Chapter 4

Results & Discussion

4.1 Plant materials and sample preparation

Ayurvedic formulations tend to show relevance in modern medicine. Kumari and Baghel (2017) standardized *Panchaguna Taila*, an Ayurvedic medicated oil, and converted it into ointments and gels to enhance usability for wound management. Talekar et al. (2017) validated the wound healing potential of a polyherbal through *in vitro* and *in vivo* assays, illustrating its scientific basis and efficacy. *Securinega leucopyrus* has been widely studied for its antimicrobial and wound-healing. Ajmeer et al. (2014, 2018) reported its efficacy in diabetic foot ulcers, emphasizing its potential as a single-herb treatment. Unlike single-herb formulations, polyherbal combinations leverage the complementary and potentiating effects of various bioactive compounds. Polyherbal formulations are gaining recognition due to their enhanced therapeutic efficacy, often attributed to the synergistic action of their phytoconstituents. The plants selected for this study, *Securinega leucopyrus*, *Vitex negundo L.*, *Acacia catechu*, and *Azadirachta indica*, have a long history of use in traditional medicine. They are chosen based on their documented pharmacological properties, which include antimicrobial, anti-inflammatory, and wound-healing activities, critical for managing infections and promoting tissue repair. The plants were rationally selected for their scientifically proven biological attributes. They were all collected, dried and collected for formulation preparation.

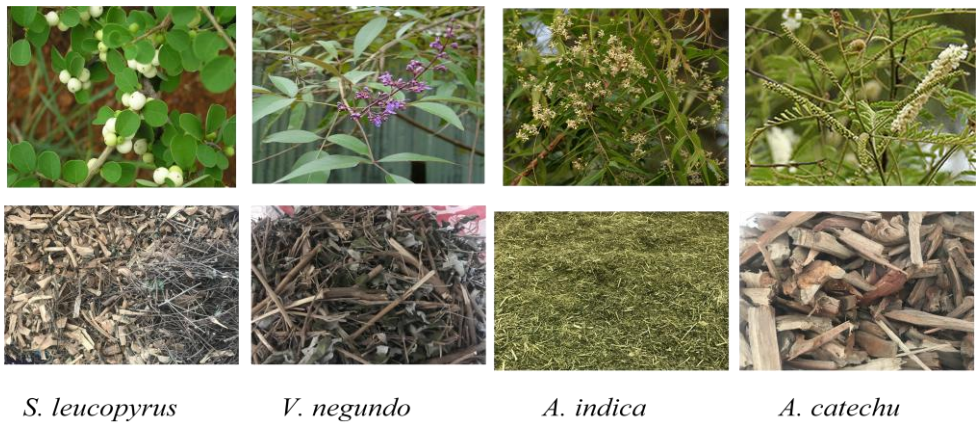

Fig 1: The dried plant parts of Securinega leucopyrus, Vitex negundo, Azadiracta indica and Acacia catechu were identified, dried, powdered and used for preparation.

4.2 Preparation of the Ayurvedic polyherbal formulation

Polyherbal decoctions are known for their rich phytochemical profiles, including flavonoids, alkaloids, saponins, tannins, and phenolics. These compounds exhibit antioxidant, anti-inflammatory, antimicrobial, and immunomodulatory properties, which are essential for their therapeutic potential. Thakuria et al. (2018) emphasized the importance of phytochemical screening of medicinal plants in assam, noting that decoctions often serve as a concentrated source of these bioactives, tailored to specific therapeutic needs Pharmacological applications

Step 1: Preparation of Kwath:

The following steps outline the preparation process for a decoction of a plant extract using powdered plant material.

Preparation of Plant Material

The dried plant samples were first ground into a fine powder using an appropriate grinding technique. The powder was then weighed to ensure accurate dosing for the extraction process.

Preparation of Aqueous Extract

The plant powder was added to distilled water in a clean, heat-resistant container. The mixture was then heated at a low flame to avoid the degradation of temperature-sensitive compounds. The heating process was performed with constant stirring to ensure even heat distribution and prevent the powder from settling at the bottom, which could lead to uneven extraction.

Concentration of Extract

The solution was continuously monitored as it was heated. The volume of the aqueous extract was reduced by boiling, typically until it reached about One-fourth of its original volume. This reduction in volume concentrates the bioactive compounds, ensuring a more potent extract. The process may take several hours, depending on the initial volume and the specific plant material being used.

Filtration of the Extract

After the decoction process, the hot mixture was filtered to remove any residual plant particles. A sterilized cloth or fine mesh sieve was used for this purpose. The cloth or

sieve should be sterile to prevent contamination, ensuring the safety and purity of the final extract.

Collection of Filtrate

The filtrate, which contains the water-soluble compounds from the plant, was collected in a clean, sterile container. Care was taken to avoid contamination during this step, ensuring the extract remains free from any foreign particles or microorganisms.

Filtrate Preservation

The collected filtrate was then preserved in sterile containers to maintain the integrity of the extract. Preservation techniques may include refrigeration or freezing, depending on the intended use of the decoction. Sterility and proper storage conditions are crucial to prevent the growth of microorganisms and maintain the potency of the extract for subsequent analysis or application.

Fig 2: The process of Kwath formation

Multiple studies have focused on polyherbal formulations with potent anti-inflammatory, antioxidant, and antimicrobial properties. Such formulations, including polyherbal emulsions and ointments, have been validated for their efficacy in preclinical and clinical trials, indicating a reduction in wound size and microbial load [134], [139], [144].

Step 2: Preparation of Taila (Polyherbal formulation)

Fig 3: The process of Tailum formation

Addition of Raw Powdered Plant Material

To further enrich the formulation and increase the concentration of bioactive compounds, 10% of the total plant powder is added to the oil and aqueous mixture. This addition ensures that more of the plant's active compounds are incorporated into the extract. The powder may contain both water-soluble and fat-soluble substances, contributing additional therapeutic value to the formulation.

Oil Decoction Process

The mixture of oil, aqueous extract, and added plant powder undergoes a decoction process. The oil is heated gently to allow the plant compounds to be extracted into the oil phase. During this process, the volume of the mixture is gradually reduced to match the original volume of oil added. The reduction ensures the concentration of active compounds within the oil, intensifying the potency of the final extract. The decoction process also allows for the full extraction of oil-soluble compounds such as essential oils, resins, and lipophilic phytochemicals.

Filtration of Polyherbal Oil Extract

After the decoction, the resulting oil extract is filtered to remove any residual plant matter. The filtration process is typically performed using a fine mesh or sterilized cloth to ensure the oil is free from unwanted particulate matter. The filtered oil now contains concentrated bioactive compounds from both the oil and aqueous phases, as well as from the additional plant material.

Final Polyherbal Oil formulation

The filtered oil, now rich in the therapeutic compounds extracted from both the aqueous decoction and the oil decoction process, constitutes the final polyherbal formulation. This formulation can be used for various medicinal, therapeutic, or cosmetic applications, leveraging the synergistic effects of the combined bioactive compounds.

4.3 Physiological Properties

The various physicochemical parameters that were determined as per The Unani Pharmacopoeia of India- viz. Acid value, Saponification value, pH, Viscosity, Specific gravity, Refractive index, Organoleptic property and Relative density [203]

S. No	Parameters	Observation	Comments [202]
1	Color	Green color	The green hue suggests the presence of chlorophyll or other plant-derived pigments. This may also indicate antioxidant properties, which are common in polyherbal formulations due to bioactive phytochemicals
2	Odor	Nutty smell	A nutty aroma can stem from specific herbal constituents or oils, which often contribute to the sensory appeal of the formulation, enhancing user compliance
3	Specific gravity	0.912kg/m^3	This value is in line with oils used in topical or therapeutic formulations. It ensures the proper flow and spreadability, essential for applications like creams or ointments
4	pH	6.40	The slightly acidic pH makes the formulation compatible with human skin, which has a natural pH range of 4.5–6.5. This reduces the risk of irritation and makes it suitable for topical use
5	Acid value	5.61	The acid value measures free fatty acids, reflecting the product's freshness and stability. A lower value indicates minimal lipid oxidation, essential for maintaining the formulation's efficacy over time
6	Saponification value	191mgKOH/g	This parameter suggests the presence of triglycerides or oils in the formulation, which can act as emollients or moisturizers in skincare applications. It also hints at potential uses in soap-making
7	Irritation test	No irritation	The absence of irritation in testing underscores the formulation's safety for dermal applications, making it a viable candidate for use in sensitive skin treatments or cosmetic products
8	Relative density	0.966 kg/m^3	Similar to specific gravity, this parameter reflects the formulation's physical characteristics, ensuring consistency and usability

Table 1: Physiological properties of Polyherbal formulation

These attributes align with modern polyherbal formulations designed for therapeutic or cosmetic use. Recent research highlights the importance of such evaluations to ensure safety, efficacy, and consumer acceptance. For further refinement, stability testing and detailed phytochemical analyses could strengthen the formulation's positioning in the market [201].

4.4 Phytochemical analysis

4.4.1 Qualitative analysis of polyherbal formulation:

The phytochemical analysis of *Securinega leucopyrus*, *Vitex negundo*, *Acacia catechu*, and *Azadirachta indica* provides valuable insight into their therapeutic potential, and recent studies have continued to affirm the relevance of these plants in traditional and modern medicine. for example, the consistent presence of phenolic compounds across all plant extracts (Gupta et al., 2024; Sharma et al., 2024) supports their role as potent antioxidants and anti-inflammatory agents. Recent findings have further emphasized the importance of phenolics in preventing oxidative stress-related diseases, including cardiovascular disorders (Patel et al., 2024; Mishra et al., 2023).

Flavonoids, identified in all the plants tested, contribute significantly to the anti-inflammatory and anticancer properties of the formulation (Kumar et al., 2024; Verma et al., 2023). The presence of specific flavonoids like luteolin in *Vitex negundo* is particularly important in modulating immune responses and preventing chronic diseases. This underscores the therapeutic value of polyherbal formulations in disease prevention and management.

Tannins and alkaloids, both identified in these plants, have been shown to offer antimicrobial benefits (Jadhav et al., 2023; Singh et al., 2023). Their presence further enhances the polyherbal formulation's application in combating microbial infections, particularly in topical and systemic treatments.

Saponins, absent in *Securinega leucopyrus* but present in the other plants and oils, continue to gain attention for their cholesterol-lowering and immune-boosting properties (Tiwari et al., 2023; Gupta et al., 2024). These compounds are particularly relevant in cardiovascular and metabolic health, where they may play a role in regulating lipid profiles.

Finally, terpenoids, abundant across all the plants, remain a major focus due to their broad-spectrum antimicrobial, anti-inflammatory, and anticancer activities (Mishra et al., 2023; Kumar et al., 2024). Terpenoids such as azadirachtin from *Azadirachta indica* have been highlighted for their effectiveness in treating microbial infections and managing skin disorders.

in conclusion, the polyherbal formulation developed from *Securinega leucopyrus*, *Vitex negundo*, *Acacia catechu*, and *Azadirachta indica* remains a promising avenue for addressing various health concerns, with comprehensive evidence supporting its broad-spectrum therapeutic properties (Gupta et al., 2024; Sharma et al., 2024). The synergistic effects of these bioactive compounds validate the importance of traditional knowledge in modern phytotherapy.

Table 2: Qualitative data of Polyherbal formulation

Bioactives	Extracts				
	S. leucopyrus	***V. negundo***	***A. indica***	***A. catechu***	**Oil**
Phenolics	**+ve**	**+ve**	**+ve**	**+ve**	**+ve**
Flavonoids	**+ve**	**+ve**	**+ve**	**+ve**	**+ve**
Tannins	**+ve**	**+ve**	**+ve**	**+ve**	**+ve**
Alkaloids	**+ve**	**+ve**	**+ve**	**+ve**	**+ve**
Terpenoids	**+ve**	**+ve**	**+ve**	**+ve**	**+ve**
Saponins	**-ve**	**+ve**	**+ve**	**+ve**	**+ve**
Glycosides	**-ve**	**+ve**	**+ve**	**+ve**	**+ve**

4.5 Extraction of phytochemicals

Polar solvents

4.5.1 Water

From the soxhlet extraction, crude plant extract was made. A thimble bag was used to pack and extract approximately 5 grammes of all four plant powders in a 4:1:1:1 ratio using 250–300 millilitres of water. Here, the extraction solvent was R/O or drinking water. The extraction procedure was carried out for 14–20 hours, or until the extractor's syphon tube's solvent turned colourless. once the solvent has turned colourless, let it cool and then store it in the refrigerator.

Fig 4: Extraction of Phytochemicals in Water

4.5.2 Methanol

Take 5 grammes of the powdered medicinal plant that was listed in Table 1. Consider the 4:1:1:1 ratio of powdered medicinal plants. then evenly compacted the plant powder to create a thimble-sized bag. A subsequent extraction of this thimble bag was carried out using 250–300 cc of methanol as a solvent. Five to six hours were spent on the extraction procedure before the solvent in the syphon tube turned colourless. Let the device cool down as soon as the syphon tube's thimble turns colourless. Pour the extract into the sterile bottle and refrigerate until it cools.

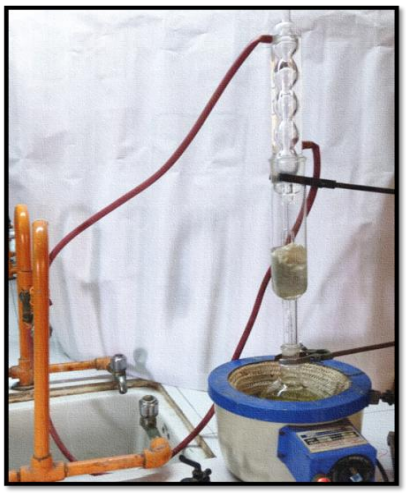

Fig 5: Extraction of Phytochemicals in Methanol

Non polar solvents

4.5.3 Ethanol

Four powdered medicinal plants, as listed in Table 1 above, were combined in a 4:1:1:1 ratio. in accordance with the ratio, 5 grammes of powder were taken, and a thimble bag of this powder was made. Between 250 and 300 millilitres of ethanol were used as the extraction solvent. The soxhlet extraction cycle lasted four to six hours, or until the thimble in the syphon tube turned colourless. Let it cool until it loses colour. once it has cooled, gather the extract in a bottle and keep it somewhere cool.

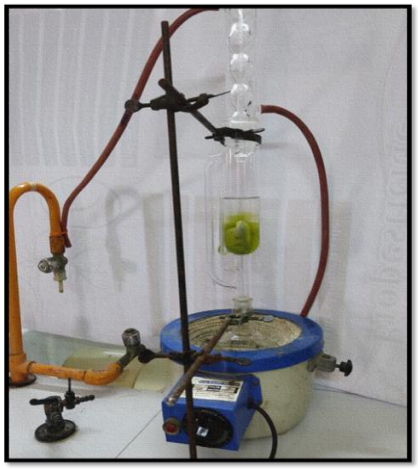

Fig 6: Extraction of Phytochemicals in Ethanol

4.5.4 Petroleum ether

The solvent used in the soxhlet extraction was petroleum ether. A thimble bag containing approximately 5 grammes of plant powder was made and placed within a syphon tube. The extraction process took place for 5 to 6 hours, or until the tube turned colourless. Approximately 250 to 300 millilitres of solvent were used for the extraction. The extract was taken, cooled, collected in a bottle, and kept in a cool place.

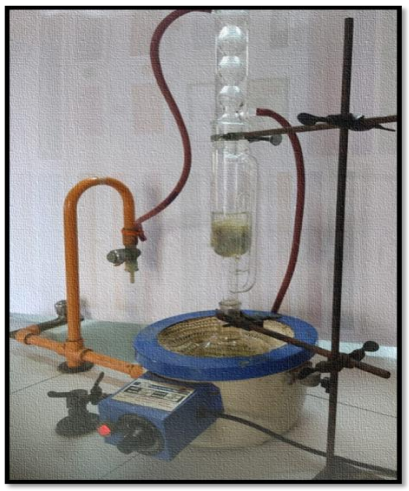

Fig 7: Extraction of Phytochemicals in Petroleum Ether

Semi polar solvents

4.5.5 Hexane

Five grammes of each of the four powdered medicinal plants were taken in the 4:1:1:1 ratio and a thimble bag was made. Dissolve with 250–300 millilitres of hexane. The extraction cycle was repeated using hexane as the solvent until the solvent inside the thimble bag was colourless. Permit the extract to cool. Once cooled, store it in the pack bottle and maintain a cool environment.

Fig 8: Extraction of Phytochemicals in Hexane

4.5.6 Chloroform

Crude plant extracts were prepared by Soxhlet extraction. Five grams of all vegetable powders were taken in a ratio of 4:1:1:1 and made into sewn bags. This was extracted

with 250-300 mL of chloroform. The solvent used was chloroform. The extraction process was carried out for 10 to 15 hours until the thimble in the siphon tube became colorless. While the solvent cooled, it was stored in a bottle at a cool temperature.

Fig 9: Extraction of Phytochemicals in Chloroform

4.5.7 Storage of extracts

After the extraction of plant material, the extracts are stored in a well closed sterile container or blue cap bottles can also be used. Collect all the different extracts in different blue cap bottles and label it & stored in refrigerator or cold room. This extract can further be used to check its quantitative estimation, anti-microbial activity, etc.

					
Methanol	**Ethanol**	**Hexane**	**Petroleum Ether**	**Chloroform**	**Water**

Fig 10: Storage of Phytochemical Extract

4.6 Quantitative analysis of plant extract:

Based on qualitative analysis of phytochemicals, quantitative analysis was also dOne on major phytochemical that were the terpenoids and flavonoids. Terpenoids being One of the

foremost bioactive compounds in plants. Likewise, Flavonoids in recent studies, considered as an essential compOnent in a variety of therapeutics, pharmaceutical, medicinal and cosmetic applications. Therefore, Quantitative analysis for the terpenoid and flavonoid was performed with the mixture of plant extract of Neem *(Azadirachta indica)*, Nirgundi *(Vitex negundo)*, Katupila *(Securinega leucopyrus)*, and Khadir *(Acacia catechu)* prepared in ratio of 4:1:1:1.

Studis of Sahu et al. (2023) and Yadav et al. (2023) report that plant extracts with high levels of phenolics and flavonoids exhibit strong antioxidant activities.

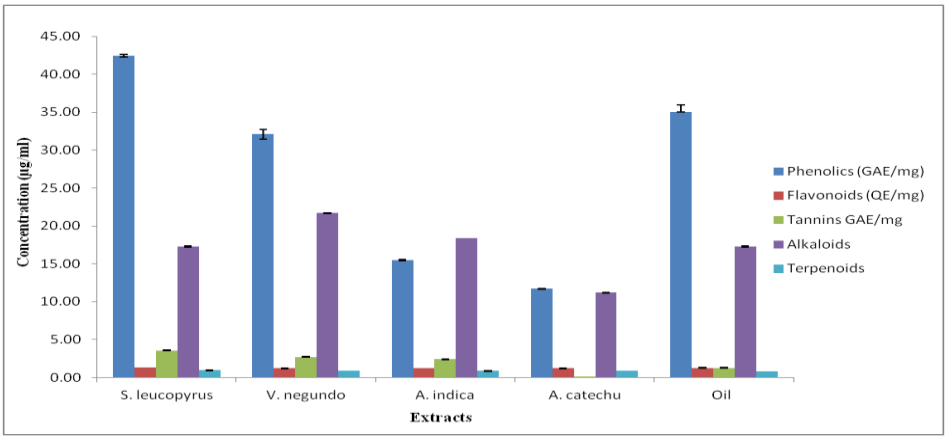

Fig 11: Data of Phytochemical Tests

Each extract is represented on the x-axis (S. leucopyrus, V. negundo, A. indica, A. catechu, and oil), while the y-axis indicates concentration (µg/ml). The graph uses different colored bars to represent each Phytochemical. Key observations are:

1. ***S. leucopyrus*** appears to have the highest concentration of phenolics, which are known for their antioxidant properties. This plant also shows a substantial presence of alkaloids.
2. ***V. negundo*** has high concentrations of alkaloids and phenolics, followed by moderate levels of terpenoids.
3. ***A. indica*** and ***A. catechu*** show lower concentrations across the board, especially in phenolics and alkaloids, compared to the other plants.
4. **Oil** (which likely refers to a specific plant oil extract) shows relatively high concentrations of phenolics and alkaloids, but low levels of flavonoids and tannins.

4.7 GCMS Analysis:

GC-MS analysis was outsourced to *Saurashtra University, Rajkot, at the Department of Pharmacy*, to facilitate the in-depth study of phytochemicals with potential roles in chronic wound healing. The data presented includes both a Total Ion Chromatogram (TIC) and a list of peaks identified, alongside their retention times, area percentages, and names of the compounds associated with each peak. Key observations from the GC-MS data are the compounds identified (e.g., **2**,4-Decadienal, Nonacosane, γ-Sitosterol) are known for their antioxidant, anti-inflammatory, and antimicrobial properties, suggesting that the oil sample might possess these bioactive characteristics. Sesquiterpenes like Sesamin are known for their antioxidant properties, while sterols like γ-Sitosterol are implicated in cholesterol-lowering effects.

Recent studies on essential oils and plant extracts have shown that compOnents like 2,4-Decadienal and γ-Sitosterol possess significant biological activities, such as:

- 2,4-Decadienal has been recognized for its potential antimicrobial and antioxidant properties, playing a role in reducing oxidative stress and microbial growth (Bazaid et al., 2023).
- Nonacosane, a saturated hydrocarbon, is known for its antioxidant and anti-inflammatory effects, contributing to the stability and therapeutic potential of oils (Singh et al., 2023).
- γ-Sitosterol, a plant sterol, has recently been studied for its lipid-lowering and anti-inflammatory effects, which make it valuable in managing cardiovascular diseases (Khan et al., 2023).

This GC-MS analysis is a crucial tool for profiling and identifying the chemical constituents of plant oils, which can guide their potential uses in medicine, cosmetics, and food industries. Furthermore, with the increasing interest in natural products as therapeutic agents, this type of chemical profiling is becoming more common to validate the pharmacological efficacy of plant-based compounds.

Fig 12: GC-MS Data Report

Department of Pharmaceutical Sciences, Saurashtra University, Rajkot.

GCMS Report

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Dilution Factor : 1
Vial # : 1
Injection Volume : 1.00
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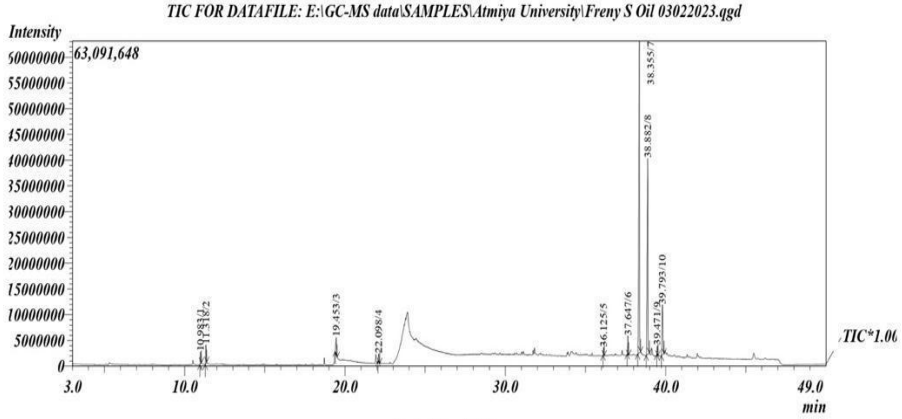

Peak Report TIC

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2	11.318	11.290	11.380	7186247	1.80	3655378	2,4-Decadienal, (E,E)-
3	19.453	19.385	19.510	10370685	2.60	3357930	n-Hexadecanoic acid
4	22.098	22.060	22.165	4523490	1.13	1549951	9-Octadecenoic acid (Z)-, methyl ester
5	36.125	36.095	36.165	2783325	0.70	1572810	Nonacosane
6	37.647	37.605	37.695	7115607	1.78	3613356	Tetratetracontane
7	38.355	38.250	38.435	198706028	49.84	60516211	(+)-Sesamin
8	38.882	38.785	38.985	122721801	30.78	38033141	5-(((1R,3aR,4S,6aR)-4-(Benzo[d][1,3]dioxc
9	39.471	39.435	39.520	4241700	1.06	1810591	Tetratetracontane
10	39.793	39.730	39.900	35758818	8.97	9724352	.gamma.-Sitosterol
				398653701	100.00	126208359	

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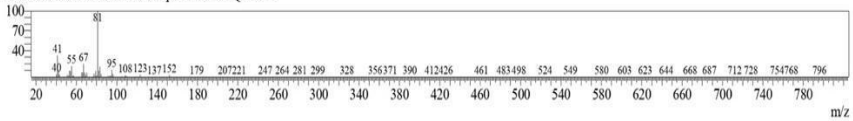

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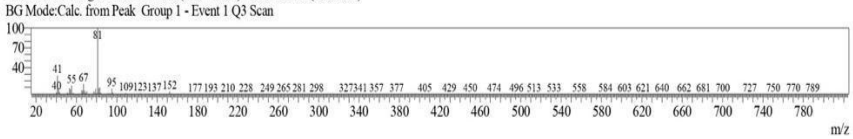

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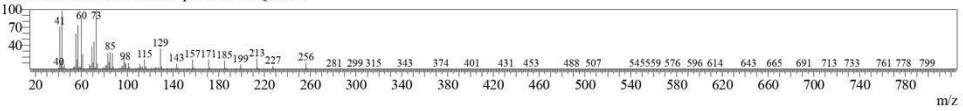

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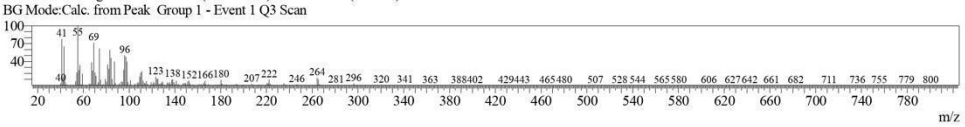

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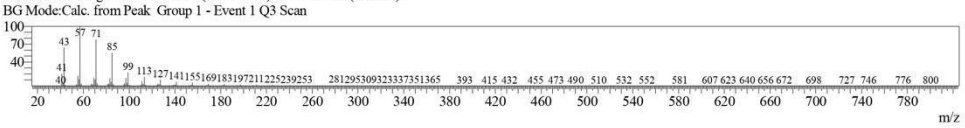

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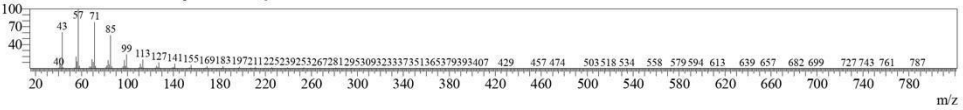

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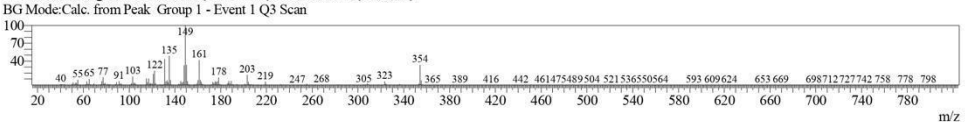

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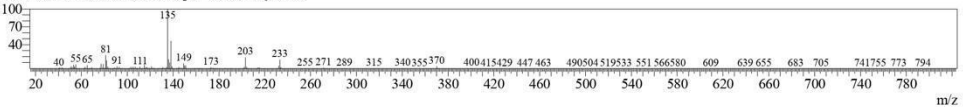

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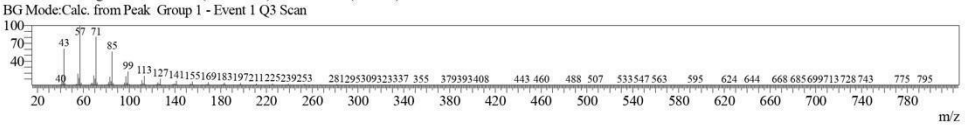

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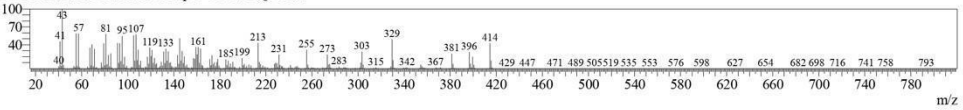

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Method

[Comment]

===== Analytical Line 1 =====

[AOC-30/20i+s]

# of Rinses with Presolvent	:5
# of Rinses with Solvent(post)	:5
# of Rinses with Sample	:3
Plunger Speed(Suction)	:High
Viscosity Comp. Time	:0.2 sec
Plunger Speed(Injection)	:High
Syringe Insertion Speed	:High
Injection Mode	:Normal
Pumping Times	:5
Inj. Port Dwell Time	:0.3 sec
Terminal Air Gap	:No
Plunger Washing Speed	:High
Washing Volume	:8uL
Syringe Suction Position	:0.0 mm
Syringe Injection Position	:0.0 mm
Solvent Selection	:All A,B,C

[GC-2010]

Column Oven Temp.	:50.0 °C
Injection Temp.	:250.00 °C
Injection Mode	:Split
Flow Control Mode	:Linear Velocity
Pressure	:103.5 kPa
Total Flow	:24.3 mL/min
Column Flow	:1.75 mL/min
Linear Velocity	:48.0 cm/sec
Purge Flow	:5.0 mL/min
Split Ratio	:10.0
High Pressure Injection	:ON
High Press. Inj. Pressure	:250.0 kPa
High Press. Inj. Time	:2.30 min
Carrier Gas Saver	:OFF
Splitter Hold	:OFF

Oven Temp. Program

Rate	Temperature(°C)	Hold Time(min)
-	50.0	2.00
10.00	200.0	10.00
10.00	300.0	10.00

< Ready Check Heat Unit >	
Column Oven	: Yes
SPL1 LQ	: Yes
MS	: Yes
< Ready Check Detector(FTD/BID) >	
< Ready Check Baseline Drift >	
< Ready Check Injection Flow >	
SPL1 LQ Carrier	: Yes
SPL1 LQ Purge	: Yes
< Ready Check APC Flow >	
< Ready Check Detector APC Flow >	
External Wait	:No
Equilibrium Time	:1.0 min

[GC Program]

[GCMS-TQ8040]

IonSourceTemp	:230.00 °C
Interface Temp.	:250.00 °C
Solvent Cut Time	:3.00 min
Detector Gain Mode	:Relative to the Tuning Result
Detector Gain	:1.00 kV +0.20 kV
Threshold	:0

[MS Table]

--Group 1 - Event 1--	
Compound Name	:Z1
Start Time	:3.00min
End Time	:50.00min
Acq. Mode	:Q3 Scan
Event Time	:0.300sec
Scan Speed	:3333
Start m/z	:40.00
End m/z	:800.00
Q1 Resolution	: -
Q3 Resolution	: -

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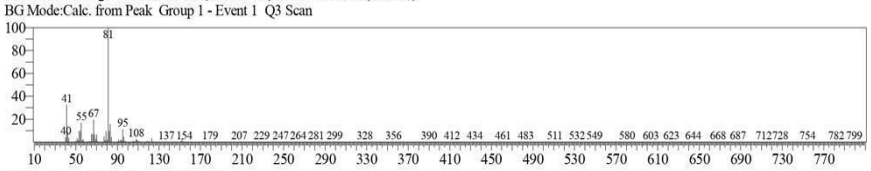
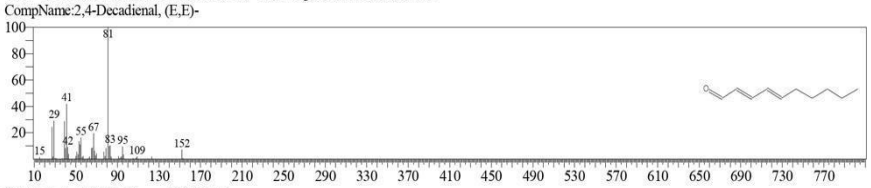
Sample Inlet Unit :GC

[MS Program]
Use MS Program :OFF

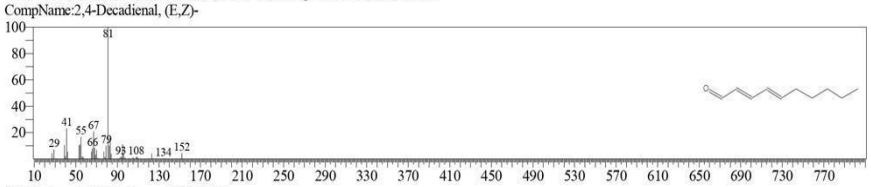
Library
<< Target >>
Line#:1 R.Time:10.985(Scan#:1598) Retention Index:1876 MassPeaks:417
RawMode:Averaged 10.980-10.990(1597-1599) BasePeak:81.10(665648)
BG Mode:Calc. from Peak Group 1 - Event 1 Q3 Scan



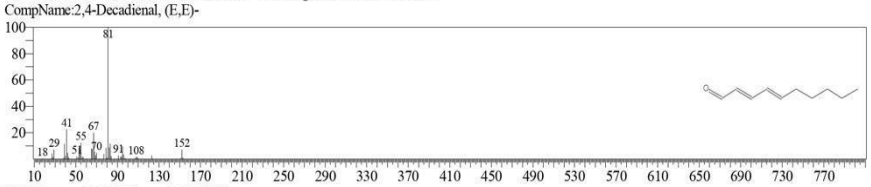
Hit#:1 Entry:28849 Library:NIST17.lib
SI:95 Formula:C10H16O CAS:25152-84-5 MolWeight:152 RetIndex:1220
CompName:2,4-Decadienal, (E,E)-



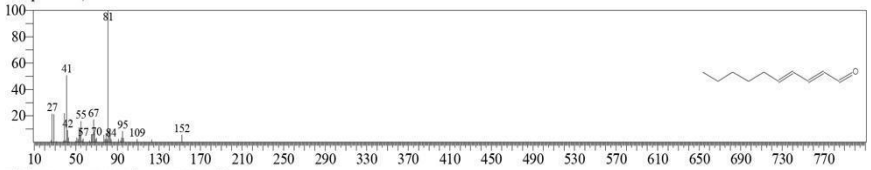
Hit#:2 Entry:28852 Library:NIST17.lib
SI:95 Formula:C10H16O CAS:25152-83-4 MolWeight:152 RetIndex:1220
CompName:2,4-Decadienal, (E,Z)-



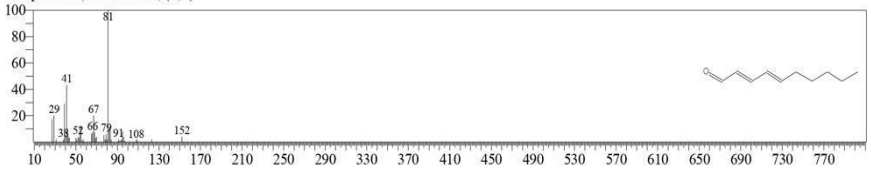
Hit#:3 Entry:29072 Library:NIST17.lib
SI:94 Formula:C10H16O CAS:25152-84-5 MolWeight:152 RetIndex:1220
CompName:2,4-Decadienal, (E,E)-



Hit#:4 Entry:28850 Library:NIST17.lib
SI:94 Formula:C10H16O CAS:2363-88-4 MolWeight:152 RetIndex:1220
CompName:2,4-Decadienal



Hit#:5 Entry:29069 Library:NIST17.lib
SI:94 Formula:C10H16O CAS:25152-84-5 MolWeight:152 RetIndex:1220
CompName:2,4-Decadienal, (E,E)-



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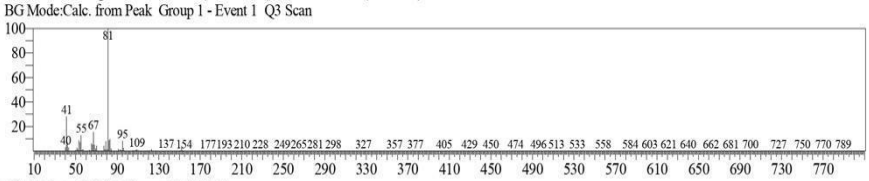
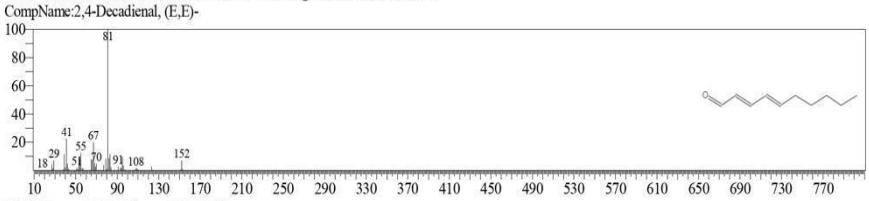
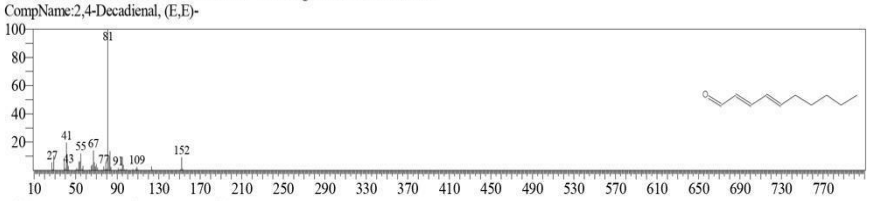
<< Target >>
Line#:2 R.Time:11.320(Scan#:1665) Retention Index:1903 MassPeaks:359
RawMode:Averaged 11.315-11.325(1664-1666) BasePeak:81.05(1204632)
BG Mode:Calc. from Peak Group 1 - Event 1 Q3 Scan



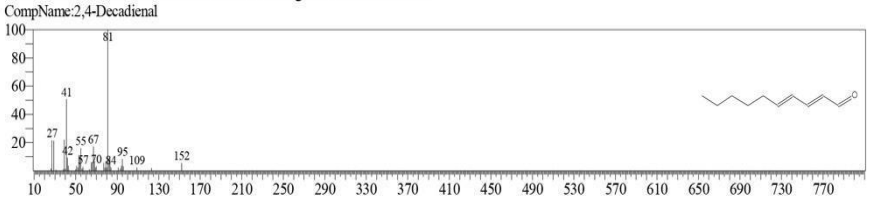
Hit#:1 Entry:29072 Library:NIST17.lib
SI:96 Formula:C10H16O CAS:25152-84-5 MolWeight:152 RetIndex:1220
CompName:2,4-Decadienal, (E,E)-



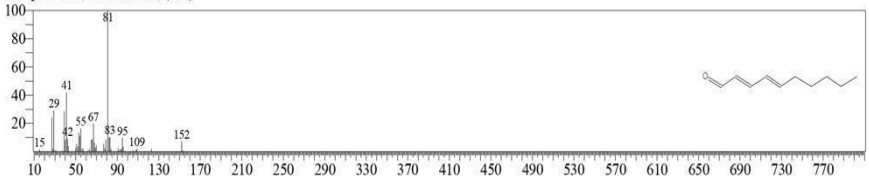
Hit#:2 Entry:29071 Library:NIST17.lib
SI:95 Formula:C10H16O CAS:25152-84-5 MolWeight:152 RetIndex:1220
CompName:2,4-Decadienal, (E,E)-



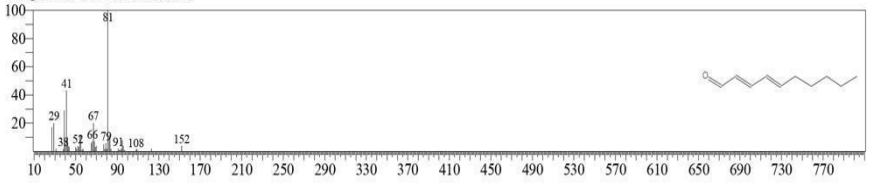
Hit#:3 Entry:28850 Library:NIST17.lib
SI:94 Formula:C10H16O CAS:2363-88-4 MolWeight:152 RetIndex:1220
CompName:2,4-Decadienal



Hit#:4 Entry:28849 Library:NIST17.lib
SI:94 Formula:C10H16O CAS:25152-84-5 MolWeight:152 RetIndex:1220
CompName:2,4-Decadienal, (E,E)-



Hit#:5 Entry:29069 Library:NIST17.lib
SI:94 Formula:C10H16O CAS:25152-84-5 MolWeight:152 RetIndex:1220
CompName:2,4-Decadienal, (E,E)-



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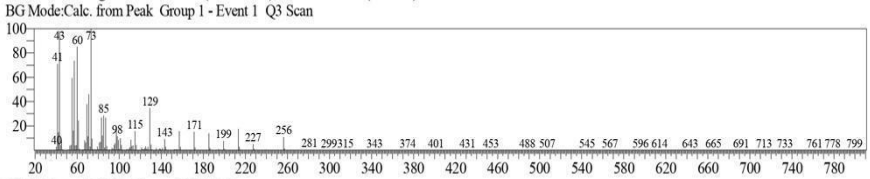
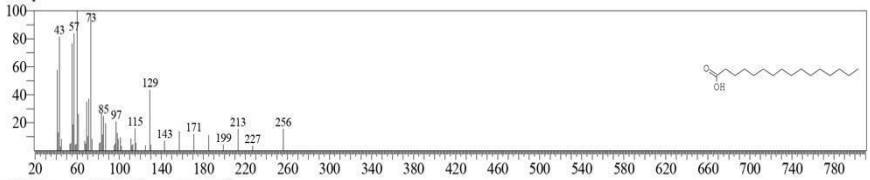
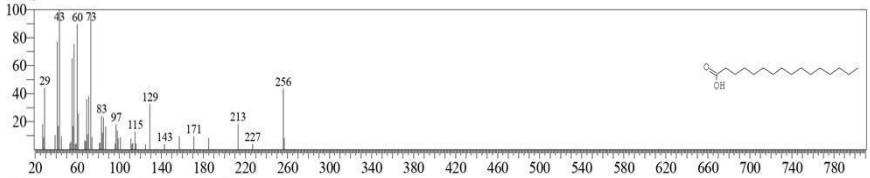
<< Target >>
Line#:3 R.Time:19.455(Scan#:3292) Retention Index:2465 MassPeaks:434
RawMode:Averaged 19.450-19.460(3291-3293) BasePeak:73.05(292265)
BG Mode:Calc. from Peak Group 1 - Event 1 Q3 Scan



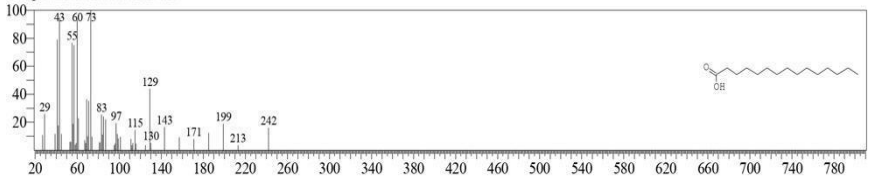
Hit#:1 Entry:129354 Library:NIST17.lib
SI:96 Formula:C16H32O2 CAS:57-10-3 MolWeight:256 RetIndex:1968
CompName:n-Hexadecanoic acid



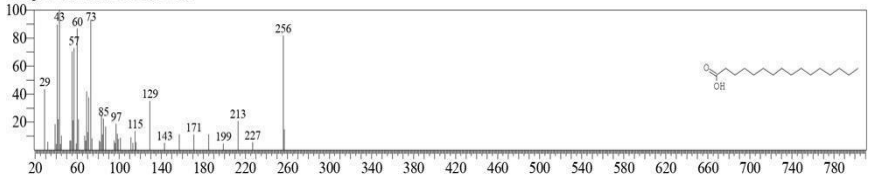
Hit#:2 Entry:129339 Library:NIST17.lib
SI:95 Formula:C16H32O2 CAS:57-10-3 MolWeight:256 RetIndex:1968
CompName:n-Hexadecanoic acid



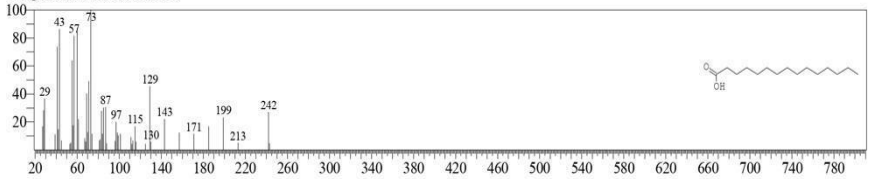
Hit#:3 Entry:114633 Library:NIST17.lib
SI:94 Formula:C15H30O2 CAS:1002-84-2 MolWeight:242 RetIndex:1869
CompName:Pentadecanoic acid



Hit#:4 Entry:129338 Library:NIST17.lib
SI:94 Formula:C16H32O2 CAS:57-10-3 MolWeight:256 RetIndex:1968
CompName:n-Hexadecanoic acid



Hit#:5 Entry:114684 Library:NIST17.lib
SI:94 Formula:C15H30O2 CAS:1002-84-2 MolWeight:242 RetIndex:1869
CompName:Pentadecanoic acid



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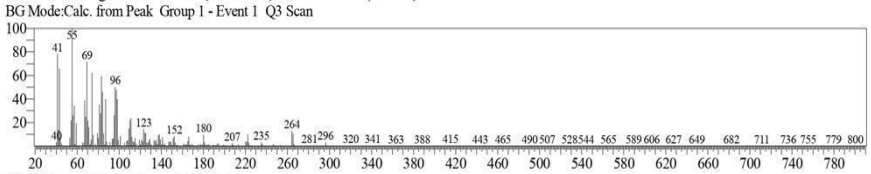
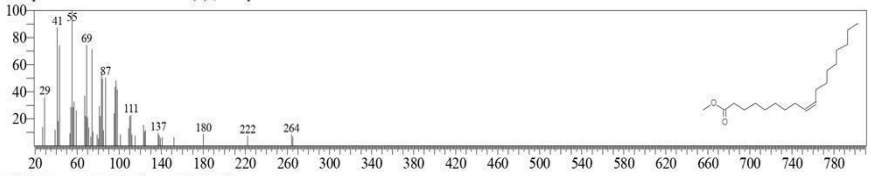
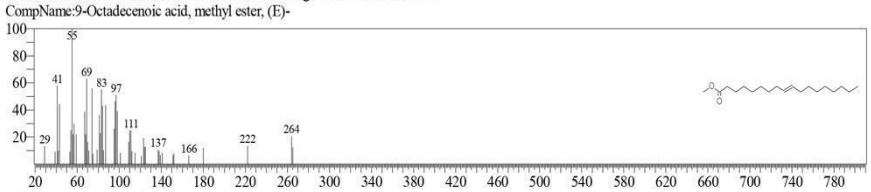
<< Target >>
Line#:4 R.Time:22.100(Scan#:3821) Retention Index:2646 MassPeaks:490
RawMode:Averaged 22.095-22.105(3820-3822) BasePeak:55.10(104885)
BG Mode:Calc. from Peak Group 1 - Event 1 Q3 Scan



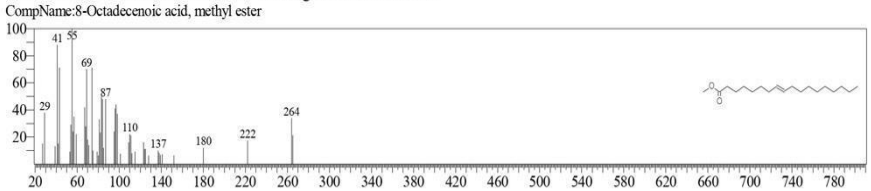
Hit#:1 Entry:172446 Library:NIST17.lib
SI:96 Formula:C19H36O2 CAS:112-62-9 MolWeight:296 RetIndex:2085
CompName:9-Octadecenoic acid (Z)-, methyl ester



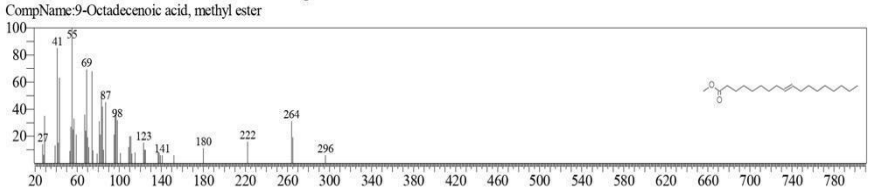
Hit#:2 Entry:172449 Library:NIST17.lib
SI:95 Formula:C19H36O2 CAS:1937-62-8 MolWeight:296 RetIndex:2085
CompName:9-Octadecenoic acid, methyl ester, (E)-



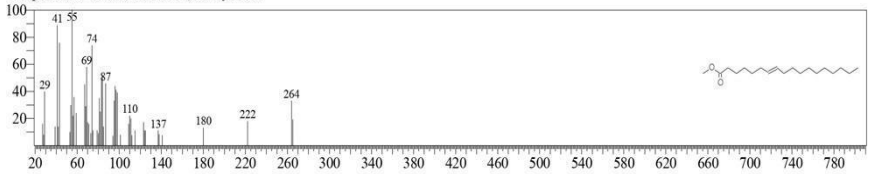
Hit#:3 Entry:172366 Library:NIST17.lib
SI:95 Formula:C19H36O2 CAS:2345-29-1 MolWeight:296 RetIndex:2085
CompName:8-Octadecenoic acid, methyl ester



Hit#:4 Entry:172372 Library:NIST17.lib
SI:95 Formula:C19H36O2 CAS:2462-84-2 MolWeight:296 RetIndex:2085
CompName:9-Octadecenoic acid, methyl ester



Hit#:5 Entry:172369 Library:NIST17.lib
SI:95 Formula:C19H36O2 CAS:57396-98-2 MolWeight:296 RetIndex:2085
CompName:7-Octadecenoic acid, methyl ester



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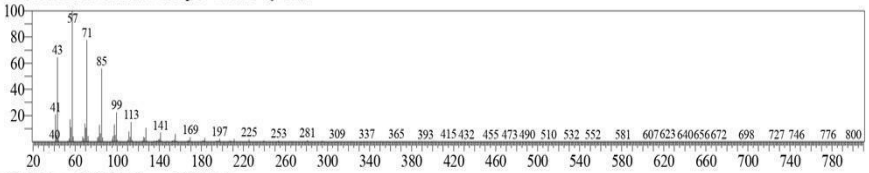
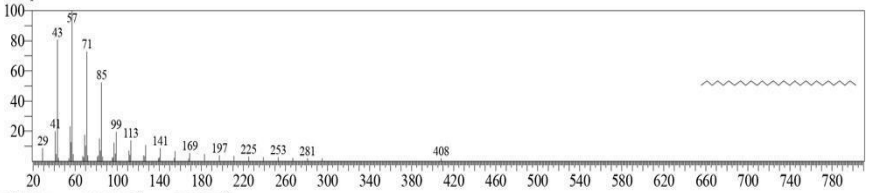
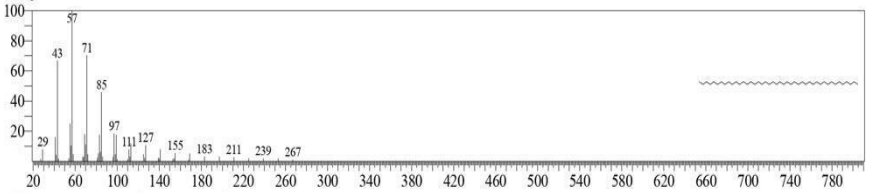
<< Target >>
Line#:5 R.Time:36.125(Scan#:6626) Retention Index:3754! MassPeaks:431
RawMode:Averaged 36.120-36.130(6625-6627) BasePeak:57.15(255294)
BG Mode:Calc. from Peak Group 1 - Event 1 Q3 Scan



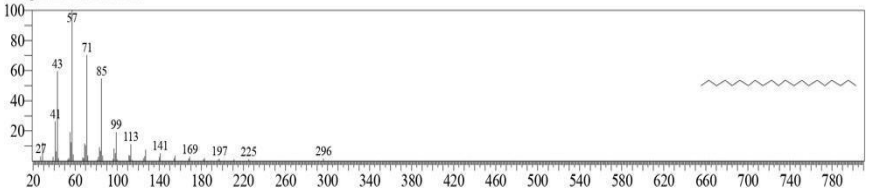
Hit#:1 Entry:269359 Library:NIST17.lib
SI:96 Formula:C29H60 CAS:630-03-5 MolWeight:408 RetIndex:2904
CompName:Nonacosane



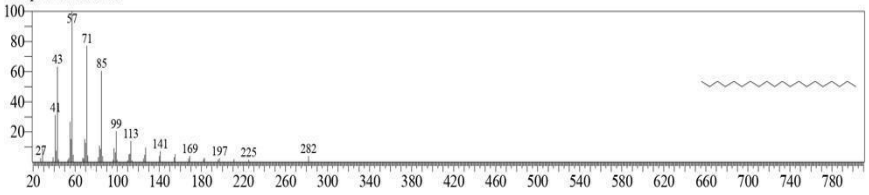
Hit#:2 Entry:303838 Library:NIST17.lib
SI:96 Formula:C44H90 CAS:7098-22-8 MolWeight:618 RetIndex:4395
CompName:Tetratetracontane



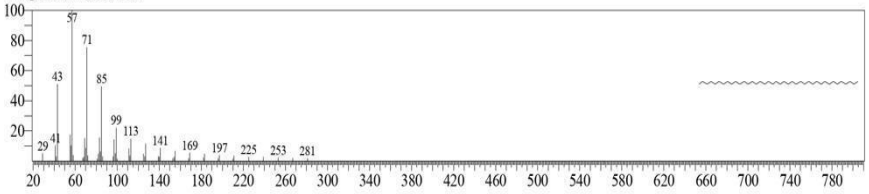
Hit#:3 Entry:172573 Library:NIST17.lib
SI:96 Formula:C21H44 CAS:629-94-7 MolWeight:296 RetIndex:2109
CompName:Heneicosane



Hit#:4 Entry:157187 Library:NIST17.lib
SI:95 Formula:C20H42 CAS:112-95-8 MolWeight:282 RetIndex:2009
CompName:Eicosane



Hit#:5 Entry:301740 Library:NIST17.lib
SI:95 Formula:C40H82 CAS:4181-95-7 MolWeight:562 RetIndex:3997
CompName:Tetracontane



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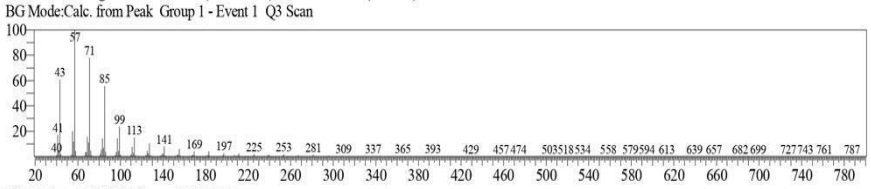
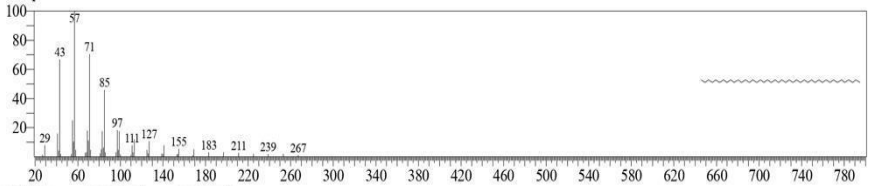
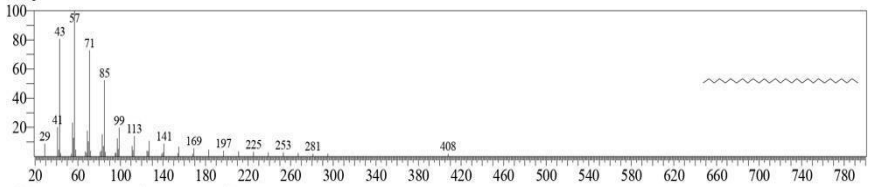
<< Target >>
Line#:6 R.Time:37.645(Scan#:6930) Retention Index:3880! MassPeaks:417
RawMode:Averaged 37.640-37.650(6929-6931) BasePeak:57.15(580719)
BG Mode:Calc. from Peak Group 1 - Event 1 Q3 Scan



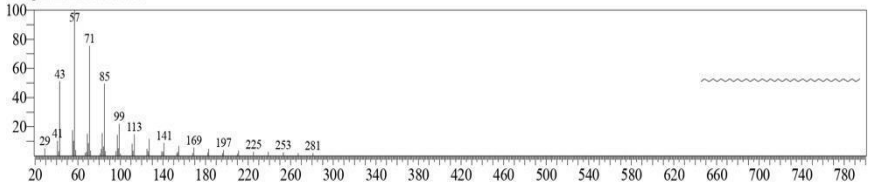
Hit#:1 Entry:303838 Library:NIST17.lib
SI:97 Formula:C44H90 CAS:7098-22-8 MolWeight:618 RetIndex:4395
CompName:Tetratetracontane



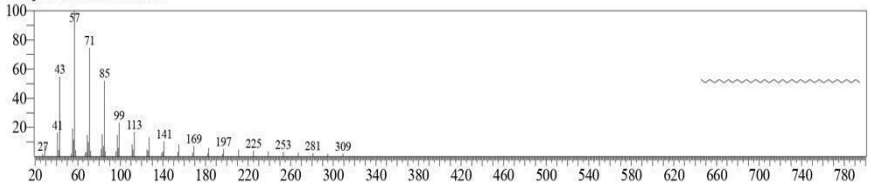
Hit#:2 Entry:269359 Library:NIST17.lib
SI:96 Formula:C29H60 CAS:630-03-5 MolWeight:408 RetIndex:2904
CompName:Nonacosane



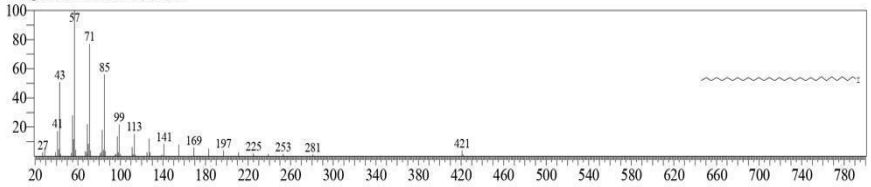
Hit#:3 Entry:301740 Library:NIST17.lib
SI:96 Formula:C40H82 CAS:4181-95-7 MolWeight:562 RetIndex:3997
CompName:Tetracontane



Hit#:4 Entry:296990 Library:NIST17.lib
SI:96 Formula:C36H74 CAS:630-06-8 MolWeight:506 RetIndex:3600
CompName:Hexatriacontane



Hit#:5 Entry:300810 Library:NIST17.lib
SI:95 Formula:C30H61I CAS:0-00-0 MolWeight:548 RetIndex:3418
CompName:Triacontane, 1-iodo-



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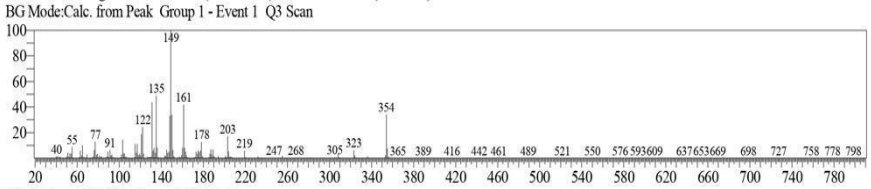
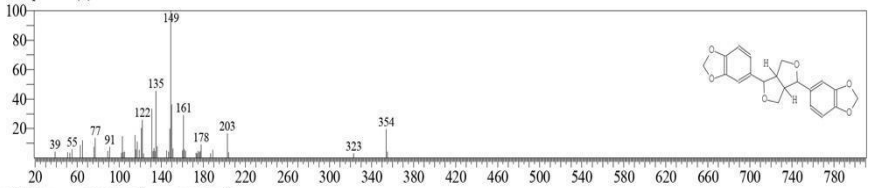
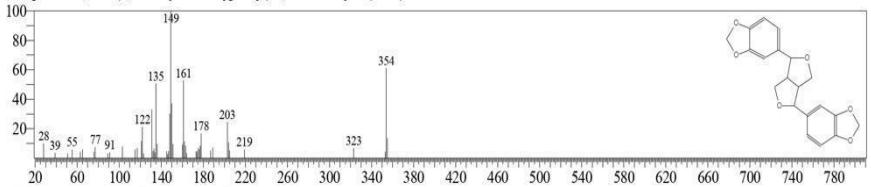
<< Target >>
Line#:7 R.Time:38.355(Scan#:7072) Retention Index:3939! MassPeaks:569
RawMode:Averaged 38.350-38.360(7071-7073) BasePeak:149.05(7395645)
BG Mode:Calc. from Peak Group 1 - Event 1 Q3 Scan



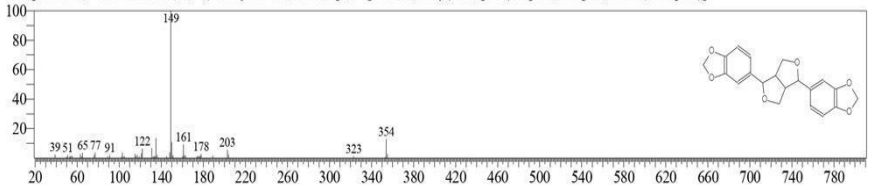
Hit#:1 Entry:231939 Library:NIST17.lib
SI:93 Formula:C20H18O6 CAS:607-80-7 MolWeight:354 RetIndex:2797
CompName:(+)-Sesamin



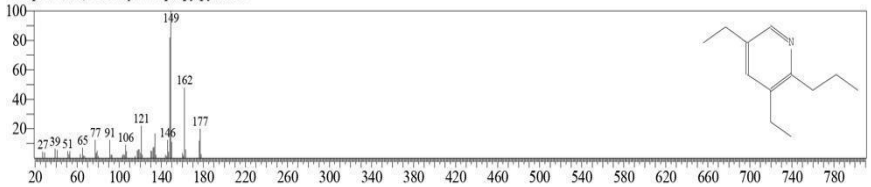
Hit#:2 Entry:231941 Library:NIST17.lib
SI:91 Formula:C20H18O6 CAS:7076-24-6 MolWeight:354 RetIndex:2797
CompName:2,6-Bis(3,4-methylenedioxyphenyl)-3,7-dioxabicyclo(3.3.0)octane



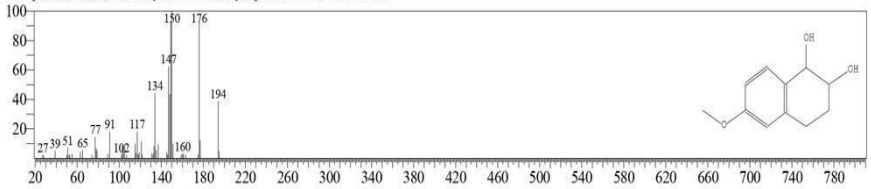
Hit#:3 Entry:231940 Library:NIST17.lib
SI:74 Formula:C20H18O6 CAS:133-03-9 MolWeight:354 RetIndex:2797
CompName:1,3-Benzodioxole, 5,5'-(tetrahydro-1H,3H-furo[3,4-c]furan-1,4-diyl)bis-, [1S-(1.alpha.,3a.alpha.,4.beta.,6a.alpha.)]-



Hit#:4 Entry:49502 Library:NIST17.lib
SI:66 Formula:C12H19N CAS:4808-75-7 MolWeight:177 RetIndex:1411
CompName:3,5-Diethyl-2-n-propylpyridine



Hit#:5 Entry:65026 Library:NIST17.lib
SI:66 Formula:C11H14O3 CAS:65272-94-8 MolWeight:194 RetIndex:1732
CompName:1,2,3,4-Tetrahydro-6-methoxynaphthalene-1,trans-2-diol



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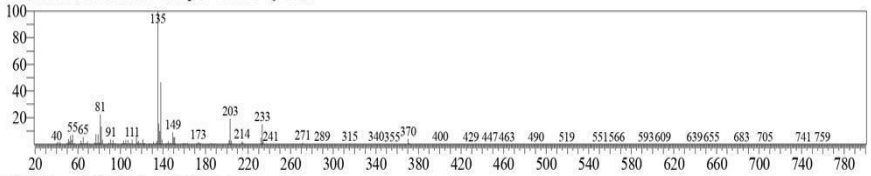
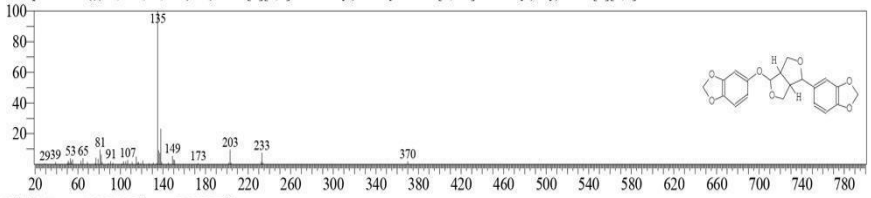
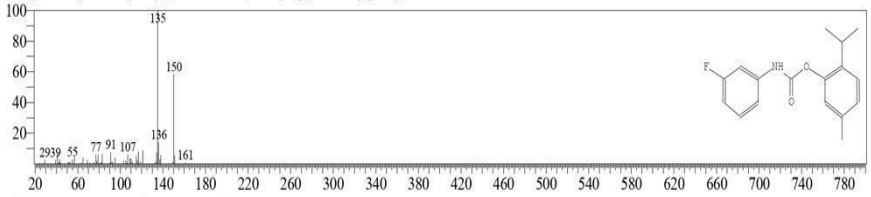
<< Target >>
Line#:8 R.Time:38.880(Scan#:7177) Retention Index:3983! MassPeaks:554
RawMode:Averaged 38.875-38.885(7176-7178) BasePeak:135.15(8326530)
BG Mode:Calc. from Peak Group 1 - Event 1 Q3 Scan



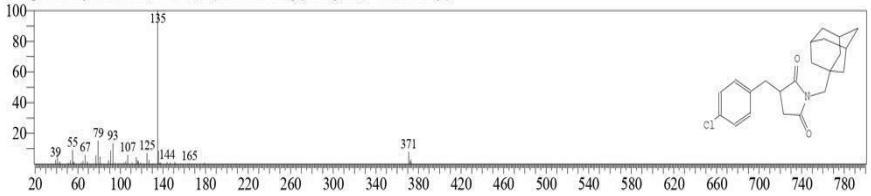
Hit#:1 Entry:245100 Library:NIST17.lib
SI:89 Formula:C20H18O7 CAS:526-07-8 MolWeight:370 RetIndex:2873
CompName:5-(((1R,3aR,4S,6aR)-4-(Benzo[d][1,3]dioxol-5-yl)hexahydrofuro[3,4-c]furan-1-yl)oxy)benzo[d][1,3]dioxole



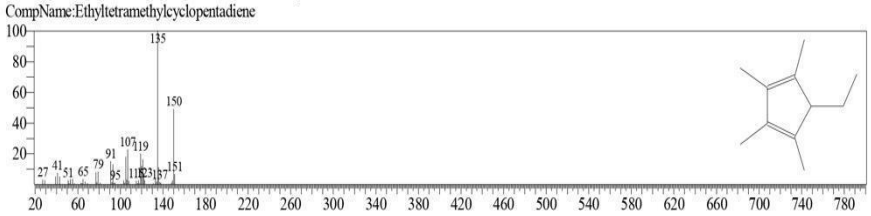
Hit#:2 Entry:162533 Library:NIST17.lib
SI:72 Formula:C17H18FNO2 CAS:0-00-0 MolWeight:287 RetIndex:2168
CompName:(3-Fluorophenyl)carbamic acid, 2-isopropyl-5-methylphenyl ester



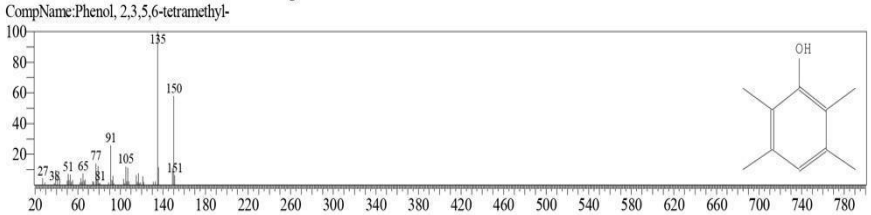
Hit#:3 Entry:246343 Library:NIST17.lib
SI:67 Formula:C22H26ClNO2 CAS:0-00-0 MolWeight:371 RetIndex:2962
CompName:Pyrrolidine-2,5-dione, 1-(adamantan-1-yl)methyl-3-(4-chlorobenzyl)-



Hit#:4 Entry:27489 Library:NIST17.lib
SI:66 Formula:C11H18 CAS:57693-77-3 MolWeight:150 RetIndex:1082
CompName:Ethyltetramethylcyclopentadiene



Hit#:5 Entry:27406 Library:NIST17.lib
SI:66 Formula:C10H14O CAS:527-35-5 MolWeight:150 RetIndex:1354
CompName:Phenol, 2,3,5,6-tetramethyl-



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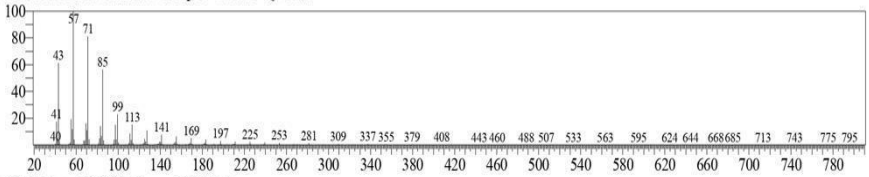
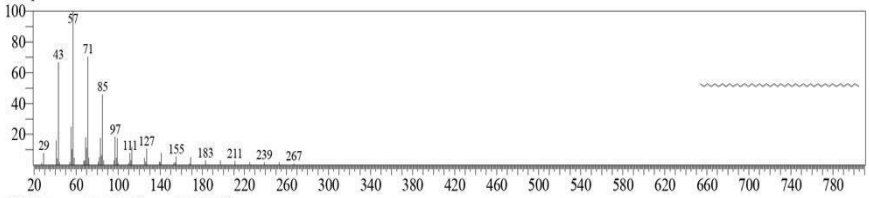
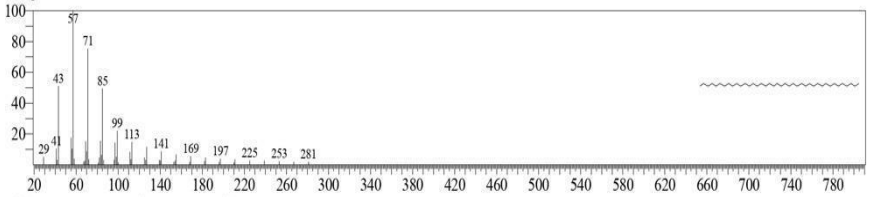
<< Target >>
Line#:9 R.Time:39.470(Scan#:7295) Retention Index:4032! MassPeaks:424
RawMode:Averaged 39.465-39.475(7294-7296) BasePeak:57.10(289883)
BG Mode:Calc. from Peak Group 1 - Event 1 Q3 Scan



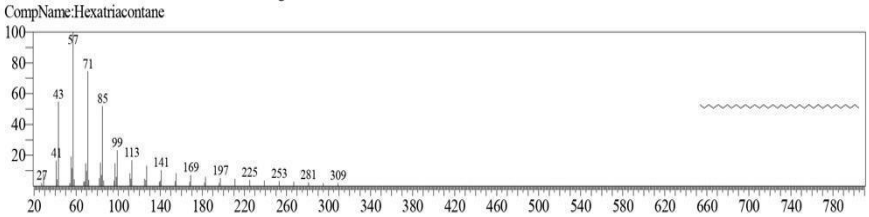
Hit#:1 Entry:303838 Library:NIST17.lib
SI:97 Formula:C44H90 CAS:7098-22-8 MolWeight:618 RetIndex:4395
CompName:Tetratetracontane



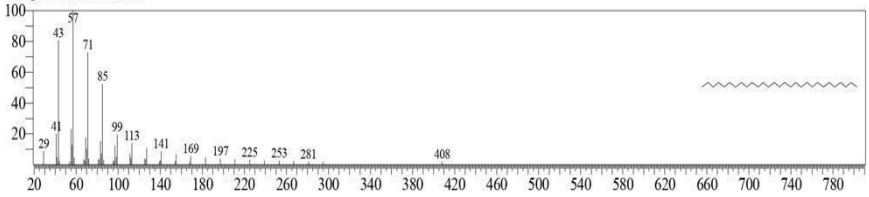
Hit#:2 Entry:301740 Library:NIST17.lib
SI:96 Formula:C40H82 CAS:4181-95-7 MolWeight:562 RetIndex:3997
CompName:Tetracontane



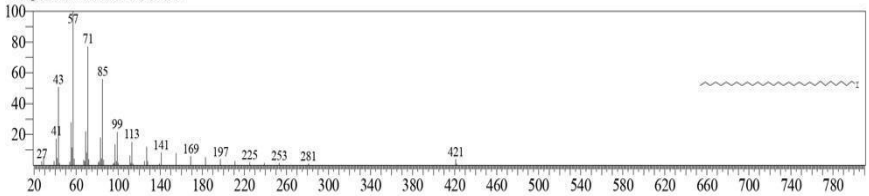
Hit#:3 Entry:296990 Library:NIST17.lib
SI:96 Formula:C36H74 CAS:630-06-8 MolWeight:506 RetIndex:3600
CompName:Hexatriacontane



Hit#:4 Entry:269359 Library:NIST17.lib
SI:96 Formula:C29H60 CAS:630-03-5 MolWeight:408 RetIndex:2904
CompName:Nonacosane



Hit#:5 Entry:300810 Library:NIST17.lib
SI:95 Formula:C30H61I CAS:0-00-0 MolWeight:548 RetIndex:3418
CompName:Triacontane, 1-iodo-



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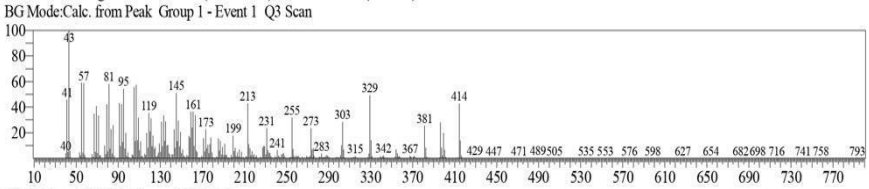
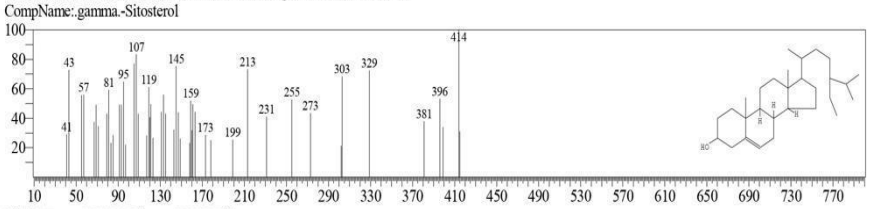
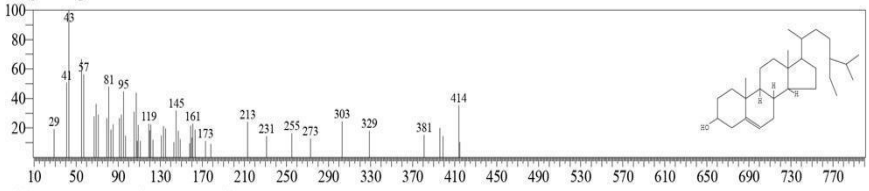
<< Target >>
Line#:10 R.Time:39.795(Scan#:7360) Retention Index:4058! MassPeaks:524
RawMode:Averaged 39.790-39.800(7359-7361) BasePeak:43.10(374535)
BG Mode:Calc. from Peak Group 1 - Event 1 Q3 Scan



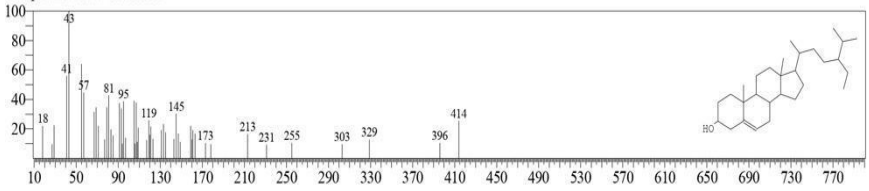
Hit#:1 Entry:272488 Library:NIST17.lib
SI:90 Formula:C29H50O CAS:83-47-6 MolWeight:414 RetIndex:2731
CompName:.gamma.-Sitosterol



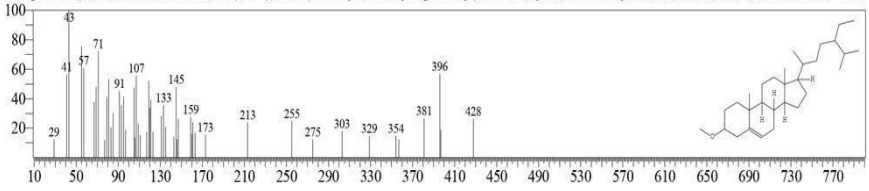
Hit#:2 Entry:272462 Library:NIST17.lib
SI:89 Formula:C29H50O CAS:83-47-6 MolWeight:414 RetIndex:2731
CompName:.gamma.-Sitosterol



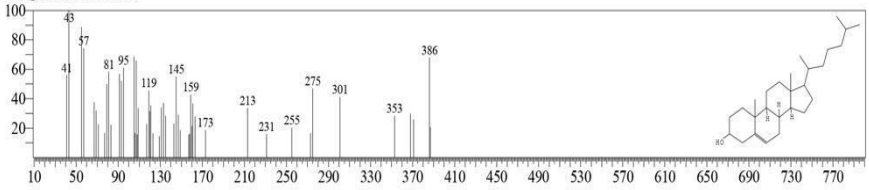
Hit#:3 Entry:272461 Library:NIST17.lib
SI:85 Formula:C29H50O CAS:83-46-5 MolWeight:414 RetIndex:2731
CompName:.beta.-Sitosterol



Hit#:4 Entry:278305 Library:NIST17.lib
SI:84 Formula:C30H52O CAS:20194-50-7 MolWeight:428 RetIndex:2680
CompName:(3S,8S,9S,10R,13R,14S,17R)-17-((2R,5R)-5-Ethyl-6-methylheptan-2-yl)-3-methoxy-10,13-dimethyl-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetrac



Hit#:5 Entry:257167 Library:NIST17.lib
SI:79 Formula:C27H46O CAS:57-88-5 MolWeight:386 RetIndex:2596
CompName:Cholesterol



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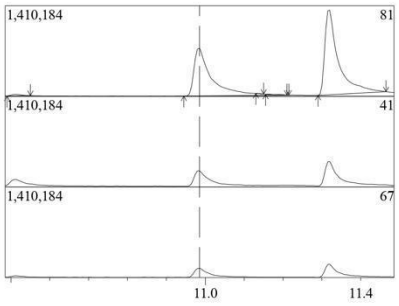
Quantitation Results

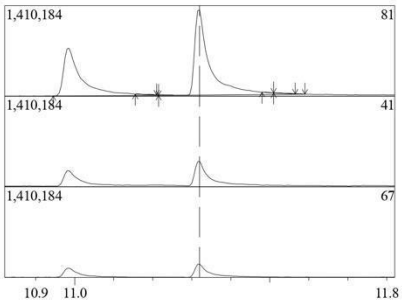
Analyzed : 03-Feb-23 3:35:42 PM
Data File : E:\GC-MS data\SAMPLES\Atmiya University\Freny S Oil 03022023.qgd



ID#:1 Mass:81.00
Type:Target
Name:2,4-Decadienal, (E,E)-

R.T.:10.983
Area:2324020
SI:99
Conc.:0.00000ppm

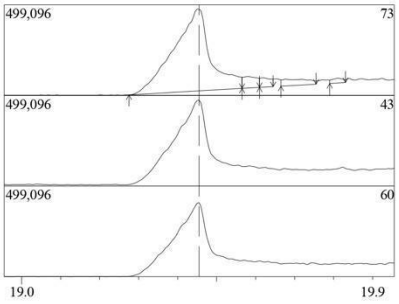
#	Mass	Area	Ion Ratio(%)	Setting(%)	Ref.Band(%)	Range(%)	Criteria
1	41.00	225826	31.6	32.7	30	2.7 - 62.7	Pass
2	67.00	136650	19.1	19.5	30	0.0 - 49.5	Pass



ID#:2 Mass:81.00
Type:Target
Name:2,4-Decadienal, (E,E)-

R.T.:11.318
Area:3641127
SI:99

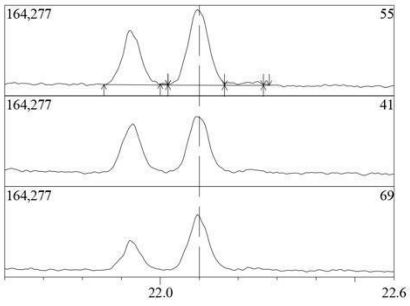
#	Mass	Area	Ion Ratio(%)	Setting(%)	Ref.Band(%)	Range(%)	Criteria
1	41.00	352402	28.1	28.3	30	0.0 - 58.3	Pass
2	67.00	193647	15.4	15.5	30	0.0 - 45.5	Pass



ID#:3 Mass:73.00
Type:Target
Name:n-Hexadecanoic acid

R.T.:19.452
Area:2989342
SI:99
Conc.:0.00000ppm

#	Mass	Area	Ion Ratio(%)	Setting(%)	Ref.Band(%)	Range(%)	Criteria
1	43.00	399035	97.6	98.1	30	68.1 - 128.1	Pass
2	60.00	345937	84.6	85.2	30	55.2 - 115.2	Pass



ID#:4 Mass:55.00
Type:Target
Name:9-Octadecenoic acid (Z)-, methyl ester

R.T.:22.096
Area:508407
SI:97

#	Mass	Area	Ion Ratio(%)	Setting(%)	Ref.Band(%)	Range(%)	Criteria
1	41.00	98298	74.2	78.1	30	48.1 - 108.1	Pass
2	69.00	94487	71.3	71.5	30	41.5 - 101.5	Pass

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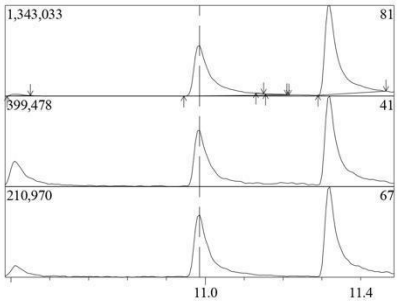
Quantitation Results

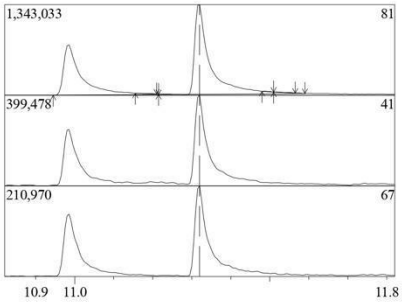
Analyzed : 03-Feb-23 3:35:42 PM
Data File : E:\GC-MS data\SAMPLES\Atmiya University\Freny S Oil 03022023.qgd



ID#:1 Mass:81.00
Type:Target
Name:2,4-Decadienal, (E,E)-

R.T.:10.983
Area:2324020
SI:99
Conc.:0.00000ppm

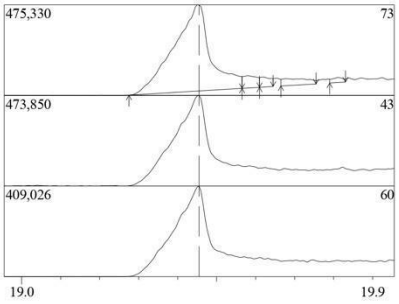
#	Mass	Area	Ion Ratio(%)	Setting(%)	Ref.Band(%)	Range(%)	Criteria
1	41.00	225826	31.6	32.7	30	2.7 - 62.7	Pass
2	67.00	136650	19.1	19.5	30	0.0 - 49.5	Pass



ID#:2 Mass:81.00
Type:Target
Name:2,4-Decadienal, (E,E)-

R.T.:11.318
Area:3641127
SI:99

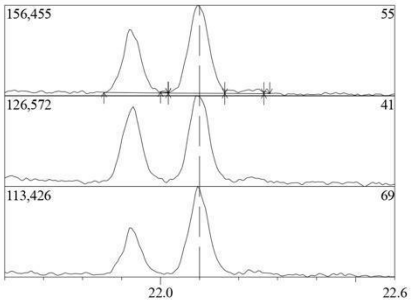
#	Mass	Area	Ion Ratio(%)	Setting(%)	Ref.Band(%)	Range(%)	Criteria
1	41.00	352402	28.1	28.3	30	0.0 - 58.3	Pass
2	67.00	193647	15.4	15.5	30	0.0 - 45.5	Pass



ID#:3 Mass:73.00
Type:Target
Name:n-Hexadecanoic acid

R.T.:19.452
Area:2989342
SI:99
Conc.:0.00000ppm

#	Mass	Area	Ion Ratio(%)	Setting(%)	Ref.Band(%)	Range(%)	Criteria
1	43.00	399035	97.6	98.1	30	68.1 - 128.1	Pass
2	60.00	345937	84.6	85.2	30	55.2 - 115.2	Pass



ID#:4 Mass:55.00
Type:Target
Name:9-Octadecenoic acid (Z)-, methyl ester

R.T.:22.096
Area:508407
SI:97

#	Mass	Area	Ion Ratio(%)	Setting(%)	Ref.Band(%)	Range(%)	Criteria
1	41.00	98298	74.2	78.1	30	48.1 - 108.1	Pass
2	69.00	94487	71.3	71.5	30	41.5 - 101.5	Pass

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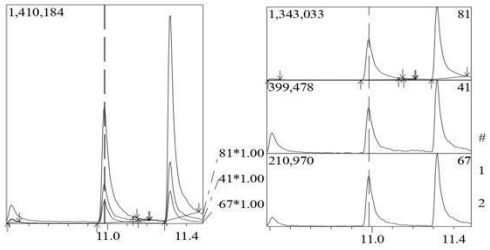
Quantitation Results

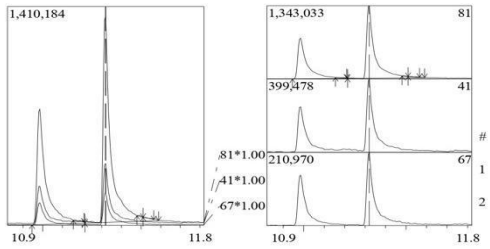
Analyzed : 03-Feb-23 3:35:42 PM
Data File : E:\GC-MS data\SAMPLES\Atmiya University\Freny S Oil 03022023.qgd



ID#:1 Mass:81.00
Type:Target
Name:2,4-Decadienal, (E,E)-

R.T.:10.983
Area:2324020
SI:99
Conc.:0.00000ppm

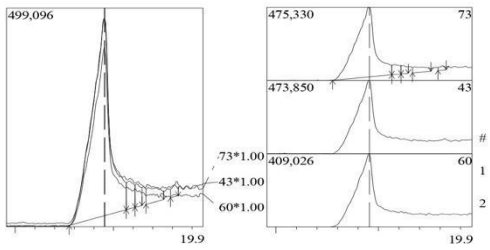
#	Mass	Area	I.Ratio(%)	Setting(%)	Ref.Band(%)	Range(%)	Criteria
1	41.00	225826	31.6	32.7	30	2.7 - 62.7	Pass
2	67.00	136650	19.1	19.5	30	0.0 - 49.5	Pass



ID#:2 Mass:81.00
Type:Target
Name:2,4-Decadienal, (E,E)-

R.T.:11.318
Area:3641127
SI:99

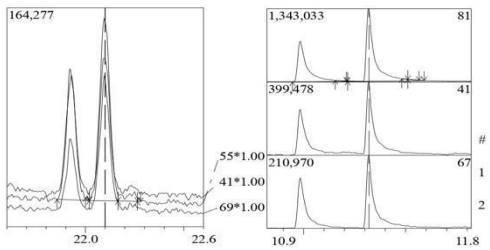
#	Mass	Area	I.Ratio(%)	Setting(%)	Ref.Band(%)	Range(%)	Criteria
1	41.00	352402	28.1	28.3	30	0.0 - 58.3	Pass
2	67.00	193647	15.4	15.5	30	0.0 - 45.5	Pass



ID#:3 Mass:73.00
Type:Target
Name:n-Hexadecanoic acid

R.T.:19.452
Area:2989342
SI:99
Conc.:0.00000ppm

#	Mass	Area	I.Ratio(%)	Setting(%)	Ref.Band(%)	Range(%)	Criteria
1	43.00	399035	97.6	98.1	30	68.1 - 128.1	Pass
2	60.00	345937	84.6	85.2	30	55.2 - 115.2	Pass



ID#:2 Mass:81.00
Type:Target
Name:2,4-Decadienal, (E,E)-

R.T.:11.318
Area:3641127
SI:99

#	Mass	Area	I.Ratio(%)	Setting(%)	Ref.Band(%)	Range(%)	Criteria
1	41.00	352402	28.1	28.3	30	0.0 - 58.3	Pass
2	67.00	193647	15.4	15.5	30	0.0 - 45.5	Pass

4.8 *In vitro* Antioxidant Activity

Decoctions derived from plants with hypoglycemic and antioxidant properties have been shown to address complications of diabetes, including delayed wound healing. Patel et al. (2019) discussed the mechanistic insight into diabetic wounds and emphasized the need for bioactive-rich formulations like decoctions to target molecular pathways involved in impaired healing. Recent studies have highlighted the importance of hydrogen peroxide scavenging in preventing cellular damage.

Kumari et al. (2023) demonstrated that plant-derived antioxidants effectively scavenge H_2O_2, protecting cellular compOnents from oxidative stress, which may support anti-aging and anti-inflammatory properties in therapeutic applications.

Patel et al. (2023) emphasizes the significance of the reducing power assay as a reliable measure of antioxidant potential. Strong reducing agents like flavonoids and phenolics in plant extracts can donate electrons, neutralizing free radicals and breaking oxidative chain reactions that could otherwise damage cellular structures. Higher reducing power correlates with stronger antioxidant potential, which is vital in conditions like diabetes, where free radical-induced damage is common.

Ghosh et al. (2023) reveal that the phosphomolybdenum assay is One of the most comprehensive tests for total antioxidant capacity, as it reflects various mechanisms like electron donation**,** metal chelation**,** and even hydrogen atom transfer. The antioxidant activity indicated by this assay has been linked to the protection of biomolecules from oxidative damage in the context of aging, inflammatory diseases, and neuroprotection.

DPPH radical scavenging assay

This assay is valuable for gauging the general antioxidant capacity of the formulation [29][61]. At lower concentrations (0.625–1.25 µg/mL), both ascorbic acid and Polyherbal formulation show low scavenging activity (~20%). At intermediate concentrations (1.87–2.5 µg/mL), PHF performs slightly better than ASC, with a visible gap between the orange and blue bars. At higher concentrations (3.125–3.75 µg/mL), both substances show very high scavenging activity (>90%), with PHF slightly outperforming ASC. PHF appears to have slightly better antioxidant activity than ASC, especially at intermediate and higher concentrations. This graph demonstrates that both substances exhibit significant antioxidant properties that improve with increasing concentration. PHF consistently shows marginally higher activity than the standard ASC, suggesting it might be a promising antioxidant.

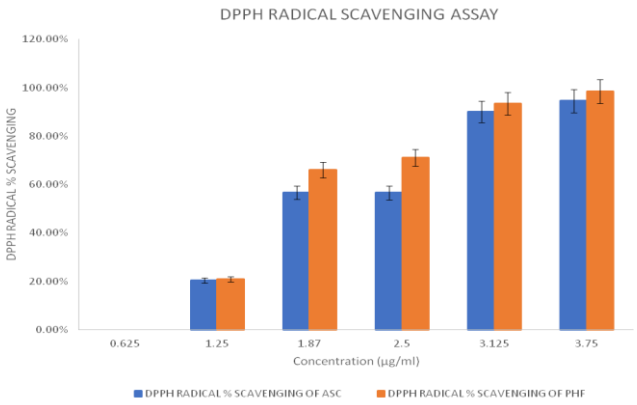


Fig13: Antioxidant analysis by DPPH assay of oil.

4.8.1 Hydrogen peroxide scavenging assay:

The graph illustrates the hydrogen peroxide scavenging activity (%) for two substances, ascorbic Acid (in red) and Oil (in green), at different concentrations (5, 10, 15, 20, and 25 mg/ml). in case of ascorbic Acid, which is used as positive control, The scavenging activity increases consistently with concentration, reaching nearly 100% at 25 mg/ml. At 25 mg/ml, the scavenging activity of the oil approaches around 80%, which indicates its significant, though less potent, antioxidant activity compared to ascorbic acid. ascorbic acid demonstrates nearly complete hydrogen peroxide scavenging (close to 100%) at higher concentrations, reflecting its role as a standard antioxidant. The oil also shows good scavenging activity, with values reaching about 80% at 25 mg/ml, suggesting the presence of bioactive compounds with antioxidant potential. The oil's considerable scavenging activity implies it could serve as a natural antioxidant source, albeit less potent than ascorbic acid. The oil's antioxidant properties may be attributed to bioactive compounds like polyphenols, flavonoids, or other secondary metabolites.

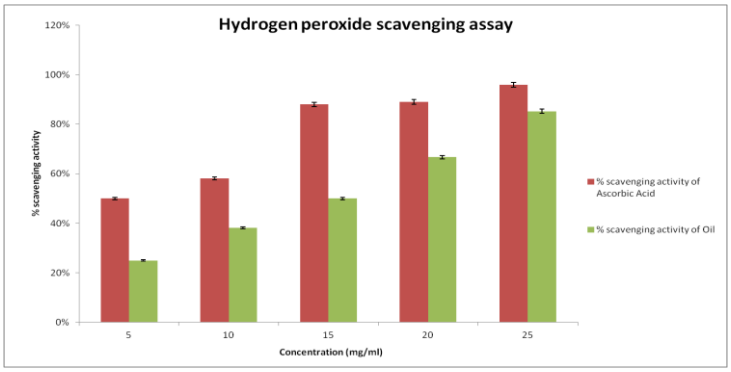


Fig 14: Antioxidant analysis by Hydrogen peroxide scavenging assay of oil.

4.8.2 Reducing power assay:

The graph represents the reducing power assay results for **ascorbic Acid** (red bars) and **Oil** (blue bars) at increasing concentrations (1, 1.5, 2, 2.5, and 3 mg/ml). The reducing power is measured as absorbance, indicating the ability of the tested substances to donate electrons and act as antioxidants. The oil consistently exhibits higher reducing power than ascorbic acid across all concentrations. At 3 mg/ml, the reducing power of the oil reaches a maximum value of approximately 1.2, indicating significant antioxidant potential. The oil demonstrates superior reducing power compared to ascorbic acid, suggesting it contains potent electron-donating compounds that contribute to its antioxidant activity. The concentration-dependent increase in reducing power for both substances highlights their effectiveness as antioxidants at higher doses. The reducing power of a substance is an important measure of its ability to counteract oxidative stress. The higher reducing power of the oil implies it could play a significant role in protecting cells from oxidative damage. The oil's activity could be attributed to the synergistic effects of its bioactive compounds, which may include both lipophilic and hydrophilic antioxidants. Exploring the stability, bioavailability, and synergistic effects of the oil's compOnents could enhance its application potential.

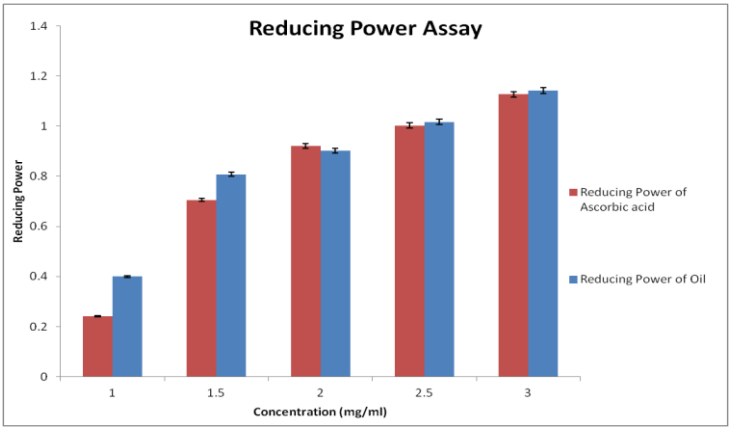


Fig 15: Antioxidant analysis by Reducing Power assay of oil.

4.8.3 Antioxidant activity by phosphomolydenum assay:

The phosphomolybdenum assay reveals that both ascorbic acid and the oil exhibit excellent antioxidant activity. The oil consistently exhibits slightly higher antioxidant activity than ascorbic acid at all concentrations. This highlights the oil's potential as a powerful natural

antioxidant, warranting further investigation into its bioactive compounds and practical applications. At 2.5 mg/ml, ascorbic acid shows approximately 88% reduction, demonstrating its high antioxidant capacity, while the oil achieves around 90% reduction, surpassing ascorbic acid. The oil shows a marginally higher total antioxidant activity compared to ascorbic acid across all concentrations indicating capable of reducing molybdenum effectively. The oil could be explored for its use in dietary supplements, functional foods, cosmetics, and pharmaceutical formulations aimed at combating oxidative stress.

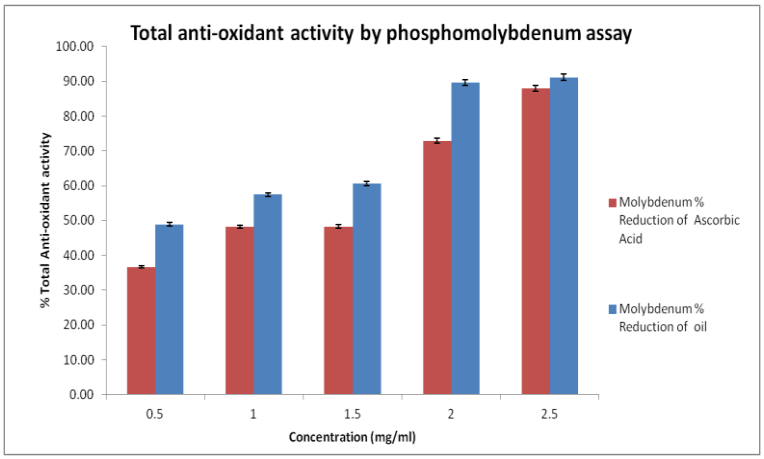


Fig 16: Antioxidant analysis by phosphomolydenum assay of oil.

4.9 Molecular Biology assay

The M5S fibroblast cell line is commonly used in research to mimic skin tissue characteristics and behavior *in vitro*. as *Krause et al. (2023)* highlight, fibroblast function in diabetic environments is altered by elevated glucose levels, underscoring the importance of using the M5S cell line to model the healing challenges faced by diabetic patients. Thus, the M5S cell line offers a reliable platform for evaluating how various treatments can impact skin cell functions in such pathological settings.

As *Prasad et al. (2022)* demonstrate, high glucose levels can induce ROS production, which accelerates cellular damage, impairs cell function, and worsens wound healing in diabetic conditions. This *in vitro* hyperglycemic model provides a controlled system for studying how therapeutic formulations can reduce oxidative stress and improve wound healing efficacy, mimicking the challenges diabetic patients face.The induction of **hyperglycemia** in these fibroblast cell lines is crucial for simulating the diabetic environment, where elevated blood

glucose levels contribute to oxidative stress, impaired cell function, and delayed wound healing.

Lee et al. (2023) show, promoting the transition from the resting phase (G0) to the active S-phase, where DNA replication occurs, is essential for effective tissue repair. This additional layer of analysis complements the MTT assay by offering a detailed understanding of the formulation's molecular effects on cell cycle progression, providing evidence for its efficacy in enhancing wound healing. Flow cytometry allows for more detailed analysis of **cell cycle progression** and **proliferation**. This technique enables researchers to precisely measure the distribution of cells across different stages of the cell cycle (G0/G1, S, G2/M), giving insights into how the polyherbal formulation influences cellular division and growth. *for diabetic patients, accelerating the transition from the resting phase (G0) to active proliferation (S-phase) is key to effective wound healing.* This assay complements the MTT assay by providing a more granular understanding of how the formulation promotes or inhibits cell proliferation at a molecular level.

Zhang et al. (2023) emphasize the importance of reducing ROS in diabetic wound healing, as ROS accumulation leads to cellular damage, inhibits fibroblast function, and impairs tissue repair processes. A decrease in ROS levels upon treatment with the polyherbal formulation would indicate its antioxidant properties, which could protect the fibroblasts from oxidative stress and enhance wound healing. The results from this assay would provide strong evidence for the formulation's potential in managing oxidative damage, a key complication in diabetic wounds. The ROS assay is crucial for assessing the antioxidant potential of the formulation. in hyperglycemic conditions, high levels of ROS cause cellular damage, impairing various functions including wound healing. Measuring ROS levels in treated cells allows researchers to evaluate whether the Ayurvedic polyherbal formulation can reduce oxidative stress and prevent cellular damage caused by ROS. A reduction in ROS levels would suggest that the formulation possesses strong antioxidant properties, which is essential for managing complications in diabetic wounds and promoting better tissue repair.

Research by *Ali et al. (2023)* shows that diabetic conditions often impair phagocytic activity, which in turn delays wound healing. The polyherbal formulation's ability to enhance phagocytosis could help improve infection control, reduce bacterial load in wounds, and accelerate healing. This suggests the formulation's potential to boost immune response and

tissue repair, further promoting efficient wound healing in diabetic patients. Phagocytosis is an important process in wound healing and immune response, as it involves the engulfment of pathogens and debris by immune cells like macrophages. Impaired phagocytosis in hyperglycemic conditions can delay wound healing and increase the risk of infection. The formulation's ability to enhance phagocytosis could improve wound clearance and reduce infection rates in diabetic wounds, accelerating the healing process.

Patel et al. (2023) indicate that promoting fibroblast migration can speed up tissue regeneration and wound healing, especially in diabetic wounds where this process is hindered. Cell migration is a key event in the wound healing process, where cells move into the wound site to aid tissue repair. The results from this assay will help determine whether the formulation can enhance fibroblast migration, thereby accelerating wound healing in diabetic conditions.

4.9.1 Cell Proliferation assay by MTT

In here, the cells were treated with Kwath and Oil, the cell proliferation was attained. The optimum concentration of Kwath and oil in normal cells is 30µl in normal cells at 4hrs. in case of Hyperglycaemic cells, Kwath showed optimum cell proliferation at 150 µl at 4hrs. in case of Hyperglycaemic cells, Oil showed significant proliferation at both 0.5 µl and 1 µl at 4hrs with the level of significance*p<0.05, #p<0.1, which is quite low concentration and in less time

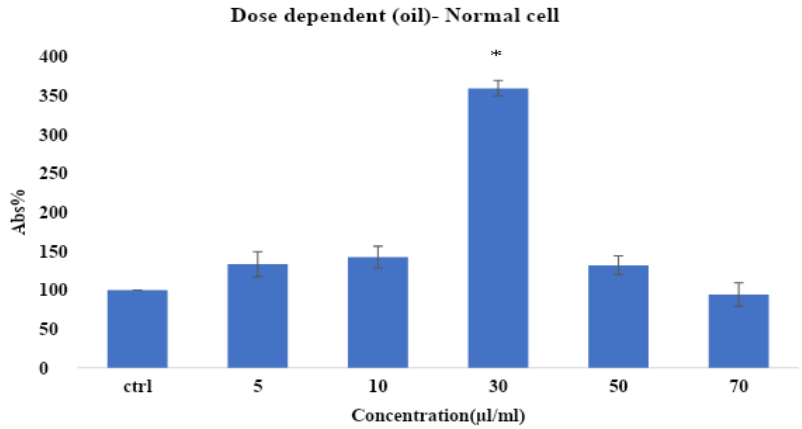


Fig 17: Cell proliferation assay (Dose Dependent) in Normal cell line.

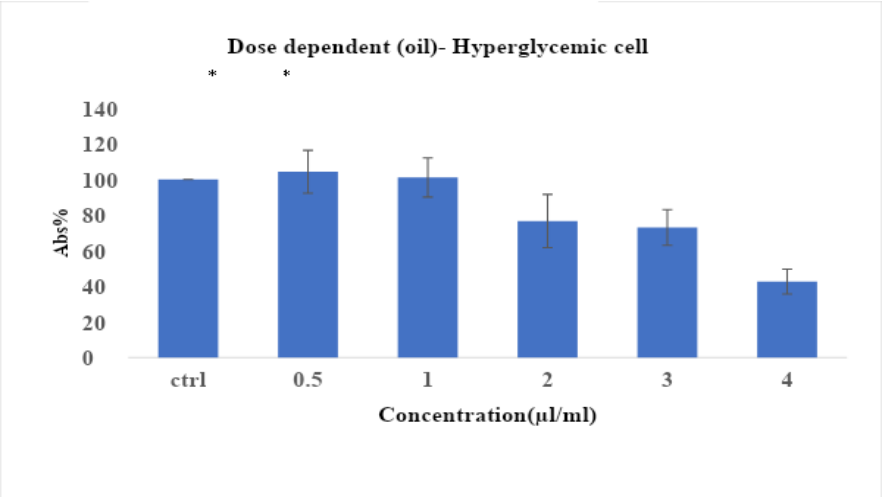


Fig 18: Cell proliferation assay (Dose Dependent) in Hyperglycemic cell line.

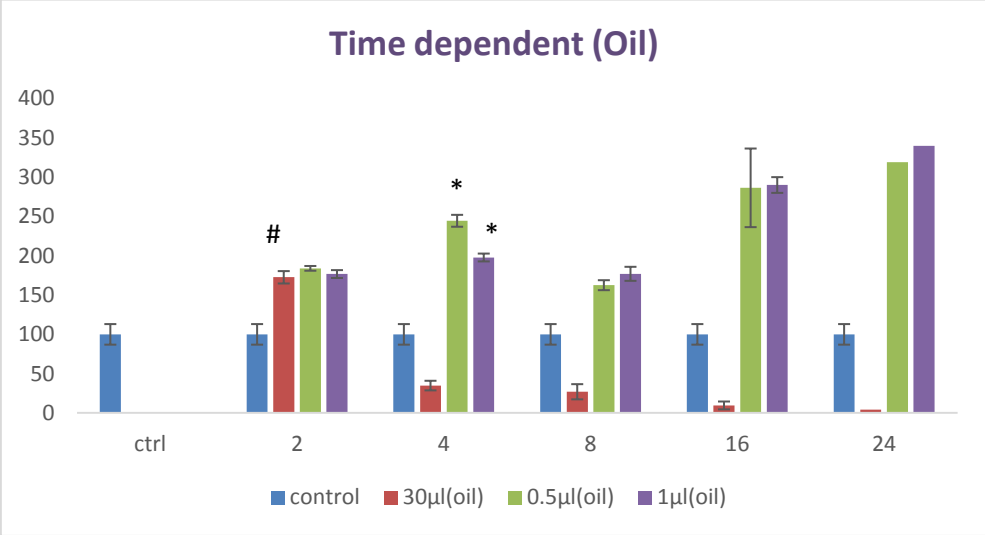


Fig 19: Time dependent cell proliferation assay in Normal cell

4.9.2 Quantification of intracellular reactive oxygen species (ROS) assay:

The images represent fluorescence microscopy images of cells stained for ROS. The green fluorescence indicates the presences of ROS. The green fluorescence represents a stressful condition that remains almost similar in case of Control while in case of cells treated with oil, because of the potent antioxidant property, the ROS are scavenged. Hence the number of cells under stress is significantly less. Cells with intracellular ROS are less fluorescent.

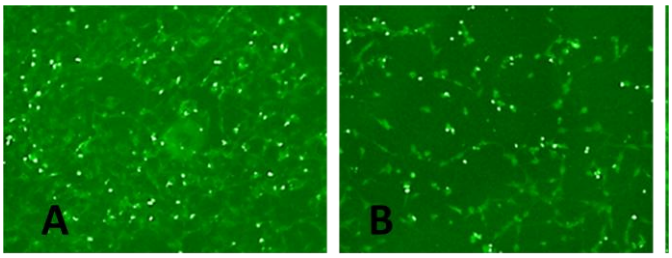


Fig 20: ROS generation in normal cell. Cells were treated with various doses of formulation for 2 hr and analysed by confocal and fluorescence microscopy. Representative images for (A) are Control and it gives green color fluorescence (B) Cells treated with 30µl oil.

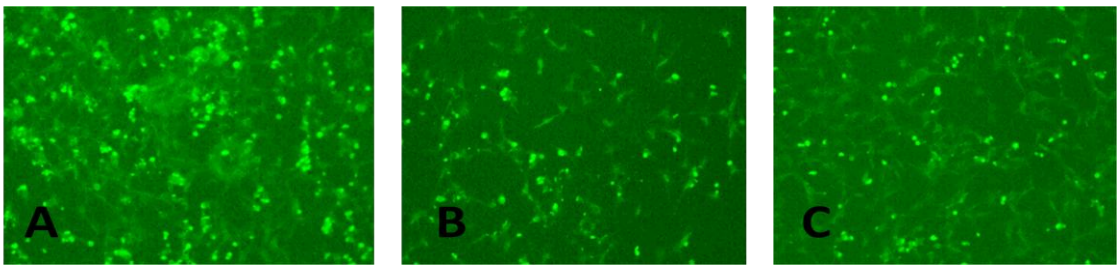


Fig 21: ROS generation in hyperglycaemic cells was (A) Control gives green color fluorescence (B) and (C) Cells were treated with formulation (0.5µl, 1µl) showed less green fluorescence.

4.9.3 Cell Proliferation by Flow Cytometer

In this study, the MTT assay was used to assess the polyherbal formulation's impact on fibroblast proliferation in both normal and hyperglycemic conditions. The results suggest that **Kwath** (an aqueous herbal formulation) and **Oil** (likely an extract) both promoted cell proliferation, with the most significant effects seen with Kwath at 150 µL in hyperglycemic cells. The **oil** also showed notable cell proliferation at lower concentrations (0.5 µL and 1 µL) in hyperglycemic cells, with statistical significance at $p < 0.05$ and $p < 0.1$, respectively. These findings align with *Nair et al. (2023),* who report that herbal formulations can stimulate fibroblast proliferation and enhance wound healing in diabetic models by counteracting the inhibitory effects of hyperglycemia on cell function. The findings suggest that the polyherbal formulation's ability to accelerate fibroblast proliferation, even at lower concentrations and shorter times, is a promising indicator of its potential to improve wound healing in diabetic patients. Statistically, the data obtained by flow cytometry assay shows the treatment with formulation to the normal cell exhibited % of live cells 94.70% which is almost same as control cells 95.03%. This suggests that the treatment with formulation is not toxic to the normal cells. in contrast when the hyperglycaemic cells were treated with

formulation, the results showed % of live cells 75.93%, respectively which is significantly less toxic compared to control 61.30%

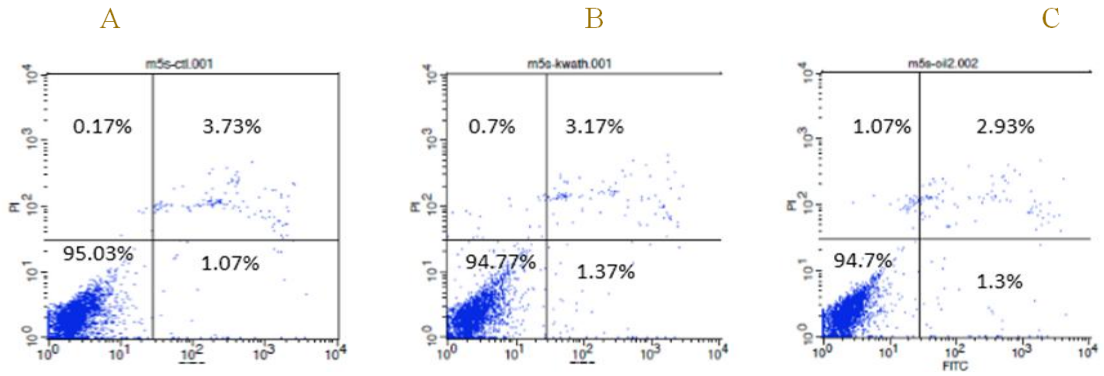


Fig 22: FACS performed in normal cells. (A) The concentration of live cells in control is 95.03%, the cells undergoing pro-apoptosis are 3.73%, (B) Cells treated with formulation are 94.7% showing similar result, the pro-apoptosis cell decrease to 3.17%.

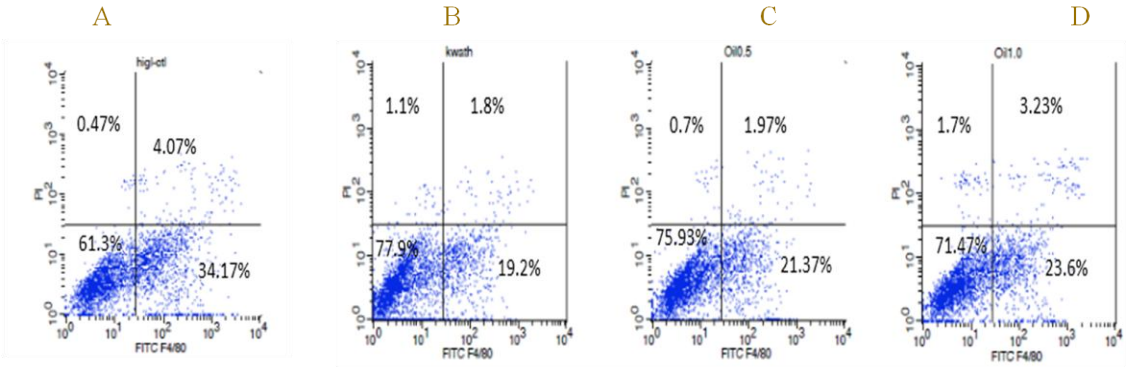


Fig 22: FACS performed in hyperglycaemic cells. (A) The concentration of live cells in control is 61.3%, cells treated with 0.5 µl (B) & 1 µl (C) formulation are 75.93% and 71.47% respectively. Under control condition the cells undergoing pro-apoptosis are 4.07%, while in 0.5 µl & 1 µl formulation treated cell decreases to 1.97% & 3.23% respectively.

4.9.4 Phagocytosis assay

Phagocytosis assay shows the treatment with formulation (0.5µl, 1µl) on macrophages cultivated high glucose α-DMEM media. Our results demonstrate that the formulation significantly enhanced the phagocytic activity of macrophages at concentrations of 0.5 µl and 1 µl, as evidenced by the increased green fluorescence intensity in treated cells compared to the control. The control group, which received no treatment, exhibited a baseline level of

phagocytic activity, as indicated by the presence of green fluorescence.cell compare to control the intensity of fluorescence is high

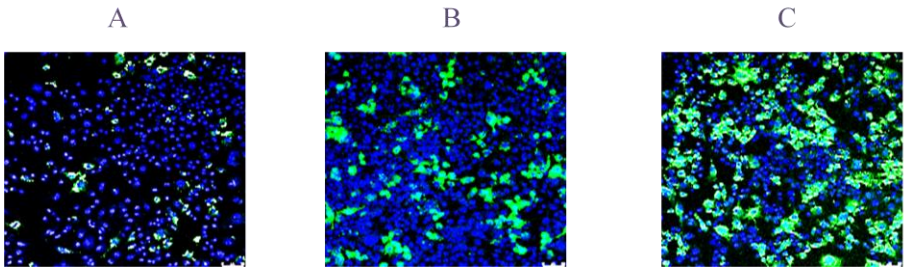


Fig 24: Phagocytosis activity in green fluorescence showed (A) control shows green fluorescence representing the phagocytic activity by macrophage. (B) and (C) Macrophage were treated with formulation and showed significant phagocytic activity at 0.5µl and 1µl concentration.

The inference can be the formulation may directly stimulate macrophage activation, leading to increased phagocytic receptor expression and enhanced phagocytic capacity. The formulation may possess antioxidant properties that mitigate oxidative stress, which may enhance macrophage phagocytic activity. The formulation might attract more macrophages to the site of infection or inflammation, thereby increasing the overall phagocytic capacity of the tissue.

4.9.5 *In vitro* cell migration assay

In vitro cell migration assay shows that formulation accelerated cell migration in normal cell and hyperglycaemic cell in 18 hr. Observation showed cell elongation of dendrites in fibroblast and migration of border cells are indicative of invasiveness. Thus, the formulation proves to be effective for the cell migration and filling of gap was evident both in normal as well as hyperglycaemic cells. The formulation may stimulate cell proliferation, leading to an increased number of cells available for migration. The formulation might influence the organization and dynamics of the actin cytoskeleton, which is essential for cell motility. The formulation may upregulate the expression of key proteins involved in cell migration, such as matrix metalloproteinases (MMPs) and integrins. The formulation may decrease cell adhesion to the extracellular matrix, facilitating cell detachment and migration.

Cell migration assay in Normal cell lines:

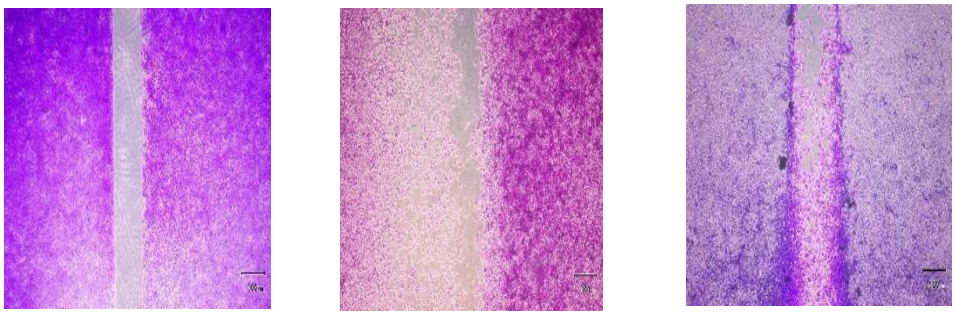


Fig 25: Cell migration assay in normal cells (A) At 0 hr control, no migration was observed, (B) At 18hr control, cell migration elongation of dendrites in fibroblast and migration of border cells are indicative of invasiveness. (C) formulation treated samples showed the significant migration of cells was observed.

Cell migration assay in Hyperglycemic cell lines

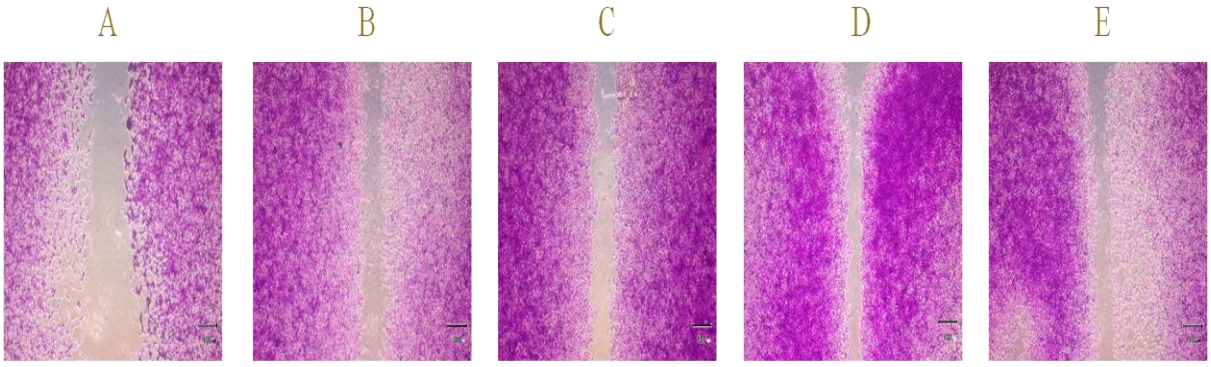


Fig 26: Cell Migration assay in hyperglycaemic Cell (A) At 0 hr control, no migration was observed, (B) At 18hr control, cell migration elongation of dendrites in fibroblast and migration of border cells are indicative of invasiveness. (C) and (D) When treated with different concentrations of formulation i.e. 0.5µl and 1µl, gap filling started via cell migration.

Comprehensive Evaluation of formulation's Efficacy

Sharma et al. (2023) have demonstrated that a combination of these assays- cell proliferation, ROS quantification, phagocytosis, and cell migration is crucial for evaluating the therapeutic potential of natural products, as it provides a holistic view of their effects on various cellular processes involved in wound healing. Each assay measures different aspects of cellular behavior that are critical for wound healing, particularly in the challenging environment created by hyperglycemia. This multi-faceted approach ensures that the formulation is evaluated from various angles, providing a clear picture of its potential efficacy in treating diabetic wounds and related complications.

4.10 Clinical Trials

The clinical trials conducted in this study provided valuable insights into the therapeutic potential of the polyherbal formulation, specifically focusing on its effects in managing diabetic wounds. The trials were meticulously conducted under the supervision of medical officers and surgeons, ensuring adherence to medical standards and patient safety protocols. The methodology followed in this study aligns with current practices for clinical trials evaluating wound healing in diabetic patients. The presence of a monitoring team throughout the process is critical for assessing both the efficacy and safety of any treatment, especially in a patient population with compromised healing capacity like diabetics (Goswami et al., 2022).

Out of the 30 patients enrolled, data from 22 patients were analyzed, while 8 patients either discontinued treatment or had to be excluded due to complications unrelated to the wound care intervention. It is not uncommon for clinical trials involving chronic conditions such as diabetes to face dropout rates, often due to comorbidities or disease progression (Badi et al., 2023). This highlights the importance of understanding the broader context of patient health when conducting trials involving diabetic patients, as other factors like cardiovascular disease or diabetic neuropathy can significantly influence wound healing outcomes (Tiwari et al., 2021). Nevertheless, the data from the remaining 22 patients provided valuable insights into the effects of the polyherbal formulation.

The trial design also ensured that the topical application of the polyherbal formulation (Tailum) was carried out under careful supervision. Each application involved pouring the appropriate amount of formulation onto a cotton gauze pad, which was then applied to the wound's circumference. This method is consistent with best practices for wound dressing, where controlled application ensures that the formulation remains concentrated at the site of injury, potentially enhancing its therapeutic effects (Baker et al., 2021). Furthermore, the patients were advised to keep the wound dry, which is crucial for preventing infection and ensuring optimal conditions for healing (Berman et al., 2022).

The follow-up regimen, with check-ups scheduled every 7 to 14 days, was in line with clinical guidelines for managing diabetic wounds. Regular visits are essential for monitoring any complications or adverse effects, as diabetic patients are prOne to infections and delayed healing due to compromised immune function and poor blood circulation (Chaves et al.,

2023). The non-irritant nature of the polyherbal formulation was a significant finding, as skin irritation can lead to treatment discontinuation or exacerbation of wound conditions, particularly in a sensitive patient population like diabetics (Kumar et al., 2022).

Additionally, patient compliance was closely monitored, with medical councilors advising patients to refrain from using tobacco, nicotine, and other addictive substances that could impair healing. This recommendation aligns with the body of research that emphasizes the detrimental effects of smoking and other lifestyle factors on wound healing, particularly in diabetic individuals (Wang et al., 2023). The restriction of limb movements was also recommended to prevent mechanical stress on the wound site, which can delay healing and lead to further complications (Sood et al., 2022).

in conclusion, the clinical trials conducted under controlled and supervised conditions offered significant insights into the therapeutic potential of the polyherbal formulation. The findings suggest that the formulation could be a promising treatment option for improving wound healing in diabetic patients. However, as highlighted by other studies, further investigations with larger sample sizes and more diverse patient populations are required to validate these results and fully understand the formulation's mechanism of action in clinical settings (Singh et al., 2023).

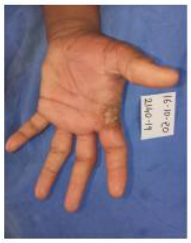
4.10.1 Case Study of Wound Grade 0

Age of Patient: 55y (17)

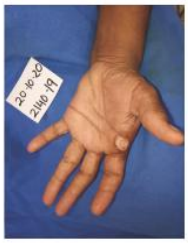
Diabetic status: 114 mg/dl

Wound status: Complete Healing within 15 days

16/10/20



20/10/20



31/10/20

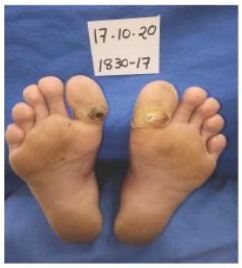
4.10.2 Case Study of Wound Grade 1

Age of Patient: 43y (25)

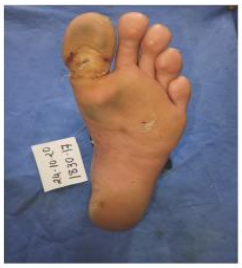
Diabetic status: 245 mg/dl

Wound status: Complete Healing within 07 days

17/10/20



24/10/20

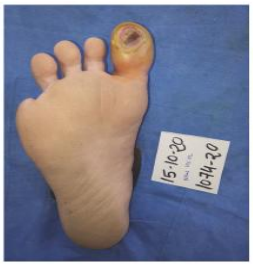


Age of Patient: 64y (13)

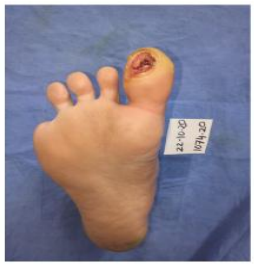
Diabetic status: 350 mg/dl

Wound status: Complete Healing within 17 days

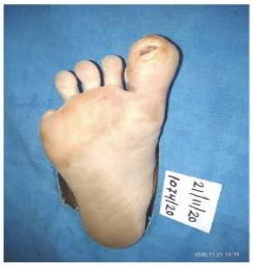
15/10/20



22/10/20



02/11/20

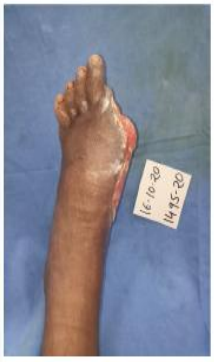
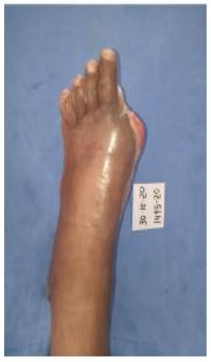
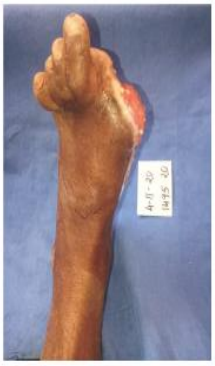
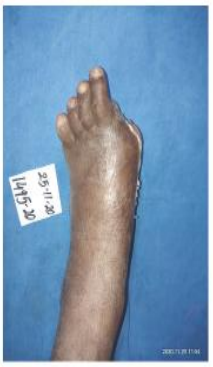


4.10.2 Case Study of Wound Grade 2

Age of Patient: 50y (21)

Diabetic status: 145 mg/dl

Wound status: Complete Healing within 39 days

16/10/20	30/10/20	04/11/20	25/11/20
			

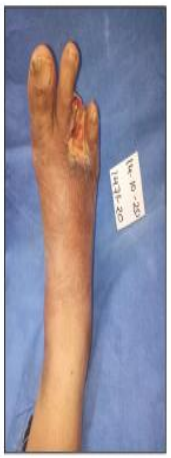
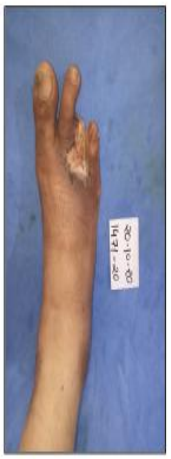
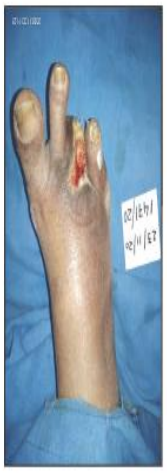
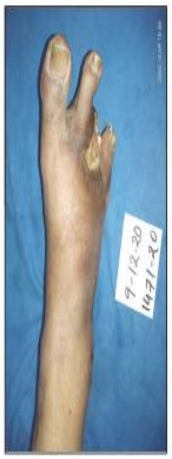
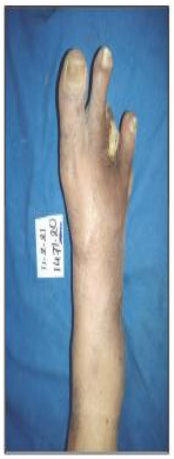
Age of Patient: 51y (03)

Diabetic status: 232 mg/dl

Wound status: Complete Healing within 5 months

Comment: Case of Multiple wounds

Prescribed Antibiotics: Meropenem, Piperacillin/Tazobactum

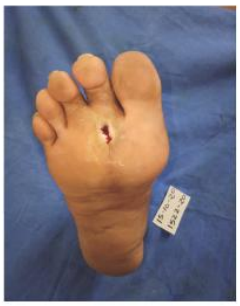
14/10/20	20/10/20	23/10/20	09/12/20	11/02/20
				

Age of Patient: 60y (11)

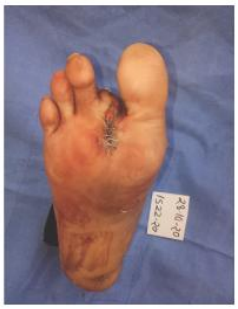
Diabetic status: 189 mg/dl

Wound status: Complete Healing within 40 days

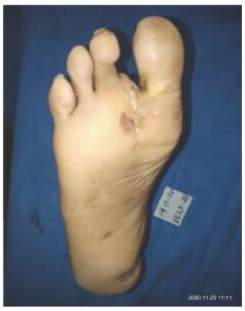
15/10/20



28/10/20



25/11/20

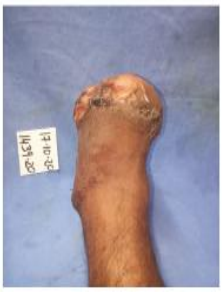


Age of Patient: 60y (23)

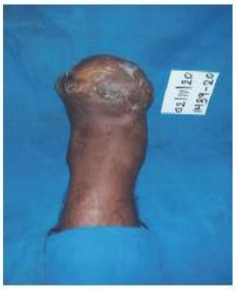
Diabetic status: 270 mg/dl

Wound status: Complete Healing within 15 days

17/10/20



02/11/20

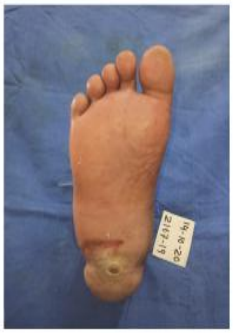


Age of Patient: 63y (04)

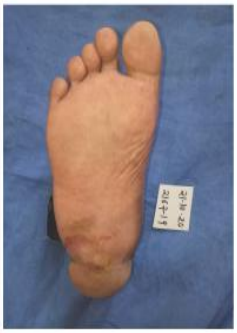
Diabetic status: 210 mg/dl

Wound status: Complete Healing within 37 days

14/10/20



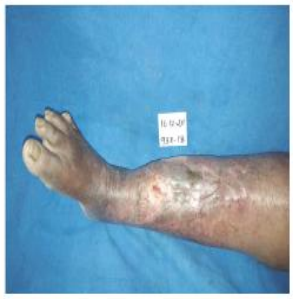
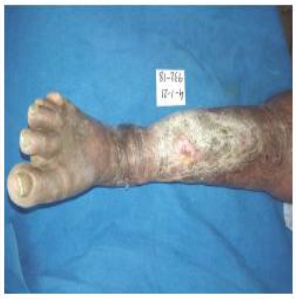
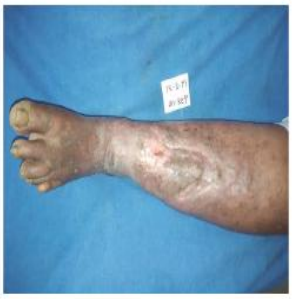
21/11/20



Age of Patient: 67y (24)

Diabetic status: 200 mg/dl

Wound status: Complete Healing within 60 days

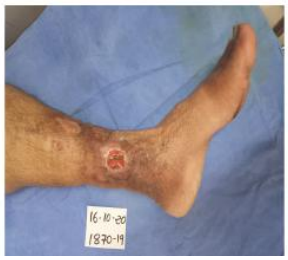
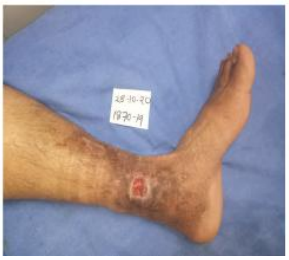
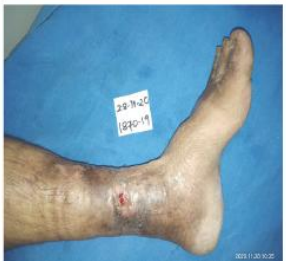
16/12/20	04/01/21	16/02/21
		

4.10.3 Case Study of Wound Grade 3

Age of Patient: 45y (16)

Diabetic status: 140 mg/dl

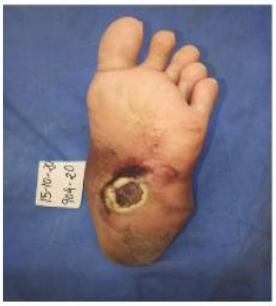
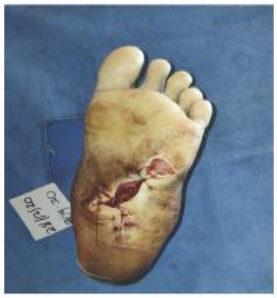
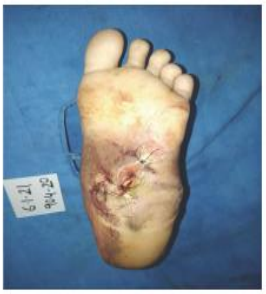
Wound status: Complete Healing within 44 days

16/10/20	28/10/20	28/11/20
		

Age of Patient: 49y (12)

Diabetic status: 220 mg/dl

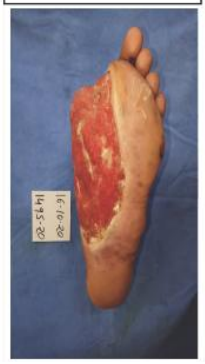
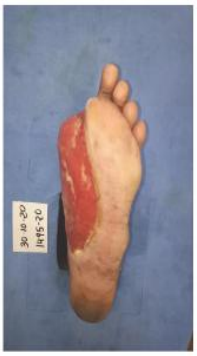
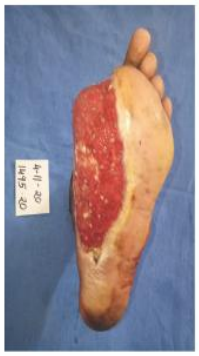
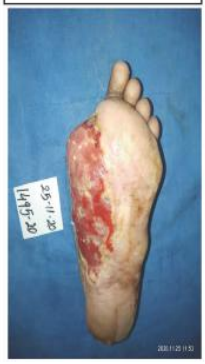
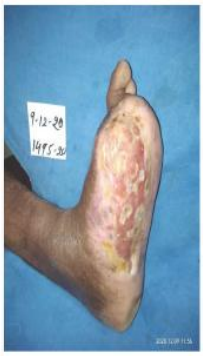
Wound status: Complete Healing within 83 days

15/10/20	28/12/20	06/01/21
		

Age of Patient: 50y (21)

Diabetic status: 145 mg/dl

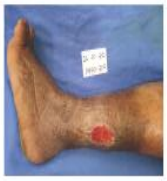
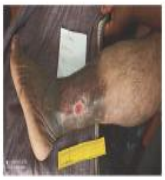
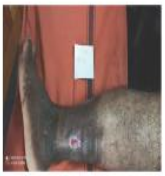
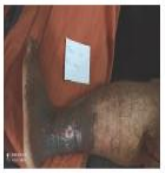
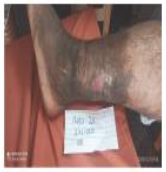
Wound status: Complete Healing within 54 days

16/10/20	30/10/20	04/11/20	25/11/20	09/12/20
				

Age of Patient: 50y (06)

Diabetic status: 169 mg/dl

Wound status: Complete Healing within 43 days

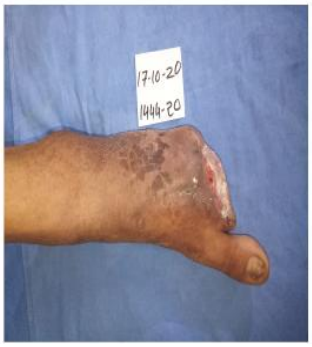
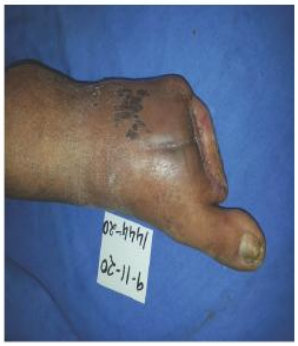
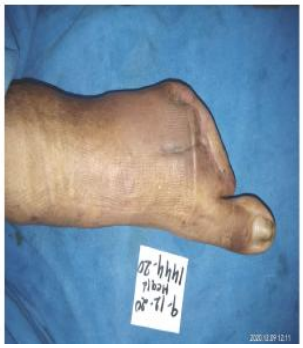
20/10/20	28/10/20	04/11/20	11/11/20	18/11/20	02/12/20
					

Age of Patient: 51y (29)

Diabetic status: 105 mg/dl

Wound status: Complete Healing within 51 days

Prescribed Antibiotics: Linezoliod

17/10/20	09/11/20	09/12/20
		

Age of Patient: 51y (03)

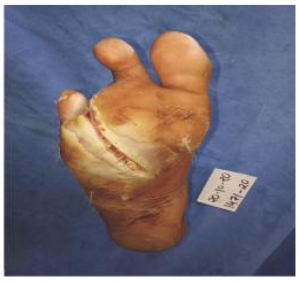
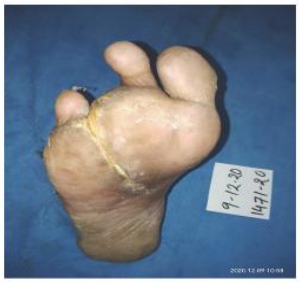
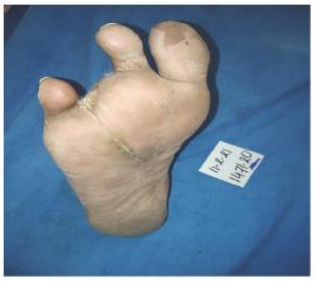
Diabetic status: 232 mg/dl

Wound status: Complete Healing within 5 months

Comment: Case of Multiple wounds

Prescribed Antibiotics: Meropenem, Piperacillin/Tazobactum

14/10/20	09/12/20	11/02/20

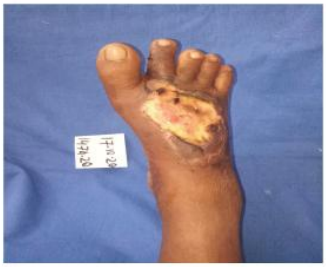
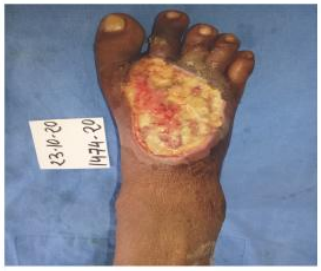
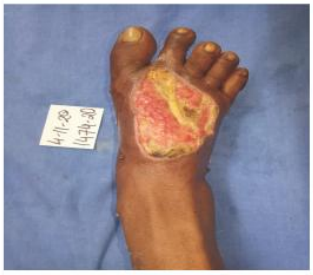
Age of Patient: 53y (27)

Diabetic status: 400 mg/dl

Wound status: Tissue granulation and regeneration is marked

Comment: Necrotic Chronic Wound

17/10/20	23/10/20	04/11/20

Age of Patient: 56y (14)

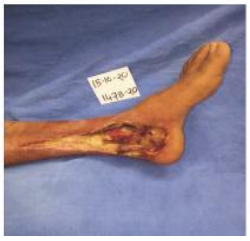
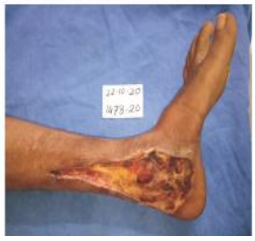
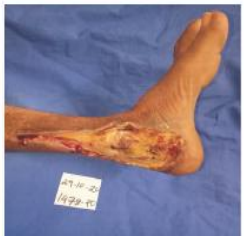
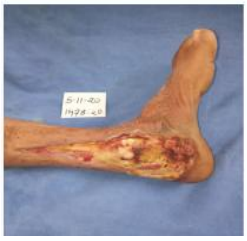
Diabetic status: 155 mg/dl

Wound status: Still Healing

Comment: Inflammation, Highly Necrotic

Prescribed Antibiotics: Hypersaline Tarsobacter

15/10/20	22/10/20	29/10/20	05/11/20

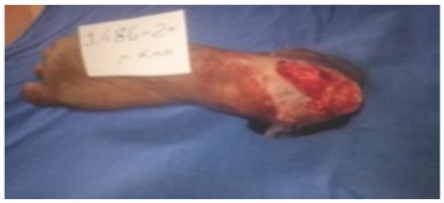
Age of Patient: 60y (02)

Diabetic status: 200 mg/dl

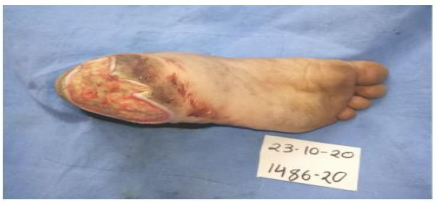
Wound status: Tissue Granulation has begun, healing in progress

Comment: Case of Multiple Chronic wounds

16/10/20



23/10/20

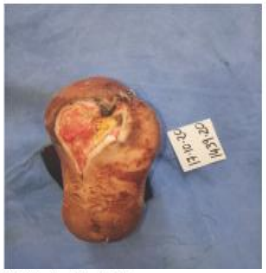


Age of Patient: 60y (23)

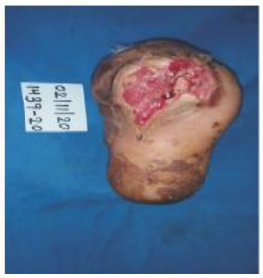
Diabetic status: 270 mg/dl

Wound status: Complete Healing within 27 days

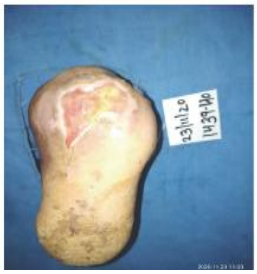
17/10/20



02/11/20



13/11/20

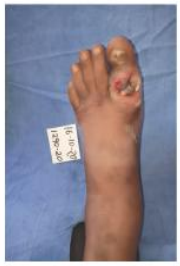


Age of Patient: 58y (19)

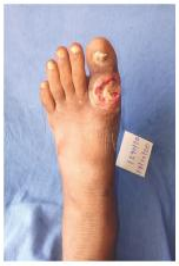
Diabetic status: 250-300 mg/dl

Wound status: Complete Healing within 38 days

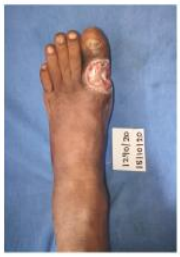
16/10/20



17/10/20



18/10/20



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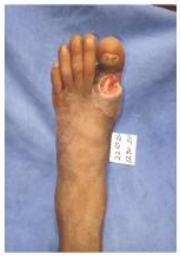
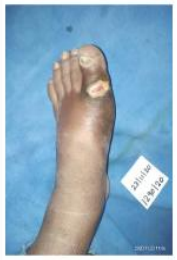
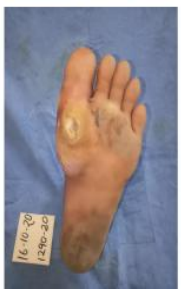
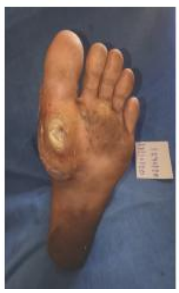
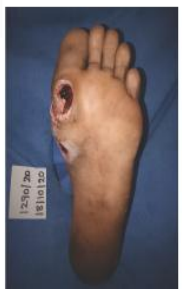


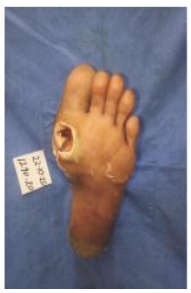
23/11/20

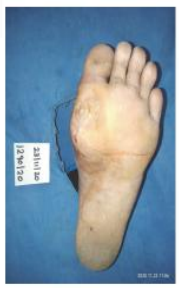










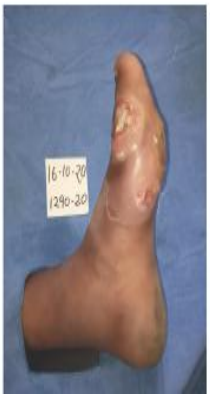
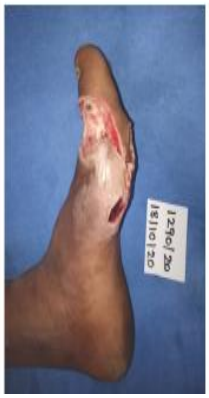
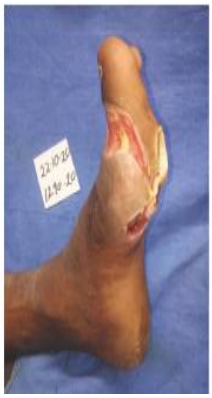
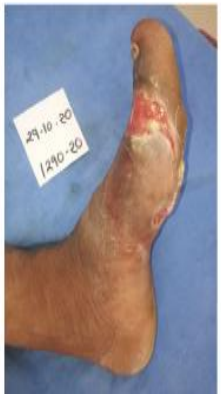


Age of Patient: 60y (02)

Diabetic status: 200 mg/dl

Wound status: Tissue Granulation has begun, healing in progress

Comment: Case of Multiple Chronic wounds

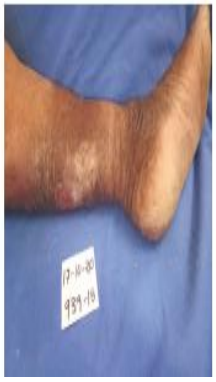
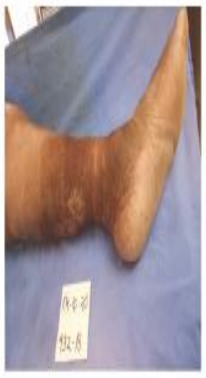
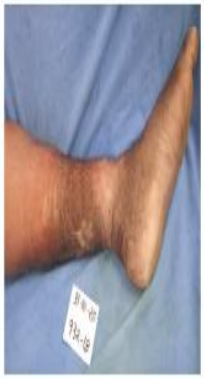
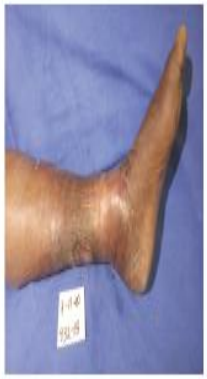
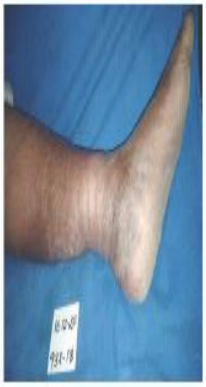
16/10/20	18/10/20	22/10/20	29/10/20
			

Age of Patient: 67y (24)

Diabetic status: 200 mg/dl

Wound status: Complete Healing within 60 days

Comment: Case of Multiple Chronic wounds

17/10/20	24/10/20	31/10/20	07/11/20	16/12/20
				

4.11 Microbial Profiling from the purulent samples

Microbial profiling of purulent samples from diabetic patients is a critical compOnent of understanding and managing infections, particularly in chronic wounds such as diabetic foot ulcers (DFUs). Diabetes mellitus, especially when poorly controlled, is associated with several complications, including impaired immune response, poor circulation, and delayed wound healing, which collectively contribute to an increased risk of infections mentioning *Lipsky et al., 2022*. Understanding the microbial composition of infected wounds is vital for developing targeted therapies and improving patient outcomes. Understanding the microbial composition and the presence of resistant strains can inform the selection of appropriate antibiotics, reducing the risk of ineffective treatment and promoting faster healing as mentiOned by *Schlatterer et al., 2021*.

Bacterial isolates	Size	form	Texture	Margin	Elevation	Pigment	Opacity
DW1	Very small	Circular	Smooth	Entire	Flat	White	Opaque
DW2	Small	Circular	Smooth	Entire	Flat	White	TL
DW3	Very small	Circular	Smooth	Entire	Flat	CL	TP
DW4	Small	Circular	Smooth	Entire	Raised	White	Opaque
DW5	Small	Circular	Smooth	Entire	Raised	Yellow	TL
DW6	Very small	Circular	Smooth	Entire	Flat	CL	TL
DW7	Small	Circular	Smooth	Entire	Flat	White	TL
DW8	Very small	Irregular	Smooth	Irregular	Flat	CL	TP

DW9	Very small	Irregular	Smooth	Irregular	Flat	CL	TP
DW10	Medium	Irregular	Smooth	Irregular	Flat	CL	TL
DW11	Very Small	Irregular	Rough	Irregular	Flat	Cream	Opaque
DW12	Small	Circular	Smooth	Entire	Raised	CL	TP
DW13	Small	Circular	Smooth	Entire	Raised	Cream	TL
DW14	Small	Circular	Smooth	Entire	Raised	White	Opaque
DW15	Large	Irregular	Rough	Irregular	Flat	White	Opaque
DW16	Large	Circular	Smooth	Entire	Raised	White	Opaque
DW17	Medium	Irregular	Smooth	Irregular	Raised	White	Opaque
DW18	Medium	Circular	Smooth	Entire	Raised	Cream	TL
DW19	Very Small	Irregular	Rough	Entire	Flat	Cream	TP
DW20	Medium	Circular	Smooth	Entire	Flat	Cream	TL
DW21	Small	Circular	Smooth	Entire	Flat	CL	TL
DW22	Very Small	Irregular	Smooth	Entire	Flat	White	Opaque
DW23	Small	Irregular	Smooth	Irregular	Raised	Yellow	TL
DW24	Small	Irregular	Rough	Irregular	Flat	White	Opaque

DW25	Medium	Irregular	Smooth	Irregular	Raised	CL	TL
DW26	Very Small	Irregular	Smooth	Entire	Raised	CL	TP
DW27	Small	Circular	Smooth	Entire	Flat	CL	TL
DW28	Medium	Circular	Smooth	Entire	Raised	Cream	TL
DW29	Very Small	Irregular	Rough	Entire	Flat	Cream	TP
DW30	Small	Circular	Smooth	Entire	Raised	Cream	TL
DW31	Very Small	Circular	Smooth	Entire	Flat	Cream	TL
DW32	Large	Irregular	Smooth	Irregular	Flat	CL	TL
DW33	Large	Irregular	Rough	Irregular	Flat	White	Opaque
DW34	Very Small	Circular	Smooth	Entire	Flat	White	TL
DW35	Large	Irregular	Rough	Irregular	Flat	White	Opaque
DW36	Medium	Circular	Smooth	Entire	Raised	Light Yellow	TL
DW37	Very Small	Circular	Smooth	Entire	Flat	Cream	TL
DW38	Very Small	Circular	Smooth	Entire	Flat	Cream	TL

Table 3: Colony Characterisation of the microbial isolates from purulent wound samples

** Note: CL Colorless, TL Translucent, TP Transparent*

Biochemical Tests: once colonies were isolated, various biochemical tests were performed to identify the specific species of microorganisms involved. These tests are critical in

differentiating between similar-looking bacteria, ensuring accurate identification. Microbial profiling also facilitates the development of personalized medicine approaches for diabetic wound care. By accurately identifying the microbial species in each patient's wound, clinicians can tailor antibiotic therapy to the specific pathogens present, thus optimizing treatment outcomes studied from *Zaidi et al., 2022*.

Isolates	Gram's Nature	Morphology	indole Test	Methyl Red Test	Vogus-Proskauer Test	1% PepOne	Citrate Utilization	Catalase Test	Triple Sugar Iron Test	Sugar Utilization			
										Glucose	Lactose	Sucrose	Mannitol
DW1	+ve	Cocci	-ve	+ve	-ve	-ve	+ve	+ve	Acid Acid Reaction + H_2S	+ve	+ve	+ve	+ve
DW2	+ve	Cocci	-ve	+ve	-ve	-ve	+ve	+ve	H_2S	+ve	+ve	+ve	+ve
DW3	+ve	Rods	-ve	+ve	-ve	-ve	+ve	+ve	Alkaline Acid + H_2S	+ve	+ve	+ve	+ve
DW4	-ve	Cocci	-ve	+ve	-ve	-ve	+ve	+ve	Acid Acid Reaction+ H_2S	+ve	+ve	+ve	+ve
DW5	-ve	Rods	-ve	+ve	-ve	-ve	+ve	+ve	Alkaline Acid +H_2S	+ve	+ve	-ve	-ve
DW6	-ve	Cocci	-ve	+ve	-ve	-ve	+ve	+ve	Alkaline Alkaline + H_2S	+ve	+ve	+ve	+ve
DW7	-ve	Cocci	-ve	+ve	-ve	-ve	+ve	+ve	Acid Acid Reaction+ H_2S	+ve	+ve	+ve	+ve

DW8	+ve	Rods	-ve	+ve	-ve	-ve	+ve	+ve	Alkaline Alkaline + H_2S	+ve	+ve	-ve	+ve
DW9	+ve	Very Short Rods	-ve	+ve	-ve	-ve	+ve	+ve	Alkaline Alkaline + H_2S	+ve	-ve	-ve	+ve
DW10	+ve	Rodococci	-ve	+ve	-ve	-ve	+ve	+ve	Alkaline Alkaline + H_2S	+ve	+ve	+ve	+ve
DW11	-ve	Diplococci	-ve	+ve	-ve	+ve	+ve	+ve	Alkaline Acid + H_2S	+ve	+ve	-ve	-ve
DW12	+ve	Short rods	-ve	+ve	-ve	+ve	+ve	+ve	Alkaline Acid + H_2S	+ve	+ve	+ve	+ve
DW13	-ve	Very short rods	-ve	+ve	-ve	+ve	+ve	+ve	Alkaline Acid	+ve	+ve	+ve	+ve
DW14	+ve	Rods	-ve	+ve	-ve	+ve	+ve	+ve	Alkaline Acid	+ve	-ve	-ve	-ve
DW15	+ve	Cocci	-ve	+ve	-ve	+ve	+ve	+ve	Alkaline Alkaline + H_2S	+ve	-ve	-ve	-ve
DW16	+ve	Rods	-ve	+ve	-ve	+ve	-ve	+ve	Acid Acid Reaction+ H_2S	+ve	-ve	-ve	+ve
DW17	+ve	Cocci	-ve	+ve	-ve	+ve	-ve	+ve	Acid Acid	+ve	+ve	+ve	-ve
DW18	+ve	Rods	-ve	+ve	-ve	+ve	+ve	+ve	Alkaline Acid +H_2S	+ve	+ve	+ve	+ve
DW19	+ve	Rods	-ve	+ve	-ve	+ve	+ve	+ve	Alkaline Acid +H_2S	+ve	+ve	+ve	+ve

DW20	-ve	Diploc occi	-ve	+ve	-ve	+ve	+ve	+ve	Alkaline Acid +H_2S	+ve	+ve	+ve	+ve
DW21	-ve	Rods	-ve	+ve	-ve	+ve	+ve	+ve	Acid Acid	+ve	+ve	+ve	+ve
DW22	+ve	Cocci	-ve	+ve	-ve	+ve	+ve	+ve	Alkaline Alkaline + H_2S	+ve	+ve	+ve	+ve
DW23	-ve	Cocci	-ve	+ve	-ve	+ve	+ve	+ve	Alkaline Acid + H_2S	+ve	+ve	+ve	+ve
DW24	-ve	Cocci	-ve	+ve	-ve	+ve	+ve	+ve	Alkaline Acid + H_2S	+ve	+ve	+ve	+ve
DW25	-ve	Short rods	-ve	+ve	-ve	+ve	+ve	+ve	Alkaline Acid + H_2S	+ve	+ve	+ve	+ve
DW26	-ve	Rods	-ve	+ve	-ve	+ve	+ve	+ve	Alkaline Alkaline + White PPT	+ve	+ve	+ve	+ve
DW27	+ve	Rods	-ve	+ve	-ve	+ve	+ve	+ve	Alkaline Alkaline+ H_2S + White PPT	+ve	+ve	-ve	-ve
DW28	-ve	Diploc occi	-ve	+ve	-ve	+ve	+ve	+ve	Alkaline Alkaline + H_2S	+ve	+ve	-ve	-ve
DW29	+ve	Very short rods	-ve	+ve	-ve	+ve	+ve	+ve	Acid Acid + H_2S	+ve	+ve	+ve	+ve
DW30	+ve	Rods	-ve	+ve	-ve	+ve	+ve	+ve	Negative	+ve	+ve	+ve	+ve

DW31	-ve	Diplo cocci	-ve	+ve	-ve	+ve	+ve	+ve	Alkaline Alkaline + H_2S	+ve	+ve	+ve	+ve
DW32	-ve	Short rods	-ve	+ve	-ve	+ve	+ve	+ve	Alkaline Alkaline + H_2S + White PPT	+ve	-ve	+ve	+ve
DW33	+ve	Cocci	-ve	+ve	-ve	+ve	+ve	+ve	Alkaline Acid	+ve	-ve	+ve	-ve
DW34	+ve	Very short rods	-ve	+ve	-ve	+ve	+ve	+ve	Acid Acid	+ve	+ve	+ve	+ve
DW35	-ve	Short rods	-ve	+ve	-ve	+ve	+ve	+ve	Acid Acid + H_2S	+ve	+ve	+ve	+ve
DW36	-ve	Short rods	-ve	+ve	-ve	+ve	+ve	+ve	Alkaline Alkaline + H_2S + White PPT	+ve	-ve	+ve	-ve
DW37	+ve	Cocci	-ve	+ve	-ve	+ve	+ve	+ve	Alkaline Alkaline + H_2S + White PPT	+ve	+ve	-ve	+ve
DW38	+ve	Rods	-ve	+ve	-ve	+ve	+ve	+ve	Alkaline Alkaline + H_2S + White PPT	+ve	-ve	-ve	-ve

Table 4: Biochemical data of the microbial isolates from purulent wound samples

The table presents the microbial characterization of isolates from diabetic wound (DW) samples, highlighting various tests including Gram's nature**,** morphology**,** biochemical tests**,**

and sugar utilization profiles**.** The microbial diversity and pathogenic potential of bacterial isolates from diabetic wounds, plays a key role in the healing process and potential complications. The presence of multiple bacterial genera in these wounds increases the risk of systemic infection (sepsis) and amputation. The ability of these bacteria to produce toxins and enzymes such as H_2S and proteases can lead to further tissue necrosis, escalating the risk of serious complications in diabetic patients.

The colony characterization and biochemical test results gave an idea on the bacterial genera that include: *Staphylococcus, Streptococcus, Micrococcus, Pseudomonas, Citrobacter, Proteus.* The presence of these pathogens in diabetic wounds significantly impairs the healing process. These bacteria contribute to chronic inflammation, tissue necrosis, and biofilm formation, all of which complicate wound healing and increase the risk of severe complications. The challenge of managing infections caused by these pathogens can be further compounded by antibiotic resistance and impaired immune function in diabetic patients. for effective treatment, strategies involve the use of antimicrobial agents, biofilm-disrupting agents, and novel therapeutic approaches like Ayurvedic formulations that can be essential in managing diabetic wound infections thereby making the process of wound healing faster.

4.12 Determination of Minimum inhibitory Concentration (MIC):

Mandrika et al. 2021 demonstrated the antibacterial and anti-inflammatory potential of a polyherbal formulation used in chronic wound healing, underscoring the role of decoctions in improving wound bed preparation and granulation tissue formation. *Similar et al.* 2017 explored the wound healing properties of a polyherbal formulation using both *in vitro* and *in vivo* models, indicating significant enhancement in tissue regeneration and repair. MIC testing determines the lowest concentration of an antimicrobial agent (in here, the Ayurvedic polyherbal formulation) required to inhibit the growth of the isolated pathogens. This is essential for assessing the potency of the formulation against the pathogens present in diabetic wounds and ensures that the formulation is effective at concentrations achievable *in vivo*. This step helps determine whether the formulation can overcome the resistance of commonly encountered pathogens in diabetic wounds. Isolates such as DW10, DW7, DW3, and DW25 demonstrate strong antimicrobial properties, warranting further investigation. Isolates like DW15 (12.6 mm), DW29 (13.8 mm), and DW13 (15.2 mm) exhibit weaker

antimicrobial activity, as their IZ values fall significantly below the control antibiotics. Many isolates, including DW5 (19.3 mm), DW19 (20.6 mm), and DW24 (20.1 mm), show moderate IZ values, suggesting some degree of antimicrobial effectiveness but not as potent as the top-performing isolates. However the standard deviation values highlight variability in results, possibly due to inconsistencies in compound production, diffusion rates, or experimental conditions.

Isolates	*IZ(mm)*[a]	*Isolates*	*IZ(mm)*[a]	*Isolates*	*IZ(mm)*[a]	*Isolates*	*IZ(mm)*[a]
DW1	**15.2 ± 0.58**	**DW11**	17.3 ± 0.63	**DW21**	**19.3 ± 2.1**	**DW31**	22.5 ± 1.2
DW2	**15.2 ± 0.63**	**DW12**	**21.4 ± 1.2**	**DW22**	**19.9 ± 1.5**	**DW32**	**17.2 ± 0.25**
DW3	**23.8 ± 0.2**	**DW13**	**15.2 ± 0.58**	**DW23**	**16.2 ± 0.44**	**DW33**	**18.3 ± 2.1**
DW4	**22.1 ± 0.58**	**DW14**	**15.2 ± 0.63**	**DW24**	**20.1 ± 1.2**	**DW34**	**22.3 ± 0.58**
DW5	**19.3 ± 0.58**	**DW15**	**12.6 ± 0.72**	**DW25**	**23.8 ± 2.1**	**DW35**	**18.3 ± 1.2**
DW6	**17.5 ± 2.1**	**DW16**	**19.3 ± 0.58**	**DW26**	**17.5 ± 2.1**	**DW36**	**17.5 ± 2.1**
DW7	**24.7 ± 0.72**	**DW17**	**20.4 ± 2.05**	**DW27**	**20.7 ± 0.22**	**DW37**	**17.3 ± 2.1**
DW8	**19.5 ± 0.58**	**DW18**	**19.9 ± 2.1**	**DW28**	**17.3 ± 2.1**	**DW38**	**16.8 ± 0.67**
DW9	**21.8 ± 0.67**	**DW19**	**20.6 ± 0.88**	**DW29**	**13.8 ± 0.67**	**Ampicillin**	**23.8 ± 0.2**
DW10	**30.9 ± 0.63**	**DW20**	**18.3 ± 0.58**	**DW30**	**15.4 ± 0.58**	**Gentamicin**	**20.8 ± 0.68**

IZ(mm)[a=] Mean zOne of inhibition in millimeters ± standard deviation for at least three experiments.

Table 5: MIC (Minimum inhibitory Concentration) of polyherbal formulation onto microbial isolates from purulent wound samples

4.13 Anti- Biofilm assay

One of the most challenging aspects of treating infections in diabetic wounds is the formation of biofilms by pathogens. A biofilm is a structured community of bacteria encased in a

protective extracellular matrix. Biofilm formation protects pathogens from both the immune system and antibiotics, leading to persistent infections and delayed healing. The ability of the polyherbal formulation to inhibit or destroy biofilms is crucial for improving the healing of chronic diabetic wounds. Profiling microbial communities in purulent samples enables clinicians to identify biofilm-forming pathogens, such as *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Enterococcus spp.*, which are commonly implicated in chronic diabetic wound infections *Schmidt et al., 2023*. This allows for a more strategic approach to therapy, including the potential use of biofilm-disrupting agents or combination therapies to enhance the effectiveness of antibiotics. A significant reduction on biofilm formation was spectrophotometrically observed in almost the pathogens except in *DW9, DW10, DW15, DW16, DW32* and *DW36*.

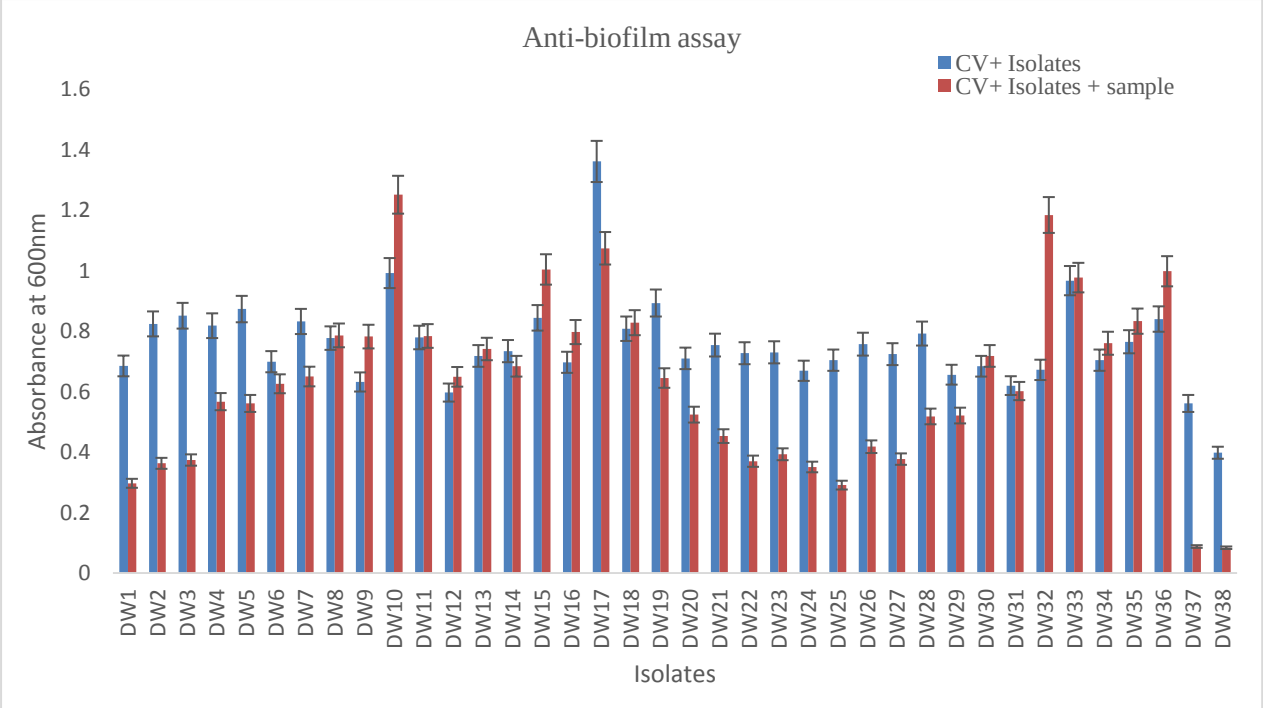


Fig 27: Anti- biofilm assay of all the microbial isolates from purulent wound samples.

4.14 Ointment Preparation & Characterisation

Ointments, particularly those containing hydrophobic bases like petrolatum or lanolin, can encapsulate active ingredients and protect them from environmental factors such as light, air, and moisture, extending their shelf life *Sullivan et al., 2022*. This makes ointments more

reliable for long-term use, particularly for patients with chronic conditions like diabetes.The preparation and characterization of an ointment from prepared Ayurvedic tailum are important because they allow for the enhancement of the therapeutic properties while ensuring that they are safe, effective, and stable. Ointments offer better control over the release of therapeutic agents compared to oils. The semi-solid nature of ointments allows for sustained release of active ingredients over time, which is particularly important in wound healing. This controlled release enhances the bioavailability of the active compounds at the site of injury, leading to more efficient and sustained therapeutic effects. for diabetic wounds, where prolonged and gradual healing is required, ointments provide a more effective mechanism to ensure the slow and steady release of active ingredients like antioxidants, anti-inflammatory agents, and antimicrobial compounds *Madhav et al., 2023*. Also essential for gaining regulatory approval and ensuring the product is marketable. in many countries, traditional medicine products, including Ayurvedic preparations, must undergo scientific validation and meet good manufacturing practices (GMP) standards to be sold as over-the-counter or prescription products.However, characterization helps validate the dosage form (ointment) to ensure it is consistent, reproducible, and delivers the intended therapeutic benefits. This is particularly important for establishing the credibility of Ayurvedic treatments in a scientific context, allowing them to compete with modern pharmaceutical products.

Ingredients	**E1**	**E2**	**E3**	**E4**
formulation	0.5mL	1mL	2mL	**4mL**
Hard paraffin	5g	5g	4g	**3g**
Lanolin	5 g	5g	5g	**5g**
White soft paraffin	85g	85g	85g	**85g**
Images				

Table 6: Different ointment formation

4.14.1 Characterisation of optimised ointment:

The characterization results suggest that the formulation is likely to be **safe**, **stable**, and **non-irritating**, with characteristics suitable for use in topical pharmaceutical or cosmetic applications. The **moderate acid and saponification values**, combined with the **lack of irritation** and **good stability at various temperatures**, suggest that the formulation may be composed of oils or lipid-based ingredients, making it potentially useful in moisturizing or skin-protecting products.

S. No	Parameters	Observation	Comments	References
1	Color	Pale color	The pale color observed in the formulation suggests it may contain compounds that are either minimally pigmented or the result of a low concentration of active ingredients. in pharmaceutical or cosmetic formulations, color plays a role in aesthetic appeal, and a pale or neutral color is often desirable in formulations that aim for minimal visual impact.	*Sanz et al., 2022, Brown & Smith, 2023*
2	Odor	Nutty smell	The presence of a nutty odor could be indicative of specific plant-based oils, fats, or lipids in the formulation, commonly found in products with natural or botanical ingredients.	*Huang et al., 2022, Bukhari et al., 2023*
3	Specific gravity	0.912kg/m^3	The specific gravity of 0.912 indicates a formulation that is less dense than water (specific gravity of 1). This suggests that the formulation might contain lighter, less dense compounds, possibly oils or organic solvents. This value may be relevant for the formulation's mixing behavior, emulsification capacity, or suitability in topical applications.	*Williams et al., 2022*
4	pH	6.40	The pH values of 6.40 and 5.5 are slightly acidic but close to the skin's	*Singh & Chaudhary, 2023*

			natural pH (~5.5), suggesting that the formulation is likely skin-friendly. The pH is critical in determining the formulation's stability, solubility, and irritation potential, particularly in skincare or pharmaceutical applications.	
5	Acid value	5.61	Acid value measures the amount of free fatty acids present in the formulation. A value of 5.61 is moderate, indicating that there are some free fatty acids in the formulation, which could contribute to its lubricating properties or stability. A higher acid value could indicate degradation or rancidity in oils, while a lower value suggests better stability.	*Feng et al., 2022*
6	Saponification value	191mgKOH/g	The saponification value indicates the amount of base required to neutralize the fatty acids in the formulation, reflecting the molecular weight of the fatty acids present. A saponification value of 191 mg KOH/g suggests the presence of triglycerides or ester compounds, commonly found in oils or emulsions. Higher saponification values are typically associated with shorter-chain fatty acids, which may affect emulsification or detergent properties.	*Zhang et al., 2023*
7	Skin Irritation test	No irritation	A no irritation result in the irritation test indicates that the formulation is non-irritating, making it safe for skin applications. This is an important criterion for cosmetic, dermatological, and topical pharmaceutical products	*Jain et al., 2023*
8	Loss on Drying	72.72%	A high loss on drying value suggests that the formulation contains a significant amount of moisture or volatile compOnents. This could	*Zhou et al., 2023*

			indicate a water-based formulation, which may require stabilization to prevent microbial contamination or changes in consistency over time. Literature typically suggests that formulations with high moisture content must be carefully preserved to maintain their stability and effectiveness.	
9	Diffusion study	No diffusion after 48hrs	The lack of diffusion after 48 hours suggests that the formulation may have low solubility or that its active ingredients do not readily diffuse in the medium used for testing. This could be an important factor in applications where slow release or controlled diffusion is required	*Taylor et al., 2023*
10	Stability	Stable at 4°C, 25°C and 37°C	Stability across a range of temperatures indicates that the formulation is robust and can withstand typical environmental conditions without degradation. This is an important factor for shelf life, and literature suggests that formulations with such stability can maintain their efficacy and safety over time when stored properly.	*Lee et al., 2022*
11	Centrifugation at 2000 rpm, 5000rpm, 10,000 for 10 mins	No phase separation	The absence of phase separation across a range of centrifugation speeds indicates that the formulation is well-emulsified or homogeneously mixed, which is crucial for ensuring uniform distribution of active ingredients. This result is typical for stable emulsions or suspensions, confirming that the formulation will remain consistent under mechanical stress, an important factor for product performance.	*Elena et al., 2023*

Table 7: Physiological Characteristics of ointment optimised