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Synthesis, Characterization, Investigation of Luminol-Chemiluminescence Enhancement by Selective Agents and Evaluation through appropriate techniques

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ABSTRACT: A Chemiluminescent substance called Luminol has attracted a lot of interest from scientists across a variety of disciplines because of its exceptional quality and many uses. The luminol compound appears to be a white to pale-yellow crystalline solid that is soluble in most polar organic solvents but insoluble in water. It is a chemical that exhibits Chemiluminescence with a blue-glow when mixed with an appropriate oxidizing agent. The forensic investigators use Luminol to detect trace amounts of blood at crime scenes as it reacts with the iron in hemoglobin. Biologists use it in a cellular assay to detect copper iron and cyanides as well as specific proteins. It is noted and carried out that the recent development studies in luminol-based techniques, including creation of fresh luminol compounds and highlighting the necessity for ongoing investigation and optimization of luminol properties to improve its efficiency and broaden its utility in other scientific fields. In the present study, the chemical compound luminol was synthesized by suitable methods subsequently noted its solubility, physical and chemical properties. The synthesized compound was characterized by using UV-Visible Spectrophotometer and FTIR techniques for confirmation of spectral data. The enhancement of luminol-chemiluminesence was carried out with selective oxidizing agents and emission of luminescence was recorded at specific wavelength. The compound luminol was investigated for identification of specific amino acids by TLC and blood stain by appropriate technique.

KEYWORDS: Luminol, Chemiluminescence, Oxidizing agents

I. INTRODUCTION:

Luminol is a chemical compound that displays Chemiluminescence properties with a blue-glow when it is mixed with an appropriate oxidizing agent. Luminol compound is white-to-pale-yellow crystalline solid that is soluble in most polar organic solvents, but it is insoluble in water. Luminol is one of the most widely studied chemiluminescent molecules. Besides that, it is synthesized and spectroscopic properties were significantly investigated. The emission of light requires both H_2O_2 and oxidant enhancer to produce excited 3-aminophthalate **(1).** The oxidation of Luminol creates a brilliant blue light that combines with the iron in hemoglobin, allowing forensic experts to detect extremely minute quantities of blood. Luminol is a compound with chemiluminescent qualities that is used in a variety of applications. A free-radical mechanism has been proposed, but the mechanism of oxidant enhancement, increased luminescence in the presence of the oxidant is still not fully comprehended yet. Ferric enzymes such as horseradish-peroxidase and luciferin enhance luminescence **(2).** Many applications were reported in different fields such as detection of ion concentrations in aqueous solutions, monitoring levels of hydrogen peroxide dependent reactions, detection of blood at crime scenes in forensic science, immunoassays, nucleic acid assays, metabolic pathways monitoring and as a biological active agent. The luminol is also frequently utilized in cellular luminescence and a sensitive approach for the identification of chemical substances, including drugs and heavy metals **(3).**

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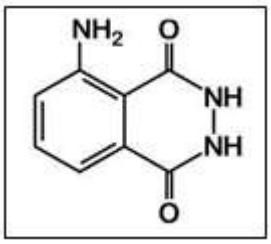

Fig.1. Chemical Structure of Luminol (5-Amino-2, 3-Dihydrophthalazine-1, 4-Dione)

The mechanism of the luminol light-emitting process has appeared in the figure **(1).** The most well-known and commonly used chemiluminescent reagent is Luminol ($C_8H_7N_3O_2$). The bicyclic chemical was given the moniker luminol because of its unique chemiluminescent properties **(4).** The present work would involve synthesis of luminol, noted its solubility, physical and chemical properties. The synthesized compound was characterized by selective spectral studies by UV-Visible Spectrophotometer and FTIR **(5).** The enhancement of luminol-chemiluminescence was carried out with selective oxidizing agents and emission of luminescence was recorded at specific wavelength. The compound luminol was investigated for identification of specific amino acids TLC method and blood stain by appropriate technique **(6).**

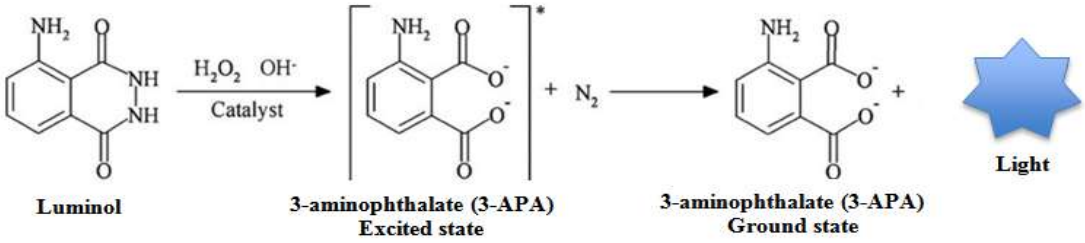

Fig.2. The luminol-Chemiluminescence (light-emitting mechanism)

II. METHODOLOGY

MATERIALS AND METHODS:

Chemicals: The chemicals used in this work were of high purity, they were analytical grade and all of them were purchased from sigma Aldrich. The synthesized compound was tested by using standard melting point apparatus and pH determination by standard pH papers. The UV-Visible spectrophotometer were recorded the spectra using Shimadzu in the parent organization similarly the FTIR spectra were recorded suing Shimadzu spectrophotometer in Sophisticated Test and Instrumentation Centre at Cochin University of Science & Technology, Cochin, Kerala. (2.1gms) 1.3-nitrophthalic acid, (1.5gms) Hydrazine sulfate, (2.1gms) Sodium acetate, (12.5gms) Sodium dithionate, (5gms) Sodium hydroxide, (12.5ml) – Glycerin, (5%) of NaOH Solution, other oxidizing agents like Hydrogen Peroxide, Potassium ferricyanide, Sodium hypo chlorate, Horseradish peroxidase, Amino acids, organic solvents and DD Water for required quantity **(7).**

Glassware's, Equipments and Instruments: Borosilicate glassware's, Weighing balance, **S**tandard pH papers, Thermometers, Vacuum filtration unit, Thermal magnetic stirrer, Melting Point apparatus, TLC Plates & Chamber, UV-Vis Spectrophotometer, FTIR Spectrophotometer.

Synthesis of Luminol: In a standard 250ml beaker, added 3-nitrophthalic acid, hydrazine sulfate and sodium acetate was added as a required quantity to the beaker. To that, 15ml water was added and stirred to get a clear solution. Glycerin was added to the above mixture and heated the solution until it reaches 200^0C. Initially water evaporates away and then the contents in the flask changed its color to dark yellow orange. The reaction temperature is maintained at 200^0C for 10 min, until the formation of intermediate 3-nitrophthalohydrazide. The content in the beaker is made cool down to room temperature and add 50ml water to dissolve all the byproduct compounds. The intermediate 3-nitrophathalohydrazine is not dissolved in water then vacuum filtration was applied to collect the crude form of nitrophthalohydrazide. Inorder to reduce the nitro group, add the crude 3-nitrophthalohydrazide to 40ml 5% NaOH solution by magnetic stirring. To get the clear deepened solution, add 12.5g of sodium dithionite (exothermic reaction) color turns to palebrowncolor and precipitation appears formation of Luminol. To the product, more water

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was added to dissolve the water soluble by products and unreacted compound followed by vacuum filtration to collect crud form of luminol **(7)**.

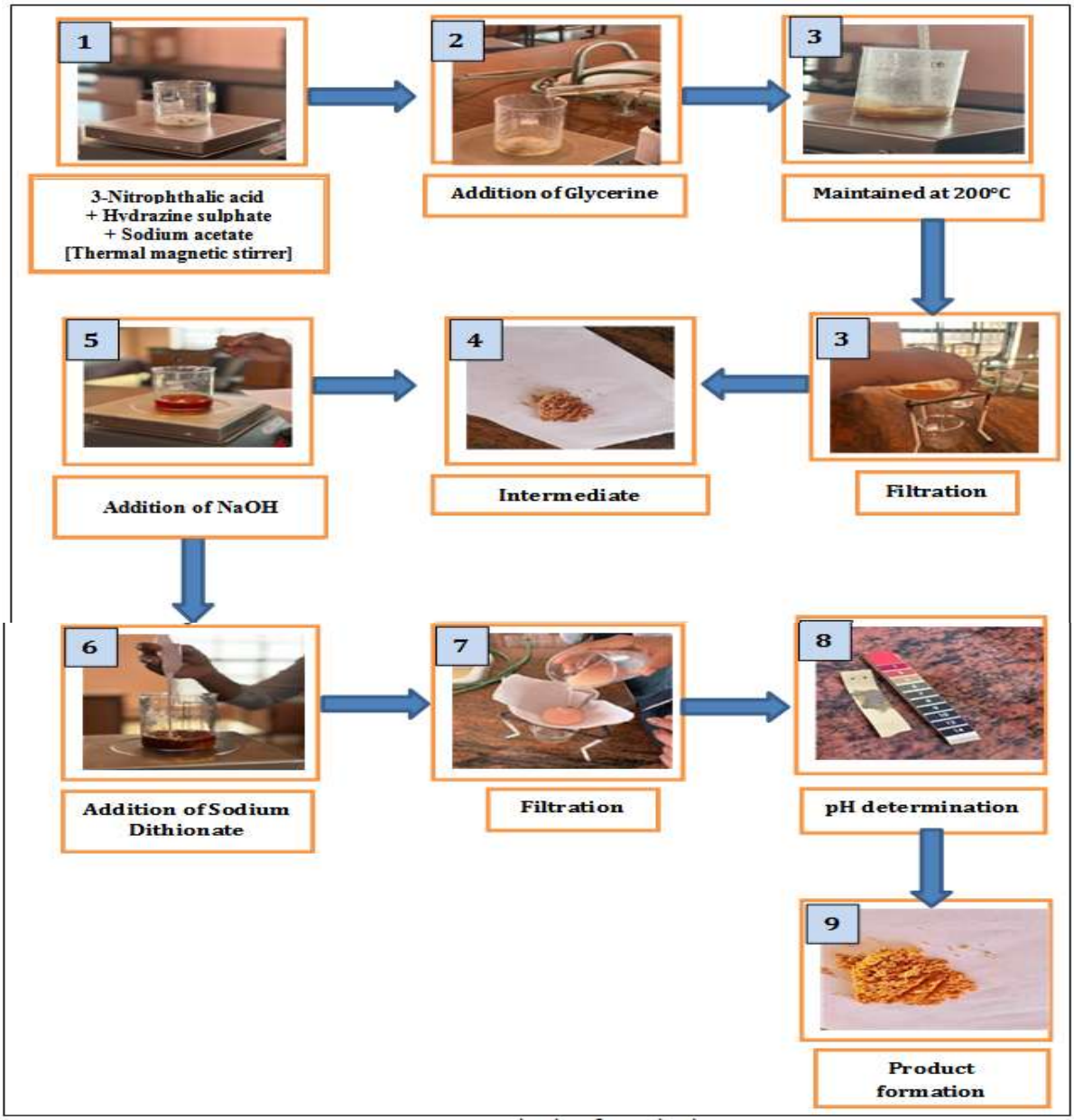

Fig.3. Synthesis of Luminol

Confirmation test for Luminol by its properties: The luminol confirmation was noted by its solubility, physical and chemical properties. The luminol confirmation by testing pH of the compound was found to be neutral using a standard pH indicator paper **(8)**

Confirmation test for Luminol by Spectral data: The synthesized compound was characterized by selective spectral studies by UV-Visible Spectrophotometer, noted its specific WL, FTIR Spectrophotometer and noted its active functional group for Chemiluminescence **(9)**.

Confirmation Test for Luminol-Chemiluminescence: To record the confirmation of Chemiluminescence, mix the solutions of A and B in equal proportions (Solution A, Luminol in

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5%NaOH and Solution B, Oxidizing agents (H_2O_2, Bleach and Potassium Ferricyanide) **(10)**. From the combination reaction, it was noted that, the electrons in Luminol get excited to higher energy state when it is oxidized and return to ground state by emitting photons (wavelength of photons determine the blue light) and lead to intense CL emission at 425 nm **(Fig. 2)**

Confirmation Test for Luminol-Chemiluminescence enhancement by selective Oxidizing Agents: The enhancement of luminol-Chemiluminescence was carried out with selective oxidizing agents and emission of luminescence **(4)** was recorded at specific wavelength **(Table. I,II,III).**

Confirmation Test for Luminol-Chemiluminescence by Selective methods: The enhancement of luminol-Chemiluminescence was carried out with selective oxidizing agents and emission of luminescence was recorded at specific wavelength. The compound luminol was investigated for identification of specific amino acids by TLC method and blood stain by appropriate technique **(11)**.

III. RESULTS:

Table- I, II & III: Comparison of Chemiluminescence Property of Luminol by using different oxidizing agents

Sl. No	Luminol + Oxidizing Agents	Chemiluminescence	Duration in Seconds
1.	Std. hydrogen peroxide	Luminescence	15-20
2.	Potassium periodate	Non-Luminescence	NIL
3.	Copper sulphate	Non-Luminescence	NIL
4.	Manganese dioxide	Non-Luminescence	NIL
5.	Sodium hypochlorite	Slight-Luminescence	5-10

Table I: Chemiluminescence of single oxidizing agents

Sl. No	Luminol + OxidizingAgents	Chemiluminescence	Duration in Seconds
1.	H_2O_2+ Potassium ferricyanide	Strong Luminescence	15-20sec
2.	H_2O_2 + Sodium hypochlorite	Strong Luminescence	15-20sec
3.	H_2O_2+ Manganese dioxide	Weak Luminescence	1-5sec
4.	H_2O_2+ Copper sulphate	Weak Luminescence	1-5sec
5.	H_2O_2 + Potassium periodate	Weak Luminescence	1-5sec
6.	H_2O_2 + Potassium permanganate	Weak Luminescence	1-5sec

Table II: Comparison of Chemiluminescence using double oxidizing agents

Sl. No	Luminol + Oxidizing Agents	Chemiluminescence	Duration in Seconds
1.	H_2O_2 + $CuSO_4$ + Potassium periodate	Weak Luminescence	2-4
2.	H_2O_2 + Sodium hypochlorite + $KmNO_4$	Weak Luminescence	5-7
3.	H_2O_2 + Potassium ferricyanide + Sodium Hypochlorite + HRP	Intense Luminescence	60-120
4.	H_2O_2 + Potassium ferricyanide + MnO_2	Weak Luminescence	2-4
5.	H_2O_2 + MnO_2 + Potassium periodate	Weak Luminescence	1-3

Table III: Comparison of Chemiluminescence using three oxidizing agents

HORSERADISH PEROXIDASE-THE ILLUMINATING ENME:

Horseradish peroxidase (HRP) is an enzyme that catalyzes the oxidation of luminol to produce light. HRP is a stable enzyme, maintaining its activity over a wide range of temperature and pH levels. HRP can be conjugated to specific antibodies or molecules, enabling targeted detection and reducing background noise. HRP is a metalloenzyme that catalyzes the oxidation of

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organic substrates using hydrogen peroxide. It's versatile in catalyzing diverse chemistry, including chromogenic or fluorescent reactions **(12)**.

Sl. No	Luminol + Oxidizing Agents	Chemiluminescence	Duration In Seconds
1.	H_2O_2 + Potassium Ferricyanide + Sodium Hypochlorite + Horseradish Peroxide	Intense Luminescence	60-120

Table IV: Prominent combination of selective oxidizing agents enhancing Luminol-Chemiluminescence

SPECTRAL ANALYSIS:

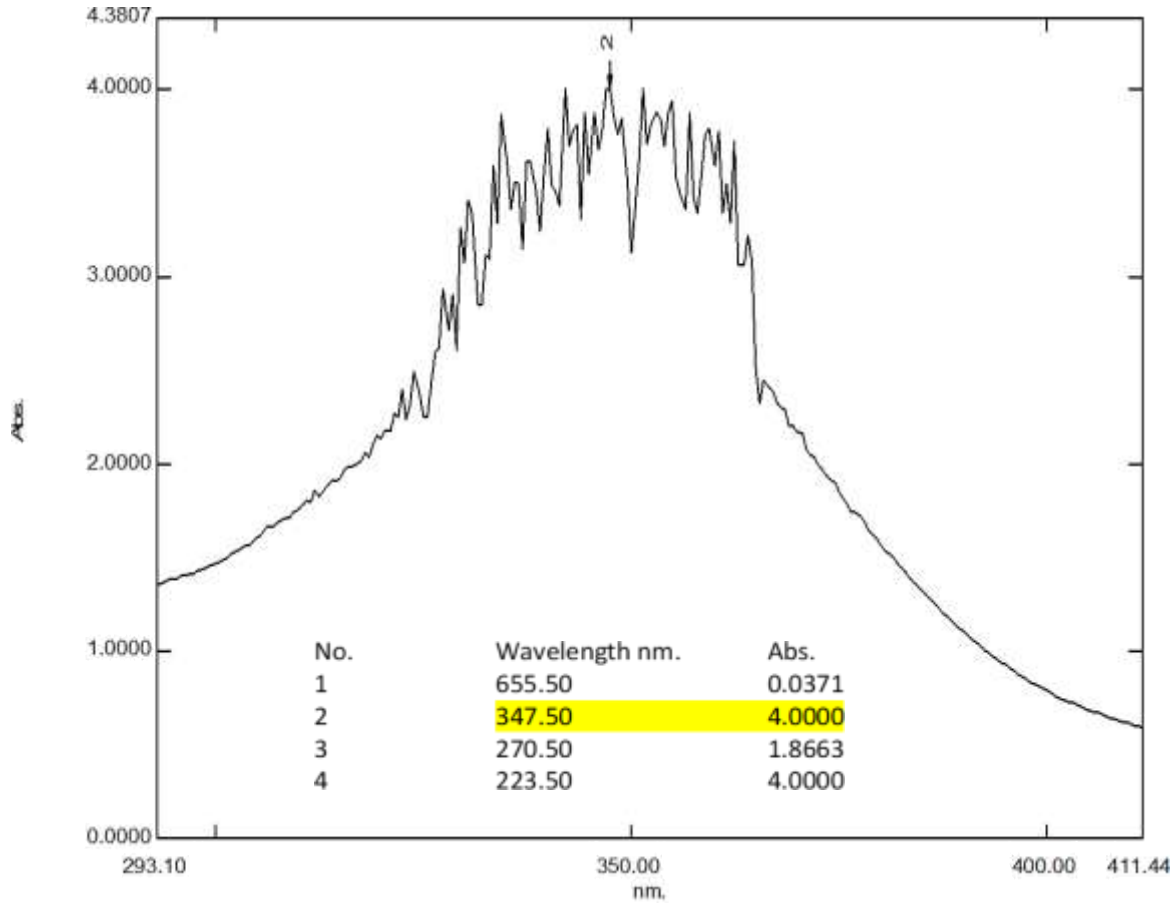

Fig.4: UV-Vis Spectra- WL of Luminol was found to be 347nm

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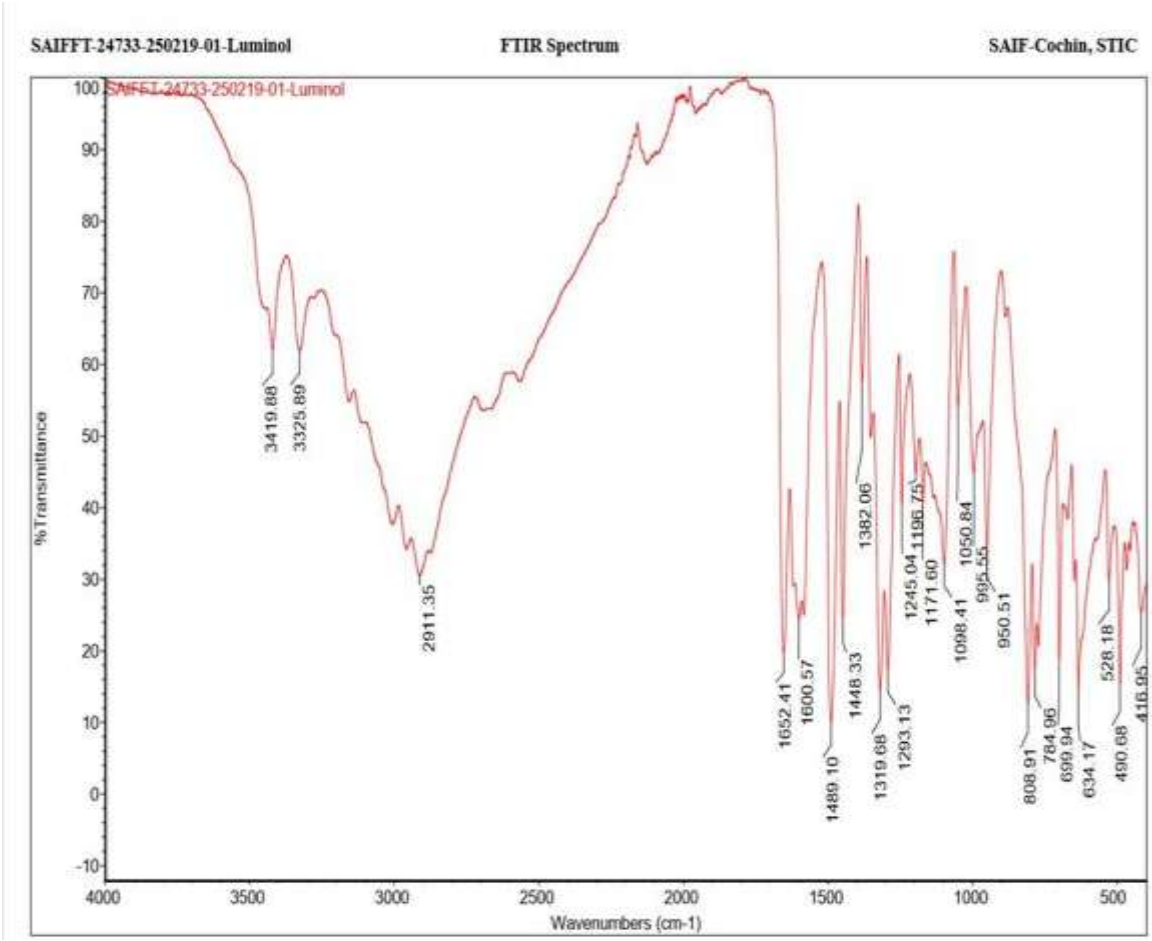

Fig.5: FTIR Spectra of Luminol

C=C AROMATIC (1600.57), C=O KETONE (1652.41), NH_2 (3419.88), N-H STRETCH (3325.89)

EVALUATION STUDIES:

Amino acid detected by Ninhydrin (Std)

Amino acid detected by Luminol (Test)

Fig.6 Identification of Aminoacid by TLC method

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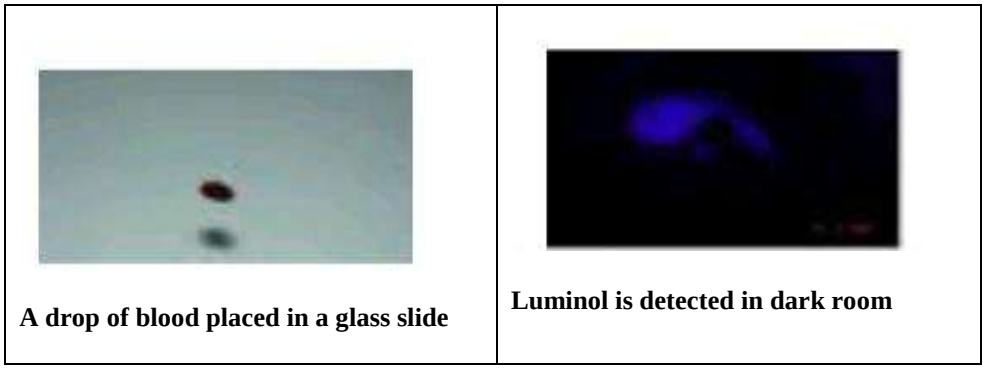
Investigation of Amino acids by Thin Layer Chromatography:

TLC plates and selective amino acid samples (Histidine, Tryptophan) were prepared as per the standard procedure by dissolving them in suitable mobile phase (n-butanol:glacial acetic acid: water 4:1:5). TLC plates were developed and dried. The test sample luminol sprayed in the darkroom to the TLC plates, the results were observed that blue-green fluorescence indicates the presence of amino acid **(13)**.



A drop of blood placed in a glass slide | **Luminol is detected in dark room**

Fig.7 Identification of Blood Stain by Glass plate method

Investigation of Blood Stain:

The investigation of blood stain was performed as per standard procedure. The standard luminol solution was prepared with 5% of NaOH. Then, Place a drop of blood on the glass slide kept for some duration. The glass plate was tested by spraying the luminol solution with prominent oxidizing agent together in a equal proportion to in the dark environment. The reaction on the glass plate was recorded as appearance of blue green glow, which indicates the presence of blood stain on the glass plate **(14)**.

IV. DISCUSSION:

The targeted luminol compound was synthesized by suitable procedure **(Fig.1)** and confirmation was noted by its solubility, physical and chemical properties. The luminol confirmation by testing $_{P}H$ of the compound was found to be neutral using a standard pH indicator paper). The synthesized compound was characterized by selective spectral studies by UV-Visible Spectrophotometer, noted its specific WL, FTIR Spectrophotometer, the presence of specific functional groups were identified which indicates the successful conversion of the starting material to the desired molecule was indeed synthesized (**Fig.4&5).** To record the confirmation of Chemiluminescence, mix the solutions of A and B in equal proportions (Solution A, Luminol in 5%NaOH and Solution B, Oxidizing agents (H_2O_2, Bleach, Potassium Ferricyanide and HRP). From the combination reaction, it was noted that, the electrons in Luminol get excited to higher energy state when it is oxidized and return to ground state by emitting photons (wavelength of photons determine the blue light) and lead to intense CL emission at 425 nm **(Fig. 2)**

Table-I: The Luminol-Chemiluminescence enhancement property was carried out with synthesized compound luminol and selective single oxidizing agents such as Std. hydrogen peroxide, Potassium periodate, Copper sulphate, Manganese dioxide, Sodium hypochlorite. The outcome of reaction was observed that luminol with Std. hydrogen peroxide gives luminescence for duration of 15 to 20 seconds. Luminol with sodium hypochlorite gives slight luminescence as 5 to 10 Sec duration. But, the reaction with luminol and Potassium periodate, copper sulphate and manganese dioxide does not produce luminescence **(Table. I).**

Table-II: The Luminol-Chemiluminescence enhancement property was carried out with synthesized compound luminol with two oxidizing agents, Hydrogen peroxide (first oxidizing agent) as common and selective oxidizing agents (second oxidizing agent) such as Potassium ferricyanide, Sodium hypochlorite, Manganese dioxide, Copper sulphate Potassium periodate. Potassium permanganate. The outcome of reaction was observed that luminol with Std. hydrogen peroxide

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as first oxidizing agent with different oxidizing agents as second oxidizing agent. The reaction mixture of luminol, hydrogen peroxide and potassium ferricyanide and luminol, hydrogen peroxide and Sodium hypochlorite gives strong luminescence for 15 to 20 sec duration. The other reaction mixture of Luminol, hydrogen peroxide with second oxidizing agent Manganese dioxide produced weak luminescence for about 1-5 sec duration. Luminol, hydrogen peroxide with Copper sulphate dioxide produced weak luminescence for about 1-5 sec duration, Luminol, hydrogen peroxide with Potassium periodate dioxide produced weak luminescence for about 1-5 sec duration, Luminol, hydrogen peroxide with Potassium permanganate dioxide produced weak luminescence for about 1-5 sec duration was recorded **(Table. II).**

Table-III & IV: The Luminol-Chemiluminescence enhancement property was carried out with synthesized compound luminol with multiple oxidizing agents, Hydrogen peroxide (first oxidizing agent) as common, selective oxidizing agents (other oxidizing agents) such as Potassium periodate, Sodium hypochlorite, Potassium ferricyanide, Manganese dioxide, Copper sulphate. The outcome of reaction was observed that luminol with Std. hydrogen peroxide as first oxidizing agent with different oxidizing agents as second oxidizing agent. The reaction mixture of luminol, hydrogen peroxide, copper sulphate and potassium periodate gives weak luminescence for 2 to 4 Sec duration. The luminol, hydrogen peroxide, Sodium hypochlorite and potassium permanganate gives weak luminescence for 5 to 7 sec duration. The luminol, hydrogen peroxide, Potassium ferricyanide and Manganese dioxide gives weak luminescence for 2-4 sec duration. The luminol, Manganese dioxide and Potassium periodate gives weak luminescence for 1-3 sec duration. The luminol, hydrogen peroxide, Potassium ferricyanide, Sodium hypochlorite and Horseradish peroxidase enzyme gives strong luminescence for about 60-120 sec duration was noted as prominent combination mixture to enhance the Luminal-Chemiluminescence **(Table. III & IV).**

TLC plates and selective amino acid samples were prepared as per the standard procedure by dissolving them in suitable mobile phase.TLC plates were developed and dried. The test sample luminol sprayed to the TLC plates in the darkroom, the results were observed that blue-green fluorescence indicates the presence of amino acid **(Fig.6)**.

The investigation of blood stain was performed as per standard procedure. The standard luminol solution was prepared with 5% of NaOH. Then, Place a drop of blood on the glass slide kept for some duration. The glass plate was tested by spraying the luminol solution with prominent oxidizing agent together in an equal proportion in the dark environment. The reaction on the glass plate was recorded as appearance of blue green glow, which indicates the presence of blood stain on the glass plate **(Fig.7)**.

V. CONCLUSION:

In the present study, the targeted luminol compound was synthesized by suitable procedure followed by physical and chemical properties were observed through standard methods. Characterization of synthesized luminol was carried out through spectral studies by UV-Vis spectroscopy and FTIR Spectroscopy and noted the presence of active functional groups. The Luminol-Chemiluminescence enhancement was performed through standard methods with combination of selective oxidizing agents. The research findings reflects that, the prominent combination of luminol with multiple oxidizing agent mixture shows enhancement of strong Luminol-Chemiluminescence and were selected for further evaluation studies for the identification of amino acids was performed by TLC method and blood stain investigation was done by appropriate method. A fascinating phenomenon with significant applications is Luminol-Chemiluminescence. The light produced through chemical reactions has transformed scientific research, advancing our knowledge in many investigations Thus, the future advancements in the field of study should lead to even more fascinating discoveries enabling scientists to observe dynamic research activities and uses for Luminol-Chemiluminescence in real-time.

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