Biological activity of the South African Traditional "miraculous plant" used as an alternative treatment for ear infections The topic should state clearly the plant species

ABSTRACT

Aims: The study aimed to investigate the *Kalanchoe luciae* Raym-Hammet plant species used for the treatment of ear infections and determine the antifungal activity against the selected fungal pathogens.

Study Design:

Place and Duration of Study:

Kalanchoe luciae Raym. -Hammet leaf materials w

Methodology:

The plant species used to combat ear infections was selected from the database in the Ethnomedicinal Plant's laboratory of over 300 medicinal plants used for the treatment of various diseases in humans, in Limpopo province, South Africa. In this study, mature and young leaves of K. luciae were extracted with acetone and methanol. The leaves were squeezed separately to for the extractraction of juice. from the leaves. The leaf extracts were tested for antifungal activity against *Aspergillus fumigatus*, *Cryptococcus neoformans*, and *Candida albicans* using a serial dilution assay.

Results:

Methanol and juice extracts had the highest antifungal activity against *A. fumigatus* with minimum inhibitory concentrations (MIC) of 0.04 and 0.10 mg/ml. *Cryptococcus neoformans* was most susceptible to acetone mature leaf extract, followed by

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both mature and young leaf extracts at a mean MIC value of 0.09 mg/ml and 0.16 mg/ml, respectively. In the bioautography assay, there were no active compounds observed in all extracts against the tested fungal pathogens.
Conclusion:
Kalanchoe luciae can serve as a potential plant that could be used as a source of antifungal compounds and hence ~~that~~ can be incorporated as an ~~into~~ antimicrobial drug production.

Keywords: Antifungal activity; Ear infection; Kalanchoe luciae; Minimum inhibitory concentration; Fungal pathogen.

1. INTRODUCTION

Kalanchoe luciae ~~Raym-Hammet~~ belongs to the family Crassulaceae and is one of the genera that is related closely to Saxifragaceae (Milad et al., 2014). The plant species is found in Limpopo, Mpumalanga, and KwaZulu-Natal provinces. It grows in the bushveld, in open, rocky situations, and on exposed hilltops. There is an estimate of roughly 15 species of Kalanchoe that are endemic to South Africa (Crouch et al., 2016). *Kalanchoe luciae* is native to ~~originates from~~ South Africa (Feakins and Sessions, 2010). Alternative names for *Kalanchoe* are *Bryophyllum* and *Cotyledon* (Milad et al., 2014). Crassulaceae is a family of dicotyledons that embodies mainly succulent herbs but tends to be miniature shrubs or trees in certain genera and species (Milad et al., 2014).

Succulent herbs have the ability to store water in their leaves and stems (Sazhina et al., 2014). The majority of these plants are used in horticulture as ornamental plants. ~~for decoration~~ (Milad et al., 2014). *Kalanchoe luciae* forms a basal rosette of large rounded, fleshy leaves, which are greyish cream with red margins. Plants reach about 0.8 -1.3 m, and the erect, upward-facing, tightly arranged leaves are without petioles. The rosettes send up dense inflorescences 1-1.3 m tall, which are coated with a white powder. *Kalanchoe luciae* is used in

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traditional medicine to treat. The leaves of *Kalanchoe* species are used for the treatment of nausea, intestinal worms, ulcers, and fever (Silalahi et al., 2015). In Limpopo and Mpumalanga provinces, K. luciae is used for the treatment of ear infections in humans.

An ear infection (otitis) is an inflammation of the ear caused by bacteria, fungi, or viruses (Aldhaher et al., 2018; Hegde et al., 2021). Over 90% of children are affected by ear infections with 20% of the cases being chronic (Ryan et al., 2020). Furthermore, it is estimated that 60% of cases of ear infections may lead to hearing impairment or death in extreme cases (Hailu et al., 2016). Fungal ear infections include otomycosis. The most common aetiological agents of otomycosis are Aspergillus and Candida species (Aneja et al., 2010). Specifically, Aspergillus fumigatus and Candida albicans are the most common fungal pathogens of the ear (Prasad et al., 2014). Symptoms of otomycosis include pain, itching, aural discharge, fullness, tinnitus, and hearing impairment (Javidnia et al., 2022). However, ear infections caused by Aspergillus and Candida species are treated using antifungal drugs such as clotrimazole cream, ketoconazole cream, and cresylate otic drops (Ayub et al., 2015). Some of these drugs are less effective. In addition, these drugs have side effects including dizziness, dry mouth, headache, high blood pressure, and sedation (Ayub et al., 2015).

The overall emergence of antibiotic resistance has led to investigations of different options for the treatment of ear infections (Kocoglu et al., 2019). The increasing failure of chemotherapeutics and antibiotic resistance exhibited by pathogenic microbial infectious agents has led to the screening of several plants for their potential antimicrobial activity (Aneja et al., 2012). The emergence of multi-drug-resistant pathogens threatens the effectiveness of many existing chemotherapeutic agents. In recent years, the overuse and misuse of antibiotics have caused a growing number of Staphylococcus bacteria to evolve into disease-

causing “superbugs” resistant to drugs like methicillin and vancomycin (Aneja et al, 2012). The increasing resistance of fungal pathogens to antibiotics such as erythromycin, penicillin, tetracycline, vancomycin, ciprofloxacin, and cefotaxime is a major threat to public health (Park et al., 2008).

The invention of a new antimicrobial drug or ear drop solution that can eliminate all pathogens at once could be a solution. Most treatments for ear infections are Western medicine, hence the need to investigate a different approach using traditional medicine. Local people use different medicinal plants and approaches to treat diseases, including ear infections, most of which have not yet been scientifically investigated (Mahmoudian-Sani et al., 2017). In Limpopo province, local people and traditional health practitioners use medicinal plants to combat various ailments in humans (Machaba et al., 2024). Plant-natural products may provide an alternative source for the development of new drugs that could be used to combat ear infections (Azhar et al., 2015). Therefore, there is a need to focus research on traditional medicine since they can provide novel drugs that are effective, cheap, and non-toxic (Machaba et al., 2024). No reports on the antifungal activity of Kalanchoe luciae have been reported in the available literature. In this study, we evaluated the antifungal properties of leaf extracts of *K. luciae* against Aspergillus fumigatus, Cryptococcus neoformans, and *Candida albicans.*

~~**2. Material and methods / experimental details / methodology (arial, bold, 10 font, left aligned, caps)**~~

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2. Methods and materials

2.1 ethical considerations

The bushbuckridge local municipality granted permission for the collection of plant materials from their natural habitat.

2.2 plant selection and collection

The plant species used in this study were selected from an existing database of ethnomedicinal plants used in the mpumalanga and limpopo provinces available at the university of limpopo, department of biodiversity. The leaves of plant species were collected at gottenburg village, bushbuckridge local municipality, mpumalanga province (figure 1). The leaf materials were ~~was~~ collected from S~~s~~eptember to N~~n~~ovember 2020. The voucher specimen (sc01) of the plant species was prepared and deposited at the university herbarium. The leaf materials were stored in an open mesh orange bags at room a temperature of 25 °c to ensure efficient drying of the material.

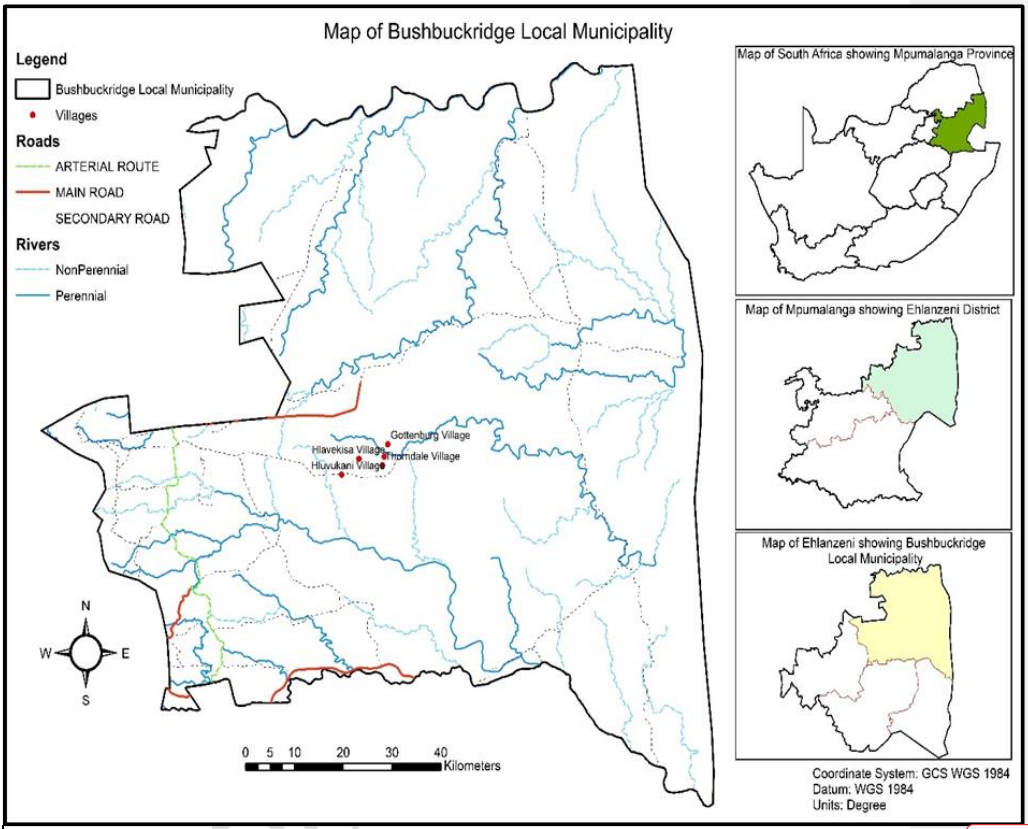

Figure 1: Ma map showing the study area, bushbuckridge local municipality, mpumalanga province (chauke, 2023).

Figure 1: a map showing the study area, bushbuckridge local municipality, mpumalanga province (chauke, 2023).

2.3 Pplant extraction

Plant materials (mature and young) was were washed with distilled water and allowed to dry at room temperature. The leaves were placed in an oven and heated at 40°c for 20 minutes. The leaves were sliced into tiny bits and ground

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using a pestle and mortar. Each ground plant material (75 g) were ~~was~~ extracted separately with 300 ml of acetone and methanol. The conical flasks were shaken vigorously for 30 minutes on a shaking machine) at a speed of 150 rpm. The plant extracts were filtered separately using whatman no.1 filter paper. The supernatants were decanted into labelled beakers. The process was repeated three times and the extracts were combined. The solvents were removed under a stream of cold air at room temperature. A freeze dryer was used to remove water from aqueous extracts. Before phytochemical analysis and bioassay testing, crude extracts were re-dissolved in acetone. Acetone ~~i~~was proven to be not toxic to the tested microorganisms (eloff, 1998).

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2.3.1 ~~t~~ Traditional method

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2.3.1.1 ~~J~~juice extraction

The leaves (5.38 g) were washed with water and allowed to dry at room temperature. The leaves were placed in an oven and heated at 40°c for 20 minutes (figure 2). The leaves were allowed to cool up for 30 minutes and were squeezed to remove 20 ml of the juice. The juice was transferred into schott bottles.

2.4 ~~P~~phytochemical analysis

Thin layer of chromatography (tlc) plates were developed and used to determine the chemical components of the plant extracts. The plates were developed using three different eluent solvents: ethyl acetate: methanol: water [emw] (polar), chloroform ethyl acetate formic acid [cef] (intermediate polarity/acidic), and benzene: ethanol: ammonia hydroxide [bea] (non-polar/basic) (kotze and eloff, 2002). Ten microliters (10 µl) of each plant extract were loaded on the tlc plates and developed using three eluent solvent systems. Separated chemical compounds were visualized under ultraviolet light at 254 and 360 nm using a camac universal uv lamp tl-600. For the chemical compounds not visible under uv light, vanillin–sulphuric acid spray reagent (stahl, 1969) was used for detection.

2.5 Ffungal strains and inoculum quantification

Aspergillus fumigatus (atcc 204305), candida albicans (atcc 10231), and cryptococcus neoformans (atcc 32264) were grown on sabouraud dextrose (sd) agar for a week at 35°c and subcultured into sd broth under aseptic conditions in a biosafety cabinet using a delta lab sterile swab. Viable cells of each fungal culture were counted using a haemocytometer (aberkane et al., 2002). For micro-dilution and bioautography assay, the final concentration of inoculum was adjusted to 1.0×106 cells/ml, respectively.

2.5.1 Mmicro-dilution assay

The microplate method was used to assess the minimum inhibitory concentration (mic) of each plant extract (eloff, 1998). The plant extracts were tested in triplicate in each assay. The assays were repeated in their entirety to confirm the results. The plant extracts (100 µl) were loaded into 96 microtiter plates and serially diluted two-fold with water (eloff, 1998). Hundred microliters (100 µl) of fungal culture were added to the microplate and incubated for 24 and 48 hrs (figure 2). Acetone was used as a negative control and amphotericin b as a positive control. As a growth indicator, 40 µl of 0.2 mg/ml p-iodonitrotetrazolium violet (int) dissolved in water was added to the microplate wells. Covered microtiter plates were incubated for 24 and 48 hours at 35°c and 100% relative humidity. The lowest extract concentration at which fungal growth was suppressed was regarded as the mic.

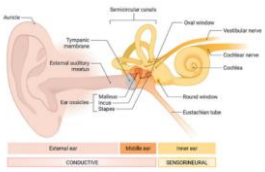
2.5.2 Bbioautography assay

Each plant extract (10 µl) was loaded onto the tlc plates and eluted with bea, cef, and emw. The plates were kept in a fume hood overnight to allow the solvents to evaporate. Fungal cultures were grown on sabouraud dextrose (sd) agar for 3-5 days. Cultures were transferred into sd broth from agar with sterile swabs. The developed tlc plates were sprayed with a concentrated suspension containing c. 1.0 x 106 cells/ml of actively growing fungi of a. Fumigatus, c. Albicans, and c.

Neoformans until fully drenched. To detect antifungal compounds, the plates were incubated for a night or longer at 35°c in a dark chamber with 100% relative humidity. The plates were sprayed with 2 mg/ml int solution and incubated for up to 24 hours. White areas indicate where reduction of int to the coloured formazan did not take place due to the presence of antifungal compounds that inhibited the growth of the fungi.

2.5 data analysis

The documented data were analysed using descriptive and inferential statistics with microsoft excel 2020. Percentages and frequencies were portrayed in tables and figures to interpret the findings of the study.



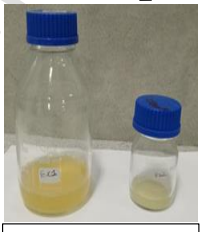
Structure of ear (Payne and Wong, 2022).

Kalanchoe luciae Raym-Hammet used to combat ear infection

Mature leaves

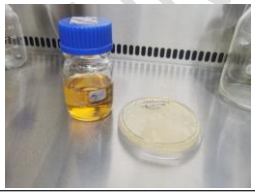
Young leaves

Plant extraction

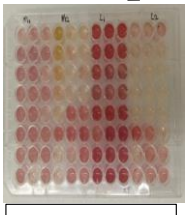


Leaf juice

Antifungal activity



Fungal culture: *Candida albicans*



Microtitre plate

figure 2: schematic representation of the leaf extraction processes of *kalanchoe luciae*. Labe the figures and give an

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illustration of each

3. Results and discussion

Percentage of mass extracted from the plant material
Acetone extracted a large quantity (7.62 g) of plant material as compared to the solvent (figure 3). The leaves of *Kkalanchoe luciae* raym-hammet are significant in size with a single mature leaf weighing 75g while a young leaf weighs 5.38g. The major contributory factor to their weight is the high level of water content they possess which can be easily squeezed out once the leaf is warmed. Young leaves produced a greater yield of extract as compared to the mature leaves with the greatest yield being 7.62% extracted using acetone. The findings suggest that the differences in phytochemical composition between mature and young leaves depend on the type of plant species being studied. Acetone extracted a large quantity (7.62 g) of plant material as compared to the solvent. It is of utmost essentiality to choose a suitable solvent because it affects the selectivity, chemical configuration, and functional characteristics of the final product (jacotet-navarro et al., 2018). The yield of plant extract extraction depends on the solvent's polarity (sineiro et al., 2008). Previously, it was reported that plant extraction using different solvents is more efficient with polar organic solvents such as acetone, methanol, and ethanol (kumoro et al., 2009).

Extractions using organic solvents are more consistent in antimicrobial activity as compared to extractions using water (gurjar et al., 2012). Young leaves produced a greater yield of extract as compared to the mature leaves with the greatest yield being 7.62% extracted using acetone. Other researchers found that young leaves contained the highest phenolic content than mature leaves (chena et al., 2018). In another study, it was found that polyalthia longifolia sonn. Mature leaves have significant amounts of chemical compounds as compared to young leaves (ojewuyi et al., 2014).

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The type of plant material, solvent, and extraction method are the determinants of the quality of a plant extract (gurjar et al., 2012). The efficiency of extraction methods differs due to factors such as the condition of plant material (wet or dry, size); time spent grinding; ph, temperature, type, and the amount of solvent (gurjar et al., 2012). Fresh and ground samples are less preferred as compared to dried and powdered samples because dry and powdered samples produce higher yields of extracts due to increased surface area (azwanida, 2015).

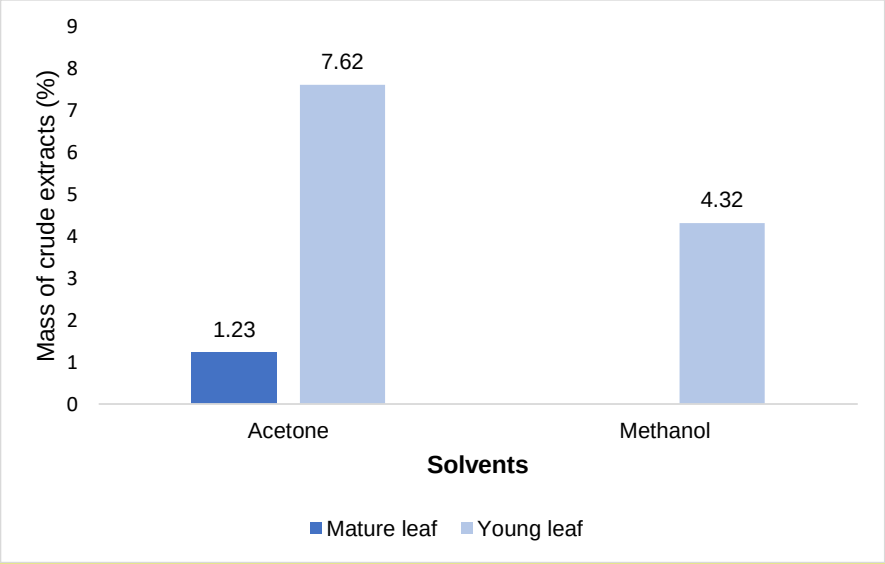

Fig. 3. Percentage mass of material extracted with acetone and methanol from young (5.38 g) and mature leaves (75 g) of *kalanchoe luciae* species.

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Phytochemical analysis

The eluent systems used in this study have different polarities viz. Ethyl acetate: methanol: water [emw] (polar/ neutral); chloroform: ethyl acetate: formic acid [cef] (intermediate polarity/acidic); and benzene: ethanol: ammonia hydroxide [bea] (non-polar/basic) (kotze and eloff, 2002). Phytochemical screening of the different extracts of the plant has shown the presence of different compounds. The chemical components were visible in all plant extracts separated with emw and bea. In tlc chromatograms separated with bea and cef, only two were visible under ultraviolet light.

In tlc chromatograms developed in bea, a few chemical components were observed in the juice extract from young leaves. Although traditional health practitioners use leaf juice or water extracts to treat ear infections. However, there were no chemical components present in these extracts. The possible reason may be that the compounds were not dissolved because the leaf juice was not mixed with other chemicals as compared to the other extracts, whereby the solvents acetone and methanol were used. In this study, the leaves were first heated in an oven set at 40°c for 20 minutes before extraction of the juice.

In the phytochemical analysis, similar chemical components with an rf value of 0.69 were observed in young leaf acetone and methanol extracts of k. Luciae. In tlc chromatograms developed in cef, one compound was visible in young leaf acetone extract with an rf value of 0.72. The rf values were calculated by dividing the distance travelled by the compound by the distance travelled by the solvent. The compounds were at the same level in the chromatograph which when calculated had the same retention factor value (rf). Despite its widespread use by folk medicinal practitioners to treat a variety of diseases, k. Luciae is reported in literature mainly for its different medicinal uses but no phytochemical analysis has been conducted specifically for this plant species. However, other plant species belonging to the same genera have been investigated for their phytochemical constituents

and antibacterial activity. The plant k. Luciae may have some or all of the chemical constituents found in the genus kalanchoe, but these would require further study to identify the chemical constituents of *k. Luciae*.

Tlc was used to investigate the chemical components of different plant extracts. The tlc plates were separated using bea, cef, and emw solvent systems. Phytochemical screening of the different extracts of the plant has shown the presence of various compounds. The compounds can be seen by the different colour changes portrayed by isolated compounds because of their reaction with vanillin (suleimana et al., 2010). For instance, the presence of terpenes is portrayed by a red or blue colour when sprayed with vanillin (suleimana et al., 2010) and pink colour for glycosides. Despite the fact that traditional health practitioners use leaf juice or water extracts, scientific evidence has shown that the use of water for plant material extraction naturally limits the type, amount, and number of compounds extracted (eloff, 1998). This could be the probable explanation for why there is no isolated compound of leaf juice extract in the chromatograms. The possible reason may be that the compounds were not dissolved because the leaf juice was not mixed with other chemicals as compared to the other extracts whereby the solvents acetone and methanol were used. Another factor that affects the phytochemical analysis of plant extracts is the ratio of the dry extract to the solvent used before spotting (aremu et al., 2010).

A seasonal collection of the plant material plays a vital role and also those plants that are exposed to sunlight contain high flavonoid content compared to those growing in the shade (muzitanoa et al., 2011). In this study, the leaves were first heated in an oven set at 40°c for 20 minutes before extraction. A study conducted by muzitanoa et al (2011) using the leaf juice extract of kalanchoe pinnata supported the heating of the juice because it optimizes active flavonoid recovery.

The r_f values were calculated by dividing the measured distance travelled by the compound and dividing it by the distance travelled by the solvent. The compounds were at the same level in the chromatograph which when calculated had the same retention factor value (rf). The rf value of the unknown compound can be compared with the rf value of a known compound thereby promoting the identification of an unknown compound in a mixture (sasidharan et al., 2011).

Chemical constituents such as flavonoids, sterols, anthocyanins, coumarins, and bufadienolides have been isolated specifically from kalanchoe genus (singab et al., 2011). Other researchers reported that the leaves of k. Daigremontiana contain very high levels of tocotrienols which are compounds of the vitamin e family (kruk et al., 2011).

Determining antifungal activity

Micro-dilution assay

The antifungal activity of plant extracts was tested against *a. Fumigatus*, *c. Albicans*, and *c. Neoformans* (table 1). All plant extracts were active against the tested fungal pathogens. The mature leaf acetone extract inhibited *c. Neoformans* at 0.09 mg/ml while the young leaf methanol extract inhibited a. Fumigatus at 0.04 mg/ml. The methanol young leaf extract and leaf juice had the greatest inhibitory effect with mic values of 0.31 and 0.02 mg/ml. The mature leaf acetone leaf extract had excellent activity with mic value of 0.02 after 24 hours of incubation. Traditional health practitioners use water to extract plant material, since water is not toxic and is the preferred extractant available. In general, *c. Neoformans* is the most susceptible as compared to other fungal pathogens. The antimicrobial activity of *k. Luciae* has not been clearly stated in the literature. In the current study, the findings indicate that the young leaves had excellent activity as compared to the mature leaves. Amphotericin b seemed to have no inhibitory activity against all three pathogens. Amphotericin b (amb) had low antifungal activity against the

tested fungal pathogens with an mic value of 2.5 mg/ml, respectively.

Bioautography assay

Bioautography was used to determine the number of active compounds present in different plant extracts. In the current study, tlc chromatograms were developed in cef, bea, and emw. No active compounds were observed in all plant extracts against the tested fungal pathogens. This suggests that some of the active compounds have evaporated during the drying period.

The plant extracts were active against the tested fungal pathogens with mic values ranging between 0.02 and 2.5 mg/ml (table 1). Traditional health practitioners use water to extract plant material, since water is not toxic and is the preferred extractant available. In general, c. Neoformans is the most susceptible to leaf extracts k. Luciae. Furthermore, a significant factor influencing the susceptibility of test organisms is the duration of incubation time. Moreover, some plant extracts' decreased antifungal activity after longer incubation time (tlaamela et al., 2024). The mature acetone extracts reduced the antifungal activity to 2.5 mg/ml after a longer incubation time. Plant extracts with the least mic values can be a great source of bioactive compounds with antifungal activity (mahlo et al., 2010).

The antimicrobial activity of *k. Luciae* has not been clearly stated in the literature. A literature review showed that different extracts of several kalanchoe species have been proven to have antimicrobial, antiulcer, analgesic, antihyperglycemic, cardiovascular, and anti-inflammatory effects (singab et al., 2011). Other plants of the same family (crassulaceae) or genus (kalanchoe) have been investigated for their minimum inhibitory concentration against a range of well-known bacterial and fungal pathogens. Cotyledon orbiculata of the crassulaceae family also commonly used to

treat ear infections has been tested against some pathogens causing ear infections including c. Albicans, e. Coli and s. Aureus whereby the mic value of the petroleum ether leaf extract was 6.25 mg/ml, 3.13 mg/ml and 3.13mg/ml respectively (aremu et al., 2010). The highest mic value of 6.25 mg/ml against c. Albicans observed in the study by aremu et al (2010) coincides with the overall results obtained in this study, that c. Albicans was the least susceptible to the plant extracts. However, the results obtained in this study of *c. Albicans* are more effective than those observed in the study by aremu et al (2010). Other studies elaborate that pathogenic fungi including *c. Albicans* (aremu et al., 2010) are resistant to the majority of plant extracts (buwa and van staden, 2006). In the current study, the findings indicate that the young leaves had excellent activity as compared to the mature leaves. This can be justified by a study conducted by shuib et al (2015), whereby the young leaves of melicope ptelefolia were found to have a high content of phenolics which had higher antimicrobial activity as compared to mature leaves.

In the current study, amphotericin b seemed to have no inhibitory activity against all three pathogens. Amphotericin b (amb) had low antifungal activity against the tested fungal pathogens with an mic value of 2.5 mg/ml. This antibiotic is used to treat systemic fungal infections (benincasa et al., 2011). However, a recent study records that amb inhibits the growth of *c. Albicans* at a concentration of 0.12 µg/ml and miltefosine at 1µg/ml (de bastiani et al., 2020). Amphotericin is one of the drugs highly recommended for treating infections caused by *c. Neoformans*. In a study conducted by rossi et al. (2020), the minimum concentration of amphotericin b that restricts the growth of c. Neoformans is 0.03 µg/ml. Furthermore, it was also reported that amb was active against *a. Fumigatus* with mic value of 3.15 µg/ml (campione et al., 2020).

Bioautography assay

Bioautography was used to determine the number of active compounds present in different plant extracts. In the current study, tlc chromatograms were developed in cef, bea, and emw. No active compounds were observed in all plant extracts against the tested fungal pathogens. This suggests that some of the active compounds have evaporated during the drying period.

In the bioautography assay, no active compounds were observed in tlc chromatograms developed in cef, bea, and emw. This suggests that some of the active compounds have evaporated during the drying period. Another possible reason is that some of the eluents such as ammonia may have inhibited the fungal growth (eloff et al., 2008). The lack of active compounds in some plant extracts could be due to the presence of antifungal compounds in very small quantities, denaturation, evaporation, and photo-oxidation of active compounds (akinsanya et al., 2017).

Table 1. Minimum inhibitory concentration (mic) of acetone, methanol, leaf juice extracts of *kalanchoe luciae,* and the positive control, amphotericin b, against the tested fungal pathogenic fungi. The results show the average of three replicates (standard deviation was 0 in all cases).

mic (mg/ml)								
kalanchoe luciae								
Fungi	**Time (hrs)**	**Extracts**						**Amphotericin b**
		Acetone		**Methanol**		**Leaf juice**		
		Mature leaves	**Young leaves**	**Mature leaves**	**Young leaves**	**Mature leaves**	**Young leaves**	
Aspergillus fumigatus	24	0.08	0.04	0.02	0.04	0.31	0.04	2.5
	48	2.5	0.31	2.50	0.04	0.31	0.16	2.5
Average		1.29	0.18	2.52	0.04	0.31	0.10	2.5
Candida albicans	24	2.5	0.04	0.31	0.02	0.31	0.02	2.5
	48	2.5	2.50	1.25	0.31	0.31	0.31	2.5
Average		2.5	1.27	0.78	0.17	0.31	0.17	2.5
Cryptococcus neoformans	24	0.02	0.02	0.02	0.31	0.16	0.16	2.5
	48	0.16	0.63	2.50	0.63	0.16	0.16	2.5
Average		0.09	0.33	1.26	0.47	0.16	0.16	2.5

Amphotericin b was used as a positive control. Extractants: acetone and methanol

Conclusion

Kalanchoe luciae is used to treat ear infections by the local people and traditional health practitioners in gottenburg village, bushbuckridge local municipality, mpumalanga province. Traditional health practitioners use leaf juice extract directly from the leaves of k. Luciae to treat ear infections. Antifungal activity of the young and mature acetone, methanol, and juice extracts was investigated against the selected fungal pathogens. All plant extracts were active against the tested fungal pathogens. The ability of the leaf juice extract to inhibit fungal growth without the addition of other chemicals or aids makes it a potential source of ear drops.

Young leaves had the lowest minimum inhibitory concentration against a. Fumigatus, c. Albicans, and c. Neoformans. This implies that the young leaf contains phytochemical compounds that have the highest antifungal activity as compared to mature leaves. From the results obtained in the study, it is clear that young leaves are the most effective against fungal pathogens, which shows that they contain compounds that have antimicrobial properties. Kalanchoe luciae extracts have the potential to be used as a source of antifungal compounds that can be used to treat ear infections and other diseases caused by c. Neoformans and a. Fumigatus.

Phytochemical analysis of the leaf extracts shows that the plant contains different compounds with the most visible in acetone and methanol young leaves. However, the compounds in the juice extract could not be separated. This may be because no organic solvent was used. Kalanchoe luciae can serve as a potential source of antifungal compounds that can be used to develop a novel drug that could be used to treat ear infections. This serves as clear scientific proof that the plant in the study is indeed effective, which proves its extensive use to treat a variety of ear infections by traditional health practitioners in the bushbuckridge local municipality. Therefore, the study supports the traditional use of this plant to treat ear infections and its potential use in antimicrobial drug production.

ETHICAL APPROVAL (WHERE EVER APPLICABLE)

THE BUSHBUCKRIDGE LOCAL MUNICIPALITY GRANTED PERMISSION FOR THE COLLECTION OF PLANT MATERIALS FROM THEIR NATURAL HABITAT.

REFERENCES

Aberkane A, Cuenca-Estreila M, Gomez-Lopez A, Petrikkou E, Mellado E, Monzon A, Rodriguez-Tudela JL. Comparative evaluation of two different methods of inoculum preparation for antifungal susceptibility testing of filamentous fungi. Journal of Antimicrobial Chemotherapy. 2002: 50: 719–722.

Akinsanya AM, Ting S., Sanusi, O. Extraction methods and TLC-bioautography for evaluation of antimicrobial activities of endophytic bacteria from medicinal plants. LASU Journal of Medical Sciences. 2017: 2(1): 1-7.

Aldhaher ZA, Hassan HF, Al-Jassim ZG, Mahmood MA. Bacterial isolates and antibiotic susceptibility of ear infections in Iraqi patients. International Journal of Biosciences. 2018:13(1): 292–297.

Aneja KR, Sharma C, Radhika J. Fungal infection of the ear: A common problem in the north-eastern part of Haryana. International Journal of Pediatric Otorhinolaryngology. 2010: 74: 604–607.

Ayub M, Islam A, Moiz A, Fahad M. Acute Otitis Media: Identification of causative pathogens with antimicrobial comparative efficacy. Journal of Applied Pharmacy. 2015: 7(2).

Aremu AO, Ndhlala AR, Fawole OA, Light ME, Finnie JF, Van Staden J. In vitro pharmacological evaluation and phenolic content of ten South African medicinal plants used as anthelmintics. South African Journal of Botany. 2010: 76,:558–566.

Azhar MF, Aziz A, Haider MS, Nawaz MF, Zulfiqar MA. Exploring the ethnobotany of Haloxylon recurvum (KHAR) and Haloxylon salicornicum (LANA) in Cholistan desert, Pakistan. Pakistan Journal Sciences. 2015: 52(4): 1085-1090.

Azwanida NN. A Review on the Extraction Methods Use in Medicinal Plants, Principle, Strength and Limitation. Medicinal and Aromatic Plants 2015: 4: 196.

Benincasa M, Pacor S, Wu W, Prato M, Bianco A, Gennaro R. Antifungal Activity of Amphotericin B Conjugated to Carbon Nanotubes. American Chemical Society. 2011: 5(1): 199–208.

Buwa LV, van Staden J. Antibacterial and antifungal activity of traditional medicinal plants used against venereal diseases in South Africa. Journal of Ethnopharmacology. 2006: 103(1): 139-42.

Campione E, Gaziano R, Doldo E, Marino D, Falcon M, Lacovelli F, Tagliaferri D, Pacello L, Bianchi L, Lanna C, Aurisicchio, L., Centofanti, F., Francesco, P.D, Principe, ID Bufalo, FD, Locatelli, F, Pistoia., Marra, E., Orlandi, A. (2020). Antifungal effect of all-trans retinoic acid against Aspergillus fumigatus in vitro and in pulmonary aspergillosis in vivo model. Antimicrobial agents and chemotherapy, 65(3).

Chauke S.Antimicrobial properties and phytochemical analysis of medicinal plants used for the treatment of ear infections. 2023. MSc Dissertation, University of Limpopo.

Chena X. Maa, Z., & Kitts, D.D. (2018). Effects of processing method and age of leaves on phytochemical profiles and bioactivity of coffee leaves. Food Chemistry, 249, 143-153.

Crouch, N.R., Smith, G.F., Walters, M., Figueiredo, E. (2016). Kalanchoe winteri Gideon F.SM., N.R.Crouch & Mich.Walters (Crassulaceae), a new species from the Wolkberg Centre of Endemism. South Africa. Bradleya, 34, 217-224.

Debta, P., Swain, S.K., Lenka, S. & Sahu, M.C. (2020). Otomycosis: A comprehensive review. Indian Journal of Forensic Medicine and Toxicology, 14(4), 8429.

De Bastiani, F.W.M.S., Spadari, C.C., De Matos, JKR., Salata, G.C., Lopes, L.B., & Ishida, K. (2020). Nanocarriers Provide Sustained Antifungal Activity for Amphotericin B and Miltefosine in the Topical Treatment of Murine Vaginal Candidiasis. Front. Microbiology, 10, 2976.

Eloff JN. Which extractant should be used for the screening and isolation of antimicrobial components from plants? Journal of Ethnopharmacology. 1998: 60(1): 1–8.

Eloff JN, Katerere DR, McGaw LJ. The biological activity and chemistry of the southern African Combretaceae. Journal of Ethnopharmacology. 2008:.119(3): 686-699.

Feakins SJ, Sessions AL.Crassulacean acid metabolism influences D/H ratio of leaf wax in succulent plants. Organic Geochemistry 2010. 41: 1269–1276.

Gurjar M., Ali S, Akhtar M, Singh KS. Efficacy of plant extracts in plant disease management. Agricultural Sciences. 2012: 3: 425-433.

Hailu D, Mekonnen D, Derbie A, Mulu W, Abera B. Pathogenic bacteria profile and antimicrobial susceptibility patterns of ear infection at Bahir Dar Regional Health Research Laboratory Center. Ethiopia. Springer Plus. 2016: 5(1): 1–6.

Hamre HJ, Fischer M, Heger M, Riley D, Haidvogl M, Baars, E, Bristol E, Evans M, Schwar, R, Kiene, H. Anthroposophic vs. conventional therapy of acute respiratory and ear infections: a prospective outcomes study. Wien Klin Wochenschr 2005. 8: 256–268.

Hegde, G., Subapriya, S. and Vairamuthu, S. (2021). Update on microbial profile and drug sensitivity of canine otitis. The Pharma Innovation Journal, 10(3), 549–551.

Jacotet-Navarro M, Laguerre M, Fabiano-Tixier A, Tenon M, Feuillere N, Bily A, Chema F. What is the best ethanol-water ratio for the extraction of antioxidants from rosemary? Impact of the solvent on yield, composition, and activity of the extracts. Electrophoresis. 2018: 0: 1–11.

Javidnia J, Ghotbi Z, Ghojoghi A, Solhjoo K, Alshahni MM, Jeddi SA, Ahmadi B, Nouripour-Sisakht S, Ansari S, Shokoohi, G. Otomycosis in the South of Iran with a high prevalence of tympanic membrane perforation: A hospital-based study. Mycopathologia: 2022: 187(2): 225–233.

Kocoglu E, Kalcioglu MT, Uzun L, Zengin F, Celik S, Serifler, S., Gulbay H, Gonullu, N. In Vitro Investigation of the Antibacterial Activity of Nigella sativa Oil on Some of the Most Commonly Isolated Bacteria in Otitis Media and Externa. The Eurasian Journal of Medicine. 51(3),:247-51.

Kotze M, Eloff JN. Extraction of antibacterial compounds from *Combretum microphyllum* (Combretaceae). South African Journal of Botany: 2002: 68(1): 62–67.

Kumoro AC, Hasana M, Sinsign. (2009). Effects of solvent properties on the Soxhlet extraction of diterpenoid lactones from Andrographis paniculata leaves. (2009)Science Asia, 35, 306-309.

Kruk, J., Pisarski, A., & Szymanska, R. (2011). Novel vitamin E forms in leaves of Kalanchoe daigremontiana and Phaseolus coccineus. Journal of plant physiology 168, 2021-2027.

Machaba TC, Mahlo SM, & Eloff J.N. (2024). Antifungal and antioxidant properties of medicinal plants used against fungal infections. Journal of Medicinal Plants for Economic Development ISSN: (Online), 2616-4809, (Print) 2519-559X.

Mahmoudian-Sani, M. R., Hashemzadeh-Chaleshtori, M., Asadi-Samani, M., & Luther, T. (2017). A Review of Medicinal Plants for the Treatment of Earache and Tinnitus in Iran. International Tinnitus Journal, 21(1), 44-49.

Milad, R., El-Ahmady, S., & Singab, A.N. Genus Kalanchoe (Crassulaceae): A Review of Its Ethnomedicinal, Botanical, Chemical and Pharmacological Properties. European Journal of Medicinal Plants, 4(1), 86-104.

Muzitanoa MF, Bergonzi, MC, De Meloa GO Lage CLS Biliac AR, Vincieri, F.F, Rossi-Bergmannd, B., & Costa, S.S. Influence of cultivation conditions, season of collection and extraction method on the content of antileishmanial flavonoids from Kalanchoe pinnata. Journal of Ethnopharmacology, (2011). 133, 132-137.

Ojewuyi, O.B., Ajiboye, T.O., Adebanjo, E.O., Balogun, A., & Mohammed, A.O. (2014). Proximate composition, phytochemical and mineral contents of young and mature Polyalthia longifolia Sonn. leaves. Fountain Journal of Natural and Applied Sciences, 3(1), 10 – 19.

Park MK., Jung, M.H., Kang, H.J., Woo, J., Lee, H., Jung, H.H., Hwang, S.J., & Chae, S.W. (2008). The changes of MRSA infections in chronic suppurative otitis media. Otolaryngology-Head and Neck Surgery, 139, 395-398.

Prasad SC., Kotigadde, S., Shekhar, M, Thada, N.D, Prabhu P., Souza, T.D. & Prasad, K.C. (2014). Primary otomycosis in the Indian subcontinent: Predisposing factors, microbiology, and classification. International Journal of Microbiology, 2014, 636493.

Ryan F, Nasamran C., Pak K, Draf C, Fisch K.M., Webster, N., & Kurabi, A.Single-cell transcriptomes reveal a complex cellular landscape in the middle ear and differential capacities for acute response to infection. Frontiers in Genetics, 11, 358.

Sasidharan, S., Chen, Y., Saravanan, D., Sundram, KM, & Lath, LY. (2011). Extraction, isolation and characterisation of bioactive compounds from plants' extracts. African Journal of Traditional, Complementary and Alternative Medicine, 8(1), 1-10.

Sazhina NN, Lapshinb PV, Zagoskinab NV, Korotkovac EI, Misin VM. A Comparative Analysis of the Antioxidant Activity of Kalanchoe Juices. Russian Journal of Bioorganic Chemistry: 2014:. 40 (7), 771–776.

Singab, A.NB, Sherweit, El-Ahmady, H, Labib RM, ekry, SS. Phenolics from Kalanchoe marmorata Baker, Family Crassulaceae. Bulletin of faculty of pharmacy, 4(2011):.9, 1-5.

Silalahi M, Nisyawati, Walujo EB. Supriatna, Mangunwardoyo, W. (2015). The local knowledge of medicinal plants trader and diversity of medicinal plants in the Kabanjahe traditional market, North Sumatra, Indonesia. Journal of Ethnopharmacology.175, 432–443.

Shuib NA, Iqbal A, Sulaiman FA., Razak, I., & Susanti, D. (2011). Antioxidant and antibacterial activities of Ruta andustifolia extract. Jurnal Teknologi, 77 (2), 101–105.

Tlaamela DM, Mahlo, SM, McGaw LJ, 2023. Antifungal activity and toxicity of bioactive compounds isolated from the leaf of *Ximenia caffra* Sond. Marchurch. natalnsis. Journal of Medicinal Plants for Economic Development (Print) 2519-559X ISSN: (Online). 2616-4809.