Phytochemical Characterisation and Biological activities of *Rosmarinus Officinalis* Leaves

Ayesha Anwar[1], Shafia Arshad[2], Amina Arif[1], Asma Naeem[3], Rida Tanveer[2], Uzma Bashir[2], Zeemal Seemab Amin[4] and Saba Abbas[5]

[1]Deaprtment of Basic and Applied Chemistry, Faculty of Science and Technology, University of Central Punjab, Lahore

[2]University College of Conventional Medicine, Faculty of Medicine and Allied Health Sciences, The Islamia University, Bahawalpur

[3]Universityty of the Punjab, Lahore Pakistan.

[4]School of Biochemistry, Minhaj University Lahore, Pakistan

[5]School of Medical Lab Technology, Minhaj University Lahore

Abstract:

Reactive oxygen species (ROS) can cause hemolysis, resulting in disorders including sickle cell anemia and thalassemia. The relationship between hemolysis and venous thrombosis is complex and encompasses both traditional thrombotic risk factors and unique prothrombotic processes connected to hemolysis. Free radicals produced by autoxidation reactions of sugars and sugar adducts to protein and by autoxidation of unsaturated lipids in plasma and membrane proteins are potential causes of oxidative stress and damage to proteins in diabetes. The present study aimed to evaluate the biological potentials, antidiabetic (α-amylase inhibition assay) antioxidant (DPPH assay), and hemolytic and thrombolytic activity of herbal plant *Rosmarinus officinalis.* In this research shade dried and powdered plant material was used. Components of *Rosmarinus officinalis* L. were extracted using methanol by maceration process from which different fractions with different polarity solvents (*n*-hexane, chloroform, ethyl acetate) were prepared to analyze biological activities. In the current study antidiabetic activity of *R. officinalis* crude extracts were assessed with different fractions like methanol, n-hexane, chloroform, and ethyl acetate exhibiting higher percentage of inhibition are 76.27± 0.031, 69.91± 0.027, 75± 0.05, 73.30± 0.041, respectively. DPPH scavenging activity has shown a comparative less percentage of inhibition of different fractions like methanol, n-hexane, chloroform, and ethyl acetate are 71.45± 0.012, 58.18± 0.022, 64.02± 0.015, 58.34± 0.031 at higher concentration of 0.1mg. In thrombolytic activity fractions of methanol, *n*-hexane, chloroform and ethyl acetate showed the highest percentage of lysis at the concentration of 0.1mg/ml is 55.6±0.03, 63.5± 0.06, 53.2± 0.03, 59.9± 0.02. Still, hemolytic activity showed less percentage of

inhibition 17.96± 0.04, 7.784± 0.05, 22.15± 0.05, 6.58± 0.07 respectively at the higher concentration of 0.1mg from all other biological activities. Reported biological activities were analyzed statistically, which showed p <0.05 shows the significance of activities. From a future perspective *Rosmarinus officinalis* L. can be further used for *in vivo* studies for drug toxicity evaluations.

Keywords: *Rosmarinus officinalis* L, Antioxidant, Antidiabetic, Anti-hemolytic, Anti-thrombotic

1. Introduction:

Reactive oxygen species (ROS) play a significant part in the development of disorders such thalassemia, glucose-6-phosphate dehydrogenase deficiency, and sickle cell anemia by causing oxidative damage and hemolysis (Juan, Pérez de la Lastra et al. 2021). Due to their high membrane concentrations of polyunsaturated fatty acids (especially linoleic and arachidonic acids) and O2 transport linked to redox-active hemoglobin molecules, which are powerful ROS promoters, red blood cells (RBCs) are the main targets of free radicals. Oxidation reduces the amount of protein in membranes, damages RBCs, and interferes with microcirculation. It may also contribute to hemolysis (Gwozdzinski, Pieniazek et al. 2021). The physiopathology of thrombosis in many diseases, such as autoimmune hemolytic anaemia, thrombotic thrombocytopenic purpura, and paroxysmal nocturnal hemoglobinuria, would likely be impacted by hemolysis. Better therapy to prevent the side effect of thrombosis will be made possible by a better understanding the harmful effects of hemolysis. Blood loss has long been used to assess the effects of antioxidants and free radical damage. Detecting oxidising or anti-oxidizing substances is helpful (Delvasto-Nuñez, Jongerius et al. 2021).

The relationship between hemolysis and venous thrombosis is complex and encompasses both traditional thrombotic risk factors and unique prothrombotic processes connected to hemolysis. The physiological defenses against the toxicity of iron from the haem group of hemoglobin are overpowered by pathological hemolysis. Free hemoglobin's pro-oxidant, pro-inflammatory, and NO-scavenging properties impact the three components of Virchow's triad by constricting blood vessels, triggering coagulation, and harming endothelial cells (Lizarralde-Iragorri and Shet 2020).

A fundamental channel connecting several pathways for the generation of problems in diabetes may be oxidative stress (Ighodaro 2018). Increased non-enzymatic glycosylation (glycation) and autoxidative glycosylation are two mechanisms that may contribute to increased oxidative stress in diabetes. Other mechanisms include metabolic stress brought on by changes in energy metabolism, changes in the activity of

the sorbitol pathway, changes in the level of inflammatory mediators and the status of antioxidant defense systems, and localised tissue damage brought on by hypoxia and ischemic hypo-perfusion injury (Rehman, Aatif et al. 2022).

The herbal plant *Rosmarinus officinalis* called the rosemary plant, is part of the Lamiaceae family and arises in the maditerrean area. Rosemary leaves are useful for their medicinal properties as they relieve joints and muscles pain and have anti-hemolytic, antithrombotic, antioxidant and antidiabetic effects (De Oliveira, Camargo et al. 2019). Therefore, present study aims to evaluate the following biological potentials of herbal plant *Rosmarinus officinalis.*

2. Methodology:

2.1 Collection of plant:

R. officinalis were collected from Swat green nursery in pots and verified by Botanist. Experimental work was performed in Biochemistry lab, Faculty of Science and Technology, University of Central Punjab, Lahore.

2.2 Extraction and fractionation:

The plants were washed to remove the dirt and unwanted material. Then the plant was dried for 5-6 days under the shade and then the dried plant was ground in electrical grinder. The maceration method was used to extract 50 g of plant powder was soaked in 500 ml of ethanol in the conical flask. The flask was kept in shaking incubator for 10 days at 37degree. The contents in conical flask were first filtered with muslin cloth and later filtered with Whatman No. 1 filter paper. The filtrate was evaporated in rotary evaporator at 40-degree colour was noticed and was stored in refrigerator at 4 degrees until use (Fauzi, Tan et al. 2020).

2.3 Antioxidant activity

With slight modifications, the 2.2 diphenyl-1-picrylhydrazyl (DPPH) technique was employed to assess the extract's capacity to scavenge free radicals (Rašković, Milanović et al. 2014). To make the DPPH solution, 50mL of ethanol were added to 1mg of DPPH and stirred. To create the various quantities of sample solution, 3mg of each semisolid form of extract were dissolved in 1mL of DMSO solution. The solution was diluted to create a range of doses, including 0.03, 0.06 and 0.1 mg/mL using 10% DMSO. After adding 100 µl of the DPPH solution to the ELISA plate, 100 µl of the sample solution was added. ELISA plate was placed in an incubator set at 37 °C for 35 minutes with aluminium foil covering it. A 10 percent DMSO solution was utilized as a control. ELISA was used to measure the absorbance at 630 nm to assess the antioxidant activity. Ascorbic acid served as the reference and its absorbance compared to fractions was evaluated (Kamdem, Adeniran et al. 2013).

The activity of radical scavenging was calculated using the formula below.

$$\%\ Radical\ Scavenging\ Activity = \frac{A - B}{A} \times 100$$

A is absorbance of DPPH; B is absorbance of sample.

2.4 Antidiabetic Activity

The alpha-amylase inhibitory activity was measured using the starch iodine technique described by (Ononamadu, Ezeigwe et al. 2020) . Amylase solution was made using a 0.025 mg/ml concentration. 195 µl of DMSO containing various fractions of the methanolic extract of *R. officinalis* was combined with 10 ml of the -amylase solution. After that, the mixture was placed in an incubator at 37 ° C for 10 minutes. Each dilution received 50 µl of a 1 percent starch solution after being incubated for 10 minutes. It was then kept in the incubator for another hour at 37° C. 0.1ml of a solution containing 1 percent iodine was added after re-incubation. 200 µl of each sample was applied to an ELISA plate to measure absorbance. Using ELISA, absorbance at 630 nm was noted.

Metformin was used as the control to compare its absorbance to the fractions. The percent inhibition was calculated using the formula below.

$$\%\ Inhibition = \frac{Absorbance\ of\ control - Absorbance\ of\ control}{Absorbance\ of\ control} \times 100$$

2.5 Thrombolytic Activity

4 mL of blood was extracted from a healthy participant's vein and put into five already-weighed 0.5 mL-capacity microcentrifuge tubes (Hussain, Islam et al. 2014). These tubes were once more weighed and kept in incubator for 45 minutes at 37° C. after the clot had formed. The whole serum was removed after incubation without causing a clot to break. To determine the weight of the blood clot, each tube was weighed once again.

$$Weight\ of\ clot = (weight\ of\ tube\ containing\ clot - weight\ of\ tube\ alone$$

Each tube containing the pre-weighed clot received an addition of 100 µL of each fraction of the methanolic extract of the chosen plant. As a positive control for thrombolytic activity, 100 µl of the enzyme streptokinase was used, and 100 µl of distilled water was utilized as a negative control. Then, to check for clot lysis, all tubes were incubated for 1 hour and 30 minutes at 37° C. The fluid that had been released after the 90-minute incubation period was collected, and all the tubes were weighed once more to determine whether or not the weight difference following the disruption of the clot was sufficient. By calculating the difference in weights between before and after the lysis of the clot, clot lysis % was discovered.

$$\%\ Thrombosis = (weight\ of\ clot\ before\ lysis - weight\ of\ clot\ after\ lysis) \times 100$$

2.5 Hemolytic Activity

Blood was centrifuged for five minutes at 1500rpm after being squeezed from a healthy person and put into a sterilized EDTA tube to prevent it from coagulating (Kumar, Karthik et al. 2011). Three times a NaCl solution solution was used to wash the pellet three times after discarding the supern After A tablet of 1 tablet in 100mL of PBS solution was dispersed in 20mL of PBS solution. The dilutions were mixed in Eppendorfs with 180l of blood cell suspension. For 35

minutes, tubes were shaken at 37 degrees Celsius in an incubator. After incubation, tubes were immediately put on ice for 5 minutes. The Eppendorf were then centrifuged for 5 minutes at 1500rpm. A fresh Eppendorf was carefully filled with 900 µL of PBS solution and around 100 µl of carefully removed Eppendorf supernatant for dilution. Approximately 200 µL of this solution was placed in an ELISA plate and the absorbance at 630nm was measured. The positive control was Triton X-100. Triton X-100 was made in various quantities, and its absorbance was measured on ELISA at 630nm to determine where maximal lysis occurred. DMSO was used as a negative control.

Following formula was used to measure hemolytic assay

$$\%\ Hemolytic\ assay = \frac{Abs.of\ sample - Abs.of\ DMSO}{Abs.of\ Triton\ X - 100} \times 100$$

3. Results and Discussion:

3.1 Anti-oxidant Activity

The DPPH radical scavenging activity is one of the most widely used method for screening of antioxidant activities of different extract of *R officinalis*. Antioxidant activity of crude extracts were assessed by following the DPPH assay. The results were analysed statistically. At the concentration of 0.03mg all the four fractions (methanol, *n*-hexane, chloroform and ethyl acetate) showed small value that are 67.36±0.051, 55.34± 0.032, 59.18± 0.034, 55.90± 0.026, respectively shown in Table 3.1, figure 3.1. But with increasing concentration the values of anti-oxidant activity were also increased. And at the concentration of 0.1 mg all four fractions (methanol, *n*-hexane, chloroform and ethyl acetate) give their best result and the value obtained is 71.45± 0.012, 58.18± 0.022, 58.34± 0.031, 64.02± 0.015. The anti-oxidant activity depends on capability of electron donor to scavenge DPPH a stable free radical upon which colour changes from deep violet to yellow. In current study, methanol and chloroform exhibit high DPPH scavenging and anti-oxidant activity 71.45± 0.012 and 64.02± 0.015, respectively as compared to the other fractions at concentration 0.1 mg/ml. Comparatively, less activity was shown by *n*-hexane and ethyl acetate fraction 58.18± 0.022 and 58.34± 0.031, respectively. These results were compared with the literature and confirmed that crude extract of *R. officinalis* show great anti-oxidant activity (Jordán, Lax et al. 2013).

Table 3.1: DPPH Radical Scavenging activity of *R. officinalis.*

Sr. No	Concentration in mg/mL	% DPPH inhibition , Mean ± S.D, FRACTIONS			
		Methanol	*n*-hexane	Ethyl acetate	Chloroform
1.	0.03	67.36±0.051	55.34± 0.032	55.90± 0.026	59.18± 0.034
2.	0.06	69.36±0.026	56.92± 0.019	56.51± 0.017	61.18± 0.052

3	0.1	71.45± 0.012	58.18± 0.022	58.34± 0.031	64.02± 0.015

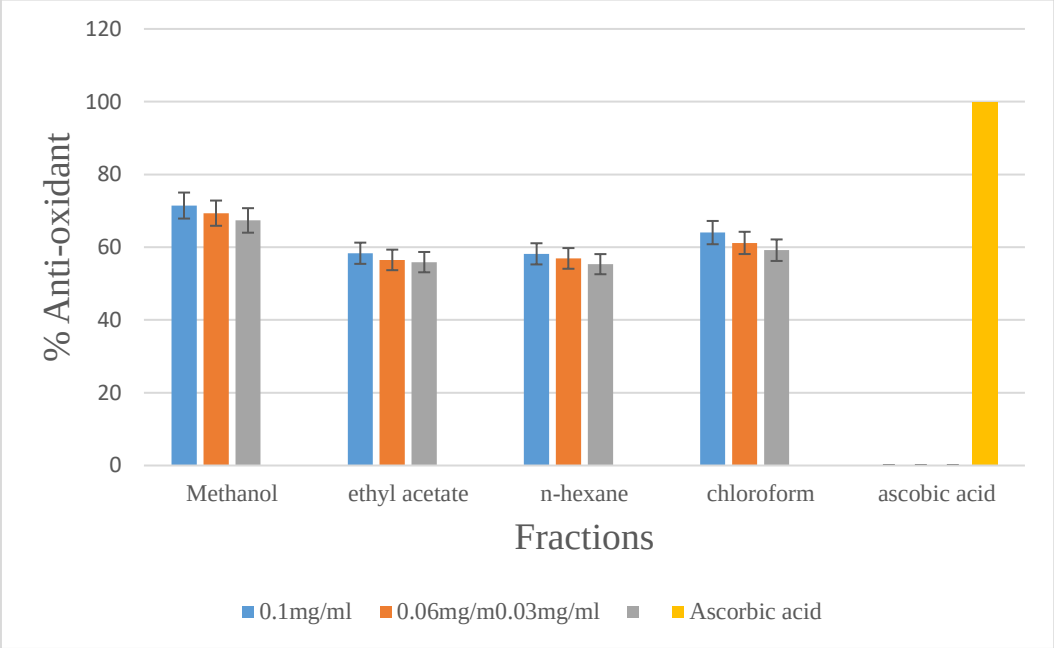

Figure 3.1: Graphical representation of DPPH radical scavenging activity of fractions of *R. officinalis.*

3.2 Anti-diabetic Activity

Antidiabetic activity depends on the α-amylase assay as its inhibition was determined using the starch iodine method, which metformin was used as standard. Standard exhibited its maximum value of 92.74. At the concentration of 0.03 mg all four fractions (methanol, *n*-hexane, chloroform and ethyl acetate) of *R. officinalis* shows the minimum value of inhibition that are 70.50±0.021, 66.31± 0.045, 72.45± 0.031, 70.31± 0.052 shown in Table 3.2, figure 3.2. Whereas with increasing concentration of the sample, values of inhibition also increased. And highest value was observed at highest concentration of 0.1 mg for all the four fractions (methanol, *n*-hexane, chloroform and ethyl acetate) that are 76.27± 0.031, 69.91± 0.027, 75± 0.05, 73.30± 0.041, respectively.

The results showed that with increasing the concentration, % inhibition was also increased and maximum values were obtained at concentration 0.1 mg/ml compared to 0.03 mg/ml, which is the lowest concentration used in this study. The maximum value was shown by methanol extract (76.27± 0.031) and comparatively less activity was shown by *n*-hexane fraction (69.91± 0.027). *R. officinalis* has a high inhibition rate (60%) according to (Funke and Melzig 2006). Higher rosmarinic acid content was associated with greater amylase inhibition (Correia, Mccue et al. 2004).

Table 3.2: Antidiabetic activity of different extracts *R. officinalis.*

Sr.No	Concentration in mg/mL	% Amylase inhibition , Mean ± S.D, FRACTIONS			
		Methanol	*n*-hexane	Ethyl acetate	Chloroform
1.	0.03	70.50±0.021	66.31± 0.045	70.31± 0.052	72.45± 0.031
2.	0.06	71.18±0.012	68.85± 0.016	72.03± 0.017	73.94± 0.023
3	0.1	76.27± 0.031	69.91± 0.027	73.30± 0.041	75± 0.05

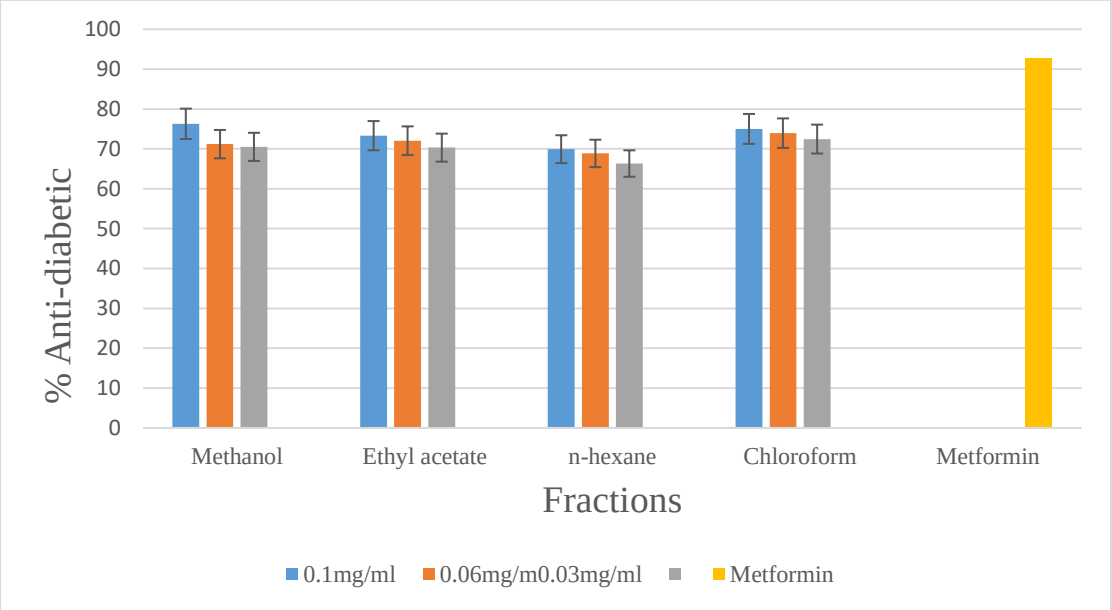

Figure 3.2: Graphical representation of α-amylase inhibition assay in fractions of *R. officinalis.*

3.3 Haemolytic Activity

Different fractions of plant (methanol, *n*-hexane, chloroform and ethyl acetate) of *R. officinalis* showed variable percentage of cell lysis. Triton X-100 was used as positive control and exhibited maximum hemolysis of 100 ± 0.002. The haemolysis percentage that was shown at the concentration of 0.03 mg by all four fractions of *Rosmarinus officinalis* L. (methanol, *n*-hexane, chloroform and ethyl acetate) are 11.97±0.01, 5.38± 0.03,11.97± 0.01, 5.38± 0.03 shown in Table 3.3, figure 3.3. And with decreasing concentration the value of percentage haemolysis is also

decreased. At the concentration of 0.1 mg/ml all the four fractions (methanol, *n*-hexane, chloroform and ethyl acetate) of *R. officinalis* showed very significant results with values 17.96± 0.04, 7.784± 0.05, 22.15± 0.05, 6.58± 0.07, respectively.

Results shows that with decreasing the concentration the value of percentage hemolysis also decreased in all fractions. The minimum value was shown by ethyl acetate (6.58± 0.07) and maximum value was shown by chloroform (22.15± 0.05).

Table 3.3: Hemolytic assay of different fractions of *R. officinalis*.

Sr.No	Concentration in µg/mL	% Hemolysis , Mean ± S.D, FRACTIONS			
		Methanol	*n*-hexane	Ethyl acetate	Chloroform
1.	0.03	11.97±0.01	5.38± 0.03	5.38± 0.03	11.97± 0.01
2.	0.06	12.17±0.03	6.58± 0.04	5.98± 0.05	15.5± 0.02
3	0.1	17.96± 0.04	7.78± 0.05	6.58± 0.07	22.15± 0.05

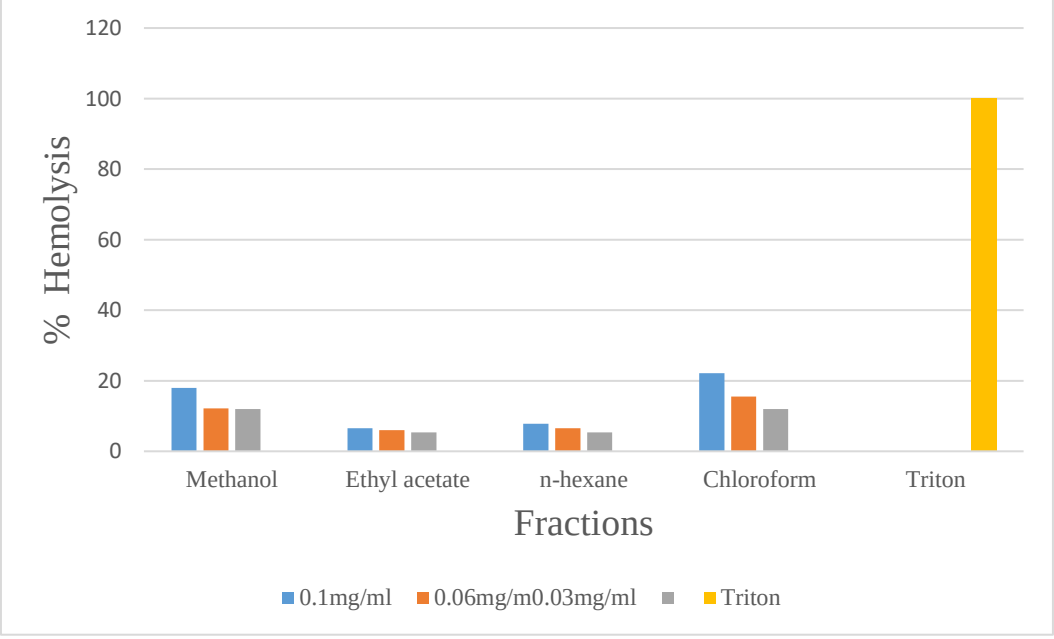

Figure 3.3: Graphical representation of hemolytic activity in fractions of R. *officinalis.*

3.4 Thrombolytic Activity

Different fractions of *R. officinalis* were treated with clot to determine its thrombolytic potential. Streptokinase was taken as positive control, possessing maximum clot lysis of 86.2%. Fraction of methanol, *n*-hexane, chloroform and ethyl acetate showed highest percentage of lysis at the concentration of 0.1mg/ml that is 55.6±0.03, 63.5± 0.06, 53.2± 0.03, 59.9± 0.02 shown in Table 3.4, figure 3.4. But as samples' concentration decreased, clot lysis percentage values were also decreased. At the concentration of 0,03 mg/ml crude extract and its three fractions (*n*-hexane, ethyl acetate, chloroform) showed less values which were 48.3±0.023, 55.9± 0.013, 49.9± 0.041, 44.9± 0.052, respectively. All the fractions showed their best result at the highest concentration. The maximum value was given by *n*-hexane (63.5± 0.061) and chloroform showed less activity (53.2± 0.031).

Table 3.4: Thrombolytic activity of different extracts of *R. officinalis*.

Sr.No	Concentration in mg/mL	% Clot lysis , Mean ± S.D, FRACTIONS			
		Methanol	*n*-hexane	Ethyl acetate	Chloroform
1.	0.03	48.3±0.023	55.9± 0.013	49.9± 0.041	44.9± 0.052
2.	0.06	50.2±0.012	59.4± 0.052	53.6±0.037	49.9± 0.015
3.	0.1	55.6±0.036	63.5± 0.061	59.9± 0.021	53.2± 0.031

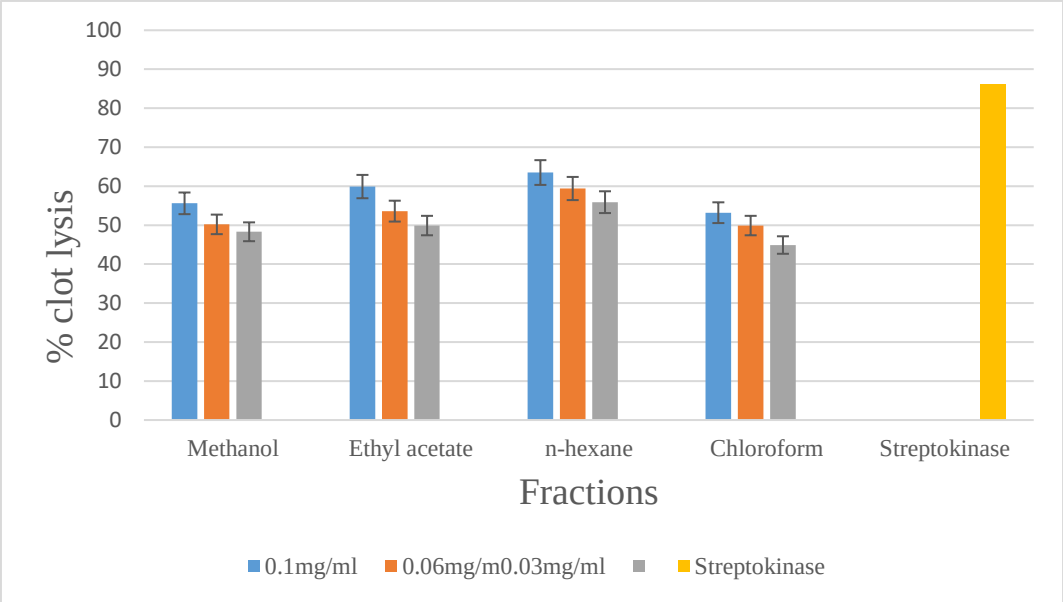

Figure 3.4: Graphical representation of thrombolytic activity of crude methanol extract and its three fractions of *R. officinalis.*

4. Conclusion:

This research study concludes that the herbal plant named *R. officinalis* has anti-oxidant, antidiabetic, and other biological activities (anti-inflammatory, anti-bacterial, hemolytic and thrombolytic activity). The cytotoxicity of *R. officinalis* methanol extract and its fractions was examined using *in vitro* hemolytic activity against human RBCs and showed that methanol and chloroform have a slight cytotoxic effect. Highest anti-oxidant and antidiabetic activity was shown by methanol extract. Best thrombolytic activity was shown by n-hexane fraction. This study concludes that the selected plant exhibits good anti-oxidant and other biological activities. The results were analysed statistically. Reported biological activities shown $p < 0.05$ which shown the significance of activities. As a future perspective, *Rosmarinus officinalis* L. can be further used for *in vivo* drug toxicity evaluations.

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Declarations

Ethics approval and consent to participate

Not applicable

Availability of data

All the data is available for the reader, please contact the corresponding authors.

Competing interests

The authors have no conflict of interest.

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