

ECO-FRIENDLY SYNTHESIS AND ANTICANCER EVALUATION OF DIBENZALACETONE COMBINED WITH LAWSONIA INERMES EXTRACT

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ABSTRACT

In recent years, natural product-based compounds have gained significant attention in medicinal chemistry due to their diverse biological activities. In the present study, a novel compound was synthesized by combining dibenzalacetone with the plant extract of *Lawsonia inermis* through a green synthesis approach. The synthesized compound was successfully characterized using spectroscopic techniques such as FT-IR and UV-Visible spectroscopy, confirming the presence of key functional groups and extended conjugation. The biological potential of the compound was evaluated through in-vitro anticancer studies using the MTT assay against cancer cell lines. The results demonstrated a dose-dependent cytotoxic effect, with a notable reduction in cell viability and an IC₅₀ value indicating significant anticancer activity. The enhanced bioactivity may be attributed to the synergistic effect of the dibenzalacetone framework and bioactive phytochemicals such as lawsone present in *Lawsonia inermis*. Overall, the study suggests that the synthesized compound is a promising candidate for further investigation in anticancer drug development and pharmaceutical applications.

KEYWORDS: DBA, Anti - Cancer, *Lawsonia Inermis*, in vitro -MTT Assay method

1. INTRODUCTION

Dibenzalacetone (DBA) is an important α,β -unsaturated ketone characterized by the presence of two aromatic benzene rings connected through a conjugated carbonyl system. It is commonly synthesized via the Claisen-Schmidt condensation reaction between benzaldehyde and acetone in the presence of a base such as sodium hydroxide. Due to its extended π -conjugated system, dibenzalacetone exhibits strong absorption in the UV-Visible region and distinct electronic transitions. This structural feature not only contributes to its physicochemical properties but also makes it a valuable intermediate in organic synthesis. Additionally, DBA is widely used in materials science applications such as UV-absorbing agents in sunscreens, and as a ligand in organometallic chemistry, particularly in palladium-based catalytic systems.

Beyond its chemical significance, dibenzalacetone has attracted attention for its diverse biological activities. Studies have reported its antioxidant, antimicrobial, anti-inflammatory, and especially anticancer, properties. 1-6 These multifunctional biological properties make dibenzalacetone a promising scaffold for the development of novel pharmaceutical agents, particularly in cancer research.

Lawsonia inermis (henna) is a well-known medicinal plant rich in bioactive compounds such as lawsone, flavonoids, tannins, and phenolic compounds, which contribute to its wide range of pharmacological activities. Traditionally used for dyeing and treating skin-related ailments, henna has been scientifically validated for its antimicrobial, antioxidant, anti-inflammatory, and anticancer properties. Its extracts have shown the ability to inhibit the growth of various pathogens and cancer cell lines by inducing apoptosis and oxidative stress. The combination of dibenzalacetone with *Lawsonia inermis* extracts may result in enhanced biological activity due to synergistic effects. Therefore, such hybrid compounds are considered promising candidates for further investigation in anticancer drug development and medicinal chemistry research.

Several studies have demonstrated the wide range of biological activities of *Lawsonia inermis* (henna), highlighting its importance in medicinal research. 7- 28 The antibacterial potential of henna leaf extracts against pathogens such as *Staphylococcus aureus* and *Escherichia coli* has been reported, primarily due to the presence of lawsone and phenolic compounds. In addition, strong antioxidant activity has been observed through free radical scavenging assays, attributed to flavonoids and polyphenols that protect against oxidative stress. Cytotoxic and anticancer studies have revealed that henna extracts can inhibit cancer cell proliferation, likely due to bioactive compounds such as naphthoquinones. Anti-inflammatory, antifungal and antimicrobial properties have also been widely documented, supporting its traditional medicinal use. Phytochemical investigations confirm the presence of important constituents like flavonoids, tannins and alkaloids, which

contribute to these biological effects. Furthermore, studies have shown wound healing, antidiabetic, analgesic and anti-inflammatory activities, along with effectiveness against various microbial strains, including *Mycobacterium tuberculosis*. Overall, these findings suggest that *Lawsonia inermis* is a rich source of bioactive

compounds with significant therapeutic potential, particularly in antimicrobial, antioxidant and anticancer applications.

2. MATERIALS AND METHODS:

2.1 Preparation of Dibenzalacetone:

Procedure :

Dibenzalacetone was synthesized by taking 10 mL of freshly distilled benzaldehyde and 20 mL of acetone in a conical flask. The flask was placed in a cold water bath and 2.5 mL of sodium hydroxide solution was added dropwise with constant stirring while maintaining the temperature around 30 °C. After complete addition, the reaction mixture was stirred continuously for 2 hours to ensure completion of the condensation reaction. The mixture was then acidified with dilute hydrochloric acid and transferred to a 250 mL separating funnel. About 20 mL of chloroform or ether was added, shaken thoroughly and the organic layer was separated; this extraction process was repeated twice for maximum recovery. The mixture was then cooled in an ice-water bath, leading to the formation of dibenzalacetone, which initially appeared as a fine emulsion and gradually crystallized as yellow crystals. The remaining solvent was distilled under reduced pressure and the fraction boiling at 150 °C was collected. Finally, the pale yellow crystals were washed with cold water, dried and recrystallized using ethanol to obtain pure dibenzalacetone. The synthesized compound was obtained in the form of pale yellow crystals, indicating the formation of the desired product. The experiment yielded approximately 4 g of the product, which is in good agreement with the expected yield. The melting point of the obtained crystals was found to be 112 °C, which supports the purity and proper formation of the compound.(Figure -1)

SYNTHESIS OF DIBENZALACETONE

Claisen–Schmidt Condensation of Benzaldehyde and Acetone

REACTION SCHEME

Benzaldehyde (2 mol) + Acetone (1 mol) $\xrightarrow{30\text{ }^{\circ}\text{C}, 2\text{ Hours}}$ Dibenzalacetone (Pale Yellow Crystals)

PROCEDURE

- 1**

Take 10 mL of freshly distilled benzaldehyde and 20 mL of acetone in a conical flask.
- 2**

Place the flask in a cold water bath.
- 3**

Add 2.5 mL of sodium hydroxide solution dropwise with constant stirring. Maintain the temperature around 30 °C.
- 4**

After complete addition, stir the reaction mixture continuously for 2 hours.
- 5**

Acidify the mixture with dilute hydrochloric acid.
- 6**

Transfer the mixture to a 250 mL separating funnel. Add about 20 mL of chloroform or ether, shake thoroughly.
- 7**

Allow the layers to separate and collect the organic layer. Repeat the extraction twice.
- 8**

Cool the mixture in an ice-water bath. Dibenzalacetone separates as yellow crystals.
- 9**

Distill the remaining solvent under reduced pressure. Collect the fraction boiling at 150 °C.
- 10**

Wash the crystals with cold water, dry and recrystallize using ethanol to obtain pure dibenzalacetone.

RESULT

Purified Dibenzalacetone (Pale Yellow Crystals)

OBSERVATION & YIELD

The synthesized compound was obtained as **pale yellow crystals**, indicating the formation of the desired product.

Yield : Approximately 4 g

Melting Point : 112 °C

CONCLUSION

Dibenzalacetone was successfully synthesized by Claisen–Schmidt condensation. The product was obtained in **good yield** with a melting point of 112 °C, confirming its purity and proper formation.

2.2 Purification of Dibenzalacetone

Purification of Dibenzalacetone after synthesis is essential to obtain a product of high purity suitable for further characterization. Several purification methods can be employed based on the specific characteristics of the compound and the nature of impurities present. Here are some commonly used purification methods for dibenzalacetone:

2.3 Recrystallization:

Recrystallization is a widely used method for purifying organic compounds based on differences in solubility at different temperatures. Dibenzalacetone is typically soluble in organic solvents such as ethanol or methanol at elevated temperatures but less soluble at lower temperatures.

The crude dibenzalacetone is dissolved in a minimum volume of hot solvent, and the solution is allowed to cool slowly. As the solution cools, dibenzalacetone crystallizes out while impurities remain in solution or precipitate separately. The resulting crystals are then collected by filtration, washed with cold solvent to remove any residual impurities, and dried to yield pure dibenzalacetone.

2.4 Column Chromatography:

Column chromatography is a versatile purification technique based on differences in the affinity of compounds for a stationary phase (such as silica gel) and a mobile phase (solvent).

The crude dibenzalacetone mixture is dissolved in a suitable solvent and loaded onto a column packed with stationary phase material.

As the solvent is passed through the column, compounds with different affinities for the stationary phase are separated, with dibenzalacetone eluting from the column earlier than impurities.

Fractions containing pure dibenzalacetone are collected, concentrated, and further characterized.

2.5 Thin-Layer Chromatography (TLC):

Thin-layer chromatography is a rapid and cost-effective method for assessing the purity of dibenzalacetone and monitoring the progress of purification processes.

A small amount of the crude dibenzalacetone sample and reference compounds are spotted onto a TLC plate coated with a thin layer of adsorbent material.

The plate is then placed in a developing chamber containing a solvent system that migrates up the plate via capillary action. After development, the plate is visualized under UV light or treated with a suitable staining reagent to visualize the separated compounds.

The R_f (retention factor) values of dibenzalacetone and impurities can be compared, and the purity of dibenzalacetone can be assessed based on the intensity and position of its spot relative to impurity spots.

2.6 Crystallization from Mixed Solvent Systems:

Crystallization from mixed solvent systems involves the use of solvent mixtures to control the crystallization process and improve the purity of dibenzalacetone.

By adjusting the composition of the solvent mixture and the cooling rate, it is possible to enhance the selectivity of dibenzalacetone crystallization while minimizing the co-precipitation of impurities.

This method is particularly useful when conventional recrystallization techniques alone are insufficient to achieve the desired purity.

2.7 Preparative High-Performance Liquid Chromatography (HPLC):

Preparative HPLC is a powerful purification technique capable of isolating large quantities of pure compounds. The crude dibenzalacetone mixture is injected onto an HPLC column, where it is separated into individual components based on their interactions with the stationary phase and mobile phase.

Fractions containing pure dibenzalacetone are collected, concentrated, and further characterized.

2.8 Characterization of Dibenzalacetone:

The FT-IR spectra were obtained using the BRUKER ALPHAFT-IR MB 102 spectrophotometer, in the 4000-400 cm^{-1} region on KBr pellets. The ^1H (500 MHz) and ^{13}C (125 MHz) NMR spectra were performed on the BRUKER ADVANCE spectrometer, using dimethylsulfoxide ($(\text{CD}_3)_2\text{SO}$).

2.9 Biological Activity of Dibenzalacetone:

The anticancer activity of dibenzalacetone was evaluated by in-vitro MTT Assay Method from the Royal BioTech laboratory, Chennai.

In-Vitro Anticancer Activity (Colon Cancer (Ht29) Cell Line) Minimal Essential Media Preparation

Weighed 9.5 grams of MEM dissolved in 950ml of pre-sterilized double distilled water mixed well. Sodium hydrogen

carbonate 2.2 grams is dissolved in 50ml of pre-sterilized double distilled water mixed well. Both bottles were sterilized at 15 lbs, 121 $^{\circ}\text{C}$, for 15 min. Allow to cool down at room temperature. 0.3grams of Lglutamine was weighed and dissolved in 10ml of pre-sterilized double distilled water and poured. Antibiotics – streptomycin, penicillin G and amphotericin B, 1mg of each was weighed. Each antibiotic was dissolved separately in 1ml of pre-sterilized double distilled water. pH was checked and adjusted to 7.2 - 7.4 with Diluted HCL. All the contents were syringe filtered. Stored at 4 $^{\circ}\text{C}$.

10% Fetal Calf Serum (FBS)

10ml FBS was syringe filtered and added 90ml of MEM media. One aliquot of the prepared MEM is kept for 2 days at 37 and checked for sterility, pH drop and floating particles.

Check the contamination and store at +4 $^{\circ}\text{C}$. **Trypsin, PBS, Versene, Glucose solution: (TPVG) Trypsin:**

Weigh 0.14 g of trypsin accurately; dissolve in 10 ml sterile distilled water for ½ hr. Filter through membrane filter. Store at -20 °C.

0.2% EDTA (Versene)

Weigh 200 mg of EDTA accurately. Dissolve in 10 ml of sterile distilled water. Autoclave at 15 lbs / 15 min.

Glucose

Weigh 0.5g of Glucose accurately, dissolve in 5 ml of sterile distilled water and filter through Whatman filter paper and autoclave at 15 lbs / 15 min.

1XPBS (phosphate buffer saline)

1× Phosphate Buffered Saline (PBS) was prepared by dissolving 8 g of sodium chloride, 0.2 g of potassium chloride, 1.15 g of disodium hydrogen phosphate, and 0.2 g of potassium dihydrogen phosphate in 800 mL of double or triple distilled water. The solution was mixed thoroughly until all the components were completely dissolved. The pH of the solution was then carefully adjusted to 7.2 using appropriate acid or base. After pH adjustment, the final volume was made up to 1 litre with distilled water. The prepared buffer solution was stored at room temperature for further use.

TPVG solution make up to 100mL

A trypsin–EDTA working solution was prepared by mixing 84 mL of 1× PBS with 10 mL of trypsin and 10 mL of 0.2% EDTA solution. To this mixture, 5 mL of glucose and 0.1 mL of penicillin and streptomycin were added to enhance cell viability and prevent microbial contamination. The antibiotics were added from a streptomycin stock solution (1 mg/mL) to ensure proper concentration. All the components were mixed thoroughly under sterile conditions to obtain a uniform solution. The prepared solution was then distributed into 5 mL aliquots and stored at –20 °C for future use.

Preparation of MTT :

MTT solution was prepared by dissolving 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl tetrazolium bromide in 1× PBS to obtain a final concentration of 5 mg/mL, and the solution was mixed thoroughly until completely dissolved for use in cell viability assays.

SUB CULTURING OF CELL LINES

Before subculturing, the medium and TPVG (trypsin–versene–glucose) solution were brought to room temperature. The tissue culture flask was carefully observed for cell growth, morphology, degeneration, pH, and turbidity, and a suitable flask was selected for splitting. The mouth of the flask was wiped with cotton soaked in spirit to maintain aseptic conditions. The spent medium was discarded, and the cells were washed twice with MEM medium to remove residual serum. Subsequently, 4 mL of TPVG solution pre-warmed to 37 °C was added to the flask and allowed to act for 1–2 minutes to facilitate cell detachment. The TPVG was then discarded, and 5 mL of 10% MEM medium was added. The cells were gently pipetted to break up clusters and obtain a uniform cell suspension (passaging). Finally, 20 mL of growth medium was added to the tissue culture flask, and the cells were transferred into 96-well plates for further experiments.

2.10 Preparation of dibenzalacetone–Lawsonia inermis compound

The synthesis of the dibenzalacetone–Lawsonia inermis compound was carried out using a simple and eco-friendly procedure. Initially, 5 g of powdered henna leaves (*Lawsonia inermis*) were taken in a beaker, and ethanol was added to extract the bioactive constituents. The mixture was placed on a magnetic stirrer and continuously stirred at room temperature for 4–5 hours to ensure efficient extraction. In a separate beaker, 5 g of dibenzalacetone was dissolved in ethanol to obtain a uniform solution. This solution was then added dropwise to the henna extract with constant stirring, allowing proper interaction between the components. As the reaction progressed, a precipitate was formed, indicating the formation of the desired compound. The precipitate was then carefully filtered using a crucible to separate it from the reaction mixture. Finally, the



SYNTHESIS OF DIBENZALACETONE – *Lawsonia inermis* COMPOUND



The synthesis of the dibenzalacetone–*Lawsonia inermis* compound was carried out using a simple and eco-friendly procedure. Initially, **5 g of powdered henna leaves (*Lawsonia inermis*)** were taken in a beaker, and **ethanol** was added to extract the bioactive constituents. The mixture was placed on a **magnetic stirrer** and continuously stirred at room temperature for **4–5 hours** to ensure efficient extraction.

In a separate beaker, **5 g of dibenzalacetone** was dissolved in ethanol to obtain a uniform solution. This solution was then added **dropwise** to the henna extract with constant stirring, allowing proper interaction between the components. As the reaction progressed, a **precipitate was formed**, indicating the formation of the desired compound. The precipitate was then carefully **filtered using a crucible** to separate it from the reaction mixture. Finally, the collected solid was **dried in a hot air oven** to obtain the purified dibenzalacetone–*Lawsonia inermis* compound in dry form.

EXPERIMENTAL PROCEDURE - STEP BY STEP

1


5 g of powdered *Lawsonia inermis* (henna leaves) taken in a beaker.

2


Ethanol added to the powdered leaves and mixed well.

3


Mixture stirred on a **magnetic stirrer** at room temperature for 4 to 5 hours.

4


In another beaker, **5 g of dibenzalacetone** dissolved in ethanol.

5


Dibenzalacetone solution **added drop by drop** to the henna extract with constant stirring.

6


At the end of the reaction, a **precipitate** is formed.

7


The precipitate was **filtered** through a **crucible**.

8


Finally, the precipitate was **dried** in a **hot air oven**.

FINAL PRODUCT


Dibenralacetone – *Lawsonia inermis* Compound

Figure -2 Synthesis of Dibenralacetone–*Lawsonia inermis* hybrid compound

collected solid was dried in a hot air oven to obtain the purified dibenzalacetone–*Lawsonia inermis* compound in dry form.(Figure -2)

2.11 CHARACTERIZATION OF DBA WITH LAWSONIA INERMES:

The Expected of the FT-IR spectrum of the synthesized compound a broad absorption band observed around $3400\text{--}3500\text{ cm}^{-1}$ is attributed to the O–H stretching vibration, indicating the presence of hydroxyl groups derived from *Lawsonia inermis*. The band observed around $1650\text{--}1700\text{ cm}^{-1}$ corresponds to the C=O stretching vibration of the carbonyl group present in the dibenzalacetone structure. The peaks around $1580\text{--}1600\text{ cm}^{-1}$ are assigned to aromatic C=C stretching vibrations. In addition, the absorption bands observed in the region $1200\text{--}1300\text{ cm}^{-1}$ correspond to C–O stretching vibrations. These characteristic peaks confirm the presence of important functional groups and support the successful formation of the dibenzalacetone–*Lawsonia inermis* compound.

The expected UV–Visible spectrum of the synthesized dibenzalacetone–*Lawsonia inermis* compound the spectrum showed an absorption band in the UV region around $270\text{--}280\text{ nm}$, which is attributed to the $\pi \rightarrow \pi^*$ transition of the aromatic ring and conjugated double bond system present in the compound. Another absorption band observed in the region of $330\text{--}350\text{ nm}$ may be assigned to $n \rightarrow \pi^*$ transition associated with the carbonyl (C=O) group. The presence of these absorption bands confirms the extended conjugation and aromatic structure in the synthesized compound. These results support the successful formation of the dibenzalacetone–*Lawsonia inermis*.

2.12 BIOLOGICAL ACTIVITY OF DBA with LAWSONIA INERMES:

The Anticancer activity of DBA with lawsonia inermes was evaluated in vitro MTT Assay method.

Source of chemical and reagents

Dulbecco's Modified Eagle's Medium, streptomycin, penicillin-G, L-glutamine, phosphatebuffered saline,

3-(4,5

dimethylthiazol-2-yl)-2,5 diphenyltetrazoliumbromide, 2'7'diacetyl dichloro fluorescein, sodium dodecyl sulphate, trypan blue, trypsin-EDTA, ethylene diamine tetra acetic acid, acridine orange, ethidium bromide, rhodamine-123, triton X-100, ethanol, dimethyl sulfoxide (DMSO), and bovine serum albumin were purchased from Sigma Aldrich Chemicals Pvt. Ltd (India). All other chemicals used were of analytical grade, purchased from Hi media Laboratories Pvt. Ltd., India.

Cell culture maintenance

Breast cancer(MCF-7) cell lines were procured from the Cell repository of National Centre for Cell Sciences (NCCS), Pune, India. Dulbecco's Modified Eagle Media (DMEM) was used for maintaining the cell line, which was supplemented with 10% Fetal Bovine Serum (FBS). Penicillin (100 U/ml), and streptomycin (100 µg/ml) were added to the medium to prevent bacterial contamination. The medium with cell lines was maintained in a humidified environment with 5% CO₂ at 37°C.

MTT assay

The cytotoxicity of lawsonia inermes on MCF-7 cells was determined by the method of Mosmann, (1983).

Principle

The yellow 3-(4,5dimethylthiazol-2-yl)-2,5diphenyltetrazoliumbromide (MTT) is reduced by mitochondrial dehydrogenase of viable cells yielding a measurable purple formation product. Viable cells contain NAD(P) H-dependent reductase, which reduce the MTT reagent to formazan, with a deep purple colour. Formazan crystals are then dissolved using solubilizing solution and absorbance is measured at 500-600 nm by plate reader.

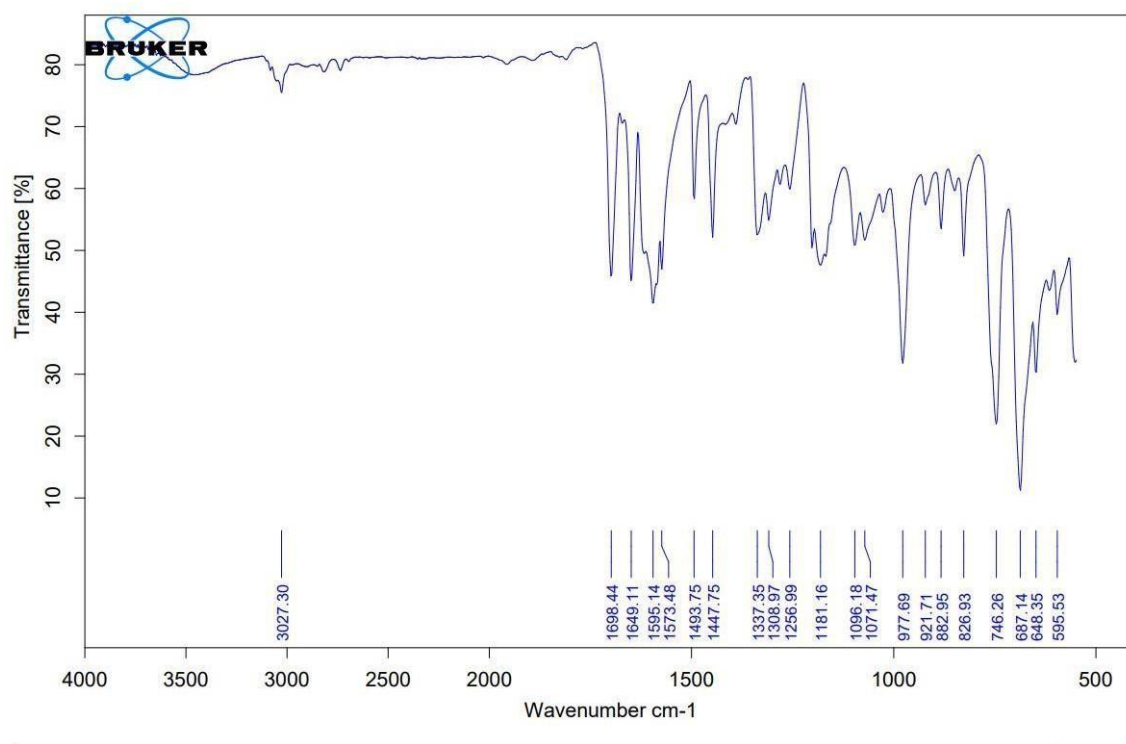


Figure 03: FT-IR SPECTRUM OF DBA

Aromatic C-H stretching vibrations are observed at approximately 3027 cm⁻¹. The stretching vibration of the carbonyl group (C=O) appears as a peak around 1698 cm⁻¹. Aliphatic C-H stretching vibrations are detected at approximately 2850 cm⁻¹. The absorption band at 1447 cm⁻¹ corresponds to the stretching vibration of -CH₂-groups.

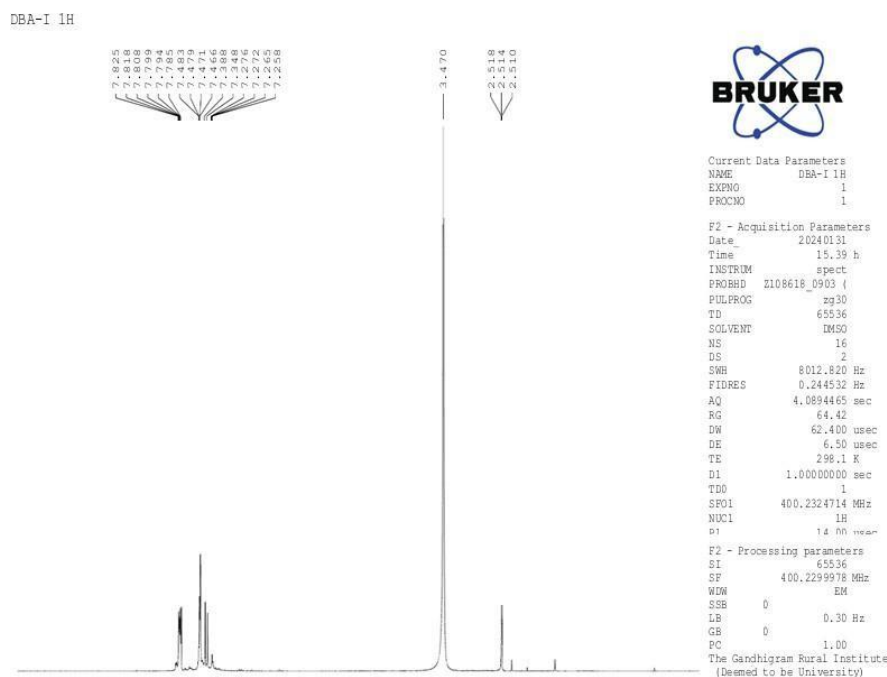


Figure 04: 1H NMR SPECTRUM OF DBA

3.2 H1 NMR Spectroscopy

The Synthesized Dibenzalacetone are characterized by H1 NMR Spectrum.(Figure 4) The Interpretation of this compound is given below.

Aromatic Protons (δ 7.25 - 7.82 ppm): A prominent multiplet in this range was observed, attributed to the aromatic protons on the benzene rings. The signals appeared broadened due to coupling with neighbouring protons. The exact chemical shifts within this range may vary depending on factors such as solvent polarity and ring substitution patterns.

Alpha-Carbon Proton Adjacent to Carbonyl Group (δ 2.5 - 3.47 ppm):

A multiplet centered around δ 2.5 - 3.47 ppm was observed, corresponding to the proton on the alpha-carbon adjacent to the carbonyl group. This multiplet arises due to coupling with neighbouring protons, including those on the benzene rings and neighbouring methylene groups. The chemical shift and splitting pattern provide insights into the chemical environment and connectivity of this proton.

3.3 C13 NMR Spectroscopy

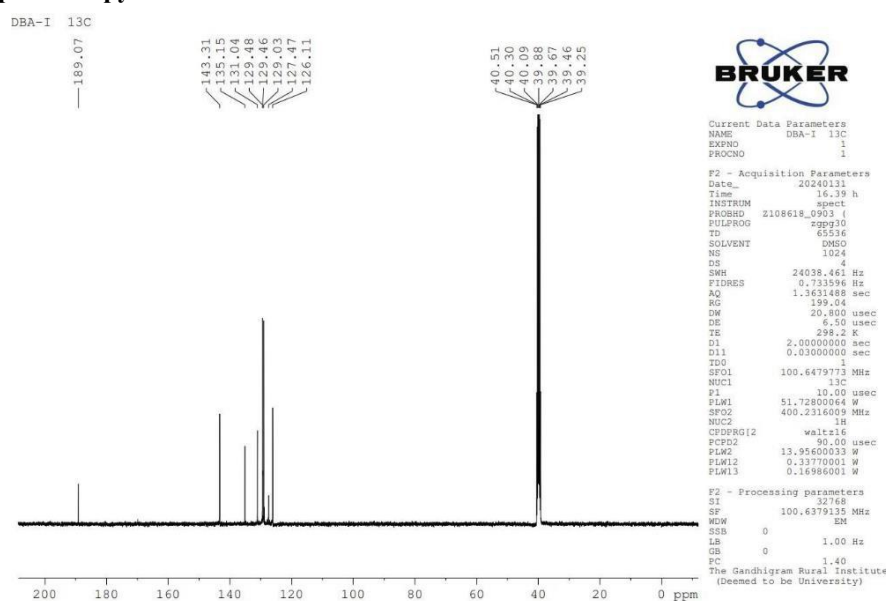


Figure 05: 13C NMR SPECTRUM OF DBA

The Synthesized Dibenzalacetone are characterized by C13 NMR Spectrum.(Figure 5) . The Interpretation of this compound is given below.

Carbonyl Carbon (C=O) (δ 189 ppm): A sharp peak was observed in the spectrum around δ 189 ppm, corresponding to the carbonyl carbon (C=O) of the molecule. The precise chemical shift of this peak depends on factors such as molecular environment and any conjugation effects with adjacent functional groups.

Aromatic Carbons (δ 126 - 143 ppm): Several peaks were observed in the range of δ 126 - 143 ppm, indicative

of the carbon atoms within the benzene rings of dibenzalacetone. The chemical shifts of these peaks vary depending on the degree of substitution and the presence of electron-withdrawing or electron-donating groups.

Aliphatic Carbons (δ 39 - 40 ppm):

Signals corresponding to aliphatic carbons, including those in the alkene and alkyl chains, were observed in the spectrum within the range of δ 0 - 70 ppm. The alpha-carbon adjacent to the carbonyl group may appear around δ 39 - 40 ppm due to the electron-withdrawing effect of the carbonyl group.

3.4 Biological screening of Dibenzalacetone:

In Biologically Related Anticancer Activity was done By In-Vitro Anticancer Activity (Colon Cancer (HT29) Cell Line by MTT Assay method.

IN-VITRO ANTICANCER ACTIVITY (COLON CANCER (HT29) CELL LINE)

An MTT assay was performed to determine the anticancer activity of Dibenzalacetone. Prominent morphological aberrations were clearly noticed, and this is indicative of cancer cell growth inhibition and cell death after 48 h of Dibenzalacetone when compared to the untreated (control) cell.

MTT assay

The cancer activity of samples on HT29 cells were determined by the MTT assay (Mosmann et al.,1983)27. Cells (1×10^5 /well) were plated in 0.2 ml of medium/well in 96-well plates. Incubate at 5 % CO₂ incubator for 72 hours. Then, added various concentrations of the samples in 0.1% DMSO for 48 hrs at 5 % CO₂ incubator. View the images under Inverted microscope 40X and take the photos. After removal of the sample solution and 20 μ l/well MTT reagent was added. Incubate at dark for 4 to 6 hrs. After incubation add 1 ml of DMSO. Viable cells were determined by the absorbance at 540nm. 50% inhibition of cell viability (IC₅₀) value was determined graphically. The effect of the samples on the proliferation of HT29 cells was expressed as the % cell viability, using the following formula:

Calculation

$$\% \text{ cell viability} = \text{A540 of treated cells} / \text{A540 of control cells} \times 100\%$$

Based on the results presented above, there was a clear dose-dependent response to Dibenzalacetone. It is evident that Dibenzalacetone is more effective in inducing cytotoxicity in HT29 colon cancer cells compared to the untreated control cells, with an IC₅₀ concentration of 21.84 μ g.(Table 1)

S. No	Concentration μ g/ml	Absorbance 540nm	% cell Viability
1	1000	0.03	2.5
2	500	0.09	7.6
3	250	0.14	11.9
4	125	0.19	16.2
5	62.5	0.39	33.3
6	31.2	0.51	43.5
7	15.6	0.78	66.6
8	7.8	1.07	91.4
9	DMSO	1.17	100
10	Control Cells	1.17	100

The cytotoxic activity of the sample was evaluated at different concentrations ranging from 1000 to 7.8 μ g/ml, and the results were expressed in terms of absorbance at 540 nm and percentage cell viability. A clear concentration-dependent effect was observed, where higher concentrations exhibited lower cell viability. At 1000 μ g/ml, the absorbance was 0.03 with only 2.5% cell viability, indicating strong cytotoxic activity. As the concentration decreased, the cell viability gradually increased, reaching 91.4% at 7.8 μ g/ml with an absorbance of 1.07. The standard control (DMSO) and untreated control cells showed maximum absorbance of 1.17 with 100% cell viability, confirming normal cell growth. Overall, the results demonstrate that the sample exhibits significant cytotoxic effects at higher concentrations and reduced toxicity at lower concentrations.(Figure -5)

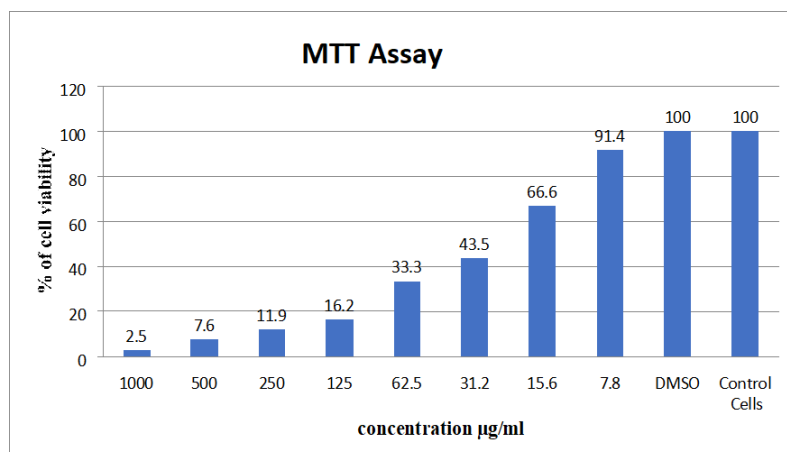
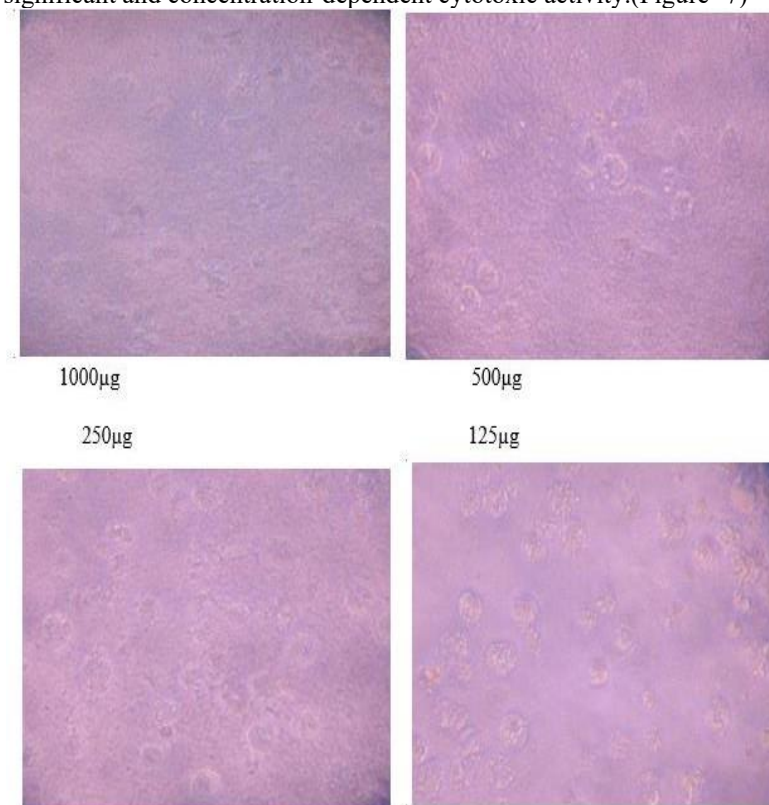
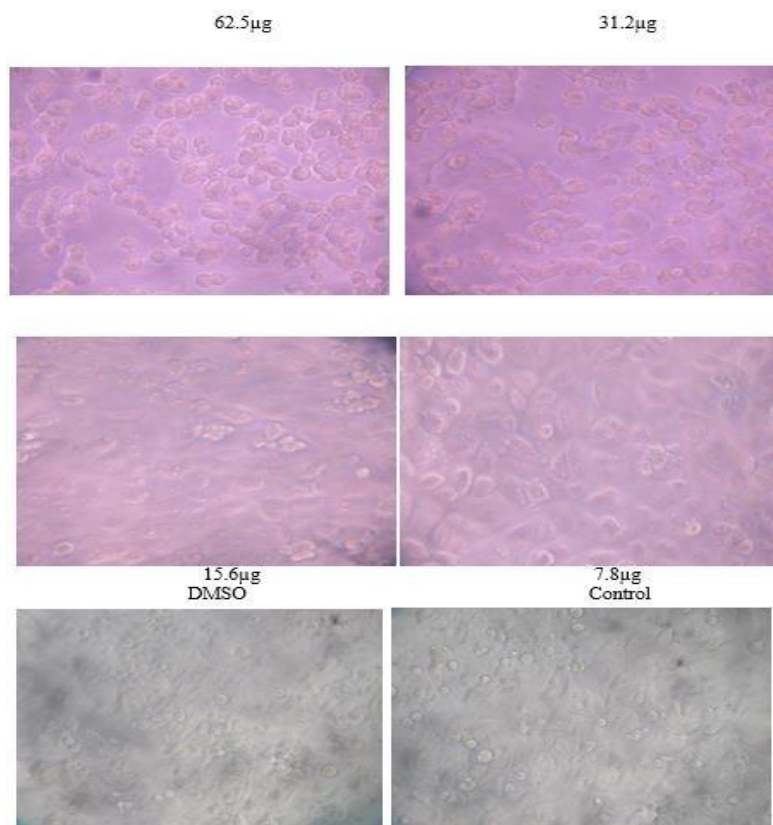


Figure 06: MTT ASSAY OF DBA

The bar graph(Figure -6) illustrates the effect of different concentrations of the test sample on cell viability. A clear inverse relationship between concentration and cell viability is observed. At higher concentrations (1000 and 500 $\mu\text{g/ml}$), the percentage of cell viability is very low, indicating strong cytotoxic activity of the compound. As the concentration decreases, there is a gradual increase in cell viability, showing reduced toxic effects on the cells. At the lowest concentrations (15.6 and 7.8 $\mu\text{g/ml}$), the cell viability approaches near-normal levels, comparable to the control. The control and DMSO-treated cells exhibit 100% viability, confirming that the solvent does not affect cell growth. Overall, the graph demonstrates a dose-dependent cytotoxic effect, where the compound is highly effective at higher concentrations and less toxic at lower concentrations.

The cytotoxic activity of the test compound was evaluated across a range of concentrations from 1000 μg to 7.8 μg , along with DMSO and control groups. The results demonstrated a clear dose-dependent response, where higher concentrations (1000 μg and 500 μg) exhibited strong cytotoxic effects with significantly reduced cell viability. As the concentration decreased through 250 μg , 125 μg , 62.5 μg , and 31.2 μg , a gradual increase in cell viability was observed, indicating reduced toxicity.(Table 1) At the lowest concentrations (15.6 μg and 7.8 μg), the cells showed high viability, approaching that of the DMSO and control groups, which maintained normal cell growth. The IC_{50} value of 21.84 μg indicates that the compound possesses notable cytotoxic potential, as a relatively low concentration is sufficient to inhibit 50% of cell viability. Overall, the findings confirm that the compound exhibits significant and concentration-dependent cytotoxic activity.(Figure -7)





IC50 value =21.84µg

Figure 07: Anticancer activity of DBA in various concentration

The synthesized Dibenzalacetone with lawsonia inermes was characterized by the following analytical assay

3.5 FT(IR) SPECTRUM OF COMPOUND DBA WITH LAWSONIA INERMES:

The FT-IR spectrum of the synthesized compound shows a broad absorption band at 3288 cm^{-1} , which is attributed to the phenolic O–H stretching vibration originating from the Lawsonia inermis m. The peaks observed at 2915 and 2848 cm^{-1} correspond to C–H stretching vibrations. A strong absorption band at 1728 cm^{-1} indicates the presence of a carbonyl (C=O) group of an α,β -unsaturated ketone, confirming the dibenzalacetone framework. The bands appearing at 1621 and 1593 cm^{-1} are assigned to conjugated C=C and aromatic ring stretching vibrations. The peaks around 1510 – 1415 cm^{-1} correspond to aromatic skeletal vibrations and C–H bending modes. Further peaks at 1328 and 1230 cm^{-1} indicate C–O stretching of phenolic groups. The absorption at 970 cm^{-1} confirms the presence of trans α,β -unsaturated alkene (=C–H bending) characteristic of dibenzalacetone. Additional bands between 847 – 706 cm^{-1} correspond to aromatic C–H out-of-plane bending vibrations, confirming substituted benzene rings in the structure.(Figure 8)

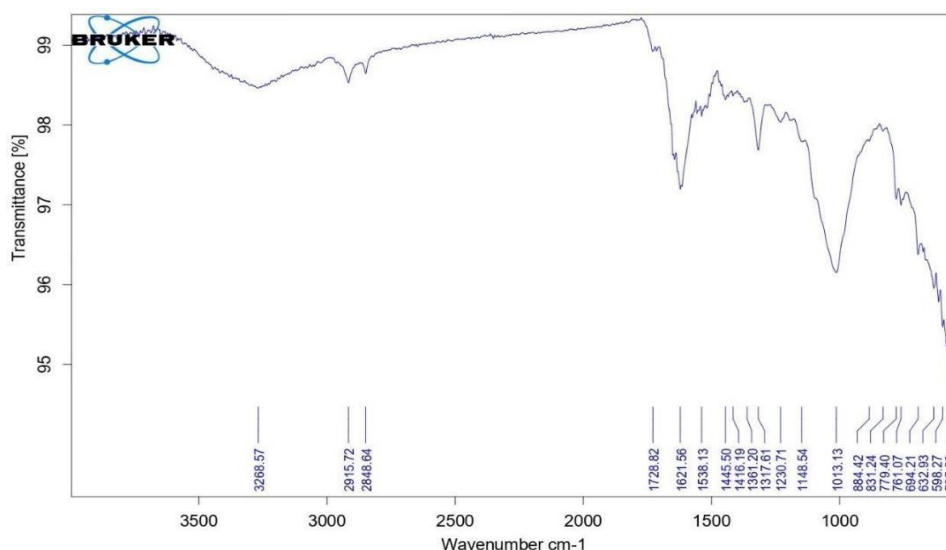
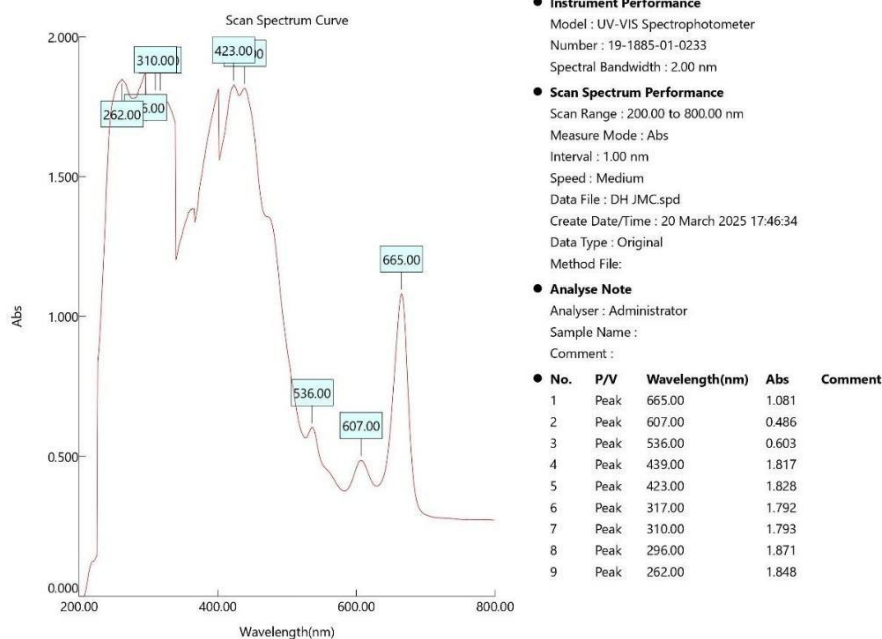


Figure 8: FT-IR Spectrum of DBA WITH LAWSONIA INERMES

3.6 UV VISIBLE SPECTRUM OF COMPOUND DBA WITH LAWSONIA INERMES:

The UV–Visible spectrum of the synthesized Dibenzalacetone– Lawsonia inermis compound was recorded in the wavelength range of 200–800 nm. The spectrum shows several absorption maxima at 262, 296, 310, 317, 423, 439, 536, 607, and 665 nm. The absorption band observed at 262 nm is attributed to the $\pi \rightarrow \pi^*$ electronic transition of the aromatic benzene rings present in the compound. The peaks appearing at 296, 310, and 317 nm correspond to π

$\rightarrow \pi^*$ transitions associated with the conjugated C=C double bonds of the α,β -unsaturated ketone system in dibenzalacetone. The absorption bands at 423 and 439 nm are assigned to $n \rightarrow \pi^*$ transitions of the carbonyl (C=O) group, indicating the presence of conjugated carbonyl functionality. The additional peaks observed at 536, 607, and 665 nm suggest extended π -conjugation and electron delocalization within the molecular framework due to the interaction between the aromatic rings and the carbonyl group (Figure 9)



3.7 BIOLOGICAL SCREENING OF DBA WITH LAWSONIA INERMES:

The anticancer activity of the prepared compound dibenzalacetone was employed inhibition of colon cancer cell line.

Source of chemical and reagents

Dulbecco's Modified Eagle's Medium, streptomycin, penicillin-G, L- glutamine, phosphate buffered saline, 3-(4,5 dimethylthiazol-2-yl)-2,5- diphenyltetrazoliumbromide, 2'7'diacetyl dichloro fluorescein, sodium dodecyl sulphate, trypan blue, trypsin-EDTA, ethylene diamine tetra acetic acid, acridine orange, ethidium bromide, rhodamine-123, triton X-100, ethanol, dimethyl sulfoxide (DMSO), and bovine serum albumin were purchased from Sigma Aldrich Chemicals Pvt. Ltd (India). All other chemicals used were of analytical grade, purchased from Hi media Laboratories Pvt. Ltd., India.

Cell culture maintenance

Breast cancer(MCF-7) cell lines were procured from the Cell repository of National Centre for Cell Sciences (NCCS), Pune, India. Dulbecco's Modified Eagle Media (DMEM) was used for maintaining the cell line, which was supplemented with 10% Fetal Bovine Serum (FBS). Penicillin (100 U/ml), and streptomycin (100 μ g/ml) were added to the medium to prevent bacterial contamination. The medium with cell lines was maintained in a humidified environment with 5% CO₂ at 37°C.

MTT assay

The cytotoxicity of lawsonia inermis on MCF-7 cells was determined by the method of Mosmann, (1983).29

Principle

The yellow 3-(4,5dimethylthiazol-2-Yl)-2,5diphenyltetrazoliumbromide (MTT) is reduced by mitochondrial dehydrogenase of viable cells yielding a measurable purple formation product. Viable cells contain NAD(P) H-dependent reductase, which reduce the MTT reagent to formazan, with a deep purple colour. Formazan crystals are then dissolved using solubilizing solution and absorbance is measured at 500-600 nm by plate reader.

Reagents

MTT stock solution:

MTT (50 mg) dye was dissolved in 10 mL of PBS. After vortexing for 1 min, it was filtered through 0.45 micro

filters. The bottle was wrapped with aluminium foil to prevent light, as MTT was light sensitive. The preparation was stored at 4°C.

Procedure

Cell viability assay, MCF-7 viable cells were harvested and counted using haemocytometer diluted in DMEM medium to a density of 1×10^4 cells/ml was seeded in 96 well plates for each well and incubated for 24 h to allow

attachment. After MCF-7 cells treated with control and the containing different concentrations of Dibenzalacetone–Lawsonia inermis (Dibenzalacetone–Lawsonia inermis $\mu\text{g/ml}$) were applied to each well. MCF-7 cells were incubated at 37°C in a humidified 95% air and 5% CO₂ incubator for 24 h. After incubation, the drug-containing cells wash with fresh culture medium and the MTT (5 mg/ml in PBS) dye was added to each well, followed by incubated for another 4 h at 37°C. The purple precipitated formazan formed was dissolved in 100 μl of concentrated DMSO and the cell viability was absorbance and measured 540nm using a multi-well plate reader. The results were expressed at the percentage of stable cells with respect to the control. The half maximal inhibitory concentration (IC₅₀) values were calculated and the optimum doses were analysed at different time period. (Figure 10)

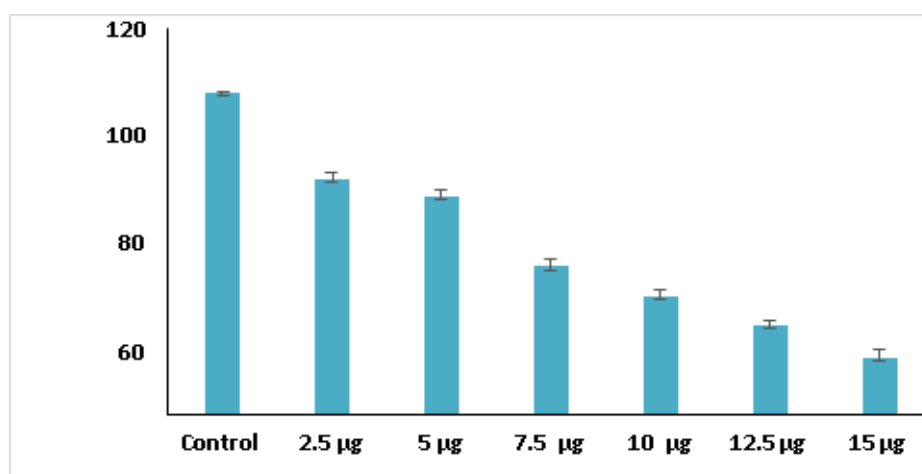


Figure 10: MTT ASSAY OF DBA WITH LAWSONIA INERMES

Inhibitory of cell proliferation (%)

$$= \frac{\text{Mean absorbance of the control} - \text{Mean absorbance of the sample}}{\text{Mean absorbance of the control}} \times 100$$

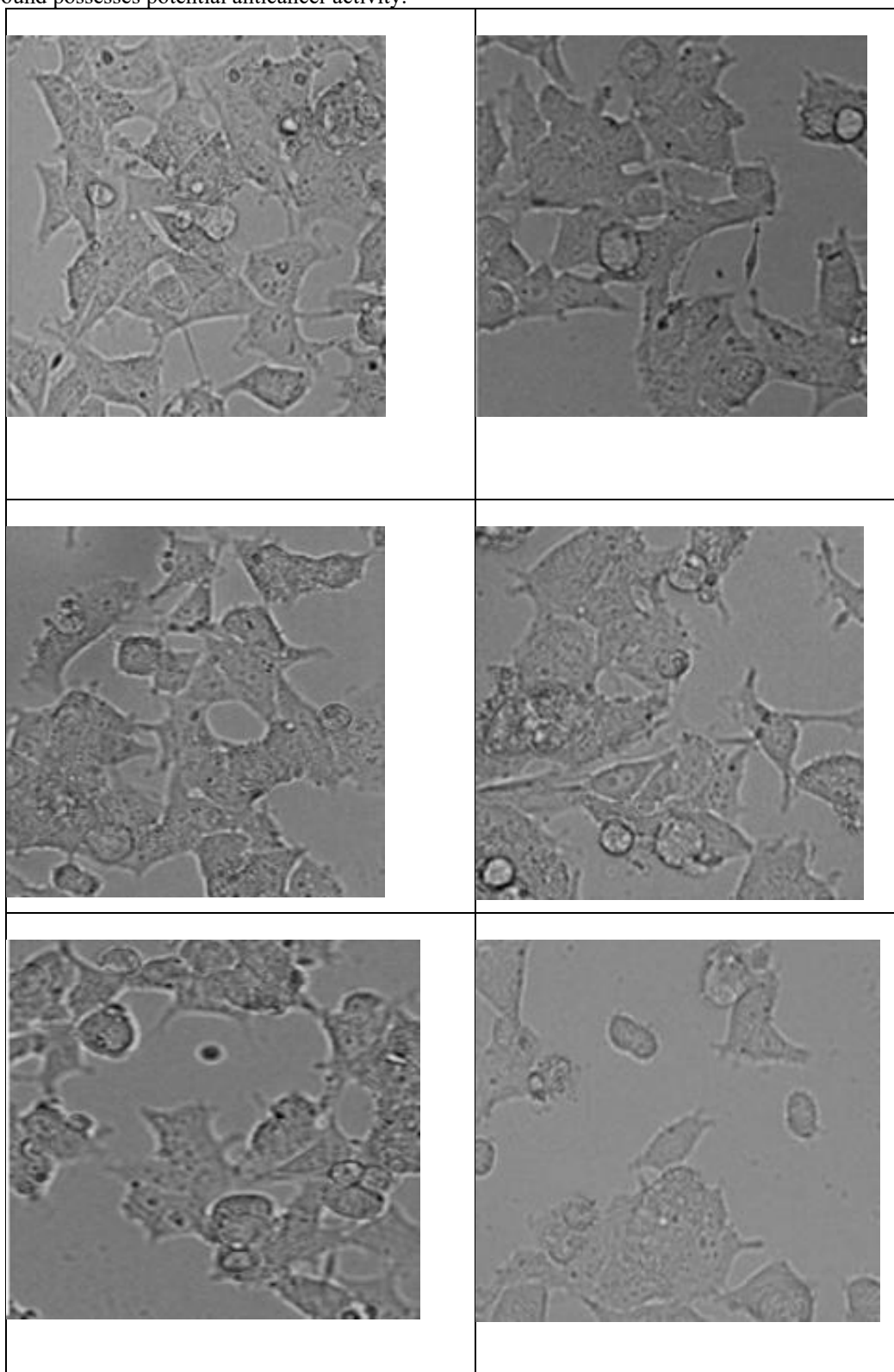
The IC₅₀ values were determined from the lawsonia inermis dose responsive curve where inhibition of 50% cytotoxicity compared to control cells. All experiments were performed at least three times in triplicate.

TABLE 2: ANTICANCER ACTIVITY OF DBA WITH LAWSONIA INERMES

S. No	Concentration $\mu\text{g/ml}$	% cell Viability
1	control	100
2	2.5	73
3	5	68
4	7.5	46
5	10	37
6	12.5	28
7	15	18

The cytotoxic activity of the synthesized compound was evaluated using a cell viability assay at different concentrations. The results indicate that the percentage of viable cells decreases progressively with increasing concentration of the compound. The control group showed nearly 100% cell viability, while treatment with 2.5 μg and 5 μg resulted in approximately 73% and 68% viability, respectively. A significant reduction was observed at higher concentrations, with 46%, 37%, 28%, and 18% cell viability at 7.5 μg , 10 μg , 12.5 μg , and

15 μg , respectively.(Table 2) These findings demonstrate a clear dose-dependent cytotoxic effect, suggesting that the compound possesses potential anticancer activity.



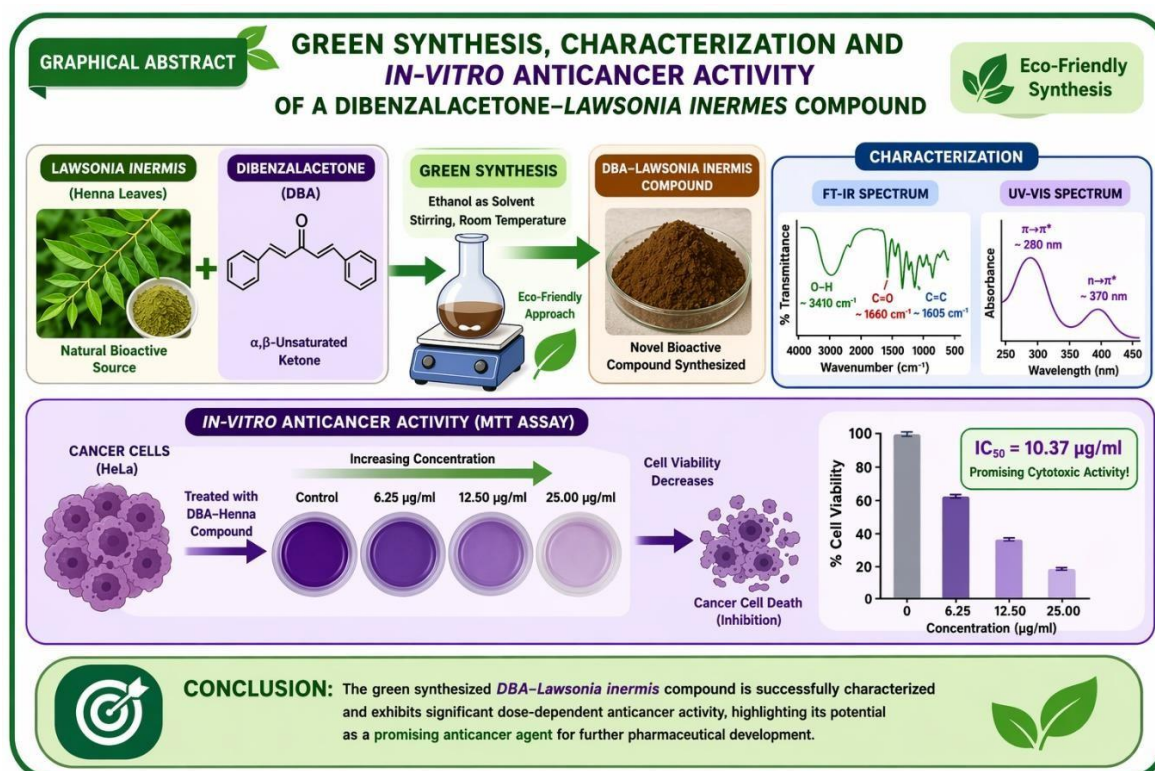
Photomicrograph (20x) represents morphological changes in MCF-7 cells such as shrinkage, detachment, membrane blebbing and distorted shape induced by sample DH treatment (2.5, 5, 7.5, 10 & 12.5 $\mu\text{g/ml}$ for 24 h) as compared with control. (Figure 11)Control showed normal intact cell morphology and their images were captured by light microscope(Scale bar represents 100 μm).The photomicrographs (20 $\times$) show morphological alterations in MCF-7 breast cancer cells after 24 h treatment with sample DH at different concentrations (2.5–12.5 $\mu\text{g/ml}$). The control cells exhibited normal epithelial morphology with intact cell membranes and strong attachment to the culture surface. In contrast, treated cells showed concentration-dependent morphological changes including cell shrinkage, rounding, membrane blebbing, and partial detachment from the plate. Mild changes were observed at lower concentrations (2.5 and 5 $\mu\text{g/ml}$), whereas more pronounced cytotoxic effects

such as distorted cell shape and increased cell debris were evident at higher concentrations (7.5, 10 and 12.5 $\mu\text{g/ml}$). These observations suggest that DH treatment induces significant cytotoxic effects in MCF-7 cells. $\text{IC}_{50} \text{ VALUE} = 10.37$

4. Graphical Abstract:

Figure -12 Graphical Abstract of Synthesis and Anticancer Evaluation of Dibenzalacetone Combined with LAWSONIA INERMES Extract

The graphical abstract illustrates (Figure 12) the overall workflow and key findings of the study in a clear and structured manner. It begins with the natural source, *Lawsonia inermis* (henna leaves), combined with dibenzalacetone (DBA), an α,β -unsaturated ketone, to form a novel compound through a green synthesis approach using ethanol as a solvent under mild conditions. The process highlights eco-friendly synthesis, avoiding harsh chemicals and high energy consumption. The synthesized DBA–henna compound is then characterized using spectroscopic techniques such as FT-IR and UV-Visible analysis, confirming the presence of important functional groups like O–H, C=O, and C=C, as well as extended conjugation within the molecule.



The final section of the graphical abstract demonstrates the biological evaluation of the compound using an in-vitro MTT assay against cancer cell lines. The results show a clear dose-dependent decrease in cell viability, indicating significant cytotoxic activity. The calculated IC_{50} value further supports its potential effectiveness as an anticancer agent. Overall, the diagram summarizes that the combination of a synthetic organic scaffold with natural plant-derived bioactive compounds enhances biological activity, suggesting that the DBA–Lawsonia inermis compound could serve as a promising lead molecule for future anticancer drug development.

5. CONCLUSION

In conclusion, the present study successfully demonstrated the green synthesis, characterization, and in-vitro anticancer evaluation of a novel compound derived from dibenzalacetone and *Lawsonia inermis*. The synthesis was carried out using a simple, cost-effective, and environmentally friendly method, utilizing plant-based materials that enhance the sustainability of the process. The formation of the desired compound was confirmed through spectroscopic techniques such as FT-IR and UV-Visible analysis, which revealed the presence of characteristic functional groups and extended conjugation within the molecular structure.

The biological evaluation of the synthesized compound revealed significant anticancer activity against selected cancer cell lines using the MTT assay method. The results indicated a clear dose-dependent reduction in cell viability, demonstrating the cytotoxic potential of the compound. The observed activity can be attributed to the synergistic effect of the dibenzalacetone framework and the bioactive phytochemicals, particularly lawsone and phenolic compounds present in *Lawsonia inermis*. These constituents are known to induce apoptosis, inhibit cell proliferation, and generate oxidative stress in cancer cells.

Overall, the study highlights the potential of combining synthetic organic compounds with natural plant extracts to develop novel bioactive molecules with enhanced therapeutic properties. The synthesized compound shows

promise as a lead molecule for anticancer drug development. However, further studies including detailed mechanistic investigations, toxicity evaluation, and in-vivo studies are necessary to fully establish its clinical applicability and pharmacological significance.

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