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RESEARCH ARTICLE
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EVALUATION OF THE TOXICITY, ANTIOXIDANT, AND ANTIMITOTIC IMPACT OF *MORINGA OLEIFERA* USING *IN-VITRO*, *EX-VIVO*, AND *IN-VIVO* MODELS.

Pravin D Badhe[1*], Pranali D Bangaiya[2], Rutuja N Mule[3], Prashant Mali[4], Rutuja M Shinde[5], Swarda V Kamble[6], Leena Pathak[7] and Ashwini P Badhe[8]

[1*,2,3,4,5,6]Sinhgad College of Pharmacy, Vadgaon (BK) Pune Maharashtra India
[1,8]Swalife Biotech Ltd, Ireland
[7]Jaihind College of Pharmacy, vadgaon Sahani, Junnar, Pune

***Corresponding Author-** Dr Pravin Badhe
Email: drbadhepravin@gmai.com

Abstract
Moringa Oleifera, a tropical plant, is characterized by its noteworthy nutritional and medicinal attributes. The present study involved the extraction of *Moringa Oleifera* leaves utilizing the n-hexane solvent. Employing this methodology, a preliminary investigation of phytochemical constituents, organic functional group identification, toxicological assessment through brine shrimp and chicken eye experiments, and antimitotic evaluation using earthworms were conducted. Phenols Test
The phytochemical screening indicated the presence of terpenoids, fats, steroids, tannins, glycoside and phenols in the n-hexane extract of *moringa oleifera* bark. Similarly, the n-hexane extract of *moringa oleifera* leaves exhibited positive results for phenols, terpenoids, tannins, glycosides, saponins, and fats. Infrared spectral data analysis revealed distinctive bonds in the extracts, with the leaves extract exhibiting C-H, C=O, C=C, C-C, and C-O bonds indicative of aliphatic and aromatic compounds. Conversely, the bark extract showcased N-H, O-H, C=O, C=C, and N-O bonds, suggesting the presence of aromatic or aliphatic phenols, alcohols, and nitrogen-containing compounds.
The brine lethality assay demonstrated a proportional increase in lethality with escalating extract concentrations. However, no ocular toxicity was observed in the chicken eye experiments. Notably, the hexane extract of *moringa oleifera* leaves exhibited pronounced antimitotic activity, underscoring its potential application in cancer treatment due to this specific biological activity

Keywords: *moringa oleifera*, antimitotic activity, phenols, terpenoids, tannins, glycosides, saponins

1.0 INTRODUCTION

In the vast world of plant diversity, *moringa oleifera* stands out as an exceptional treasure trove, revealing a captivating story of both nutritional richness and medicinal significance. Belonging to the Moringaceae family, this plant showcases a spectrum of valuable bioactive components, including vitamins, phenolic acids, flavonoids, isothiocyanates, tannins, and saponins, all contributing to its impressive nutritional profile (Olushola, 2006).
This botanical wonder is particularly renowned for its adaptability and usefulness. Its various parts – flowers, roots, leaves, and bark – have played important roles in nutritional supplements, culinary practices, and even the production of cosmetics (Ayotunde et al., 2011; Moyo et al., 2012). As we

delve into the exploration of *Moringa oleifera*, we uncover not only its historical significance but also its contemporary relevance in the realms of health and nutrition

Extensive studies have spotlighted *Moringa oleifera's* efficacy in addressing a spectrum of health concerns, including hypercholesterolemia, high blood pressure, diabetes, and even cancer (Olushola, 2006). The leaves, in particular, have been scrutinized for their potential therapeutic applications, showcasing the plant's multifaceted pharmacological landscape. Beyond its nutritional and medicinal contributions, *Moringa oleifera* stands as an agricultural asset, adapting seamlessly to the climates of Asia and Africa, and holding historical significance as a dietary supplement and remedy (Paikra et al., 2017).

1.1 Plant Taxonomy

Delving into the systematic classification of *Moringa oleifera* within the taxonomic hierarchy provides a comprehensive understanding of its botanical identity. Positioned within the Kingdom Plantae, Subkingdom Tracheobionta, Superdivision Spermatophyte, and other taxonomic levels, *Moringa oleifera* stands as a testament to the intricate botanical diversity encapsulated within its taxonomic framework (Chukwuebuka, Egbuna, 2015).

1.2 Morphology

The morphological dimensions of *Moringa oleifera* contribute to its adaptability and aesthetic allure. As a small, fast-growing tree, it ascends to heights ranging from 10 to 12 meters, adorned with spreading branches and distinctive tripinnate leaves. The visual appeal of the plant is complemented by its delicate flowers and fruits, underscoring not only its aesthetic significance but also its functional diversity (Chukwuebuka, E., 2015; Mohlala et al., 2023; Shamim et al., 2018

A nuanced exploration into the chemical constituents of *Moringa oleifera* reveals the intricate biochemical basis underpinning its pharmacological efficacy. From the vibrant quercetin and kaempferol in the flowers to the vital oleic acid, isothiocyanates, and β-D-Glucopyranoside in the seeds, and the bioactive glucomoringin, benzyl isothiocyanate, and glucosinolates in the leaves, the plant's chemical diversity forms the bedrock of its therapeutic potential (Razis et al., 2014; Shaji et al., 2021).

This intricate molecular tapestry not only reflects the plant's adaptability to diverse environmental challenges but also underscores its potential pharmacological applications. The varied chemical constituents contribute not merely to the plant's survival but serve as a reservoir for compounds with promising therapeutic properties. The quercetin and kaempferol found in the flowers, for instance, have been associated with antioxidant and anti-inflammatory effects, amplifying the potential health benefits of *Moringa oleifera* (Razis et al., 2014). The presence of oleic acid and β-D-Glucopyranoside in the seeds hints at potential cardiovascular benefits, further enhancing the plant's holistic profile as a natural health resource.

Moreover, the bioactive compounds in the leaves, including glucomoringin and benzyl isothiocyanate, have been linked to antitumor and anti-inflammatory activities (Shaji et al., 2021). This not only aligns with the historical uses of *Moringa oleifera* in traditional medicine but also resonates with contemporary scientific investigations into its potential role in cancer treatment. The glucosinolates present in the leaves add another layer of complexity, as these compounds have been associated with antimicrobial and anti-cancer properties, augmenting the plant's pharmacological repertoire (Shaji et al., 2021).

As we embark on a journey to explore the biological activities of *Moringa oleifera*, the intricate chemical composition unveiled in this section sets the stage for a comprehensive investigation. The subsequent sections delve into meticulous phytochemical analyses, toxicity assessments, and antimitotic properties, utilizing non-polar extracts. Through innovative model systems, including brine shrimp, chicken eyes, and earthworms, this study endeavors to bridge the botanical characteristics of *Moringa oleifera* with practical applications, presenting an expansive canvas for the exploration of its potential (Marcus et al., 2015; Nandiyanto et al., 2019; Haris Niksic et al., 2021; Suryawanshi et al., 2018; K. Rajamanikkam et al., 2019). The chemical constituents, like characters

in a complex narrative, beckon us to unravel the secrets held within *Moringa oleifera*, promising a saga of discovery in the realms of health and medicine.

2.0 Material and Methods

2.1 Material

In the pursuit of our experimental objectives, various essential instruments were employed. These included a Rotary Shaker, Rotary Evaporator, and an FT-IR Spectrometer (Model-Alpha T, Bruker, Germany) for distinct stages of the experiment. Precise measurements were facilitated by a Single Pan Electronic Balance (AY-120, Shimadzu, Japan).

For data interpretation and analysis, Opus version-7.5 software was employed to interpret IR spectra, while GraphPad Prism aided in the interpretation of plots and conducted ANOVA.

The materials under investigation encompassed Moringa oleifera leaf powder, procured from World of Nature in Balewadi. Other necessary chemicals were sourced from Sigma Aldrich India.

This delineation provides a thorough overview of the instruments, software, and materials enlisted throughout the experimental course, offering clarity on the resources employed in our research endeavor

2.2 Methods

2.2.1 Extraction:

In the extraction procedure, a 250 mL Iodine flask was employed, wherein 100 g of Moringa leaf powder was meticulously combined with 250 mL of n-hexane. The resulting mixture was thoroughly homogenized, and to safeguard the experimental surface, foil paper was employed. Subsequently, the prepared extract was subjected to a 24-hour agitation period on a rotary shaker. Following this, filtration was performed, and the solvent was then evaporated from the solution utilizing a rotary evaporator.

Precision in the process was ensured by weighing the Round Bottom Flask (RBF) both before and after the filtration step. Post-evaporation, the RBF was weighed again to ascertain the yield. The addition of the solvent (n-hexane) to the RBF, followed by subsequent placement in a freezer, was executed in accordance with the methodology outlined by Rozi, Parhat, et al. (2019). This systematic extraction procedure was implemented to obtain the desired components from the Moringa leaf powder for further analysis.

2.2.2 Phytochemical tests

In the assessment of phytochemical constituents within medicinal plants, a qualitative investigation is imperative to discern the presence of bioactive components associated with therapeutic properties. Various tests were conducted to identify specific phytochemical elements:

1. Alkaloids Test: A mixture of 1 ml of 1% HCl and 3 ml of the dried extract in a test tube was prepared. Subsequently, a portion of the extract was combined with a few drops of Mayer's reagent (potassium mercuric iodide), resulting in the formation of a creamy white precipitate.

2. Phenols Test: A combination of 0.5 cm^3 of the extract with 4 cm^3 of distilled water was heated and filtered. The filtrate was then treated with 3 cm^3 of neutral FeCl3 solution, yielding a brown coloration indicative of the presence of phenols.

3. Terpenoids Test: An assay involving 0.5 ml of the extract, 2 ml of chloroform, and a careful addition of 3 ml of concentrated H2SO4 was conducted. A reddish-brown coloration at the interface signaled the presence of terpenoids.

4. Tannins Test: A blue-green precipitate was obtained by combining 2 drops of 5% FeCl3 with 1 ml of the extract.

5. Glycosides Test: A solution comprising 5 ml of 50% H2SO4 and 5 ml of the extract was heated, and to this, 5 ml of Fehling's solutions A and B were added. The emergence of a brick-red precipitate indicated the presence of glycosides.

6. Saponins Test: A mixture of 5 ml of the extract and 10 ml of distilled water was vigorously shaken, resulting in the formation of a soapy-like foam, indicative of the presence of saponins.
7. Flavonoids Test: Combining 2 drops of concentrated HCl, a few magnesium turnings, and 1 ml of the extract produced a color change to pink, red, or purple, signifying the presence of flavonoids.
8. Proteins and Amino Acids Test (Xanthoproteic Test): The addition of conc. nitric acid to the extract resulted in the formation of a yellow color, indicating the presence of proteins.
9. Steroids Test: A combination of 1 ml of the crude plant extract with 10 ml of chloroform, followed by the addition of concentrated sulfuric acid, resulted in a red upper layer, indicating the presence of steroids.
10.Carbohydrates Test (Molisch's Test): The filtrate obtained by combining the extract with distilled water was treated with two drops of alcoholic α-naphthol solution, leading to the formation of a violet ring at the junction, indicating the presence of carbohydrates.
11.Fats and Fixed Oils Test: Conducted through a filter paper press test, where extracts were pressed onto filter paper, and results were observed.
12.Phytosterols Test (Liebermann–Burchard's Test): The extract, chloroform, and conc. sulfuric acid were combined, resulting in the formation of a brown ring at the junction, indicating the presence of phytosterols (Marcus, A. C., et al., 2015; Sudha, Ramalingam, et al., 2021)

2.2.3 FTIR (Fourier Transform infrared spectroscopy)

The Fourier Transform Infrared Spectroscopy (FTIR) procedure, a well-established method for the rapid and accurate detection and analysis of organic compounds, was employed in this study to elucidate the chemical structure of substances.

KBr (Potassium Bromide) Pellet Method:

In the preparation of the KBr pellet, meticulous cleaning procedures were undertaken to ensure the elimination of any contaminants. The die components were cleaned with either chloroform or lubricant spray, followed by thorough drying with tissue. The mortar and pestle, utilized for sample preparation, were similarly cleaned with chloroform or acetone and dried. A small portion of the sample was placed in the mortar, ensuring an appropriate quantity to avoid oversaturation. Dry KBr was then added to the mortar, and the ingredients were ground using the pestle. Continuous mixing ensued for an additional minute or two to achieve a homogeneous mixture.
Due to the hygroscopic nature of potassium bromide, which absorbs moisture from the surroundings and forms bands in specific IR regions when finely powdered, the Pellet Press was employed. The ground sample mix was transferred into the cavity of the die, ensuring even distribution. The amount of powder used was determined by the desired pellet thickness, with caution to avoid excessive quantities that could lead to thickening. The pellet press was placed in the chamber and rotated to disperse particles. Subsequently, the entire die set was secured in the hydraulic pellet press, and pressure was applied by pulling the lever until snug. After releasing the pressure, the die was lifted out, and the pellet was ejected by removing the bottom plunger.

Application of Sample on KBr Pellet:

A small drop of the compound was placed on one of the KBr plates, and the second plate was positioned on top, followed by a quarter turn to achieve an even film. The plates, containing the sample, were then inserted into the sample holder for spectrum analysis. In instances of concentrated samples, the plates were separated, and one side was wiped clean before reassembly (Grdadolnik, J., 2002; van de Weert, et al., 2001; Neel R, S., 2023).

2.2.4 *In vitro* Antioxidant Assay

DPPH Assay:

The DPPH assay was conducted using a Microplate Reader (BMG LABTECH Instrument). In each well of the 96-well plate, a reaction mixture was prepared, comprising solutions of the test molecules,

aqueous methanol, and 70% ethanol as a blank, containing DPPH radicals. The reaction mixture was allowed to stand for 60 minutes in the dark. The reduction of DPPH radicals was quantified by measuring the absorption at 517 nm (Badhe P, et al 2022).

Hydroxyl Radical Scavenging Activity Assay:
This assay, adopted with slight modifications from a previous study (Pravin B, et al 2023), was performed using test tubes and later transferred to a 96-well plate for absorbance readings. Initially, a mixture was prepared, including 3.6 mM deoxyribose, 0.1 mM EDTA, 0.1 mM L- ascorbic acid, 1 mM H2O2, and 0.1 mM Iron(III) chloride hexahydrate (FeCl3.6H2O). Subsequently, 10 µM of the test molecules were added to this mixture, and the volume was adjusted to 1 mL with 25 mM phosphate buffer at pH 7.4. The mixture was then incubated for 1 hour at 37°C.
After incubation, 500 µl of 1% Thiobarbituric acid and 500 µl of 1% Trichloroacetic acid were added to the mixture, followed by heating in a water bath (80°C) for 20 minutes and subsequent cooling. The absorbance was measured at 532 nm. The control reaction lacked the test sample for baseline comparison. This assay provides insights into the hydroxyl radical scavenging activity of the tested compounds, contributing to the evaluation of their antioxidant potential.

2.2.5 Brine Shrimp Lethality Test (BSLT):
Artificial sea water was meticulously prepared by dissolving 27g of a commercially available salt mixture in 900ml of distilled water. Artemia salina eggs were introduced into a small tank for hatching, incubated under a halogen lamp for 24 hours, allowing the shrimp to hatch and mature into nauplii. The hatched shrimp, attracted to light, were collected, and ten nauplii were counted macroscopically against a lighted background using a graduated Pasteur pipette. These nauplii were then transferred to a test tube.
A 1ml extract was dissolved in 100ml of water for serial dilutions, creating different concentrations for testing. The experiment was conducted with three replicates for each treatment, with 10 nauplii per replicate. Each test tube was maintained under illumination for 24 hours, aligning with established protocols (Obi, O. O. B., 2018; Niksic, H., et al., 2021; Suryawanshi, V., 2020).

2.2.6 Chicken Eye Irritation or Damage Test:
Source of Chicken Eyes: Eyes were procured from chickens at a slaughterhouse, ensuring they were only obtained from healthy animals deemed suitable for the human food chain. Chickens approximately 7 weeks old, weighing between 1.5 - 2.5 kg, were selected for this study.
Collection and Transport of Eyes to the Laboratory: Immediately after neck incision for bleeding, heads were removed, and intact heads were swiftly transported from the slaughterhouse to the laboratory to minimize deterioration and bacterial contamination. The time between collection and placing eyes in the superfusion chamber was kept to a minimum, typically within two hours. Heads were transported at ambient temperature in plastic boxes humidified with tissues moistened with isotonic saline.
Selection Criteria and Number of Eyes Used in the ICE: Eyes displaying high baseline fluorescein staining or corneal opacity scores were rejected. Each treatment group, including a positive control with at least three eyes and a negative control or solvent control (if using a solvent other than saline) with at least one eye, was established.
Preparation of the Eyes: Upon removal from the orbit, a visible portion of the optic nerve was left attached. Eyes were placed on an absorbent pad, and after removing the nictitating membrane and connective tissue, they were mounted in a stainless steel clamp with the cornea positioned vertically. The clamp was then transferred to a chamber of the superfusion apparatus, ensuring the entire cornea received isotonic saline drip. The superfusion chambers were temperature-controlled at 32 ± 1.5°C. Eyes were examined with a slit lamp microscope to ensure no damage during dissection, and corneal thickness was measured at the corneal apex. Eyes meeting specified criteria were incubated for 45 to 60 minutes to equilibrate them to the test system prior to dosing (Dutok, Carlos, et al., 2015; Prinsen,

et al., 2017).

2.2.7 Antimitotic Assay: Earthworm Regeneration Assay

In this investigation, the antimitotic potential of a known or unknown compound was assessed utilizing the earthworm regeneration assay. Healthy adult earthworms with consistent sizes, ranging from 1.0 to 1.5 g in body weight, were meticulously selected for the experiments. Using a sterile scalpel blade, earthworms were incised at the 30th segment from the mouth, and the posterior segments were amputated. The anterior parts of the body, inclusive of the clitellum region, were segregated into separate beds and monitored at 24-hour intervals. Particular attention was given to the amputation region to observe wound healing and/or the formation and growth of the blastema.

For the experimental procedure, earthworms were injected between the clitellum and the amputation region with 10 µL of either distilled water or an aqueous drug solution. Injections were administered at 24-hour intervals for either 3 or 7 consecutive days. Each treatment involved five earthworms, and the entire experiment was conducted a minimum of three times to ensure reliability and consistency in the results (Rajamanikkam, K., et al., 2019; Rajesh, C., et al., 2019). This earthworm regeneration assay serves as a valuable tool for evaluating the antimitotic effects of the tested compounds.

3.0 Statistical Analysis:

Statistical analysis was conducted using Graph Pad Prism 9 software. To assess differences between the treatment groups and the positive control, a one-way analysis of variance (ANOVA) was employed, followed by Tukey's test for post hoc comparisons. This rigorous statistical approach enabled the comprehensive evaluation of any observed variations among the experimental groups.

4.0 Results and Discussion:

4.1 Extraction yield:

We performed the extraction using n-hexane of moringa oleifera (Rozi, P., et al.,2019; Missio, A. L., et al., 2017). We got the extract yield of 2.5gm.

4.2 Phytochemical analysis:

Table 1 Phytochemical test result of n-hexane moringa oleifera extract

Sr.No.	Test	**Observation**
1	Phenols Test	+
2	Terpenoid Test	+
3	Tannin Test	+
4	Glycoside Test	+
5	Saponnin Test	+
6	Fat	+

According to Marcus, A. C., et al., Pathak, I. et al., tannin, phenols, terpenoid, glycoside, saponnin, fat present in n-hexane moringa oleifera extract.

In the current study extract was subjected to phytochemical analysis to ascertain the various secondary metabolites present in plants under study. Tannin, phenols, terpenoid, glycoside, saponnin, fat present in n-hexane moringa oleifera extract.

The hexane extract of Moringa oleifera contains a variety of bioactive compounds, including phenolic compounds, terpenoids, tannins, glycosides, saponins, and fatty compounds. The hexane-extracted oil content of Moringa oleifera seeds has been reported to range from 38.00 to 42.00% (Anwar & Bhanger, 2003).

Moreover, the total phenolic content of Moringa oleifera seed oil extract has been determined spectrophotometrically using the Folin-Ciocalteu method, indicating the presence of phenolic compounds in the seed oil (Farhan et al., 2021).

These findings suggest that the hexane extract of Moringa oleifera is rich in bioactive compounds with potential health benefits.

Furthermore, the n-hexane extract and the ethanol extract of Moringa leaves have been identified as potential sources of bioactive compounds with anticancer activities (Junias, 2021). Overall, the hexane extract of Moringa oleifera contains a diverse array of bioactive compounds, including phenolic compounds, terpenoids, tannins, glycosides, saponins, and fatty compounds, with potential antioxidant, antimicrobial, anticancer, and cytotoxic activities.

4.3 FTIR (Fourier transform infrared spectroscopy):

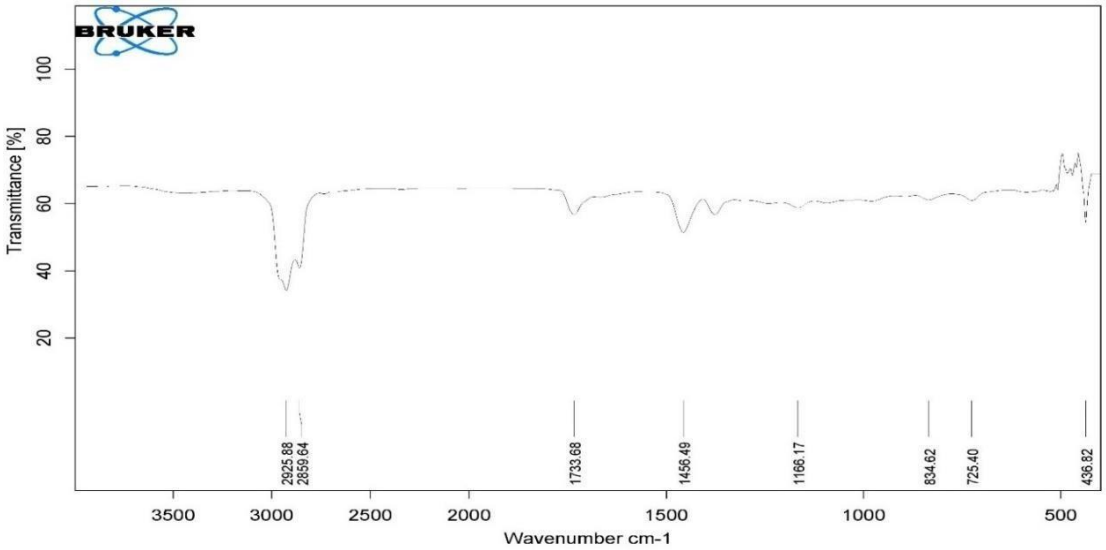

Fig.1 FTIR analysis of n hexane *moringa oleifera* extract

Table 2 Bond and functional group present in n-hexane extract of moringa oleifera

Sr. No	Region in cm-1	Type of vibration	Functional Group
1	2925.88	C-H stretch	Hydrocarbons, Alkanes, All aldehydes
2	2859.64	C-H stretch	Alkane, C-O-CH3
3	1733.68	C=O stretch	B-diketone, Cyclohexanone, A,B-unsaturated esteres(5-member), Aryl esters
4	1456.49	C=C stretch C-C stretch	Hydrocarbons, Aromatics
5	1166.17	C-O stretch	Tertiary alcohols C-O-C
6	834.62	C-H deformation	Disubstituted Aromatic comp. (para)
7	725.40	C-H deformation	Monosubstituted Alkenes
8	436.82	C-C stretch	Cycloalkane

At the range of 2925.88 cm-1 which is associated with Hydrocarbons, Alkanes, All aldehydes with C-H stretch, range of 2859.64 cm-1 which is associated with Alkane, C-O-CH3 with C- H stretch, range of 1733.68 cm-1 which is associated with B-diketone, Cyclohexanone, A,B- unsaturated esteres(5-member), Aryl esters with C=O stretch, range of 1456.49 cm-1 which is associated with Hydrocarbons, Aromatics with C=C, C-C stretch, the range of 1166.17cm-1 which is associated with Tertiary alcohols with C-O stretch, range of 834.62cm-1 which is associated with Disubstituted Aromatic comp. (para) with C-H deformation, range of 725.40cm-1 which is associated with Monosubstituted Alkenes with C-H deformation, range of 436.82cm-1 which is associated with Cycloalkane with C-C stretch(Shanmugavel, G., et al., 2018; Khalid, S., et al., 2023).
In FTIR analysis it was observed that hexane extract contains C-H, C=O, C=C, C-C, C-O, C- H , C-C bonds which indicates presence of Hydrocarbons, Alkanes, aldehydes, B-diketone, Cyclohexanone, A,B-unsaturated esteres(5-member), Aryl esters, Hydrocarbons, Tertiary alcohols, Disubstituted Aromatic comp, Monosubstituted Alkenes, Cycloalkane respectively(Marcus, A. C., et al., 2015;).
The hexane extract of Moringa oleifera has been widely studied for its diverse bioactive compounds

and potential applications. Saini et al. (2016) emphasized the nutritional and therapeutic significance of phytochemicals present in Moringa oleifera, indicating the potential presence of these compounds in the hexane extract (Saini et al., 2016). Additionally, found that the aqueous extract of Moringa oleifera had a high content of total phenolic compounds, which may also be present in the hexane extract (El-Gammal et al., 2017). This suggests that the hexane extract of Moringa oleifera may contain phenolic compounds, contributing to its potential antioxidant properties.

Furthermore, the study by Pandey et al. (2020) highlighted the efficacy of Moringa oleifera leaves hexane extract in improving water quality parameters, indicating the diverse applications of the hexane extract beyond its use in obtaining bioactive compounds (Pandey et al., 2020). This demonstrates the versatility of the hexane extract of Moringa oleifera in different fields, including environmental and water treatment applications.

In conclusion, the hexane extract of Moringa oleifera contains a diverse array of bioactive compounds, including oil content, phenolic compounds, and other phytochemicals. Its potential applications extend beyond the pharmaceutical and nutraceutical industries to include environmental and water treatment fields.

4.4 In vitro Antioxidant Assay

4.4.1 DPPH Assay:

DPPH scavenging activity of *Moringa oleifera*

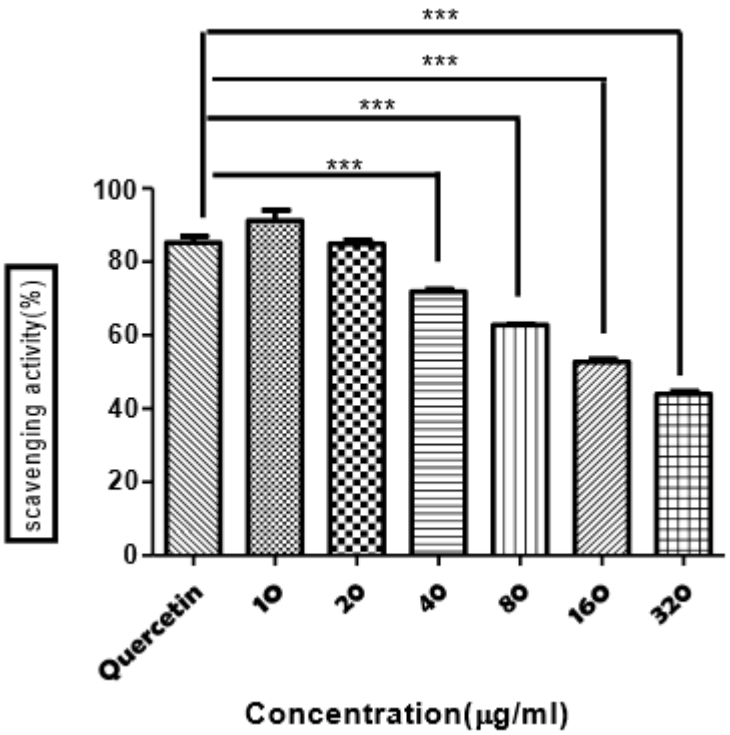

Fig. 2. DPPH assay of *Moringa oleifera* was tested to identify the free radical scavenging activity. The p value was less than 0.0001. ANOVA was performed followed by the Tukey test

The hexane extract of *Moringa oleifera* has been investigated for its antioxidant properties, particularly in relation to its ability to scavenge DPPH (2,2-diphenyl-1-picrylhydrazyl) radicals. Studies have demonstrated the antioxidant activity of Moringa oleifera leaf extracts through the reduction of DPPH radicals Sreelatha & Padma (2009). Additionally, the antioxidant activity of the Moringa oleifera flower extract was measured using the DPPH assay, showing a 59.65% scavenging activity (Jaglan, 2023). Furthermore, the ethanolic extract of Moringa leaves from Cameroon exhibited high DPPH scavenging activity, indicating its potent antioxidant properties (Segwatibe et al., 2023).

Moreover, the hexane extract of Moringa oleifera seeds has been shown to possess antioxidant activity, as evidenced by its concentration-response relationship in scavenging DPPH free radicals (Ali et al., 2020). Additionally, the antioxidant activity of Moringa oleifera leaves was evaluated using the DPPH assay, providing insights into its potential health benefits ("The Antioxidant and Antibacterial Activity of Moringa oleifera Extracts against some Foodborne

Pathogens", 2021). Furthermore, the hexane extract of combined Moringa oleifera plant materials demonstrated enhanced purification efficacy in comparison to individual extracts, highlighting its potential as a natural coagulant with antioxidant properties (Alam et al., 2020).
These findings collectively indicate that the hexane extract of Moringa oleifera possesses significant antioxidant activity, as demonstrated by its ability to scavenge DPPH radicals. This antioxidant potential holds promise for various applications in the pharmaceutical, nutraceutical, and environmental fields.

4.4.2 Hydroxyl Radical Scavenging Activity Assay:

Hydroxyl radical scavenging activity of *Moringa oleifera*

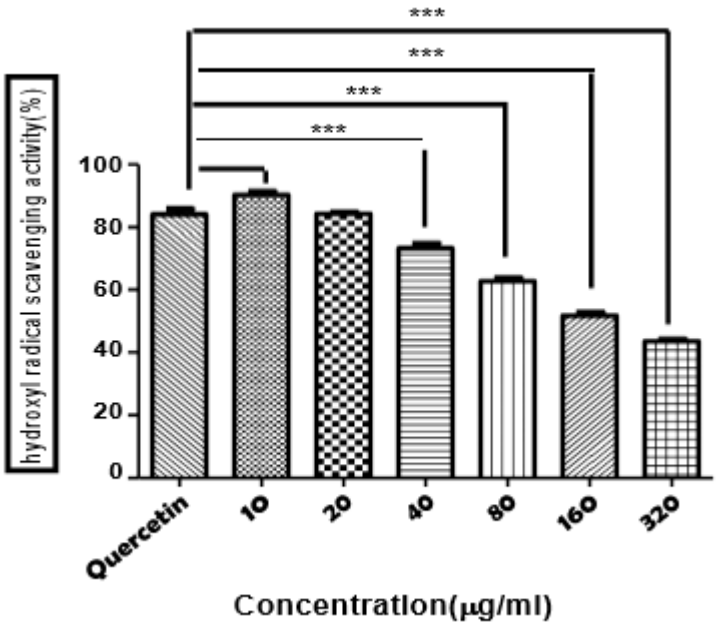

Fig. 3. Hydroxyl radical scavenging activity assay of *Moringa oleifera* was tested to identify the free radical scavenging activity. The p value was less than 0.0001. ANOVA was performed followed by the Tukey test

The hydroxyl radical scavenging activity of the hexane extract of Moringa oleifera has been a subject of interest in various studies. Siddhuraju & Becker (2003) investigated the scavenging effect of different solvent extracts from various Moringa leaf samples on superoxide radical (O2•-). Additionally, Sreelatha & Padma (2009) reported that the free radical scavenging effect of Moringa oleifera leaf extract was comparable to that of reference antioxidants, indicating its potential as a natural source of antioxidants. Furthermore, Muzammil et al. (2023) assessed the free radical scavenging effects of Moringa oleifera leaf extracts using the DPPH assay, demonstrating its antioxidant potential.

Moreover, the study by Parasuraman et al. (2021) evaluated the hydroxyl radical scavenging activity of methanolic and ethanolic extracts of seeds of Macrotyloma uniflorum, providing insights into the scavenging abilities of plant extracts. Additionally, Nnadi & Igbokwe (2022) highlighted the hydroxyl

radical scavenging abilities of phenolic extract from Moringa oleifera leaves, further emphasizing its potential antioxidant properties. Furthermore, Santos et al. (2012) demonstrated the antioxidant activity of Moringa oleifera tissue extracts, showing the highest free radical scavenging activity with an IC50 value of 49.30 µg/mL in the DPPH assay.

These studies collectively indicate that the hexane extract of Moringa oleifera possesses significant antioxidant potential, as evidenced by its ability to scavenge hydroxyl radicals. This antioxidant activity holds promise for various applications in the pharmaceutical, nutraceutical, and food industries.

4.4.3 Brine shrimp lethality test result (BSLT):

Control 25 µg/ml 50 µg/ml 75 µg/ml 100µg/ml

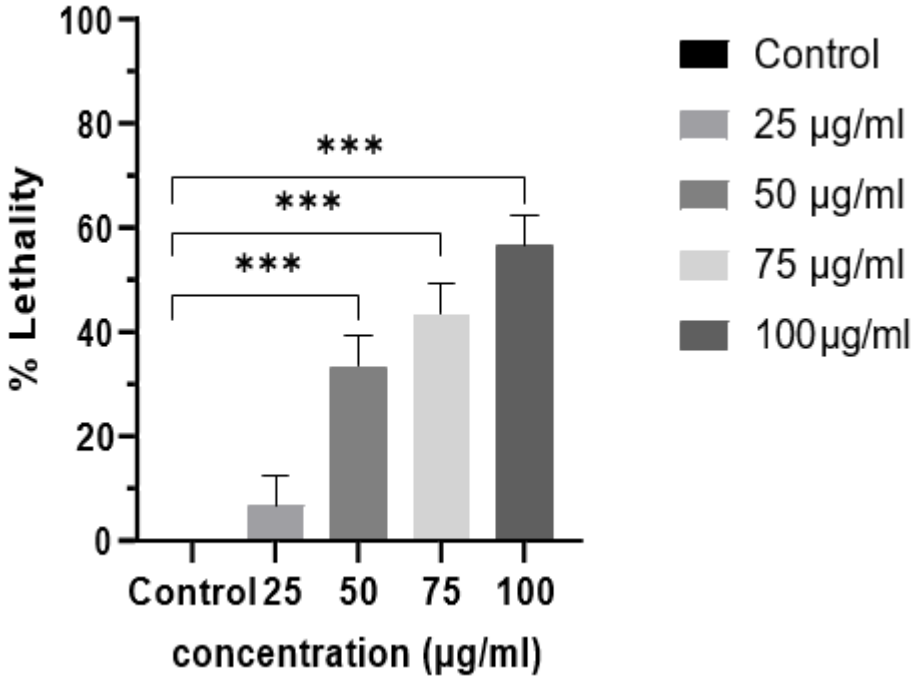

Fig.4 Graph represents effect of control and hexane extract of *Moringa oleifera* on brine shrimp after 24hr (50 µg/ml,100 µg/ml, 200 µg/ml,400 µg/ml). Data is analyzed by one way ANOVA followed by Tukey's Multiple Comparison test, where ***$p \leq 0.001$ when compared with Control group.

The brine shrimp fatality assay is a quick, low-cost, and straightforward bioassay for evaluating plant extracts bioactivity (Sarah, Q. S., et al., 2017). The brine shrimp lethality assay (BSLA) is a straightforward and low-cost bioassay that is used to evaluate the potency of phytochemicals found in plant extracts. The results of the current investigation showed that the lethality's degree was directly related to extract concentration. All of the brine shrimp in the control group lived after 24 hours of monitoring. Even still, low concentrations had the lowest lethality rates and high concentrations had the highest lethality. It was shown that in larger concentrations of the treatment extracts, the shrimps started to die only after 8 hours and perished completely after 24 hours (Waghulde, S., et al., 2019;).

LC50 obtained from the brine shrimp analysis of moringa oleifera hexane extract was observed to be 129.7 µg.

All the experimental results in brine shrimp lethality test (BSLT) were as mean±SD of three measurements. The percent of mortality (% M) of the brine shrimp nauplii was calculated for each concentration. The median lethal concentration (LC50) of the test samples were determined from the twenty-four hours count using the probit analysis method(Obi, O. O. B., 2018; Niksic, Haris, et al.,

2021). Statistical analysis done by using one way anova.

4.4.4 Ex-vitro chicken eye toxicity test observation: -

Control Saline 50 µg/ml 100 µg/ml

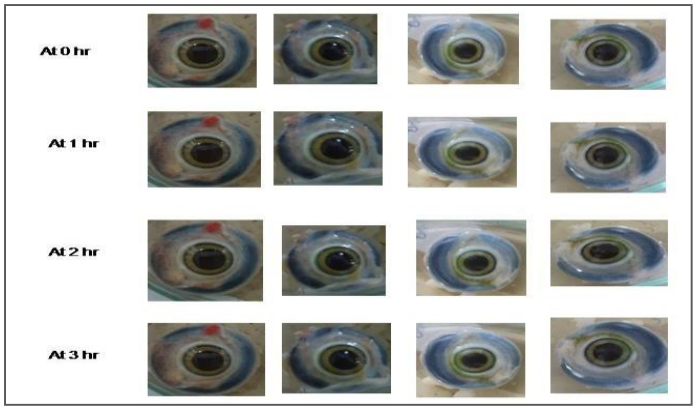

Fig. 5 Representative Eye toxicity images of the control and treated group

We observe that there is no damage of eye at concentration 50 µg/ml and 100 µg/ml from 0 to 3 hours after applying the concentrations. Performing in-vitro eye study extract observe that no ocular corrosion or severe irritation to the eye, So no toxicity was observed to the eyes(Dutok, Carlos, et al., 2015; Prinsen, Menk K., et al., 2017)

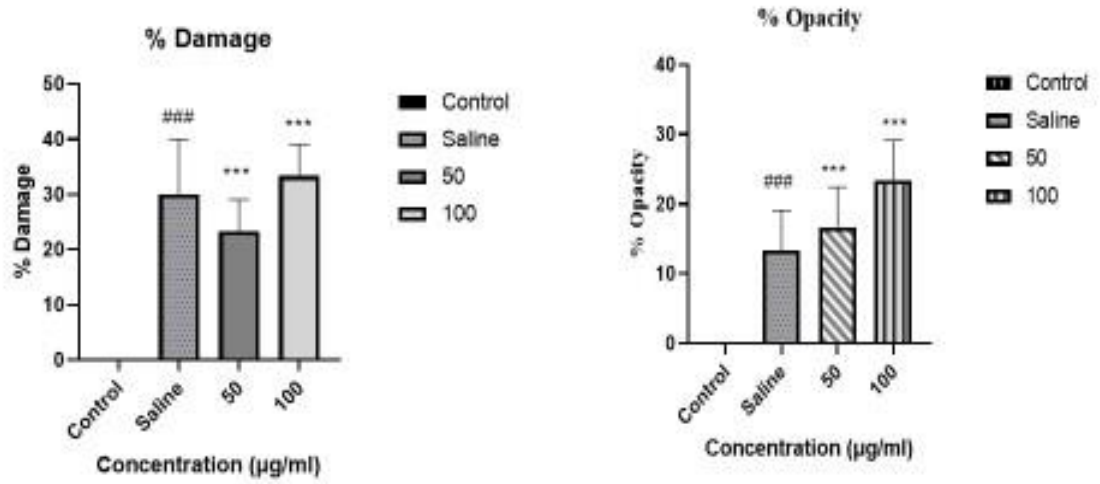

Fig.6. Effect of Control, Standard (saline) and Hexane extract of *Moringa oleifera* on chicken eye after 3hr (50 µg/ml, 100 µg/ml,). Data is analyzed by one-way ANOVA followed by Tukey's Multiple Comparison test, where $^{###}P \leq 0.001$ when compared with the Control group and $***p \leq 0.001$ when compared with the Standard that is saline.

The OECD 438 guideline has been used by the industry to screen items early in the product development cycle to identify product compositions that might have an unsatisfactory eye irritation/corrosion profile (OECD 438 Lowther et al. and Das, S. K., et al. 2017).
Damage, and opacity were employed as the parameters to evaluate the toxicity of the formulation on the chicken eye. Each parameter was scored on a scale from 100 to 0, with a score of 100 signifying severe eye injury and a score of less than 100 signifying less eye damage.
In the present study, according to the damage parameter for the hexane extract of *moringa oleifera*, maximum damage was seen in the chicken eye treated with a 100 µg/ml concentration of both extracts when compared to the control, while only minor damage was seen in the one treated with a 50 µg/ml

concentration of both extracts. However, this suggests that the 100 µg/ml non-polarcinn extract formulation of moringa oleifera was to some degree hazardous. The opacity parameter evaluated the eyeball's transparency or lack thereof. After 3 hours of observation, the 100 µg/ml concentration had the highest level of opacity of both the extract.

4.4.5 Antimitotic assay result:

Fig.7 Inhibition of blastema development in Eudrilus eugeniae by *moringa oliefera* extract.

Hexane extract of *moringa oliefera* was injected similarly for 7 consecutive days and the development of blastema was observed after day 7 at concentrations 160ug/ml. extract of Moringa oliefera was injected every 24 h for a period of 7 days and the development of blastema was not observed after day 7 at concentrations of 320ug/ml to 640ug/ml**.**

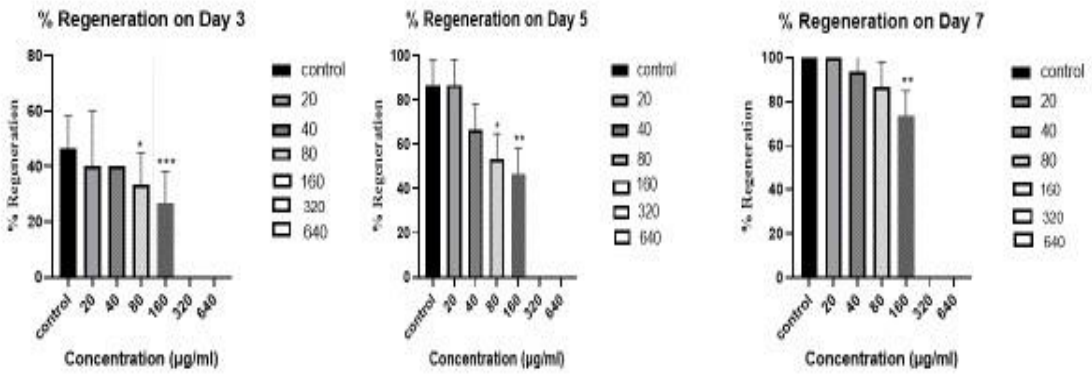

Fig. 8 % Regeneration of Earthworms on Day 3, Day 5 and Day 7 at different concentrations of *Moringa oleifera* leaf extract (20µg/ml, 40 µg/ml, 80 µg/ml, 160µg/ml, 320µg/ml, 640µg/ml).

The control and treatment worms were injected with distilled water and extract formulation once in 24 hrs for a period of 7 days. Development of blastema was observed on 3^{th},5^{th} and 7^{th} day at 20µg/ml, 40 µg/ml, 80 µg/ml, and 160µg/ml, and no blastema development was observed at 320 µg/ml and 640µg/ml. Data is analyzed by one way ANOVA followed by Tukey's Multiple Comparison tests, where *(*P ≤ 0.05, **P ≤ 0.001 ***P ≤ 0.0001*) compared with the control group. According to Khazir et al. (2014), substances derived from plants or other natural resources may be employed as medications or as leads for potential cancer treatment components. Plant- derived antimitotic chemicals are now employed as chemotherapeutic agents to treat cancer. Since more than 35 years ago, taxol and its variants have been used to treat non-small-cell lung cancer, ovarian cancer, and breast cancer. A combination of chemotherapeutic medications can stop cell division (Mukhtar et al., 2014).

One of the problems with chemotherapy is the development of drug resistance to antimitotic drugs (Van et al., 2015). To find new active components that can treat cancer, more chemicals need to be investigated globally. Even with modest facilities, we need a simple technique to screen more compounds globally. Here, we present a technique for prescreening chemicals for their antimitotic potential using earthworms. Aqueous extracts of A. calamus rhizomes and the widely accessible antimitotic drug colchicine were used to validate this approach.

An easy in vivo animal model for investigating antimitotic drugs is earthworms. It is predicted that blastema formation after wound repair will require active mitotic cell division in the earthworm's cut regions. Therefore, utilizing the ability of severed earthworms to regenerate, it is feasible to assess the potential of antimitotic medications. (Subramanian et al., 2017; (Rajamanikkam, K., at al.,2019). The anterior and posterior parts of the segmented earthworm E. eugeniae are separated by a clitellum. In this species, blastema regeneration from severed regions has been documented (Samuel et al., 2012; (Rajesh, C., et al., 2021).

In the present study, three days following amputation is when regeneration of the blastema from amputated regions, which necessitates quick mitotic cell division, is typically seen. The antimitotic capability of the compounds was confirmed using the ability of severed regions to regenerate. For a period of seven days, injections of sterile distilled water (control), *moringa oleifera* extract were given once every 24 hours. All control worms underwent blastema regrowth after 3, 5, and 7 days. In the case of *moringa oleifera*, blastema regeneration was seen in earthworms treated with *moringa oleifera* extract at concentrations 20µg/ml,40µg/ml,80µg/ml,160µg/ml, and regeneration was not seen in earthworms treated with *moringa oleifera* extract with concentrations of 320µg/ml and 320µg/ml upto 7 days. This proves that hexane extract of *moringa oleifera* with antimitotic action. Thus, this assay could be utilized to confirm the existence of unidentified antimitotic chemicals.

5. CONCLUSION

In this study, non-polar fractions were isolated from the Moringa oleifera extract through the application of hexane as a solvent during the preparation process. Subsequent phytochemical analysis revealed the presence of a diverse array of compounds within the extract, including proteins, lipids, tannins, glycosides, terpenoids, saponins, and phenolic compounds. Fourier Transform Infrared (FTIR) spectroscopy provided spectral data characterized by a wide range of absorption peaks. Through the interpretation of these peaks, it was possible to deduce the types of molecular vibrations and infer the presence of specific functional groups. The safety of the extract was evaluated using an ex-vivo model involving isolated chicken eyes, which demonstrated no adverse effects. The cytotoxicity of the extract was assessed via the brine shrimp lethality assay, revealing a dose-dependent increase in lethality. This correlation was graphically represented, showcasing a direct relationship between extract concentration and mortality rate. Given its antimitotic properties, the non-polar extract of Moringa oleifera holds potential for therapeutic applications in cancer treatment, attributable to its capacity to inhibit cell division.

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