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BIOLOGICAL CONTRA CHEMICAL DIVERSITY AND THEIR PRACTICAL, INSECTICIDAL ASPECTS IN LICHENOLOGY

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Arthur Macharia Muhoro

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The Doctoral School

Name of Doctoral School: Doctoral School of Biological Sciences

Discipline: Biological Sciences

Head of School: Dr Zoltán Nagy
Professor, DSc
Department of Plant Physiology and Plant Ecology
Institute of Biological Sciences
Faculty of Agricultural and Environmental Sciences
Hungarian University of Agriculture and Life Sciences

Supervisor: Dr Edit Farkas
Scientific advisor, DSc
HUN-REN Centre for Ecological Research
Institute of Ecology and Botany

.....	.....
Dr. Zoltán Nagy	Dr. Edit Farkas
Approval of the Head of Doctoral School	Approval of the Supervisor

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LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
ATSB	Attractive Toxic Sugar Bait
Bti	*Bacillus thuringiensis israelensis*
DMSO	Dimethyl sulfoxide
EDTA	Ethylenediaminetetraacetic Acid
EMMs	Estimated marginal means
FA	Fumarprotocetraric acid
HPLC-PDA	High-performance liquid chromatography, including a photodiode array detector
HPTLC	High-performance thin-layer chromatography
IRS	Indoor residual spraying
ITNs	Insecticides treated bed nets
KEMRI-CDC	Kenya Medical Research Institute- Centre for Disease Control and Prevention
LSMs	Lichen secondary metabolites
MLR	Multi-linear regression
PTFE	Polytetrafluoroethylene
QGIS	Quantum Geographic Information System
TSB	Toxic sugar bait
UA	Usnic acid
UHPLC-PDA-MS	Ultrahigh performance liquid chromatography photodiode array detection-mass spectrometry
WHOPES	World Health Organization Pesticide Evaluation Scheme

1. INTRODUCTION

Lichens are associations of fungi (mycobiont) and algae and/or cyanobacteria (photobiont) and another indeterminate number of microscopic organisms as a complex ecosystem (HAWKSWORTH and GRUBE 2020). However, this definition has limitations according to other scientists who insist on that mycobiont and phycobiont are the main elements to be used in defining lichens at this stage (SANDERS 2024).

This symbiotic relationship results in unique and different morphological features and the production of unique chemicals known as lichen secondary metabolites (LSMs) that are not produced by the individual partners when they live in isolation. At least a thousand LSMs are produced by lichens specifically produced by the mycobiont. The groups of chemicals produced consist mostly of depsides, depsidones and dibenzofurans that are distributed on the cortex and also in the medulla of the lichen thalli (STOCKER-WÖRGÖTTER 2008, ELIX 2022).

Though the biological role of LSMs is still insufficiently known, it has been established that among several other uses, e.g., photoprotection against high radiation (MCEVOY et al. 2006, BECKETT et al. 2021). Insecticidal activity has also been justified in several cases (MOLNÁR and FARKAS 2010, BHATTACHARYYA et al. 2016). LSMs protect lichens from herbivory (NIMIS and SKERT 2006), furthermore, both enantiomers of usnic acid, a frequent dibenzofuran in the cortical layer of lichens (*Cladonia foliacea* and *Ramalina farinacea*), exhibited strong larvicidal activity against the third and fourth instar larvae of *Culex pipiens,* the house mosquito (CETIN et al. 2008).

About 249 million cases of malaria were documented in 85 countries in the world in 2022. In African regions, 95% of malaria-related deaths are recorded annually (580,000). Among them 80% are children (WHO 2024). Human malaria caused by *Plasmodium falciparum* is nearly endemic in Africa. Nearly all malarial deaths are caused by *P. falciparum*, and 95% of such cases occur in Africa (WHO 2021). In Sub-Saharan Africa, almost 100% of cases were due to *P. falciparum*, whereas in most other regions where malaria occurs, other, less virulent plasmodial species predominate.

The primary vectors of *P. falciparum* in western Kenya are *Anopheles gambiae* (s.s.), *An. arabiensis* and *An. funestus* (ROBI et al. 2010, STEVENSON et al. 2012, WIEBE et al. 2017). Other secondary vectors have also been documented with less importance in this respect (MUSTAPHA et al. 2021)

The most effective methods for the control of malaria are mainly to prevent and reduce mosquito bites. The current tools in use include indoor residual spraying, use of insecticide treated bed nets, application of larvicides on breeding grounds, such as *Bacillus thuringiensis israelensis* (Bti) and genetic modification of mosquito vectors (TIZIFA et al. 2018, KARUNARATNE and SURENDRAN 2022). The use of insecticide-treated wall lining nets containing deltamethrin was also evaluated in Bundalangi region of western Kenya as part of the MSc research of the candidate with promising results (MUHORO 2013). Despite the applications of the above tools, malaria still exists in Kenya and in particular the western parts due to resistance to chemicals used as insecticides causes infections in high numbers. Human behaviour as well as the behaviour of vectors added also to this situation frustrating the enormous efforts in eliminating malaria from western Kenya (KARUNARATNE et al. 2018, MACHANI et al. 2020, LINDSAY et al. 2021, NG'ANG'A et al. 2021, OWUOR et al. 2021, ODERO et al. 2024). Examples of countries that have achieved "zero malaria" and thus declared malaria-free, should be followed and their methods and protocols should be applied in other regions (BADMOS et al. 2021). However, there is a need to explore other potential vector control methods such as the use of biopesticides to overcome the limitations of the current methods (SOUGOUFARA et al. 2020).

LSMs of insecticide properties may act in natural defence as potential biopesticides. Some studies indicate the application of LSMs for the control of various insect stages that transmit human diseases or cause damage to agricultural products. This includes control of insects such as mosquitoes and weevils that have exhibited larvicidal and adulticidal effects through bioassay laboratory experiments (EMMERICH et al. 1993, VINAYAKA et al. 2009, EMSEN et al. 2012, YILDIRIM et al. 2012, DA SILVA et al. 2023) and can be integrated in or replace the use of the synthetic insecticides. However, other biological roles are yet to be discovered (HAGER et al. 2008) and there is a limited knowledge on the use of LSMs as pesticides and malaria controlling agents. A systematic review was necessary to reveal the existing knowledge on the use of LSMs for the control of malaria vectors (MUHORO and FARKAS 2021) that further research could be planned in this important field. Their potential to control insects can be further applied to insect vectors particularly due to their low toxicity to the environment and non-target organisms like bees and other insect pollinators.

The knowledge of lichens in Africa is limited, even in Kenya, though East Africa is one of the best studied regions of the continent due to the synthesising work of SWINSCOW and KROG (1988). However, there are evidence of the increasing knowledge based on several publications (ALSTRUP and APTROOT 2005, ALSTRUP and CHRISTENSEN 2006, YESHITELA et al. 2009, FARKAS and FLAKUS 2015, BJELLAND et al. 2017, KANTELINEN et al. 2021,

FRYDAY et al. 2022, KAASALAINEN et al. 2023). It would be important to summarise the existing knowledge on lichens in Kenya and promote their identification particularly the lichens rich in biologically active LSMs. This would enhance their preservation, collection and identification. The parmelioid group has special importance, since it contains a large number of lichen species with potential bioactive compounds.

Usnic acid has been widely studied and has been confirmed to have unique biological characteristics (COCCHIETTO et al 2002). As a cortical pigment it is generally known to protect algae from extreme radiation due to its localisation in the cortical layer (SOLHAUG et al. 2003, VERES et al. 2022a, XU et al. 2022). It has also been shown that UA has other possible biological applications, such as antimicrobial larvicidal effects, and anticancer activities (DIEU et al. 2020, GALANTY et al. 2021, MUHORO and FARKAS 2021, KULINOWSKA et al. 2023).

It has been known since 1967, that UA is a chiral molecule and occurs in two forms (enantiomers) in lichens in nature (BENDZ et al. 1967, KINOSHITA et al. 1997), mentioning that the lichen *Flavoparmelia caperata* produces exclusively (+)-UA. According to previous research referred in the review of GALANTY et al. (2019) there are fields where the (+)-UA ("right-handed" form) is reported to have higher efficacy, while in other applications the (-)-UA ("left-handed" form) is more effective, therefore the bioactive potential of the enantiomers needs further studies. There are generally limited data on the produced amount of LSMs (FARKAS et al. 2020b). Since *F. caperata* grows both in Europe and in the tropics (WIRTH et al 2013, SWINSCOW and KROG 1988), both the chiral nature and the concentration can be checked in specimens originate from these areas. Furthermore, it can be assumed that macroclimatic environmental conditions (SOLHAUG et al. 2003, VERES et al. 2020, 2022a, 2022b, SINGH et al. 2021), temperature, radiation and humidity in European and African habitats (RICHARDS 1952, RICKLEFS 2008, OLOU *et al.* 2019) could result in different concentrations produced in European and African samples, since the production of lichen secondary metabolites – similarly to other physiological processes – is influenced by these conditions.

The lichen *Cladonia foliacea* is relatively frequent in Central Europe and Hungary (WIRTH et al. 2013, VERSEGHY 1994, as *Cladonia convoluta* (Lam.) P. Cout.). (-)-UA was previously isolated from Italian (CAVALLORO et al. 2021) and French samples (BÉZIVIN et al. 2004), however it needs confirmation if *C. foliacea* is conservative in only producing the (-)-UA isomer, and the quantitative variation of UA production is also less known (FARKAS et al. 2020b).

Since *An. gambiae* is the leading vector of malaria parasites in Kenya, and both (+)-UA and (-)-UA are regarded as effective as insecticides, furthermore (-)-UA might be even more

effective than the other enantiomer, bioassay experiments using (+)-UA and (-)-UA or crude extract containing (-)-UA on target organism *An. gambiae* mosquito seems to be very promising. This could overcome the limitation of current challenges of synthetic insecticides where the target *An. gambiae* has developed resistance (MUNYWOKI et al. 2021). Few studies have attempted to shade more light on the impact of lichen compounds on the larval stages of the mosquitoes and have shown to be successful (VINAYAKA et al. 2009, KHADER et al. 2018, LOGANATHAN et al. 2023). Knowledge on the role of LSMs as stomach poison on adult *An. gambiae* mosquitoes is limited and therefore its study may lead to novel results.

2. OBJECTIVES TO ACHIEVE

2.1. General objectives

The general objectives of the current PhD dissertation are

- to study the diversity of lichens with insecticidal potential and their lichen secondary metabolites, the amount and chiral property of usnic acid (UA) in *Cladonia foliacea* and *Flavoparmelia caperata*,
- to determine the insecticidal potential of the pure usnic acid and the acetone crude extract of *Cladonia foliacea* as an oral pesticide against *Anopheles gambiae* mosquitoes.

2.2. Specific objectives

The specific objectives of the current PhD dissertation are

1. to review the current literature on lichens and insecticidal activities of their lichen secondary metabolites with specific attention to the life stages of mosquitoes and their role in malaria control,
2. to study the biological and chemical diversity of LSM-rich lichen taxa of East Africa and compile a revised identification key of the lichen species of the parmelioid clade in Kenya to promote their collection, research and future studies of their biological role and application,
3. to confirm the chiral property (+) and determine the amount of usnic acid in *F. caperata* (Parmeliaceae, lichenized Ascomycota) from samples collected in Kenya and Europe,
4. to confirm the chiral property (-) and determine the amount of usnic acid and fumarprotocetraric acid (FA) in *Cladonia foliacea* from Central and Southern Europe,
5. to determine the insecticidal potential of (+)-usnic acid as an oral pesticide against *Anopheles gambiae* mosquitoes,
6. to determine the insecticidal potential of (-)-usnic acid and fumarprotocetraric acid containing acetone extract of *Cladonia foliacea* as an oral pesticide against *Anopheles gambiae* mosquitoes.

3. LITERATURE OVERVIEW

3.1. Lichen species and their LSMs tested for bioactivity on insect vectors of human diseases and for antiprotozoal activity

Insects are known to be the most successful organisms on Earth. Their role in the ecosystem plays a significant impact on the ecosystem (DUFFUS et al. 2021, VERMA et al. 2023). However, many insects are known to harm humans and other animals. When they bite, they can act as vectors of microbial agents like viruses, protozoans and helminths in humans and domestic animals (RODHAIN 2015). They feed on vital parts of plants, hence causing damage to crops and thus reducing agricultural yields (MANOSATHIYADEVAN et al. 2017). Among the most notorious mosquito genera are *Aedes*, *Anopheles*, and *Culex*. They are known to transmit microbial agents that cause malaria, dengue fever, yellow fever, Japanese encephalitis, chikungunya, and filariasis diseases (LEE et al. 2018). Thus, controlling the insect population or preventing insects from biting is critical in preventing disease transmission.

Synthetic insecticides have been used for insect control for many years (OBEREMOK et al. 2015) These chemicals are toxic and they act as ovicidal, larvicidal and adulticidal agents. However, synthetic chemicals have several limitations such as high costs, environmental pollution, harmful effects on non-target organisms, and contamination of food and water exposing humans and non-target animals to their toxic effects (FRAMPTON et al. 2006, STARKS et al. 2012). Furthermore, with prolonged exposure to synthetic chemicals, insects have developed resistance, therefore necessitating the search for alternative insecticides that are less likely to promote resistance (DENHOLM and DEVINE 2013, OBEREMOK et al. 2015).

Therefore, to overcome limitations associated with dependence on synthetic insecticides, prospecting for biopesticides such as those derived from microorganisms (bacteria, fungi) and lichens are a promising alternative for insect control in integrated pest management strategies due to their safety on the environment (NANAYAKKARA et al. 2005).

The bioactivity of several LSMs has been investigated and confirmed to offer alternative potential therapeutic and insecticidal roles (EINARSDÓTTIR et al. 2010, MOLNÁR and FARKAS 2010, GOGA et al. 2018, SACHIN et al. 2018), therefore further understanding of the specific metabolites and susceptibility levels of the target insect pests are required to further increase our knowledge in applied lichenology.

From the 27 journal publications surveyed, the number of lichen species tested for the biological activities on insects was 57 in total (MUHORO and FARKAS 2021). Four species were tested on the protozoan diseases transmitted by insects. A total of 61 lichen species were investigated to determine either insecticide or antiprotozoal activity. Those that exhibited a high

mortality effect between 91% to 100% are presented in Table A1 (Appendix A2). NANAYAKKARA et al. (2005) applied 48 species on larvae of *Aedes aegypti*, however, only those were selected that had a high mortality effect above 91%. Table A1 (Appendix A2) contains the names of lichens and the most important data of the tests (concentration of the known LSM or the crude extract of the lichen; mortality rate ranging from 50 to 100%) for comparison.

These lichens contain the following 15 LSMs (Table A2 (Appendix A2)): (atranorin), 1'chloropannarin, (erythrin), evernic acid, (galbinic acid), gyrophoric acid, (lecanoric acid, norstictic acid, orsellinic acid), psoromic acid, pannarin, (salazinic acid, sekikaic acid), usnic acid, and vulpic acid. Only 7 of them were tested on insect vectors or protozoa. Eight of the listed compounds (given in brackets) were identified by chromatography, but several of them were occasionally applied together and therefore it is not known which was the effective component. In other cases pure LSMs were used without mentioning the species from which the LSM was extracted.

Only a limited number (27) of relevant publications found from five research databases indicates that, despite the wide application of lichen secondary metabolites in various fields, there is limited research on their application as potential biopesticides in controlling insect vectors and protozoan parasites (MOLNÁR and FARKAS, 2010). However, usnic acid has been widely studied including its effect on insects and protozoans. Bioassay result of the potential of the following LSMs: diffractaic acid, gyrophoric acid and salazinic against the larval stages of mosquitoes indicated their possible consideration as insecticides as per the WHO standard of 80% mortality (CETIN et al. 2008, NANAYAKKARA et al. 2005, KARTHIK et al. 2011, KHADER et al. 2018). Antiprotozoal effects of evernic acid, 1′-chloropannarin, pannarin, psoromic acid, and vulpic acid have also been confirmed as potential agents in search for alternative chemotherapy agents (FOURNET et al. 1997, LAUINGER et al. 2013).

The most investigated stage of the mosquito was the second larval stage which is in line with the WHO guidelines for larvicide bioassay (WHO 2005). Also, different stages of the protozoa were tested, for example (+)-usnic acid extracted from *Cladonia substellata* had a physiological effect by causing lysis and flagellar pockets on the developmental stages of *Trypanosoma cruzi* (amastigote, epimastigote and trypomastigote). Usnic acid exhibited strong activity against the liver stages of *Plasmodium* species (FOURNET et al. 1997, DE CARVALHO et al. 2005, CETIN et al. 2008, VINAYAKA et al. 2009, CETIN et al. 2012, LAUINGER et al. 2013). *In vitro* and *in vivo* studies on protozoa that cause leishmaniasis using usnic acid show that LSMs can be an effective source of future chemotherapy. Other protozoa parasites such as *Toxoplasma gondii* and *Trichomonas vaginalis* have been shown to be susceptible to usnic acid, however, they are not transmitted by insect vectors (WU et al. 1995).

The mode of action of usnic acid has been confirmed to cause membrane disruption of the trypomastigote stage in laboratory animals such as mice (*Mus musculus*) (DE CARVALHO et al. 2005). When administered orally, subcutaneously, or intralesional, the parasite load and size of the lesion were reduced by 72.28% and 43.34%, respectively. Further studies (DE CARVALHO et al. 2005) on the side effects of usnic acid were investigated, while no side effects were detected in mice after treatment with usnic acid. Other research (LAUINGER et al. 2013) has indicated potential toxic effects on the liver. Liver enlargement has also been confirmed after evernic, vulpic, and psoromic acid treatment (LAUINGER et al. 2013) Hepatotoxic effect has been confirmed in rats at dose above 6 μM where it caused reduction in respiratory control and adenosine triphosphate synthesis (PRAMYOTHIN et al. 2004). UA has also been confirmed to be toxic to brine shrimps (*Artemia salina*) and further recommended that additional studies would solve this challenge by developing selective derivatives of UA (BOMFIM et al. 2009). However, in studies involving humans, usnic acid has been tested to determine its safety on topical application and has been confirmed to be safe (GALANTY et al. 2021).

The applied doses of lichen extracts in several studies on insect vectors and the protozoan parasites were wide, ranging from c. 5–100 g/ml of LSMs and c. 1000–5000(–20,000) g/ml (FOURNET et al. 1997, KHADER et al. 2018). This variation can be better understood, if the natural concentrations of LSMs in lichen thalli ranging from c. 0.1% to 10% (–30%) of their dry weight is considered. This high concentration of the crude extract is also justified by the amount of the LSMs measured in the thalli of *C. foliacea*, UA (11.61±0.29 mg/g) and FA (2.45±0.21 mg/g) (FARKAS et al. 2020b, BOKHORST et al. 2024).

The following results describe the complexity of these studies. KARTHIK et al. (2011), for example, investigated insecticidal activities of *Leucodermia leucomelos* (L.) Kalb containing atranorin and salazinic acid on mosquito larvae (2nd and 3rd larval stages of *Aedes aegypti* with 20 larvae tested) using different concentrations at 1000, 1500, and 2000 g/ml when mortality was recorded after 24 h. The mortality differed by concentration: the highest mortality was 80% at 1000 g/ml for the 2nd instar larvae and the lowest was 50% for the 3rd instar larvae at 1.5 mg/ml. The susceptibility was 80% for 3rd instar larvae and 100% for 2nd instar larvae, while at 2000 g/ml survival rate was 0% indicating 100% mortality, hence 2000 g/ml is the best concentration to kill 2nd instar larvae of *A. aegypti* if these results are compared to 10% dimethyl sulfoxide (DMSO) as a control in the investigation protocol (VINAYAKA et al. 2009).

Bioassay studies against the 3rd and 4th instar larvae of the house mosquito (*Culex pipiens*) using enantiomers of usnic acid were also confirmed to be dose dependent based on observed mortality (CETIN et al. 2008). LSMs from various taxonomic groups showed that bioactivity exhibited at LC50 was as follows: atranorin – 0.52 μg/ml, 3-hydroxyphysodic acid – 0.97 μg/ml,

gyrophoric acid –0.41 µg/ml, (+)-usnic acid – 0.48 µg/ml indicating that gyrophoric acid showed the highest toxicity (CETIN et al. 2012).

The main difference noted between the two optical enantiomers of usnic acid is based on the fact that they exist naturally in various species of lichens and this may cause considerable differences in efficacy as described and confirmed in a series of bioactivity analyses (KINOSHITA et al. 1997, GALANTY et al. 2019). The studies were based on effects on insect vector hence this topic is worthy of further investigation.

Globally, approximately 18,000 to 20,000 lichen species are known (KIRK et al. 2008), however, only a small fraction of them have been investigated on their role as insecticide and antiprotozoan efficacy. This large number of lichens are also known to produce around 1000 LSMs (STOCKER-WÖRGÖTTER 2008), but only a limited number have been studied for use in controlling insect vectors and the diseases they transmit. This suggests that other lichen species and their compounds may be effective that are yet to be explored. More attention should be focused on testing their biological activities because of the diversity in their chiral nature and their derivatives that has been shown to affect their biological activity (MOLLINEDO 2008, FELCZYKOWSKA et al. 2019, GALANTY et al. 2019).

To further advance the use of lichens that exhibit biological activities against insects, more field surveys and testing on diverse species of insects are needed. These would help to determine if the bioactive metabolites of these lichens can be developed into commercial products for controlling insect vectors and managing diseases caused by parasitic protozoa.

Their environmental safety and efficacy can be enhanced by current novel microencapsulation techniques (MIE et al. 2012, GOVINDARAJAN et al. 2016, KUMAR et al. 2020, MACEDO et al. 2021). These natural products could become a valuable tool for disease and pest management.

3.2. Bioassay and field studies on the control of mosquitoes using insecticidal agents in sugar bait

There are over 140 *Anopheles* species of mosquitoes and at least 8 species are considered as efficient vectors of human malaria (COETZEE 2004, SINKA et al. 2010). The two primary vectors of malaria in Africa are *Anopheles gambiae* and *An. arabiensis* (WHITE 1974). They are predominantly anthropophilic and anthropophagic and prefer to rest and bite indoors (endophagic insects) (DEGEFA et al. 2017). The primary vectors in western Kenya are *An. gambiae* (s.s.), *An. arabiensis* and *An. funestus*. However, other secondary vectors have also been documented recently in Kenya, they include *An. coustani* and *An. pharoensis* (ROBI et al. 2010, STEVENSON et al. 2012, WIEBE et al. 2017, MUSTAPHA et al. 2021).

Anopheles gambiae Giles 1902 is considered the main vector of *Plasmodium falciparum* that is nearly endemic in sub-Saharan Africa. It is more successful as a vector due to its high vectorial capacity, longevity and strong preference to feed on humans while they rest indoors (zoophilic and endophagic) (BASS et al. 2007, SINKA et al. 2010, WIEBE et al. 2017, AKPAN et al. 2018).

The most frequently used method that is considered effective in controlling mosquitoes to reduce malaria transmission is to prevent or reduce their biting frequency (TIZIFA et al. 2018). Currently, the most common tools used to control mosquitoes and reduce their bite include genetic modification, the killing of the larval stages and destruction of their aquatic habitats by use of larvicides, the use of insecticide-treated bed nets (ITNs), indoor residual spraying (IRS), natural predators and entomopathogenic fungi (SCHOLTE et al. 2004, KARUNARATNE and SURENDRAN 2022).

However, despite the continuous use and over dependence of these methods, the target vectors have developed resistance to the active ingredients of the toxic agents. Other challenges associated with overdependence of the methods include human behaviour in adherence to the proper use of the ITNs, and the behaviour of the mosquitoes when exposed to the toxic chemical in the ITNs and on the wall after IRS interventions. Climate changes has also negatively affected the positive outcomes of eliminating and controlling mosquitoes (BHATT et al. 2015, KARUNARATNE et al. 2018, MACHANI et al. 2020, LINDSAY et al. 2021, NG'ANG'A et al. 2021, OWUOR et al. 2021, KRIPA et al. 2024, ODERO et al. 2024).

Non-human malaria hosts have also been investigated to determine the possibility of humans acquiring infections from mosquitoes that get infected by biting various animals. It has been found that this possibility exists, and it is considered as a challenge in malaria control and elimination (ANTINORI et al. 2021). Special attention should be paid to this and the current existing malaria vector control methods must be improved and new ones should be discovered to attain the expected intervention outcomes (SOUGOUFARA et al. 2020). One such potential method is the Toxic sugar bait (TSB) which uses the sugar-seeking behaviour of both male and female mosquitoes. It works on the attract and kill principle that has an application that dates back to 77 CE (SIPPY et al. 2020). The application of using boric acid and arsenic to control termites and mosquitoes has been evaluated and this forms the premise of the bioassay to test for alternative ingredients of the TSB in an aqueous state (ESENTHER and BEAL 1974, XUE and BARNARD 2003, FIORENZANO et al. 2017, SIPPY et al. 2020).

This natural predisposition of insects to seek sugar as a source of energy has been used to control other insect vectors of medical importance, such as sandflies and blackflies (LEWIS and DOMONEY 1966, FOSTER 1995, BURGIN and HUNTER 1997, MÜLLER et al. 2010a,

MÜLLER et al. 2010b). The principle of a successful bioassay to attract and kill mosquitoes is based on the fact that both male and female mosquitoes will occasionally seek and consume sugar during their lifetime. Females will seek blood from humans or other vertebrates to obtain protein as a nutritional requirement for their egg production (STONE et al. 2011). The preference for specific types of sugar such as glucose, and fructose among *An. gambiae* has been confirmed to influence its ecological fitness and survival. This has been confirmed in a recent baseline study in western Kenya (MANDA et al. 2007, STONE et al. 2011, OMONDI et al. 2022).

Synthetic insecticides have been utilized as toxic agents in TSB and the results of these studies have shown to be effective in killing male and female mosquitoes when boric acid, dinotefuran ivermectin have been applied as ingredients (QUALLS et al. 2015, REVAY et al. 2015, TENYWA et al. 2017, DIARRA et al. 2021, FRASER et al. 2021, KUMAR et al. 2023). However, when used outdoors, these chemicals may have a negative effect to the environment and other non-target organisms (FIORENZANO et al. 2017, REZENDE-TEIXEIRA et al. 2022). Therefore, the use of more safe ingredients can solve the challenge associated with the use of synthetic insecticides as active toxic agents. Promising results where such natural ingredients have been used to control mosquitoes include microencapsulated garlic oil, eugenol, spinosyns, erythritol, *Bacillus thuringiensis israelensis* (Bti) and sodium ascorbate. Their safety has been confirmed and the use of plant-based toxic agents emphasized (JUNNILA et al. 2015, REVAY et al. 2015, MCDERMOTT et al. 2019, DAVIS et al. 2021, ALOMAR et al. 2022, REZENDE-TEIXEIRA et al. 2022, BAKER et al. 2023).

4. MATERIALS AND METHODS

4.1. Study of literature on application of lichens as an insecticide

To determine the current literature on LSMs as an insecticide in English for preparing the literature review (MUHORO and FARKAS 2021), the following public databases were used: Google Scholar, PubMed, Recent literature on lichens, Scopus and Web of Science. The keywords used were “lichen secondary metabolites” AND” insect vectors” AND” human diseases” AND (“insecticidal” OR” bioassay” OR, “bioactive”) and (“insect vectors of parasitic” AND” human” AND” protozoa” AND” diseases”) according to guidelines by KHAN et al. (2003). The papers were exported in comma separated values as CSV format files, and the suitability of the paper to be reviewed was based on the following inclusion and exclusion criteria. Only publications in English that addressed LSMs as insecticides against vectors of human parasitic protozoan diseases were included. Duplicate articles and those that did not meet these criteria were excluded.

After the inclusion criteria, the following guiding topics of the review were investigated and used to determine the biological role of lichens as insecticides against vectors of human diseases.

1. Lichen species and their LSMs tested for bioactivity on insect vectors of human diseases and for antiprotozoal activity
2. Groups of insect vectors and parasitic protozoa tested on effectiveness of LSMs
3. Methods of extraction of LSMs for bioassay on insect vectors and antiprotozoal activity
4. Dosage, methods, application of LSMs and their physiological and morphological effects on protozoa
5. Effective LSMs documented by toxicity tests

4.2. Lichens studied and collected for taxonomical, chemical and bioassay studies

Literature from the first published book in Kenya on Macrolichens of East Africa and other current published literature on parmelioid clade were used (SWINSCOW and KROG, 1988, KIRIKA et al. 2016a, 2016b, 2017) for preparing a revised key to parmelioid species (FARKAS and MUHORO 2022). Information regarding the habitat, distribution, taxonomic identity, nomenclature, morphological characters and chemical identification were collected on lichen species that belong to the parmelioid clade (178) of the following genera: *Bulborrhizina*, *Bulbothrix*, *Canoparmelia*, *Cetrelia*, *Crespoa*, *Flavoparmelia*, *Flavopunctelia*, *Hypotrachyna*, *Melanelixia*, *Myelochroa*, *Parmelia*, *Parmelinella*, *Parmotrema*, *Pseudoparmelia*, *Punctelia*, *Relicina*, *Remototrachyna* and *Xanthoparmelia*. Specimens deposited in herbarium VBI (THIERS 2024) were used for preparing illustrations and listed in Appendix 3.

Further, other earlier undetermined lichens previously collected from various localities in Kenya and Tanzania were identified often together with their lichenicolous fungi and published in FARKAS et al. (2023) are listed in Appendix 4.

Flavoparmelia caperata (L.) Hale (30 samples) were collected in Hungary (by E. Farkas, L. Lőkös, A. M. Muhoro, N. Varga), Serbia (by L. Lőkös), Kenya (P. M. Kirika, H.T. Lumbsch, G. Mugambi, A. M. Muhoro) and Tanzania (by E. Knox, T. Pócs) from its typical habitats between 24 October 1987 and 5 September 2022 (Figs 1–2). However, the time of collection and elevation was not considered in selecting samples for this study which might be important in the process of production of LSMs. It was necessary to revise the samples before they were investigated. These samples were revised by E. Farkas, L. Lőkös and A. M. Muhoro by using a Nikon SMZ18 stereo microscope (Nikon Corporation, Tokyo, Japan), as well as an Olympus SZX7 stereo microscope (Olympus Corporation, Tokyo, Japan) and chemically by analysing LSMs by HPTLC. Voucher specimens are deposited in Lichen Herbarium VBI at the Institute of Ecology and Botany of HUN-REN CER, (Vácrátót, Hungary) and in Herbarium EA (Nairobi, Kenya) (abbreviations follow THIERS 2024). More information about the samples of lichens used and their locations in this study are in Table A3 (Appendix A2).

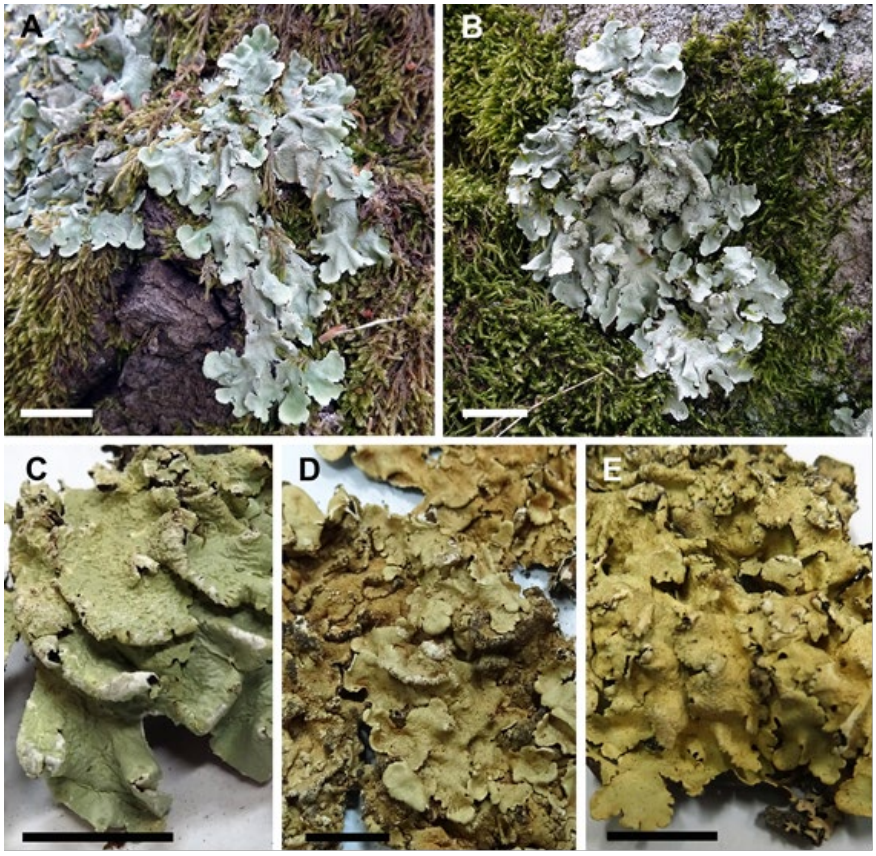

Fig. 1. *Flavoparmelia caperata* thalli, in its habitats in Hungary: **A**, on the bark of Quercus sp. (sample 4); **B**, on andesite rock (near sample 11); herbarium specimens: **C**, from Kenya (VBI 6044, sample 19); **D**, **E**, from Tanzania (VBI 6169 – sample 20, VBI 6251 – sample 23). Scale bars: 1 cm. (Photo E. Farkas). (Farkas et al. 2024a)

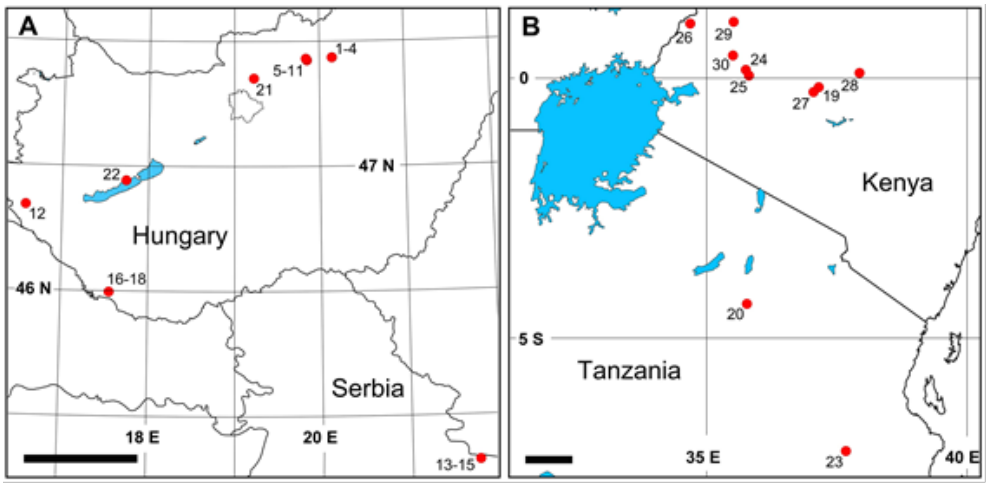

Fig. 2. Collecting sites of the investigated *Flavoparmelia caperata* samples (Table A3). **A**, in Hungary and Serbia; **B**, in Kenya and Tanzania. Scale bars = 100 km. (FARKAS et al. 2024a)

Cladonia foliacea (Huds.) Willd. (29 samples) used for chiral chromatography and quantitative analysis were collected from Hungary, Albania and North Macedonia between 16 June 2013 and 9 June 2022 (Figs 3–4). Their identity was confirmed by E. Farkas, L. Lőkös and N. Varga. During their collection, important characteristics of their habitats were also recorded such as habitat type (acidic soil or basic calcareous soil), elevation and date of collection (Table A4 (Appendix A2)).

For bioassay studies thalli of *C. foliacea* were collected from Hungary [Pest County, Vácrátót, Tece, along the 'red line' tourist route (Ág-dűlő), in open sandy grassland from the soil. Lat.: 47.702358° N; Long.: 19.224312° E].

Fig. 3. Habitat photograph of the lichen *Cladonia foliacea.* **a**, Thalli in a typical lowland steppe habitat in Hungary; **b**, **c,** specimens with round lobes, **d**, **e**, specimens with divided lobes. Photo E. Farkas. (FARKAS et al. 2024b)

Voucher specimens of *C. foliacea* were deposited in Lichen Herbarium VBI (at the Institute of Ecology and Botany of HUN-REN CER, Vácrátót, Hungary) Vácrátót, Hungary) (abbreviation follow THIERS 2024, continuously updated).

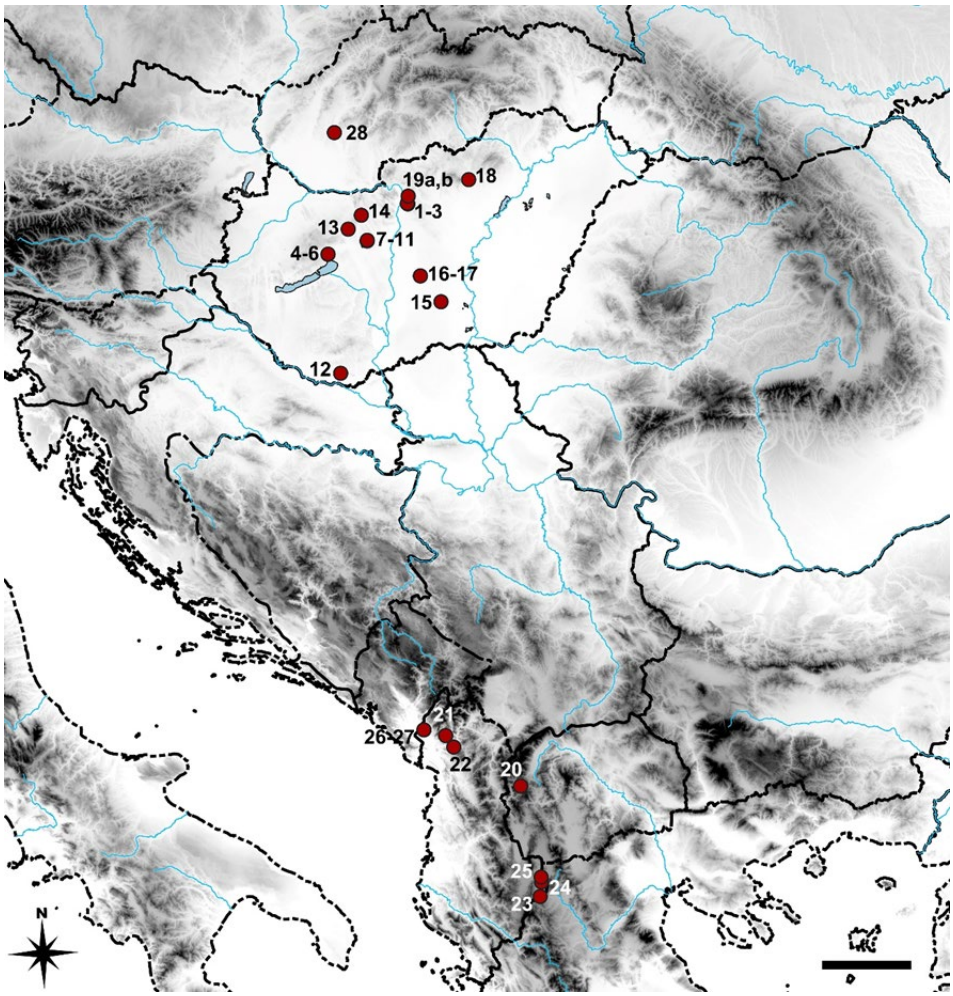

Fig. 4. Collecting sites of the investigated *Cladonia foliacea* samples (Table A4, for CLF1–CLF28 only numbers 1–28 are indicated) in Slovakia, Hungary, Albania and North Macedonia. Scale = 100 km. (FARKAS et al. 2024b)

4.3. Microscopic investigations

Morphological characters of the main diagnostic parts of the lichens were studied by use of a dissecting light microscope (Nikon SMZ18 stereomicroscope) and Nikon Eclipse/NiU compound microscope. Microscopic anatomical features were studied by wet mount preparation of hand-cut sections. Micrographs of the observed features were recorded using a Nikon Fi3 camera with NIS-Elements BR ML software.

4.4 Chemical investigations

4.4.1. Spot test (K, C and P tests)

To identify the chemical substances the following reagents were applied on the upper cortex or the medulla of the lichen thallus individually or in combination with additional reagents. A color change was observed, recorded and compared with standard identification references (ORANGE et al. 2010). They are indicated in the Table 1 below. Removal of the upper cortex to expose the medulla enables detection of lichen substances within the medulla.

Table 1. List of reagents used to determine chemical substances present in lichens

Name of reagent	Type of test
Potassium hydroxide (KOH) solution (10%)	**K test**
Commercial bleach or sodium hypochlorite (diluted to 5%)	**C test**
Para-phenylenediamine (PD) solution (in ethanol or water)	**P test**:

4.4.2. Qualitative determination of LSMs by high-performance thin layer chromatography (HPTLC)

Qualitative analysis of the LSMs was performed by high performance thin layer chromatography (HPTLC) according to the standard methods described by ARUP et al. (1993), MOLNÁR and FARKAS 2011). CAMAG horizontal chamber 10×10 cm (DONAU LAB Kft., Budapest, Hungary), CAMAG TLC Plate Heater III (DONAU LAB Kft., Budapest, Hungary), and HPTLC chromatographic plates were used (10×10 cm, Merck, Kieselgel 60 F254), most often solvent C was applied consisting of toluene and acetic acid in the ratio of 20:3, v/v. Other additional pieces of equipment in this analysis include a CAMAG ultraviolet lamp cabinet with tubes 254 nm and 366 nm. About 0.25 mm^2 size lobes of the lichen thalli were removed and placed in Eppendorf tubes containing acetone to extract the lichen substances. Lichen compounds were identified by comparing migration rates with the controls (atranorin, zeorin and norstictic acid) from the lichens *Pleurosticta acetabulum* (Neck.) Elix & Lumbsch (norstictic acid) and *Leucodermia boryi* (Fée) Kalb (atranorin, zeorin).

4.4.3. Quantitative determination of LSMs by HPLC-photodiode array detector (PDA)

To determine the amount of UA and FA, High-performance liquid chromatography system (HPLC, Alliance e2695, Waters Corporation, Milford, MA, United States), with a photodiode array detector (PDA, 2998, Waters Corporation, Milford, MA, United States) was used as described by JI and KHAN (2005) and the steps were as follows:

1. About 1.5–2 g lichen thalli (*F. caperata* and *C. foliacea)* was homogenised and pulverised and about 50 mg of the entire amount (10–20(–30) g/sample) was suspended in 10 ml pure acetone by placing it in an ultrasonic water bath for 20 minutes.
2. The suspensions were then centrifuged at 8500 g for 20 min, and the supernatant was filtered through a Cronus Ø 25 mm PTFE syringe filter (0.22 µm)
3. For calibration purposes, standard stock solutions (1 mg/ml) were made from reference standards for fumarprotocetraric acid and usnic acid (Phytolab GmbH & Co. KG, Vestenbergsgreuth, Germany) and usnic acid UA (Sigma Aldrich Kft., Budapest, Hungary) dissolved in dimethyl sulfoxide. The lichen metabolites were quantified according to a five-point (5, 10, 20, 50, 100 µg/ml) calibration.
4. For chromatographic separation Phenomenex Luna C18 (150×4.6 mm, 5 µm) column was used, and 10 µl of the filtered supernatant were injected. The temperature was 40 °C in the column oven and 5 °C in the sample cooler.
5. For the baseline separation of LSMs, a gradient elution program was used. Solvent A consisted of orthophosphoric acid and deionised (Milli-Q ultrapure) water (0.5:99.5, v/v), and solvent B contained ortho-phosphoric acid and acetonitrile (0.5:99.5, v/v). All the chemicals used were HPLC grade.
6. The linear gradient started with a 60% A solvent after the volume decreased to 10% within 20 min and then to 0.5% in 30 s after which the volume remained constant for 9.5 min. The volume of solvent A was changed back to 60% within 1 min.
7. The flow rate of solvents was 1 ml/min. The lichen metabolites were detected (n = 5), FA at 240 nm and UA at 280 nm.

4.4.4. Determination of usnic acid enantiomers

Determination of usnic acid enantiomers was performed according to chiral chromatographic methods described by XU et al. (2022). Samples were analysed in the University of Iceland (Faculty of Pharmaceutical Sciences, Reykjavik).

4.5. Preparation of distribution maps

Distribution maps indicating the main sampling points were prepared using Quantum Geographic Information System (QGIS) 3.18.2 ‘Zürich’ version 2020.

4.6 Bioassays

4.6.1 Procedure for breeding *Anopheles gambiae* (Kisumu strain)

This study used the *Anopheles gambiae* Giles, 1902 mosquito Kisumu reference laboratory strain (MUNYWOKI et al. 2021) (Fig. 5). It has been colonized in the Kisumu insectary laboratory (KEMRI-CDC) since 1954. It is known to be free from any insecticide-resistant mechanism. Therefore, it is ideal for this study. It was necessary to breed the mosquitoes from the egg to the adult stage. The following steps were undertaken:

1. Females were fed on bovine blood prepared by dissolving 300 µl of Ethylenediaminetetraacetic Acid (EDTA) in 1 litre of distilled water to prevent coagulation. The blood was passed through a strainer to remove the fibres and collected in a bottle ready for feeding. Feeding was done using a membrane feeding system (Hemotek PS5) (Fig. 6) and the temperature was set at 37°C. An improvised membrane obtained from the large

intestine of a cattle was peeled and placed over the blood holder and firmly secured with a rubber band. The feeding equipment was turned on and the feeder was placed over the cage in contact with the net so that the mosquitoes could access the membrane.

2. Males and females were fed on a 10% sugar solution soaked in cotton wool that was placed over the cage on a net so that the mosquitoes could access the sugar.
3. After feeding a Petri dish containing soaked cotton wool, filter paper on top was placed inside the cage. This served as an egg-laying pad.
4. The filter paper was removed and placed in a hatching container with spring water.
5. After hatching the larvae were fed on brewers' yeast and the water was changed daily.
6. When the larvae attained the pupal stage, they were collected using a pipette and transferred to another container with water. This was placed in a mosquito cage for more time to develop into pupae and emerge as adults.
7. They were fed on sugar before they were subjected to bioassay.

Fig. 5. *Anopheles gambiae* mosquito (photo J.J Kosgei)

Fig. 6. Membrane feeding system (photo A.A. Muhoro)

4.6.2. Extraction and concentration of crude extract of *Cladonia foliacea*

4.6.2.1. Extraction process

C. foliacea lichen thalli (c. 200 g) were cleaned and dried at room temperature and pulverized using an electric blender. Samples were stored in the fridge at 4°C. Extraction was performed according to the method described by MOHAMMADI et al. (2020) at Egerton University, Department of Biochemistry and Molecular Biology. Soxhlet apparatus (Fig. 7) was assembled, temperature was set at 60°C. The lichen material was introduced in a thimble and acetone (50ml) was introduced. The extraction cycle lasted for 6 hours.

Fig. 7. Soxhlet apparatus (photo A.A. Muhoro)

Fig. 8. Vacuum evaporator (photo A.A. Muhoro)

4.6.2.2. Concentration process of the crude extract

To concentrate the crude extract, a rotary evaporator (Fig. 8) under reduced pressure was used. The rotation speed of the evaporating flask and temperature of the heating bath was set at 115 rpm and 60°C, respectively. A final crude extract was obtained and allowed to dry for 12 hours. After weighing, 1.97 g of the amount was collected and kept in the fridge for further bioassay procedure.

4.6.3. Preparation of usnic acid test solutions and sugar bait

The toxic agent used in this study was (+)-usnic acid (IUPAC name: 2,6-diacetyl-7,9-dihydroxy-8,9b-dimethyl-1,3(2H,9bH)-dibenzo-furandione). The chemical structure of both (+)- and (-)-usnic acid enantiomers is shown in Fig. 9. Usnic acid is slightly soluble in water (POPOVICI et al. 2022). (+)-usnic acid (Fig. 10) was supplied by Phytolab (Phytolab GmbH & Co. KG, Vestenbergsgreuth, Germany), where its purity was determined (chromatographic purity 10%, absolute purity 99%).

1. Usnic acid stock solution was prepared by dissolving 500 mg pure (+)-usnic acid in 10 ml of acetone and covered with parafilm to prevent evaporation. This was labelled as 50 mg/ml Usnic Acid (UA) stock solution
2. 10% cane sugar solution (Mumias brand) was prepared by mixing 90 ml of rainwater with 10 g brown sugar.
3. 2% food dye solution was prepared by dissolving 2g of food dye in 98ml water.
4. The following dilutions and a negative control were prepared as follows

- 5 mg/ml usnic acid solution: 0.3 ml of the UA stock solution was mixed with 2.7 ml of acetone and labelled as 5 mg/ml usnic acid.
- 10 mg/ml usnic acid solution: 0.6 ml of UA stock solution was mixed with 2.4 ml of acetone and labelled as 10 mg/ml usnic acid.
- 15 mg/ml usnic acid solution: 0.9 ml of UA stock solution was mixed with 2.1 ml of acetone and labelled as 15 mg/ml usnic acid.
- Replicates: each solution (5, 10, and 15 mg/ml) was divided into 3 equal volumes of 1.0 ml, creating 3 replicates for each concentration.

5. 5 ml of the 10% sugar solution and 0.5 ml of the 2% food dye solution were added to each replicate of all concentrations. The contents of each replicate were mixed thoroughly.
6. Preparation of Negative Controls: Negative control was prepared by mixing 5.5 ml of the 10% sugar solution, 0.5 ml of the 2% food dye solution, and 0.8 ml of acetone.
7. Each test solution and the negative control was applied to prepared cotton wool balls, allowing 30 minutes for the acetone to evaporate.

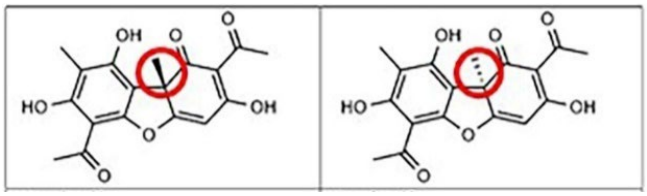

Fig. 9. Molecular structure of (+)-usnic acid (left) and (-)-usnic acid (right).

Fig. 10. PhytoLab reference substance (+)-usnic acid used in bioassay.

4.6.4. Preparation of crude extract toxic bait

Since the major LSMs of *Cladonia foliacea* is fumarprotocetraric and usnic acid (SMITH et al. 2009, WIRTH et al. 2013), the crude extract of *C. foliacea* is also known to contain UA and FA (CETIN et al. 2008). It is slightly soluble in water.

At the start of the experiments, it was necessary to prepare the stock solution, therefore, the first stock solution was a 50 mg/ml crude extract of *C. foliacea*. For monitoring the mortality rate, further concentrations (70, 80 and 90 mg/ml) were used. The preparation of another stock solution is also described below and that was further diluted.

To prepare 20 ml of 50 mg/ml *C. foliacea* extract, 1000 mg of the extract was dissolved in 20 ml acetone and covered using parafilm to prevent evaporation. It was labelled as 50 mg/ml of *C. foliacea* extract (CFE). This was further diluted:

1. 0.6 ml of CFE was added to 2.4 ml acetone and labeled as 10mgml *C. foliacea* extract
2. 1.2 ml of CFE was added to 1.8 ml acetone, label it as 20 mg/ml *C. foliacea* extract
3. 1.8 ml of CFE was added to 1.2 ml acetone and labelled as 30 mg/ml *C. foliacea* extract

4. 2.4 ml of CFE was added to 0.6 ml acetone and labelled as 40 mg/ml *C. foliacea* extract
5. 3.0 ml of CFE was labelled as 50 mg/ml *C. foliacea* extract
6. Each of the above concentrations was divided into to 3 equal volumes of 1.0 ml to make 3 replicates of each concentration. (10, 20, 30, 40, and 50 mg/ml *C. foliacea* extracts).
7. To each replicate in all the concentrations 5.5 ml of 10% sugar solution and 0.5 ml of 2% food dye solution were added and mixed.
8. Five (5) negative controls were prepared by mixing 5.5 ml 10% sugar solution, 0.5 ml 2% food dye solution, and 2.4 ml acetone and labelled as a negative control.
9. Each of the replicates and negative controls prepared above were introduced onto a cotton wool ball and waited up to 30 minutes until the acetone evaporated.
10. Paper cups were labelled according to the concentrations above e.g., 10 mg/ml R1, R2, and R3, 20mg/ml R1, R2, and R3 30 mg/ml R1, R2, and R3 40 mg/ml R1, R2, and R3 and 50 mg/ml R1, R2, and R3 (where R1-R3 indicated the number of replicates).
11. 5 male and 5 female non-blood-fed mosquitoes were aspirated using a mosquito aspirator and blown into experimental cups. The preparation was left undisturbed for 1 hour for the acclimatization of the mosquitoes before the introduction of the toxic agent in or on the paper cups.
12. The cotton wool containing the mixture of dye, sugar, and *C. foliacea* extract for each concentration was introduced on top of the net of respective paper cups according to the corresponding concentrations (Fig. 11).
13. The timer was started and recorded any mortalities after 4 hours and 24 hours.
14. Dead mosquitoes with visible dyes in the abdomen were recorded in the datasheet.
15. The date and physiological status of the mosquito are recorded before the start and termination of each experiment.

To prepare 7.8 ml of 90 mg/ml *C. foliacea* extract, 704 mg of the extract was dissolved in 7.8ml acetone and covered using parafilm to prevent evaporation. It was labeled as 90 mg/ml *C. foliacea* extract (CFE) (stock solution) It was preserved for future use by using parafilm to prevent evaporation and contamination.

1. 2.2 ml of CFE was mixed with 0.8 ml acetone and labelled as 70 mg/ml CFE.
2. 2.6 ml UAE was mixed with 0.4 ml acetone and labelled as 80 mg/ml CFE.
3. 3.0 ml UAE was labelled as 90 mg/ml CFE.
4. Each of the above (70, 80, and 90 mg/ml) CFE solution were divided into 3 equal volumes of 1.0 ml to make 3 replicates of each concentration.
5. To each replicate in all the concentrations 5.5 ml of 10% sugar solution was added.
6. To each replicate in all the concentrations 0.5 ml 2% food dye solution was added.
8. Three negative controls were prepared by mixing 5.5 ml 10% sugar solution + 0.5 ml 2% food dye solution + 0.8 ml acetone and label it as a negative control.
9. Each replicate and negative controls prepared above were placed onto a cotton wool ball. Acetone was allowed to evaporate for 30 minutes.
10. Paper cups were labelled according to the concentrations above e.g., 70 mg/ml R1, R2, and R3, 80 mg/ml R1, R2, and R3 and 90 mg/ml R1, R2, and R3.

4.6.5. Exposure to toxic sugar bait and determination of mortality rate

A protocol and further laboratory procedures for the novel attractive toxic sugar bait (ATSB) bioassay using adult mosquito candidates were worked out and approved by the scientific and ethics review unit of the KEMRI (SERU) protocol number SERU04–06–423/4610. It

represents an important contribution to the World Health Organization Pesticide Evaluation Scheme (WHOPES). Bioassay laboratory experimental tests were conducted at Kenya Medical Research Institute (KEMRI-CDC) laboratories in Kenya according to ALLAN (2011) and STEWART et al. (2013) (Fig. 11) with slight modifications as it is described below.

Before the start of the experiment, mosquitoes were denied sugar for 14 hours, however, water was provided. Only mosquitoes that exhibited a high affinity for feeding were used and care was taken not to cause any injury during aspiration and blowing out of the mosquitoes into the paper cups.

The paper cups were labelled as follows according to the concentrations 5 mg/ml: R1, R2, R3, 10 mg/ml: R1, R2, R3 and 15 mg/ml: R1, R2, R3. (where R represent the replicate number).

Five male and five female non-blood-fed mosquitoes were introduced into each experimental cup.

Mosquitoes were allowed to acclimatise for 1 hour, and any dormant mosquitoes were removed and replaced taking care that the sex ratio was maintained.

Cotton wool containing the mixture of dye, sugar, and different concentrations of usnic acid was placed on top of the net in each paper cup, corresponding to the appropriate concentrations.

The timer was started, and mosquito mortalities were recorded at 4 hours, 24 hours, 48 hours and 72 hours.

Only dead and knocked-down mosquitoes (unable to stand or showing no wing movement) were removed after 4 and 24 hours to observe the ingested poison.

The dyed dead mosquitoes were observed using a light microscope. Their sex was determined based on antenna morphology, counted and recorded on the datasheet.

The remaining mosquitoes were left for delayed mortality observation over the next 72 hours.

Fig. 11. Bioassay experiment.

4.7. Statistics

All data generated in this study were subjected to statistical analysis for visualization and interpretation. Depending on the specific research question, a suitable application was selected.

Variations of usnic acid content among samples of *F. caperata* from East Africa in Tanzania and Kenya and in Europe from Hungary and Serbia were compared by one-way analysis of variance (ANOVA) using R software version 3.6.3 of 2020.

Before the data were analysed, its normality was checked by a Q-Q plot and the Shapiro-Wilk normality test to ensure it followed a normal distribution. However, samples with extremely high values were considered outliers and therefore, excluded from further analysis to avoid skewness that could negatively influence the conclusion. In determining whether there was any significant difference in the amount of usnic acid from the specimens of *F. caperata* from sampled areas, $p < 0.05$ was considered significant.

To compare the concentration of two LSMs UA and FA from *C. foliacea* specimens, data was first checked for normality. T-test was used pairwisely for comparing concentrations of samples in the R software program version 3.6.3 of 2020.

To determine the effect of pure (+)-usnic acid the data were analysed using a multiple linear regression model approach that included main effects for Concentration (Conc.), Sex, and Time, as well as interaction terms of Conc.*Time, Conc.*Sex, and Time*Sex. A

Posthoc test was then conducted on the significant main effects and interaction terms using the emmeans function from the emmeans R package. All analyses were performed in RStudio using base R (version 4.3.3). The specific code for the model used was: Model <—lm(TotalDeaths ~ Conc + Time + Sex + Conc*Time + Conc*Sex + Time*Sex, group_data).

Code for the Posthoc analysis: *lsmeans < -emmeans(model, ~ Conc*); lsmeans_df <— as.data.frame(lsmeans); *lsmeansT < -emmeans(model, ~ Time)*; lsmeansT_df <— as.data.frame(lsmeansT); lsmeansCT < -emmeans(model, ~ Conc:Time); lsmeansCT_df <— as.data.frame(lsmeansCT); lsmeans_df; lsmeansT_df; lsmeansCT_df.

To determine the effects of *C. foliacea* extract, three-way factorial ANOVA with two-way interactions was conducted using the aov() function in base R, assessing the effects of concentration (Conc.), time, and sex on mortality, this was decided because of the size of our data and the kind of combinations we wanted to observe. Following ANOVA, residual normality was evaluated using the Shapiro-Wilk test (shapiro.test() function in base R), revealing a significant deviation from normality ($W = 0.94656$, $p = 0.0041$). Homogeneity of variances across groups was examined with the levene Test function from the car package (v3.1.2; FOX and WEISBERG 2019), which indicated a violation for concentration (Conc.: $F(8, 63) = 3.64$, $p = 0.0015$) and a marginal violation for time ($F(3, 63) = 2.68$, $p = 0.05$), while no significant variance heterogeneity was observed for sex ($F(1, 63) = 0.40$, $p = 0.486$). Despite these violations, we proceeded with ANOVA due to the small sample size and the lack of non-parametric alternatives capable of analyzing the interaction effects of interest. To substantiate the robustness of our ANOVA analysis, effect sizes (η^2) of the independent variables used in this selected model using the effect-size (v0.8.9; BEN-SHACHAR et al. 2020) R package was checked. Post-hoc comparisons were across significant groups and interactions were performed using the emmeans() and cld() functions to group the significant differences in the levels of our variables from the emmeans (v1.10.3; Lenth, 2024) and multcompView (v0.1-10; GRAVES et al. 2024) packages, respectively. Estimated marginal means (EMMs) were calculated to provide the average total mortality adjusted for other covariates in the model, where EEMs represent the mean numbers of mosquitoes died in the 3-3 replicates of the various treatments during the experiments.

Visualization of results was completed using the ggplot2 R package (v3.5.1; WICKHAM, 2016). All statistical analyses were conducted in R Statistical Software (v4.4.0; R CORE TEAM, 2024). The estimated marginal means (EMMs) of mortality for various concentration levels and exposure times were analyzed.

5. RESULTS AND THEIR DISCUSSION

5.1. Current literature studies on insecticidal and antiprotozoal activities of lichens and lichen secondary metabolites

Our review (MUHORO and FARKAS 2021) on the insecticidal and antiprotozoal properties and role of LSMs on insect vectors and their transmitted protozoal diseases to humans summarised records from 27 literature sources. It confirmed that 7 lichen secondary metabolites from 32 lichen species (Table A1 and A2 (Appendix A2); Table 1 in MUHORO and FARKAS 2021) were characterised with insecticidal or antiprotozoal activity: 1′chloropannarin, evernic acid, gyrophoric acid, psoromic acid, pannarin, usnic acid, and vulpic acid.

57 lichen species containing LSMs with potential insecticidal properties were tested on *Aedes, Anopheles* and *Culex* mosquito species. The larval stages of *A. aegypti*, *An. stephensi*, *C. pipiens* and *C. quinquefasciatu*s were mainly investigated as presented in the Table A1 (Appendix A2) where only those records of 28 lichen species are shown that exhibited a high mortality effect (between 91% to 100%). There were no studies reviewed that showed investigation of the effects of LSMs or their extracts on eggs and the adult stages of the mosquitoes. The review also revealed that the *An. gambiae* complex responsible for malaria transmission in Kenya was never tested. Usnic acid was widely investigated as a potential insecticide. The three main larval stages tested were 2nd, 3rd (VINAYAKA et al. 2009, KHADER et al. 2018) and 4th (CETIN et al. 2008).

The three main solvents used in extracting the lichen crude extracts were methanol, acetone and chloroform. The choice of the solvent used is greatly based on the solubility of the LSMs that affects the yield of the extract after concentration (BOMFIM et al. 2009, MOREIRA et al. 2016). Toxicological assay (using the crustacean brine shrimp *Artemia salina*) to determine the ecological impact of the UA (2 to 10 ppm) was determined and found to be unsafe (BOMFIM et al. 2009). This indicates that the use of UA is not recommended in aquatic ecosystems. Therefore, a more selective synthetic derivative of the UA can solve the toxicological challenges of UA. Further nanoparticle-based compounds known to be biologically active can be modified to exploit their biological potential as shown in several studies (MIE et al. 2012, GOVINDARAJAN et al. 2016, DHANESH GANDHI et al. 2019, KUMAR et al. 2020).

5.2 Revised taxonomic key of the parmelioid clade in Kenya

East African fruticose and foliose lichens were studied in detail through the joint efforts of Dougal Swinscow and Hildur Krog in the 1970s and 1980s, resulting in a synthesis in 1988 (SWINSCOW and KROG 1988). The taxonomy and nomenclature of taxa treated in their identification book ‘Macrolichens of East Africa’ have been significantly changed as a result of

molecular genetic studies carried out during the last decades in several taxonomic groups, especially in the family *Parmeliaceae* (e.g., BLANCO et al. 2004, 2006, CRESPO et al. 2007, 2010, 2011, DIVAKAR et al. 2010, THELL et al. 2012, LEAVITT et al. 2018, GREWE et al. 2020). This species-rich lichen family is widely distributed in the Southern Hemisphere and its largest clade, the parmelioid clade, contains one tenth of the lichen species known worldwide (with c. 1800 spp. (KIRK et al. 2008, THELL et al. 2012)). The group is rich in lichen secondary metabolites with various bioactive and other potential roles. However, the identification of these taxa is difficult since the only key to Macrolichens of East Africa (SWINSCOW and KROG 1988) needs to be revised. In an attempt to update a considerable part of the key, we were concentrating on parmelioid taxa in Kenya (FARKAS and MUHORO 2022). This family is not only rich in species, but also characterized by an enormous diversity in its LSMs (DIVAKAR and UPRETI 2005).

Additionally, our knowledge of the various biological and other roles of these unique substances has also increased (MOLNÁR and FARKAS 2010, NGUYEN et al. 2013, PETROVA et al. 2021). However, little information is available regarding the application of LSMs in terms of their potential insecticidal and antiprotozoal activity (MUHORO and FARKAS 2021). Since Parmeliaceae is largely found in the Southern Hemisphere with main distribution centres being in Southern Africa, South America and Australia (ELIX 1993), field collectors in these regions frequently meet representatives of this taxonomic group. In the case of Kenya, 178 (Appendix 5) of the c. 900 lichen species belong to the parmelioid clade (KROG and SWINSCOW 1987, SWINSCOW and KROG 1988, HALE 1990, STAIGER and KALB 1995, ALSTRUP and APTROOT 2005, ALSTRUP and CHRISTENSEN 2006, ARCHER et al. 2009, ALSTRUP et al. 2010, KIRIKA et al. 2012, 2016a, 2016b, 2016c, 2017, , 2019, LÜCKING and TIMDAL 2016, BJELLAND et al. 2017, KANTELINEN et al. 2021, KIRIKA and LUMBSCH 2021). All contain green alga photobiont.

The following morphological characteristics were checked among the revised parmelioid species: growth form – foliose, not umbilicate, thallus corticate above and below, adnate or loosely attached to substrate, rhizines – present, medulla – solid, colour – grey, yellowish green or brownish, fruitbody if present – apothecium with thalline exciple, ascospores – simple, pycnida – laminal.

Some of the important morphological characters are illustrated: lamina, lobe margin, lower and upper surface (Fig. 12), cilia and rhizines (Fig. 13).

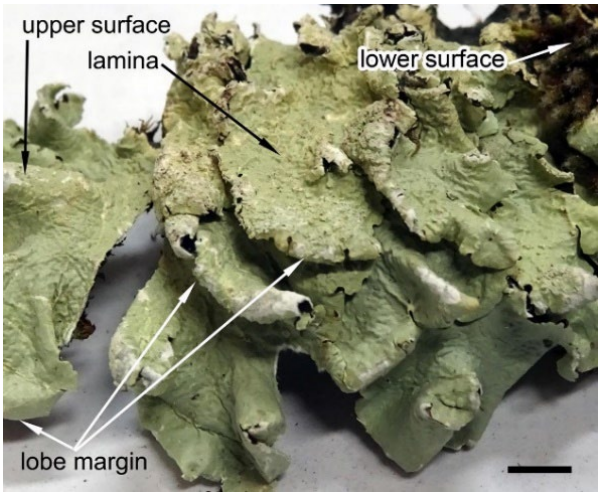

Fig. 12. Parts of a foliose thallus indicated by arrows: upper and lower surface, lamina, lobe margin (*Flavoparmelia caperata*, A. M. Muhoro 21/01). Scales = 1 mm.

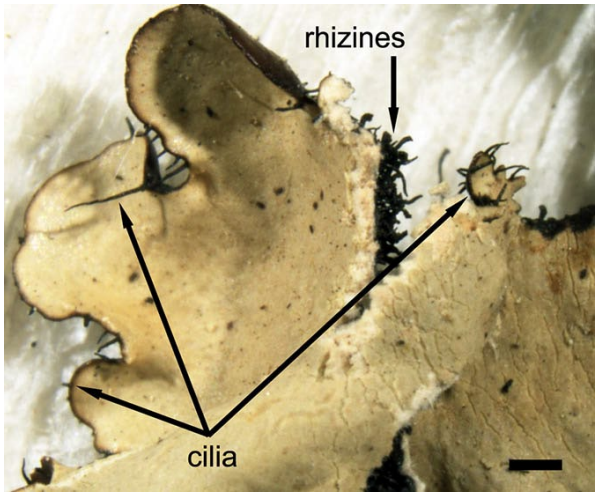

Fig. 13. Appendages on the thallus: cilia, rhizines (*Parmotrema ultralucens*, VBI 2217). Scale = 0.5 mm.

The 178 species after nomenclatural revision belong to the genera *Bulborrhizina* (1), *Bulbothrix* (9), *Canoparmelia* (9), *Cetrelia* (1), *Crespoa* (1), *Flavoparmelia* (4), *Flavopunctelia* (2), *Hypotrachyna* (37), *Melanelixia* (1), *Myelochroa* (1), *Parmelia* (2), *Parmelinella* (1), *Parmotrema* (64), *Pseudoparmelia* (2), *Punctelia* (9), *Relicina* (4), *Remototrachyna* (1) and *Xanthoparmelia* (29).

The dichotomous main key (Appendix 6 – detail of the key presented) leads to species where the genus has only a small number of representatives (maximum 4 species), but otherwise to genera. Larger genera are treated separately after the main key. *Bulborrhizina* (1) is treated in the generic key (*Bulbothrix* s. lat.) together with *Bulbothrix* (9); *Canoparmelia* (9) and *Pseudoparmelia* (2) are also treated in the same key.

5.3. New distribution records of lichens from East Africa

New distribution records of lichen and lichenicolous fungi species were identified from East African samples (FARKAS et al. 2023). Their chemical analysis confirmed the presence of LSMs with bioactive properties. *Bulbothrix kenyana*, *Chrysothrix xanthina*, *Lobaria discolor*, *Parmotrema durumae* and *P. taitae* were discovered as new for Tanzania; *Usnea abissinica* and *U. sanguinea*, furthermore lichenicolous fungi *Didymocyrtis* cf. *melanelixiae*, *Lichenoconium erodens* and *Spirographa lichenicola* are new for Kenya (Table 2).

Further 29 taxa were listed in alphabetical order (FARKAS et al. 2023). Here below the new distribution records are discussed only. Lichenicolous fungi are indicated by a # sign. New record for East Africa are signed by **.

Table 2. New distribution records of lichens and lichenicolous fungi in Kenya and Tanzania with lichen secondary metabolites (those with potential insecticidal properties indicated in bold).

Species of lichen/Fungi	Country	location and collectors	LSMs/Host
Bulbothrix kenyana	Tanzania (Tanga	Tanga by E. Farkas (1986)	atranorin and salazinic acid (with *Chrysothrix xanthina*)
Chrysothrix xanthina	Tanzania	Arusha by T. Pócs & J. Kjelland-Lund (1988), E. Farkas (1986)	calycin and pinastric acid
Lobaria discolor	Tanzania	Tanga by E. Farkas (1986)	fatty acid and **gyrophoric acid**
Parmotrema durumae	Tanzania	Tanga by E. Farkas (1986)	alectoronic acid, atranorin, and α-collatolic acid
P. taitae	Tanzania	Tanga by E. Farkas (1986)	atranorin and **fumarprotocetraric acid**
Usnea abissinica	Kenya	Nyeri County by A.M. Muhoro (2021)	**usnic acid** and salazinic acid
U. sanguinea	Kenya	Nyeri County by A.M. Muhoro (2021)	**usnic acid**
Didymocyrtis cf. melanelixiae	Kenya	Nyeri County by A.M. Muhoro (2021)	on *Parmotrema austrosinense*
Lichenoconium erodens	Kenya	Nyeri County by A.M. Muhoro (2021)	on *P. austrosinense*
Spirographa lichenicola	Kenya	Nyeri County by A.M. Muhoro (2021)	on *P. austrosinense* and *P. reticulatum*

Bulbothrix kenyana Kirika, Divakar & Lumbsch

The species *Bulbothrix kenyana* (Fig. 14) was described on the basis of an extended, worldwide molecular genetic study a few years ago (KIRIKA et al. 2017). While identifying the specimen from the East Usambara Mts (VBI 6180), a careful investigation of the most important characters (emaculate upper surface, pale brown lower surface, marginal cilia reduced to bulbate nodules) resulted in the recognition of this species as a new distribution record from Tanzania and the taxonomic revision of a specimen earlier identified as *B. isidiza* (VBI 1691). Both *B. isidiza* and *B. kenyana* contain atranorin and salazinic acid and the ascospore sizes are overlapping: 10–15 × 5–7 µm in *B. isidiza* (SWINSCOW and KROG 1988), 7.5–15.0 × 5.0–7.5 µm (10.30–11.75 × 6.00–7.00 µm, ± SD = 1.8–1.7 × 1.3–4.0 µm, n = 40) in *B. kenyana* (KIRIKA et al. 2017), while in our specimens are the following: (10)–11.7–(13.8) × (4.7)–5.9–(6.9) µm ± 1.22 × 0.75 (n = 16) (VBI 6180) and (10.6)–12–(14) × (4.4)–5.7–(7) µm ± 1.7 × 1.22 (n = 10) (VBI 1691).

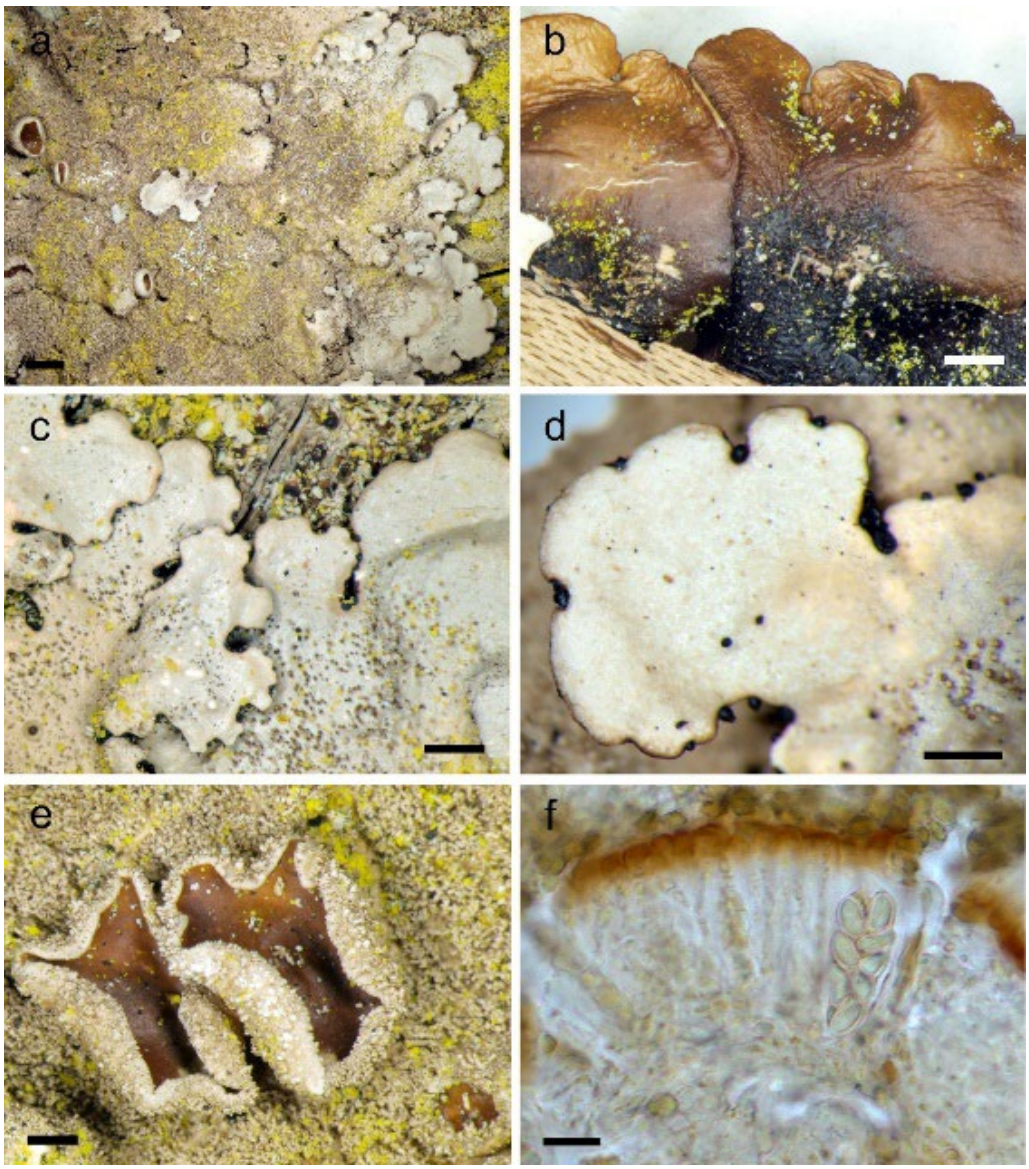

Fig. 14. *Bulbothrix kenyana*: a), a part of the thallus richly covered by isidia, with a few apothecia and marginal lobes, b), a pale brown lower surface with a papillate to naked zone towards the margin, c) –d), marginal lobes with cilia reduced to bulbate nodules and with often brown tipped isidia on the lamina, e), two large apothecia and a small one on the thallus richly covered with isidia and with granules of *Chrysothrix xanthina,* f), asci and ascospores in section (a–c, e, f: VBI 6180, with *Chrysothrix xanthina*; d: VBI 1691). Scales: a = 2 mm, b, c, e = 1 mm, d = 500 µm, f = 10 µm.

Chrysothrix xanthina (Vain.) Kalb

This crustose-leprose species consists of fine granules (20–50 µm) and contains a yellow pigment, pinastric acid (all investigated specimens) and rarely also another yellow pigment (calycin) is produced in tropical specimens (VBI 6246). It has been mentioned from Kenya, but probably new for Tanzania (KALB 2001). More recent studies consider it as a tropical and temperate region species growing on bark of both coniferous and deciduous trees, also on lignum

and seldom on sheltered, somewhat shaded sandstone (HARRIS and LADD 2008; ELIX and KANTVILAS 2007).

**#*Didymocyrtis cf. melanelixiae* (Brackel) Diederich, R.C. Harris & Etayo

The shape (ellipsoid to subspherical) and size (4.8–5 × 3.2–3.5 µm, length/width>1.4) of the conidia – having one bigger or two smaller guttules – are in the range of the original description (BRACKEL 2011). The species has a wide distribution area and it occurs mainly on parmelioid lichens (ERTZ et al. 2015), from East Africa it was found on *Parmotrema austrosinense* also together with #*Lichenoconium erodens*, on *Parmotrema reticulatum*.

**#*Lichenoconium erodens* M.S. Christ. & D. Hawksw.

This fungus is highly pathogenic on various lichen hosts (HAWKSWORTH 1977; DARMOSTUK 2019) causing bleached, black marginated necrotic spots on the surface of the host. In the middle of each spot there are several pycnidia containing brown, spherical conidia. It seems to be common also in East Africa.

Lobaria discolor (Bory ex Delise) Hue

Corticolous in montane forest at c. 2,200 m altitude – rare in East Africa (Kenya, Madagascar, Reunion). It was also found in South America, more frequently in Japan, Indonesia, Australia (SWINSCOW and KROG 1988, GBIF 2023). It is new for Tanzania.

Parmotrema durumae (Krog & Swinscow) Krog & Swinscow

Saxicolous on dry, exposed rock at 1,750 to 2,100 m altitude, uncommon. Earlier it was found in Kenya and Uganda, also in South Africa and Madagascar (SWINSCOW and KROG 1988). It is new for Tanzania.

Parmotrema taitae (Krog & Swinscow) Krog & Swinscow

Earlier it was known from Kenya as saxicolous on granitic rock at 2,000 m altitude (SWINSCOW and KROG 1988). The here presented new records from Tanzania are both saxicolous and ramicolous.

**#*Spirographa lichenicola* (D. Hawksw. & B. Sutton) Flakus, Etayo & Migdl.

This parasitic fungus causes bleached patches with a greyish circle on the surface of the host. The main axis of the Y-shaped conidia is 8.2–10.2 × 1.6 µm, arms (=appendages) 4.1–4.4 µm (n = 50) are agreeing well with data presented in the original description (cf. 6–11.5 × 1.2 µm, appendages 2.5–6 µm – HAWKSWORTH 1976). Although FLAKUS et al. (2019) concluded mainly from their molecular genetic results in South America that *Spirographa* species are strongly host-specific (at the generic level of the host), it is confirmed also by the current East

African records occurring on various *Parmotrema* species. This species can be widespread also in East Africa.

Usnea abissinica Motyka

On trunks of *Erica acrophya*, probably at c. 3,000 m altitude, a single locality was known from Ethiopia (SWINSCOW and KROG 1988). New distribution record for Kenya.

Usnea sanguinea Swinscow & Krog

Locally common in Arusha National Park (Tanzania) on branches of trees and shrubs in open woodland at 1,500 to 2,600 m altitude (SWINSCOW and KROG 1988). New distribution record for Kenya.

5.4. Analyses of LSMs by chromatographic methods in *Flavoparmelia caperata*

LSMs in the lichen *Flavoparmelia caperata* were analysed by various chromatographic methods, HPTLC, chiral chromatography and HPLC-PDA (FARKAS et al. 2024a).

LSMs were identified in 28 samples collected from Africa (Kenya and Tanzania) and Europe (Hungary and Serbia) by HPTLC method. The presence of usnic acid, protocetraric and caperatic acid in samples is shown in Fig. 15 against controls (atranorin, zeorin and norstictic acid).

This result confirms assumptions based on literature data (SWINSCOW and KROG 1988, VERSEGHY 1994, BRODO et al. 2001, NASH and ELIX 2002, DIVAKAR and UPRETI 2005, AWASTHI 2007, SMITH et al. 2009, WIRTH et al. 2013, BRODO 2016).

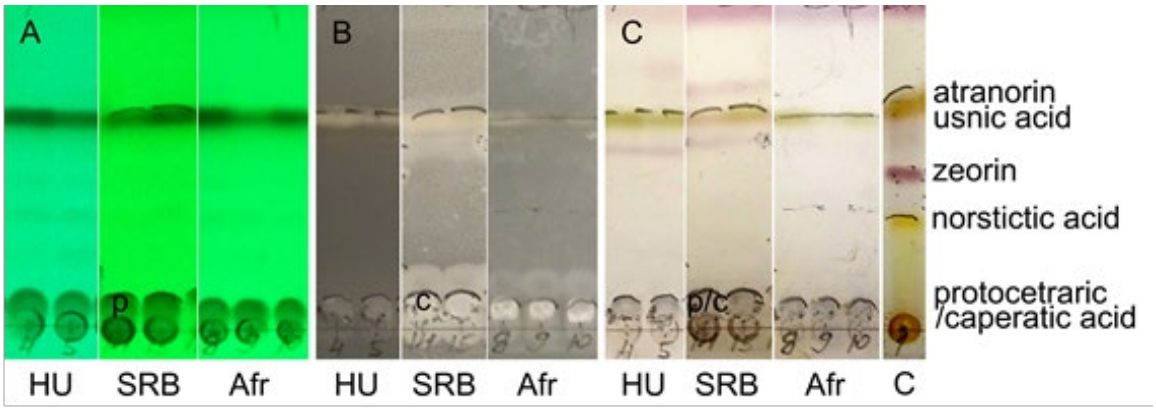

Fig. 15. Usnic acid, protocetraric acid (p) and caperatic acid (c) sampled from *Flavoparmelia caperata* specimens collected in Europe: Hungary (HU), Serbia (SRB), and Africa (Afr) is presented on developed HPTLC plates (in solvent system C according to ARUP et al. 1993) with control containing atranorin, zeorin and norstictic acid (C): **A**, under UV 254 nm; **B**, sprayed with water; **C**, in daylight after sprayed with 10% sulphuric acid and charring.

Analysis of specimens (13) collected in Europe and Africa using a chiral chromatographic method indicates that only (+)-UA was confirmed in specimens as shown in chiral chromatogram (Fig. 16). The measured concentrations are ranging from 5.08 to 26.43 mg/g dry wt in European samples (12) and 20.27 mg/g dry wt in a sample from Kenya (Table 3).

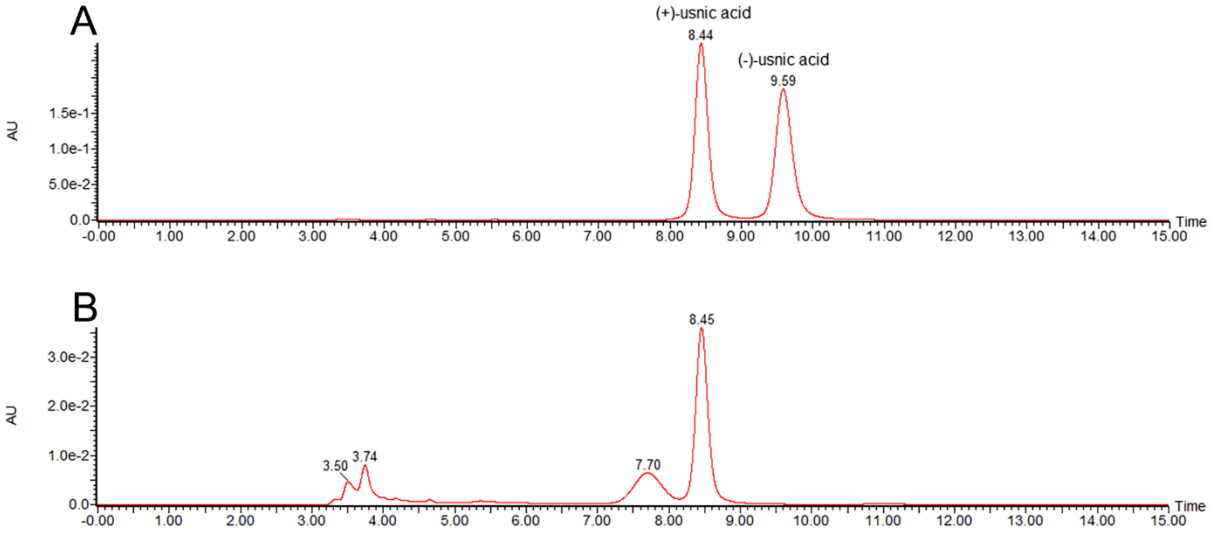

Fig. 16. Usnic acid enantiomers in the lichen *Flavoparmelia caperata*. **A**, the chiral HPLC chromatogram showing separation of usnic acid enantiomer standards: (+)-usnic acid eluting at 8.44 min and (-)-usnic acid eluting at 9.59 min; **B**, the chromatogram showing only (+)-usnic acid present in *F. caperata*.

The usnic acid content (mg/g dry wt) was measured by HPLC-PDA in all 28 samples. The content of usnic acid shows a substantial variation in both continents, ranging from 5.21 to 19.23 mg/g dry wt (mean = 9.90 ± 2.52) in Europe and from 6.15 to 23.54 mg/g dry wt (mean = 9.79 ± 2.29) in Africa (Table 3). The comparison between continents did not result in significant differences ($p = 0.91$). Since there was no significance difference ($p < 0.05$) (Fig. 17) in the concentration of UA in the samples from the two continents, it can be explained by the supposedly similar microclimatic conditions of the habitats (within macroclimatically different sites) that are probably consistent with the specific niche requirements of *F. caperata* as it is further discussed below.

Since UA is a cortical pigment that protects lichens from solar radiation (SOLHAUG et al. 2003, VERES et al. 2022a), it can be concluded that the amount of solar radiation might reach similar levels in the two regions having a major role in the production of UA. Thus, macroclimatic conditions (MOREAU 1938, VAN ZINDEREN BARKER 1962) supposedly play less role in that compared to microclimatic differences where they grow (RICHARDS 1952, RICKLEFS 2008, HEILMANN-CLAUSEN et al. 2014).

Table 3. Concentrations (with standard deviation) of usnic acid measured by HPLC-PDA (n= 28) and (+)-usnic acid measured by chiral chromatography (n = 13) in the collected *Flavoparmelia caperata* samples.

Sample nr	**Usnic acid*** [mg g^{-1} dry weight]	**(+)-usnic acid**** [mg g^{-1} dry weight]
1	12.35 ± 1.99	-
2	9.03 ± 4.17	-
3	8.66 ± 2.68	-
4	12.95 ± 3.51	-
5	8.47 ± 0.17	17.90 ± 0.43
6	8.78 ± 0.20	18,23 ± 0.19
7	11.48 ± 1.00	9.77 ± 0.07
8	11.22 ± 0.84	19.13 ± 0.79
9	7,71 ± 1.26	15.54 ± 0.79
10	9.04 ± 0.54	15.37 ± 0.45
11	9.28 ± 0.20	13.23 ± 0.22
12	6.83 ± 0.79	5.08 ± 0.28
13	14.43 ± 0.36	17.84 ± 0.47
14	8.92 ± 0.92	9.27 ± 0.42
15	9.92 ± 0.90	-
16	19.23 ± 1.61	26.43 ± 0.24
17	14.73 ± 2.22	-
18	-	13.59 ± 0.50
19	-	20.27 ± 1.44
20	10.62 ± 0.38	-
21	9.33 ± 1.30	-
22	5.21 ± 0.30	-
23	23.54 ± 0.58	-
24	9.55 ± 0.96	-
25	11.94 ± 1.06	-
26	12.89 ± 1.25	-
27	9.96 ± 0.63	-
28	10.30 ± 0.35	-
29	6.15 ± 0.76	-
30	6.92 ± 0.98	-

* Mean concentration measured by HPLC-PDA. ** Mean concentration measured by chiral HPLC.

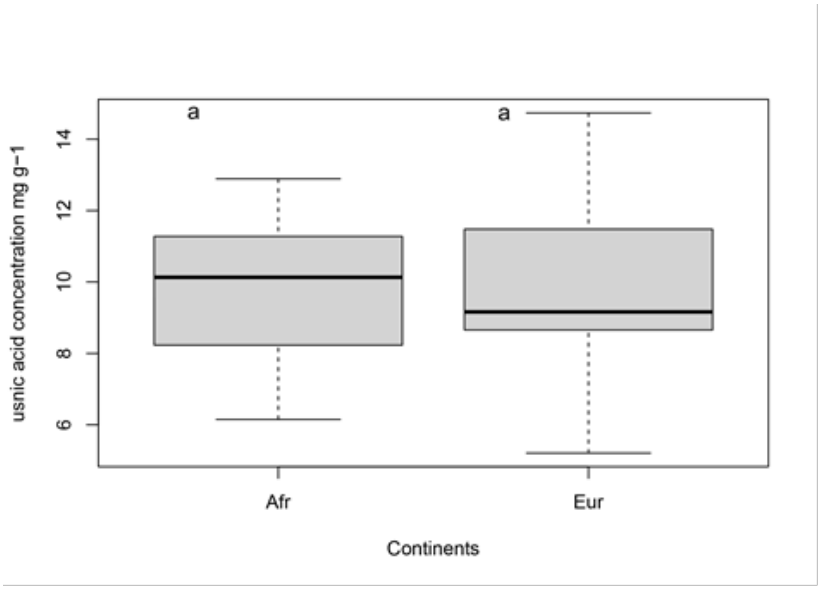

Fig. 17. Usnic acid concentrations [mg/g dry wt] in the lichen *Flavoparmelia caperata* measured in Europe (Eur) and Africa (Afr) by HPLC-PDA. The lines represent the minimum and maximum values, the box represents the 25 and 75% of the data, the thick line represents the median. Continents indicated by the same letter are not significantly different at 95% confidence.

In Europe (temperate region) *F. caperata* grows in lower altitudes (below 2000 m a.s.l.) while in Africa (tropics) it grows between 1500 and 3600 m (SWINSCOW and KROG 1988, VERSEGHY 1994, SMITH 2009, WIRTH et al. 2013). The habitats at these different elevations could be associated with similar microclimatic conditions, hence exhibit specific and suitable ecological requirements of *F. caperata* in both continents. However, further studies on the effect of other environmental conditions and the influence of genetic background of *F. caperata* using larger sample size from various geographical regions may shed light on the additional details of LSM production in this species.

5.5. Analysis of LSMs fumarprotocetraric acid (FA) and (-)-usnic acid (UA) in *Cladonia foliacea* from Central and Southern Europe by chromatographic methods

LSMs in the lichen *C. foliacea* were analysed by various chromatographic methods: HPTLC, chiral chromatography and HPLC-PDA (FARKAS et al. 2024b).

C. foliacea samples collected from Hungary (20) and Slovakia (1), North Albania (7) and North Macedonia (1) were first checked for the presence of UA and FA by HPTLC method. The two LSMs were confirmed in these samples (Fig. 18).

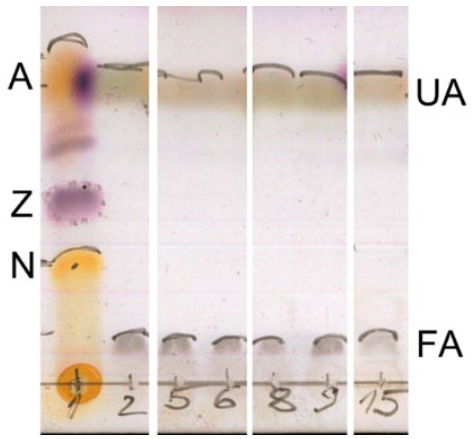

Fig. 18. Characteristic spots of lichen secondary metabolites of *Cladonia foliacea* samples on a high performance thin layer chromatography plate developed in solvent system C according to ARUP et al. (1993). Abbreviations: A = atranorin, Z = zeorin, N = norstictic acid (controls); UA = usnic acid, FA = fumarprotocetraric acid.

It was shown that (-)-UA was produced by *C. foliacea* and confirmed in the 29 samples using the chiral chromatographic method (Fig. 19). At the current limit of detection (ca. 18 mg/ml) (+)-UA was not detected. There was a substantial variation in the quantity of (-)-UA (6.88 to 34.27 mg/g lichen dry wt) therefore, the isomer may constitute 3.4% lichen dry wt (Table 4).

The FA content of the 29 samples was detected by HPLC-PDA. The content of FA (1.44–9.87 mg/g dry wt) was lower than that of UA (Table 4).

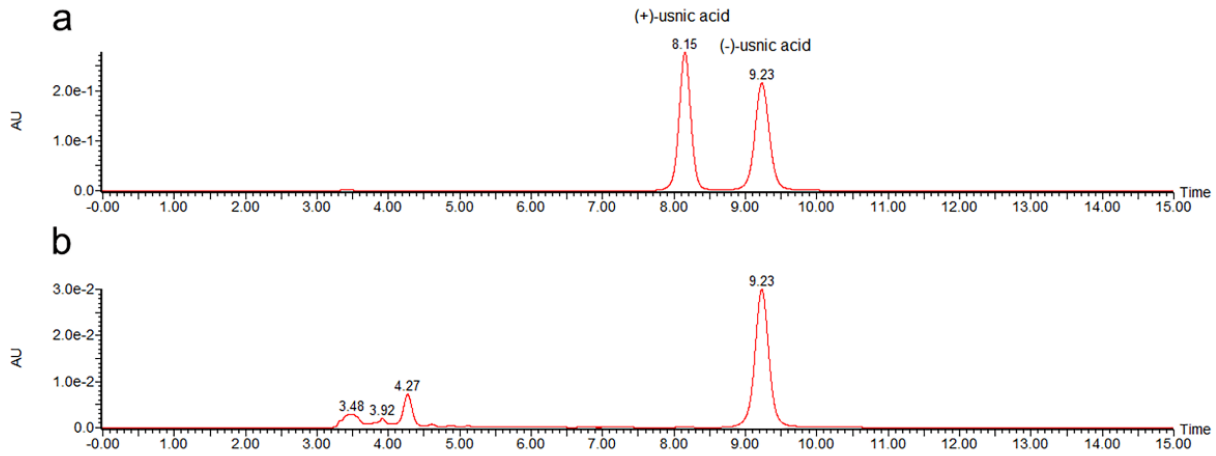

Fig. 19. Chiral separation of the usnic acid enantiomers. **a**, The chiral HPLC chromatogram showing separation of usnic acid enantiomer mixture standard: (+)-usnic acid eluting at 8.15 min and (-)-usnic acid eluting at 9.23 min, **b**, a sample chromatogram showing only (-)-usnic acid present in *Cladonia foliacea.*

Determination of the content and confirmation of (-)-UA in *C. foliacea* in specimens collected in Hungary and nearby countries was first attempted in this study using chiral chromatography method (XU et al. 2022). Our wider sampling provided more support for the enantiomer production pattern in the lichen, and also provided direct evidence that (+)-UA is not present in detectable amounts.

Table 4. Mean concentrations (with standard deviation) of fumarprotocetraric acid (n=5) and (-)-usnic acid (n=3) in the collected samples.

Sample ID	**Fumarpotocetraric acid*** [mg/g d. w.]	**(-)-usnic acid**** [mg/g d. w.]
CLF1	5.36 ± 0.63	24.56 ± 1,13
CLF2	4.57 ± 0.58	34.27 ± 2,43
CLF3	5.54 ± 0.90	17.04 ± 0,41
CLF4	4.70 ± 0.41	21.58 ± 0,49
CLF5	4.09 ± 0.48	21.95 ± 1,19
CLF6	5.04 ± 0.37	10.24 ± 0,46
CLF7	5.05 ± 0.46	12.36 ± 0,53
CLF8	5.45 ± 0.27	8.05 ± 0,40
CLF9	4.34 ± 0.24	11.96 ± 0,51
CLF10	3.64 ± 0.38	11.11 ± 0,25
CLF11	4.60 ± 2.05	10.35 ± 0,37
CLF12	6.06 ± 1.62	19.51 ± 1,15
CLF13	4.41 ± 0.30	11.96 ± 0,73
CLF14	5.30 ± 0.87	15.35 ± 0,55
CLF15	3.29 ± 0.36	24.06 ± 0,70
CLF16	1.94 ± 0.14	7.37 ± 0,32
CLF17	1.71 ± 0.21	16.33 ± 0,68
CLF18	3.03 ± 0.73	6.88 ± 0,26
CLF19a	1.44 ± 0.07	16.37 ± 0,59
CLF19b	2.45 ± 0,04	30.55 ± 0,44
CLF20	5.28 ± 0.78	16.14 ± 0,23
CLF21	9.87 ± 2.27	19.87 ± 0,50
CLF22	4.60 ± 0.62	15.04 ± 0,22
CLF23	3.85 ± 0.57	17.38 ± 0,70
CLF24	4.90 ± 0.58	9.83 ± 0,19
CLF25	6.78 ± 0.59	21.27 ± 0,27
CLF26	5.98 ± 0.48	20.68 ± 0,13
CLF27	5.80 ± 0.19	13.35 ± 0,45
CLF28	7.02 ± 0.29	11.59 ± 0,22

* Mean concentration measured by HPLC-PDA. ** Mean concentration measured by chiral HPLC.

Previously isolated (-)-UA from Italian (CAVALLORO et al. 2021) and French samples (BÉZIVIN et al. 2004), supports that *C. foliacea* is conservative in only producing the (-)-UA

isomer. The quantitative variation in these lichen metabolites is large (5–6-times difference between the lowest and the highest) in both UA (6.88 to 34.27 mg/g dry wt) and FA (1.44 to 9.87 mg/g dry wt) content among the various tested lichen specimens. There was also up to two-fold differences between the specimens collected in the same or very near sites (e.g., samples CLF1–3, CLF4–6; CLF16–17, CLF19a–19b or CLF26–27).

Previous studies that differ with the results of this study show that FA content is higher ($p < 0.0001$) in specimens obtained from mountain areas than those collected in lowland and UA concentration was significantly higher in specimens obtained from lowlands than those obtained from mountain areas ($p<0.0001$) (VERES et al. 2022a). The possible explanation for these differences could be due to the sampling strategies of the two studies where studies by VERES et al. (2022a) were conducted at regular intervals of six months over a period of 3 years in Hungary and in the current study the samples were not collected at regular time intervals and seasons. Sampling was also done at a broader geographical range. This indicates that the concentration of UA and FA is determined by temporal and environmental factors.

5.6.Mortality of *Anopheles gambiae* measurements against different concentrations of (+)-UA enantiomer

The impact of ingested (+)-UA containing toxic sugar bait (UA TSB) on *Anopheles gambiae* was determined by the number of dead mosquitoes counted and expressed as a percentage in comparison to the control (MUHORO et al. 2024b). The concentration of the UA TSB and the period of exposure as predictors were evaluated as indicated in Fig. 20. UA TSB affected both male and female mosquitoes. Higher mortality (50%) was observed at 15mg/ml in 4 hours, however, the mortality rate declined when exposure was extended to 72 hours (Fig. 20). Post-exposure mortality compared to the duration of exposure at the highest concentration (15 mg/ml), when increased, did not result to a significant difference in mortality, however, there is a considerable difference when compared to 5, 10 and 15 mg/ml. Despite the observed low mortality (about 30%) after 4 hours of exposure, the mortality rates were much lower after 24, 48 and 72 hrs. Furthermore, the 15 mg/ml concentration. caused the highest mortality after 4 hours exposure. This indicates that acute toxicity was associated with 15 mg/ml compared to 5 and 10 mg/ml where more than 30% mortality was achieved after 4 hrs treatment. At 10 mg/ml, mortality was sustained from 4 hours to 24 and 48 hours at 36.7%.

Notably, in all treatments, there was an increased high mortality rate at 4, 24 and 48 hours post-exposure. Low mortalities were observed after 72 hours of exposure compared to the control.

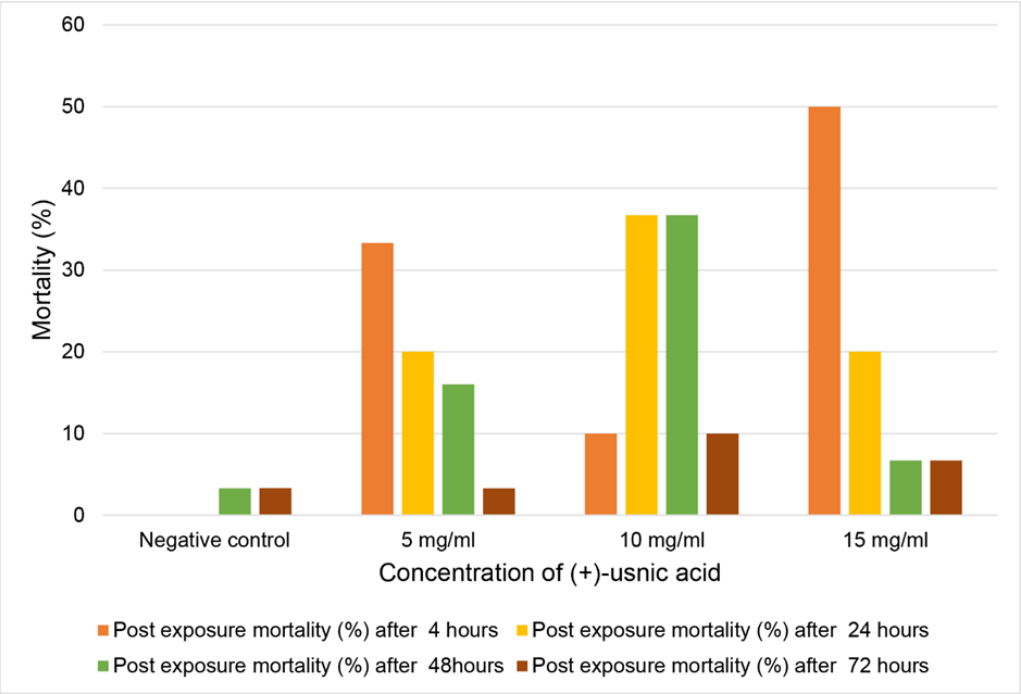

Fig. 20. Post-exposure mortalities (%) of *Anopheles gambiae* after 4, 24, 48, and 72 hours in different concentrations of (+)-UA.

Since both male and female consumed the UA TSB, their levels of susceptibility were shown to be different after 72 hours post exposure bioassay. At 4 hours and 15 mg/ml concentration, mortality among females were 60% while the male mortality was 40% (Fig. 21). The mortalities suggest a higher intake of the UA TSB and susceptibility to the UA TSB. At 5 mg/ml and 15 mg/ml concentrations, mortality for both males and females was 20% after 24 hours. A higher death rate (48%) among males were recorded at 10 mg/ml after 48 hours while female mortality was 48% after 24 hours at 10mg/ml. Extending post exposure time to 72 hours did not increase mortality for male and female mosquitoes since the mortality was less than 13%. Higher mortality of males was observed after 48 h compared to females in all three treatments.

Acute toxicity using 4% boric has been confirmed in other studies where it caused 100% mortality of *Aedes aegypti* and *An. stephensi* within 24 hours in laboratory bioassay (KUMAR et al. 2022). Boric acid has also been shown to be toxic to both males and females in equal proportions, but decreased after 24 hours. However, fewer males readily took the toxic agent compared to females (BARBOSA et al. 2019). In another study by ALLAN (2011) female *Culex quinquefasciatus* were reported to be less susceptible than males (ALLAN 2011). Deltamethrin has demonstrated to be toxic when used as ingredient in ATSB against *Ae. aegypti.* After 24 hours of exposure its impact was 8.33% to 97.44% mortality. Since the impact of synthetic insecticide have demonstrated to be effective insecticide ingredient in ATSB, considering its potential negative impact to the environment and on the non-target organism, several insect orders such as

Hymenoptera, Lepidoptera, *Coleoptera*, *Diptera*, *Hemiptera* and *Orthoptera* can be affected (QUALLS et al. 2015). Other biological ingredients have been evaluated with promising results based on field and laboratory results. The active biological ingredients used include eugenol (QUALLS et al. 2015), microencapsulated garlic oil (JUNNILA et al. 2015), *Bacillus thuringiensis israelensis* (*Bti*) (DAVIS et al. 2021) and spinosyns (actinobacterial secondary metabolites, fermentation-derived insecticides) (ALOMAR et al. 2022). Application of *Bti* ingredient in TSB was shown to cause higher mortality after 48 hours (97% for *Ae. aegypti*, 98% for *Ae. albopictus,* and 100% for *Cx. quinquefasciatus*), when these results are compared to synthetic insecticides in TSB, *Bti* has optimum mortality after 24 hours. The use of nano formulated oral TSB has been shown to overcome this limitation. This claim has been confirmed in several applications of nano-formulated toxic agents used in ATSB can prolong the efficacy of the insecticide. Nano-formulated toxic agents have been demonstrated to be safe for non-target invertebrates (BENELLI, 2016, DHANESH GANDHI et al. 2019, LOGANATHAN et al. 2023). Specific example is the application of nano-ATSB cypermethrin with prolonged efficacy after 72 hours of delayed mortality bioassay on *An. gambiae* (FARHAN et al. 2024). Delayed mortality (93.3%–100.0%) for up to 7 days has been confirmed among field collected male and female *Ae. albopictus* mosquitoes when boric acid is used as toxic agent in TSB (CHIU et al. 2024).

Based on the promising results of the current new mosquito control method (ATSB), this study aimed at exploring the potential of (+)-UA as oral TSB to kill both male and female *An. gambiae*. It provides the knowledge that increasing concentrations of (+)-UA has the potential to kill target mosquitoes from 4 hours to 72 hours post-exposure and the degree of susceptibility of both male and female mosquitoes under laboratory conditions. The susceptibility of laboratory-reared *An. gambiae* to (+)-UA as an oral insecticide via TSB has not been evaluated since botanical biopesticide has not received much attention as an alternative oral toxic agent. This study demonstrated its promising potential as an effective oral insecticide. Despite the positive results of this study, there were several limitations, all experiments were conducted under laboratory conditions, thus it did not use field-collected or resistant mosquitoes for bioassay experiments. Furthermore, the effects of (+)-UA on non-target organisms were not determined. No further mortality recordings were performed after 72 hours of exposure. No other sources of meal were provided to determine feeding preferences among male and female mosquitoes.

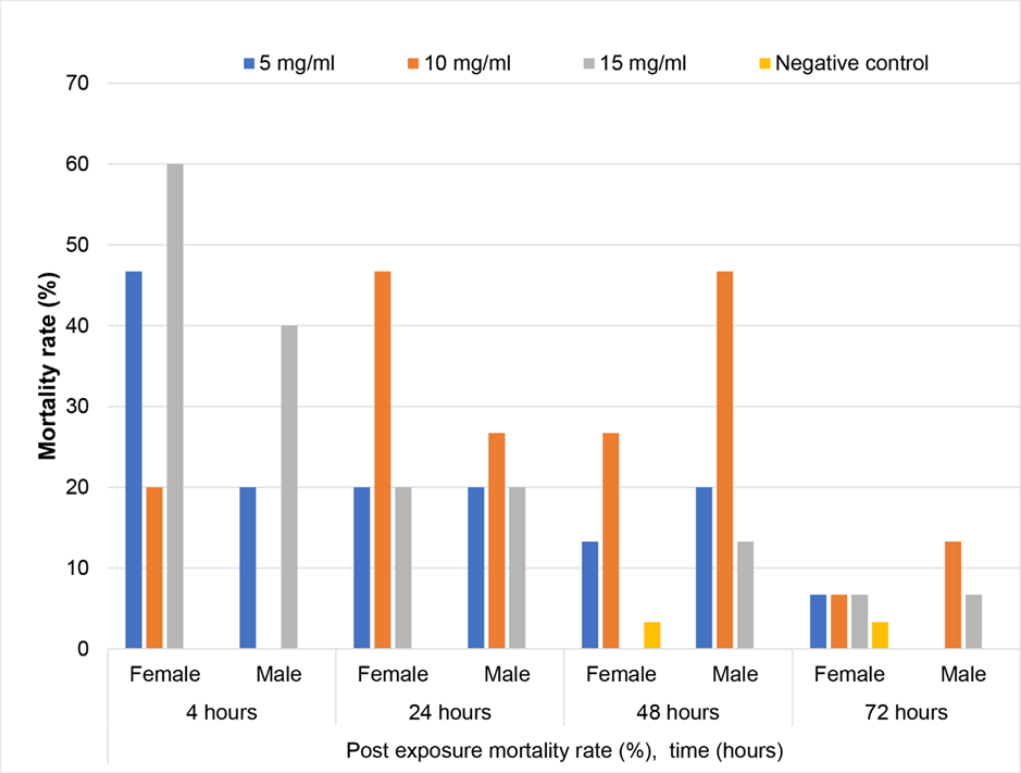

Fig. 21. Mortality (%) of both male and female *Anopheles gambiae* after oral ingestion of (+)-UA.

5.7. Mortality of *Anopheles gambiae* measurements against different concentrations of *Cladonia foliacea* extract containing FA and (-)-UA enantiomer

Bioassay experiments were conducted on *Anopheles gambiae* Kisumu strain mosquitoes (MUHORO et al. 2024a). Mortality rates were measured after ingesting TSB with lichen extracts *C. foliacea* containing FA and (-)-UA in different concentrations. The effects of concentration and time of exposure on *An. gambiae* mortality were found to be statistically significant, with concentration showing a large effect size ($F(8,36) = 5.837$, $p < 0.0001$, $\eta^2 = 0.56$) and time also indicating a substantial effect ($F(3,36) = 4.611$, $p = 0.00787$, $\eta^2 = 0.28$). As sex did not significantly influence mortality ($F(1,24)=0.889$, $p = 0.3614$), it was excluded from the model. The interaction between concentration and time (Conc:Time) was statistically significant with a large effect size ($F(24,36) = 2.559$, $p = 0.00526$, $\eta^2 = 0.63$), while other two-way interactions lacked significance (Conc:Sex ($F(8,24) = 0.531$, $p = 0.8215$); Time:Sex ($F(3,24) = 1.984$, $p = 0.1432$) and were therefore removed from the further analysis (in the new model).

According to the three-way ANOVA results, concentration and exposure time have statistically significant effects on the total mortality of adult *An. gambiae*, indicating that mortality levels vary significantly with changes in concentration and exposure duration. However, sex does not have a statistically significant effect on mortality, suggesting that male and female *An. gambiae* show similar susceptibility to the *C. foliacea* extract.

Exposure durations of 24 hours (M = 1.444, 95% CI = [0.952, 1.937]) and 48 hours (M = 1.222, 95% CI = [0.729, 1.715]) had significantly higher mean mortality compared to other exposure times and were marked with the letter "b," and with a slightly higher mortality observed at 24 hours than at 48 hours (Fig. 22, Table 5).

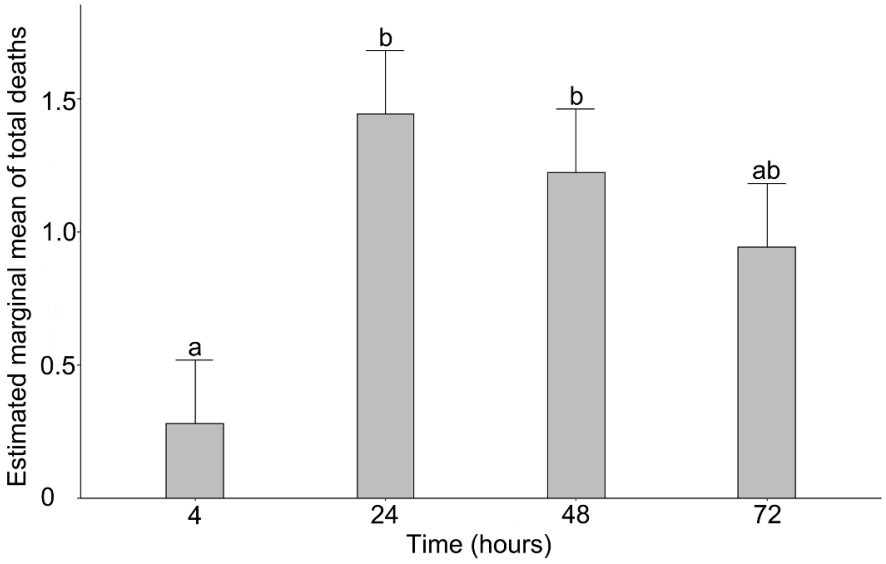

Fig. 22. Mean mortality after exposure to *Cladonia foliacea* extract from 4 hours to 72 hours. Similar letters signal no statistical difference between the groups at 95% confidence.

Table 5. Results of analysis of variance (ANOVA) followed by Tukey-HSD post-hoc-test on mortality effect between 4 and 72 hours after *Anopheles gambiae* ingested *Cladonia foliacea* extract sugar bait. Results are averaged over the levels of concentration and sex. 95% of confidence level was used.

Exposure time (hours)	emmean	SE	df	Lower CL	Upper CL	Group
4	0.278	0.239	24	–0.215	0.771	a
72	0.944	0.239	24	0.452	1.437	ab
48	1.222	0.239	24	0.729	1.715	b
24	1.444	0.239	24	0.952	1.937	b

Mortality of *An. gambiae* was observed in the concentartions range between 10 mg/ml to 90 mg/ml of *C. foliacea* extract. The Estimated marginal means (EMMs) of mortality at 50 mg/ml (M = 2.375, 95% CI = [1.658, 3.092]) was significantly higher than at other concentration levels (Fig. 23, Table 6). These results indicate dose and time-dependent mortality response.

The interaction between a concentration of 50 mg/ml and 24 hours of exposure showed the highest mean mortality (M = 6.0, 95% CI = [4.52, 7.48]) marked with the letter ‘b’ (Fig.23, Table 6). These results indicate dose and time-dependent mortality response.

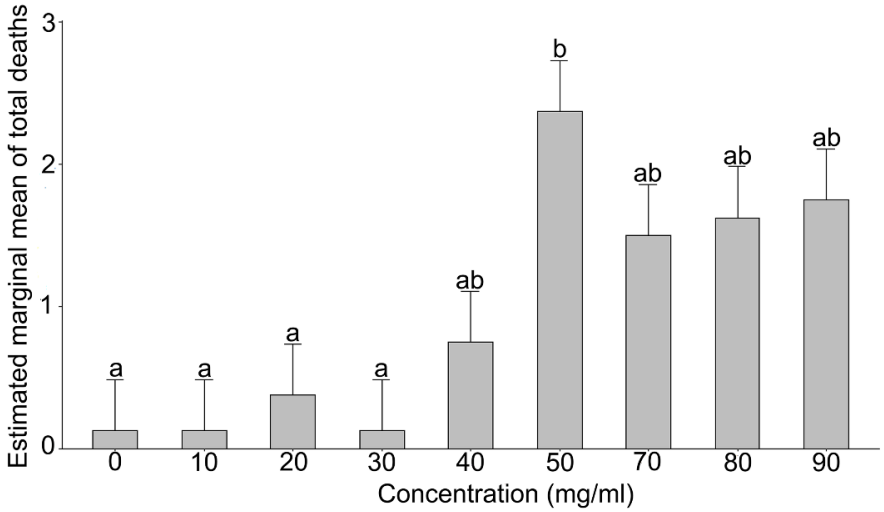

Fig. 23. Mean separation of the significant effects of *Cladonia foliacea* extract concentration on the average total *An. gambiae* mortality. (Concentrations sharing similar letter "a" are not significantly different in terms of total mortality).

Interaction combinations with similar mortality outcomes were assigned the same letter, indicating non-significant differences between them (e.g., "a" or "ab") (Fig. 24, Table 6).

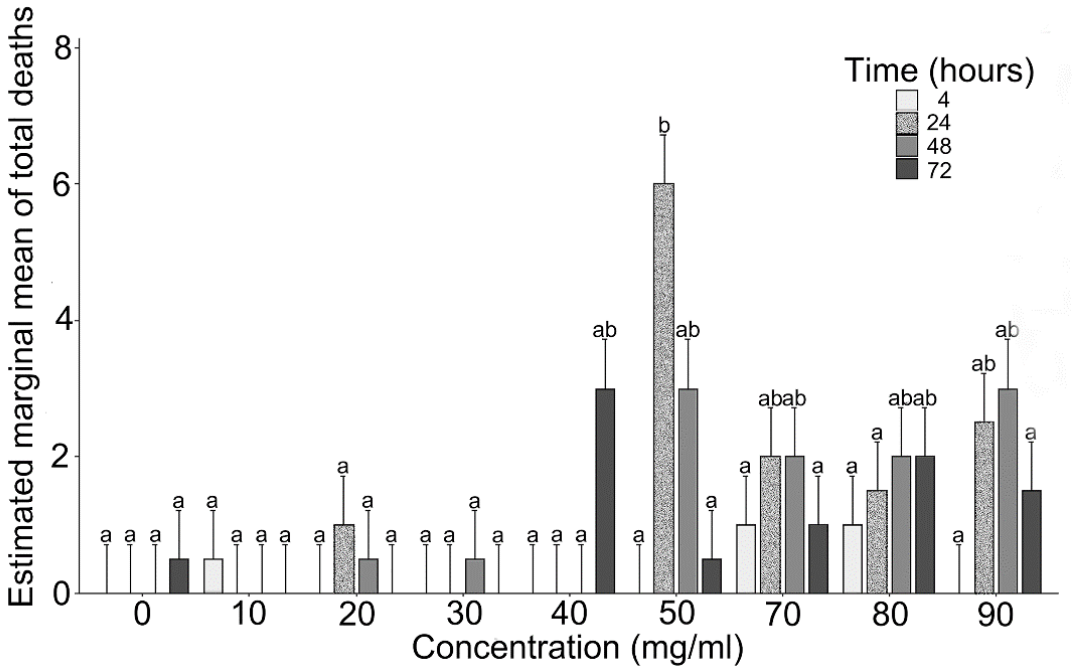

Fig. 24. Effect of concentration of *Cladonia foliacea* extract on mortality of male and female mosquitoes: Estimated marginal means after 4, 24,48, and 72 hours. (Concentrations by exposure time sharing similar letters are not significantly different regarding average total mortality.).

The findings of this laboratory study indicate that adult male and female *An. gambiae* are susceptible to *C. foliacea* lichen extract because of higher mortality observed in the test experiment

compared to the control. This observation can be explained by the mosquitoes' innate behaviour to search for sugar after emergence, indicating that oral poison in a 10% sugar solution can be used as bait to kill both male and female mosquitoes. Male and female mosquitoes have been observed searching and feeding on flower nectar in their natural bait, and field studies have investigated this behaviour of mosquitoes to meet their nutritional needs and confirmed various sugars in their abdomens (GOUAGNA et al. 2010). A study in western Kenya found that sugar-fed males outnumbered females and newly emerged mosquitoes fed rather on sugar than blood-fed females, and that the local *Anopheles* mosquitoes were more attracted to *Mangifera indica* than to other sugar sources (OMONDI et al. 2022, YALLA et al. 2023).

Table 6. Results of analysis of variance (ANOVA) followed by tukey-HSD post-hoc test to test the effect of different concentrations of *Cladonia foliacea* extract after oral administration to *Anopheles gambiae*. Results are averaged over the levels of exposure time and sex. 95% of confidence level was used.

Concentration (mg/ml)	emmean	SE	df	Lower CL	Upper C	Group
0 (negative control)	0.125	0.358	24	-0.6143	0.864	a
10	0.125	0.358	24	-0.6143	0.864	a
20	0.375	0.358	24	-0.3643	1.114	a
30	0.125	0.358	24	-0.6143	0.864	a
40	0.75	0.358	24	0.0107	1.489	ab
50	2.375	0.358	24	1.6357	3.114	b
70	1.5	0.358	24	0.7607	2.239	ab
80	1.625	0.358	24	0.8857	2.364	ab
90	1.75	0.358	24	1.0107	2.489	ab

Using 2- to 4-day-old adult mosquitoes in this bioassay was appropriate because newly emerged males and females seek sugar for energy compared to the blood-fed females. This important concept has been successfully applied in the field targeting to kill mosquitoes by use of attractive toxic sugar bait (MÜLLER et al. 2010a, 2010b). A study in Israel has demonstrated that the availability of sugar sources affects population dynamics and the vectorial capacity of *A. sergentii* (GU et al. 2011). Therefore, the availability of sugar during the initial and subsequent life of a mosquito is an important factor in their life cycle.

The bioassay results showed a dose-related mortality response, ingested *C. foliacea* extract exhibited a significantly higher lethal effect on male and female mosquitoes at concentrations above 30 mg/ml to 90 mg/ml, however, a significantly higher effect was observed at 50 mg/ml compared to controls. Equally, increasing the exposure time at concentrations between 10 to 30 mg/ml has no significant effect on mortality, therefore, when considering the most toxic concentration to kill more mosquitoes, a dose that yields maximum mortality of test mosquitoes should be selected and the concentration that kills more number within a short period would also be an important factor to consider. Therefore, if this concept were applied, acute toxicity to the

target mosquitoes means the mosquitoes will be killed sooner and reduce their lifespan and reduce transmission of the malaria parasite. In other studies, the effect of (–)-usnic acid on larval stages of *Aedes aegypti* exhibited a dose-response relationship. Exposure time and mortality were positively correlated (KOC et al. 2021). Oral toxicity of extracts of *C. foliacea* on *Sitophilus granaries* has also demonstrated dose and time response mortality effects where high concentration and longer exposure time resulted in a significantly higher mortality rate (EMSEN et al. 2012). These findings are in agreement with the current study.

Both male and female mosquitoes readily ingested the extract despite the extract being known to contain antiherbivore substances. This was denoted by the distended abdomen with the red dye hence the food dye can effectively indicate the feeding rate when advanced methods like fluorescence microscopy are inaccessible. Results according to Sandra A. Allan also agree with our study by using 2% food dye and observing the abdominal status with the help of the light microscope (ALLAN 2011). Their study used 1 ml of the toxic solution, in our experiment, the volume was increased to 5 ml which could be sustained for up to 72 h limiting any possibility of recording false positive deaths (ALLAN 2011).

The current study did not focus on measuring the amount of the secondary metabolites in *C. foliacea* since it was initially determined by FARKAS et al. (2020a, 2020b, 2024b) from samples obtained from the same location of Tece, Vácrátót, Hungary. Use of acetone and 2% food dye as negative control did not cause high mortalities of mosquitoes to warrant discarding any of the test results during the bioassay tests. Therefore, our study recommends that the following negative controls are safe for mosquitoes and can be made by mixing 5.5 ml 10% sugar solution, 0.5 ml 2% food dye solution and 0.8 ml acetone as a negative control for bioassay studies using organic plant-based metabolites as an oral insecticide.

This study was the first attempt to determine the mortality effects of LSMs on adult *An. gambiae.* We have demonstrated that the acetone extract of *C. foliacea* that is known to contain (–)-usnic acid with insecticidal potential is toxic to newly emerged male and female adult of *An. gambiae* when used as an oral poison in toxic sugar bait. Fumarprotocetraric acid and 9′-(O-methyl)protocetraric acid are also present in the crude extract, and may add to the effect of (–)-usnic acid. Both male and female *An. gambiae* showed a dose-related response at a concentration of up to 90 mg/ml and the mortality rate was time-dependent for up to 72 h. The ingestion of *C. foliacea* extract at 50 mg/ml and a post-exposure period of 24 to 48 h had a maximum effect on the mortality rate of targeted male and female *An. gambiae*. Therefore, it was shown that the *C. foliacea* extract when used as oral toxic sugar bait effectively killed the target mosquitoes and had insecticidal potential. Therefore, it can be recommended as an alternative biological toxic agent in

the current trials and future studies on attractive targeted sugar bait as a vector control tool in malaria-endemic regions where *An. gambiae* is the main vector of malaria transmission.

To enhance existing vector control tools within integrated vector management, our novel findings may open an avenue for the application of LSMs with insecticidal properties as oral poison in the form of toxic sugar bait (TSB).

6. CONCLUSIONS

If the number of lichen species known worldwide (c. 18–20,000 – KIRK et al. 2008) is compared to the number of insecticides justified so far on insect vectors and parasitic protozoa carried by them, it is obvious that there are potentially many more efficacious species. Similarly, the number of applicable LSMs of the existing c. 1000 (STOCKER-WÖRGÖTTER, 2008) should be higher than those tested so far. Furthermore, investigation of the optical isomers of usnic acid and its insecticidal role has been initiated by recent studies (FARKAS et al. 2024a, 2024b, MUHORO et al. 2024a, 2024b). Successful insecticidal application of crude extract of *C. foliacea* containing LSMs (UA and FA) in addition to other substances has been confirmed by MUHORO et al (2024a). The review by MUHORO and FARKAS (2021) strongly revealed that very little information is available on the application of LSMs to determine their efficacy in the field compared to laboratory tests as well as toxicological information when using LSMs on non-target organisms in the environment. Higher vertebrates should also be considered for widening the range of taxa where LSMs are applied and their effects are controlled. There is therefore the need to use those lichens that have been identified as having the highest biological activity against insects and perform field survey for consideration as new tools in insect vectors control and in management of parasitic protozoal diseases for commercial production in the market. In general, the publications reviewed contained more detailed information on antiprotozoal application. These were more thorough studies based on recent sophisticated instrumental, ultrastructural and metabolomic studies (DE CARVALHO et al. 2005, LAUINGER et al. 2013). It is necessary to also consider recent methods in analysis for application, too, for example the possibilities of microencapsulation (where the active substances are protected by encapsulation and their activity kept constant after this process) in order to facilitate environmental protection and sustainable development (BARBAT et al. 2013, BENELLI, 2016, DHANESH GANDHI et al. 2019). This will supplement the existing control and management tools of human diseases, vectors of human and animal diseases and non-communicable and communicable diseases when further studies are done on several lichen groups that have demonstrated bioactive potentials.

Lichenologically, Kenya is one of the best studied countries in Africa. The search word ‘Kenya’ in the database ‘Recent literature on lichens’ (CULBERSON et al. 2022) resulted in 79 papers out of 52,174, while 645 papers were found for ‘tropical’ (563) + ‘tropics’ (82) or 805 for ‘Africa’ in 2022. Thus c. 10% of publications from Africa originate from Kenyan material. However, discoveries of species new to science can be expected since tropical and African lichens are generally understudied. Since our knowledge of lichenicolous fungi in East Africa is still limited (FARKAS and FLAKUS, 2016, SUIJA et al. 2018), research into possible host species is

very important. The key prepared (FARKAS and MUHORO 2022) will support further field studies and the identification work that follows, and thus contribute to a better knowledge of both lichens and their lichenicolous fungi in Kenya and East Africa (FRYDAY et al. 2022), as well as promote conservation studies and the practical use of bioactive lichen secondary metabolites.

More than half (23 species) of the 42 species identified from East-African collections in 2022 (FARKAS et al. 2023) belonged to the parmelioid clade, thus the recently published identification key (FARKAS and MUHORO 2022) could be tested on both Kenyan and Tanzanian specimens. Only *Parmotrema eciliatum* was not treated in that key (FARKAS and MUHORO 2022), however, it was possible to identify almost all further species by the former key of SWINSCOW and KROG (1988). Our work led to confirming distribution records of the parmelioid and other groups. The occurrence of *Bulbothrix kenyana* (recently described in KIRIKA et al. 2017) in Tanzania widens the distribution area of the species known so far from various localities in Kenya. Further new distribution records were also detected: *Chrysothrix xanthina, Lobaria discolor, Parmotrema durumae* and *P. taitae* were discovered as new from Tanzania; *Usnea abissinica* and *U. sanguinea* are new for Kenya. Future field studies in these and other countries in East Africa may result in further interesting records that widens our knowledge in this field. The lichenicolous fungi *Didymocyrtis* cf. *melanelixiae, Lichenoconium erodens* and *Spirographa lichenicola* are presented for the first time from East Africa. Since SUIJA et al. (2018) and (ZHURBENKO 2021, 2022) described several new lichenicolous species from Kenya and Tanzania recently, further taxonomic novelties are expected by a more detailed study in this promising region.

Although no significant differences were found in the *Flavoparmelia caperata* populations of the geographical areas investigated from Europe and Africa with regard to the concentration of UA, there were populations and specimens with high amounts of UA in each country (Hungary, Serbia, Kenya and Tanzania) and continent (FARKAS et al. 2024a). Therefore, if a study on bioactivity of locally available lichen material is to be carried out, *F. caperata* can be selected for further research in different geographical regions. Given that the chiral nature of the UA found in the studied species has been confirmed here as (+)-UA, further study with an increased dataset applying HPLC-PDA only may clarify other details on production conditions and extraction efficiency.

The paper (FARKAS et al. 2024b) published on quantitative variation of LSMs in the lichen *Cladonia foliacea* combined phytochemical, phylogenetic and statistical analyses, in order to investigate the association between environmental / biological variables and LSM contents in their thalli. It was confirmed that *C. foliacea* thalli collected from Hungary, Albania and North

Macedonia contain (-)-UA enantiomer, that can supply a source of bioactive substance, a potential raw material for further application.

Laboratory experiment on the susceptibility of *An. gambiae* to 5, 10 and 15 mg/ml (+)-UA when administered as oral sugar bait has first been demonstrated by our recent study (MUHORO et al. 2024b). Mortality for both males and females is higher in the first 4 h of exposure and continues up to 72 h. Therefore, this study has confirmed that (+)-UA can be used as an ingredient in the novel attractive toxic sugar bait for the control of malaria vectors in the current effort to search for new tools to reduce malaria transmission in African countries.

It was shown (MUHORO et al. 2024a) that the *C. foliacea* extract containing FA and (-)-UA, when used as oral toxic sugar bait effectively killed the target mosquitoes. Therefore, it can be recommended as an alternative biological toxic agent in the current trials and future studies on attractive targeted sugar bait as a vector control tool in malaria-endemic regions where *An. gambiae* is the main vector of malaria transmission.

To enhance existing vector control tools within integrated vector management, these novel findings may open an avenue for the application of lichen secondary metabolites with insecticidal properties as oral poison in the form of toxic sugar bait (TSB).

7. NEW SCIENTIFIC RESULTS

1. An identification key to the 178 species of the parmelioid clade in Kenya, based on updated nomenclature, was produced to support the practical work in collecting and selecting certain parmelioid lichens for further research.

2. New distribution records were found in East Africa. The lichens *Bulbothrix kenyana*, *Chrysothrix xanthina*, *Lobaria discolor*, *Parmotrema durumae* and *P. taitae* were discovered as new for Tanzania; *Usnea abissinica* and *U. sanguinea,* furthermore lichenicolous fungi *Didymocyrtis* cf. *melanelixiae, Lichenoconium erodens* and *Spirographa lichenicola* are new for Kenya.

3. The presence and concentration of usnic acid enantiomers were established from earlier not studied geographical areas. The presence of lichen secondary metabolite (+)-usnic acid (UA) enantiomer was confirmed in samples of *Flavoparmelia caperata* originating from two continents: Europe and Africa. Substantial variation in the content of (+)-UA analysed by high-performance liquid chromatography (HPLC-PDA) was observed, but no significant differences between European (with 5.21 to 19.23 mg/g dry wt) and African (with 6.15 to 23.54 mg/g) samples were found. The presence of lichen secondary metabolites (-)-usnic acid (UA) enantiomer and fumarprotocetraric acid (FA) was confirmed in samples of *Cladonia foliacea* originating from Central and Southern Europe. The concentration ranges of UA (6.88 to 34.27 mg/g dry wt) and FA (1.44 to 9.87 mg/g dry wt) were established.

4. A protocol and further laboratory procedures for the novel attractive toxic sugar bait (ATSB) bioassay using adult mosquito candidates were worked out and approved by the scientific and ethics review unit of the KEMRI (SERU) protocol number SERU04–06–423/4610. It represents an important contribution to the World Health Organization Pesticide Evaluation Scheme (WHOPES).

5. For the first time it was revealed that oral administration of lichen secondary metabolite (+)-usnic acid when combined with sugar solution can kill male and female adult *Anopheles gambiae* mosquitoes endemic in Western Kenya. The efficacy was dose and time-dependent.

6. For the first time it was shown that acetone extract of the lichen *Cladonia foliacea* (containing (-)-usnic acid enantiomer and fumarprotocetraric acid) applied as oral toxic sugar bait effectively killed both male and female *Anopheles gambiae.* Consequently, it is a promising biological oral toxic agent to be incorporated in the novel attractive toxic sugar bait for mosquito control to prevent malaria transmission.

8. SUMMARY

The symbiotic relationship between fungi and photosynthetic algae and cyanobacteria forming lichens results in unique morphological and chemical characteristics. Their unique substances known as lichen secondary metabolites (LSMs), mostly depsides and depsidones produced by the fungal partner and located in the medulla and cortex of the lichen thallus. Their role is not fully understood, however, they are known to protect the algae from UV radiation and deter herbivores, such as insects. LSMs – most frequently usnic acid – with insecticidal activity have been evaluated and confirmed to be promising alternative biopesticides. There is limited knowledge on lichens in Kenya, therefore lichens with potentially bioactive (among them of insecticide nature) secondary metabolites require further studies. It is assumed that LSMs can be applied to control the *Anopheles gambiae* mosquito, the primary vector of *Plasmodium falciparum* (causing 95% of malaria-related deaths in Africa) in western Kenya, which has developed resistance against synthetic insecticides. Both (+)- and (-)-usnic acid is present in nature and have been applied against mosquito larvae, but not against *An. gambiae* and not against adult mosquitoes. Therefore, the aim of the dissertation was (1) to study the diversity of lichens with insecticidal potential and their lichen secondary metabolites, the amount and chiral property of usnic acid (UA) in two relatively frequent lichens *Cladonia foliacea* (in Europe) and *Flavoparmelia caperata* (in Africa and in Europe); (2) to determine the insecticidal potential of the pure usnic acid and the acetone crude extract of *Cladonia foliacea* as an oral pesticide against *Anopheles gambiae* mosquitoes.

Based on 27 journal publications a total of 61 lichen species were found that were investigated to determine either for insecticide or antiprotozoal activity. These lichens contain 15 LSMs but only 7 of them were tested on insect vectors or on protozoa, others could be present when crude extracts of lichens were used.

The key of the parmelioid clade was revised and included 178 species from Kenya, resulting an important tool that will aid in lichen collection since lichens in Kenya are understudied. Identification work resulted in further new geographical distribution records of lichens and the lichenicolous fungi in East Africa (Tanzania and Kenya): the lichens *Bulbothrix kenyana*, *Chrysothrix xanthina*, *Lobaria discolor*, *Parmotrema durumae* and *P. taitae* were discovered as new for Tanzania; *Usnea abissinica* and *U. sanguinea,* furthermore lichenicolous fungi *Didymocyrtis* cf. *melanelixiae, Lichenoconium erodens* and *Spirographa lichenicola* are new for Kenya.

Enantiomer nature being (+)-UA in *F. caperata* and (-)-UA in *C. foliacea* was confirmed. Geographical and ecological variability of the lichen *F. caperata* did not show any significant

impact on the UA levels between samples obtained from Europe and Africa indicating similar production levels (5.21–23.54 mg/g dry wt) under different environmental factors. The concentration of UA (6.88 to 34.27 mg/g dry wt) of *C. foliacea* samples from Southern and Central Europe was also established.

(+)-UA and extract of *C. foliacea* containing (-)-UA was found to be effective in control of malaria vector *An. gambiae* when administered as sugar bait. The ability to kill the mosquito was based on the concentration and time after ingestion of the toxic agent. Higher mortality was associated with high concentration especially when pure usnic acid was used. Crude extract had impact when used in high concentration. Both male and female mosquitoes were susceptible.

There is a need to test and determine how field collected mosquitoes would respond to the UA. Successful application of UA enantiomers could certainly add to the current existing mosquito control methods as a potential toxic ingredient in toxic sugar bait.

APPENDICES

Appendix 1: Bibliography

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Appendix 2: Tables

Table A1. Effect of LSMs and crude lichen extract on larval stages of mosquito mortality among *Aedes, Anopheles* and *Culex* species.

Lichen species	Concentration of LSM or crude extract (µg/mL)	Mortality effect (LC_{50})	References
Cladonia coniocraea	≤5,000*	91–100%	(NANAYAKKARA et al. 2005)
Cladonia foliacea	10***	100%	(CETIN et al. 2008)
Dirinaria applanata	≤5,000*	91–100%	(NANAYAKKARA et al. 2005)
Everniastrum sp.	≤5,000*	91–100%	(NANAYAKKARA et al. 2005)
Hypogymnia sp.	≤5,000*	91–100%	(NANAYAKKARA et al. 2005)
Lepraria atrotomentosa	≤5,000*	91–100%	(NANAYAKKARA et al. 2005)
Leptogium papillosum	81.1*, 89.1**, 9.0****	100%	(KHADER et al. 2018)
Leucodermia leucomelos	1,000–2,000*	(50–)100%	(KARTHIK et al. 2011)
Leucodermia leucomelos	≤5,000*	91–100%	(CETIN et al. 2008)
Myriotrema spp.	≤5,000*	91–100%	(NANAYAKKARA et al. 2005)
Notoparmelia erumpens	341.0*, 112.0**, 9.3****	100%	(KHADER et al. 2018)
Ocellularia sp.	≤5,000*	91–100%	(NANAYAKKARA et al. 2005)
Parmeliella sp.	≤5,000*	91–100%	(NANAYAKKARA et al. 2005)
Parmelina tiliacea	≤5,000*	91–100%	(NANAYAKKARA et al. 2005)
Parmotrema chinense	≤5,000*	91–100%	(NANAYAKKARA et al. 2005)
Parmotrema kamatti	296.3*, 153.3**, 13.2****	100%	(KHADER et al. 2018)
Parmotrema reticulatum	417.1*, 102.1**, 10.2****	100%	(KHADER et al. 2018)
Parmotrema reticulatum	≤5,000*	91–100%	(NANAYAKKARA et al. 2005)
Parmotrema tinctorum	≤5,000*	91–100%	(NANAYAKKARA et al. 2005)
Parmotrema tinctorum	201.1*, 156.2**, 5.3****, (660*)	100%	(FOURNET et al. 1997, VINAYAKA et al. 2009)
	5,000*	25%	
Ramalina conduplicans	10,000*	40%	(KUMAR et al. 2010)
	20,000*	85%	
Ramalina farinacea	5***	100%	(CETIN et al. 2008)
	5,000*	50%	
Ramalina hossei	10,000*	70%	(KUMAR et al. 2010)
	20,000*	100%	
Ramalina nervulosa	1000*	100%	(VINAYAKA et al. 2009)
Ramalina pacifica	830*	100%	(VINAYAKA et al. 2009)
Ramalina usnea	150*	96.6%	(MOREIRA et al. 2016)

Roccella montagnei	640.9*, 127.4**, 7.0****, (830*)	100%	(VINAYAKA et al. 2009, KHADER et al. 2018)
Roccella montagnei	≤5,000*	91–100%	(NANAYAKKARA et al. 2010)
Stereocaulon sp.	≤5,000*	91–100%	(NANAYAKKARA et al. 2010b)
Usnea galbinifera	760*	100%	(VINAYAKA et al. 2009)
Usnea sp.	≤5,000*	91–100%	(NANAYAKKARA et al. 2005)

* = *A. aegypti*, ** = *A. stephensi*, *** = *C. pipiens*, **** = *C. quinquefasciatus*.

Table A2. Lichen species and LSMs/ crude extract were evaluated for larvicidal or antiprotozoal effects. (N/A = not available.)

Lichen species	**LSM**	**Bioactivity**	**References**
Cladonia coniocraea (Flörke) Spreng.	crude extract	larvicidal	(NANAYAKKARA et al. 2005)
Cladonia foliacea (Huds.) Willd.	(-)-usnic acid	larvicidal	(CETIN et al. 2008)
Cladonia substellata Vain.	(+)-usnic acid	antiprotozoal activity	(DE CARVALHO et al. 2005)
Dirinaria applanata (Fée) D.D. Awasthi	crude extract	larvicidal	(NANAYAKKARA et al. 2005)
Erioderma leylandi (Taylor) Müll. Arg**.**	1′chloropannarin	antiprotozoal activity	(FOURNET et al. 1997)
Everniastrum sp.	crude extract	larvicidal	(NANAYAKKARA et al. 2005)
Hypogymnia sp.	crude extract	larvicidal	(NANAYAKKARA et al. 2005)
Lepraria atrotomentosa Orange & Wolseley	crude extract	larvicidal	(NANAYAKKARA et al. 2005)
Leptogium papillosum (de Lesd.) C.W. Dodge	crude extract	larvicidal	(KHADER et al. 2018)
Leucodermia leucomelos (L.) Kalb (syn. *Heterodermia leucomelos* (L.) Poelt)	crude extract (atranorin, salazinic acid)	larvicidal	(NANAYAKKARA et al. 2005, KARTHIK et al. 2011)
Myriotrema spp. (2)	crude extract	larvicidal	(NANAYAKKARA et al. 2005)
Notoparmelia erumpens (Kurok.) A. Crespo, Ferencová & Divakar (syn. *Parmelia erumpens* Kurok.)	crude extract	larvicidal	(KHADER et al. 2018)
Ocellularia sp.	crude extract	larvicidal	(NANAYAKKARA et al. 2005)
Parmeliella sp.	crude extract	larvicidal	(NANAYAKKARA et al. 2005)
Parmelina tiliacea (Hoffm.) Hale	crude extract	larvicidal	(NANAYAKKARA et al. 2005)
Parmotrema chinense (Osbeck) Hale & Ahti	crude extract	larvicidal	(NANAYAKKARA et al. 2005)
Parmotrema kamatti Patw. & Prabhu	crude extract	larvicidal	(KHADER et al. 2018)
Parmotrema reticulatum (Taylor) M. Choisy (syn. *Rimelia reticulata* (Taylor) Hale & A. Fletcher)	crude extract	larvicidal	(NANAYAKKARA et al. 2005)

Parmotrema tinctorum (Despr. ex Nyl.) Hale	crude extract (lecanoric acid, orsellinic acid)	larvicidal	(NANAYAKKARA et al. 2005, VINAYAKA et al. 2009, KHADER et al. 2018)
Protousnea malacea (Stirt.) Krog	(+)-usnic acid	antiprotozoal activity	(FOURNET et al. 1997)
Psoroma pallidum Nyl.	pannarin	antiprotozoal activity	(FOURNET et al. 1997)
Ramalina conduplicans Vain.	crude extract (salazinic, sekikaic acid, usnic acid, (tannins, steroids))	larvicidal	(KUMAR et al. 2010)
Ramalina farinacea (L.) Ach.	usnic acid	larvicidal	(CETIN et al. 2008)
Ramalina hossei Vain.	crude extract (sekikaic acid, usnic acid, (tannins, terpenoids))	larvicidal	(KUMAR et al. 2010)
Ramalina nervulosa (Müll. Arg.) Abbayes	crude extract (sekikaic acid, usnic acid)	larvicidal	(VINAYAKA et al. 2009)
Ramalina pacifica Asahina	crude extract (salazinic acid, usnic acid)	larvicidal	(VINAYAKA et al. 2009)
Ramalina usnea (L.) R. Howe	usnic acid	larvicidal	(MOREIRA et al. 2016)
Roccella montagnei Bél.	crude extract (erythrin, lecanoric acid)	larvicidal	(NANAYAKKARA et al. 2005; VINAYAKA et al. 2009; KHADER et al. 2018)
Stereocaulon sp.	crude extract	larvicidal	(NANAYAKKARA et al. 2005)
Usnea galbinifera Asahina	crude extract (galbinic acid, norstictic acid)	larvicidal	(VINAYAKA et al. 2009)
Usnea sp.	crude extract	larvicidal	(NANAYAKKARA et al. 2005)
N/A	evernic acid	antiprotozoal activity	(LAUINGER et al. 2013)
N/A	gyrophoric acid	larvicidal	(CETIN et al. 2012)
N/A	psoromic acid	antiprotozoal activity	(LAUINGER et al. 2013)
N/A	(+)-usnic acid	antiprotozoal activity	(LAUINGER et al. 2013)
N/A	vulpic acid	antiprotozoal activity	(LAUINGER et al. 2013)

Table A3. List of collected *Flavoparmelia caperata* specimens with their main collecting data (date of collection, altitude, COUNTRY. Site, habitat, GPS coordinates), herbarium specimen registration number.

Sample nr	Date of collection	Altitude [m a.s.l.]	Collecting sites	Herbarium _nr*
1	30.05.2020.	250	HUNGARY. Heves County. Mátra Mts (SE side), valley of stream „Hátsó-Tarnóca" at SW side of Mt „Sózó-tető", ca 2 km N of Kisnána. On bark (*Quercus petraea*). Lat.: 47.868593° N; Long.: 20.148331° E	VBI_6029
2	30.05.2020.	250	HUNGARY. Heves County. Mátra Mts (SE side), valley of stream „Hátsó-Tarnóca" at SW side of Mt „Sózó-tető", ca 2 km N of Kisnána. On bark (*Quercus petraea*). Lat.: 47.868593° N; Long.: 20.148331° E	VBI_6029
3	30.05.2020.	250	HUNGARY. Heves County. Mátra Mts (SE side), valley of stream „Hátsó-Tarnóca" at SW side of Mt „Sózó-tető", ca 2 km N of Kisnána. On rock and soil. Lat.: 47.868593° N; Long.: 20.148331° E	VBI_6030
4	30.05.2020.	255	HUNGARY. Heves County. Mátra Mts (SE side), valley of stream „Hátsó-Tarnóca" at SE foot of Mt „Kopasz-hegy", ca 2 km N of Kisnána. On bark (*Quercus* sp). Lat.: 47.868281° N; Long.: 20.146347° E	VBI_6031
5	05.09.2021.	370	HUNGARY. Heves County. Mátra Mts (SW side), Mt Világos-hegy (Kopasz-hegy), ca 4.0 km NNW of Gyöngyöstarján. On bark (*Quercus cerris*). Lat.: 47.846228° N; Long.: 19.851541° E	VBI_6032
6	05.09.2021.	370	HUNGARY. Heves County. Mátra Mts (SW side), Mt Világos-hegy (Kopasz-hegy), ca 4.0 km NNW of Gyöngyöstarján. On bark (*Quercus cerris*). Lat.: 47.846228° N; Long.: 19.851541° E	VBI_6033
7	05.09.2021.	580	HUNGARY. Heves County. Mátra Mts (SW side), Mt Világos-hegy (Kopasz-hegy), ca 4.0 km NNW of Gyöngyöstarján, along the 'green triangle' tourist route. On bark (*Quercus petraea*). Lat.: 47.856740° N; Long.: 19.840504° E	VBI_6034
8	05.09.2021.	580	HUNGARY. Heves County. Mátra Mts (SW side), Mt Világos-hegy (Kopasz-hegy), ca 4.0 km NNW of Gyöngyöstarján, along the 'green triangle' tourist route. On mossy bark (*Quercus petraea*). Lat.: 47.856740° N; Long.: 19.840504° E	VBI_6035
9	05.09.2021.	585	HUNGARY.Heves County. Mátra Mts (SW side), Mt Világos-hegy (Kopasz-hegy), ca 5.4 km NNW of Gyöngyöstarján, along the 'green triangle' tourist route. On rock. Lat.: 47.856812° N; Long.: 19.840791° E	VBI_6036
10	05.09.2021.	635	HUNGARY.Heves County. Mátra Mts (SW side), Mt Világos-hegy (Kopasz-hegy), ca 5.4 km NNW of Gyöngyöstarján, along the 'green triangle' tourist route. On rock. Lat.: 47.858014° N; Long.: 19.841340° E	VBI_6037
11	05.09.2021.	680	HUNGARY. Heves County. Mátra Mts (SW side), Mt Világos-hegy (Kopasz-hegy), ca 5.6 km NNW of Gyöngyöstarján, along the 'green triangle' tourist route. On rock. Lat.: 47.859535° N; Long.: 19.838879° E	VBI_6038
12	12.06.2021.	190	HUNGARY. Zala County. Göcsej, Lenti, Mt Cser-hegy (S side), mixed forest in "Szemere-lakosi-dűlő", ca 1.5 km ENE of Kerkabarabás. On bark (*Quercus cerris*). Lat.: 46.682048° N; Long.: 16.575839° E	VBI_6039
13	30.06.2022.	102	SERBIA. Brnjica, along stream Brnjica. On bark (*Prunus* sp). Lat.: 44° 38' 19.19" N; Long.: 21° 45' 10.03" E	VBI_6040
14	30.06.2022.	102	SERBIA. Brnjica, along stream Brnjica. On bark (*Alnus glutinosa*). Lat.: 44° 38' 19.19" N; Long.: 21° 45' 10.03" E	VBI_6041

15	30.06.2022.	102	SERBIA. Brnjica, along stream Brnjica. On bark (*Prunus* sp). Lat.: 44° 38' 19.19" N; Long.: 21° 45' 10.03" E	VBI_6040
16	11.08.2022.	125	HUNGARY. Somogy County, Duna–Dráva National Park, Barcsi-ősborókás, Nagy-Berek ca 3 km NW of Darány. On bark (*Quercus* sp.). Lat.: 45.990993° N, Long.: 17.555412° E	VBI_6042
17	11.08.2022.	125	HUNGARY. Somogy County, Duna–Dráva National Park, Barcsi-ősborókás, Nagy-Berek ca 3 km NW of Darány. On bark (*Quercus* sp.). Lat.: 45.990993° N, Long.: 17.555412° E	VBI_6042
18	11.08.2022.	125	HUNGARY. Somogy County, Duna–Dráva National Park, Barcsi-ősborókás, Nagy-Berek ca 3 km NW of Darány. On bark (*Quercus* sp.). Lat.: 45.990993° N, Long.: 17.555412° E	VBI_6043
19	14.08.2021.	2,454 m	KENYA. Nyeri county, at the foot of Mt. Kenya: c. 1 km from Naro Moru entry gate to Kenya wildlife service in Gitinga village, from bark, twigs and branches of trees in tropical rainforest. Lat.: 0° 10’ 25.84” S; Long.: 37° 9’ 3.40” E	VBI_6044
20	24.10.1987.	1,700 m	TANZANIA. Pienaar S Heights, S of Babati, Bereku F. R., Mist effected miombo on the ridge.	VBI_6169
21	05.09.2022.	134	HUNGARY. Pest County. Vácrátót, Tece, along the ’red line’ tourist route (Ág-dűlő), in open sandy grassland. On bark. Lat.: 47.702358° N; Long.: 19.224312° E	VBI_6045
22	03.09.2022.	114	HUNGARY. Balatonakali, in the fores patch behind the camp “Kapuvári tábor”. On bark (*Quercus* sp). 46.883282° N, 17.737843° E.	VBI_6055
23	25.10.1988.	2,350-2,500	TANZANIA. S Uluguru Mts in Morogoro District. Secondary grassland dominated by *Panicum luckwangulense* with scattered *Agauria salicifolia* and *Myrica salicifolia* trees. Ramicolous on *Agauria* ct.	VBI_6251
24	21.01.2013.	2,137	KENYA. Baringo County, Eldama Ravine Lembus Forest off Eldama Ravine-Eldoret Road. In remnant montane forest. On bark. Lat.: 0.16°N, Long: 35.75°E	dupl EA 2835
25	22.01.2013.	2,168	KENYA. Baringo County, Esageri Hill, 9 km from Esageri Market off Eldama Ravine - Nakuru Road. In a remnant montane forest. On bark. Lat. 0.05° N, Long. 35.81° E	dupl EA 2922
26	29.03.2014	3,008	KENYA. Western Kenya, Trans-Nzoia County, Mt. Elgon National Park. In *Olea-Juniperus-Podocarpus* forest. On bark. Lat.: 01° 03' N Long: 34°41' E	dupl EA 4246
27	10.04.2014	2,022	KENYA. Nyeri county, Mt. Kenya, Gathiuru Forest, Naromoru, Bantu Lodge area. In disturbed *Juniperus-Podocarpus-Olea* forest. On bark. Lat.: 00° 16' S Long: 37° 03' E,	dupl EA 4425
28	22.01.2015.	1,995	KENYA. Nyeri County, Mt. Kenya area, Naromoru, On shrubs and trees in pasture land. On bark. Lat.: 0° 06'N, Long: 37°56' E	dupl EA 4755
29	04.06.2016.	2,709	KENYA. Elegeyo-Marakwet County, Cherangani Hills, Embobut Forest, Montane forest.On bark. Lat.: 01° 05' N, Long: 35° 31' E	dupl EA 5123
30	26.10.2021	2,462	KENYA. Elgeyo Marakwet County, Keiyo South Sub-County, Kaptagat forest, Riparian vegetation, dry patch in plantation forest. On bark. Lat.: 00.43667°N, Long: 35.50362° E	dupl EA 5671

* Herbarium abbreviation according to Thiers 2024.

Table A4. List of collected specimens of *Cladonia foliacea* with their main collecting data (date of collection, altitude, country, site, habitat, GPS coordinates; collector and determinator), herbarium specimen registration numbers.

Sample ID	Date of collection	Altitude [m a.s.l.]	Collecting site; collector and determinator	VBI*_nr
CLF1	12.04.2014.	270	HUNGARY, Pest County. Fót, Mt Fóti-Somlyó, plateau area at SE side, on calcareous soil in open sandy grassland. Lat.: 47.622460° N; Long.: 19.218871° E; E. Farkas, L. Lőkös	VBI_6002
CLF2	12.04.2014.	265	HUNGARY, Pest County. Fót, Mt Fóti-Somlyó, plateau area at central part, on calcareous soil in rocky grassland. Lat.: 47.627751° N; Long.: 19.210869° E; E. Farkas, L. Lőkös	VBI_6003
CLF3	12.04.2014.	265	HUNGARY, Pest County. Fót, Mt Fóti-Somlyó, plateau area at central part, on calcareous soil in rocky grassland. Lat.: 47.627751° N; Long.: 19.210869° E; E. Farkas, L. Lőkös	VBI_6004
CLF4	03.05.2014.	185	HUNGARY, Veszprém County. Balaton-felvidék, Mt Mogyorós-hegy (NW side), ca 1.0 km NE of Litér, on calcareous soil in open rocky grassland. Lat.: 47.102248° N; Long.: 18.019568° E; E. Farkas, L. Lőkös	VBI_6005
CLF5	03.05.2014.	185	HUNGARY, Veszprém County. Balaton-felvidék, Mt Mogyorós-hegy (NW side), ca 1.0 km NE of Litér, on calcareous soil in open rocky grassland. Lat.: 47.102248° N; Long.: 18.019568° E; E. Farkas, L. Lőkös	VBI_6006
CLF6	03.05.2015.	188	HUNGARY, Veszprém County. Balaton-felvidék, Mt Mogyorós-hegy (NW side), ca 1.1 km NE of Litér, on calcareous soil in karst bush-forest. Lat.: 47.103124° N; Long.: 18.020481° E; E. Farkas, L. Lőkös	VBI_6007
CLF7	27.08.2016.	188	HUNGARY, Fejér County. Velence Mts, Mt Új-hegy (SW side) at Sukoró, along the 'yellow cross' tourist route („Nadapi út"), on acidic soil in open rocky grassland. Lat.: 47.242787° N; Long.: 18.605325° E; E. Farkas, L. Lőkös	VBI_6008
CLF8	27.08.2016.	193	HUNGARY, Fejér County. Velence Mts, Mt Új-hegy (SW side) at Sukoró, along the 'yellow cross' tourist route („Nadapi út"), on acidic soil in open rocky grassland. Lat.: 47.243180° N; Long.: 18.604463° E; E. Farkas, L. Lőkös	VBI_6009
CLF9	27.08.2016.	196	HUNGARY, Fejér County. Velence Mts, Mt Új-hegy (S side) at Sukoró, along the 'yellow cross' tourist route („Nadapi út") between Sukoró and Nadap, at an exposed site on acidic soil in pine forest. Lat.: 47.245265° N; Long.: 18.607907° E; E. Farkas, L. Lőkös	VBI_6010
CLF10	27.08.2016.	196	HUNGARY, Fejér County. Velence Mts, Mt Új-hegy (S side) at Sukoró, along the 'yellow cross' tourist route („Nadapi út") between Sukoró and Nadap, in shade on acidic soil in pine forest. Lat.: 47.245265° N; Long.: 18.607907° E; E. Farkas, L. Lőkös	VBI_6011
CLF11	27.08.2016	225	HUNGARY, Fejér County. Velence Mts, Mt Új-hegy (S side) at Sukoró, along the 'yellow cross' tourist route („Nadapi út") between Sukoró and Nadap, on acidic soil in pine forest. Lat.: 47.249208° N; Long.: 18.606851° E; E. Farkas, L. Lőkös	VBI_6012
CLF12	20.10.2013.	226	HUNGARY, Baranya County. Mts. Villányi-hegység, Mt. Kis-hegy, 1.4 km SE of Csarnóta on calcareous soil.. Lat.: 45°53'05.7" N; Long.: 18°13'41.8" E; E. Farkas, N. Varga	VBI_6013
CLF13	20.03.2022.	309	HUNGARY, Fejér County. Vértes Mts (SW side), Mt Keskeny-hegy, ca 1.4 km NWN of Csákberény, on	VBI_6014

			calcareous soil in open rocky grassland. Lat.: 47.361838° N; Long.: 18.318804° E; L. Lőkös	
CLF14	26.03.2022.	343	HUNGARY, Komárom-Esztergom County. Gerecse Mts (SW side), Mt Zuppa-tető, ca 2.0 km SE of Szárliget, along the 'blue triangle' tourist route, on calcareous soil in open rocky grassland. Lat.: 47.505643° N; Long.: 18.515455° E; L. Lőkös	VBI_6015
CLF15	04.07.2020.	107	HUNGARY, Bács-Kiskun County. Great Hungarian Plain, Kiskunság, „Monostori erdő", ca 1.7 km ESE of Alsómóricgát, on calcareous soil in open sandy grassland. Lat.: 46.618510° N; Long.: 19.708816° E; E. Farkas, L. Lőkös	VBI_6016
CLF16	04.07.2020.	109	HUNGARY, Bács-Kiskun County. Great Hungarian Plain, Kiskunság, ca 3.0 km WSW of Fülöpháza, on calcareous soil in open sandy grassland. Lat.: 46.884610° N; Long.: 19.403767° E; E. Farkas, L. Lőkös	VBI_6017_1
CLF17	04.07.2020.	109	HUNGARY, Bács-Kiskun County. Great Hungarian Plain, Kiskunság, ca 3.0 km WSW of Fülöpháza, on calcareous soil in open sandy grassland. Lat.: 46.884610° N; Long.: 19.403767° E; E. Farkas, L. Lőkös	VBI_6017_2
CLF18	30.05.2020.	240	HUNGARY, Heves County. Mátra Mts (SE side), valley of stream „Első-Tarnóca" near „Úsztatói erdészház", ca 1.6 km N of Kisnána, on acidic soil in rocky grassland. Lat.: 47.866252° N; Long.: 20.145268° E; E. Farkas, L. Lőkös	VBI_6018
CLF19a	08.06.2022.	134	HUNGARY, Pest County. Vácrátót, Tece, along the 'red line' tourist route (Ág-dűlő), on calcareous soil in open sandy grassland. Lat.: 47.702358° N; Long.: 19.224312° E; E. Farkas	VBI_6019
CLF19b	08.06.2022.	134	HUNGARY, Pest County. Vácrátót, Tece, along the 'red line' tourist route (Ág-dűlő), on calcareous soil in open sandy grassland. Lat.: 47.702358° N; Long.: 19.224312° E; E. Farkas	VBI_6019
CLF20	16.06.2013.	1,250	NORTH MACEDONIA, Polog Statistical Region, Mavrovo and Rostuša Municipality, Mavrovo. On calcareous soil. Lat.: 41.652188° N; Long.: 20.736456° E; L. Lőkös	VBI_6020
CLF21	05.05.2015.	220	ALBANIA, Shkodër District (Rrethi i Shkodrës); in the valley of stream Përroi i Çibunit E of village Prekal; on calcareous soil in scrubland. Lat.: 42.179080° N; Long.: 19.721794° E; L. Lőkös	VBI_6021
CLF22	06.05.2015.	735	ALBANIA, Pukë District (Rrethi i Pukës); Mount Rras' e Qerretit above village Qerret; in serpentine rocky area, on acid soil in in pine woodland. Lat.: 42.060272° N; Long.: 19.831513° E; L. Lőkös	VBI_6022
CLF23	11.05.2015.	1,035	ALBANIA, District of Devoll (Rrethi i Devollit); on the hills above village Ponçarë; in dry grasslands, on flysh soil. Lat.: 40.521564° N; Long.: 20.965064° E; L. Lőkös	VBI_6023
CLF24	12.05.2015.	910	ALBANIA, District of Devoll (Rrethi i Devollit); on the mount between village Tren and lake Liqeni i Prespës së Vogël; on calcareous soil in rocky grasslands. Lat.: 40.669596° N; Long.: 20.986839° E; L. Lőkös	VBI_6046
CLF25	01.05.2017.	1,105	ALBANIA, Korçë County, Mt "Golines" at village Rakickë; on calcareous soil in rocky grasslands. Lat.: 40.719132° N; Long.: 20.983602° E; L. Lőkös	VBI_6047
CLF26	30.03.2015.	78	ALBANIA, District of Shkodër (Rrethi i Shkodrës); in the valley of river Përroi i Thatë at village Lashaj, along the road nr. E 762, on calcareous soil in open dry rocky grasslands. Lat.: 42.238806° N; Long.: 19.423560° E; L. Lőkös	VBI_6048

CLF27	30.03.2015.	78	ALBANIA, District of Shkodër (Rrethi i Shkodrës); in the valley of river Përroi i Thatë at village Lashaj, along the road nr. E 762, on calcareous soil in open dry rocky grasslands. Lat.: 42.238806° N; Long.: 19.423560° E; L. Lőkös	VBI_6027
CLF28	12.11.2013.	383	SLOVAKIA. Nitra Region, Mt. Zobor, at c. 4.5 km N of Nitra, on calcareous soil in rocky grassland. Lat.: 48°20'56.6" N; Long.: 18°05'32.7" E; E. Farkas, N. Varga	VBI_6028

* Herbarium abbreviation VBI (https://sweetgum.nybg.org/science/ih/herbarium-details/?irn=124855).

Appendix 3: Collection data of specimens used for preparing the illustrations to the identification key (FARKAS and MUHORO 2022)

Bulbothrix isidiza (Nyl.) Hale. Tanzania: Morogoro Region: Northern Uluguru Mts, near the town of Morogoro, valley leading S from Bigwa Mission to Lupanga peak, on E-facing slope, alt. 1100–1200 m, from the bark of Dahlbergia lactea in dry rocky woodland, 1988, T. Pócs 88191/P (VBI 1691).

Canoparmelia texana (Tuck.) Elix & Hale (as Pseudoparmelia texana (Tuck.) Hale). Tanzania: Arusha Region: Ngorongoro Conservation Area, NE rim of Ngorongoro Crater, inner slope, NW of Oljoro Nyuki, alt. 2220 m, ramicolous in mature, mist affected, heavily grazed Acacia lahai stand, very rich in epiphytes, 1989, T. Pócs, A. Kijazi, P. Murphy 89011/PB, det. H. Krog, rev. E. Farkas (VBI 2308).

Flavopunctelia flaventior (Stirt.) Hale (as Punctelia flaventior (Stirt.) Krog). Tanzania: Arusha Region: Ngorongoro Conservation Area, NE rim of Ngorongoro Crater, inner slope, NW of Oljoro Nyuki, alt. 2220 m, ramicolous in mature, mist affected, heavily grazed Acadia lahai stand, very rich in epiphytes, 1989, T. Pócs, A. Kijazi, P. Murphy 89011/X, det. H. Krog, rev. E. Farkas (VBI 2321).

Hypotrachyna vexans (Zahlbr. ex W. L. Culb. & C. F. Culb.) Divakar et al. (as Cetrariastrum vexans (Zahlbr.) W. L. Culb. & C. F. Culb.). Tanzania: Mbeya Region: Southern Highlands, Poroto Mts, W of Isongole Village, SE of Ngozi Crater, alt. 2000 m, from bark of Hagenia sp. in montane rainforest, 1989, T. Pócs, E. Farkas, H. Krog 89128/H, det. H. Krog, rev. E. Farkas (VBI 1741).

Parmelinella schimperiana Kirika & Divakar (as Pseudoparmelia wallichiana (Taylor) Krog & Swinscow). Tanzania: Arusha Region: Mt Meru, W slope, on the ridge above Laikinoi, alt. 2600 m, corticolous in Juniperus-Podocarpus usambarensis forest, 1988, T. Pócs & Helsinki Univ. Bot. Dept. 88296/L, det. H. Krog, rev. E. Farkas (VBI 2309).

Parmotrema tinctorum (Nyl.) Hale. Tanzania: Tanga Region: Lushoto District, West Usambara Mts, W slopes of Gonja Hill, 5 km E of Mgwashi Village, alt. 1600–1700 m, from bark in montane evergreen forest, 1988, T. Pócs, H. Krog 88205/LC, det. H. Krog (VBI 1741).

Parmotrema ultralucens (Krog) Hale. Tanzania: Tanga Region: Lushoto District, East Usambara Mts, Hunga stream valley, below Derema Village, alt. 840 m, rupicolous on granitic river-bed rocks, 1987, K. Pócs 87037/U, det. H. Krog (VBI 2217)

Appendix 4: Locality data and the detected lichen secondary metabolites of lichens represent new distribution records

Taxa are listed in alphabetical order. Lichenicolous fungi are indicated by #. New distribution data are indicated by * (for Kenya or Tanzania), ** (for East Africa) (FARKAS et al. 2023.

**Bulbothrix kenyana* Kirika, Divakar & Lumbsch Tanzania: Tanga Region, East Usambara Mts, roadside and secondary vegetation between Amani Research Station and Greenway's forest house, lignicolous at 8 5 0 -9 0 0 m a.s.l., E. Farkas 86209/B , 2 5-26.10.1986 (VBI 6180 - atranorin, salazinic acid, with Chrysothrix xanthina); Morogoro Region, Northern Uluguru Mts, near the town of Morogoro, valley leading S from Bigwa Mission to Lupanga peak, on E-facing slope, from bark of Dahlbergia lactea in dry rocky woodland at 1,100-1,200 m a.s.l., T. Pócs 88191/P , 28.09.1988 (VBI 1691, as Bulbothrix isidiza (Nyl.) Hale - atranorin, salazinic acid). new distribution record from Tanzania

**Chrysothrix xanthina* (Vain.) Kalb Tanzania: Arusha Region. W slope of Mt Meru, Pinus patula plantation along Laikinoi road above Olmotonyi village, corticolous on Pinus, at 2,110 m a.s.l., T. Pócs & J. Kjelland-Lund 88293/LA 13. 12.1988 (VBI 6249 - pinastric acid); Tanga region, West Usambara Mts, Shagayu Forest Reserve, Acacia m earnsii secondary forest plantation NW of Mlalo Mission, corticolous at 1,450-1,500 m a.s.l., E. Farkas 86200/LA , 20.10.1986 (VBI 6247 - pinastric acid); Roadside and secondary vegetation between Amani Research Station and Greenway's forest house, lignicolous at 8 5 0 -9 0 0 m a.s.l., E. Farkas 86209/D , 2 5-26.10.1986 (VBI 6246 - calycin, pinastric acid); Former Marvera Forest Reserve E of Marvera Tea Estate, 6 km of Amani, very degraded forest fragment with cardamom and other plantations, corticolous at 1,000-1,800 m a.s.l., E. Farkas & T. Pócs 86244/CP, 12.11.1986 (VBI 6248 - pinastric acid); Iringa Region, Mufindi Highlands, Colin Congdon's Garden in Mufindi, corticolous on Pinus at 1,860 m a.s.l., E. Farkas 89129/LA, 12.04.1989 (VBI 6250 - pinastric acid)

***Didymocyrtis cf. melanelixiae* (Brackel) Diederich, R.C. Harris & Etayo Kenya: Nyeri county, at the foot of Mt Kenya, c. 1 km from Naro Moru entry gate to Kenya wildlife service in Gitinga village, from bark, twigs and branches of trees in tropical rainforest, 0°10'25.84" S, 37°9'3.40" E, 2,454 m a.s.l., A.M. Muhoro 21/01, 14.08.2021 (on Parm otrem a austrosinense - VBI 6210, VBI 6215 with Lichenoconium erodens, on Parmotrema reticulatum - VBI 6224).

***#Lichenoconium erodens* M.S. Christ. & D. Hawksw. Kenya: Nyeri county, at the foot of Mt Kenya, c. 1 km from Naro Moru entry gate to Kenya wildlife service in Gitinga village, from bark, twigs and branches of trees in tropical rainforest, 0°10'25.84" S, 37°9'3.40" E, 2,454 m a.s.l., A.M. Muhoro 21/01, 14.08.2021 (on Parm otrem a austrosinense (Figure 2b) - VBI 6217, VBI 6215 - with Didymocyrtis cf. melanelixiae, on Parm otrem a cooperi - VBI 6207, on Parm otrem a reticulatum - VBI 6190, VBI 6218, VBI 6223). — Tanzania: Tanga Region, East Usambara Mts, Mlinga Peak area, moist and moderately mossy forest around the peak (with Podocarpus milanjianus, Memecylon greenw ayi), on Podocarpus twigs 115 Acta Bioi. Piant Agriensis 11(1): 107-128 between 9 0 0-1,050 m a.s.l., E. Farkas & A. Borhidi 86230a/BC, 05.11.1986 (on Parm otrem a durum ae - VBI 6240); Southern Highlands, on Brachystegia bark near Sao Hill sawmills at 1,920 m a.s.l., Kata Pócs 86174/BA , 23-25.12.1986 (on Parm otrem a andinum - VBI 6170).

**Lobaria discolor* (Bory ex Delise) Hue Tanzania: Tanga region, West Usambara Mts, Shagayu Forest Reserve, SE range of Shagein, corticolous in montane evergreen forest at 1,850-2,050 m a.s.l., E. Farkas 86205/G , 22.10.1986 (VBI 6167 - fatty acid, gyrophoric acid)

**Parmotrema durumae* (Krog & Swinscow) Krog & Swinscow Tanzania: Tanga Region, East Usambara Mts, Kwamkoro F. R., SE of Kwamkoro Tea Estate, heavily logged intermediate rainforest of Ocotea usambarensis, C ephaiosphaera, Variodendron, etc. at 9 0 0 -1,030 m a.s.l., E. Farkas 86214/N , 28.10.1986 (VBI 6163 - alectoronic acid, atranorin, a-collatolic acid); Mlinga Peak area, moist and moderately mossy forest around the peak (with Podocarpus milanjianus, Memecylon greenw ayi), on Podocarpus twigs between 9 0 0 -1,050 m a.s.l., E. Farkas & A. Borhidi 86230a/BC, 86230a/B , 05.11.1986 (VBI 6240, VBI 6242 - atranorin, alectoronic acid, a-collatolic acid).

**Parmotrema taitae* (Krog & Swinscow) Krog & Swinscow Tanzania: Tanga Region, East Usambara Mts, Kwamkoro F. R., SE of Kwamkoro Tea Estate, heavily logged intermediate rainforest of Ocotea usambarensis, C ephaiosphaera, Variodendron, etc., ramicolous at 9 0 0 -1,030 m a.s.l., E. Farkas 86214/NA, 28.10.1986 (VBI 6162 - atranorin, fumarprotocetraric acid); Mlinga Peak, rocky outcrops near the southern summit between 1,020-1,060 m a.s.l., E. Farkas 86229/A , 86229/AB, 86229/ABB, 86229/AA, 86229/AAA, 05.11.1986 (VBI 6231, VBI 6233, VBI 6237 - atranorin, fumarprotocetraric acid, VBI 6232, VBI 6234 - atranorin, fatty acid, fumarprotocetraric acid).

***#Spirographa lichenicola (D. Hawksw. & B. Sutton) Flakus, Etayo & Migdl. Kenya: Nyeri county, at the foot of Mt Kenya, c. 1 km from Naro Moru entry gate to Kenya wildlife service in Gitinga village, from bark, twigs and branches

of trees in tropical rainforest, 0°10’25.84” S, 37°9’3.40” E, 2,454 m a.s.l., A.M. Muhoro 21/01, 14.08.2021 (on Parm otrem a austrosinense - VBI 6225, on Parm otrem a reticuiatum - VBI 6226).

**Usnea abissinica* Motyka Kenya: Nyeri county, at the foot of Mt Kenya, c. 1 km from Naro Moru entry gate to Kenya wildlife service in Gitinga village, from bark, twigs and branches of trees in tropical rainforest, 0°10’25.84” S, 37°9’3.40” E, 2,454 m a.s.l., A.M. Muhoro 21/01, 14.08.2021 (VBI 6192 - usnic acid, salazinic acid, VBI 6214 - usnic acid, 2 fatty acids, protocetraric acid).

**Usnea sanguinea* Swinscow & Krog Kenya: Nyeri county, at the foot of Mt Kenya, c. 1 km from Naro Moru entry gate to Kenya wildlife service in Gitinga village, from bark, twigs and branches of trees in tropical rainforest, 0°10’25.84” S, 37°9’3.40” E, 2,454 m a.s.l., A.M. Muhoro 21/01, 14.08.2021 (VBI 6196 - usnic acid, VBI 6211, VBI 6212, VBI 6213, VBI 6227, VBI 6228, VBI 6229, VBI 6230 - usnic acid, 2 fatty acids, protocetraric acid).

Appendix 5: Updated list of species in parmelioid clade known from Kenya (Supplement of FARKAS and MUHORO 2022)

Updated list of species in parmelioid clade known from Kenya	Selected synonyms
Bulborrhizina africana Kurok.	
Bulbothrix goebelii (Zenker) Hale	
Bulbothrix hypocraea (Vainio) Hale	
Bulbothrix isidiza (Nyl.) Hale	
Bulbothrix kenyana Kirika, Divakar & Lumbsch	
Bulbothrix meizospora (Nyl.) Hale	
Bulbothrix sensibilis (Steiner & Zahlbr.) Hale	
Bulbothrix sublaevigatoides (Dodge) Kirika, Divakar & Lumbsch	Bulbothrix tabacina (Mont.& v.d.Bosch) Hale
Bulbothrix suffixa (Stirton) Hale	
Bulbothrix ventricosa (Hale & Kurok.) Hale	
Canoparmelia amazonica (Nyl.) Elix & Hale, Mycotaxon 27: 278 (1986)	Pseudoparmelia amazonica (Nyl.) Hale
Canoparmelia caroliniana (Nyl.) Elix & Hale	Pseudoparmelia caroliniana (Nyl.) Hale
Canoparmelia concrescens (Vain.) Elix & Hale	
Canoparmelia ecaperata (Müll. Arg.) Elix & Hale	Pseudoparmelia ecaperata (Müll. Arg.) Hale
Canoparmelia nairobiensis (J. Stein. & Zahlbr.) Elix & Hale	Pseudoparmelia nairobiensis (Steiner & Zahlbr.) Hale
Canoparmelia pustulescens (Kurok.) Elix	Pseudoparmelia pustulescens (Kurok.) Hale
Canoparmelia rodriguesiana (Hue) Elix	Pseudoparmelia rodriguesiana (Hue) Hale
Canoparmelia somaliensis (Müll. Arg.) Elix & Hale	Pseudoparmelia somaliensis (Miill. Arg.) Hale
Canoparmelia texana (Tuck.) Elix & Hale	Pseudoparmelia texana (Tuck.) Hale
Cetrelia braunsiana (Müll. Arg.) W. Culb. & C. Culb.	
Crespoa crozalsiana (B. de Lesd. ex Harm.) Lendemer & B.P. Hodk.	Pseudoparmelia crozalsiana (Harm.) Hale
Flavoparmelia caperata (L.) Hale	Pseudoparmelia caperata (L.) Hale
Flavoparmelia pachydactyla (Hale) Hale	Pseudoparmelia pachydactyla (Hale) Hale
Flavoparmelia rutidota (Hook. f. & Taylor) Hale	Pseudoparmelia rutidota (J. D. Hook. & Taylor) Hale
Flavoparmelia soredians (Nyl.) Hale	Pseudoparmelia soredians (Nyl.) Hale
Flavopunctelia flaventior (Stirt.) Hale	Punctelia flaventior (Stirton) Krog
Flavopunctelia praesignis (Nyl.) Hale	Punctelia praesignis (Nyl.) Krog
Hypotrachyna afrorevoluta (Krog & Swinscow) Krog & Swinscow	
Hypotrachyna brevirhiza (Kurok.) Hale	
Hypotrachyna catawbiensis (Degel.) Divakar, A. Crespo, Sipman, Elix & Lumbsch	
Hypotrachyna costaricensis (Nyl.) Hale	
Hypotrachyna croceopustulata (Kurok.) Hale	
Hypotrachyna damaziana (Zahlbr.) Krog & Swinscow	
Hypotrachyna densirhizinata (Kurok.) Hale	
Hypotrachyna ducalis (Jatta) Hale	
Hypotrachyna endochlora (Leighton) Hale	
Hypotrachyna fissicarpa (Kurok.) Hale	
Hypotrachyna formosana (Zahlbr.) Hale	
Hypotrachyna gondylophora (Hale) Hale	
Hypotrachyna horrescens (Taylor) Krog & Swinscow	
Hypotrachyna immaculata (Kurok.) Hale	
Hypotrachyna kenyana Kirika, Divakar & Lumbsch	
Hypotrachyna laevigata (Sm.) Hale	
Hypotrachyna leiophylla (Kurok.) Hale	
Hypotrachyna meridionalis Kirika, Divakar & Lumbsch	
Hypotrachyna meyeri (Zahlbr.) Streim.	
Hypotrachyna microblasta (Vainio) Hale	
Hypotrachyna minarum (Vainio) Krog & Swinscow	
Hypotrachyna neodissecta (Hale) Hale	
Hypotrachyna nyandaruaensis Kirika, Divakar & Lumbsch	
Hypotrachyna orientalis (Hale) Hale	
Hypotrachyna polydactyla (Krog & Swinscow) Nash	
Hypotrachyna producta Hale	
Hypotrachyna revoluta (Flörke) Hale	
Hypotrachyna rockii (Zahlbr.) Hale	
Hypotrachyna scytophylla (Kurok.) Hale	
Hypotrachyna sinuosa (Sm.) Hale	
Hypotrachyna sorocheila (Vain.) Divakar, A. Crespo, Sipman, Elix & Lumbsch	
Hypotrachyna spathulata (Kurok.) Krog & Swinscow	
Hypotrachyna spumosa (Asah.) Krog & Swinscow	
Hypotrachyna subfatiscens (Kurok.) Krog & Swinscow	
Hypotrachyna sublaevigata (Nyl.) Hale	
Hypotrachyna swinscowi (Hale) Krog & Swinscow	

Hypotrachyna vexans (Zahlbr. ex W.L. Culb. & C.F. Culb.) Divakar, A. Crespo, Sipman, Elix & Lumbsch	
Melanelixia subaurifera (Nyl.) O. Blanco, A. Crespo, Divakar, Essl., D. Hawksw. & Lumbsch	Melanelia subaurifera (Nyl.) Essl.
Myelochroa aurulenta (Tuck.) Elix & Hale	Hypotrachyna aurulenta (Tuck.) Krog & Swinscow
Parmelia saxatilis (L.) Ach.	
Parmelia sulcata Taylor	
Parmelinella schimperiana Kirika & Divakar	part of Parmelinella wallichiana (Taylor) Elix & Hale (syn: Pseudoparmelia wallichiana (Taylor) Krog & Swinscow), identified earlier from Kenya
Parmotrema abessinicum (Krempelh.) Hale	
Parmotrema aldabrense (Dodge) Hale	
Parmotrema andinum (Müll Arg.) Hale	
Parmotrema apricum (Krog & Swinscow) Krog & Swinscow	
Parmotrema araucariarum (Zahlbr.) Hale	
Parmotrema austrosinense (Zahlbr.) Hale	
Parmotrema bangii (Vainio) Hale	
Parmotrema cetratum (Ach.) Hale	
Parmotrema cooperi (Steiner & Zahlbr.) Sérus.	
Parmotrema crinitum (Ach.) M. Choisy	
Parmotrema cristiferum (Taylor) Hale	
Parmotrema cryptoxanthum (des Abb.) Hale	
Parmotrema defectum (Hale) Hale	
Parmotrema dilatatum (Vainio) Hale	
Parmotrema direagens (Hale) Hale	
Parmotrema durumae (Krog & Swinscow) Krog & Swinscow	
Parmotrema epileucum (Hale) Kirika, Divakar & Lumbsch	Pseudoparmelia epileuca (Hale) Hale
Parmotrema erubescens (Stirton) Krog & Swinscow	
Parmotrema eunetum (Stirton) Hale	
Parmotrema gardneri (Dodge) Sérus.	
Parmotrema hababianum (Gyelnik) Hale	
Parmotrema hololobum (Hale) Hale	
Parmotrema indicum Hale	
Parmotrema jacarandicola (Krog & Swinscow) Krog & Swinscow	
Parmotrema kwalense (Krog & Swinscow) Krog & Swinscow	
Parmotrema leonis Krog & Swinscow	
Parmotrema lobulascens (Steiner) Hale	
Parmotrema lophogenum (des Abb.) Hale	
Parmotrema louisianae (Hale) Hale	
Parmotrema maclayanum (Müll. Arg.) Hale	
Parmotrema mellissii (Dodge) Hale	
Parmotrema nilgherrense (Nyl.) Hale	
Parmotrema parahypoptorum (W. Culb.) Hale	
Parmotrema pardi (Krog & Swinscow) Krog & Swinscow	
Parmotrema perlatum (Huds.) M. Choisy	Parmotrema chinense (Osbeck) Hale & Ahti
Parmotrema permutatum (Stirton) Hale	
Parmotrema pigmentiferum (Krog & Swinscow) Krog & Swinscow	
Parmotrema pilosum (Stizenb.) Krog & Swinscow	
Parmotrema poolii (Dodge) Krog & Swinscow	
Parmotrema praesorediosum (Nyl.) Hale	
Parmotrema pseudocrinitum (des Abb.) Hale	
Parmotrema pseudograyanum (Hale) Sérus.	
Parmotrema ravum (Krog & Swinscow) Sérus.	
Parmotrema reticulatum (Taylor) M. Choisy	Rimelia reticulata (Taylor) Hale & A. Fletcher
Parmotrema rimulosum (Dodge) Hale	
Parmotrema sancti-angelii (Lynge) Hale	
Parmotrema soyauxii (Müll. Arg.) Hale	
Parmotrema stuhlmannii (C.W. Dodge) Krog & Swinscow	
Parmotrema subarnoldii (des Abb.) Hale	
Parmotrema subcoloratum (Hale) Hale	
Parmotrema subisidiosum (Müll. Arg.) Hale	
Parmotrema subschimperi (Hale) Hale	
Parmotrema subsumptum (Nyl.) Hale	
Parmotrema subtinctorium (Zahlbr.) Hale	
Parmotrema sulphuratum (Nees & Flotow) Hale	

Parmotrema taitae (Krog & Swinscow) Krog & Swinscow	
Parmotrema tinctorum (Nyl.) Hale	
Parmotrema tsavoense (Krog & Swinscow) Krog & Swinscow	
Parmotrema uberrimum (Hue) Hale	
Parmotrema ultralucens (Krog) Hale	
Parmotrema umbrosum (Krog & Swinscow) Krog & Swinscow	
Parmotrema nyasense (C. W. Dodge) R. S. Egan comb. nov. [MB#844542]	Parmotrema xanthinum (Müll. Arg.) Hale
Parmotrema zimbabwense (Hale) Kirika, Divakar & Lumbsch	Pseudoparmelia zimbabwensis (Hale) Hale
Parmotrema zollingeri (Hepp) Hale	
Pseudoparmelia singularis Krog & Swinscow	
Pseudoparmelia usambarensis (Steiner & Zahlbr.) Krog & Swinscow	Parmotrema usambarense (J. Steiner & Zahlbr.) Buaruang et al. (invalid)
Punctelia borreri (Sm.) Krog	
Punctelia neutralis (Hale) Krog	
Punctelia punctilla (Hale) Krog	
Punctelia reddenda (Stirton) Krog	
Punctelia rudecta (Ach.) Krog	
Punctelia semansiana (W. Culb. & C. Culb.) Krog	
Punctelia stictica (Duby) Krog	
Punctelia subpraesignis (Nyl.) Krog	
Punctelia subrudecta (Nyl.) Krog	
Relicina abstrusa (Vainio) Hale	
Relicina echinocarpa (Kurok.) Hale	
Relicina limbata (Laurer) Hale	Pseudoparmelia sphaerospora (Nyl.) Hale
Relicina malaccensis (Nyl.) Kirika, Divakar & Lumbsch	Pseudoparmelia malaccensis (Nyl.) Hale
Remototrachyna rhabdiformis (Kurok.) Divakar & A. Crespo	Hypotrachyna rhabdiformis (Kurok.) Hale
Xanthoparmelia africana Hale	
Xanthoparmelia amplexula (Stirton) Elix & Johnston	
Xanthoparmelia annexa (Kurok.) Elix	Pseudoparmelia annexa (Kurok.) Hale
Xanthoparmelia atroventralis (Hale) Hale	
Xanthoparmelia austroafricana (Stirton) Hale	
Xanthoparmelia australasica D.J. Galloway	
Xanthoparmelia congensis (B. Stein) Hale	
Xanthoparmelia cylindriloba M.D.E. Knox	
Xanthoparmelia diadeta (Hale) Hale	
Xanthoparmelia endochrysea (Müll. Arg.) Hale	
Xanthoparmelia glomerulata Krog & Swinscow	
Xanthoparmelia hypoleia (Nyl.) Hale	
Xanthoparmelia kenyana (Essl.) O. Blanco, A. Crespo, Elix, D. Hawksw. & Lumbsch	Neofuscelia kenyana (Essl.) Essl.
Xanthoparmelia kiboensis (Dodge) Krog & Swinscow	
Xanthoparmelia krogiae Hale & Elix	Pseudoparmelia endochromatica Krog & Swinscow; Parmelia adplanata Müll. Arg.
Xanthoparmelia lusitana (Nyl.) Krog	
Xanthoparmelia meruensis Krog & Swinscow	
Xanthoparmelia mexicana (Gyelnik) Hale	
Xanthoparmelia nakuruensis (Essl.) O. Blanco, A. Crespo, Elix, D. Hawksw. & Lumbsch	Neofuscelia nakuruensis (Essl.) Essl.
Xanthoparmelia phaeophana (Stirton) Hale	
Xanthoparmelia rogersii Elix & J. Johnst.	
Xanthoparmelia salkiboensis Hale	
Xanthoparmelia subramigera (Gyelnik) Hale	
Xanthoparmelia subtortula (Hale) Elix	Pseudoparmelia subtortula (Hale) Hale
Xanthoparmelia tasmanica (Hook. f. & Taylor) Hale	
Xanthoparmelia treurensis Hale, T.H. Nash & Elix	
Xanthoparmelia tinctina (Maheu & A. Gillet) Hale	
Xanthoparmelia verrucigera (Nyl.) Hale	
Xanthoparmelia weberi (Hale) Hale	

Appendix 6: Details (pages 303-305) of the identification key from publication FARKAS and MUHORO (2022)

Key to species of the parmelioid clade in Kenya

Parmelioid species share the following characters within *Parmeliaceae*: photobiont green alga; foliose growth form, not umbilicate, thallus corticate above and below, adnate or loosely attached to substratum; presence of rhizines; medulla solid, grey, yellowish green or brownish; if present, fruiting body apothecium with thalline exciple; simple ascospores; laminal pycnidia.

Further keys lead to species of the genera *Bulborrhizina* (1) and *Bulbothrix* (9), *Canoparmelia* (9) and *Pseudoparmelia* (2), *Hypotrachyna* (37), *Parmotrema* (64), *Punctelia* (9) and *Xanthoparmelia* (29) and follow the main key.

1 Pseudocyphellae present, may occur on both sides . . . 2
Pseudocyphellae absent, cortex continuous on both sides . . . 8

2(1) Pseudocyphellae may occur on both upper and lower surfaces in the genus but this species has pseudocyphellae on upper surface only, together with granular and coralloid isidia on large wide lobes, lower side is black with black rhizines; cortex K+ yellow, atranorin; medulla K–, C–, KC+ pink, Pd–, alectoronic acid, α-collatolic acid . . . **Cetrelia braunsiana** (Müll. Arg.) W. L. Culb. & C. F. Culb.
Pseudocyphellae present on upper surface, thallus otherwise . . . 3

3(2) Thallus grey; cortex K+ yellow, atranorin . . . 4
Thallus yellow-green or brown; cortex K–, with or without usnic acid . . . 6

4(3) Pseudocyphellae mostly linear effigurate, reticulate, seldom punctiform, lobes sublinear; medulla K+ red, Pd+ orange, salazinic acid . . . 5
Pseudocyphellae punctiform to suborbicular, lobes rotund . . . **Punctelia** (9 species)

5(4) Pseudocyphellae linear effigurate, cylindrical isidia present with brown tips; lobes 1–3 mm wide, underside black; rhizines simple to bifurcate; lignicolous at *c.* 3000 m alt. . . . **Parmelia saxatilis** (L.) Ach.
Pseudocyphellae linear, reticulate, often developing into soredia; lobes 2–5 mm wide, underside black; rhizines simple to squarrose; saxicolous at 3500–4200 m alt. . . . **Parmelia sulcata** Taylor

6(3) Thallus olive to reddish brown; isidia present, cylindrical and punctiform soredia originate from pseudocyphellae, lower side brown to black with scattered simple rhizines; cortex without usnic acid, HNO_3–; medulla C+ red, lecanoric acid . . . **Melanelixia subaurifera** (Nyl.) O. Blanco *et al.*
Thallus yellow-green, pseudocyphellae punctiform to elongate; cortex with usnic acid; medulla C+ red, lecanoric acid . . . 7

7(6) Soredia present, soralia marginal linear or laminal punctiform; underside black, with a brown and glossy, broad, naked marginal zone; rhizines few, scattered, usually black but towards the margin pale brown or with white tips; apothecia common, with sorediate thalline exciple; ascospores long ellipsoid, 15–18 × 6–8 µm. . . . **Flavopunctelia flaventior** (Stirt.) Hale
Soredia and isidia absent; underside black, glossy brown or white mottled in a broad marginal zone; rhizines short, inconspicuous; apothecia often numerous towards the centre of the thallus, with a pseudocyphellate thalline exciple; ascospores ellipsoid, 12–15 × 8–10 µm . . . **Flavopunctelia praesignis** (Nyl.) Hale

8(1) Thallus yellow, yellow-green or brown . . . 9
Thallus grey . . . 18

9(8) Thallus brown, grey-brown; upper cortex HNO_3+ blue-green, without usnic acid . . . **Xanthoparmelia** pr. p.
Thallus yellow, yellow-green, pale yellowish grey; upper cortex HNO_3–, with usnic acid . . . 10

10(9) Bulbate cilia present at lobe margin, lobes pale yellow . . . 11
Lobe margin without cilia, yellow-green to green (pale yellow in *Relicina abstrusa*) . . . 14

11(10) Isidia absent . . . 12
Cylindrical isidia present . . . 13

12(11) Thallus yellow-green to green, with 1–2 mm wide sublinear, dichotomous lobes; underside black; cortex with usnic acid; medulla K+ yellow, C+ pale yellow to orange (unknown substance) and Pd+ deep orange (unknown substance, 'echinocarpic acid'); apothecia laminal, thalline exciple pilose; ascospores subglobose, 6–8 × 3–5 µm . . . **Relicina echinocarpa** (Kurok.) Hale
Lobes 3–6 mm wide, yellow-green to green, medulla pale yellow; apothecia laminal, thalline exciple crenate; ascospores subglobose, 8–10 × 7–8 µm; undetermined pigment and substances, ±atranorin (trace); corticolous at 1500–2200 m alt. . . . **Relicina limbata** (Laurer) Hale

13(11) Lobes 1–2 mm wide, pale yellow, marginal cilia well developed, clearly bulbate; medulla white; apothecia absent in East African specimens; cortex, usnic acid; medulla K+ red, Pd+ orange, norstictic acid; corticolous in mangroves at 0–300 m alt. . . . . **Relicina abstrusa** (Vain.) Hale

Lobes 0.8–1.5 mm wide, bright yellow to yellow-green, ±eciliate; rhizines sometimes protruding beyond the lobe margins; medulla pigmented faintly yellow, especially near the upper cortex; apothecia common, up to 2 mm diam., thalline margin entire or crenate, disc flat; ascospores 8–10 × 4–6 µm; cortex, usnic acid; medulla Pd+ orange-red, protocetraric acid, ±fatty acid; corticolous on twigs and branches in mangroves, 0–500 m alt. . . . . **Relicina malaccensis** (Nyl.) Kirika *et al.*

14(10) Lobes rounded 15

Lobes elongated . . . . . **Xanthoparmelia** pr. p.

15(14) Laminal soralia present 16

Laminal soralia absent, dactyls present or absent 17

16(15) Lobes 5–10 mm wide, imbricate in central parts, soralia starting from individual pustules but soon coalescing to cover larger areas; underside black with a fairly wide brown, naked marginal zone; rhizines simple, slender, dense or scattered, black, often tipped with white or brown; apothecia not seen in East African material; pycnoconidia weakly bifusiform, 5–6 µm long; cortex, usnic acid, ±atranorin (trace); medulla Pd+ orange-red, protocetraric acid, caperatic acid; corticolous at 1500–3600 m alt. . . . . **Flavoparmelia caperata** (L.) Hale

Lobes 1–5 mm wide, soralia punctiform, becoming confluent over larger areas; underside black, with a narrow, brown marginal zone; rhizines simple, black to brown, some tipped with white; apothecia and pycnidia not seen in material from Kenya; cortex, usnic acid; medulla K+ red, Pd+ orange, salazinic acid; corticolous, lignicolous, or saxicolous at 1100–2700 m alt. . . . . **Flavoparmelia soredians** (Nyl.) Hale

17(15) Lobes 1–2.5 mm wide, dactyls laminal, crowded in central parts of the thallus, mainly closed but occasionally becoming eroded or with a small perforation at the apex; underside velvety black to the margins; rhizines short, black; apothecia rare, up to 2 mm diam., with a thick, crenate thalline margin; spores 10–16 × 5–8 µm; pycnoconidia filiform, 10–12 µm long; cortex, usnic acid; medulla Pd+ orange-red, protocetraric acid; saxicolous at 1750 m alt. . . . . **Flavoparmelia pachydactyla** (Hale) Hale

Lobes 5–8 mm wide, without dactyls; underside black, with a narrow, brown, naked marginal zone; rhizines simple, black or tipped with white; apothecia not seen in East African material; pycnoconidia weakly bifusiform, 7–8 µm long; cortex, usnic acid, ±atranorin (trace); medulla Pd+ orange-red, protocetraric acid, caperatic acid; saxicolous at 3600 m alt. . . . . **Flavoparmelia rutidota** (Hook. f. & Taylor) Hale

18(8) Upper surface mostly maculate 19

Upper surface mostly emaculate 20

19(18) Lobes large, rotund, wider than 0.5 cm, upper cortex often reticulately cracked; maculae may occur… . . . . . **Parmotrema** pr. p. (65 species)

Lobes narrower than 0.5 cm, rotund, subrotund or sublinear; emaculate species may occur . . . . . **Canoparmelia** (9 species) and **Pseudoparmelia** (2 species)

20(18) Bulbate cilia present at lobe margin . . . . . **Bulborrhizina** (1 species) and **Bulbothrix** (9)

Lobe margin without bulbate cilia 21

21(20) Lobes large, rotund, wider than 0.5 cm . . . . . **Parmotrema** pr. p. (65 species)

Lobes narrower, rotund or elongated, narrower than 0.5 cm 22

22(21) Thallus with secalonic acid A yellow pigment 23

Thallus without secalonic acid A yellow pigment . . . . . **Hypotrachyna** (37 species)

23(22) Medulla yellow to salmon pink, pigment K–; lobes sublinear to irregular, 3–5 mm wide, pale grey; rhizines mainly simple but a few branched, some growing out horizontally from the margins; pustular soralia and open dactyls laminally and submarginally situated; cortex K+ yellow, atranorin; medulla, triterpenoids, secalonic acid A . . . . . **Myelochroa aurulenta** (Tuck.) Elix & Hale

Medulla white; lobes irregularly to subirregularly branched, 3–8 mm wide, margins ciliate; upper surface grey, grey-green, usually pruinose; isidia cylindrical, mostly simple, also branched; lower surface black with brown papillate margins; rhizines black, simple, evenly distributed; apothecia 1–5 mm; ascospores 5–10 × 5–7.5 µm; pycnidia not seen in material from Kenya; cortex K+ yellow, UV–, secalonic acid A and atranorin; medulla K+ yellow turning red, C–, KC–, Pd+ orange-red, UV–, salazinic acid . . . . . **Parmelinella schimperiana** Kirika & Divakar

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