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PHYTOCHEMICAL INVESTIGATION, AND ANTIMICROIBIAL ACTIVITY OF LICHEN SPECIES *Usnea orientalis (Motyka)*

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ABSTRACT:

This study explores the phytochemical composition, thin-layer chromatography (TLC) profiles, and antibacterial activity of *Usnea orientalis* motyka extracts using various solvents: acetone, petroleum ether, chloroform, and methanol. Phytochemical analysis reveals that acetone is the most versatile solvent, extracting a wide range of compounds including alkaloids, saponins, glycosides, flavonoids, triterpenes, and steroids. Methanol also extracts multiple phytochemicals but is less comprehensive than acetone. Petroleum ether predominantly extracts alkaloids and glycosides, while chloroform selectively extracts proteins and tannins. Carbohydrates were not detected in any of the extracts. TLC analysis indicates that acetone extracts show the highest mobility of compounds with an Rf value of 0.64, reflecting a strong affinity for the mobile phase. Methanol extracts have the highest Rf value of 0.78, demonstrating the greatest mobility, whereas petroleum ether and chloroform extracts exhibit lower Rf values of 0.54 and 0.71, respectively. Antibacterial sensitivity tests show that gentamicin and piperacillin effectively inhibit *Staphylococcus aureus* and *Pseudomonas aeruginosa*, respectively. Gentamicin also inhibits *Klebsiella pneumoniae*, though to a lesser extent, while ampicillin is effective against *Escherichia coli*. The DMSO control confirms that the observed effects are attributable to the antibiotics used. Among the *Usnea orientalis* extracts, the acetone extract exhibits notable dose-dependent antibacterial activity against *Pseudomonas aeruginosa*, with inhibition zones increasing from 22 mm at 25 µl to 40 mm at 100 µl. The methanol extract maintains stable antibacterial activity with minimal variation in inhibition zone across concentrations, while the hydro methanol extract shows no antibacterial activity.

KEYWORDS: *Usnea orientalis*, phytochemical analysis, thin-layer chromatography (TLC), antibacterial activity, acetone extract,

INTRODUCTION:

Natural products have long held a pivotal role in microbiological research due to their unique and intricate molecular architectures. Over the course of their evolutionary history, natural products have developed traits closely tied to biological processes in both animal and plant

organisms. These substances are renowned for their complex structures, which often include unusual functional group configurations, strained ring systems, and other distinctive characteristics that make them highly appealing for scientific exploration.[1]

The significance of natural products extends beyond their structural complexity. There are several key reasons for the sustained interest in natural product chemistry. First and foremost, natural products serve as invaluable lead compounds in the development of new medications. Their unique molecular frameworks often provide a starting point for the synthesis of novel drugs. Additionally, studying natural products offers insights into potential biological mechanisms, shedding light on the molecular origins and underlying causes of various disorders.[2]

Moreover, the isolation and characterization of natural products have driven the advancement of cutting-edge analytical and spectroscopic techniques. Methods such as HPTLC, NMR spectroscopy, and MS have been refined and developed in response to the challenges posed by the study of these complex molecules. Furthermore, the total synthesis of natural products presents an ongoing challenge that continues to inspire the discovery of new reagents and chemical reactions.[3]

Herbal medicine, which has been used for centuries as both food and medicine, remains an area of significant interest. Humans have long relied on medicinal plants to treat various ailments, and the availability of indigenous botanical flora has greatly influenced the development and use of herbal products. The appeal of herbal medicine lies in its affordability, accessibility, and ease of administration. Research has demonstrated that many herbal plants contain a diverse array of chemical constituents, some of which have been shown to inhibit bacterial growth and treat a variety of harmful diseases. The utilization of antimicrobial compounds derived from natural vegetation holds the potential to impact pollution control and the management of microbial infections in plants, humans, and animals.[4]

The quest for new therapeutic compounds is an ongoing and urgent endeavor. Despite the vast array of current medications, the need for the discovery and development of new agents remains critical. It is estimated that effective treatments are available for only about one-third of all known human diseases, including cancer, AIDS, autoimmune disorders, and other serious conditions. In developing nations, the enhancement of herbal medicine serves not only to preserve ancient traditions but also as an alternative solution to pressing health concerns. The growing resistance of pathogens to many commonly used antibiotics has intensified the search for novel antimicrobial compounds. These new agents are essential to combat infections and address the challenges posed by antibiotic resistance and the side effects associated with

currently available antimicrobial drugs.[5-7]

MATERIAL AND METHODS:-

Collection and authentication of plant material

Usnea orientalis specimens were collected from the biodiverse Nagzira Wildlife Sanctuary in Maharashtra, India, In February 2023, these sample were preserved and sent to the CSIR-National Botanical Research Institute in Lucknow for authentication. Upon verification, the voucher specimen numbers PDSH/LWG/Authentication/2023-24/03 and PDSH/LWG/Authentication/2023-24/14, dated 29/05/2023, were assigned and duly deposited at the institute for future reference and further scientific study.

Preparation of plant extract

For the extraction of *Usnea orientalis* (Motyka), sixteen grams of air-dried thallus, including both vegetative and fruiting parts, were initially washed thoroughly with tap water followed by distilled water to remove any impurities. After washing, the thalli were carefully pat-dried. The dried thalli, weighing 16.04 grams, were subjected to successive solvent extraction using a Soxhlet apparatus.

The first extraction was carried out using 500 mL of petroleum ether (60-80°C) for 25 to 26 cycles. Following this, the remaining plant material was dried and extracted sequentially with chloroform, acetone, and methanol, each solvent being used until exhaustive extraction was achieved. The remaining plant material was macerated in water for three days, followed by filtration through Whatman No. 1 paper. The crude extract was then obtained by evaporating the solvents under reduced pressure using a rotary evaporator.[8-10] The final yield of the dried crude extract was 15.04 grams. Per cent yield of crude extract was calculated according to the equation below:

Per cent yield (%) = (Dry weight of extract/Dry weight of sample) X 100.

= 15.04/16.04

= 93.76

Preliminary phytochemical analysis of *Usnea orientalis motyka* Lichen extract: -

The extract of *Usnea orientalis* (Motyka) underwent a comprehensive phytochemical investigation to identify the presence of various bioactive compounds. This screening aimed to detect key phytochemicals, including alkaloids, tannins, saponins, flavonoids, triterpenoids, steroids, glycosides, proteins, and carbohydrates. Each of these classes of compounds is known for its potential biological activity and therapeutic value.

The analysis was conducted using standard and widely accepted methodologies. These

methods involve specific chemical tests that react with the compounds present in the extract, producing observable changes, such as color shifts or precipitate formation, which indicate the presence of the targeted phytochemicals. The successful identification of these bioactive compounds highlights the potential pharmacological significance of *Usnea orientalis* and provides a foundation for further research into its medicinal properties.[11-14]

Table 1: Preliminary Phytochemical Investigation of *Usnea orientalis motkya*

Test	Procedure	Observation	Result
Alkaloids (Dragendorff's)	Extract mixed with 1% HCl, steamed, then Dragendorff's reagent added.	Reddish-brown precipitate.	Alkaloids present.
Carbohydrates	Extract boiled with Benedict's reagent.	No reddish brown precipitate.	Carbohydrates absent.
Glycosides	Extract mixed with acetic acid/$FeCl_3$ and H_2SO_4.	Brown ring at interphase.	Cardiac glycosides present.
Tannins	Extract mixed with ferric chloride solution.	No blue color.	Hydrolysable tannins absent.
Triterpenoids (Salkowski)	Extract shaken with chloroform and sulfuric acid added.	Reddish-brown color.	Triterpenoids present.
Steroids (Liebermann-Burchard)	Extract mixed with acetic anhydride and sulfuric acid added.	Blue-green ring.	Steroids present.
Flavonoids	Extract mixed with NaOH solution.	Yellow to orange color.	Flavonoids present.
Proteins (Xanthoproteic)	Extract boiled with nitric acid.	No orange color.	Proteins absent.
Saponins (Frothing)	Extract shaken with distilled water.	Stable foam formation.	Saponins present.

Thin Layer Chromatography of *Usnea orientalis Motkya* Extract

Begin by preparing the TLC plate, ensuring it is clean and activated by briefly heating it in an oven. Dissolve a small amount of *Usnea orientalis* extract and usnic acid standard in methanol to create concentrated solutions. Using a capillary tube or micropipette, spot both the extract

and standard onto the baseline of the TLC plate.[15-18]

Mix toluene, ethyl acetate, and formic acid in a 6:3:1 ratio to prepare the mobile phase, and pour it into the TLC chamber. Place the TLC plate in the chamber, ensuring the baseline stays above the solvent. Let the solvent rise by capillary action, then remove the plate when the solvent front is near the top. After drying, visualize the spots under UV light or with a staining reagent. Calculate the Rf values by comparing the spot distances to the solvent front. Match the Rf value and spot appearance with the usnic acid standard to confirm its presence in *Usnea orientalis* motyka.

Antibacterial Activity of Usnea *orientalis Motkya* Extracts

The antibacterial activity of *Usnea orientalis* (Motyka) was evaluated using the Kirby-Bauer well diffusion method, a widely recognized technique for assessing the efficacy of antimicrobial agents. The test organisms included four bacterial strains: *Staphylococcus aureus* (Gram-positive), *Escherichia coli* (Gram-negative), *Pseudomonas aeruginosa* (Gram-negative), and *Klebsiella pneumoniae* (Gram-negative). These bacteria were freshly cultured for 24 hours to reach a turbidity equivalent to 0.5 McFarland standards, ensuring consistent and reliable results. The cultures were then inoculated onto sterile, solidified Mueller Hinton Agar (MHA) plates using the lawn culture technique, which involves uniformly spreading the bacterial suspension across the agar surface with sterile cotton swabs.[19-25]

Three extracts of *Usnea orientalis*—methanol, acetone, and hydro-methanol—were tested for antibacterial activity. Stock solutions of each extract were prepared at 1 mg/mL in acetone, methanol, and chloroform. MHA plates were prepared by boring three wells with an 8 mm sterile cork borer, and each well was filled with 25 µL, 50 µL, and 100 µL of the extract solutions. The plates were left at room temperature for 2-3 hours to allow diffusion, then incubated at 37°C for 24 hours. The antibacterial activity was determined by measuring the inhibition zones around each well.[26-30]

Control plates were also prepared to validate the results. DMSO (100 µL) was used as a negative control to ensure that the solvent did not influence bacterial growth. Additionally, standard antibiotic discs were employed as positive controls to compare the efficacy of the extracts against known antibiotics.[31] For *E. coli*, an Ampicillin disc (10 mcg) was used; for *Pseudomonas aeruginosa*, Piperacillin (100 mcg) was applied; for *S. aureus*, Penicillin G (10 units) was utilized; and for *Klebsiella pneumoniae*, Gentamicin (10 mcg) was the antibiotic of choice.[32-35]

RESULT AND DISCUSSION

Preliminary phytochemical analysis

The table 2 provides an overview of the phytochemical composition of *Usnea orientalis motyka.* extracts obtained using various solvents, including acetone, petroleum ether, chloroform, and methanol. It details the presence or absence of specific phytochemicals in each extract.

Tannins are absent in both acetone and petroleum ether extracts but are present in chloroform and methanol extracts, indicating their higher solubility in polar solvents. Alkaloids are found in the acetone, petroleum ether, and methanol extracts but are absent in the chloroform extract, suggesting that these compounds have varying solubility across polar and non-polar solvents. Saponins appear exclusively in the acetone extract, indicating their preference for acetone as a solvent. Glycosides are present in all extracts, reflecting their solubility in a wide range of solvents and their diverse chemical nature. Flavonoids are found only in the acetone extract, showing that acetone is particularly effective in extracting these compounds. Proteins are detected solely in the chloroform extract, suggesting that chloroform is selective for proteinaceous compounds. Triterpenes, similar to flavonoids, are present only in the acetone extract, reinforcing acetone's suitability for extracting these compounds. Carbohydrates are absent in all extracts, indicating that the solvents used may not be appropriate for their extraction. Steroids are found in both acetone and methanol extracts, but not in the petroleum ether or chloroform extracts, suggesting their solubility in moderately polar solvents.

Overall, this phytochemical analysis underscores the differential solubility of bioactive compounds in *Usnea orientalis* based on the solvent used. Acetone proves to be the most versatile solvent, extracting a wide array of phytochemicals such as alkaloids, saponins, glycosides, flavonoids, triterpenes, and steroids. In contrast, petroleum ether is less effective, primarily extracting alkaloids and glycosides. Chloroform and methanol extracts are rich in tannins and glycosides, with chloroform also selectively extracting proteins. These insights are vital for selecting the appropriate solvent for extracting specific bioactive compounds from *Usnea orientalis* for further research or therapeutic applications.

Table 2: Phytochemical studies of *Usnea orientalis* extracts.

Phytochemicals	Acetone Extract	Petroleum Ether	Chloroform	Methanol
Tannins	-	-	+	+
Alkaloids	+	+	-	+
Saponins	+	-	-	-
Glycosides	+	+	+	+

Flavonoids	+	-	-	-
Proteins	-	-	+	-
triterpenes	+	-	-	-
Carbohydrates	-	-	-	-
Steroids	+	-	-	+

+ Indicates Presence, - Indicates Absence

TLC studies of *Usnea orientalis* extracts

The retention factor (Rf) values for the different extracts are determined by dividing the distance traveled by the compound by the distance traveled by the solvent front, which is 5.7. The results reveal that the acetone extract has an Rf value of 0.64, indicating that its compounds exhibit relatively high mobility in the TLC system. This suggests that acetone is an effective solvent for extracting *Usnea orientalis* components, as these compounds have a strong affinity for the mobile phase. In contrast, the petroleum ether extract has an Rf value of 0.54, which is lower, signifying reduced mobility of its compounds compared to those in acetone. The chloroform extract shows an Rf value of 0.71, indicating better mobility than petroleum ether but still lower than acetone. The methanol extract, with the highest Rf value of 0.78, demonstrates the greatest mobility of its compounds among the solvents tested.

Table 3: TLC results for *Usnea orientalis* extracts

Extracts	Distance Traveled by Compound (cm)	Distance Traveled by Solvent Front (cm)	Rf Value
Acetone Extract	3.7	5.7	0.64
Petro. Ether Extract	3.1	5.7	0.54
Chloroform Extract	4.1	5.7	0.71
Methanol Extract	4.5	5.7	0.78

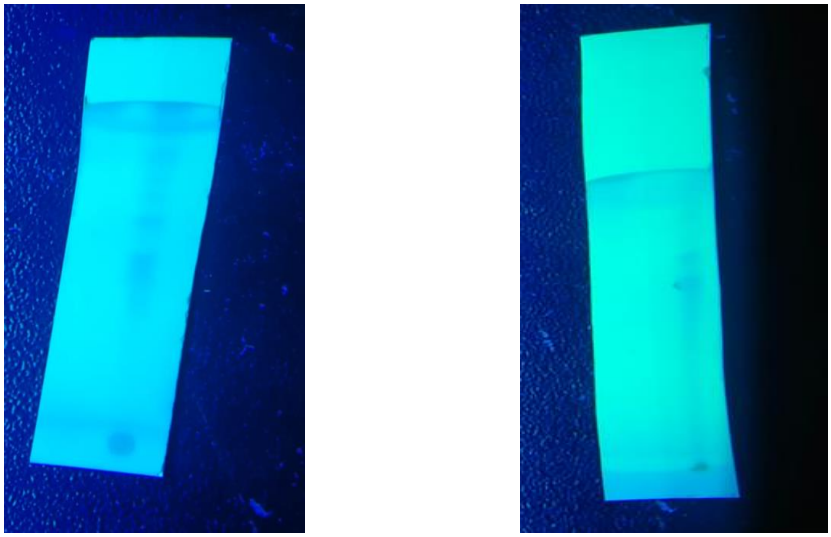


Figure 1: TLC plate of *Usnea orientalis* Motyka (acetone extract)

Antibiotics sensitivity of bacterial isolates

The table 4 illustrates the antibacterial activity of various antibiotics against different bacterial isolates, measured by the zone of inhibition in millilitre Gentamicin, tested against *Staphylococcus aureus* (SA), produced a ZOI measuring 20 mm, indicating effective antibacterial activity. In contrast, the DMSO control did not show any inhibition, confirming that the effect observed is due to the antibiotic. Similarly, piperacillin demonstrated strong activity against *Pseudomonas aeruginosa* (PA) with a zone of inhibition of 25 mm, again validating the effectiveness of the antibiotic as the DMSO control showed no inhibition.

Gentamicin also inhibited *Klebsiella pneumoniae* (KP) but with a smaller zone of 14 mm, suggesting a reduced effectiveness compared to its action on *Staphylococcus aureus*. Finally, ampicillin was effective against *Escherichia coli* (EC), producing a zone of inhibition of 17 mm. The DMSO control for all tests did not show any inhibition, ensuring that the observed effects are attributable solely to the antibiotics used.

Overall, the results highlight the varying efficacy of the antibiotics tested, with gentamicin and piperacillin showing strong inhibitory effects on their respective target bacteria, while ampicillin also proved effective against *Escherichia coli*. The differences in inhibition zones suggest that each antibiotic has specific activity profiles against different bacterial strains.

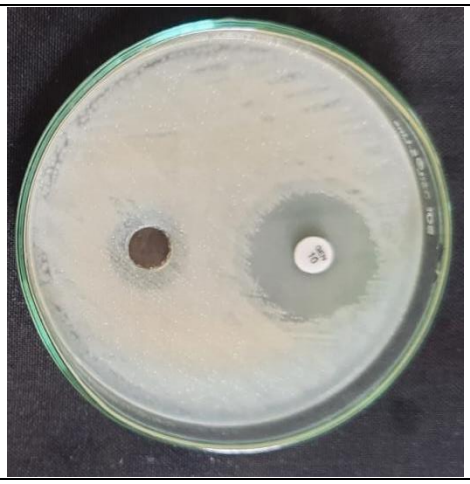
Table 4: Antibiotics sensitivity of bacterial isolates

Zone Of Inhibition in mm (Control)		
Organism	**AB**	**DMSO**
SA	20	NI
PA	25	NI

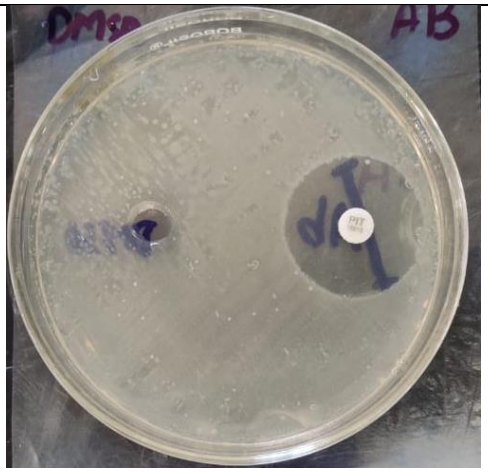
KP	14	NI
EC	17	NI

Note : SA-*Staphylococcus aureus,* PA-*Pseudomonas aeruginosa* ,KP-*Klebsiella pneumoniae* ,EC-*Escherichia coli, NI-No Inhibition*

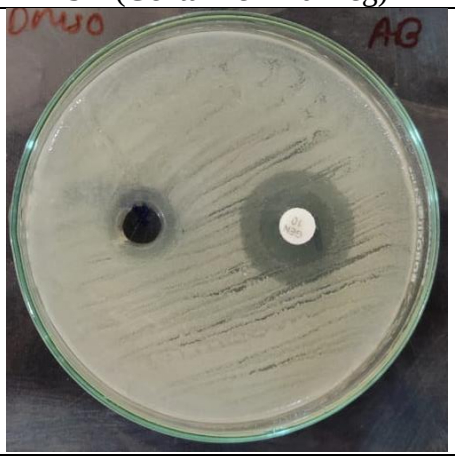
AB – Antibiotics (EC- Ampicillin 10 mcg , PA- Piperacillin – 100 mcg , SA – Gentamicin 10 mcg , KP – Gentamicin 10 mcg)



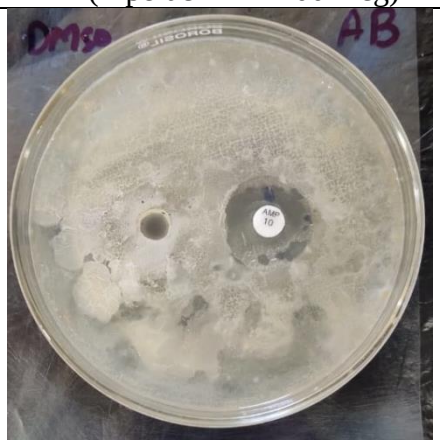
SA (Gentamicin 10 mcg)



PA (Piperacillin – 100 mcg)



KP (Gentamicin 10 mcg)



EC (Ampicillin 10 mcg)

Figure 2: Antibiotics sensitivity of bacterial isolates

Antibacterial Activity of Usnea *orientalis Motyka* Extract

The table 5 provides data on the antibacterial activity of various *Usnea orientalis* extracts against *Pseudomonas aeruginosa*, with results shown as the zone of inhibition in millilitre for different extract concentrations (25 µl, 50 µl, and 100 µl).

For the Usnea orientalis methanol extract, the antibacterial activity is consistent across the concentrations tested. The zone of inhibition remains at 22 mm for both 25 µl and 100 µl

concentrations, with a slight decrease to 20 mm at 50 µl. This indicates that while the methanol extract maintains a stable antibacterial effect, its potency does not substantially increase with higher concentrations.

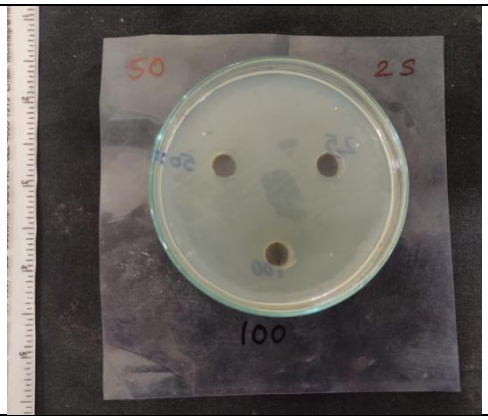
In contrast, the Usnea orientalis acetone extract shows a significant dose-dependent increase in antibacterial activity. At 25 µl, the zone of inhibition is 22 mm, which rises to 34 mm at 50 µl and further increases to 40 mm at 100 µl. This demonstrates that the acetone extract becomes more effective against *Pseudomonas aeruginosa* as the concentration increases.

The hydro methanol extract of *Usnea orientalis* does not exhibit any antibacterial activity at any concentration, as indicated by the absence of inhibition (NI). This suggests that these extracts either lacks effective antibacterial compounds or that the compounds present are ineffective against *Pseudomonas aeruginosa*.

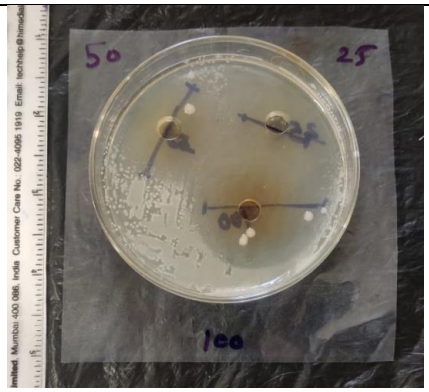
Overall, the results suggest that the acetone extract of *Usnea orientalis* motyka is the most effective in combating *Pseudomonas aeruginosa*, showing substantial improvement with higher concentrations. The methanol extract also demonstrates stable antibacterial activity but without significant enhancement at higher concentrations. The hydro methanol extract does not show any antibacterial properties. These findings highlight the acetone extract as the most promising for potential therapeutic applications against *Pseudomonas aeruginosa*.

Table 5: Antibacterial activity of Plant extracts against *Pseudomonas aeruginosa*

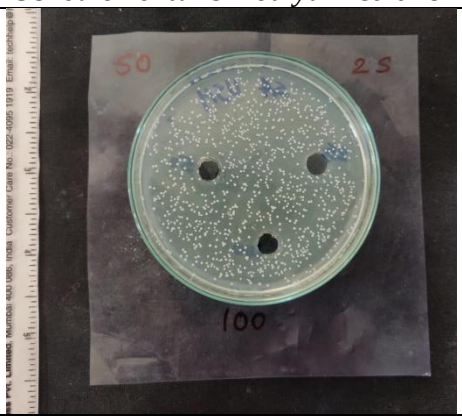
Zone of Inhibition in mm			
Pseudomonas aeruginosa	25µl	50µl	100µl
***Usnea orientalis motkya* methanol**	22	20	22
***Usnea orientalis motkya a*cetone**	22	34	40
***Usnea orientalis motkya* hydro methanol**	NI	NI	NI



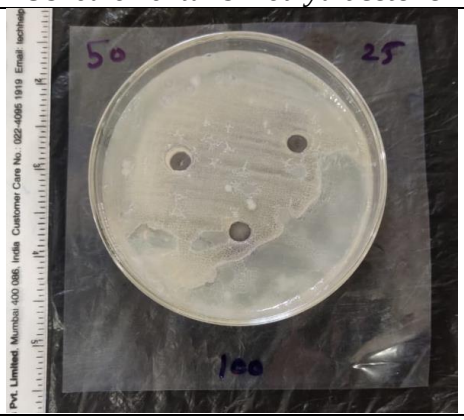
Usnea orientalis motkya methanol



Usnea orientalis motkya acetone



Usnea orientalis motkya hydro Methanol



Usnea orientalis motkya chloroform

Figure 3: Antibacterial activity of Plant extracts against *Staphylococcus aureus*

CONCLUSION

The comprehensive analysis of *Usnea orientalis* extracts reveals important insights into their phytochemical composition, TLC profiles, and antibacterial activity. The preliminary phytochemical analysis demonstrated that acetone is the most effective solvent for extracting a broad range of bioactive compounds, including alkaloids, saponins, glycosides, flavonoids, triterpenes, and steroids. In contrast, petroleum ether primarily extracts alkaloids and glycosides, while chloroform and methanol are effective for tannins and glycosides, with chloroform also selectively extracting proteins. The absence of carbohydrates in all extracts suggests that the solvents used may not be suitable for their extraction.

The TLC studies further support the efficacy of acetone, which showed a high Rf value of 0.64, indicating that its compounds have strong mobility and affinity for the mobile phase. Methanol, with the highest Rf value of 0.78, also demonstrated effective extraction properties, but acetone remains superior in terms of overall phytochemical extraction. Antibacterial sensitivity tests of various antibiotics confirmed the effectiveness of gentamicin and piperacillin against *Staphylococcus aureus* and *Pseudomonas aeruginosa*, respectively, with ampicillin also

proving effective against *Escherichia coli*. These results highlight the varying efficacy of antibiotics and emphasize the need for targeted antibiotic selection based on specific bacterial profiles. In terms of antibacterial activity of *Usnea orientalis* extracts, the acetone extract emerged as the most effective against *Pseudomonas aeruginosa*, showing significant dose-dependent enhancement in activity. The methanol extracts maintained consistent antibacterial effects, while the hydro methanol extract showed no activity. This underscores the potential of acetone extract for therapeutic applications, particularly in combating *Pseudomonas aeruginosa*. Overall, the findings emphasize the importance of solvent selection for optimizing the extraction of bioactive compounds and highlight the acetone extract's promising potential for further development in antibacterial therapies.

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AUTHORS CONTRIBUTIONS:

All authors contributed equally to this work.

CONFLICTS OF INTERESTS:

None.

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