration, vitality, motility, linearity (VCL and VSL), velocity (VAP), the amplitude of lateral head displacement (ALH), and the beat cross frequency (BCF) were automatically calculated.

Vitality analysis

Sperm vitality was assessed by SCA® CASA. Vitality is determined using BrightVit, a solution derived from nigrosine-eosin (NE), when the entry of eosin (Vital dye) into cells with dead or compromised membranes, causing them to appear pink. Meanwhile, live cells retain their natural white coloration. The inclusion of nigrosin as a background stain enhances the contrast for better distinction. BrightVit solution was made up in a hypo-osmotic medium and accordingly intact cells/cell membranes will swell, but cells with burst cell membranes will show thin straight tails and no signs of swelling.

DNA fragmentation test

The GoldCyto Sperm Kit (Goldcyto Biotech Corp) is a simple test that allows assessment of sperm DNA fragmentation in animals. The DNA fragmentation exanimated in three groups selected according to the results of epididymis semen analysis using the CASA, three samples was chosen from the control, ZNP1 and ZNP2 groups. The method is based on the Sperm Chromatin Dispersion (SCD)

(Fernández *et al*., 2005). Intact unfixed spermatozoa are immersed in an inert agarose microgel on a pretreated slide. An initial lysing treatment removes most of the nuclear proteins, and in the presence of massive DNA loops, emerging from a nucleoid from spermatozoa with central core. However, the nucleoids from spermatozoa with intact DNA either do not show a dispersion halo or the halo is minimal. Results based on statistic methods are shown using mederate plus high DNA fragmentation proportion. According to Evenson and Jost, (2002), less than 15% represents a high fertility statue, 15-30% represents a resemblance to a fertility statue, and more than 30% signifies a significant lack of fertility potential.

Measurement of oxidative stress parameters

Frozen stored testis was thawed, then 100 mg of each sample was transferred to test tubes for the determination of glutathione (GSH) using the method of Weckbecker and Cory (1988). The principle of this assay is based on the measurement of the optical density of the acid 2-nitro-5-mercapturic. The latter results from the reduction of 5,5'-dithio-bis-2- acid nitrobenzoïque (Ellman's reagent, DTNB) by groups (-SH) of glutathione. Deproteinization of the homogenate is essential in order to keep only specific thiol groups of glutathione.

The testicular total proteins were quantified according to the colorimetric method of Bradford (1976) by using the Coomassie Brilliant Blue (BBC) as a reagent and the bovine serum albumin as a standard. The BBC reacts with the protein amino groups ($-NH_2$) to form a complex of blue color. Color intensity reflects the concentration of protein which is measured at 595 nm.

Malondialdehyde (MDA) was estimated by using the method of Ohkawa and Yagi, (1979). The dosage is based on the formation of a colored pigment absorbing at 530 nm after a reaction between MDA and thiobarbituric acid in an acidic and hot environment (100°C).

The measurement of glutathione peroxidase (GPx) was realized by the method of Flohe and Günzler (1984). This method is based on the reduction of hydrogen peroxide (H_2O_2) in the presence of reduced glutathione (GSH); the latter is transformed into (GSSG) under the influence of the GPx.

Nanoparticles size

ZnONPs were measured using ZEISS GeminiSEM 500 in the Nanoteknoloji Uygulama ve Araştırma Merkezi (ERNAM) in the University of Erciyes, Kayseri, Turkey.

Statistical analysis

Statistics was realized using (MINITAB 18 Software ANOVA Tukey). Results are expressed as mean ±standard deviation. The significant test was considered at $p<0.05$.

Results

Results in Table 1 showed the effect of ZnONPs extracted from *C. monogyna* using zinc acetate with diameter less then 200nm (Fig. 1) on testis and epididymis weights. ZNP1 showed a significant decrease ($p<0.05$) in the testicular absolute weights compared to the control while ZNP2 and ZNP3 have maintained the same levels as that of the control. Compared to the control, epididymis absolute weights have decreased significantly in ZNP1, while it showed a significant increase in ZNP2, whilst that of ZNP3 has maintained almost the same level of the control.

Table 1. Absolute testicular and epididymis weights (Mean ± SD) in Wistar rats after 15 days of treatment with ZnONPs synthesized from *C. monogyna* leaves.

	Control	ZNP1	ZNP2	ZNP3
Testis (g)	1.758±0.013^{a}	1.372±0.056^{b}	1.756±0.004^{a}	1.754±0.006^{a}
Epididymis (g)	0.572±0.002^{b}	0.518±0.039^{c}	0.676±0.009^{a}	0.572±0.002^{b}

Means that do not share the same letter are significantly different, according to one-way ANOVA, followed by Tukey test.

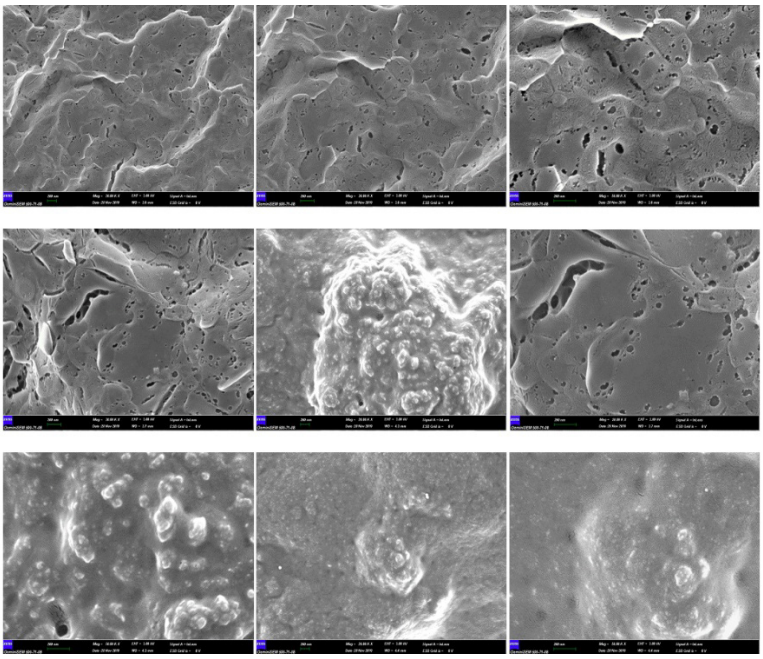

Figure 1. SEM image of zinc nanoparticles in the aqueous extract of *Crataegus monogyna* and their particles size of 200 nanometers.

Concerning the epididymis sperm parameters, a significant decrease of concentration, live sperm VCL, VCL, VAP, LIN, ALH, and BCF was observed in the ZNP1 compared to the control while in the ZNP2, the sperm concentration, VSL, LIN, ALH, and BCF have increased significantly (Table 2). Contrary, VSL and ALH levels were higher in rats of ZNP3 group compared to the control. In ZNP2 and ZNP3, both live and dead sperm have maintained the same percentages as that of the control.

The MDA concentration (Table 3) was significantly higher in ZNP1 compared to all groups. Contrary, a significant decrease in ZNP2 and ZNP3 compared to the control and ZNP1 group.

GSH showed a significant decrease in ZNP1 compared to the control while ZNP2 has demonstrated an elevation in the GSH level. In ZNP3, the level of GSH has similar as that of the control.

GPx activity illustrated a significant increase in ZNP2 group compared to the control. Though ZNP1 and ZNP3 groups have remained as that of the control.

Table 2. Epididymical semen parameters (Mean ± SD) in Wistar rats after 15 days of treatment with ZnONPs synthesized from C. monogyna leaves.

Parameter	Control	ZNP1	ZNP2	ZNP3
Concentration (Millions/ml)	82.05±1.31^{b}	26.08±0.82^{c}	88.80±0.67^{a}	82.25±1.23^{b}
Dead sperm (%)	26±0.89^{b}	31.33±1.21^{a}	24.66±1.21^{b}	26±0.89^{b}
Live sperm (%)	78.33±0.81^{a}	71.16±0.75^{b}	78.16±0.75^{a}	77.83±0.75^{a}
Motility (%)	77.14±0.88^{b}	70.74±0.45^{c}	81.23±1.009^{a}	76.342±1.51^{b}
VCL (μm/s)	88.84±0.70^{b}	74.97±0.08^{c}	92.85±0.63^{a}	88.48±0.72^{b}
VSL (μm/s)	16.27±0.16^{b}	11.03±0.45^{c}	19.47±0.60^{a}	19.13±0.78^{a}
VAP (μm/s)	39.002±0.46^{b}	30.14±0.21^{c}	45.84±0.16^{a}	39.10±0.49^{b}
LIN	29.93±0.36^{b}	16.64±0.66^{c}	31.77±0.60^{a}	29.80±0.26^{b}
ALH (μm)	4.35±0.09^{c}	4.03±0.086^{d}	4.93±0.04^{a}	4.59±0.11^{b}
BCF (Hz)	4.28±0.04^{b}	2.60±0.178^{c}	4.59±0.27^{a}	4.26±0.08^{b}

Means that do not share the same letter are significantly different, according to one-way ANOVA, followed by Tukey test.

Results in Table 4 showed that spermatozoa DNA fragmentation percentages of ZNP1 and ZNP2 are less than 15% which may indicate no effect of the doses used in our study (10 mg/kgbw and 50 mg/kgbw of ZnONPs extracted from *C. monogyna* leaves) on sperm DNA.

Table 3. Oxidative stress markers (Mean ± SD) in Wistar rats after 15 days of treatment with ZnONPs synthesized from C. monogyna leaves.

Parameter	Control	ZNP1	ZNP2	ZNP3
MDA (nmol/g tissue)	0.017±0. 00008[b]	0.018±0. 00004[a]	0.017±0. 00003[b]	0.017±0. 00001[b]
GSH (nmol/mg proteins)	0.447±0.011[b]	0.232±0.008[c]	0.549±0.011[a]	0.433±0.009[b]
GPx (µmol GSH/mg proteins)	0.055±0.002[b]	0.055±0.002[b]	0.080±0.003[a]	0.058±0.001[b]

Means that do not share the same letter are significantly different, according to one-way ANOVA, followed by Tukey test.

Table 4. Spermatozoa DNA fragmentation (%) in Wistar rats after 15 days of treatment with ZnONPs synthesized from *C. monogyna* leaves.

Parameter	Control	ZNP1	ZNP2
None fragmented DNA (%)	93	94	96.77
Fragmented DNA (%)	7	9	3.23

Discussion

In this study, the addition of zinc acetate to the *C. monogyna* leaves' extract has led to the synthesis of ZnONPs with a size of 200 nm. It is suggested that plant antioxidant compounds can reduce metals such as zinc acetate into nanoparticles (Mutukwa and Khotseng, 2022). Besides, *C. monogyna* has many active compounds such as flavones, rutin, catechin, and caffeic acid (Muradoğlu and Yıldız, 2019; Cosmulescu and Nour, 2017), which act as reducing agent and may be responsible in reducing zinc ions to ZnONPs (Shi and Jiang, 2013). Therefore, nanostructures are used in diagnosing, evaluating, and treating many health disorders (Paluszkiewicz *et al*., 2021) by targeting organs, tissues, and specific cells (Prairna *et al*., 2020). Furthermore, the absorption, distribution, and elimination of nanomaterials raise their ability to pass through cell barriers easily (Bleeker *et al*., 2013). It was believed that nanoparticles exhibit better biomedical activity than the chemically synthesized ones (Agarwal *et al*., 2018).

Results of treating rats using 10 mg/kgbw of ZnO synthesized nanoparticles using *C. monogyna* for 15 days showed a decline in sperm quality and an augmentation in oxidative stress reflected a higher level of MDA and GSH depletion. This dose has also no effect on sperm DNA fragmentation, despite it has not passed the threshold reported by Evenson and Jost (2002), who

postulated that fragmented DNA of less than 15% represent high fertility rate. It was confirmed that MWCNTs and other carbon-based nanoparticles injected to mice (5 g/kg) for 15 days didn't change the total sperm concentration, motility, and percentage of abnormal semen (Bai *et al*., 2010).

On the other hand, the administration of 50 and 300 mg/kgbw of ZnNPs during 35 days has affected sperm biology of mice (Rachid *et al*., 2008; Talebi and Moridian, 2013). Zinc nanoparticles have probably provoked testicular injuries (Boekelheide *et al*., 2000), and other nanoparticles may affect testicular tissue architecture (Iavicoli *et al*., 2013). Moreover, supplementation of 500 µL Au-NPs solution to human semen could reduce sperm motility and induce sperm DNA fragmentation (Wiwanitkit and Rojanathanes, 2009). In addition, Ag-NPs (nominal diameter of 20 ± 5 nm) may provoke a decrease in sperm concentration, and DNA damage when Wistar rats were treated with intravenous single dose of 5 mg/kg or 10 mg/kg (Gromadzka *et al*., 2012). This decline may be explained the effect of a varieties of nanoparticles on testosterone regulation (Iavicoli *et al*., 2013), via generating ROS such as superoxide ions, hydroxyl ions, singlet oxygen species, and peroxide molecules (Isık and Horzum, 2019), leading to poor sperm quality. As a result, ROS may induce apoptosis, lipid peroxidation, and DNA damage (Bardaweel *et al*., 2018) of Sertoli cells and disturb the spermatogenesis (Silva *et al.* 2022). In addition to modifying the sperm physiological function (Agarwal and Sengupta, 2020). Furthermore, sub-acute oral exposures to ZnNPs (300 mg/kg) for 14 consecutive days were stated to enhance ROS formation (Sharma *et al*., 2012), MDA augmentation, and GSH depletion. ZnONPs demonstrated effectiveness in combating various forms of cancer through the promotion of oxidative stress, enhancement of calcium influx into cancer cells, ultimately resulting in their demise (Bai *et al*., 2017).

In our finding, the rats of ZNP2 treated with 50 mg/kgbw have an increased fertility and a weaken oxidative stress, with a lower percentage of sperm DNA fragmentation while those of ZNP3 has kept sperm quality as that of the control. In addition, to having more protection of DNA structure. Likewise, NPs were suggested to have a role in activating proteins and inhibiting cell toxicity (Ali *et al*., 2009; Abbasi and Hano, 2017), while Kulandaivelu and Gothandam (2016) have found in an *in-vitro* study that silver nanoparticles at 100 µg/mL may inhibit cancerous cells through the activation of caspase-3. Similarly, ZnONPs of 1 mg/ml was not toxic and may prevent against oxidative stress (Nagajyothi *et al*., 2015).It was proven that ZnONPs using *Aloe vera* have capacity in scavenging free radicals and anticancer effect (Mahendiran *et al*., 2017). Accordingly, the ZnONPs *in vitro* (0.4, 1.6, and 6.4 µg ml) could increase the antioxidant enzymes in mice intestinal epithelium cell (Dawei, Zhisheng, and Anguo 2010) and could protect cells from ROS stress (Elshama and Abdel-Karim, 2018).

Nano-selenium supplementation (0.3 mg/kg) has proved to enhance sperm quality of goats after 90 days exposure (Shi *et al*., 2011). It was stated that the addition of zinc acetate to a plant extract may boost metal ions adsorption (Isık and Horzum, 2019). It could be argued in this circumstances that testis received ZnONPs has activated Sertoli cells for a better spermatogenesis. Also, zinc was proven to play an important role in improving spermatogenesis (Roy *et al.* 2013) and *C. monogyna* was effective in protecting against oxidative stress (Shalizar-Jalali and Malekinejad, 2011). *C. monogyna* components such as polyphenols (Muradoğlu and Yıldız, 2019), linoleic acid, oleic acid, oxalic acid bis (trimethylsilyl) ester, palmitic acid, and tetracosamethyl-cyclododecasiloxane (Bechkri *et al.,* 2017) may act as source of antioxidants and energy for better spermatozoa quality.

Conclusions

The effect of 200 nm ZnONPs synthesized from *C. monogyna* leaves' extract on sperm quality in Wistar rats was evaluated. At a dosage of 10 mg/kgbw for 15 days, fertility parameters were reduced, oxidative stress increased, and sperm DNA damage was observed. In contrast, a dosage of 50 mg/kgbw significantly improved sperm parameters, as well as levels of GSH and GPx activity. The dosage of 100 mg/kgbw had minimal impact on the parameters investigated.

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Influence of substrate particle size and detention time on mycelium-colonized sawdust efficiency for removal of faecal bacteria from slaughterhouse wastewater in batch treatment

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Abstract. Mycofiltration is a recent cost-effective biotechnology that is still under development for wastewater treatment. The use of mycelium-colonised substrate for wastewater treatment in a batch system with delayed residence time and the effect of sawdust particles has not previously been considered. Slaughterhouse wastewater is discharged untreated in many developing countries majorly due to the high cost of existing conventional treatment systems. The effect of sawdust detention time and particle size on the removal efficiency of faecal bacteria from slaughterhouse wastewater by *Pleurotus ostreatus* mycelium was assessed in the lab using mycelium colonized with sawdust of particle sizes (0.6, 1.18, 2.36 mm and unsorted particle sizes) and under varying detention times (0, 12, 24, 36, 48, 60 and 72 hours) using batch treatment procedure. Hydrogen peroxide produced during mycelium colonisation of the sawdust was also evaluated. Oxidation-reduction potential (ORP) was measured during this study to determine the oxidative capacity in each treatment reactor. The removal efficiency of *Escherichia coli* ranged from -7.9 – 77.2 % and was the highest for mycofilter with 2.36 mm sawdust particle size at 72 hours detention time (0.7 log removal).

Salmonella spp. removal efficiency ranged from 0.66 – 71 % with the highest efficiency recorded in the system with 1.16 mm also at 72 hours (0.4 log removal). Mean hydrogen peroxide concentration ranged from 0.53±0.12 (unsorted inoculated) to 25.18±1.77 mg/l (1.18 mm, 72 hours). ORP values ranged from 5.0 ±2.2 mV (raw wastewater, 24 hours) to 232±55 mV (unsorted inoculated, 72 hours). The result of this study showed that substrate particle size and detention time have roles to play in the efficiency of mycofilters using batch treatment. The concentration of hydrogen peroxide was also influenced significantly by sawdust particle size. Therefore, there is a need to further study this system in a bid to optimize its ability to remove faecal bacteria from wastewater.

Keywords: batch treatment, colonized, faecal bacteria, mycelium, mycofilter

Introduction

Mycofiltration is a recent cost-effective biotechnology that is still under development for wastewater treatment. In order to increase its effectiveness in eliminating faecal bacteria, different factors have been considered. However, these factors though promising, have not fully addressed the challenges associated with mycofiltration. The application of mycofilters as columns in multiple series for faecal bacteria removal has been investigated (Olorunfemi *et al*., 2015: Taylor *et al.*, 2015: Shekhar *et al*., 2017:Vu *et al*.,2018). However, this system can be relatively expensive to set up, thus making it unsustainable as a viable alternative to conventional treatment systems. The use of mycelium-colonised substrate for wastewater treatment in a batch system with delayed residence time as in other disinfection treatment systems has been proposed (Martinez, 2016; Umstead, 2019). This is due to the proposition that the system, apart from being phagocytic to bacteria, can also produce antibacterial compounds that need time to react with the bacteria cells (Vu *et al*., 2018). However, a previous study on the application of mycofilters made with sawdust in batch treatment for bacterial elimination did not fully identify the necessary factors for improved removal of faecal bacteria. A recent study has reported the detection of a relatively high concentration of hydrogen peroxide in mycelium colonised sawdust submerged in water (Umstead, 2019). However, the study failed to demonstrate the correlation between faecal bacteria removal and hydrogen peroxide present during batch treatment using mycofiltration. Hydrogen peroxide is an enzymatic by-product of fungi colonisation of substrates (Eichlerová *et al.*, 2006).

It has been reported that sawdust promotes high fungi mycelium colonization (Pozdnyakova *et al.*, 2018). It is also readily available compared to other substrates used for fungus cultivation and pollutant removal in mycofiltration treatment systems. The application of mycelium colonised sawdust in batch treatment system for the removal of faecal bacteria has been reported to be not relatively efficient (Umstead, 2019). There is also a scarcity of adequate knowledge of the factors responsible for the removal process.

In addition, the use of faecal bacteria indicator *E. coli*, for quantifying the removal of faecal pathogens in mycofiltration treatment needs to be fully investigated. This is because pathogenic bacteria can enter a viable but not culturable (VBNC) state, which is a virulence strategy employed to evade the attack of antimicrobial compounds (Rogers, 2012).

Slaughterhouse effluents contain high levels of faecal pathogenic bacteria, which have been reported to be poorly removed by previously used conventional and biological treatment systems (Lawal *et al.*, 2018). The application of mycofiltration with delayed residence time to remove faecal bacteria has shown promise and can be efficient for their removal from slaughterhouse effluents (Martinez, 2016). This study was done to determine the effect of sawdust particle size in mycelium-colonised sawdust for the removal of faecal bacteria from slaughterhouse wastewater in a batch treatment system.

Materials and methods

Set up of experimental bioreactors

Mycofilters were prepared by inoculating the spawn of *Pleurotus ostreatus* into particle size separated autoclaved sawdust with particle sizes of 0.6, 1.18, and 2.36 and unsorted (with particle size range of 0.6-4.75 mm). The fungi spawn was purchased from Mycofarm and Synergy Ltd, Benin City. Sawdust sorting followed the process previously described by Osarenotor et al., 2021. The inoculated sawdust was then placed in the dark to allow for incubation at room temperature and was allowed to colonise for 20 days. After colonisation, the mycelium colonised sawdust was transferred into their respective treatment bioreactors.

The wastewater used in this study was obtained from a cattle slaughterhouse located in Edo state, South of Nigeria. The facility has no wastewater treatment unit. Hence the effluents from the facility are channelled into a nearby side drain from which the water flows directly into the nearby river which is approximately 100 meters away from the slaughterhouse. Samples were collected directly from the effluent pipe leading out of the facility. 50 L of wastewater was collected and immediately transported to the laboratory.

Before the commencement of the treatment experiment, the wastewater was kept in the refrigerator at 4°C after skimming and filtering to remove large floating matter and solids.

Treatment bioreactors were 500 ml Pyrex conical flasks. The experimental design included ten reactors. Each reactor was filled with 400 ml of wastewater. The reactors were separated into five treatment groups: two reactors each with mycofilter of sawdust of particle sizes; 0.6, 1.18 and 2.36 mm, two with mycofilter prepared from unsorted sawdust and two with sawdust only and two controls (blank reactors without sawdust nor mycofilter) Inoculated media were transferred into the reactor vessels and allowed to recolonize for three days. This was done to ensure that the fungi mycelium colonises the substrate evenly following the dislodgement during the transfer process. To each setup, 25 % mycofilter was introduced as recommended by Martinez, 2016, using this method 100 g of each treatment media which is 25 % of 400 ml was added. Each reactor was monitored for treated effluent after (0, 12, 24, 36, 48, 60 and 72 hours) detention time. Samples were collected from each reactor and analysed for pH, *E. coli*, *Salmonella* spp, total dissolved solids, electrical conductivity and extracellular hydrogen peroxide. The performance of the treatment reactors was assessed using mean removal efficiency and mean log removal *E. coli* and *Salmonella* spp.

Samples analysis

Faecal bacteria analysis

Raw wastewater, as well as treated effluents, were analysed for *E. coli* and *Salmonella* spp. All samples were serially diluted and tested in triplicate using the membrane filter technique. Diluted water samples were vacuum filtered using 0.45 µm filter pads and thereafter transferred to Petri dishes containing specific media. Eosine methylene blue agar (EMB) was used for *E. coli* detection and quantification, while Salmonella-SSA was used for *Salmonella* spp. The Petri dishes were then inverted and incubated for 24-48 hours at 24-48°C.

Analysis of physiochemical parameters

Total dissolved solids (TDS), electrical conductivity, oxidation-reduction potential (ORP) and pH of the wastewater samples were analysed using the multi-parameter meter following the standard protocol prescribed by the American Public Health Association (APHA), 2005.

Hydrogen peroxide determination

The concentration of hydrogen peroxide in the treatment reactors was determined using the spectrophotometric methods previously described by El Sayed and El-Sayed, 2020 with a slight modification.

Data analysis

All statistical analysis was performed using R version 3.2.4. Multiple analysis of variance (MANOVA) was applied to check if a significant difference exists between the *E. coli* as well as *Salmonella* log removal efficiency among the treatment units. The significant difference in hydrogen peroxide concentration and ORP was also checked. Pearson's correlation coefficient was used to determine the relationship between; *E. coli* as well as *Salmonella* log removal and hydrogen peroxide concentration, ORP and pH. The relationship between hydrogen peroxide and ORP was also determined. A possible correlation between faecal bacteria indicator *E. coli* and faecal pathogen *Salmonella* during this study was also determined. All P values were considered significant at a level of 0.05. Blue colours in the visual representation output imply positive correlation while red colours imply negative correlation. Dark colour is an indication of the intensity of the correlation, the darker the colour the stronger the correlation.

Results and discussion

Effect of sawdust particle size on removal efficiency

This study was carried out to investigate the influence sawdust particle size has on the performance of mycelium-colonised sawdust for faecal bacteria removal from wastewater. Mean *E. coli* removal efficiency ranged from -7.9 ± 2.34 (unsorted uninoculated) to 77.2 ± 1.5 % (2.36 mm, 72 hours) (Tab. 1). The mean removal efficiency was the highest in the reactor with mycofilter of sawdust particle size 2.36 mm at 72 hours detention time with corresponding *E. coli* log removal of 0.7 (Fig. 1). The removal of *E. coli* in this treatment was statistically significant. The significant removal recorded in this treatment could be related to the fact that antimicrobial compounds produced during the treatment process were sufficient to deactivate the *E. coli* cells. Another possibility may be that *E. coli* are pathogenic strains that are more prone to bactericidal action compared to the non-virulent strains which are employed as indicators of faecal pollution (Taylor *et al.*, 2015). The high removal efficiency recorded in this study is in line with the result obtained by Umstead, 2019, who also used sawdust as a substrate for removing *E. coli* from synthetic storm water. However, the highest mean removal efficiency recorded in this study was lower than the 99 % reported in their study. The reason for this may be that while this study used real slaughterhouse wastewater with a mean *E. coli* load of 101 x 10^6 cfu/100 ml, the previous study used synthetic wastewater with the mean initial bacteria load 1.94 x 10^4, which is 2 log lesser. As this is the first study using mycelium colonised sawdust for the treatment of real wastewater, there was no other basis for comparison apart from the related study by Umstead, 2019.

Statistical analysis revealed that detention time also had an effect on the removal efficiency of *E. coli* in this study. It showed that faecal pollutants were most significantly removed at 72 hours. This was contrary to the study by Umstead (2019), who reported no significant difference in removal efficiency with time. The reason for this disparity may be a result of the fact that the maximum detention time in their study was 48 hours. This time may not be enough for the antimicrobials to have inhibitory action on the bacteria. As it has been reported the contact time between antimicrobial agents from fungi and bacteria is needed for effective bacteriostatic or bactericidal action (Stamets, 2005).

Table 1. Mean *E. coli* removal efficiency in the batch treatment experiment (%)

Retention time (hours)	Sawdust particle size (mm)			Control treatment		
	0.6	1.18	2.36	Unsorted inoculated	Unsorted uninoculated	Untreated wastewater
10	9.9 ±1.2	13.8 ± 4.5	13.8 ± 2.5^{c}	11.8 ± 1.4	7.9 ± 2.6	0
12	12.8 ± 2.3	22.7 ± 2.1	36.6 ± 2.1^{c}	18.8 ± 3.6	3.9 ± 2.13	-2.97 ± 2.67
24	29.7 ± 2.6	34.6 ± 2.9	45.5 ± 3.5^{c}	23.7 ± 2.6	0.99 ± 2.14	-4.9 ± 4.56
36	24.7 ± 1.7	45.5 ± 1.8	49.5 ± 3.6^{c}	-11.8 ± 3.6	0.99 ± 1.56	34.6 ± 6.7
48	33.6 ± 2.6	54.4 ± 1.5	55.4 ± 2.1^{c}	46.5 ±2.6	- 7.9 ± 2.34	4.95 ± 1.2
60	47.5 ± 2.9	63.3 ± 1.5	66.3 ±2.5^{c}	55.4 ± 2.1	-5.94 ± 2.15	9.9 ± 1.3
72	51.4 ± 2.1^{a}	66.3 ± 3.2^{a}	77.2 ± 1.5^{b}	67.3 ± 4.6^{a}	-0.99 ±1.57 ^{a}	13.8 ± 3.4^{a}

*Values in rows and column with different superscripts are significantly different (P<0.05)

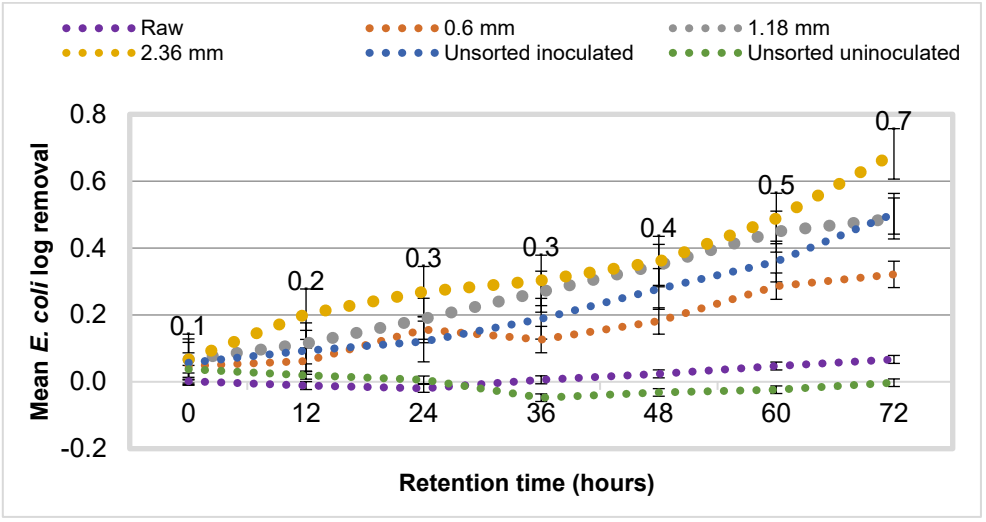

Figure 1. Mean *E. coli* log removal in the batch treatment experiment

Salmonella spp. removal

Mean *Salmonella* spp. removal efficiency in this experiment ranged from 0.66 ± 0.2 (control) to 71±3.8 % (1.18 mm, 72 hours) (Tab. 2) and was the highest in treatment with the sawdust particle size of 1.18 mm at 72 hours with a removal efficiency of 71 ±3.8 % with mean log removal of 0.4 (Fig. 2), however, this mean log removal was the same at 60 hours detention time.

Table 2. Mean *Salmonella* spp. removal efficiency in the batch removal experiment (%)

Retention time (hours)	Sawdust particle size (mm)			Control treatment		
	0.6	1.18	2.36	Unsorted inoculated	Unsorted uninoculated	Untreated wastewater
10	6.68 ± 2.34	12.7 ± 3.6[a]	10.7 ± 3.2	8.02 ± 4.3	5.3 ± 3.7	0
12	8.69 ± 3.4	18.7 ± 9.8[a]	26.08 ± 3.6	12.7 ± 3.2	2.67 ± 2.4	-2.0 ± 0.7
24	27.2 ± 2.1	29.2 ± 2.1[a]	29.2 ± 4.6	15.2 ± 5.7	-2.6 ± 0.7	-0.66 ± 0.2
36	36.9 ± 3.5	41.8 ±7.6[a]	34.14 ±7.2	24.3 ± 8.7	-10.4 ± 2.1	4.18 ± 1.1
48	44.1 ± 5.6	49.8 ± 3.2[a]	39.85 ± 3.1	37.7 ± 4.3	-7.1 ± 1.5	6.4 ± 1.4
60	53.4 ±4.6	59.9 ± 2.5[a]	48.3± 4.9	51.26 ± 6.7	-6.4 ± 0.4	7.94 ± 1.7
72	67.1 ± 7.6[a]	70.9 ± 3.8[b]	55.8 ± 3.8[a]	59.6 ± 4.7[a]	-1.50 ± 0.1[a]	12.8 ± 2.6[a]

*Values in rows and column with different superscripts are significantly different (P<0.05)

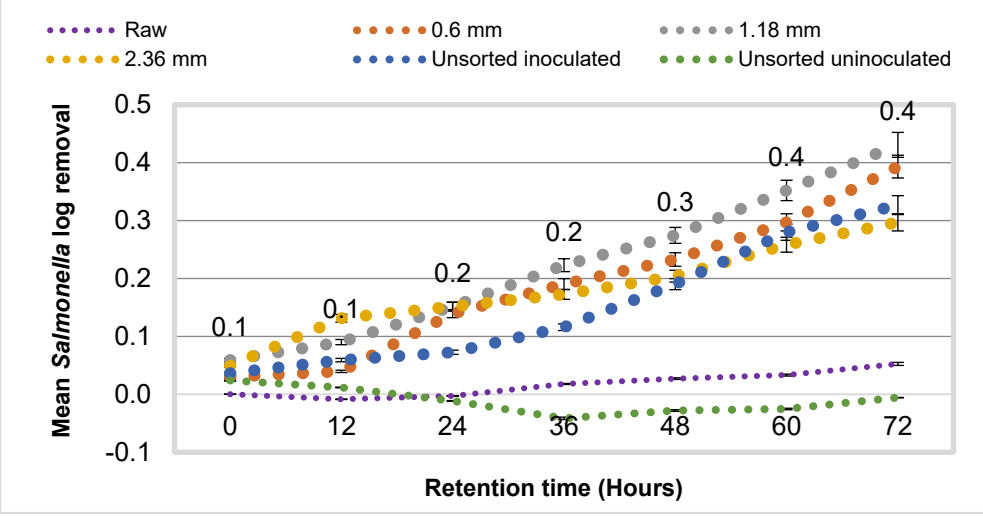

Figure 2. Mean *Salmonella* spp. log removal in the batch treatment experiment

Further statistical analysis, however, showed the mean log removal at 72 hours was significantly different from that at 60 hours ($P<0.05$). The high removal efficiency in this experimental treatment may be due to the high concentration of hydrogen peroxide detected in this treatment (see Tab. 3). Hydrogen peroxide is a potent disinfectant, therefore its presence in addition to other antimicrobials may be responsible for the significant removal efficiency recorded for *Salmonella* spp. *Salmonella* spp. is a more pathogenic bacteria compared to *E. coli* and may require a combination of antimicrobials to eliminate it (Chen *et al*., 2019). The attachment of pathogenic bacteria present in wastewater to organic matter can aid their ability to evade antimicrobial deactivation (Koivunen *et al*., 2003).

Table 3. Mean hydrogen peroxide concentration in batch removal experiment (mg/l)

Retention time (hours)	Sawdust Particle size (mm)			Control treatment		
	0.6	1.18	2.36	Unsorted inoculated	Unsorted uninoculated	Untreated wastewater
10	9.04 ± 0.4	7.97 ± 1.8	9.78 ± 1.1	2.58 ± 0.8	0	0
12	24.83 ± 1.8	25.18 ± 1.7	24.71 ± 1.8	2.46 ± 0.20	0	0
24	14.62 ±1.78	14.98 ± 1.8	14.51 ± 1.8	4.14 ± 1.63	0	0
36	7.82 ± 1.9	8.18 ± 1.7	7.70 ± 1.79	7.38 ± 1.79	0	0
48	4.42 ± 1.78	4.78 ± 1.7	4.90 ± 1.35	4.51 ± 1.67	0	0
60	2.45 ± 0.14	2.75 ± 0.20	2.41 ± 0.23	1.89 ± 0.46	0	0
72	0.99 ± 0.14	1.28 ± 0.20	0.94 ± 0.23	0.53 ± 0.12	0	0

Hydrogen peroxide quantification

The result of this study showed that hydrogen peroxide production during batch mycofiltration treatment is affected by the substrate particle size. Mean hydrogen peroxide concentration ranged from 0.53±0.12 (unsorted inoculated) to 25.18±1.77 mg/l (1.18 mm, 72 hours) (Tab. 3). Reactor treatment with sawdust of particle size 1.18 had a mean concentration of 25.18 ±177 mg/l which was the highest detected concentration in this study. Statistical analysis confirmed that the concentration of hydrogen peroxide was significantly different from other reactor treatments. The high concentration associated with this particle size may be due to the continuous high regeneration of hydrogen peroxide as the experiment progresses. As the sawdust was not used up completely, the mycelium could recolonize under an aqueous solution and produce more hydrogen peroxide. This is similar to the study by Umstaed, 2019 who reported that hydrogen peroxide production during mycelium colonization of sawdust under batch conditions is influenced by detention time and increases with time.

Effect of pH on bacteria removal

The mean pH during the treatment ranged from 1.5 ± 0.0 (unsorted inoculated at 72 hours) to 7.1± 0.1 (raw wastewater at 12 hours) (Tab. 4). The presence of various phenolic compounds during mycelium degradation of substrates is responsible for the low pH of the fungi colonised substrate. Low system pH is needed for the Fenton reaction and pollutant oxidation in the system. The relatively low pH in mycelium colonised treatments during this study are similar to the results of Vu et al.,2018, who reported low pH in broth cultures containing wood decaying fungi, *Flavodon flavus* and *Schizophyllum commune*. The mean pH value recorded in this study was however lower when compared to the mean values of 5-6 obtained in their study. This indicates that there is increased production of phenolic acids by fungi in the presence of substrates compared to artificial microbial growth media.

Table 4. Mean pH in batch removal experiment

Retention time (hours)	Sawdust Particle size (mm)			Control treatment		
	0.6	1.18	2.36	Unsorted inoculated	Unsorted uninoculated	Untreated wastewater
10	3.4 ± 0.0	2.7 ± 0.0	3.2 ± 0.0	2.4 ± 0.2	6.3 ± 0.0	6.4 ± 0.2
12	2.6 ± 0.1	3.0 ± 0.1	3.5 ± 0.1	2.2 ± 0.0	6.5 ± 0.0	7.1 ± 0.1
24	2.2 ± 0.16	2.6 ± 0.1	3.1 ± 0.1	1.8 ± 0.0	6.1 ± 0.0	6.7 ± 0.1
36	2.1 ± 0.1	2.5 ± 0.1	3.0 ± 0.1	1.7 ± 0.0	6.0 ± 0.0	6.6 ± 0.1
48	2.1 ± 0.1	2.5 ± 0.1	3.0 ± 0.1	1.7 ± 0.0	6.0 ± 0.0	6.5 ± 0.1
60	2.1 ± 0.1	2.5 ± 0.1	3.0 ± 0.1	1.7 ± 0.0	6.0 ± 0.0	6.5 ± 0.1
72	1.9 ± 0.1	2.2 ± 0.1	2.7 ± 0.1	1.5 ± 0.0	5.7 ± 0.0	6.3 ± 0.1

Influence of oxidation-reduction potential on removal of bacteria

ORP was measured during this study to determine the oxidative capacity of each treatment reactor. The result showed that mean ORP values ranged from 5.0 ±2.2 mV (raw wastewater, 24 hours) to 232±55 mV (unsorted inoculated, 72 hours) (Tab. 5). The ORP levels were relatively higher in the colonised sawdust compared to uncolonised sawdust. The high ORP is an indication of the active breakdown of hydrogen peroxide to hydroxyl radicals in the treatment systems. Hydroxyl radical generates high oxidative potential (Liu *et al*., 2020).

Correlation studies were done to determine the relationship between explanatory variables. The result revealed that there was no significant correlation between faecal bacteria indicator *E. coli* as well as *Salmonella* spp. and hydrogen peroxide (P>0.05) (Fig. 3).

Table 5. Mean ORP (mV) in batch removal experiment

Retention time (hours)	Sawdust Particle size (mm)			Control treatment		
	0.6	1.18	2.36	Unsorted inoculated	Unsorted uninoculated	Untreated wastewater
10	189.0 ± 14.5	211.3 ± 3.9	191.3 ± 4.6	232.0 ± 5.5	56.8 ± 22.3	21.0 ± 2.2
12	212.5 ± 1.3	198.8 ± 2.2	175.8 ± 1.7	234.0 ± 0.8	18.5 ± 9.3	9.0 ± 4.1
24	206.5 ± 1.3	192.8 ± 2.2	169.8 ± 1.7	228.0 ± 0.8	12.5 ± 9.3	5.0 ± 2.2
36	202.5 ± 1.3	188.8 ± 2.2	165.8 ± 1.7	224.0 ± 0.8	9.8 ± 7.8	2.5 ± 0.6
48	207.5 ± 1.3	193.8 ± 2.2	170.8 ± 1.7	229.0 ± 0.8	14.8 ± 7.8	7.5 ± 0.6
60	209.5 ± 1.3	195.8 ± 2.2	172.8 ± 1.7	231.0 ± 0.8	16.8 ± 7.8	9.5 ± 0.6
72	207.5 ± 1.3	193.8 ± 2.2	170.8 ± 1.7	229.0 ± 0.8	14.8 ± 7.8	7.5 ± 0.6

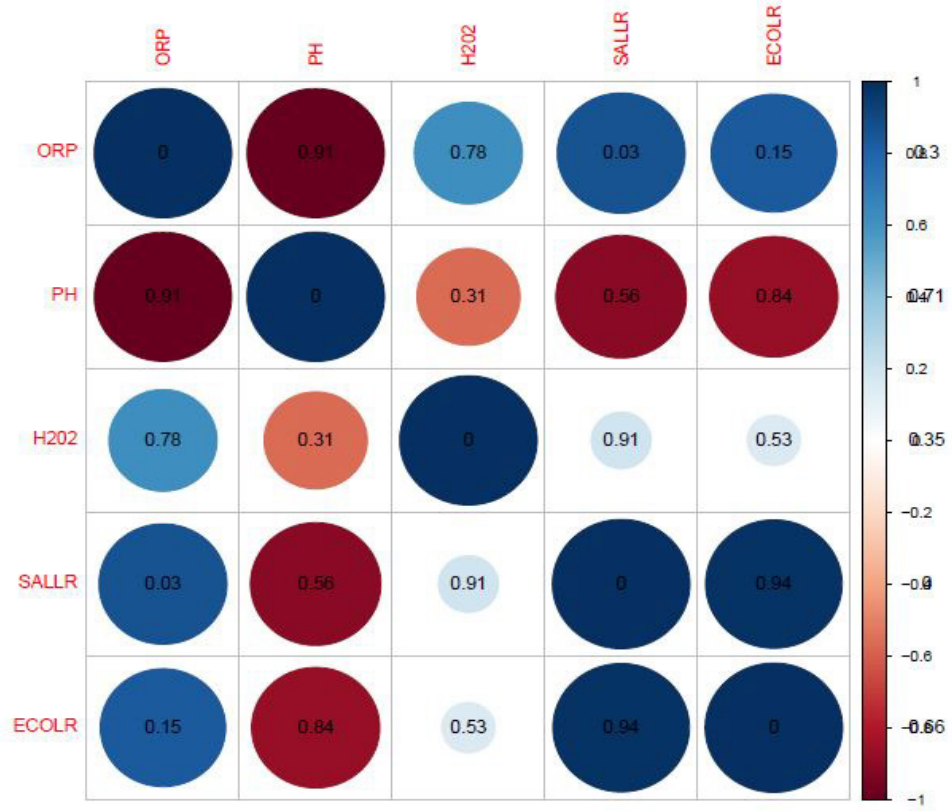

Figure 3. Visual representation of significant correlation among variables in batch treatment study

This observed non-significant correlation may be as a result of the the presence of antimicrobial compounds with bacteriostatic and bactericidal action. Phenolic compounds produced in fungal cultures have been demonstrated to have bactericidal actions against bacteria (Vu *et al*.,2018). The results are consistent with previous studies by Chen et al., 2019, who reported no significant correlation between *E. coli* removal and commercial hydrogen peroxide in their study using chemical Fenton for bacteria removal from wastewater. There was also no significant correlation between faecal indicator bacteria *E. coli* and *Salmonella* spp. during this study. This could imply that *E. coli* may not be the best indicator to establish the presence of faecal pathogens such as *Salmonella* spp. in mycofiltration studies. As previously reported, some *E. coli* strains can be subjected to a viable but not culturable state (VBNC) by some treatment systems and under that condition they may not be detectable while the actual pathogen is detected in high levels (George *et al*., 2002).

Further, the post-hoc test showed that the factors independent variables considered in this study (sawdust particle size and detention time) had an overall influence of 57 % and 74 % respectively on all dependent variables (*E. coli* and *Salmonella* spp. log removal, hydrogen peroxide concentration, pH and ORP) (Tab. 6). It also showed that the interaction between the two independent variables had an 80 % overall influence on all dependent variables. The individual size effect showed that treatment was significantly responsible for 82 and 99 % log removal in *E. coli* and *Salmonella* spp. respectively. Treatment time was also responsible for 73 and 99 % of the observed variation in *E. coli* and *Salmonella* spp. log removal respectively. Detention time was also responsible for 73 and 99 % of the observed variation in *E. coli* and *Salmonella* spp. log removal respectively.

Table 6. Estimated effect size table at 0.05 level for batch treatment experiment

Parameter	Particle size	Retention time	Particle size and retention time
Overall effect	57	74	80
E. coli log removal	82	73	60
Salmonella spp. log removal	99	99	99
Hydrogen peroxide	99	99	20
pH	99	94	84
Oxidation-reduction potential	99	56	65

Conclusion

The results of this study showed that particle size plays a role in the efficiency of mycelium-colonised sawdust used for the removal of faecal bacteria in batch treatment. While the removal of *E. coli* was most effective in treatment with sawdust of particle size 2.36, the removal of *Salmonella* spp. was the highest in the treatment system with sawdust of particle size 1.18 at 72 hours.

The study also showed that apart from sawdust particles which was the main factor considered in this study, detention time also had a significant influence on bacteria removal. This may be because antimicrobial compounds require some contact time to react with bacteria cell wall components before eventual killing of the whole bacterial cell. The concentration of hydrogen peroxide was also influenced significantly by sawdust particle size. The study showed that the removal of bacteria in batch treatment might also be influenced by phenolic compounds produced by fungi.

Therefore, there is a need to further study this system in a bid to optimize the production of hydrogen peroxide and other antimicrobial compounds that may be present for the large-scale removal of faecal bacteria from biological wastewater with a high load of these organisms. This can serve as a cheap and green eco-biotechnological way of slaughterhouse wastewater management.

Acknowledgement We wish to thank the Regional Water and Environmental Sanitation Centre, Kumasi (R.W.E.S.C.K) for providing funding for this work through support from the Ghanaian government and the World Bank under the African Centre of Excellence project.

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Antibacterial activity on methicillin-resistant *Staphylococcus aureus* (MRSA) and antioxidant properties of silver nanoparticles synthesized by *Hunteria umbellata* (K. Schum.) seeds

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Abstract. Silver nanoparticles (AgNPs) have gained significant attention over the years due to their unique physicochemical properties and diverse applications in various areas including medicine, electronics, and so on. *Hunteria umbellata* (*HU*), is a glabrous tree native to West Africa that belongs to the Apocynaceae family with various medicinal properties. The medicinal applications; anti-microbial and anti-oxidant properties of synthesized and characterized silver nanoparticles by using *HU* seeds, were studied. Aqueous and ethanol extracts of *HU* were obtained for the synthesis process. UV-Vis spectra analysis was used to reveal the distinct absorption patterns for AgNPs synthesized by ethanol and aqueous extracts. FTIR spectra exhibited characteristic transmittance peaks, indicating the presence of functional groups such as hydroxyl groups. XRD analysis confirmed the crystalline nature of AgNPs with identifiable peaks at specific planes while SEM/EDX was used to provide insights into the size distribution and morphology of AgNPs, reinforcing the data from other characterization methods. Medicinal properties were assessed through antibacterial assays using Methicillin-Resistant *Staphylococcus aureus* (MRSA) and DPPH radical scavenging activity, showcasing the potential

biomedical applications of AgNPs-*HU* complex. Comparative studies with standard compounds like ascorbic acid validated their efficacy as antioxidants. The findings of this study suggest promising antibacterial and antioxidant properties of AgNPs-*HU* complex synthesized by *HU* seeds extracts and also contribute to the understanding of nanomedicine and underscore the potential of green synthesis for biomedical applications.

Keywords: *characterization, Hunteria umbellata,* plant extracts, silver nanoparticles, synthesis.

Introduction

Over the years, from the 1960s up until today Methicillin-resistant *staphylococcu aureus* (MRSA) has continued to be disseminated globally from human to human among healthcare workers, communities, and individuals. The burden of MRSA dissemination can be attributed to geographical variation with differences that may occur from local infection practices, management of the disease, awareness of the infection, and circulating clones. The epidemiology of MRSA has seen its ravaging effect in some parts of Europe where <5% of *S. aureus* isolates from invasive cases were reported to be MRSA (Köck *et al*., 2010; Tacconelli *et al*., 2018); report in America stated that 50% of all skin and tissue infections are clinical isolates of MRSA (Styers, 2006). MRSA is predominantly endemic in Asia with over 50% of *S. aureus* traced to bloodstream infections (Mendes *et al*., 2011; Chen & Huang, 2014). Nigeria and South Africa are the leading countries with the highest MRSA figures in Africa with over <25% (Falagas *et al*., 2013) prevalence rate, owing to the incidence of HIV infection, lack of infection control practices, and availability/use of antibiotics. In Nigeria for example, MRSA is predominantly identified at the hospital laboratory by chance or cases of patients repeated treatment with antibiotics with little or no effect. Over the years, clinicians have repeatedly used combination therapy of vancomycin with β-Lactams as an alternative treatment regime for MRSA (Bal *et al*., 2017; Purrello *et al*., 2016), however, hospital cases have shown that these combination therapies may no longer be effect again to combat MRSA today because of the clinical manifestation, virulence factors and *S. aureus* new clones (staphylococcal cassette chromosome mec- SCCmec), hence the need to search for alternative treatment regimen to combat MRSA. Andrade *et al*. (2023) suggested the use of targeted Nanoparticles to fight MRSA, however, he concluded that these nanoparticles could be used via antibiotic-independent

activity and/or serve as drug delivery systems (DDSs). We believe that sylva nanoparticles (AgNPs) may be explored in the nanofabrication of antibacterial substances that can be used to combat MRSA. The use of green synthesis particularly plant-based synthesis was preferred for this study because of safety consigns and wide acceptability. *Hunteria umbellata* (*HU*) on the other hand has shown relatively reducing potential and medicinal applications which necessitated its use for this study.

A variety of techniques are combined under the umbrella of nanotechnology to work at the nanoscale to design, create, characterize, and apply materials, systems, devices, and structures (Hornyak et al., 2018). This scale usually applies to sizes less than 100 nanometers. AgNPs have garnered attention across various sectors like medicine, food, and healthcare due to their exceptional physical and chemical attributes, such as high electrical conductivity, and biological, thermal, and chemical properties (Galatage *et al.*, 2021). These distinctive traits make AgNPs suitable for applications such as antimicrobial agents, biosensor materials, and cosmetic products (Bamal *et al.*, 2021). In recent times, researchers have focused on AgNPs due to their effectiveness against a wide range of microorganisms and the emergence of drug-resistant strains against conventional antibiotics (Prasher *et al.*, 2018; Mateo & Jiménez, 2022; More *et al.*, 2023;). The synthesis of AgNPs falls into three main categories; physical methods, chemical methods, and biological methods. Physical methods involve techniques like evaporation-condensation using a tube furnace under atmospheric pressure (Bouafia *et al.*, 2021; Nguyen *et al.*, 2023). In the process of preparing AgNPs, chemical methods use organic solvents or water, whereas biological methods also referred to as "green synthesis" rely on non-toxic substances to function as reducing agents and solvents. (Kanwar *et al.*, 2021; Patel *et al.*, 2023).

Characterization is essential after AgNPs are synthesized because their physicochemical characteristics have a big impact on how they behave biologically. A comprehensive characterization of prepared nanoparticles is necessary for their efficient use in nanomedicine, healthcare, or other applications related to human welfare. Various analytical techniques such as ultraviolet spectroscopy (UV-vis spectroscopy), X-ray diffractometry (XRD), Fourier Transform Infrared Spectroscopy (FTIR), transmission electron microscopy (TEM), scanning electron microscope (SEM), Dynamic Light Scattering (DLS) analysis, energy-dispersive X-ray spectroscopy (EDS), among others, are employed to evaluate synthesized nanomaterial (Catalano *et al.*, 2021; Selvan *et al.*, 2021; Ullah *et al.*, 2024).

H. umbellata (HU), a glabrous tree native to West Africa, is characterized by broad leaves, creamy to pale yellow flowers, and yellow smooth fruits, commonly known as Erinor abeere (Yoruba) Osu (Edo), Nkpokiri (Igbo), this plant

belongs to the Apocynaceae family (Okolafor & Ekhaıse, 2021). Historically, *HU* has been used in traditional medicine to treat various ailments such as malaria, diarrhea, diabetes mellitus, gastric ulcers, and skin conditions. Recent studies have highlighted its antimicrobial (Aderele *et al.*, 2020), anti-inflammatory (Ahajumobi & Anderson, 2022), anti-diabetic (Aderele *et al.*, 2020; Okolafor & Ekhaıse, 2021) and antioxidant properties (Edosuyi *et al.*, 2018; Oboh *et al.*, 2018; Ogunlana *et al.*, 2021), particularly in extracts from its leaves, bark, fruit, pulp and seeds. A report on the synthesis of AgNPs by *HU* seeds and the application of the synthesized NPs as antibacterial and antioxidant properties is lacking in the literature which necessitated this study. This study focuses on the use of aqueous and ethanol seed extracts of *HU* as a biological precursor for the synthesis of AgNPs, and its antibacterial effect on Methicillin-Resistant *Staphylococcus aureus* (MRSA) and antioxidant properties.

Materials and methods

Sample collection and preparation

Dried seeds of *HU* were purchased at the Uwa Market, Benin City, Nigeria, in January 2024. The epicarps of the seeds were removed and air-dried to obtain constant weight. The dried seeds of *HU* were pounded using a mortar and pestle, it was then ground using a manual grinder. Subsequently, the seeds were further pulverized into a powdered form using a electronic blender (Model; KCB239K), and the pulverized seeds were stored in an airtight container.

Extraction of plant materials

Ethanolic extraction. Pulverized dried *HU* seeds (200g) were introduced into a thimble of soxhlet extractors (Model; KEX 250 (F) B00232348) and ethanol was added to a round bottom flask which was placed on the heating mantle of the soxhlet extractor, with a small amount of ethanol poured into the soxhlet extractor column to wet the thimble. The extraction process was monitored for 48 hours until completion. After the extraction, the extracted solvent was concentrated using a rotary evaporator (Model; DUAB RE100-Pro). The extracts obtained from the previous soxhlet extraction phase were transferred into round bottom flasks. Glycerol was applied to the tip of the round flat bottom flask for ease of removal. The rotary evaporator settings were adjusted to 86°C and 276 rpm, and the concentration of samples were monitored for 14 hours. The resultant crude extracts were then stored in cream jars and subjected to freeze-drying to -55°C (Model; BK-FD189). The recovered ethanol was carefully preserved in separate Winchester bottles for future use.

Aqueous extraction. Pulverized *HU* seeds (100g); were measured into a beaker and 800ml distilled water was poured and stirred using a glass stirring rod. The mixture was stirred and heated on a hot plate (Model; Heat-stir-SD162) continuously at 70°C for 3 hrs and was allowed to cool. After cooling, the mixture underwent filtration using a Teflon cloth to remove solid residues. The filtrate was then transferred into a separating funnel setup. The supernatant was subjected to further drying on a hot plate until it attained a paste-like consistency, which was then transferred to cream jars and subjected to freeze-drying for preservation (Okolafor & Ekhaıse, 2021).

Synthesis of Ag nanoparticles

Silver nitrate ($AgNO_3$) solution (1 mM) was prepared by dissolving 0.01697g silver nitrate powder in 1000 ml distilled water. One gramme (1g) of aqueous and methanol extracts of *HU* seed was carefully weighed and dissolved in 5 milliliters of Tris-HCl buffer (Tris hydroxymethyl aminomethane) and the pH was adjusted to 7.0. The prepared $AgNO_3$ solution was mixed with the dissolved *HU* extracts (1:1), and vortexed using a vortex mixer (PV-1 Grant-bio). The mixture was allowed to incubate at room temperature for 24 to 48 hrs. After the synthesis, the mixture was centrifuged using a high-speed centrifuge at 15000 rpm for 10 minutes. The supernatant cells were harvested and used for further characterization and applications.

Preparation of samples for characterization

The supernatant cells harvested from the synthesis were repeatedly washed with Tris-HCl buffer (pH 7.0) and distilled water. The purified harvest cells were smeared on a microscopic slide by emulsification using sterile deionized water. The smear was allowed to air dry on the slide and heat was fixed by passing through flame repeatedly. The heat-fixed slide was used for FTIR, XRD, and SEM characterization.

Characterization of $AgNO_3$ nanoparticles

One milliliter aliquot of the synthesis was collected daily for the UV-Vis characterization, while Trs-HCl (Ph 7.0) buffer was used as the blank. UV-Vis spectroscopy (UV-6300PC double beam spectrometer) studies were performed for analysis of reflectance or absorbance of the sample at different wavelengths (250 to 850 nm). Fourier transform infrared spectroscopy (FTIR Thermo scientific Nicolet iS5) studies were used for the analysis of chemical bonding and functional groups. X-ray diffraction (XRD, Shimadzu XDS 2400H diffractometer with Cu anode control, 40 KV, 30 M.A, optics: Automatic divergence slit), in Bragg-

Brentano configuration, using Cu-Kα radiation (1.54Å) was used for the identification of its crystalline structure and morphology. The XRD pattern was recorded in the 2θ range between 0° to 60° at a scan speed of 0.017°/14s. The particles were coated in gold and were then analyzed using scanning electron microscopy (Hitachi SU 3500 scanning microscope, Tokyo, Japan).

Antimicrobial properties

The antimicrobial properties of AgNPs-*HU* complex synthesized using aqueous and methanol extracts of *HU* were tested against clinical strains of Methicillin-Resistant *Staphylococcus aureus* (MRSA) obtained from the University of Benin Teaching Hospital (UBTH). The antimicrobial properties of the AgNPs were tested using a microplate broth dilution method described by López-Malo *et al.* (2020). The MRSA was standardized to 0.5 McFarland and amoxicillin tablet was used as the control drug. Different concentrations (78.44, 156.9, 313.8, and 627.5, mg/L) of the synthesized AgNPs were introduced into a peptone broth containing standardized MRSA incubated in a microplate at 37^0C under shaking condition for 24 hr. The bacteria inhibition by AgNPs was calculated using the following formula:

$$\%\, Bacteria\ inhibition = \frac{Absorbance\ of\ control\ drug - Absorbance\ of\ sample\ (AgNPs)}{Absorbance\ of\ control\ drug} \times \frac{100}{1} \quad (1)$$

To determine the half-maximal effective concentration (EC_{50}) of AgNPs synthesized by aqueous and methanol extracts of *HU* as an antibacterial agent, a non linear curve fitting was computed on Origin Pro 9.0 using the following derivative formula:

$$y = A1 + \frac{A2 - A1}{1 + 10^{(Logx0 - x)P}} \quad (2)$$

Where the X values are supposed to be the logarithm of the dose and LOGx0 is the center of the curve, that is, the concentration for the half response. So we can compute the EC_{50} by:

$$EC_{50} = 10^{Logx0} \quad (3)$$

Antioxidant properties

The antioxidant properties of AgNP were determined by 2,2-Diphenyl-1-picrylhydrazyl (DPPH) scavenging method (Sharma & Kumar, 2011). DPPH power (0.01 g) was dissolved in 50 ml methanol to give 100 µg/ml stock concentration. From the stock concentration, 80, 60, 40, and 20 µg/ml concentrations were

diluted while methanol served as the blank. The prepared 1g AgNPs were mixed with the varied concentration of DPPH preparation and allowed to incubate for 15 minutes. Ascorbic acid was used as the control antioxidant. The antioxidant properties of AgNPs were determined using the formula:

$$\%\ inhibition = \frac{Absorbance\ of\ control - Absorbance\ of\ test\ sample}{Absorbance\ of\ control} \times \frac{100}{1} \quad (4)$$

To determine the half-maximal effective concentration (EC_{50}) of AgNPs synthesized by aqueous and methanol extracts of *HU* as an antioxidant, a non-linear curve fitting was also computed on Origin Pro 9.0 using the derivative formula in equations 2 and 3 above.

Results

UV-Vis Spectroscopy

UV-Vis spectroscopy is one non-invasive and fast real-time method available to monitor and obtain a reliable measurement of nanoscale-related properties. The UV-VIS spectroscopy results (Figure 1) of AgNPs revealed high peaks at 450 and 500 nm at 72/24 hrs for ethanol and aqueous extracts of *HU* respectively.

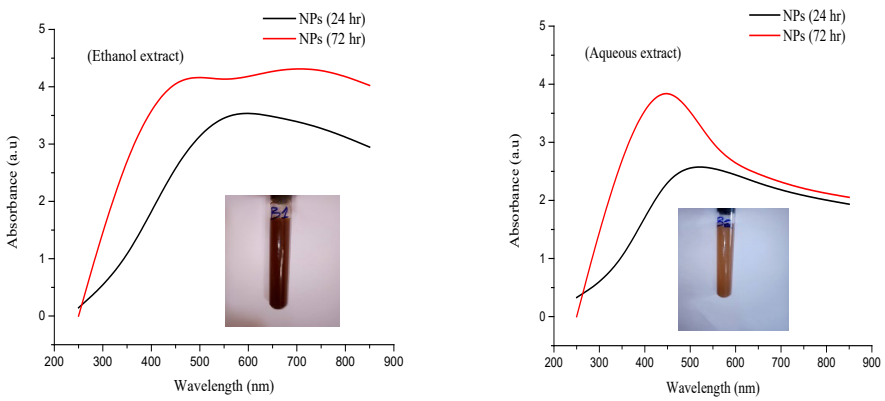

Figure 1. UV-VIS spectra of AgNPs synthesized by ethanol and aqueous extracts of *HU*.

Fourier transform infrared spectroscopy (FTIR)

The FTIR analysis provided insights into the types of molecules and the functional groups present on the surface of AgNPs synthesized. Figure 2 shows variations in light transmission at different wavelengths, indicating the involvement of various molecules in the synthesis process. High % transmission at specific wavelengths suggests the presence of molecules that might contribute to the stability and potential medicinal activity of the AgNPs synthesized by *HU*.

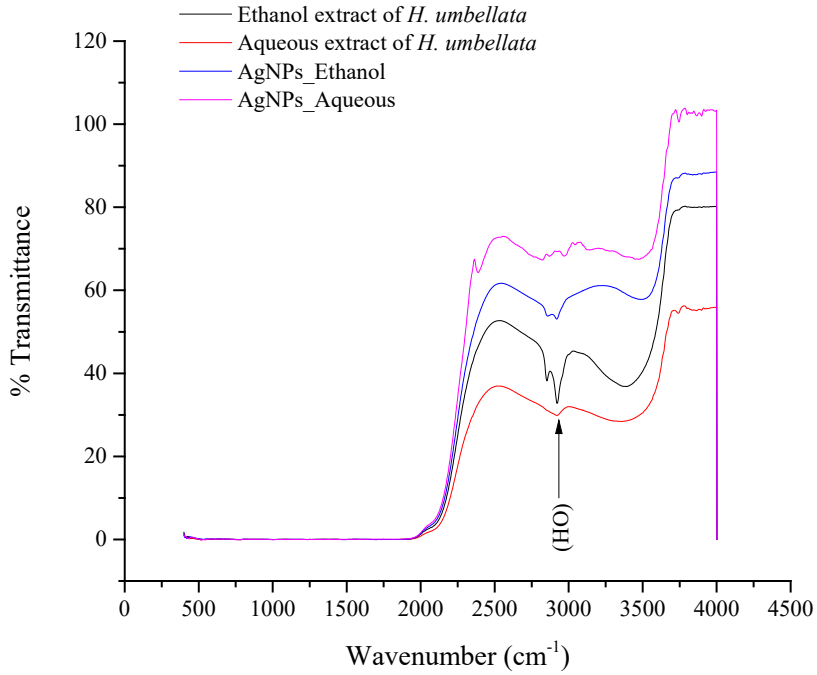

Figure 2. FTIR spectra of AgNPs synthesized by ethanol and aqueous extracts of *HU*.

X-ray diffraction spectroscopy

The X-ray diffraction (XRD) analysis has shorter electromagnetic wavelength radiation that can be used for the determination of the degree of crystallinity, and deviation from a compound of interest. Figure 3 shows the crystallinity of AgNPs by aqueous and ethanol extracts of *HU* which was confirmed by comparing with the standard Joint Committee on Powder Diffraction Standards

(JCPDS) card number 00-004-0783. The peaks at 2θ with the corresponding Miller indices (hkl) in parenthesis for AgNPs by ethanol and aqueous extracts of *HU* were 24.12 (111), 26.1 (200), 32.5 (220), 31.4 (311), 32.4 (222), 45.0 (400) and 32.3 (111), 35.0 (220), 45.0 (222), 51.4 (400) respectively.

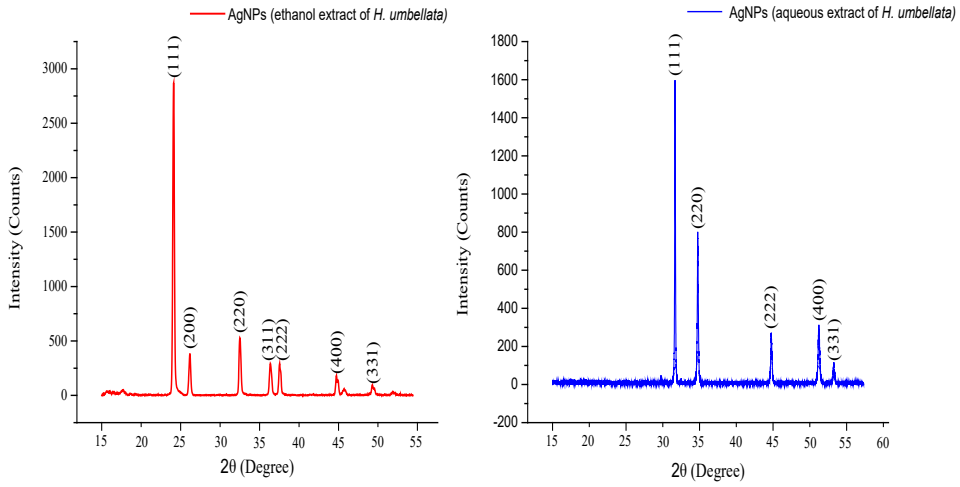

Figure 3. XRD characterization of AgNPs synthesized by ethanol and aqueous extracts of *HU*

Scanning Electron microscopy (SEM)

Scanning electron microscopy (SEM) analysis revealed information about the size distribution and shapes of the AgNPs. Figures 4 & 5 illustrate the surface features of nanoparticles synthesized by ethanol and aqueous extracts of *HU*, complementing the data obtained from other characterization techniques. The EDX characterization confirmed the crystalline and elemental composition of the AgNPs, however, other elements such as O, Na, Al, P, Ca, Cl, Cu, and Fe were captured in trace amounts after the synthesis, whereas Ag was the most prominent element.

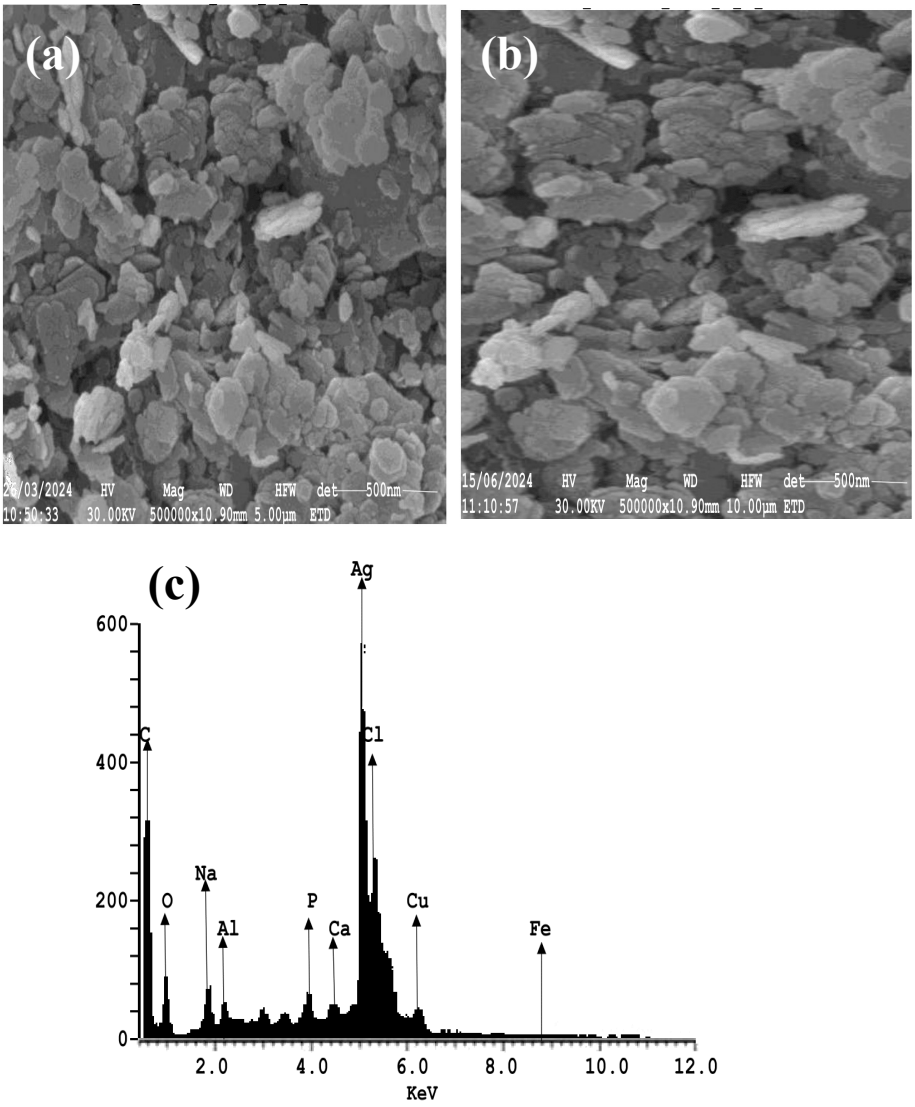

Figure 4. SEM/EDX characterization of AgNPs synthesized by ethanol extracts of *HU* (a and b: SEM angles of capture; c: EDX)

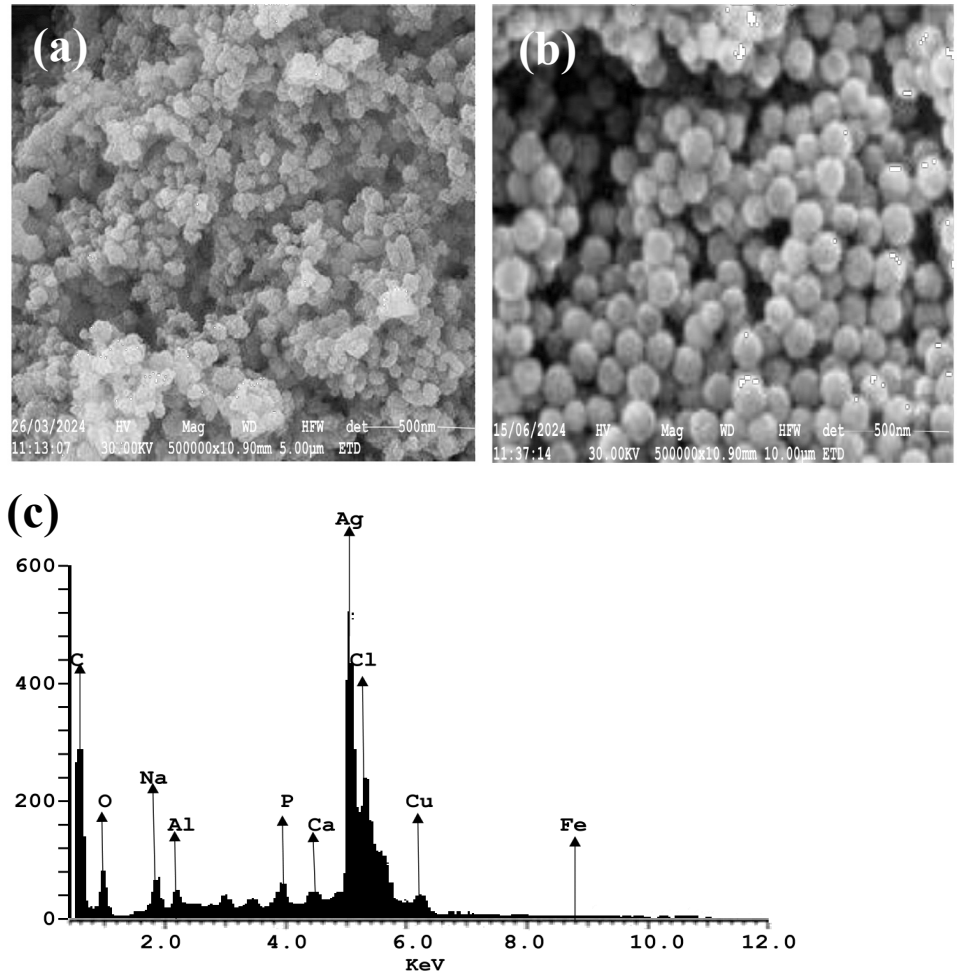

Figure 5. SEM/EDX characterization of AgNPs synthesized by aqueous extracts of *HU* (a and b: SEM angles of capture; c: EDX)

DPPH free radical scavenging activities of AgNPs-HU complex

The DPPH activity of AgNPs-*HU* complex synthesized by ethanol and aqueous extracts of *HU* showed radical scavenging activities compared to the standard (ascorbic acid). The result in Figure 6 is the linear regression equation comparing the DPPH activity at 20 to 100 µ/ml concentration. Table 1 and

Figure 7 show the ANOVA summary Table to compare the radical scavenging activity of AgNPs synthesized by ethanol and aqueous extracts of *HU*/standard and Half-maximal inhibitory concentration of radical scavenging activity of AgNPs-*HU* complex synthesized by *HU* extracts respectively.

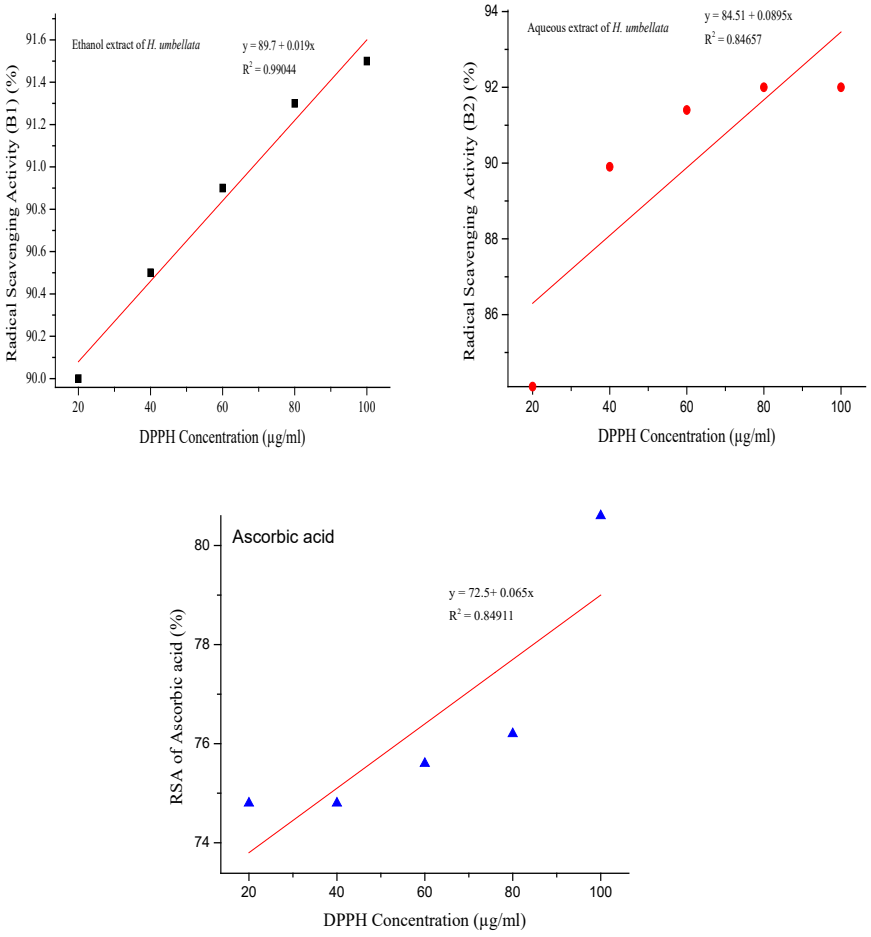

Figure 6. Radical scavenging activity of AgNPs synthesized by ethanol and aqueous extracts of *HU* and standard (ascorbic acid).

Table 1: ANOVA summary Table to compare the radical scavenging activity of AgNPs synthesized by ethanol and aqueous extracts of *HU* and standard (Ascorbic acid)

Sample		DF	Sum of squares	Mean square	F Value	Prob>F
AgNPs by ethanol extract of *H. umbellata*	Model	1	1.444	1.444	154.7	0.001
	Error	3	0.028	0.009		
	Total	4	1.472			
AgNPs by aqueous extract of *H. umbellata*	Model	1	32.04	32.04	7.588	0.07
	Error	3	12.67	4.222		
	Total	4	44.71			
RSA of Ascorbic acid	Model	1	16.9	16.9	7.752	0.069
	Error	3	6.54	2.18		
	Total	4	23.44			

Significant at $p<0.05$

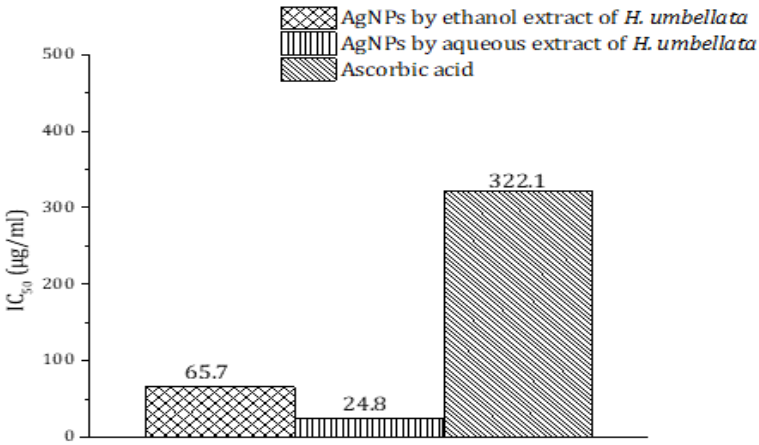

Figure 7. The half-maximal inhibitory concentration of Radical scavenging activity of AgNPs synthesized by ethanol and aqueous (B2) extracts of *HU* and standard (ascorbic acid).

Antimicrobial application

The result of the antimicrobial inhibition of MRSA by AgNPs-*HU* complex synthesized by ethanol and aqueous extracts of *HU* is presented in Figure 8 below. The result indicated that aqueous extracts of AgNPs-*HU* complex had a

percentage bacteria inhibition above 60% at a concentration of 800 mg/L whereas ethanol/aqueous extracts of HU-AgNPs complex at a concentration of 200 to 600 mg/L showed bacteria inhibition below 40% threshold. The result of Half maximal effective concentration of AgNPs-*HU* complex synthesized by ethanol and aqueous of *HU* against Methicillin-Resistant *Staphylococcus aureus* (MRSA) is also presented in Figure 9.

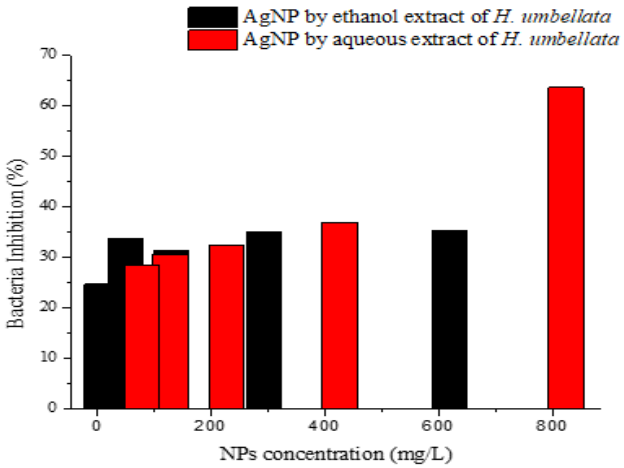

Figure 8. Antimicrobial inhibition of Methicillin-resistant *Staphylococcus aureus* (MRSA) by Ag NPs synthesized from ethanol (B1) and aqueous (B2) extracts of *HU.*

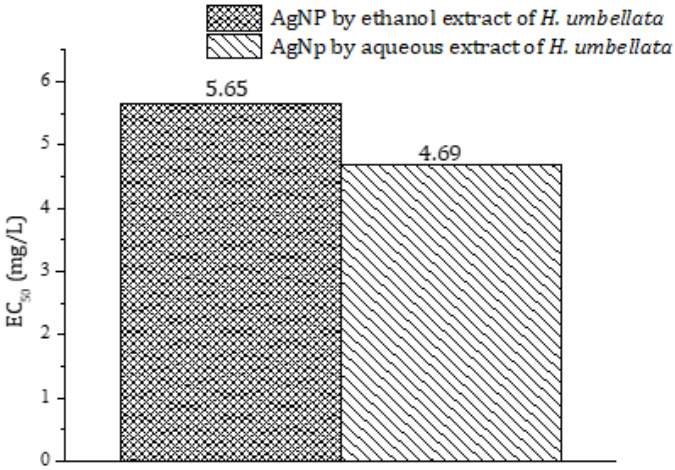

Figure 9. The half-maximal effective concentration of AgNPs synthesized by ethanol and aqueous (B2) extracts of *HU* against Methicillin-Resistant *Staphylococcus aureus* (MRSA).

Discussion

The UV-Vis characterization slightly agreed with the report of Asif *et al.* (2022) who averred that green synthesized AgNPs formed intense surface plasmon resonance (SPR) peaks at 370 – 470 nm. The SPR is a function of the morphology, shape, size, and chemical composition of AgNPs in a liquid medium. These absorption values at wavelength 370 to 470 showed how efficiently the nanoparticles were produced and how stable they were over time. The UV-Vis result revealed that ethanol extract was more stable at 24/72 hrs compared to aqueous extract. The functional groups present in the AgNPs by *HU* were -OH-stretch. A strong absorption IR band between 3200 to 3600 is regarded as OH-stretching indicating that the compound is an alcohol group (Liu, 2021). The strong OH stretching bands may indicate the presence of phenols and flavonoids (Sharma *et al.*, 2020) present in the ethanol and aqueous extracts of *HU*.

The specific crystal planes, signifying the formation of well-defined nanoparticles. Although there were no reports on the XRD characterization of AgNPs by *HU,* however the hkl crystalline planes for *Olea europaea* leaf extract (Sharma *et al.*, 2020), *Annona squamosa* leaf extract (Vivek *et al.*, 2012), *Salvia verticillata* and *Filipendula ulmaria* extracts (Mihailović *et al.*, 2023) were similar with that of *HU* in this study. It is therefore safe to state that green synthesis of AgNPs using plant extracts under XRD characterization forms similar corresponding Miller indices (hkl). The SEM micrograph of AgNPs synthesized by ethanol extracts of *HU* formed an irregular shape distribution compared to AgNPs synthesized by aqueous extracts of *HU* where the shape distribution was well defined. The shape morphology of the AgNPs by ethanol extract of *HU* may be attributed to the alcohol used for the extraction of the plant. The SEM micrograph revealed that AgNPs synthesized by aqueous extracts of *HU* are more stable however, formed agglomeration owing to the weak force binding them together. The strong EDX signal at 5.0 Kev recorded by the AgNPs synthesized by *HU* depicts strong surface plasmon resonance (SPR). SPR is an electronic technique used to monitor responses from cellular activities (Nguyen *et al*., 2015).

This study revealed near-perfect linear regression line for ethanol extracts (R^2=0.99044) and ascorbic acid (R^2= 0.84911), while AgNPs synthesized by aqueous extract of *HU* recorded a scatter graph (R^2=0.84657). The R^2 values equal to or close to 1 are regarded as a perfect linear regression relationship. The result of the comparison of the scavenging activity of AgNPs-*HU* complex affirmed that DPPH activity of AgNPs-*HU* complex synthesized by ethanol extract of *HU* ($p<0.001$) had a better scavenging activity compared to ascorbic acid ($p<0.069$) and AgNPs-*HU* complex synthesized by aqueous extract of *HU* ($p<0.07$). The IC_{50} values were lowest for AgNPs synthesized by ethanol

(IC_{50} = 65.7) and aqueous (IC_{50} = 24.5) extracts of *HU* compared to ascorbic acid (IC_{50} = 322.1). The report by Adeneye *et al.* (2011) and Abubakar *et al.* (2019) on the antioxidant properties of *HU* recorded IC_{50} values > 150 compared to the combined antioxidant properties of AgNPs-*HU* complex. We can confidently infer that the antioxidant activities of AgNPs-*HU* complex synthesized by ethanol and aqueous extracts of *HU* will be far better than the antioxidant activities of *HU* extracts alone.

The antibacterial properties of AgNPs synthesized by ethanol and aqueous extracts of *HU* against Methicillin-Resistant *Staphylococcus aureus* (MRSA) showed the same level of bacteria inhibition (<35% inhibition) for ethanol and aqueous extracts of *HU*, however, at highest concentration (>80 mg/L), the bacteria activity showed 60% bacteria inhibition. The EC_{50} values for AgNPs synthesized by ethanol and aqueous extracts of *HU* were 5.65 and 4.69 respectively. The EC50 values may replace the minimum inhibitory concentration (MIC) levels of AgNPs synthesized by ethanol and aqueous extracts of *HU.* The broth dilution method of antibacterial studies remains the best option owing to the precision of the result and accuracy of measurements. Small variations in MIC values for plant-related extracts may be attributed to the stability and microtechnique limitations (Van de Vel *et al.*, 2019).

Conclusion

The process of synthesis and characterization of AgNPs encompasses a variety of approaches, each with its own merits and drawbacks. Green synthesis using ethanol and aqueous extracts of *HU* seeds was explored in this study owing to its reducing properties and medicinal applications. Understanding the characteristics and actions of these nanoparticles is important in fully exploiting their capabilities across different domains, ranging from healthcare to environmental science. The detailed characterization (UV-VIS spectroscopy, FTIR analysis, XRD patterns, and SEM imaging) employed in this study validates the successful creation of stable and biologically effective AgNPs utilizing ethanol and aqueous extracts of *HU*. These nanoparticles exhibited promising diverse applications, particularly as an antibacterial on MRSA and as an antioxidant. We recommend further studies on *HU*-AgNPs complex as alternative treatment regimen for combating new clones of MRSA infections.

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In vivo evaluation of the therapeutic effect of *Streptococcus thermophilus* isolated from camel milk on intestinal disorders

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Abstract. This study aimed to isolate, select, and evaluate lactic acid bacteria possessing probiotic properties. Isolates obtained from camel milk from El-Oued region in Algeria were investigated for their potential effect on intestinal disorders in Wistar rats. The results relating to the selection of probiotic strains confirm that one strain, identified as *Streptococcus thermophilus* exhibited the best probiotic activity, with an important tolerance to different degrees of pH and to bile salts, and a remarkable antibacterial activity and resistance to antibiotics. During *in vivo* studies, the administration of isolated lactic acid bacteria was evaluated after inducing intestinal disorders in rats. The microscopic observations of the histological section of the intestine showed an almost complete disappearance of the damages in the intestinal structure. The haematological parameters were in agreement with the results of the histological sections.

Keywords: camel milk, intestinal disorders, lactic acid bacteria, probiotics, pathogenic bacteria.

Introduction

Lactic acid bacteria (LAB) are among the most widely used bacteria in food fermentations thanks to the production of a wide range of metabolites. Lactic acid bacteria are found in abundance in fermented milk (Mokoena, 2017; Ruiz Rodriguez *et al*., 2017). These bacteria have been used for a long time in the food industry and allow, through their metabolism, to increase the nutritional quality, organoleptic and shelf life of food (Bouguerra, 2021).

Due to their health benefits, some bacteria are widely used as probiotics, such as *Lactobacillus*, *Bifidobacterium* and *Streptococcus*, if these live probiotic bacteria are administered in sufficient quantities, they improve human or animal health (Amrouche, 2005). Probiotics are available in fermented foods such as yogurt, or as nutritional supplements that contain live bacteria for the constitution of the intestinal microbiota (Mokoena, 2017). The beneficial effects of probiotics on the health of the host are, in theory, numerous, but the scientific evidence confirming these claims requires additional investigations. Several clinical studies have already demonstrated the effectiveness of certain probiotics in the treatment of systemic and infectious diseases (Villeger, 2014).

To prove the efficacy of a probiotic strain or product, testing must be performed using increasingly complex systems, ranging from in vitro studies to in vivo animal and human studies. This work aims to highlight the importance of camel milk as a biological source of preferment probiotic autochtone strains. Similarly, this research concerns proving the therapeutic effect of these strains in intestinal disorders in Wistar rats.

Materials and Methods

Besides, pathogenic strains used in the detection of the antibacterial activity come from the Pasteur Institute in Algiers, namely: *Staphylococcus aureus* ATCC 44300; *Pseudomonas aeruginosa* ATCC 9027; *Escherichia coli* ATCC 25922.

Isolation and purification of lactic acid bacteria

Samples of raw milk were taken from a healthy female approximately three or four years old, in the region of the Wilaya of El Megheir (Setil) during February 2022. Camel milking was done in the evening under aseptic conditions to avoid contamination. The lactic acid bacteria were selectively isolated by culture on several media according to the method described by the International Milk Federation in 1999 (Badis *et al.,* 2005). The shape of the bacteria, their Gram type as well as their cell arrangement were determined after Gram staining and only Gram-positive bacteria were selected. The production of catalase by

all lactic acid isolates was detected by the addition of hydrogen peroxide at 10 V (Tabak, 2007). Temperature test makes it possible to distinguish mesophilic lactic acid bacteria from thermophilic lactic acid bacteria (Boullouf, 2016). After inoculation of the M17 broth with the pure cultures, the tubes are incubated at different temperatures (Badis *et al.,* 2005).

The cultures to be tested were inoculated on hypersaline broths containing 2%,4% and 6% NaCl for all strains. After incubation at 30 ° C. for 24 to 72 hours, bacterial growth resulted in turbidity of the culture medium (Guiraud et Galzy, 1980; Badis *et al.,* 2005).

The test of Gallery API S10 was used for biochemical identification of the lactic acid strain. The micro-tubes were inoculated with bacterial suspensions. Following anaerobic incubation at 36°C ± 2°C for 18-24 hours. positive/negative reactions were read and interpreted based on the list of identification profiles (Mehtar and Afsha, 1983).

Selection of probiotic strains

Several *in vitro* and *in vivo* tests were applied for the determination of the probiotic potential of the lactic acid strains.

Acidity tolerance. pH 3 resistance is often used in *in vitro* assays to determine stomach pH resistance. As the food stays for 3 hours, this time limit has been taken into account (Prasad *et al.,* 1998). For this purpose, active cultures (incubated for 16-18 h in M17 broth) were used. The cells were harvested by centrifugation for 10 min. At 2500 rpm, the viable microorganisms were counted after exposure to the acid state for 0.3 h of incubation at 37°C. This process is repeated three times. LAB counts were expressed in log colony-forming units per millilitre (log CFU/ml) (Benyoucef, 2019).

Bile salt tolerance. To exert their beneficial effects in the digestive tract, LAB must resist the toxicity of bile salts which was tested by following the steps of the protocol described by Ruiz *et al.* (2019). For this purpose, to estimate bile tolerance, bile salt was used to perform the bile salt tests at different percentages. The cell pellets were harvested by centrifugation, washed twice and resuspended in phosphate buffer saline (PBS at pH 8) supplemented with 0.3%, 0.5% and 1% bile salts and incubated at 37°C (Hosseini *et al.,* 2009). LAB counts were expressed in (log CFU/ml) (Benyoucef, 2019).

Antibacterial activity. The antibacterial activity of lactic acid strains against selected pathogenic strains was determined using the agar disc diffusion method (Labioui, 2009). After aerobic incubation at 36°C ± 2°C for 18-24 hours, the Petri plates were observed for a zone of inhibition around the discs (Achemchem and Abrini, 2005).

The antimicrobial susceptibility of each LAB was determined using the disk diffusion method described by Zhang *et al.* (2016) against certain antibiotics, including gentamicin (10μg/ml), ofloxacin (5μg/ml), erythromycin (15μg/ml), amoxicillin (25μg/ml), penicillin (10 μg/ml), vancomycin (30 μg/ml) and aztreonam (10μg/ml). The zone of inhibition (diameter in mm) for each antibiotic was measured and expressed as susceptible, S (≥21 mm); intermediate, I (16–20 mm) and resistance, R (≤15 mm) (Guesh *et al.,* 2019).

In vivo evaluation of probiotic properties. The 25 white Wistar rats used in this experiment were supplied from the Pasteur Institute in Algiers, as adult males of rats, with an average body weight of 250 ± 25g at the start of the experiment. In the 15 days of the adaptation phase, all rats received a normal diet. After this phase, the diet of the four target groups was modified by fasting them for 16 hours each day before intragastric gavage for one week. The rats were divided into 5 Lots of on each 5 rats, which were:

Lot 01: uninfected and untreated healthy control.

Lot 02: target rats with a disease of bacterial origin at a dose of 0.5 ml of *E. coli* diluted in a quantity of physiological water, for one week.

Lot 03: target rats with a disease of biochemical origin at a dose of 0.5mL of castor oil each day by force-feeding, for one week.

Lot 04: treatment of rats, indicated for disease of bacterial origin, with a dose of 0.5mL of a probiotic lactic acid bacteria diluted in 5ml of physiological water, for one week.

Lot 05: treatment of rats, indicated for the disease of biochemical origin, with a dose of 0.5mL of a probiotic lactic acid bacterium diluted in a quantity of physiological water (5ml), for one week.

The blood sample was taken when the rats were sacrificed (the rats were fasted for 24 hours before being sacrificed), and the blood taken from each rat was collected in EDTA tubes, and transported in a cooler in the laboratory. The blood was then used for the biochemical parameter assay, consisting in the coplete blood counts (CBC).

The realization of the histological sections of the intestines of the rats was carried out in the laboratory of the faculty and with the assistance of the laboratory of pathological anatomy of the hospital BIN OMAR JILANI of El-Oued. Organ sampling (the intestine) was performed at the end of the sacrifice of the rats after washing the organ with physiological water (NaCl 0.9 %) and then preserved in an appropriate medium (Formal 10%).

Results

Pre-identification of isolates

Macroscopic and microscopic examination. The morphology of lactic acid bacteria is an important criterion for their identification. According to the results of isolation and identification of lactic acid bacteria, their contents revealed results shown in Table 1.

Biochemical and physiological tests. The biochemical and physiological tests make it possible to better list our strains towards the appropriate genera. The results relating to the biochemical and physiological tests are represented the Table 2 and Figure 1.

Table 1. Macroscopic and microscopic observation of isolates

Code	Macromorphology (appearance of colonies)	Micromorphology (bacterial forms)	Gram staining	Assembling mode
S_1	Whitish and cream Rounded Very small	Coccus	+	Chains
S_2	Whitish Rounded Small	Coccus	+	Isolated or diplococcus
S_3	Whitish Rounded Small	Coccus	+	Diplococcus and tetracoccus
S_4	Whitish and cream Rounded or lenticular Variable sizes	Coccus	+	Bacillus

Table 2. Physiological and biochemical criteria of lactic acid bacteria strains

Parameter		S_1	S_2	S_3	S_4
Temp	10 °C	+	+	+	+
	40 °C	+	+	+	+
NACL	2 %	+	+	+	+
	4 %	+	+	+	+
	6%	+	+	+	+
Catalase test		+	+	-	-
ONPG		Yellow (+)	Yellow(+)	Yellow (+)	Yellow (+)
GLU		Yellow(+)	Yellow(+)	Yellow (+)	Yellow (+)
ARA		Yellow (+)	Yellow(+)	Yellow(+)	Yellow (+)
LDC		Yellow (-)	Orang (+)	Orang (+)	Red (+)

Parameter	S_1	S_2	S_3	S_4
ODC	Yellow (-)	Red (+)	Red (+)	Red (+)
CIT	Yellow (-)	Bleu (+)	Glass blue (+)	Glass blue (+)
H_2S	Colourless (-)	Black (+)	Black (+)	Black (+)
URE	Yellow (-)	Red (+)	Red (+)	Red (+)
TDA	Yellow (-)	Brown (+)	Brown (+)	Brown (+)

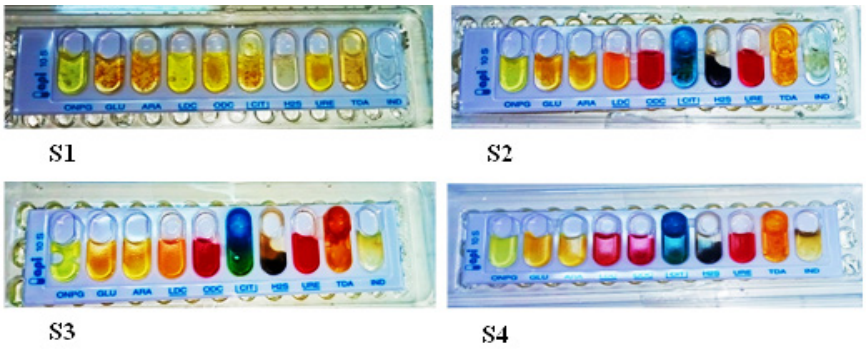

Figure1. API S10 test results.

Selection of strains with probiotic properties

Acidity tolerance. The acid resistance analysis study was carried out under acidic conditions similar to those of the stomach by exposing our strains to different pH: 2, 3 and 4 for 3 hours. The results obtained are presented in Table 3.

Table 3. Effect of acid pH on the viability of lactic acid bacteria (log CFU/ml)

Strain	pH 2		pH 3		pH 4	
	0h	3h	0h	3h	0h	3h
S_1	8.16±0.06	8.38±0.05	8.46±0.02	8.17±0.14	9.97±0.03	10.89±0.07
S_2	9.60±0.03	6.00±0.92[a]	9.31±0.1	7.92±0.16	9.15±0.4	8.42±0.1
S_3	8.54±0.08	8.70±0.09	8.32±0.04	8.77±0.9	8.68±0.01	8.97±0.14
S_4	8.47±0.17	8.47±0.02	9.15±0.22	9.73±0.08	8.85±0.02	9.97±0.03

Mean values (n=3) ± standard deviation (SD). a: highly significant difference($p<0.01$).

There has been little study on probiotic activity since it is generally assumed that *Lactococcus* do not survive during passage through the digestive tract, this is due to the low pH of the stomach, however, several recent works have suggested that *Lactococcus* can survive to reach the human or animal gastrointestinal tract (Kimoto-Nira *et al.,* 2013).

Bile salt tolerance. Bile salts are one of the barriers that probiotic bacteria must cross to gain their site of action. The results for resistance to bile salts are shown in Table 4. Bile tolerance is a determining criterion for the selection of probiotic bacteria, thus allowing survival during passage through the gastrointestinal tract and colonization of the intestinal environment (Marteau and Shamahon, 1998).

Table 4. Effect of different bile salt concentrations on the viability of lactic acid bacteria (log CFU/ml)

Strain	**Bile salts**					
	0.3%		**0.9%**		**1.5%**	
	0h	**3h**	**0h**	**3h**	**0h**	**3h**
S_1	0.95	0.80	1.07	0.20	1.22	1.04
S_2	1.06	1.22	1.20	0.80	1.26	1.10
S_3	1.29	1.23	0.10	0.99	0.50	0.70
S_4	0.58	1.31	1.25	1.10	1.12	1.24

Antibacterial activity. Antimicrobial activity is a very important property in the selection of probiotics, thus allowing the preservation of food and the prevention of gastrointestinal infections (Champomier-Verges *et al.,* 2010; Azat *et al.*, 2016).

The measurement of inhibition zones of isolated strains against selected bacteria by the method of diffusion on an agar disk has been illustrated in Figure 2.

Antibiotic sensitivity. Antibiotic sensitivity tests can be performed using various phenotypic methods. In our study, the selected lactic acid strains were tested using the standardized agar diffusion method (Charteris et *al.*, 1998). For this, eight antibiotics were used, and the results of resistance and sensitivities to the various antibiotics used in this study are presented in Table 5.

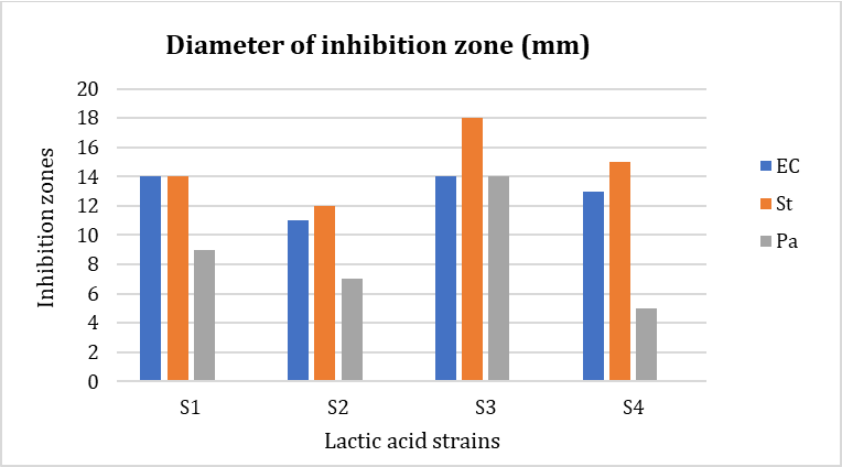

Figure 2. Diameters of inhibition zones obtained by lactic acid bacteria strains against certain pathogenic bacteria (*EC: Escherichia coli; St: Staphylococcus aureus; Pa: Pseudomonas aeruginosa*)

Table 5. Antibiogram of the selected lactic acid strains(mm)

Antibiotic	**Dose (µg)**	**S_1**	**S_2**	**S_3**	**S_4**
Amoxicillin	10	R	R	R	S
Erythromycin	30	R	R	R	R
Vancomycin	30	S	S	I	S
Gentamicin	10	S	R	S	I
Oxacillin	01	R	R	R	R
Penicillin G	10	R	R	R	R
Ofloxacin	05	S	S	S	S
Erythromycin	15	I	R	I	I

R: resistant S: sensitive I: intermediate resistant.

In vivo evaluation of the therapeutic effect on intestinal disorders

Microscopic observations. During the period of infection, we noticed the emergence of several factors, the most important of which are: underweight and diarrheal (a condition indicating that the intestine is irritated).

The histological sections of the organs (small intestines), observed by a microscope with a camera, (Optika, Italy) (objective ×10), are shown in Figure 3.

The observation of the histological section of the intestine in **Lot 1** shows a healthy structure accompanied by a healthy intestinal wall composed of the

mucosa, with villi in a healthy and normal state also a total absence of inflammation and no tissue/cell damage, with an absence of necrosis, so the histological section shows a more or less regular tissue appearance.

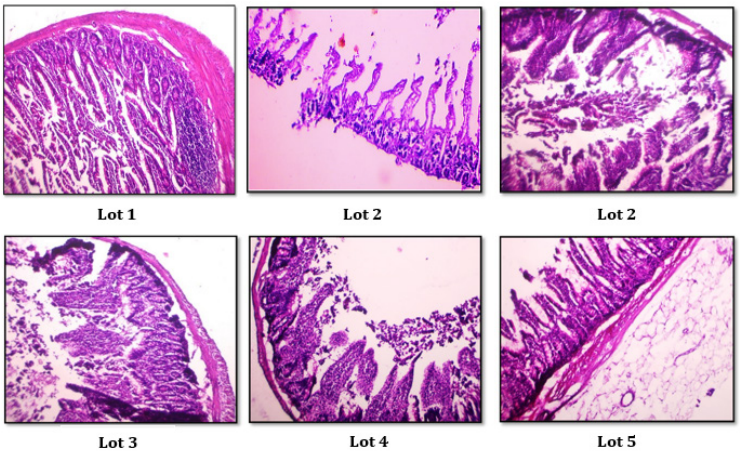

Figure 3. Microscopic observations of the histological small intestine of the rats (100X).

Likewise, microscopic observation of the histological section of the intestine of the rats of **Lot 2** and **3** shows a damaged intestinal structure and some symptoms of irritation including a decrease in the height of the villi and the presence of others destroyed, inflammation (grouping of white blood cells), some villi are necrotized in appearance. In addition to the almost total disappearance of mucous cells.

In the rats in **Lot 4** and **5** and after dissection, the signs of infection were less severe, microscopic observations of the histological sections of the small intestine of the rats show a less affected intestinal structure, and the mucosa seems to be less affected, with a decrease in the rate of inflammation, with a partial return of the height of the villi accompanied by the absence of necrosis.

We also noted a decrease in the severity of inflammation and the absence of necrotic cells and also found the healthy intestinal wall structure with their components, the microscopic study shows that the histological section of the intestine is healthy and has a normal structure compared to the damaged one.

During the period of infection, all rats received a daily dose of the pathogens for a week, the results obtained reveal a harmful effect and damage to the intestines accompanied by structural modification of intestinal behaviours. The results obtained after the treatment period of the rats confirm the beneficial and/or protective effect exerted by *Streptococcus thermophilus* against infection or contamination by whatever the origin of infection.

Haematological parameters. It was observed that the target groups (infected) ***2*** and ***3*** have an increase in the level of red blood cells (6.53 and 7.15 10^3 cel/µl) compared to the other groups (2.61 at 3.5 10^3 cel/µl). This is a symptom of an infection in the body, regardless of the cause (either a bacterial infection or the presence of inflammation). Similarly, it turned out that the third group had an increase in hematocrit levels (37.65%), which corresponded to the concentration of red blood cells in the blood.

It has been observed that the two infected groups recorded the highest values compared to the other groups regarding the number of platelets. The results were probably due to a reaction to an infection, or an inflammatory disease. It has also been observed that the two infected batches showed the highest values of white blood cells (4.95 and 5.1 10^3 cel/µl) compared to the other groups (1.5 to 3.5 10^3 cel/µl). An increase in the number of white blood cells is often due to the body's fight response against an infection. A high white blood cell count may indicate that the immune system is working well to kill pathogens.

The CBC analysis allows ennumeration of red blood cells, white blood cells, HGB, platelets count, and haematocrit measurement. It is applied for diagnosisi of inflammation, infection, and anaemia. The results are illustrated in Figure 4.

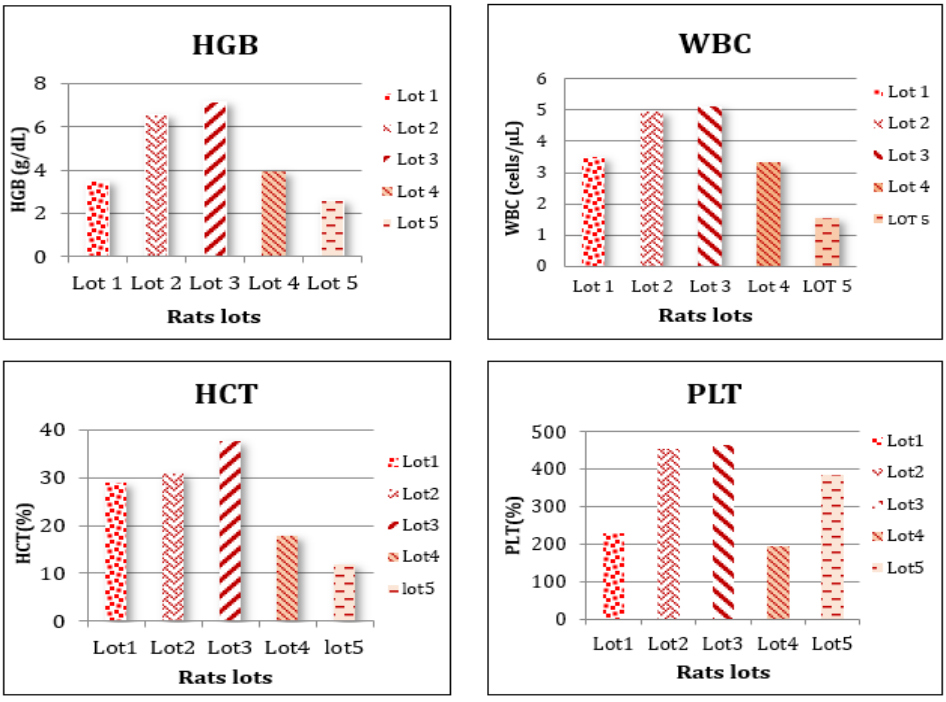

Figure 4. Average values of haematological parameter of rats in experimental groups.

Discussion

Based on morphological, microscopic and biochemical criteria; our isolates can probably be listed in the following species: S1: *Lactococcus lactis,* S2: *Pediococcus acidilactici* S3 and S4: *Streptococcus thermophilus.*

Microscopic observation after Gram staining carried out on the isolated strains shows the following characteristics: all isolates are Gram-positive, in the form of coccus or bacillus, the grouping of cells into chains or isolated or into diplococci and tetrads. Our results were similar and consistent with those obtained by Salhi *et al*., (2020) in terms of macroscopic and microscopic observations.

A significant increase in bacterial growth was noticed for most strains at a temperature of 40 °C. This confirms that lactic acid bacteria are capable of multiplication at high temperatures (Salhi *et al.,* 2020). Regarding the saline tolerance, good growth was observed at a concentration of 2% NaCl in most strains, normal growth in 4%, and little growth in 6% compared to 2% and 4%. The results indicate that probiotic bacteria have a good tolerance to physiological saline concentrations, consistent with the study by Hadj *et al.,* (2013).

The catalase test revealed a negative reaction (no gas bubbles) in the strains S3 and S4, then it is devoid of catalase, this indicates that it is a streptococcus because this bacterium does not produce the catalase enzyme. A positive catalase reaction (presence of gas bubbles) was produced by strains S1 and S2. The enzyme production acts to avoid the toxicity of H_2O_2 by breaking the water and oxygen bond.

In our study, all strains showed a strong resistance to acidity (pH 2, pH 3 and pH 4) after three hours of incubation, these results are in agreement with the results of (Bouguerra, 2021). Our results confirm that lactic acid bacteria can survive and resist deadly acid concentrations, this is consistent with the work of Mathara et *al*.;(2008). It seems that pH 3 does not affect the viability of most of the lactic acid strains tested, they are considered acid-tolerant (Muller *et al.,* 2009); Azat *et al.,* 2016).

According to our results, it turns out that all strains have a tolerance to 0.3%, 0.9% and 1.5% bile salts. Strain S1 was the most affected by the increase in the concentration of bile salts, showing an improvement in the survival rate of bile salts to 1.5%. The results are consistent with the findings of Noriega *et al.,* (2004), which confirmed that several strains of lactic acid bacteria have been stably adapted to salts bile ducts, through a gradual adaptation after growth in bile salt extracts in increasing concentration.

Regarding the antibacterial activity against indicator bacteria, the best inhibition was obtained against *Staphylococcus aureus* with an average inhibion zones of 18 mm, while lactic acid bacteria weakly inhibited *Pseudomonas aeruginosa* with an average of inhibition zones of 5 mm (Davati et *al*. 2015). The inhibitory

properties of LAB are mainly attributed to the production of organic acids, in particular lactic and acetic acids, responsible for the decrease in pH, they also affect the integrity of the cell membrane compromising the viability of the cells and leading in many cases to their lysis. The inhibition of certain pathogenic bacteria can also be associated with the exopolysaccharides secreted by the producing strains, in addition, are capable of inhibiting aerobic pathogenic bacteria by producing CO_2 which creates an anaerobic environment (Denkova et *al.*, 2017).

Most of the strains were sensitive to antibiotics, except for penicillin G, oxacillin and erythromycin, which all strains have been resistant. Several studies have shown the natural resistance of a large range of lactic acid bacteria to antibiotics (Botes et *al*.,2008). According to the results obtained, most of the tasted lactic acid strains are resistant to most antibiotics and very resistant to oxacillin, similar results were obtained by Morandi *et al*., (2013).

The results of all analyses that we obtained led us to choose the best *Streptococcus thermophilus* strain with probiotic properties, to be applied in the in vivo study.

Manjarrez-Hernandez *et al.* (2000) have shown that enteropathogenic *E. coli* (ECEP) could induce lesions (fixation /erasure) in the intestinal epithelium. However, after infection (7 days and after dissection), the almost total disappearance of the villi and the destruction of intestinal behaviours were observed (group 2), with a colour change accompanied by a bad odour (macroscopic observations).

Guergour (2011) in his study of "toxicity of *Ricinus communis* oil, mentioned the signs of poisoning by castor oil and according to the route of administration the oral route is more toxic compared to other routes, the signs are nonspecific (anuria; diarrheal; gastric haemorrhages). This is what we observed after the 3rd group received an oral injection (intragastric gavage) of castor oil. *In vitro* and *in vivo* studies show that taking probiotics reduces the colonization of the digestive tract by pathogenic bacteria and stimulates the specific immune defence response of the host by activating lymphocytes, stimulating anti-tumour activity and reducing infections (Amrouche, 2005).

Compared to the control group, the analysis of CBC confirmed the diagnosis of infection in rats as caused by both methods (infection with castor oil; by *E. coli*). This can also be due to atrophy of the intestinal villi resulting from cell damage. The parameter values of RBC, HCT, PLT and WBC in groups 2 and 3 had higher values compared to the control group. The increase in the level of RBC in the body is a symptom of an infection. The interest of this analysis is to detect possible diseases, in particular haematological, infectious and inflammatory.

The high percentage of HCT analysis may indicate the presence of a bacterial infection in the body, and the higher the analysis, the more severe the infection will be (group 3). This analysis makes it possible to find out if the disease is caused by a bacterial infection. An increase in the level of platelets (PLT) means the presence of infectious diseases, inflammatory diseases, and massive haemorrhage (groups 2 and 3).The *in vitro* and *in vivo* studies show that the selected strain of our study *Streptococcus thermophilus* has a beneficial effect on the regeneration and protection of the intestine, this is confirmed by the results of the blood analysis (CBC) of the treated groups (lot 4 and 5).

Conclusions

Probiotics are very benign microorganisms used as nutritional and medicinal supplements that exert beneficial effects on human and animal health. Certain strains of probiotics have proven long-term safety and efficacy.

From the Eimegheir region, a sample of camel milk was taken under rigorous conditions, the objective of which was to isolate and select lactic strains with probiotic properties, after carrying out identification and isolation tests of the lactic strains and evaluating their properties in vitro, followed by an in vivo study. The four strains isolated were characterized by their form of forms, gram-positive, and catalase-negative. These four isolates are retained and have undergone physiological and biochemical tests for the identification of the species. The species revealed are *Lactococcus lactis, Pediococcus acidilactici., and Streptococcus thermophilus.*

The results obtained through this study show a total disappearance of symptoms of infection and inflammation at the intestinal level after treatment with *Streptococcus thermophilus.* The results of the haematological analysis of the infected groups are in agreement with the results of the histological sections, where the results of the blood count indicated values that the infected rats present symptoms of inflammation on the other hand the treated rats presented values following the standards. Due to these results.

From these results, we conclude this work with the importance of lactic acid bacteria, which have the property of probiotics and their ability to restore the small intestine after damage, as well as their ability to resist pathogenic foreign elements inside the intestinal system.

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Ethics approval. The study protocol approach for laboratory animals followed ethical principles specified in the Declaration of Helsinki and The Council for International Organizations of Medical Sciences (CIOMS). In accordance with ethical health research standards outlined in the Algerian Executive Directive (No 10–90 JORA, dated 18 March 2004), and in compliance with the regulations of Law No. 88 – 08 issued on 26 January 1988, which addresses veterinary medicine activities and the protection of animal health (No JORA: 004 of 27-01-1988), approval for these protocols was granted.

Conflict of interest. The authors declare that they have no conflicts of interest.

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Terrestrial isopod (Crustacea, Isopoda) assemblages near a local road in a forested region from Oaş Mountains, North-Western Romania

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Abstract. Roads are in permanent expansion at the global level and have numerous negative effects, impacting even the litter-dwelling invertebrates from their vicinity. In this context, we studied the terrestrial isopod assemblages from the vicinity of a local road situated in a forested region in Oaş Mountains (North-Western Romania) with the direct collecting method, where we encountered 17 terrestrial isopod species. Most of them were native species related to forested and wet areas. We also recorded species that are endemic to the Carpathian Mountains and species that are rare in the country. Thus, we recorded *Trichoniscus provisorius*, mentioning it for the second time in the country, both distribution records being situated in North-Western Romania. The terrestrial isopod assemblages from the road edges were diverse, as the species number resembles the number previously registered in the natural areas of North-Western Romania. The synanthropic and generalist species and individuals were only a few, recorded especially in the vicinity of the Negreşti-Oaş town. Because this local road is situated in the middle of a region covered with vast and natural forests, it did not succeed in modifying the isopod assemblages, which, even on the road edges, resemble the assemblages from the region's natural habitats.

Keywords: forests, human impact, transportation infrastructure, native species, litter-dwelling invertebrates.

Introduction

Litter-dwelling invertebrates are negatively affected by the road network, a fact manifested through the increased abundance of invasive species and some native generalist species in their vicinity (e.g., Delgado *et al.* 2013). This situation is also true for other invertebrates, whose abundance is higher at a distance from the road (Gagnon *et al.* 2024). The negative influence of this transportation infrastructure can be observed at a greater scale, too, with the increase in the number of roads affecting even ecosystems (Delgado *et al.* 2013). The influence of road networks on invertebrate assemblages was also registered in the case of terrestrial isopods (Vona-Túri *et al.* 2017, 2019). They are detritivores, contributing to the decomposition processes (see in: Zimmer 2002), a fact important especially in the vicinity of roads, where heavy metals and other toxic elements accumulate (e.g. Legret & Pagotto 2006, Werkenthin *et al.* 2014, Ciazela & Siepak 2016). These elements could also accumulate in isopods, which in this way become indicators for the presence of those toxins in the environment (e.g., Dallinger *et al.* 1992, Nannoni *et al.* 2015, Ghemari *et al.* 2017). Isopod assemblages can also offer valuable information about roadsides, reflecting their negative effects by changes registered in abundance and diversity (Vona-Turi *et al.* 2017, 2019). However, these effects are not uniformly applicable to all types of infrastructure, as in the vicinity of some abandoned railways, terrestrial isopod assemblages are diverse, comprising even rare and endemic species (Pop *et al.* 2021a,b). Also, in the case of insects like Collembola, density was more reduced near roads situated in the forest than further inside the forests, at some distance from the road (Hasegawa *et al.* 2015). Spiders and beetles, and especially species linked to forests, were negatively affected by the vicinity of the highway, but the same factor seems to favor the generalist species (Knapp *et al.* 2013). Moreover, road verges are considered important habitats in the case of some grassland invertebrates (Kaur *et al.* 2019). Thus, indeed, the impact of the road network on fauna seems to differ according to the taxa (Gagnon *et al.* 2024).

In North-Western Romania, there are numerous terrestrial isopod species related to wet areas and forests, species which even dominated the assemblages in many cases (e.g., Ferenți *et al.* 2013a,b, Ianc & Ferenți 2014, Ferenți & Covaciu-Marcov 2015). Thus, because invertebrate species linked to forests are negatively affected by roads (Knapp *et al.* 2013), we hypothesized that in a region like North-Western Romania with many isopods related to forests (Ferenți *et al.* 2013a,b, Ianc & Ferenți 2014, Ferenți & Covaciu-Marcov 2015), the impact of roads crossing the forest should reflect on the terrestrial isopod assemblages living on their verges. Because, at least in the case of pollinating

insects, highway verges differ from other road-type verges, as they are more abundant on non-highway roads (Villemey *et al.* 2018), we supposed that in the case of terrestrial isopods, the negative effect of the local road from a forested region will be much reduced compared with the effects of highways and more closed with the effect of railways which does not negatively affect the isopods (Pop *et al.* 2021a,b). Therefore, we chose to study terrestrial isopod assemblages on the verge of a local road from a forested region in North-Western Romania that shelter diverse and relatively well-known terrestrial isopod assemblages (Ferenți *et al.* 2012, 2013a, b). The study's objectives were to establish the specific composition of the terrestrial isopod assemblages on the road verges and their diversity depending on their location and the surrounding habitats.

Materials and methods

Site description

The field study took place on 22 October 2022. All samples were collected on the same day from the same region. The studied region is situated in the northeastern part of Satu Mare County, in the region of Oaș Mountains, near Negrești-Oaș town. Our study focused on the terrestrial isopod assemblages on the road's verges that connect the Negrești-Oaș town with the touristic resort of local interest from Luna Șes. In the region of Luna Șes, there is a concentration of tourist offers (Herman 2012). The road runs, for the most part, parallel with the Talna River. The road passes a mountain region, the Oaș Mountains, which are volcanic (Pătrașcu 1993, Jurje *et al.* 2014, Kovacs *et al.* 2017), rich in mineral resources. Thus, there were mines in the region in the past, most of which are closed nowadays. Present industrial activities are represented by some still-functioning quarries. Even in the vicinity of the studied road, there are large and active stone quarries, but also abandoned mine openings. The region crossed by the studied road is mostly covered by vast, natural, predominantly beech forests. The forest from the Oaș Mountains and other surrounding mountain ranges are dominated by beech (Rob 2008), which was present in the region also in the past (Feurdean & Astaloș 2005). However, in the vicinity of the road surrounded by massive and compact beech forests, there are also sectors with wet areas surrounded by alders, small areas with secondary open areas with grassy vegetation, rocky areas surrounded by forests, and in the case of sector 1, also abandoned buildings.

Sampling

The terrestrial isopods were collected directly under different types of shelters present in the region (logs, stones, debris, etc.), as in other studies (Ferenți *et al.* 2013a, b, Pal *et al.* 2019, Pop *et al.* 2019). Also, we collected samples from the forest litter with the help of a litter sieve. The samples were collected from a maximum distance of 5 meters from the road edges (the edge of the asphalted part). Totally, on the road edge, we collected samples from 10 sites (10 road sectors). Sector 1 was the most downstream, situated right at the limit of Negrești-Oaș town, and sector 10 was the most upstream, situated in the region of Luna Șes touristic resort. Also, where it was possible (and in the case of most sectors, it was possible), we separated the samples from the lower edge of the road (which was situated towards the watercourse) from the ones situated on the upper edge of the road (from the opposite side and usually drier). Nevertheless, there were sectors where this separation was not possible (in the case when there were vertical concrete walls between the road and the watercourse as the road was situated right next to the water).

Species identification and data analysis

The isopods were preserved in test tubes with ethyl alcohol and subsequently determined in the laboratory according to the identification keys (e.g. Radu 1983, 1985, Farkas & Vilisics 2013, Giurginca 2022). After species identification, we calculated the percentage abundance of the isopod species, both totally and according to the sectors, roadside, and shelter types. Also, we calculated the frequency of occurrence using the same datasets. Subsequently, we calculated species diversity with the Shannon index and the similarity between the species composition of different datasets with the Jaccard index. The significance of the differences between sectors, roadsides, and shelter types was calculated with the help of Kruskal-Wallis and Mann-Whitney tests. Also, we used principal component analysis to observe the affinity of different terrestrial isopod species for different sectors, shelter types, and roadsides. The statistics were calculated in the Past software (Hammer *et al.* 2001).

Results

At the vicinity of the road between Negrești-Oaș and Luna Șes we identified 166 individuals which belonged to 17 terrestrial isopod species: *Ligidium hypnorum*, *L. germanicum*, *L. intermedium*, *Trichoniscus provisorius*, *Hyloniscus transsilvanicus*, *H. riparius*, *Cylisticus convexus*, *Porcellium conspersum*, *P. collicola*,

Protracheoniscus politus, *Trachelipus difficilis*, *T. rathkii*, *T. arcuatus*, *T. nodulosus*, *Porcellio scaber*, *Armadillidium vulgare*, *A. versicolor*. Besides those species, we also identified a female from the *Trichoniscus* genus, which could not be assigned to a species. Thus, in reality, 18 terrestrial isopod species were present on the studied road edges. The best-represented species was *P. politus*. This species registered the highest percentage abundance (18.07%), as we collected 30 individuals. *P. politus* was followed by *L. hypnorum* with a percentage abundance of 14.45% and *P. conspersum* with a percentage abundance of 10.24% (Table 1).

Table 1. Percentage abundance (P%), frequency of occurrence (f%) of terrestrial isopod species in the ten sectors (S1 – S10) of the roadside from Oaş Mountains.

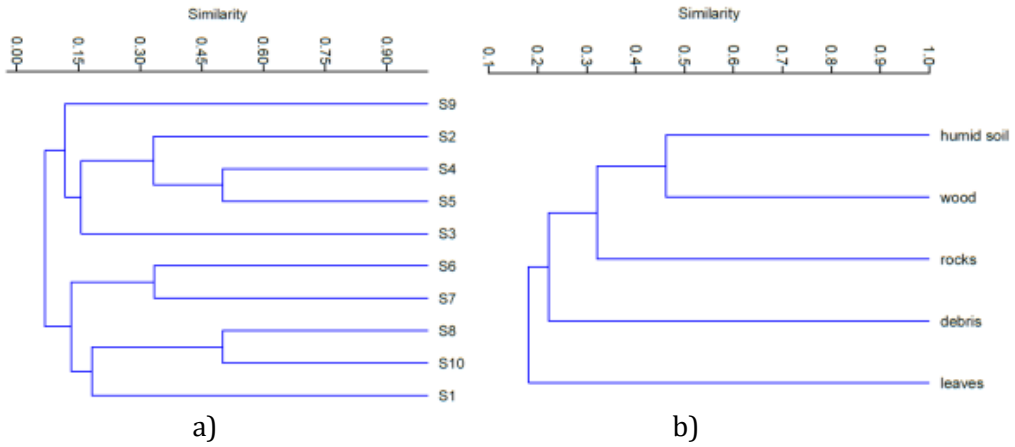
Identification		S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	P%	f%
L. hypnorum	P%	-	31.25	66.66	20.00	13.79	-	-	-	-	-	14.45	21.73
	f%	-	50.00	50.00	66.66	33.33	-	-	-	-	-		
L. germanicum	P%	-	-	-	-	-	-	-	20.83	-	30.00	6.62	8.69
	f%	-	-	-	-	-	-	-	33.33	-	50.00		
L. intermedium	P%	-	-	-	-	17.24	-	-	-	-	-	3.01	4.34
	f%	-	-	-	-	33.33	-	-	-	-	-		
Trichoniscus sp.	P%	-	-	-	-	-	-	20.00	-	-	-	0.60	4.34
	f%	-	-	-	-	-	-	50.00	-	-	-		
T. provisorius	P%	-	-	-	-	-	16.66	-	-	-	-	0.60	4.34
	f%	-	-	-	-	-	33.33	-	-	-	-		
H. transsilvanicus	P%	-	12.50	-	6.66	17.24	-	-	-	100	-	7.83	21.73
	f%	-	50.00	-	33.33	66.66	-	-	-	100	-		
H. riparius	P%	-	-	-	6.66	-	33.33	20.00	20.83	-	-	5.42	26.08
	f%	-	-	-	33.33	-	33.33	50.00	100	-	-		
C. convexus	P%	46.66	4.16	-	-	-	-	20.00	16.66	-	-	8.43	26.08
	f%	100	50.00	-	-	-	-	50.00	66.66	-	-		
P. conspersum	P%	-	35.41	-	-	-	-	-	-	-	-	10.24	4.34
	f%	-	50.00	-	-	-	-	-	-	-	-		
P. collicola	P%	6.66	-	-	26.66	-	-	-	-	-	-	3.01	8.69
	f%	50.00	-	-	33.33	-	-	-	-	-	-		

Identification		S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	P%	f%
P. politus	P%	-	-	-	26.66	44.82	33.33	-	8.33	-	45.00	18.07	21.73
	f%	-	-	-	33.33	33.33	33.33	-	33.33	-	50.00		
T. difficilis	P%	-	6.25	-	6.66	-	-	-	-	-	-	2.40	8.69
	f%	-	50.00	-	33.33	-	-	-	-	-	-		
T. rathkii	P%	-	-	-	-	-	16.66	40.00	-	-	-	1.80	8.69
	f%	-	-	-	-	-	33.33	50.00	-	-	-		
T. arcuatus	P%	-	4.16	-	-	-	-	-	4.16	-	-	1.80	8.69
	f%	-	50.00	-	-	-	-	-	33.33	-	-		
T. nodulosus	P%	6.66	-	-	-	-	-	-	-	-	-	0.60	4.34
	f%	50.00	-	-	-	-	-	-	-	-	-		
P. scaber	P%	33.33	-	-	-	-	-	-	-	-	-	3.01	4.34
	f%	50.00	-	-	-	-	-	-	-	-	-		
A. vulgare	P%	6.66	2.08	-	-	-	-	-	29.16	-	25.00	8.43	21.73
	f%	50.00	50.00	-	-	-	-	-	33.33	-	100		
A. versicolor	P%	-	4.16	33.33	6.66	6.89	-	-	-	-	-	3.61	26.08
	f%	-	100	50.00	33.33	66.66	-	-	-	-	-		
% individuals		9.03	28.91	1.80	9.03	17.46	3.61	3.01	14.45	0.60	12.04		
No. of species		5	8	2	7	5	4	4	6	1	3		
Shannon diversity		1.26	1.64	0.63	1.74	1.42	1.32	1.33	1.65	0	1.06		

The first place as frequency of occurrence was occupied by three species (*H. riparius*, *C. convexus,* and *A. versicolor*), each with a frequency in samples of 26.08%. The second place was occupied by four species (*L. hypnorum, H. transsilvanicus*, *P. politus,* and *A. vulgare*), each with a frequency of occurrence of 21.73%. The total diversity of the terrestrial isopod assemblages from the road edges was 2.54.

The differences between the terrestrial isopod assemblages from the 10 road sectors were important in terms of the number of species, the percentage abundance, the frequency of occurrence, and the diversity (Table 1).

The highest number of species (8) was registered in the case of sector 2, and the smallest number (only one species) was registered in the case of sector 9. The highest diversity (H=1.74) was recorded in sector 4, and the lowest in sector 9, where only one species was present. According to the Jaccard similarity index, the most different was sector 9, and the most resemblance was registered between sectors 4 and 5 on one side, and sectors 8 and 10 on the other side (Figure 1). According to the Kruskal-Wallis test, the differences between the assemblages from the 10 road sectors were not significant (p=0.083). Nevertheless, analyzing the sectors two by two, differences were significant between sectors 2 and 3 (p=0.007), sectors 2 and 9 (p=0.006), sectors 3 and 4 (p=0.021), sectors 3 and 8 (p=0.035), sectors 4 and 9 (p=0.016) and sectors 8 and 9 (p=0.031).



a) b)

Figure 1. Similarity of terrestrial isopod assemblages (according to the Jaccard index) between the studied sectors (a) and shelter types (b)

In the case of shelters, in the forest litter, we identified 7 terrestrial isopod species, the most abundant and frequent being *P. politus* (Table 2).

Table 2. Percentage abundance (P%), frequency of occurrence (f%), species richness, and Shannon diversity index of terrestrial isopods identified in different shelters and sides of the road.

		Shelters					Roadside	
		Leaf litter	Humid soil	Stones	Debris	Logs	Upper side	Lower side
L. hypnorum	P%	8.10	22.61	-	-	8.33	17.32	5.12
	f%	50.00	33.33	-	-	33.33	28.57	11.11
L. germanicum	P%	-	13.09	-	-	-	3.93	15.38
	f%	-	33.33	-	-	-	7.14	11.11
L. intermedium	P%	13.51	-	-	-	-	3.93	-
	f%	25.00	-	-	-	-	7.14	-
Trichoniscus sp.	P%	-	1.19	-	-	-	-	2.56
	f%	-	16.66	-	-	-	-	11.11
T. provisorius	P%	2.70	-	-	-	-	0.78	-
	f%	25.00	-	-	-	-	7.14	-
H. transsilvanicus	P%	10.81	9.52	-	12.50	-	9.44	2.56
	f%	50.00	33.33	-	50.00	-	28.57	11.11
H. riparius	P%	2.70	2.38	30.76	-	8.33	5.51	5.12
	f%	25.00	16.66	37.50	-	33.33	28.57	22.22
C. convexus	P%	-	2.38	15.38	50.00	25.00	7.87	10.25
	f%	-	16.66	25.00	50.00	66.66	21.42	33.33
P. conspersum	P%	-	20.23	-	-	-	13.38	-
	f%	-	16.66	-	-	-	7.14	-
P. collicola	P%	10.81	-	-	12.50	-	3.93	-
	f%	25.00	-	-	50.00	-	14.28	-
P. politus	P%	51.35	13.09	-	-	-	16.53	23.07
	f%	75.00	33.33	-	-	-	28.57	11.11
T. difficilis	P%	-	3.57	-	-	4.16	-	10.25
	f%	-	16.66	-	-	33.33	-	22.22
T. rathkii	P%	-	-	23.07	-	-	-	7.69
	f%	-	-	25.00	-	-	-	22.22
T. arcuatus	P%	-	2.38	-	-	4.16	2.36	-
	f%	-	16.66	-	-	33.33	14.28	-
T. nodulosus	P%	-	-	-	12.50	-	0.78	-
	f%	-	-	-	50.00	-	7.14	-

		Shelters					Roadside	
		Leaf litter	Humid soil	Stones	Debris	Logs	Upper side	Lower side
P. scaber	P%	-	-	-	-	20.83	3.93	-
	f%	-	-	-	-	33.33	7.14	-
A. vulgare	P%	-	5.95	7.69	12.50	29.16	7.08	12.82
	f%	-	33.33	12.50	50.00	33.33	21.42	22.22
A. versicolor	P%	-	3.57	23.07	-	-	3.14	5.12
	f%	-	50.00	37.50	-	-	28.57	22.22
% individuals		22.28	50.60	7.83	4.81	14.45	76.50	23.49
No. of species		7	12	5	5	7	15	11
Shannon diversity index		1.49	2.14	1.52	1.38	1.71	2.42	2.19

The assemblage diversity in the forest litter was reduced, reaching a value of only 1.49. In humid soil, we identified 12 species, the most abundant being *L. hypnorum*; in the case of frequency of occurrence, several species registered a high value (Table 2). In this type of shelter, we registered the highest species number and diversity (H=2.14). Under stones, we identified only 5 species, the diversity of the assemblages being 1.52. The same number of species was identified under debris, but the species diversity was the lowest, reaching a value of only 1.38. Under logs, we identified 7 terrestrial isopod species, with a diversity of 1.71. According to the Jaccard index, the assemblages from wet soil and under logs seem the most similar and the most different from forest litter (Figure 1).

According to the Kruskal-Wallis test, the differences between the assemblages from different types of shelters were significant (p=0.025). The Mann-Whitney test shows significant differences between the assemblages from wet soil and stones (p=0.011), between wet soil and debris (p=0.003), and between wet soil and logs (p=0.044). The differences between the assemblages on the two roadsides were significant, according to the Kruskal-Wallis test (p=0.012) and the Mann-Whitney test (p=0.013). The Principal Component Analysis showed that *H. transsilvanicus*, *L. hypnorum*, and *P. conspersum* had an affinity for the upper side of the road, and *L. germanicum* and *P. politus* for the lower part of the road (Figure 2).

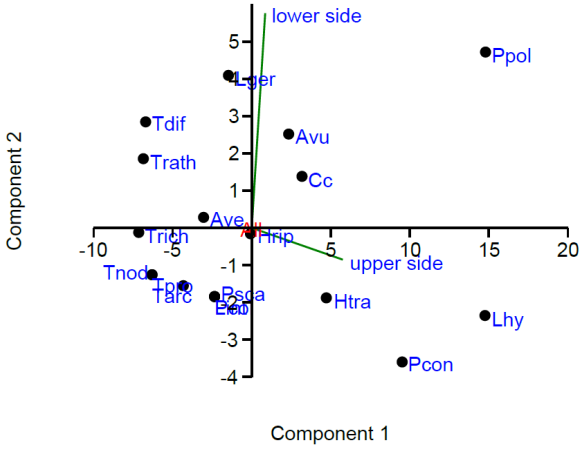
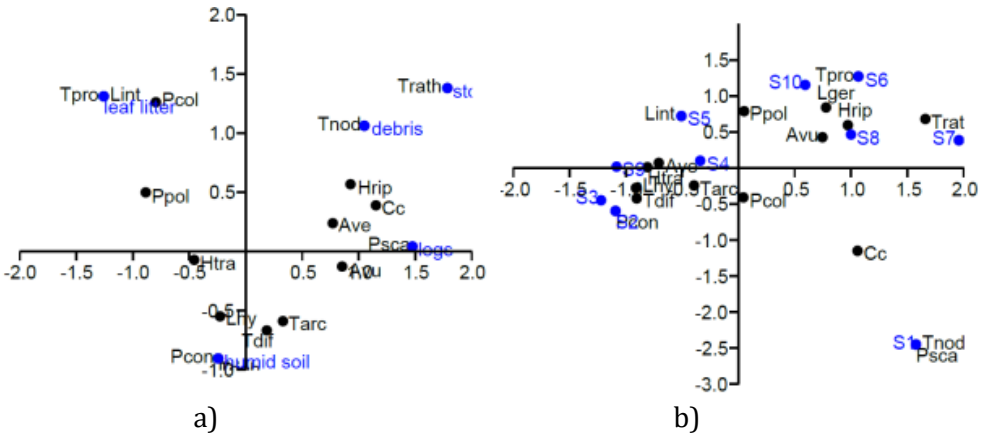


Figure 2. Principal component analysis between terrestrial isopod species and sides of the road (Lhy–*L. hypnorum*, Lger-*L. germanicum*, Lint-*L. intermedium*, Tpro–*T. provisorius*, Htra-*H. transsilvanicus*, Hrip-*H. riparius*, Cc-*C. convexus*, Pcon-*P. conspersum*, Pcol-*P. collicola*, Ppol-*P. politus*, Tdif-*T. difficilis*, Trath-*T. rathkii*, Tarc-*T. arcuatus*, Tnod-*T. nodulosus*, Psca-*P. scaber*, Avu-*A. vulgare*, Ave-*A. versicolor*)

Regarding shelters, *P. conspersum* presented an affinity for wet soil, *T. nodulosus* for debris, and more species for leaf litter (Figure 3). In the sectors, the affinity of *P. scaber* and *T. nodulosus* for sector 1 was very clear (Figure 3).



a) b)

Figure 3. Correspondence analysis between terrestrial isopod species and shelters (a)/ studied sectors (b) (for species abbreviations see Figure 2)

Discussion

The terrestrial isopod species with the highest percentage abundance on the edges of the road between Negrești Oaș and Luna Șes are native species, characteristic of wet areas and forests. This is the case of the species from the *Ligidium, Hyloniscus,* and *Porcellium* genera or the case of *T. difficilis* and *T. arcuatus*, which, according to the literature, are related to such habitats (e.g., Radu 1983, 1985, Tomescu *et al.* 2011, 2012, 2015, Giurginca 2022). Through this composition, the isopod assemblages that populate the road verges resemble the ones from natural areas situated in this region of Romania (Ferenți *et al.* 2012, 2013a,b, Ianc & Ferenţi 2014, Ferenţi & Covaciu-Marcov 2015). Unlike these, both in the case of terrestrial isopods (Vona-Túri *et al.* 2017, 2019) and other invertebrates (Knapp *et al.* 2013), road verges are populated by generalist and invasive species.

Unlike roads, railways, especially abandoned ones, shelter more diverse isopod assemblages dominated by native species, which include even rare and endemic species (Pop *et al.* 2021a,b). Thus, the degree of disturbance in the assemblages is determined by the type of transportation network and its use, but also probably by the amplitude of the impact it generates. This is proved by this secondary road with little traffic surrounded by the forests from Oaș Mountains, which had a rich terrestrial isopod fauna. Therefore, the presence of natural areas surrounding the road had important effects in maintaining the natural assemblages that are in balance with the region, just like in the case of small towns surrounded by natural habitats (Herle *et al.* 2016). The number of terrestrial isopod species present on the road edges is close to the one registered in the natural areas of Western Romania (Ferenţi *et al.* 2013a,b, Ianc & Ferenţi 2014, Ferenţi & Covaciu-Marcov 2015) or other regions in the country (Tomescu *et al.* 2011). In this study we even recorded two more species than in another study realized in more localities in the Oaș Mountains (Ferenţi *et al.* 2013b).

Porcellium conspersum is a species well represented near the road from Oaș Mountains. In other parts of its distribution range it is usually connected with old and humid natural forests (Soesbergen 1999, Berg *et al.* 2012). It was also identified in the wet soil situated at the base of alders (Berg *et al.* 2012), just like in the vicinity of the road between Negrești Oaș and Luna Șes. Even in Romania, *P. conspersum* was considered a species typical for coniferous and deciduous forests (Tomescu *et al.* 2012). In the Oaș Mountains region, *P. conspersum* has been identified previously at low altitudes but in a smaller number of individuals (Ferenţi *et al.* 2012, 2013b). In this study, however, *P. conspersum* registered a much higher percentage abundance than *P. collicola*, which is usually the better-represented species (Ferenți *et al.* 2013b) or the

only one present from the genus (Ferenţi *et al.* 2013a, Ferenţi & Covaciu-Marcov 2015). The higher abundance of *P. conspersum* is probably a consequence of the higher altitude reached by the studied road, compared with the locations previously studied in the region (Ferenţi *et al.* 2012, 2013b). This is also proved by the fact that *P. conspersum* was abundant in an area with high altitude from the southern Făgăraş Mountains (Ferenţi & Covaciu-Marcov 2016).

Even if *T. provisorius* was not mentioned in the past in Romania (Radu 1983), not being indicated even in the most recent monograph on terrestrial isopods in the country (Giurginca *et al.* 2022), there is a recent record of the species in Carei town (Pal *et al.* 2019). The identification of this species in the vicinity of the road from Oaş Mountains represents its second record in Romania. However, those two distribution records are situated in the same region (North-Western Romania), just approximately 100 km from each other. Thus, at least in North-Western Romania, the species seems well represented. Moreover, it was also mentioned in the neighbouring areas in Hungary (Vilisics *et al.* 2008, Gregory *et al.* 2009). Nevertheless, the fact that it was identified only now in a region where there are many studies on isopods (Ferenţi *et al.* 2012, 2013a,b, Ianc & Ferenţi 2014, Ferenţi & Covaciu-Marcov 2015), confirms the fact that it is a species difficult to observe, although native in the region (Pal *et al.* 2019). Even if it is considered a species largely distributed in Europe, it was only recently mentioned in other regions, too, like Slovenia (Vittori *et al.* 2023). In other cases, *T. provisorius* was identified in karst areas, at the base of sinkholes (Vilisics *et al.* 2011), unlike Oaş Mountains, which are volcanic mountains (Pătraşcu 1993, Jurje *et al.* 2014, Kovacs *et al.* 2017).

Trachelipus nodulosus was extremely rare on the road edges in Oaş Mountains (only 1 individual). In western Romania, it is a common species (Ferenţi & Covaciu-Marcov 2015, Tomescu *et al.* 2015, Pop *et al.* 2021b), even in mountain regions (Ianc & Ferenţi 2014), and in some cases, it is the most abundant species (e.g. Ferenţi *et al.* 2015, Laza *et al.* 2017). This rarity is probably a consequence of two facts. First, the region surrounding the road is covered almost completely by wide and natural forests, which are not favorable habitats for this species related to open areas (e.g. Tomescu *et al.* 2015), or even urbanized ones (e.g. Ferenţi *et al.* 2015, Laza *et al.* 2017). Second, the road was asphalted only recently, therefore, the opening cut by the road in the forest was insufficient for this species to migrate along the road, in terms of both space and time. Moreover, the single individual was encountered in sector 1, a sector which is situated on the outskirts of Negreşti Oaş town, as the species was frequently encountered under rubble in other towns (Herle *et al.* 2016). Not only *T. nodulosus* but also *P. scaber*, another synanthropic and generalist species (Radu 1985), had an affinity for sector 1, which is situated at the limit of the

town. The large surface of the natural areas in the vicinity of the road is emphasized even by the low diversity of species of the isopod assemblages found under debris. Moreover, the area shelters species linked to natural areas and endemic ones. Thus, *L. intermedium*, *H. trassilvanicus*, and *T. difficilis* are Carpathian endemic species (Radu 1983, 1985, Schmalfuss 2003, Tomescu *et al.* 2015, Giurginca 2022), also present in other cases in natural forested habitats in Romania (Pop *et al.* 2019).

The terrestrial isopod fauna linked to forests and natural areas obviously was not modified by the road, as it is predominant in the assemblages from the vicinity of the road. As animals with low mobility, terrestrial isopods were only slightly affected by the presence of the road in this case, even though, in the past, they were found killed both on roads (Ciolan *et al.* 2017, Popovici & Ile 2018, Sucea *et al.* 2023, Cupșa *et al.* 2024) and on railroads (Pop *et al.* 2023). Unlike terrestrial isopods, the negative direct impact of roads on other invertebrate groups is much higher (e.g. Baxter-Gilbert *et al.* 2015). In the Oaș Mountains, a narrow and relatively short road that crosses a natural, high-biodiversity area failed in shifting the native terrestrial isopod assemblages. Thus, in the case of this group, local roads from natural areas do not affect much the local assemblages from the vicinity of the roadside, they being resilient enough. Still, this fact must not be viewed out of context because these types of roads have numerous other negative effects, killing a large number of other invertebrates (e.g. Baxter-Gilbert *et al.* 2015).

Apparently, the scientific literature presents a notable gap of knowledge referring to the importance of transport networks as corridors for insects (Villemey *et al.* 2018). Even if our study does not focus on insects but on other arthropods, it contributes to the increase of knowledge in this direction. At the same time, our study is a particular case, in which the road did not succeed in shifting the native assemblages due to the large surface of natural areas from the vicinity of the road. Thus, generalist, non-native, and synanthropic species did not use the road as a movement corridor and, therefore, did not colonize the neighboring areas due to the fact that the road is enclosed by a sanitary corridor made up of natural habitats. Unlike this, other types of roads, like highways, have been certified as corridors for terrestrial isopods (Vona-Túri *et al.* 2017). The same situation was reported on a secondary railroad, in the vicinity of a city (Pop *et al.* 2021b). At least for the soil fauna from the area of the studied road, this equilibrium has been maintained, supporting ecosystem functioning.

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STUDIA UNIVERSITATIS BABEŞ-BOLYAI BIOLOGIA, 69, 2, 2024 (p. 193-197)

=== IN MEMORIAM ===

Vasile Muntean, PhD,
Associate professor to Faculty of Biology and Geology,
Babeş–Bolyai University

(21 June 1954 - 26 August 2024)

Some people remain in our memory through the good mood they used to convey.

He was born on June 21, 1954, in a family with ancient Transylvanian roots. The child Vasile Muntean attends the general school in his native village – Tirimia, Mureş county (1961-1969) and then the Theoretical High School no. 4 from Târgu-Mureş (1969-1973). For the young Vasile, during the period 1973-1974, military training follows; military rank: second lieutenant.

Following his passion for biology, he attended the Faculty of Biology, Geography and Geology, Biology section, at "Babeş-Bolyai" University from Cluj-Napoca (1974-1978). After this cycle, he will obtain the Bachelor's degree in Biology with the thesis entitled: "*Bacteriological and biochemical study of the biodeterioration of stone monuments*". Between 1978-1979 he followed a specialization in "Ecology and environmental protection" and the specialization paper was entitled "*Ecological study of the biodegradation of organic films applied for the protection of some materials used in industry and cultural and artistic works*".

Between 1987-1988, he followed the post-graduate course "Environmental protection", with the graduation thesis: "*The enzymatic potential of saline lakes in Romania and the need for their protection*". Since 1996, he has a PhD in biology with the doctoral thesis: "*Enzymological and microbiological studies on the sludge of saline lakes in Romania*" under the coordination of Professor Stefan Kiss, "Babeş-Bolyai" University, Cluj-Napoca.

During 2002-2003 he followed a postdoctoral programme at the Institute of Plant Molecular and Cellular Biology, Polytechnic University of Valencia, Spain, where he studied "*Post-transcriptional regulation of plant response to stress: splicing of mRNA precursors as a target of ionic toxicity of salts*".

After completing his specialization, he worked as a biologist at the Sewage Treatment Plant at the Craiova Chemical Plant (1979-1983), then for a short period as a biologist at the Biofarm Medicine Factory, Bucharest (August-September 1983), then from September 1983 to 1986 he was employed as a biologist at the Institute of Biological Sciences Bucharest, in the Bacterial and Viral Genetic Engineering Laboratory. From 1986 to 2005, he came to Cluj-Napoca at the Institute of Biological Research, in the Laboratory of Environmental Microbiology and Enzymology, covering the following stages: 1986-1988 – biologist; 1988-1990 – principal biologist; 1990-1995 – scientific researcher; 1995-1999 – senior scientific researcher III; 1999-2005 – senior scientific researcher II. In the period 2001-2002, he also served as scientific secretary of the Cluj-Napoca Biological Research Institute. At the same time, he completed his doctoral studies, obtaining his PhD degree in biology in 1996.

He started his teaching career in 2005 at the Faculty of Biology and Geology at the Department of Experimental Biology, the Microbiology discipline, as lecturer, and from 2009 he becomes an associate professor in the same discipline. Dedicated staff, he is loved by students and colleagues with whom he makes deep friendships. He coordinated dozens of bachelor's and dissertation theses. He was a tutor to several generations of students. He was a person involved in the education and learning of students, organizing with them thematic trips connected with the taught field (pharmaceutical factories, milk factories, beer, wine, wastewater treatment plants, etc.). Also, in support of the students, he contributed with four books:

1) Muntean, V., 2009, *Microbiologie generală*, Ed. Presa Universitară Clujeană, 332 p., ISBN: 978-973-610-845-7.

2) Muntean, V., 2013, *Microbiologie industrială,* Ed. Presa Universitară Clujeană, Cluj-Napoca, 440 p., ISBN: 978-973-595-507-6.

3) Carpa, R., Drăgan-Bularda, M., Muntean, V., 2014, *Microbiologie generală. Lucrări practice*, Ed. Presa Universitară Clujeană, Cluj-Napoca, 223 p., ISBN: 978-973-595-694-3.

4) Muntean, V., 2017, *Microbiologie medicală*, Ed. Presa Universitară Clujeană, Cluj-Napoca, 381 p., ISBN: 978-606-37-0129-0.

As for research, he has published over 70 articles in national and international journals. He participated with oral or poster presentations at many national and international conferences (Italy, Russia, Spain, Hungary, Germany, etc.). He was member of scientific journals in Romania (reviewer to *Studia Universitatis Babeș-Bolyai Biologia*) and member of other editorial committees (International Consortium for Salt Lake Research).

He was principal investigator in 2004-2006, for project 04-03-PED-4526, PNCDI I – BIOTECH, Biotechnologies for the remediation of waste dumps from mining operations, and participated as a member of the working group of several dozen other projects submitted by the Institute of Biological Research Cluj-Napoca, but also by the Faculty of Biology and Geology.

Vasile Muntean had four children, Vasile, Ștefan, Mihaela and Horia. All of them graduated university and are now working in Bucharest city. Vasile jr. studied at Faculty of Veterinary Medicine, University of Agricultural Sciences and Veterinary Medicine, Bucharest and now he is a veterinarian. Ștefan studied at Faculty of Automatic Control and Computers, Politehnica University of Bucharest. Mihaela studied at Faculty of Agriculture, University of Agricultural Sciences and Veterinary Medicine, Bucharest. Horia studied at Faculty of Cybernetics, Statistics and Economic Informatics, Academy of Economic Studies from Bucharest.

We met Vasile Muntean during our study years, but only got to know him better after 2004, when we became colleagues at the Faculty of Biology and Geology, Cluj-Napoca. His friendly, communicative nature combined with a sense of humor, and especially the joint research projects, made us close friends. Perhaps the most interesting common activities were recreational activities when we talked about not only science but also life problems. Also, the trips organized together with the students in various factories or at Salina Turda, or the participation in the conferences in Bistrița brought us closer and we built friendships that we will never forget. We spent over 15 years together in the same office, with joys and troubles, but we never argued. We listened to Mozart and other classics together, told jokes and worked on what needed to be worked on. Being a volcanic nature, the first sign of the disease appeared in 2015, but then, with a return of strength, he continued her activity with dedication, especially the teaching one. But the disease is unforgiving, and in January 2024, a week after we met again at the office, a stroke kept him in the beds of the Neurology hospital for 7 months, during which we understood each other only by signs. On August 25, being moved by the children to be closer to them, at the Geronto Life Med Center, Bucharest, he stopped his activity on earth forever.

We thank you Vasile, for everything you did and what you left behind!

Rest in peace!

SELECTED PAPERS

Books:

Muntean, V., 2009, Microbiologie generală, Ed. Presa Universitară Clujeană, 332 pag. ISBN: 978-973-610-845-7.

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