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Unlocking the Therapeutic Potential of Oxystelma esculentum: A Phytochemical Treasure Trove with Anticancer and Lactogenic Properties

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Abstract: This study investigates the phytochemical profile, cytotoxic effects, and lactogenic activity of *Oxystelma esculentum*, a plant renowned for its traditional medicinal uses. Preliminary phytochemical screening revealed a rich array of secondary metabolites, including alkaloids, flavonoids, glycosides, and tannins, predominantly in polar solvents such as methanol and ethanol. Spectroscopic analysis further identified key functional groups, suggesting significant bioactivity. The cytotoxic potential was evaluated against ovarian (OVCAR3), breast (T47D), and cervical (HeLa) cancer cell lines, demonstrating a concentration-dependent increase in cytotoxicity, with T47D cells exhibiting the highest sensitivity (IC50 = 42.82 µg/ml). Additionally, the brine shrimp lethality assay indicated low toxicity, while in vivo studies highlighted the extract's ability to enhance milk yield and pup growth in lactating rats, correlating with increased prolactin levels. These findings underscore *Oxystelma esculentum* as a promising candidate for natural anticancer therapies and lactation enhancement, warranting further exploration of its bioactive compounds and mechanisms of action.

Keywords: Phytochemical screening; Cytotoxicity; Anticancer activity; Lactogenic activity; Galactagogue; Prolactin; Brine shrimp lethality assay; FTIR spectroscopy; Medicinal plants; Natural products

1. Introduction

Oxystelma esculentum, a climbing plant belonging to the milkweed family (Asclepiadaceae), holds a significant place in traditional medicine and nutrition. Its geographic distribution extends across a wide range, from China to Africa, and its various parts, including the milky sap, leaves, and fruits, have been utilized to address a diverse set of health conditions. This widespread use highlights the ethnomedicinal importance of *O. esculentum* and suggests its potential therapeutic value(Pandya & Anand, 2011a).

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Oxystelma esculentum, known by various names like Dudhialata or Swallow-wort, has a rich history of traditional use across different cultures. In Ayurvedic medicine, it is considered a supplement, a rejuvenating tonic believed to promote longevity and vitality. The plant's milky sap, leaves, and roots have been employed to treat a variety of ailments. For instance, in India, the sap is often used as a digestive aid, while the leaves are applied topically to treat skin conditions like eczema and boils. In traditional Chinese medicine, the plant is valued for its antipyretic (fever-reducing) and anti-inflammatory properties. It has also been used to address respiratory issues like coughs and bronchitis. Additionally, some African communities have utilized *Oxystelma esculentum* as a natural remedy for diabetes, further highlighting its diverse therapeutic applications across different cultural contexts(Chishti et al., 2021).

Scientific investigation has revealed the diverse array of phytochemicals present in this plant, with compounds like pregnane glycosides drawing particular attention. Initial research suggests these glycosides may have potential benefits in managing obesity and suppressing appetite(Abdel-Sattar & Ali, 2022; El-Shiekh et al., 2019; Hamed et al., 2004). Additionally, studies have pointed to the plant's antidiabetic properties, along with its cooling and metabolic regulatory effects, as documented in works such as Xavier et al. (2022) and Kumar et al. (2009)(Kumar et al., 2009; Xavier et al., 2022). Extracts derived from O. esculentum have demonstrated the capacity to modulate glucose levels and enhance insulin sensitivity, further supporting its potential in addressing metabolic concerns(SOUJANYA, 2017). Beyond its metabolic benefits, O. esculentum has exhibited antimicrobial and anti-inflammatory activities in laboratory assays. The plant's potential as a source of natural antioxidants has also garnered attention due to its ability to combat oxidative stress(D Ashok et al., 2010). *Oxystelma esculentum* is rich in diverse phytochemicals with potential pharmacological properties. Key compounds include pregnane glycosides (anti-obesity and appetite-suppressant effects), flavonoids (antioxidant and anti-inflammatory), triterpenoids (anti-inflammatory, antimicrobial, and antidiabetic), and alkaloids (antimicrobial and cytotoxic). The plant also contains other bioactive compounds like cardiac glycosides, saponins, and tannins. While these findings are promising, more comprehensive studies are needed to fully understand the mechanisms of action and potential applications of these phytochemicals in human health(Pandya & Anand, 2011a; Trivedi et al., 1988).

Breastfeeding is crucial for both infant nutrition and maternal health, but some women experience challenges due to inadequate milk production(Davidove & Dorsey, 2019). Galactagogues, substances believed to enhance breast milk supply, have been sought after for centuries. While pharmaceutical options exist, concerns about side effects and safety have spurred interest in exploring natural alternatives(Ryan et al., 2024; Zizzo et al., 2021). *Oxystelma esculentum*, a plant with a long history of traditional use in various cultures, has emerged as a potential candidate due to anecdotal reports and its diverse array of bioactive compounds(Chishti et al., 2021; Pandya & Anand, 2011a). This research aims to delve into the scientific evidence supporting the galactagogue properties of *Oxystelma esculentum*, examining its traditional uses, phytochemical composition, and potential mechanisms of action. By critically evaluating existing literature and identifying areas for further investigation, this study seeks to contribute to the understanding of this plant's potential as a safe and effective galactagogue. While specific scientific studies on *Oxystelma esculentum* as a galactagogue are scarce in literature published till date, the plant's traditional uses for various health conditions indirectly suggest its potential in supporting lactation(Pandya, 2012; Pandya & Anand, 2011b; Pandya et al., 2011; Poornima et al., 2009). For instance, its documented use as a tonic in Ayurvedic medicine, promoting overall health and vitality, could indirectly contribute to improved breastfeeding outcomes. Similarly, its anti-inflammatory and antimicrobial properties might help address common issues like mastitis that can interfere with lactation. However, direct evidence of its traditional use as a galactagogue requires further investigation into ethnomedical practices. Furthermore, rigorous scientific research, including clinical trials, is necessary to confirm *Oxystelma esculentum's* galactagogue effects and establish its safety and efficacy for breastfeeding mothers.

2. Results

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2.1. Preliminary Phytochemical Analysis

Preliminary phytochemical screening of *Oxystelma esculentum* (Table 1) revealed a rich profile of secondary metabolites. The plant extracts exhibited a high content of polar compounds, including alkaloids, flavonoids, glycosides, phenolic compounds, reducing sugars, and tannins, particularly in the polar solvents methanol, ethanol, and water. Moderate levels of anthraquinones and saponins were detected in most extracts, while terpenoids were primarily found in non-polar solvents. These findings suggest that *O. esculentum* is a potential source of bioactive compounds with various pharmacological properties.

Table 1. Preliminary Phytochemical analysis of organic extracts of *Oxystelma esculentum.*

Phytochemical Constituent	Water	Methanol	Ethanol	Acetone	Chloroform	Diethyl Ether	n-Hexane	n-Butane
Alkaloids	± to ++	++ to +++	++ to +++	++ to +++	++	-	-	-
Anthraquinones	±	+++	+++	++	+	-	-	-
Flavonoids	+ to ++	+++	+++	++	+	-	-	-
Glycosides	+ to ++	+++	+++	+	-	-	-	-
Phenolic compounds	+ to ++	+++	+++	+	-	-	-	-
Reducing sugars	+++	+++	+++	+	-	-	-	-
Saponins	±	++	++	±	-	-	-	-
Tannins	+ to ++	+++	+++	+	-	-	-	-
Terpenoids	- to ±	+++	+++	++	++	++	+	+

(+++) = high concentration; (++) = Moderate presence; (+) = Low presence; (±) = Trace presence; (-) = Absent.

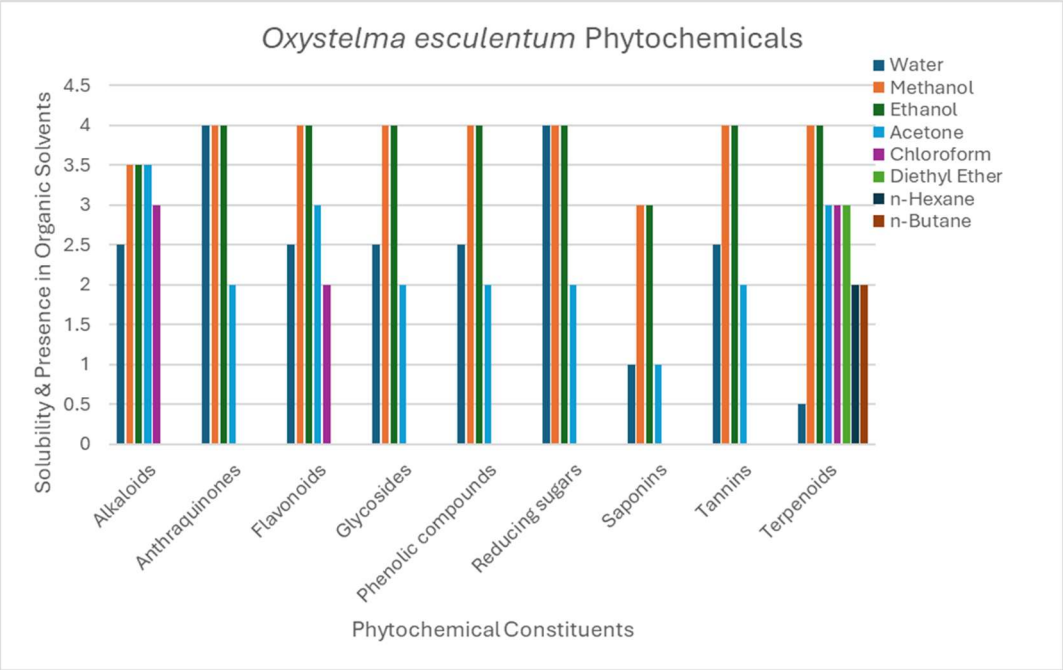

Figure 1. Solubility frequency of phytochemicals in different organic solvents.

2.2. Fourier Transform Infrared (FTIR) Analysis

2.2.1. FTIR Analysis of Hydro-Ethanolic Extract of Oxystelma esculentum

Spectroscopic analysis of ethanolic extract indicated absorption bands between 3853.81 and 3735.63 cm^{-1}, signifying the presence of organic acids characterized by the carboxyl group (-COOH).

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Additionally, absorption ranges from 3649.16 to 3594.33 cm^{-1} were observed, which are indicative of alcoholic and phenolic compounds, as evidenced by the O–H stretch. The distinct peak at 3335.54 cm^{-1} is associated with the amino group (-NH_2). An aliphatic compound presence is denoted by a peak at 2921.71 cm^{-1}, characterized by a C–H bend. Ester linkages are suggested by the peaks ranging from 1700.37 to 1590.69 cm^{-1}, which correspond to multiple bond structures (C=C, C=N, O=C-O-). The series of peaks from 1521.92 to 1021.25 cm^{-1} are representative of various functional groups, including amides, ketones, aldehydes, aromatic compounds (C–C stretch (in-ring)), and aliphatic amines (C–N stretch). Lastly, the presence of halogen compounds is confirmed by peaks between 783.70 and 609.33 cm^{-1}, which are characteristic of bonds such as C-Cl, C-F, C-I, and C-Br.

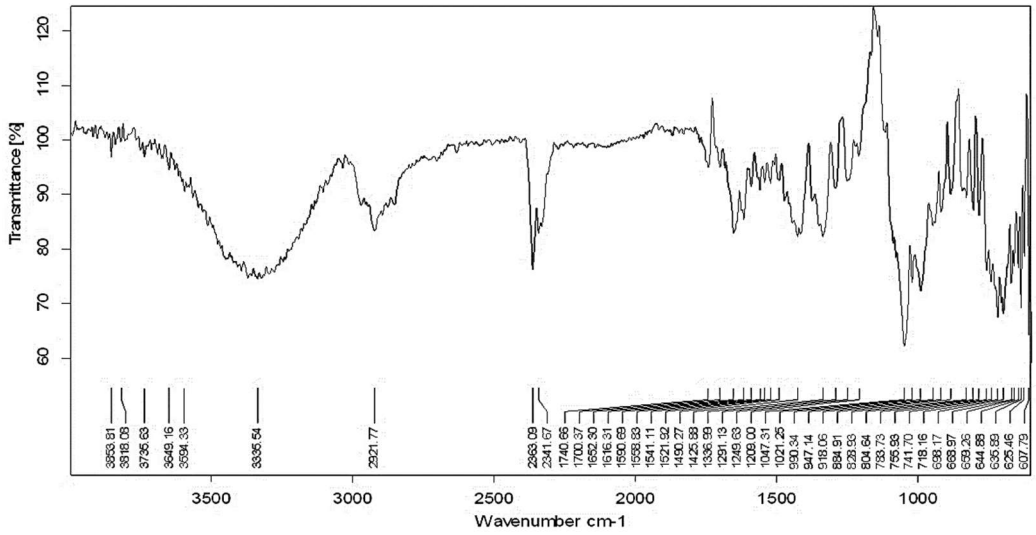

Figure 2. FTIR spectra of Hydro-Ethanolic Extract of Oxystelma esculentum.

2.2.2. FTIR Analysis of Methanolic Extract of Oxystelma esculentum

Spectroscopic analysis of methanolic extract indicated absorption bands at 3872.10-3802.24 cm^{-1}, which are characteristic of organic acids, specifically the carboxyl group (-COOH). Additionally, bands at 3392.39, 3365.42, and 3335.26 cm^{-1} were observed, suggesting the presence of amino functional groups. Aliphatic compounds were inferred from the peaks at 2918.56 and 2853.70 cm^{-1}, indicative of C–H bending vibrations. The spectrum also displayed a series of bands from 1710.20 to 1496.48 cm^{-1}, which align with the vibrational modes of ester linkages, encompassing C=C, C=N, and O=C-O- bonds. Furthermore, the presence of functional groups such as amides, ketones, aldehydes, aromatic compounds (C–C & C-O stretching within the ring structure), and aliphatic amines (C–N stretching) was deduced from the peaks at 1338.47, 1294.04, 1250.09, and 1208.96 cm^{-1}. Lastly, the range from 1020.83 to 609.33 cm^{-1} revealed the existence of aromatic alkyl halides and various carbon-halogen bonds (C-Cl, C-F, C-I, C-Br).

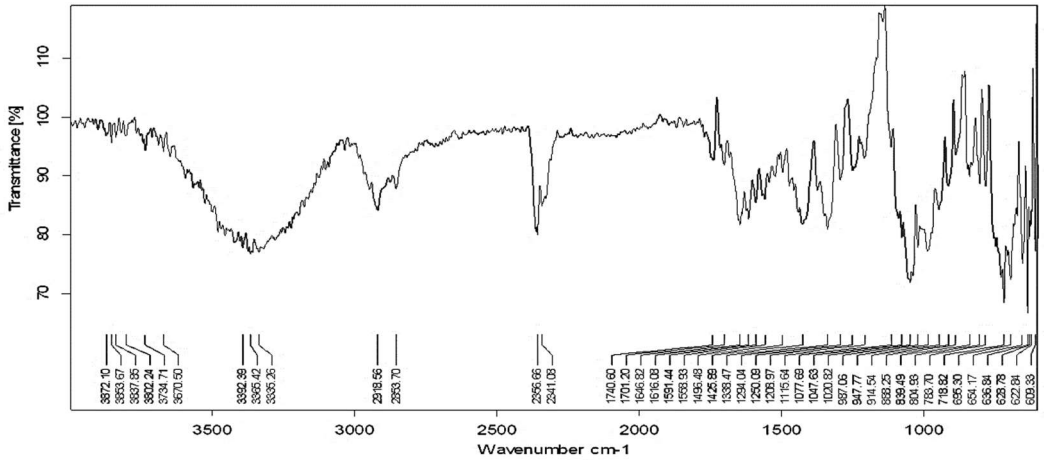

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Figure 3. FTIR spectra of Methanolic Extract of Oxystelma esculentum.

2.2.3. FTIR Analysis of Chloroform Extract of Oxystelma esculentum

Spectroscopic analysis of chloroform extract indicated absorption bands at 3901.56-3817.85 cm-1, which are characteristic of organic acids, specifically the carboxyl group (-COOH). Additionally, absorption peaks observed at 3799.39, 3734.87, and 3648.71 cm^{-1} are indicative of O-H stretching, consistent with alcohols and phenolic compounds. The presence of amino groups is suggested by the peak at 3357.02 cm^{-1}. The absorption bands at 3033.84 and 2928.19 cm^{-1} are associated with aromatic and aliphatic compounds, as evidenced by C–H bending vibrations. Ester linkages, characterized by C=C, C=N, and O=C-O- stretching vibrations, are implied by the peaks ranging from 1741.71 to 1559.19 cm^{-1}. The spectrum also displays peaks between 1496.61 to 1020.83 cm^{-1} and at 1208.96 cm^{-1}, which are representative of functional groups such as amides, ketones, aldehydes, and aromatic compounds, with in-ring C–C and C-O stretching, as well as aliphatic amines, denoted by C–N stretching. Finally, the peaks extending from 1051.24 to 607.80 cm^{-1} suggest the presence of halogenated compounds, including C-Cl, C-F, C-I, and C-Br bonds.

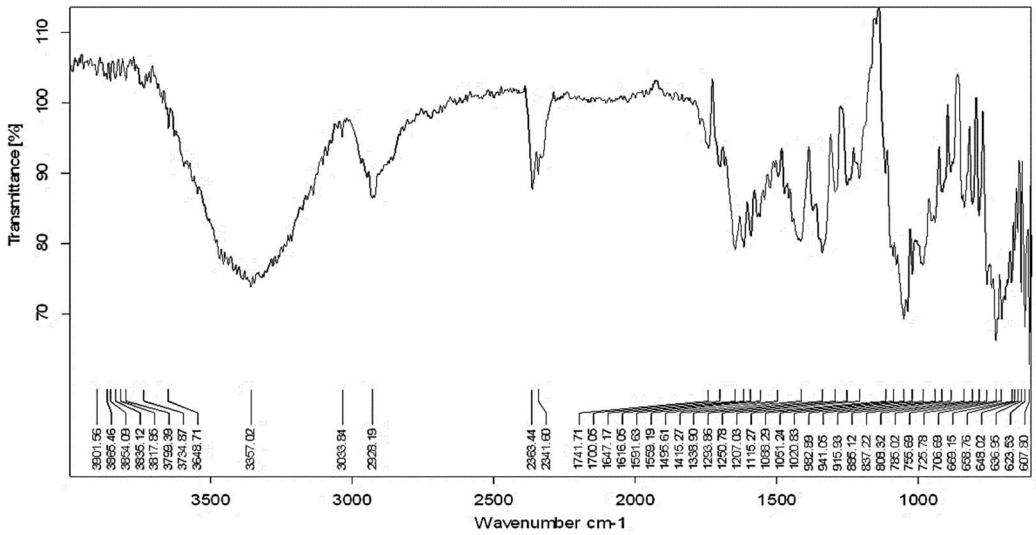

Figure 4. FTIR spectra of chloroform Extract of Oxystelma esculentum.

2.2.4. FTIR Analysis of n-Hexane Extract of Oxystelma esculentum

Spectroscopic analysis indicated absorption bands at 3916.68, 3853.98, and 3838.06 cm^{-1}, which are characteristic of organic acids, specifically the carboxyl group (-COOH). Additionally, the bands at 3674.98 and 3648.20 cm^{-1} are indicative of alcoholic and phenolic compounds, as evidenced by the O–H stretch. The absorption peak at 3364.54 cm^{-1} is associated with the amino group (-NH2). The presence of aliphatic compounds is denoted by the peaks at 2918.37 and 2850.31 cm^{-1}, which correspond to the C–H bend. The range from 1740.37 to 1590.98 cm^{-1} aligns with the ester linkage, including C=C, C=N, and O=C-O- bonds. Functional groups such as amide, ketone, aldehyde, and aromatics, as well as aliphatic amines, are represented by the peaks between 1558.32 and 1049.30 cm^{-1}, which are attributed to the C–C stretch (in-ring) and C–N stretch. Lastly, the peaks ranging from 726.06 to 608.54 cm^{-1} confirm the presence of halogen compounds, specifically C-Cl, C-F, C-I, and C-Br bonds.

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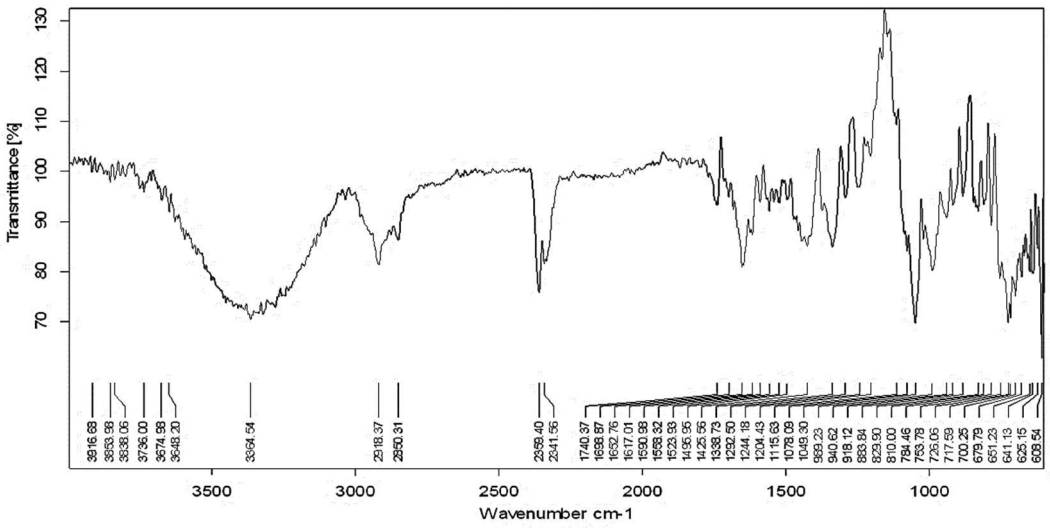
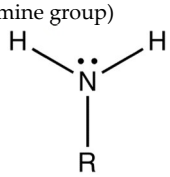


Figure 5. FTIR spectra of n-Hexane Extract of Oxystelma esculentum.

Table 2. FTIR peak values and functional groups of different organic extracts of Oxystelma esculentum.

Wave number ranges Cm^{-1} (Ethanol)	**Wave number ranges Cm^{-1} (Methanol)**	**Wave number ranges Cm^{-1} (chloroform)**	**Wave number ranges Cm^{-1} (Hexane)**	**Functional group present**	**Examples**
4000-3800 Cm^{-1} 3853.81 Cm^{-1} 3818.08 Cm^{-1}	4000-3800 Cm^{-1} 3872.10 Cm^{-1} 3802.24 Cm^{-1}	4000-3800 Cm^{-1} 3901.56 Cm^{-1} 3865.46 Cm^{-1} 3854.09 Cm^{-1} 3835.12 Cm^{-1}	4000-3800 Cm^{-1} 3916.68 Cm^{-1} 3853.98 Cm^{-1} 3838.06 Cm^{-1}	Organic acid (-COOH Group)	Tartaric acid Malic acid Lactic acid Acetic acid Citric acid etc.
3800-3500 Cm^{-1} 3735.63 Cm^{-1} 3649.16 Cm^{-1} 3594.33 Cm^{-1}	3800-3500 Cm^{-1} 3734.71 Cm^{-1} 3670.50 Cm^{-1}	3800-3500 Cm^{-1} 3799.39 Cm^{-1} 3734.87 Cm^{-1} 3648.71 Cm^{-1}	3800-3500 Cm^{-1} 3736 Cm^{-1} 3674.98 Cm^{-1} 3648.20 Cm^{-1}	i. O-H group ii. Alcoholic with phenolic compounds	**Phenolic compounds e.g.,** Vanillin Salicylic acid Pyrocatechol Resorcinol Cresol Hydroquinone Eugenol
3300-3400 Cm^{-1} 3336.54 Cm^{-1}	3300-3400 Cm^{-1} 3392.29 Cm^{-1} 3365.42 Cm^{-1} 3335.26 Cm^{-1}	3300-3400 Cm^{-1} 3357.02 Cm^{-1}	3300-3400 Cm^{-1} 3364.54 Cm^{-1}	NH_2 group (Amine group) 	Amino acids Biogenic amines Trimethylamine Aniline
2500-3000 Cm^{-1} 292.71 Cm^{-1}	2500-3000 Cm^{-1} 2918.56 Cm^{-1} 2853.70 Cm^{-1}	2500-3000 Cm^{-1} 3033.84 Cm^{-1} 2928.19 Cm^{-1}	2500-3000 Cm^{-1} 2918.37 Cm^{-1} 2850.31 Cm^{-1}	Aromatic compound with C-H Bond  Aliphatic compound with C-H bond	**Aromatic compound with C-H bond** Benzene, Toluene, Purines, Pyrimidines e.g., Monoterpenes Carvacrol Phenylpropene etc. **Aliphatic compound with C-H bond** Hexane

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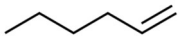
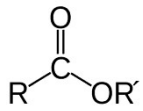
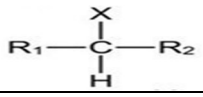
					
2000-1500 Cm^{-1} 1700.37 Cm^{-1} 1652.30 Cm^{-1} 1616.31 Cm^{-1} 1590.69 Cm^{-1} 1558.83 Cm^{-1} 1541.11 Cm^{-1} 1521.92 Cm^{-1}	2000-1500 Cm^{-1} 1710.20 Cm^{-1} 1496.48 Cm^{-1}	2000-1500 Cm^{-1} 1700.05 Cm^{-1} 1647.17 Cm^{-1} 1616.05 Cm^{-1} 1591.63 Cm^{-1}	2000-1500 Cm^{-1} 1740.37 Cm^{-1} 1698.87 Cm^{-1} 1652.76 Cm^{-1} 1617.01 Cm^{-1} 1590.98 Cm^{-1} 1558.32 Cm^{-1} 1523.93 Cm^{-1}	Ester peaks i. C=N ii. C=C iii. O ‖ C-O- 	Ethyl Propanoate Propyl Methanoate Propyl Ethanoate Methyl Butanoate.
1000-600 Cm^{-1} 783.73 Cm^{-1} 607.79 Cm^{-1}	1000-600 Cm^{-1} 783.70 Cm^{-1} 609.33 Cm^{-1}	1000-600 Cm^{-1} 658.76 Cm^{-1} 648.02 Cm^{-1} 636.95 Cm^{-1} 623.53 Cm^{-1}	1000-600 Cm^{-1} 726.06 Cm^{-1} 608.54 Cm^{-1}	Halogen with carbon group (C-X) 	C-Cl C-F C-Br C-I

FTIR Measurements of Flavonoids

In the context of Fourier-transform infrared (FTIR) spectroscopy, the analysis was conducted within a wavenumber range of 4000-650 cm^{-1}. Prior to interpretation, the spectrum underwent preprocessing; this included baseline correction to achieve a horizontal baseline and smoothing techniques to minimize noise interference. Subsequently, the processed FTIR spectrum was examined to distinguish and identify the characteristic wavenumber ranges specific to flavone and flavanone, facilitating their recognition within the spectrum.

Table 3. Five regions assigned for identification and classification of flavonoids classes.

Region	Wavenumbers (cm^{-1})	Structural identification
1	4000-3250	Presence of hydroxyl group
2	3140-3000	Presence of di-substitution
3	1670-1620	Unsaturation bonds
4	1650-1600	Conjugation of bonds

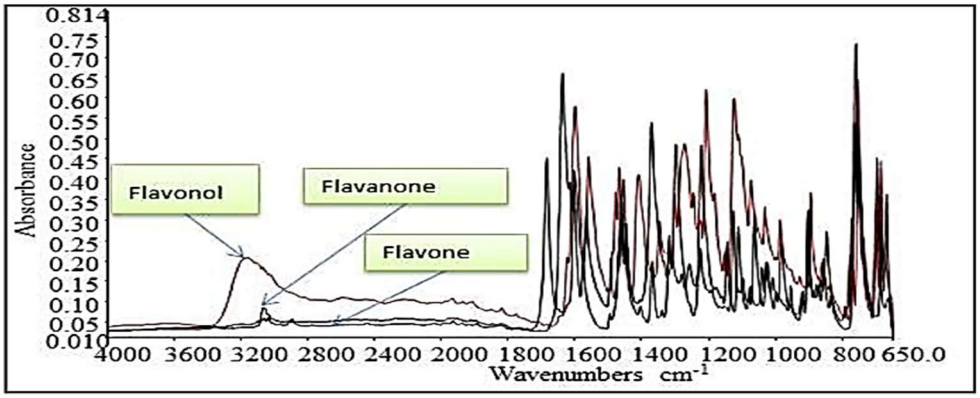


Figure 6. FTIR spectrum in mid IR range for flavonol, flavone and flavanone.

2.3. In Vitro Analysis of Plant Extract

2.3.1. Cytotoxic Effects of Oxystelma esculentum Extract

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The cytotoxic potential of *Oxystelma esculentum* plant extract was evaluated against three cancer cell lines: OVCAR3 (ovarian), T47D (breast), and HeLa (cervical). The extract was tested at various concentrations (0(control), 1, 5, 10, 20, 30, 50, and 100 µg/ml) to determine its effect on cell viability.

The results showed a concentration-dependent increase in cytotoxicity across all three cell lines. For OVCAR3 cells, the cytotoxicity ranged from 2.71% at 1 µg/ml to 79.74% at 100 µg/ml. Similarly, T47D cells exhibited cytotoxicity ranging from 2.29% at 1 µg/ml to 70.50% at 100 µg/ml. HeLa cells showed the lowest cytotoxicity, ranging from 2.01% at 1 µg/ml to 70.29% at 100 µg/ml (**Figure 7)**.

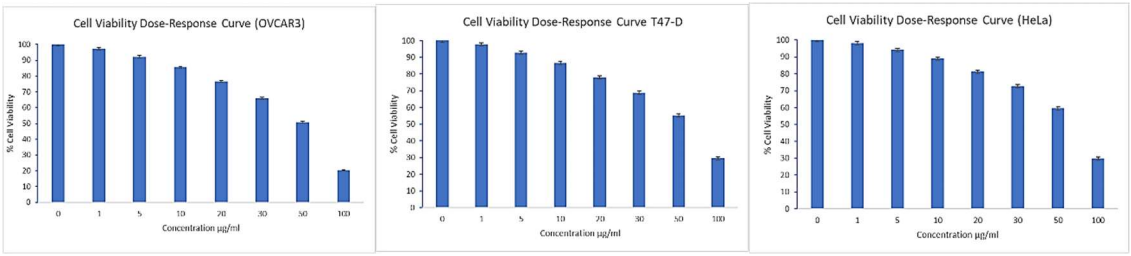

Figure 7. Cell viability Dose-response curve showing cytotoxic potential of *Oxystelma esculentum* extract against three cancer cell lines: OVCAR3 (ovarian), T47D (breast), and HeLa (cervical).

These findings suggest that Oxystelma esculentum extract possesses significant cytotoxic activity against ovarian and breast cancer cells, with moderate effects on cervical cancer cells. The extract's ability to inhibit the growth of these cancer cell lines at higher concentrations indicates its potential as a natural anticancer agent. Further studies are warranted to elucidate the underlying mechanisms of action and to evaluate its efficacy in vivo.

2.3.2. Determination of IC50 and IC80

The IC50 values indicate that the extract exhibits similar potency against both OVCAR3 and T47D cell lines, with T47D showing a slightly lower IC50 (42.82 µg/ml) compared to OVCAR3 (43.57 µg/ml). This suggests that T47D cells may be marginally more sensitive to the extract's cytotoxic effects. In contrast, HeLa cells require a higher concentration (45.42 µg/ml) to achieve the same level of inhibition, indicating a reduced sensitivity to the extract compared to the other two cell lines.

The IC80 values follow a similar trend, with T47D again being the most sensitive, requiring 72.82 µg/ml to inhibit 80% of cell growth, while OVCAR3 and HeLa require 73.57 µg/ml and 75.42 µg/ml, respectively. These results suggest that while all three cancer cell lines are affected by the extract, T47D cells are the most susceptible, followed closely by OVCAR3, with HeLa cells being the least affected (**Figure 8**).

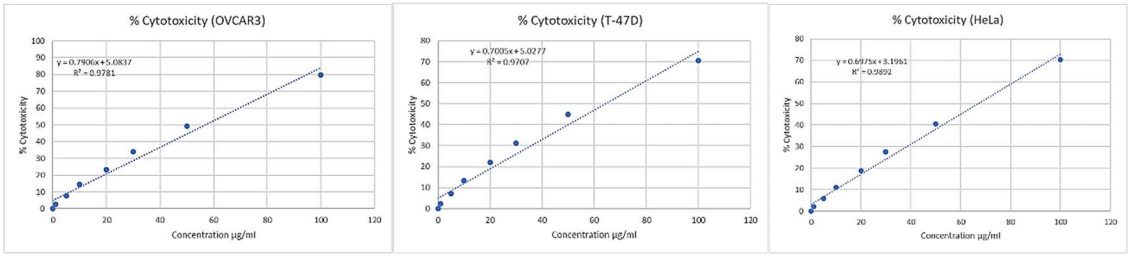

Figure 8. Determination of IC50 and IC80 through linear regression.

These findings highlight the potential of Oxystelma esculentum extract as a promising candidate for anticancer therapy, particularly against breast and ovarian cancers. The relatively low IC50 values indicate strong cytotoxic activity, warranting further investigation into the mechanisms of action and the exploration of this extract in preclinical and clinical settings. The variation in sensitivity among different cell lines also underscores the importance of tailored approaches in cancer treatment, as different tumors may respond differently to the same therapeutic agents.

2.4. In Vivo Toxicity Using Brine Shrimp Lethality Assay

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The brine shrimp lethality assay conducted with varying doses of *Oxystelma esculentum* demonstrated a dose-dependent increase in mortality rates, albeit at relatively low levels. At a concentration of 10 µg/ml, the mortality rate was recorded at 3.34%, which increased slightly to 6.67% at 100 µg/ml and further to 6.89% at 1000 µg/ml. In contrast, the standard control, etoposide, exhibited a significantly higher mortality rate of 70%. These findings (**Figure 9)** suggest that while *Oxystelma esculentum* exhibits some lethality towards brine shrimp, its effectiveness is markedly lower compared to the standard cytotoxic agent, etoposide. The results indicate that further investigation is warranted to explore the bioactive compounds present in *Oxystelma esculentum* and their potential therapeutic applications.

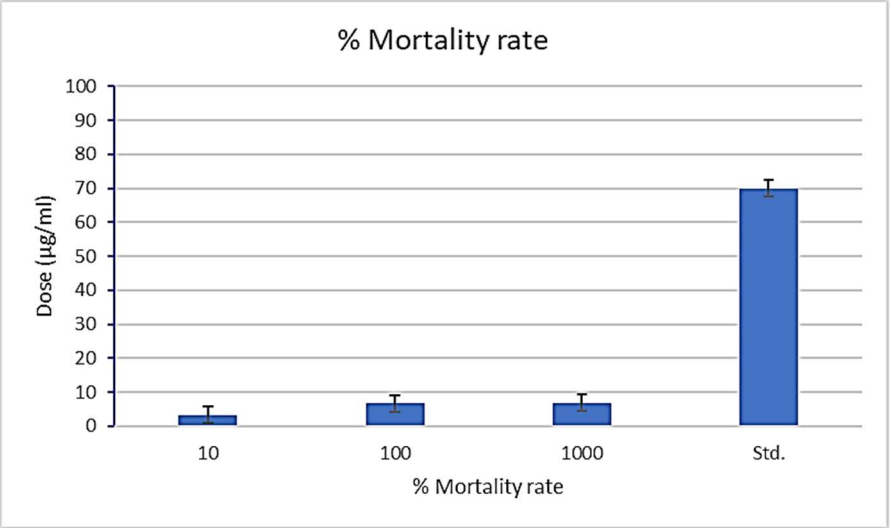

Figure 9. In vivo toxicity using Brine shrimp lethality assay.

2.5. In vivo Lactogenic Activity of Oxystelma esculentum

2.5.1. Milk Production and Yield

Differences in milk yield among four treatment groups was analyzed: G1 Extract 200, Extract 300, Extract 600, and Metoclopramide over a 15-day period. The results indicated significant differences in milk yield across the groups. Post-hoc comparisons using the Tukey HSD test revealed that Extract 600 produced the highest average milk yield (M = 150 g, SD = 0.00), significantly greater than Extract 300 (M = 135 g, SD = 0.00) and Extract 200 (M = 115 g, SD = 0.00). The Metoclopramide group showed an average yield of 140 g (SD = 0.00), which was not significantly different from Extract 600 but was significantly higher than Extract 200. These findings suggest that the type of treatment significantly influences milk production in lactating rats, with Extract 600 being the most effective in enhancing milk yield. The results underscore the potential of plant extracts as viable alternatives to conventional treatments in improving lactation outcomes. Further investigation is warranted to explore the underlying mechanisms contributing to these differences in milk yield.

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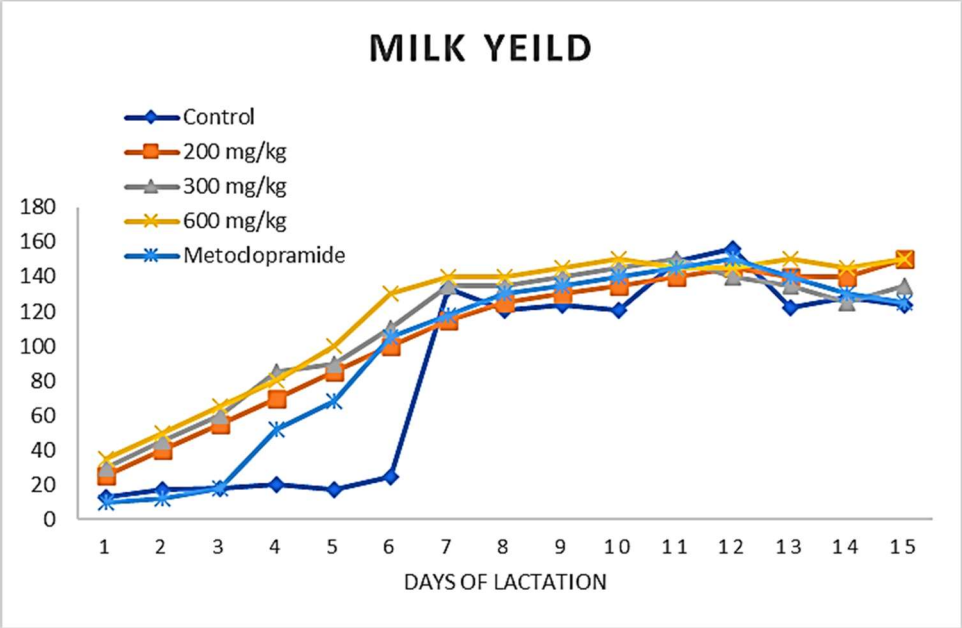

Figure 10. Effect of Oxystelma esculentum on milk yield.

2.5.2. Pup Weight and Growth Rate

The weight gain of pups over a 15-day lactation period was assessed across five treatment groups: Control, 200 mg/kg, 300 mg/kg, 600 mg/kg, and Metoclopramide. The results indicated a significant increase in pup weight over time, with all treatment groups showing growth. By Day 15, pups in the 600 mg/kg group exhibited the highest average weight (M = 50 g, SD = 0.00), significantly surpassing the Control group (M = 40 g, SD = 0.00) and the Metoclopramide group (M = 34 g, SD = 0.00). The 300 mg/kg group also showed notable growth (M = 45 g, SD = 0.00), while the 200 mg/kg group had an average weight of 43 g (SD = 0.00). The Control group consistently lagged behind the treatment groups, demonstrating the least weight gain throughout the lactation period. Statistical analysis revealed significant differences in pup weights among the groups, particularly between the 600 mg/kg treatment and the Control group ($p < 0.05$). These findings suggest that higher doses of the administered extract significantly enhance pup growth during lactation, indicating its potential effectiveness in improving growth outcomes in pups. Further studies are warranted to explore the mechanisms underlying these effects and their implications for lactation management.

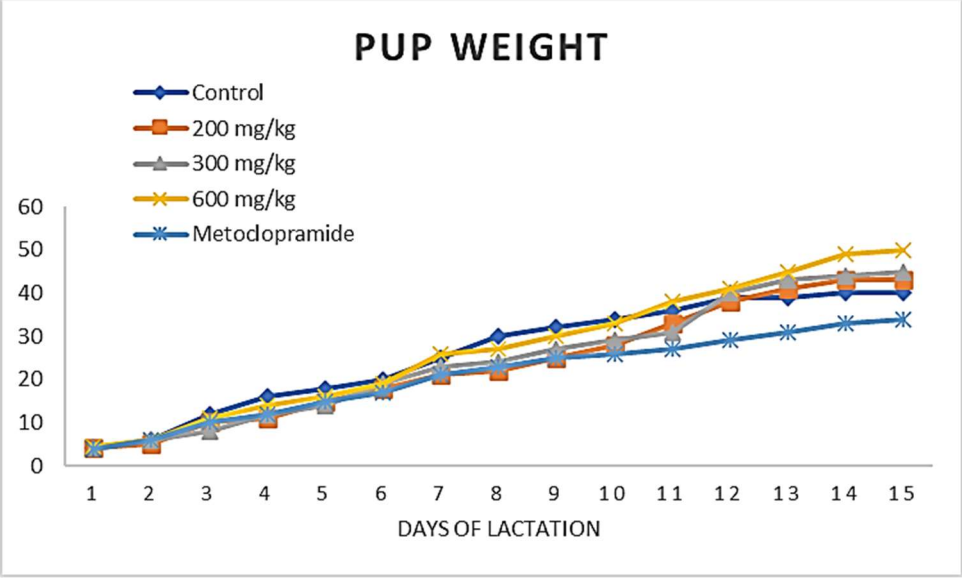

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Figure 11. Effect of Treatment groups on weight and growth of lactating pups.

2.6. Screening Of Prolactin and Its Relation to Galactagogue Activity

The evaluation of serum prolactin levels in rats following treatment with varying doses of the plant extract demonstrated a significant increase in prolactin concentration compared to the control group. The control group exhibited a mean prolactin level of 28.17 ng/mL (SD = 9.64). In contrast, the group receiving the 200 mg/kg dose of the extract showed a substantial increase, with mean prolactin levels rising to 53.07 ng/mL (SD = 8.80). As the dosage increased to 300 mg/kg, the mean prolactin level further elevated to 60.15 ng/mL (SD = 12.79), indicating a dose-dependent response. Notably, the highest dose of 600 mg/kg resulted in an impressive mean prolactin level of 84.88 ng/mL (SD = 9.30), which was significantly higher than all other groups ($p < 0.01$). The metoclopramide group also showed elevated prolactin levels (M = 74.90 ng/mL, SD = 10.80), demonstrating its known effects as a galactogogue. These findings suggest that the plant extract effectively stimulates prolactin secretion in a dose-dependent manner, with the 600 mg/kg dosage exhibiting the most pronounced effect. The results support the potential of the plant extract as a natural galactogogue, warranting further investigation into its mechanisms of action and practical applications in promoting lactation.

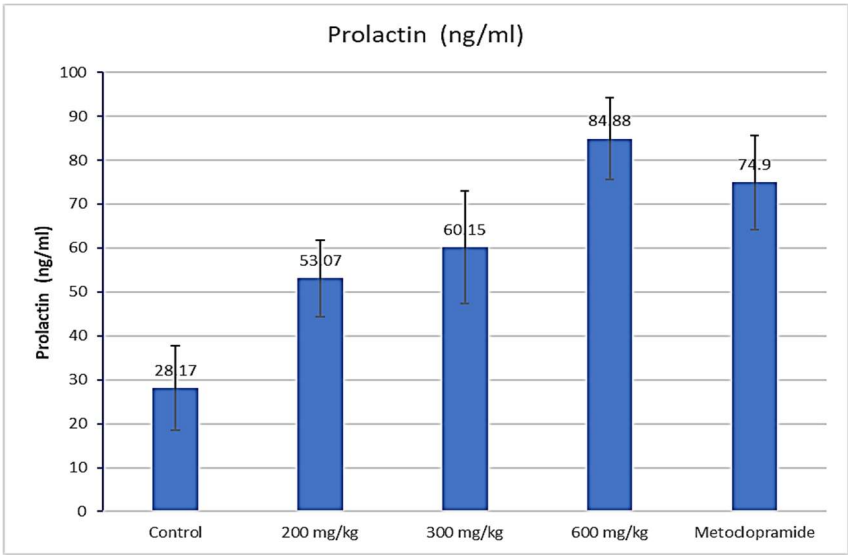

Figure 12. correlation of prolactin levels with lactogenic potential of *Oxystelma esculentum.*

3. Discussion

The preliminary phytochemical screening of Oxystelma esculentum has revealed a diverse array of secondary metabolites, indicating its potential as a source of bioactive compounds. The findings demonstrate a significant presence of polar compounds such as alkaloids, flavonoids, glycosides, phenolic compounds, reducing sugars, and tannins, particularly in polar solvents like methanol, ethanol, and water. These metabolites are known for their various pharmacological properties, which could be harnessed for therapeutic applications.

The phytochemical analysis (Table 1) indicates that O. esculentum extracts contain varying concentrations of bioactive compounds across different solvents. High concentrations of alkaloids, flavonoids, and tannins were consistently observed in methanol and ethanol extracts, suggesting these solvents are effective for extracting valuable phytochemicals. In contrast, non-polar solvents primarily yielded terpenoids, which may have distinct biological activities. The moderate presence of anthraquinones and saponins further supports the plant's potential medicinal value.

Spectroscopic analysis, particularly FTIR, provided insights into the functional groups present in the extracts. The presence of absorption bands characteristic of organic acids, amino groups, and various carbon compounds suggests a complex chemical composition that may contribute to the plant's bioactivity. The identification of ester linkages and aromatic compounds indicates that O.

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esculentum could possess antioxidant and anti-inflammatory properties, aligning with the findings of previous studies that highlighted its pharmacological potential.

The cytotoxic effects of O. esculentum were evaluated against three cancer cell lines: OVCAR3 (ovarian), T47D (breast), and HeLa (cervical). The results demonstrated a concentration-dependent increase in cytotoxicity, with the extract showing significant activity against ovarian and breast cancer cells. The IC50 values indicated that T47D cells were slightly more sensitive to the extract compared to OVCAR3, while HeLa cells exhibited the least sensitivity. These findings suggest that O. esculentum may be a promising candidate for natural anticancer therapies, particularly for breast and ovarian cancers.

The brine shrimp lethality assay indicated that while O. esculentum has some cytotoxic effects, its lethality is considerably lower than that of standard agents like etoposide. This suggests a relatively low toxicity profile, which is advantageous for therapeutic use. Furthermore, the lactogenic activity of the extract was assessed in lactating rats, revealing significant increases in milk yield and pup weight, particularly at higher doses. This underscores the potential of O. esculentum as a natural galactagogue, promoting further research into its mechanisms of action and practical applications in lactation management.

4. Materials and Methods

4.1. Collection of Plant

Specimens of *Oxystelma esculentum* were freshly gathered from the vicinity of the Shshmahi Canal, located in Bahawalpur, Punjab, Pakistan. The taxonomic verification of the collected species was conducted and authenticated by Professor Dr. Ghazallah H. Rizwani, the Director of Research at Hamdard University in Karachi, Pakistan. The specimens have been duly cataloged in the university's herbarium and bear the voucher number 144 for reference and future studies.

4.2. Preparation of Organic Extract

The plant material of *Oxystelma esculentum* was desiccated in shade at a temperature of 30°C and subsequently ground to a fine powder using a mechanical grinder. A quantity of 30 grams of this leaf powder was measured and macerated with 200 milliliters of solvent for a period of three days. Following maceration, the mixture was filtered through a Whatman No.1 filter paper, and the clear filtrate was collected. The remaining plant residue was subjected to two additional rounds of extraction, each with a three-day interval, and the resulting filtrates were also collected. These filtrates were then concentrated using a rotary evaporator (Heidolph, Germany) at 45°C to yield a dark greenish-brown semi-solid extract. This extract was prepared using organic solvents of varying polarity such as ethanol, chloroform, n-hexane, and methanol etc. Each extract was individually stored in a cool, dark place in airtight glass containers for subsequent analysis(Singh, 2008).

4.3. Preliminary Phytochemical Analysis

In the current study, various solvent extracts from *Oxystelma esculentum* underwent specific phytochemical assays to detect the presence of glycosides, alkaloids, terpenoids, flavonoids, phenols, saponins, and tannins. The evaluation utilized various organic solvents like methanol, ethanol, chloroform, and n-hexane etc. for *Oxystelma esculentum* extraction, following established protocols. This comprehensive screening is crucial for identifying bioactive compounds within the plant, which could have therapeutic applications.

4.4. Fourier-Transform Infrared (FTIR) Spectroscopy

Fourier-transform infrared (FTIR) spectroscopy, utilizing the BRUKER TENSOR 27 model from the United States, was employed to discern the chemical bond types and functional groups within both medicinal and non-medicinal botanical specimens. This technique identifies peaks corresponding to the absorption of light wavelengths by chemical bonds. For the analysis, 1.0 mg of various solvent extracts from *O. esculentum* were integrated into a potassium bromide matrix to

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achieve a semi-transparent medium. Subsequently, individual samples of ethanol, methanol, chloroform, and n-hexane extracts from *O. esculentum* were examined using the FTIR spectrometer, over a scanning range of 400 to 4000 cm^{-1}. This procedure was conducted in triplicate to ensure experimental consistency. The solvent extracts underwent scanning within a spectral range of 400-3400 nm, facilitating the identification of characteristic absorption peaks and their associated functional groups.

4.4.1. FTIR Analysis of Flavonoids

Flavonoids, a diverse group of bioactive compounds, are ubiquitously present in various fruits, seeds, vegetables, and microorganisms. These compounds are integral to human health due to their potent antioxidant, anti-inflammatory, and anticancer properties. Some studies have shown that certain flavonoid-rich foods or supplements might contribute to increased milk production. Flavonoids might influence the production of hormones like prolactin and oxytocin, which are crucial for lactation. Due to its anti-inflammatory properties, it can help reducing inflammation in the mammary tissue can support milk production. They also Increased blood flow to the breast area can enhance milk synthesis. Structurally, flavonoids are characterized by a C6-C3-C6 configuration, consisting of two phenolic rings (A and B) linked to a heterocyclic ring C. Over 4000 subgroups of flavonoids have been identified, all sharing the basic phenyl-benzopyran backbone but differing in the type, number, and position of substituents, as well as in their degree of saturation. Fourier Transform Infrared Spectroscopy (FTIR) has emerged as a prevalent tool for the isolation of bioactive compounds, including flavonoids. Although FTIR is widely accepted for its cost-effectiveness and simplicity, it has yet to facilitate the spectrum detection of individual flavonoids, a task currently performed by NMR and UV spectroscopy, which, despite their precision, are less sensitive, more complex, and costlier. Consequently, FTIR is favored for its efficiency and economic advantages in the analysis of these compounds(Oliveira et al., 2016).

4.5. In Vitro Analysis of Plant Extract

4.5.1. MTT Cytotoxicity Assay of Oxystelma esculentum Extract

The cytotoxic potential of *Oxystelma esculentum* plant extract was assessed using three cancer cell lines: OVCAR3 (ovarian), T47D (breast), and HeLa (cervical). The extract was prepared in a series of concentrations (0, 1, 5, 10, 20, 30, 50, and 100 µg/ml) to evaluate its effects on cell viability. Cells were cultured in appropriate media under standard conditions (37°C, 5% CO2) until they reached 70-80% confluence. Following this(Van Meerloo et al., 2011), the cells were treated with the plant extract for a specified duration, typically 24 hours. Cell viability was determined using the MTT assay, where viable cells convert MTT into a formazan product, which can be quantified spectrophotometrically. The absorbance was measured at 570 nm, and the percentage of cytotoxicity was calculated relative to the control group (0 µg/ml concentration). The results were analyzed to determine the concentration-dependent effects of the extract on cell viability. Statistical analysis was performed to confirm the significance of the observed cytotoxic effects across the different concentrations and cell lines. The study aimed to elucidate the potential of *Oxystelma esculentum* as a therapeutic agent in cancer treatment.

4.5.2. Calculation of Inhibitory Concentrations

To determine the IC50 and IC80 values, the data were analyzed using nonlinear regression analysis with GraphPad Prism software(Kowalska-Krochmal & Dudek-Wicher, 2021). The software generated dose-response curves by plotting the logarithm of the extract concentrations against the percentage of cell viability. The IC50 value, representing the concentration at which 50% of cell viability is inhibited, and the IC80 value, indicating the concentration at which 80% of cell viability is inhibited, were derived from the fitted curves. This approach allowed for precise quantification of the extract's cytotoxic potency across the different cell lines, facilitating comparisons of sensitivity among them.

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4.6. In Vivo Toxicity Using Brine shrimp Lethality Assay

The brine shrimp lethality assay was conducted to evaluate the cytotoxic potential of the plant extract. Brine shrimp (*Artemia salina*) eggs were hatched in a hatching chamber containing artificial seawater (38 g/L sea salt in distilled water) under constant aeration and illumination for 24 hours to obtain nauplii. The plant extract was prepared in dimethyl sulfoxide (DMSO) to create stock solutions, from which working solutions of varying concentrations (1000, 100, 10, and 1 µg/mL) were made using artificial seawater. In a 24-well microplate, ten brine shrimp nauplii were added to each well containing 2 mL of the test solution in triplicate. A negative control was included with seawater containing DMSO (not exceeding 10% v/v), and a positive control was established using a reference cytotoxic agent (e.g., potassium dichromate). The microplates were incubated at room temperature (25-27°C) under light for 24 hours. After incubation, the number of surviving nauplii was counted using a magnifying glass, with mortality defined as the absence of movement for several seconds. The percentage of mortality was calculated for each concentration, and the concentration that resulted in 50% mortality (LC50) was determined by plotting the logarithm of the concentration against the percentage of mortality using statistical software. This assay provides a rapid and effective means to assess the cytotoxicity of the plant extract(Krishnaraju et al., 2005).

4.7. In vivo Lactogenic Activity of Oxystelma esculentum

4.7.1. Animals

Rats were acquired from The Islamia University of Bahawalpur and housed in a controlled environment with a steady temperature of (21 ± 2) °C. The rodents were provided with wood shavings for bedding in plastic enclosures topped with wire mesh. The enclosures were situated in a room where the light cycle was maintained at 12 hours of light and 12 hours of darkness. The rats had access to commercial local market bought rodent chow and water ad libitum. The Board of Studies of the university sanctioned all procedures involving the animals.

4.7.2. Experiment Design

five groups of rats, each consisting of three females and one male. The experimental groups received varying doses of the plant extract: Group 1 was administered 200 mg/kg, Group 2 received 300 mg/kg, and Group 3 received 600 mg/kg. Group 4 served as a positive control, receiving metoclopramide, while Group 5 acted as a negative control, receiving normal saline. A total of 15 female rats, weighing between 200 and 350 g, were housed and mated, with the day of parturition designated as Day 1 of lactation. Within 48 hours post-partum, each lactating rat was adjusted to have six pups per litter and randomly assigned to one of the five treatment groups. The test compounds were administered orally at a dose. Treatments commenced on Day 1 and continued daily until Day 15 of lactation. Milk production was measured daily from Day 1 to Day 15, following a standardized separation protocol where pups were isolated from their dams for increasing durations before milking. The weights of the litters were recorded before and after a 60-minute suckling period to estimate milk yield, with all measurements taken using an electronic balance. This comprehensive approach allowed for a detailed analysis of milk production and weight gain among the treatment groups compared to their respective controls(Mahmood et al., 2012).

4.7.3. Prolactin Levels

At the end of the treatment period, blood samples were collected via cardiac puncture under anesthesia to measure hormone levels, specifically prolactin, which is crucial for lactation. Serum was separated and stored at -20 °C until analysis. Prolactin levels were quantified using an enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's instructions(Ozalkaya et al., 2018).

4.8. Statistical Analysis

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Statistical analysis was performed using appropriate software, with results expressed as mean ± standard deviation. Differences between groups were analyzed using one-way ANOVA followed by post-hoc tests, with p-values < 0.05 considered statistically significant. This methodology aimed to evaluate the potential galactagogue effects of the plant extract through hormonal analysis and physiological measurements in the rat model.

5. Conclusions

Overall, the comprehensive analysis of Oxystelma esculentum highlights its rich phytochemical profile and promising pharmacological properties. The significant cytotoxic effects against specific cancer cell lines, coupled with its potential as a lactation enhancer, warrant further investigation into the bioactive compounds present in this plant. Future studies should focus on elucidating the mechanisms of action and evaluating the efficacy of O. esculentum extracts in clinical settings, paving the way for its application in traditional and modern medicine.

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References

1. Abdel-Sattar, E., & Ali, D. E. (2022). Russelioside B: a pregnane glycoside with pharmacological potential. *Revista Brasileira de Farmacognosia*, *32*(2), 188-200.
2. Chishti, A. W., Fatima, B., Akram, M., Sultana, S., Amer, M. M., Pandian, R., Ozdemir, F. A., Kilic, O., Voitenkov, V., & Ekusheva, E. (2021). Phytochemical reviews about Oxystelma esculentum. *Annals of Pharma Research*, *9*(6).
3. D Ashok, K., Saha, P., Islam, A., Mazumder, U. K., & Gupta, M. (2010). Antimicrobial and lipid peroxidation inhibition activity of Oxystelma esculentum (Asclepiadaceae). *Oriental Pharmacy and Experimental Medicine*, *10*(3), 208-213.
4. Davidove, M. E., & Dorsey, J. W. (2019). Breastfeeding: A cornerstone of healthy sustainable diets. *Sustainability*, *11*(18), 4958.
5. El-Shiekh, R., Al-Mahdy, D., Hifnawy, M., & Abdel-Sattar, E. (2019). In-vitro screening of selected traditional medicinal plants for their anti-obesity and anti-oxidant activities. *South African journal of botany*, *123*, 43-50.
6. Hamed, A. I., Sheded, M. G., Shaheen, A. E.-S. M., Hamada, F. A., Pizza, C., & Piacente, S. (2004). Polyhydroxypregnane glycosides from Oxystelma esculentum var. alpini. *Phytochemistry*, *65*(7), 975-980.
7. Kowalska-Krochmal, B., & Dudek-Wicher, R. (2021). The minimum inhibitory concentration of antibiotics: Methods, interpretation, clinical relevance. *Pathogens*, *10*(2), 165.
8. Krishnaraju, A. V., Rao, T. V., Sundararaju, D., Vanisree, M., Tsay, H.-S., & Subbaraju, G. V. (2005). Assessment of bioactivity of Indian medicinal plants using brine shrimp (Artemia salina) lethality assay. *International Journal of Applied Science and Engineering*, *3*(2), 125-134.
9. Kumar, M. S., Chaudhury, S., & Balachandran, S. (2009). In Vitro Micropropagation Of Oxystelma Esculentum R. Br.–A Medicinal Herb. *International Journal of Biotechnology and Biochemistry*, *5*(2), 147-156.
10. Mahmood, A., Omar, M. N., & Ngah, N. (2012). Galactagogue effects of Musa x paradisiaca flower extract on lactating rats. *Asian Pacific Journal of Tropical Medicine*, *5*(11), 882-886.
11. Oliveira, R. N., Mancini, M. C., Oliveira, F. C. S. d., Passos, T. M., Quilty, B., Thiré, R. M. d. S. M., & McGuinness, G. B. (2016). FTIR analysis and quantification of phenols and flavonoids of five commercially available plants extracts used in wound healing. *Matéria (Rio de Janeiro)*, *21*(03), 767-779.

 doi:10.20944/preprints202409.1609.v

12. Ozalkaya, E., Aslandoğdu, Z., Özkoral, A., Topcuoğlu, S., & Karatekin, G. (2018). Effect of a galactagogue herbal tea on breast milk production and prolactin secretion by mothers of preterm babies. *Nigerian journal of clinical practice*, *21*(1), 38-42.
13. Pandya, D. (2012). PHARMACOGNOSTIC STUDY AND ESTABLISHMENT OF QUALITY PARAMETERS OF LEAVES OF OXYSTELMA ESCULENTUM.
14. Pandya, D., & Anand, I. (2011a). A Complete Review on Oxystelma esculentum R. Br. *Pharmacognosy Journal*, *3*(19), 87-90.
15. Pandya, D., & Anand, I. (2011b). Establishment of quality parameters of roots and rhizomes of Oxystelma esculentum. *Pharmacognosy Journal*, *2*(18), 27-31.
16. Pandya, D., Shah, B., Shah, M., & Kapadia, N. (2011). Pharmacognostic study and establishment of quality parameters of stems of Oxystelma esculentum.
17. Poornima, N., Umarajan, K., & Babu, K. (2009). Studies on anatomical and phytochemical analysis of Oxystelma esculentum (Lf) R. br. Ex Schltes. *Botany Research International*, *2*(4), 239-243.
18. Ryan, R. A., Hepworth, A. D., Bihuniak, J. D., & Lyndon, A. (2024). A qualitative study of breastfeeding experiences among mothers who used galactagogues to increase their milk supply. *Journal of Nutrition Education and Behavior*, *56*(3), 122-132.
19. Singh, J. (2008). Maceration, percolation and infusion techniques for the extraction of medicinal and aromatic plants. *Extraction technologies for medicinal and aromatic plants*, *67*, 32-35.
20. SOUJANYA, B. (2017). QUANTIFICATION OF LUPEOL IN SELECTED COMMERCIAL/POPULAR CULTIVARS OF MANGO (Mangifera indica L.).
21. Trivedi, R., Khare, A., & Khare, M. P. (1988). A pregnane ester tetraglycoside from Oxystelma esculentum. *Phytochemistry*, *27*(7), 2297-2300.
22. Van Meerloo, J., Kaspers, G. J., & Cloos, J. (2011). Cell sensitivity assays: the MTT assay. *Cancer cell culture: methods and protocols*, 237-245.
23. Xavier, T. F., Sabitha, R., & Balavivekananthan, S. (2022). In vitro pharmacological investigations of Oxystelma esculentum R. Br. and in silico molecular docking analysis of its leaf constituents on diabetic related target. *South African journal of botany*, *149*, 320-338.
24. Zizzo, G., Amir, L. H., Moore, V., Grzeskowiak, L. E., & Rumbold, A. R. (2021). The risk-risk trade-offs: understanding factors that influence women's decision to use substances to boost breast milk supply. *PLoS One*, *16*(5), e0249599.