Assessment of In-Vitro Hemolytic Activity of *"Myristica Fragrans Houtt"*

Amol V. Pore, Sanjay K. Bais , Gopika D. Dongare, Pranali B. Bandgar , Meher S. Sutar
Fabtech College of pharmacy, Sangola Dist – Solapur, Maharashtra.
Corresponding Author: pranalibandgar362@gmail.com

ABSTRACT

Myristica fragrans is the source of javetri, or mace, help for its therapeutic qualities and contains a number of bioactive substances. Study is designed to use spectrophotometric analysis hemolytic activity of Javetri extract. Key components like alkaloids, flavonoids, tannins, and saponins, the extract was first put through a preliminary phytochemical screening process using an appropriate solvent extraction technique Red blood cells were exposed to varying concentrations of the extract in a lab setting for the hemolytic assay. Using a spectrophotometer set ideal wavelength; liberated hemoglobin was measured to ascertain hemolysis. The experimental results were validated by using standard compounds as references. Statistical techniques were used to calculate and interpret the percentage of hemolysis. According to the results, Javetri extract exhibits significant hemolytic activity, which could be related the inclusion of particular phytochemicals. The current analysis enriches the available scientific information of Javetri's biological effects and highlights the need for additional research to assess its safety and possible therapeutic uses.

KEYWORDS: Myristica fragrans, Organic extract, phytochemical screening, Hemolytic activity, Therapeutic plants.

INTRODUCTION

Myristica fragrans (nutmeg), especially its aril known as Javetri (mace), is highly enriched with essential oils and bioactive phenolic constituents and terpenoids. These constituents are known produce various medicinal effects, including antimicrobial, antioxidant, and membrane-active properties. [1]

An essential biological metric for assessing how substances affect the integrity of red blood cell (RBC) membranes is hemolytic activity. Because the erythrocyte membrane resembles other biological membranes, it provides a straightforward and sensitive model for studying membrane stability. Hemolysis is the term for the release of hemoglobin when this membrane is disrupted by exposure to specific phytochemicals. Thus, measuring hemolytic activity offers important insights into the cytotoxic potential and safety of extracts derived from plants. [2]

A number of substances found in Javetri engage with cell membrane proteins and lipid bilayers alter permeability of membranes. At higher concentrations, these interactions may result in unwanted cell damage, contribute to therapeutic effects. These effects are frequently assessed in controlled laboratory settings using in vitro hemolytic assays, which provide a quick and accurate way to identify dose-dependent responses. [3, 4]

To balance the medicinal benefits of Myristica fragrans extract with safety concerns, it is crucial to comprehend its hemolytic behavior. Its in vitro hemolytic activity is the main focus. [5]

Definition of Hemolysis

Hemolysis represents the breakdown or destruction of red corpuscles due to disruption of cellular boundary that leads to intracellular components, mainly hemoglobin, to be released into the surrounding fluid. Numerous physical, chemical, or biological elements that jeopardize membrane integrity may cause this process. [6]

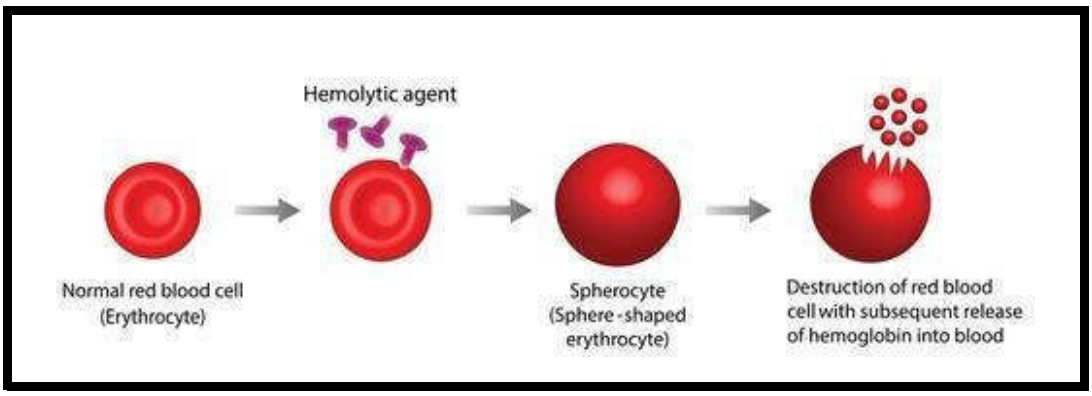

***Figure 1**: Hemolysis.*

Physiology of Red Blood Cells

Red blood cells (erythrocytes) play a vital role in transporting oxygen throughout the body tissues and carrying carbon dioxide back to the lungs. This function is performed by hemoglobin, an iron-containing protein present in RBCs. They have a biconcave disc shape, thereby improving surface availability for efficient gas exchange and allow flexibility to pass through narrow capillaries. Mature RBCs lack a nucleus and other organelles, providing more space for hemoglobin and enhancing their oxygen-carrying capacity. Their cell membrane plays an important role in maintaining shape, flexibility, and osmotic balance. [7]

Types of Hemolysis

Intravascular Hemolysis

RBCsare destroyed within arteries and veins rather than in organs like the spleen or liver. Leadsto direct release of hemoglobin in systemic circulation [8]

Extravascular Hemolysis

Is a process that primarily occurs in organs like the splenic and hepatic organs and includes destruction of red blood cells? RBCs become less flexible can readily be identified and eliminated by reticuloendothelial system macrophages when they get old, damaged, or have an irregular shape. Instead of being released straight into the bloodstream in type of hemolytic, hemoglobin is lysedinside these cells into components like bilirubin, which is subsequently sent to the liver for additional processing. Because of this, extravascular hemolysis frequently results in elevated levels of indirect (unconjugated) bilirubin, which can occasionally cause jaundice. [9]

Osmotic Hemolysis

Consequently, water passes into the red blood cells through osmosis, causing them to swell and ultimately rupture (lysis). Hemoglobin is released into the surrounding fluid. It is frequently employed in lab experiments to evaluate RBC osmotic fragility and investigate membrane stability. [10, 11, 12]

Immune Hemolysis

When antibodies mistakenly identify red blood cells (RBCs) as foreign, the body's immune system destroys them, a process as immune hemolysis. When these antibodies bind to the surface of red blood cells, they either cause intravascular hemolysis in blood vessels or extravascular hemolysis in organs such as the spleen. Hemolytic disease of the newborn, incompatible blood transfusions Anemia, jaundice, and other associated complications are possible outcomes. [13, 14]

Mechanical Hemolysis

The breakdown of red blood cells leads of trauma or physical forces is known as mechanical hemolysis. This can happen in mechanical heart valves and dialysis devices or during intense physical activity when red blood cells encounter to shear stress, turbulence, or compression. Because of the damage to the cell membrane caused by these forces, the cells burst and release hemoglobin into the blood. Mechanical hemolysis is frequently linked to medical devices or conditions that change normal blood flow and it can result in anemia. [15, 16]

Laboratory Diagnosis

Diagnosing Hemolysis involves various pathological examinations

CBC: Decreased hemoglobin and hematocrit (anemia)
Reticulocyte Count: Increased (bone marrow compensation)
Peripheral Blood Smear: Abnormal RBC shapes Lactate Dehydrogenate (LDH): Increased
Indirect (Unconjugated) Bilirubin: Increased
Haptoglobin Level: Decreased
Direct Coombs Test: Positive in immune hemolysis
Urine Examination: Hemoglobinuria (in intravascular hemolysis)
Serum Hemoglobin: Increased (in intravascular hemolysis) [17]

MATERIAL AND METHODOLOGY

1. Plant Collection and Authentication

The best time for collection of Myristica fragrance Houtt when the fruit is fully ripe and naturally splits open were collected from sangola, solapure, Maharashtra, India. The plant was authenticated by Mr. Tebhurne R.R.M.Sc.B.Ed Botony plant physiology.

Plant profile

Figure 2: *Myristica fragrance Houtt*

Synonym: Jaatishasya, Maalatiphala (seed kernel).
Biological source: Myristica fragrans Houtt. (Nutmeg) is obtained from the dried seed and aril (mace) of the fruit of the evergreen tree.
Family: Myristicaceae
Color: Dark brown (seed), bright red when fresh and yellowish-orange to reddish-brown when dried (aril/mace).
Odour: Strong, aromatic, and characteristic spicy fragrance.
Taste: Warm, slightly sweet, and strongly aromatic with a pungent spicy taste. [18]

Description: Myristica fragrans Houtt It is a medium-sized aromatic tree that produces a fleshy yellow fruit containing a single seed known as nutmeg, surrounded by a lacy red aril called

mace. The seed is hard, oval, and dark brown in color, while the aril is reddish when fresh and turns yellowish-brown on drying. Both nutmeg and mace are highly aromatic and widely used as spices and in traditional medicine for their carminative, antioxidant, and antimicrobial properties. [19]

Seedling care – Young seedlings are

Geographical Distribution

Indonesia – Native and largest producer (Maluku/Spice Islands)

India – Mainly Kerala, Karnataka, Tamil Nadu

Sri Lanka – Cultivated in humid tropical regions

Malaysia – Grown in small to moderate scale plantations

Grenada – One of the major global exporters ("Spice Island")

Caribbean Islands – Including Trinidad and Tobago, Jamaica

Brazil – Limited cultivation in tropical zones

Papua New Guinea – Small-scale production in suitable climates

Cultivation and cultivation

Climate requirement – Myristica fragrans grows well in a tropical climate that is warm, humid, and an average temperature of 20–30°C and high rainfall throughout the year.

Soil requirement – It prefers deep, well-drained, fertile loamy soil rich in organic matter. Waterlogging conditions should be avoided.

Nursery preparation –Seeds are shielded from the sun using shade nets or partial shade and are watered regularly to maintain moisture.

Transplantation – When seedlings reach sufficient height (about 20–30 cm), they are transplanted into the main field at proper spacing.

Field planting – Pits are prepared in advance and filled with topsoil and organic manure before transplanting. Proper spacing ensures good growth and aeration.

Shade management – Since it is a shade-loving plant in early stages, intercrops or shade trees are used to protect it from direct sunlight.

Irrigation – Regular irrigation is essential, especially during dry seasons, to maintain soil moisture and healthy growth.

Manuring and fertilization – Organic manure and balanced fertilizers are applied periodically to improve growth, flowering, and fruit yield.

Weeding and maintenance – Regular weeding, mulching, and pest control are done to maintain plant health.

Flowering and fruiting – The tree begins flowering after about 6–8 years, and fruits develop over several months.

Harvesting – Harvesting fruits occurs when it turn yellow and split open naturally on the tree.

Storage -Stored in airtight containers somewhere dry and cool, far from moisture and light to preserve aroma and active constituents. [20]

Chemical constituent

It contains essential oils (such as myristicin, safrole, eugenol, and elemicin), fixed oils, phenolic compounds, flavonoids, lignans, and starch.

Uses

Flavoring agent in food.
Carminative (relieves gas and bloating).
Improves digestion and appetite.
Applied asaaromatic stimulant.
Possesses antioxidant activity.
Shows antimicrobial properties.
Has an anti-inflammatory effect.
Used in traditional medicine for mild pain relief and relaxation. [21]

2. Preparation of Plant extracts Myristica fragrance Houtt

Collection and Plant Material

Javetri (mace) is harvested from ripe Myristica fragrans fruits that have naturally split open, exposing the bright red aril, indicate maximum maturity and active constituents. The fruits are either plucked or gathered right after they fall, and the mace is carefully separated from the seed. To avoid moisture contamination, collection should be done in dry conditions in the morning or later afternoon.

Cleaning and Drying

Myristica fragrans Houtt. is cleaned and dried by removing the outer fleshy pericarp after harvesting and carefully separating the mace (aril) from the seed, then washing to remove dirt and discarding any damaged or infected material. The mace and seeds are then dried separately, usually in the sun; mace dries in 10-14 days until crisp, while seeds take 4-8 weeks until the kernel shrinks within the shell. The material is turned frequently during the drying process to ensure uniform moisture removal, and proper drying is required prior to storage to prevent fungal growth and spoilage.

Grinding and Crushing

occurs after proper drying, in which the hard shells of the seeds are first broken to obtain the kernels, which are then crushed or ground into a fine powder using mechanical grinders. This process increases surface area, aiding in the extraction of active constituents and enhancing flavor and aroma; however, it must be done carefully under controlled conditions to avoid volatile oil loss and overheating, which can reduce final product quality. [22]

3. Preparation of Ethanolic Extract

Can be prepared by placing the dried powder in a Soxhlet apparatus and extracting it with ethanol as the solvent; the system is then maintained at around 60°C and allowed to run continuously for about 6-8 hours to ensure efficient extraction of active constituents; the obtained extract is then filtered and concentrated to yield the final ethanolic extract.[23]

4. Preparation of Phosphate buffer Solution

A phosphate buffer solution is made by dissolving suitable amounts of potassium dihydrogen phosphate (KH_2PO_4) and disodium hydrogen phosphate (Na_2HPO_4) in distilled water to achieve the required pH (typically 6.8–7.4). Distilled water is used to make up the final volume after the solutions are combined in the appropriate amounts and the pH is measured with a pH meter. For later use in the extraction or analysis of MyristicafragransHoutt, the prepared buffer is filtered if needed and kept in a clean, airtight container. [24]

5. Calculate the Proportions

Ascertain the buffer solution's ideal pH, which is typically between 6.8 and 7.4. Determine the necessary amounts of dibasic sodium phosphate (Na_2HPO_4) and monobasic potassium phosphate (KH_2PO_4) using the Henderson–Hasselbalch equation or standard buffer tables.

Prepare stock solution

Make stock solutions of Na_2HPO_4 (0.2 M) and KH_2PO_4 (0.2 M) by dissolving precisely weighed amounts in distilled water. Make sure it dissolves completely.[25]

Mixing

To achieve the required pH, gradually add the calculated volumes of both stock solutions into a clean container while stirring constantly.

Adjust the pH

by measuring it with an indicator paper or pH meter. Adjust by adding tiny amounts of either solution if necessary. [26]

Final Dilution (if needed)

Once the pH of the buffer is adjusted to the desired range, make up the volume with purified water and combine thoroughly.

Filtering (optional)

If required, filter the buffer solution using a sterile filter to remove any particulate matter or impurities.[27]

Sterilization (optional)

For biological applications, sterilize the buffer by autoclaving at 121°C for 15 minutes or by passing via a 0.22 µm membrane filter.

Storage

Store the prepared phosphate buffer solution in a clean, airtight container. It can be kept at room temperature for short-term use or refrigerated for longer storage.

6. Preparation of erythrocyte cells

Samples of blood are taken from healthy volunteers. The cells are washed using PBS by centrifugation. After centrifuging about 5 mL of blood, the pellet is washed and re-suspended to obtain a 0.5% erythrocyte suspension. [28]

7. Perform the in-vitro hemolytic activity test

Prepare different concentrations of Javetri extract and mix with erythrocyte suspension. Incubate and centrifuge the samples, then collect the supernatant. Measure absorbance using a UV-Visible spectrophotometer and calculate the percentage hemolysis. [29]

Phytochemical Investigation [30]

Sr. No	Name of Test	Observation	Inference
1	**Test for Phenol:** Extract Mixed with 2ml of 2% of Solution of $Fecl_3$	Blue /green color	Phenol Present
2	**Test for Saponin:** A portion of the extract was taken in a test tube and agitated strongly with water.	formation of stable foam	Saponin present
3	**Test for Tannins:** Extract Mixed with 2% of Fecl3	Black color	Tannins Present
4	**Test for Terpenoids:** The extract Mixed with chloroform, then 2ml of conc. sulphuric acid added gently and shaken gently	Reddish brown color observed in the inerphase	Terpenoids Present
5	**Test for Flavonoids:** The extract was subjected to treatment with sodium hydroxide solution.	Observation of strong yellow coloration.Which becomes colorless on the addition of dilute acid	Flavonoids Present
6	**Test for Glycoside:** A mixture of the extract and glacial acetic acid with 2% $FeCl_3$ was prepared and subsequently added to concentrated sulphuric acid in a separate test tube.	visible brown ring developed at the interface	Carbohydrate Absent
7	**Test for Protein:** The extract was carefully mixed with a 'few drops of conc. Nitric acid	Formation of yellow color	Protein Absent
8	**Test for Alkaloids:** **A) Dragandroff's test:** A few ml of filtrate,1 or 2ml Dragandroff 's reagent	Brown colored ppt	Alkaloids Present
	B) Mayer's test: To few ml of extract, 2 drops of Mayer's reagents	Cream colored ppt	Alkaloids Present
	C) Hager's test: To few ml of extract 1 or 2ml of Hager's reagent add	Yellow colored ppt.	Alkaloids Present
	D) Wagner's test: To few ml extract, a minimal volume of Wagner's reagent	Reddish brown colored ppt	Alkaloids Present

Table no. 1: *Phytochemical screening of Myristica fragrance Houtt.*

EXPERIMENTAL WROK

Requirement

Chemical used

Ethanol, PBS buffer Solution, Distilled water Triton x-100

Apparatus used:

Test Tube, Heparin Tube, Beaker, Stirrer. 5ml Syringe. Soxhlet Extractor.

Equipment used:

Centrifugation machine, Incubator, UV- Vis spectrophotometer

Procedure:

1. Procedure of phytoextract Myristica fragrans Houtt:

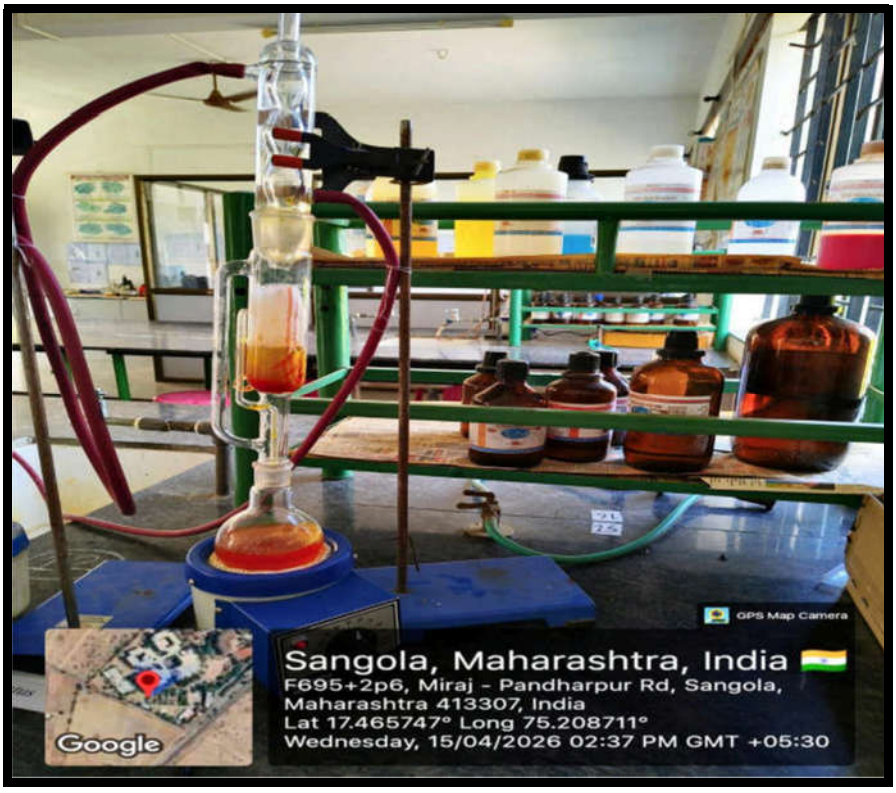

Figure 3: *Preparation of Plant Extract.*

25 g of dried, coarsely powdered sample is placed in a thimble inside the Soxhlet extractor, which is assembled with a round bottom flask containing 200–250 mL ethanol at the bottom and a condenser on top with continuous water flow. The setup is heated at about 60°C so ethanol evaporates, condenses, and repeatedly siphons through the sample for 6–8 hours until extraction is complete; the solvent is then evaporated to obtain the crude extract.[31]

2. Preliminary Phytochemical screening of Extraction

Figure 4: *Phytochemical screening.*

3. Preparation of phosphate buffer solution

To prepare a phosphate buffer solution, you need to mix monobasic sodium phosphate and dibasic phosphate in appropriate proportions to achieve the desired PH. [32]

1. Calculate buffer ratio

Use the Henderson-Hasselbalch equation to calculate the ratio of monobasic to dibasic sodium phosphate needed to achieve the desired PH. The equation is pH = pKa +log ([A-]/[HA]) . [33]

2. Prepare stock solution

Prepare separate stock solutions of monobasic sodium phosphate (0.1 M) and dibasic sodium phosphate (0.2 M) by dissolving the appropriate amounts of each salt in distilled water. Ensure complete dissolution of both solutions before use. [34]

4. Adjust pH

Check the pH of the buffer using a pH meter and adjust it to the desired value by adding small amounts of acid or base as required.

5. Storage

Store the prepared buffer solution in a clean, airtight container at room temperature or as required for further use. [35]

Figure 5: *Preparation of phosphate buffer.*

4. Preparation of erythrocyte cell

1. Collection of Blood

Draw fresh blood a tube containing an anticoagulant such as EDTA or heparin and process it within 1–2 hours.

2. Centrifugation

Spin the sample at about 3000 rpm over 10 minutes to separate the red blood cells from plasma.

3. Washing

Remove the plasma and flush the packed blood cells with isotonic saline or phosphate buffer. Repeat this step 2–3 times, centrifuging at 3000 rpm for 5 minutes each time.

4. Preparation of Suspension

Dilute the washed erythrocytes with phosphate buffer (pH 7.4) to obtain a 2% v/v cell suspension.

5. Storage

Keep the prepared erythrocyte suspension at 4°C and use it within 24 hours for accurate results [36]

Figure 6: *separation of Serum and RBCs.*

5. Hemolytic Activity Test

Preparation of RBCs

Separating erythrocytes from a blood sample by centrifugation, washing them with normal saline, and resuspending the cells to obtain a clean sample for laboratory analysis.

Washing of RBCs

Washing of red blood cells is carried out by repeatedly rinsing erythrocytes with an isotonic saline solution after centrifugation to remove plasma, proteins, and impurities, resulting in a purified cell sample for laboratory use.

Preparation of Test sample

Test samples are prepared by dissolving the required substance in saline phosphate buffer at different concentrations (25, 50, 75, and 100 g/ml). The material used may be a natural extract, synthetic compound, or pharmaceutical formulation.

Incubation with RBCs

RBC suspension is mixed with test samples and kept under physiological conditions (around 37°C, pH 7.4) for 1–2 hours. [37]

Centrifugation

After incubation, the mixture is centrifuged to separate intact cells (pellet) from lysed cells present in the supernatant.

Measurement of Hemolysis

Hemolysis is determined by measuring the absorbance of the supernatant at around 540 nm using a UV-visible spectrophotometer. The extent of red cell lysis is calculated as a percentage by comparing the test sample absorbance with positive and negative controls. [38, 39]

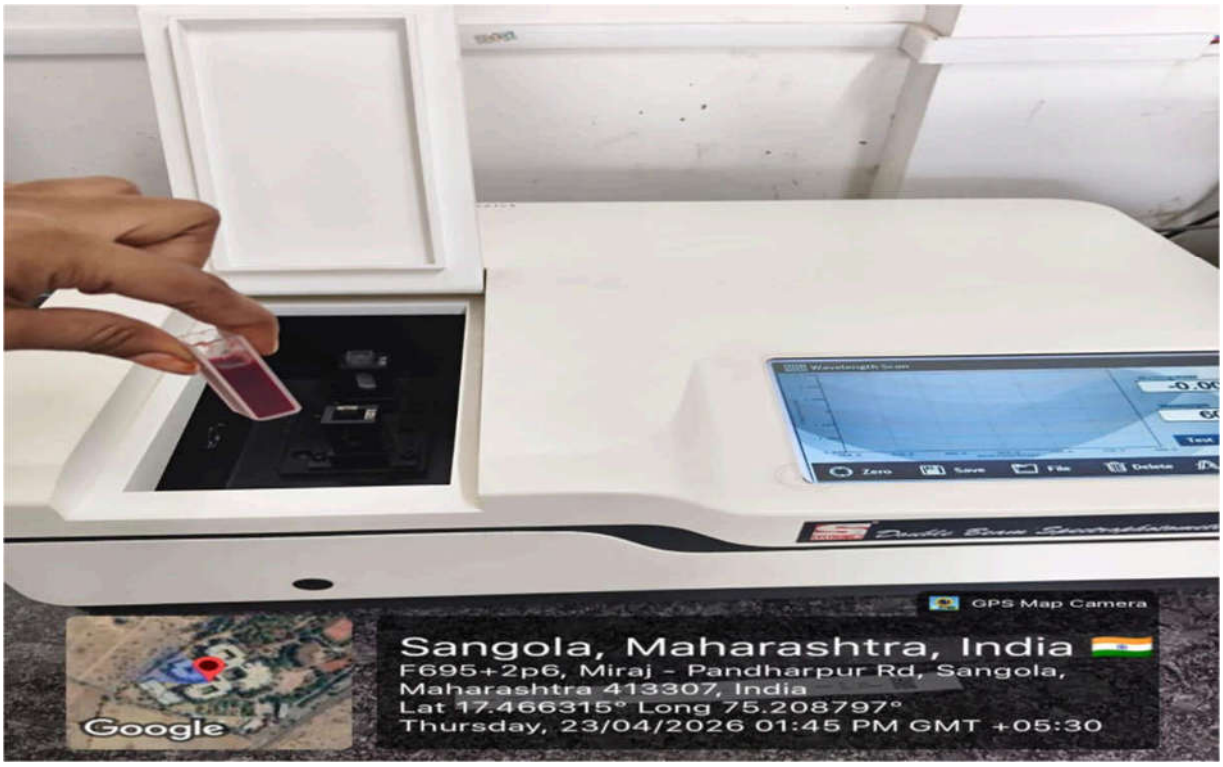

Figure 7: *Checking Absorbance of Sample.*

RESULT

The following formula used for assess proportion of Hemolysis.

% Hemolysis = [(At – An) / (Ac – An)] .100

Where,
At: Absorbance of the test sample
An: Minimum control absorbance (PBS solution)

Ac: Maximum control absorbance (Distilled water)

Observation

Sr.No	Concentration	Absorbance	Hemolysis	Protection
1	25 ml	0.042	72.14%	0.27
2	50ml	0.118	46.82%	0.53
3	75ml	0.205	18.64%	0.51
4	100ml	0.332	7.25%	0.92

Table no. 2: *Absorbance of Sample.*

Preliminary phytochemical evaluation of the ethanolic extract of Myristica fragrans confirmed the presence of several bioactive secondary metabolites. Compounds such as saponins, phenolic compounds, tannins, terpenoids, flavonoids, and alkaloids were detected in the extract, while proteins and carbohydrates were absent. These phytoconstituents are widely associated with antioxidant and protective biological activities, suggesting the therapeutic potential of the plant extract.

Sr. No	Constituents	Observation
	Ethanolic Extract	+
1	Saponins	+
2	Phenols	+
3	Tannins	+
4	Terpenoids	+
5	Flavonoids	+
6	Proteins	-
7	Carbohydrates	-
8	Alkaloids	+

Table No. 3 : *Result of preliminary phytochemical screening of Myrestica Fragarns Houtt.*

(-) indicates absence of compound

(+) indicates presence of compound

The hemolytic behavior of the ethanolic extract of Myristica fragrans was investigated using human erythrocytes, and the results were represented in terms of percentage hemolysis. The experimental findings revealed that the extract produced varying hemolytic effects depending on its concentration. A minimal hemolytic response of 7.25% was detected at 100 g/ml, whereas the extract at 75 g/ml produced 18.64% hemolysis. At 50 g/ml, a moderate erythrocyte lysis of 46.82% was recorded, while the maximum hemolytic effect of 72.14% was observed at 25 g/ml.

These results suggest that higher concentrations of the extract provided greater protection against erythrocyte membrane disruption. The biological activity of the extract may be associated with the presence of several phytoconstituents such as flavonoids, phenolic compounds, tannins, terpenoids, and alkaloids. Evaluation of hemolytic activity serves as an important parameter for determining the cytotoxic influence of plant extracts on normal red blood cells and for analyzing their membrane protective or membrane damaging potential. The intermediate hemolytic pattern observed during the investigation indicates that the extract possesses moderate cytotoxic properties toward human erythrocytes.

Sr. no	Concentration	Hemolysis %
1	25ml	72.14%
2	50ml	46.82%
3	75ml	18.64%
4	100ml	7.25%

Table no. 4: *Percentage of Hemolysis.*

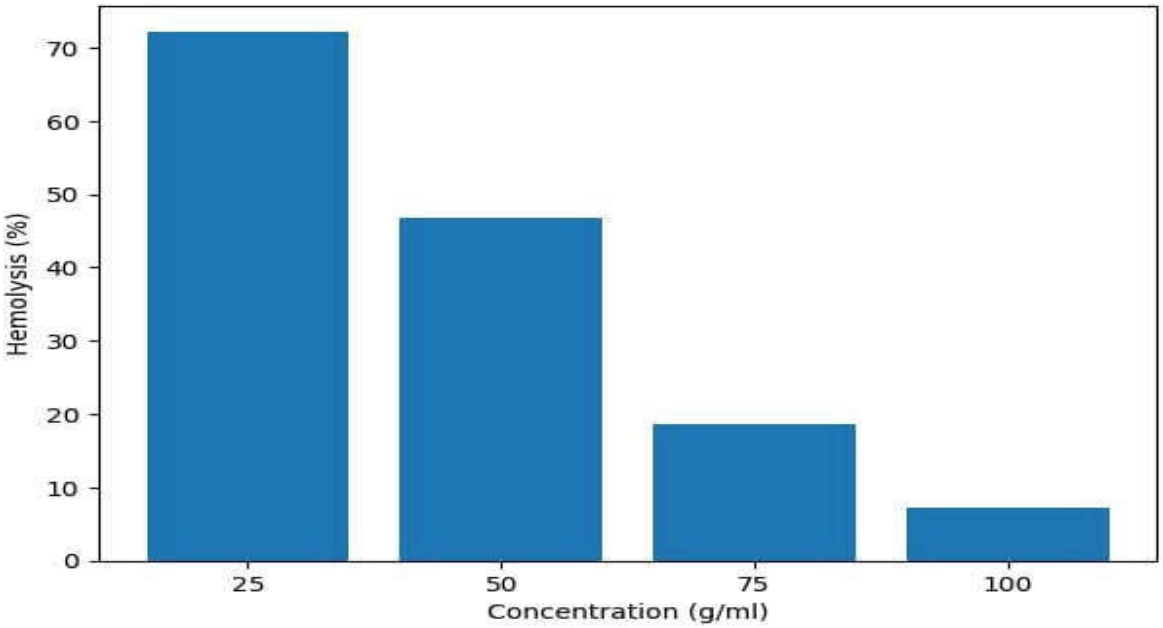

Figure 8: *Bar Graph of Hemolytic Activity of Javetri*

DISCUSSION

The current investigation was conducted to study phytochemical profi and in-vitro erythrocyte hemolytic effect of the ethanolic extract of Myristica fragrans Houtt. The phytochemical evaluation revealed the existence of several biologically constituents, tannins, phenolic compounds, terpenoids, and saponins, whereas proteins and carbohydrates were not detected. Are widely recognized for their antioxidant, protective, and therapeutic activities.

The hemolytic study demonstrated that the extract exhibited a concentration-related effect on red blood cell membranes. A greater percentage of hemolysis was observed at lower concentration levels, while higher concentrations produced reduced erythrocyte damage. The extract showed maximum hemolysis at 25 µg/mL and minimum hemolysis at 100µg/mL, indicating possible membrane eprotective behavior at elevated concentrations. This effect may be associated with the interaction of phytoconstituents with membrane proteins and phospholipid layers of erythrocytes.

The findings present investigation align with earlier reports suggesting that compounds present in Myristica fragrans possess the ability to alter membrane permeability and influence cellular integrity. Phenolic and flavonoid components may contribute to antioxidant defense, thereby minimizing oxidative stress–induced membrane injury. The moderate hemolytic response observed during the experiment indicates that the extract possesses limited cytotoxicity under controlled conditions.

CONCLUSION

That demonstrated that thehydro alcoholic extract of Myristica fragrans Houtt. (Javetri) contains various bioactive phytoconstituents including alkaloids, flavonoids, tannins, terpenoids, phenols, and saponins. Screening of plant constituents confirmed the therapeutic significance plant extract and supported its traditional medicinal use.

The in-vitro hemolytic assay revealed concentration-dependent hemolytic action of the extract on red blood cells. Higher hemolysis appeared at lower concentrations, while reduced hemolysis was found at higher concentrations, indicating possible membrane protective activity. Overall, the study suggests that Myristica fragrans Houtt Further detailed pharmacological and toxicological studies are needed to evaluate its safety and therapeutic applications.

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