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Comparative Study of Single and Co-Emulsions of Essential Oils in Wound Dressing Efficiency: preparation, characterization and burn healing application

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Abstract

This study aimed to investigate the wound healing effects of peppermint (P) single and its co-emulsion with black seed oil (B) or frankincense oil (F) through an ultrasonication process. Successfully Prepared nano-emulsions were examined through TEM, particle size analyser (PSA), and zeta potential (ZP) techniques. The produced emulsion was applied to non-woven fabric (NWF) by spraying technique. Non-woven fabric surface morphology was produced and examined through a scanning electron microscope (SEM). Antibacterial activities of emulsions treated NWF were examined against *Staphylococcus aureus* as gram-positive bacteria and *Escherichia coli* as gram-negative bacteria and their healing effect to second burn degree were examined through reduction of the burn size. Additionally, Histopathological evaluations also evaluated for burn skin samples. Nano-emulsion droplets size lies between 20 and 70 nm and are increased by storage. The Zeta potential of P/F emulsion showed the highest negative charge compared with all prepared emulsions. FTIR-ATR charts showed new peaks for oils treated NWF compared with untreated NWF sample. The P/F emulsion showed higher zeta potential measurement compared with other single or Co-emulsions. For burn healing in-vivo, P/F emulsion treated NWF showed complete healing with original skin colour compared with other groups. While histopathological evaluation showed a homogenous epidermis with more granulation tissue formation than P/B emulsion treated NWF and all single oils emulsions. Antimicrobial evaluation of co-emulsions showed higher results than single oils emulsions. The results of co-emulsion oils showed superior properties and burn healing, especially P/F co-emulsion.

Keywords: Emulsion oil; Peppermint; Black seed; Frankincense; non-woven; Burn healing

1. Introduction

Emulsion oils are a type of oil that is mixed with water to form a stable emulsion. This type of oil is often used to produce creams, lotions, and other skincare products. Studies have shown that emulsion oils can positively affect wound healing [1]. Emulsion oils are believed to help facilitate wound healing by providing a moist healing environment, which encourages new tissue growth and helps prevent scarring [2]. Additionally, emulsion oils may help provide anti-inflammatory and antibacterial benefits [3], which can help reduce the risk of infection and speed up the healing process. It is important to note that more research is needed to understand their potential benefits and how best to use them fully. Different publications reported that using a cream containing oil emulsion on diabetic foot ulcers helped improve wound healing and reduce inflammation. Another study published found that using an emulsion oil-based cream helped improve the healing of skin wounds caused by surgery [4].

Menthol oil is a natural compound that is extracted from the peppermint plant. It is commonly used in various products, including topical creams, cough drops, and chewing gum [5]. Recent studies have suggested that menthol oil and its mixtures may have potential as wound healing and antibacterial agents [6]. Many reports found that menthol oil showed antimicrobial activity against various bacterial strains, including Staphylococcus aureus and Escherichia coli [7]. Mixture of menthol oil and olive oil was shown to have antibacterial activity against methicillin-resistant Staphylococcus aureus (MRSA) in vitro. Furthermore, menthol oil has been shown to have a cooling effect on the skin and may help relieve pain and itching associated with wounds. Cream containing menthol oil was applied to skin wounds in mice, resulting in reduced inflammation, increased collagen deposition, and faster healing [8]. Overall, these studies suggest that menthol oil and its mixtures may have potential as wound healing and antibacterial agents.

Frankincense oil is a natural essential oil that is derived from the resin of Boswellia trees [9]. This oil has been traditionally used for medicinal purposes, and recent studies suggest that . A study published in the Journal of Antibiotics found that these bacteria are known to cause wound infections, and the study suggests that frankincense oil may effectively prevent these infections [9, 10]. In addition In addition, another study demonstrates that frankincense emulsion oil has wound-healing properties suggests that |Frankincense oil possesses promising potential to modulate the biological processes of inflammation and tissue remodeling in human skin [11]. Another study created wounds in rats and treated them with a hydrogel containing

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frankincense emulsion oil [12]. The treatment resulted in accelerated wound healing, reduced inflammation, and increased collagen production. Frankincense oil is also believed to have anti-inflammatory properties [13], which can further aid in the wound-healing process. In addition, the oil has been shown to have analgesic properties, which can help reduce pain associated with wounds. The studies suggest that frankincense emulsion oil may have antibacterial and wound-healing properties [14, 15].

Black seed emulsion has several antibacterial and wound-healing properties due to the presence of thymoquinone, which is its active ingredient [16]. Thymoquinone has been reported to have several health benefits, including antioxidant, anti-inflammatory, and antimicrobial properties [17]. Studies have shown that black seed emulsion has potent antibacterial activity against various Gram-positive and Gram-negative bacteria. It has been found to be effective against bacteria responsible for skin infections, such as Staphylococcus aureus and Streptococcus pyogenes. Furthermore, black seed oil emulsion has been found to promote wound healing.

From this point of view, the current work aims to screen the effect of individual and combined oils emulsions, namely menthol, black seed, and frankincense. Oils emulsions were prepared successfully using homogenizer and tween 80 and Carbpol as emulsifying agents. Produced emulsions were characterized and immobilized on nonwoven fabrics dressings to treat rat burns. Produced fabrics morphology screened using SEM while their antibacterial activities were examined against *Staphylococcus aureus* as a gram-positive bacteria and *Escherichia coli as* a gram-negative bacteria. Burns healing size and its pathology were evaluated upon using oils emulsions individually and their combinations.

2. Results and Discussion

2.1. Transmission Electron Microscope

Transmission electron microscopy was employed to examine the surface morphology and determine the droplet size of the nano-emulsions prepared using peppermint, frankincense, or black seed oils. The results showed that the essential oils in Nano emulsions belonging to peppermint were evenly dispersed as spherical particles between 46 to 88 nm (Fig. 1a), while black seed oil lay between 20.78 and 39 nm in size (Fig. 1b). The mixture size of co-emulsion peppermint and black seed emulsion (Fig. 1d) lied between 36 and 58 nm. It seems that size of co-emulsion decreases. Frankincense emulsion oil size (Fig. 1c) located between 11 to 69 nm, while its mixture with peppermint (Fig. 1e) lies between 28 to 56 nm. The size range of accepted nanoemulsion particle sizes is occupied by all of the produced droplet sizes.

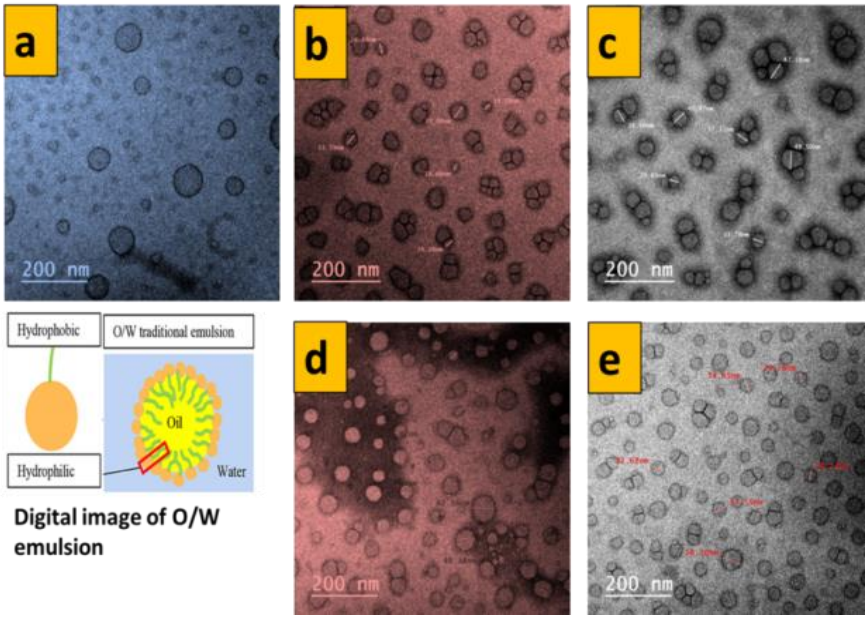


Figure 1: TEM images of: a) P, b) B, c) F, d) P/B and e) P/F. where Peppermint (P), Black seed (B), and Frankincense (F).

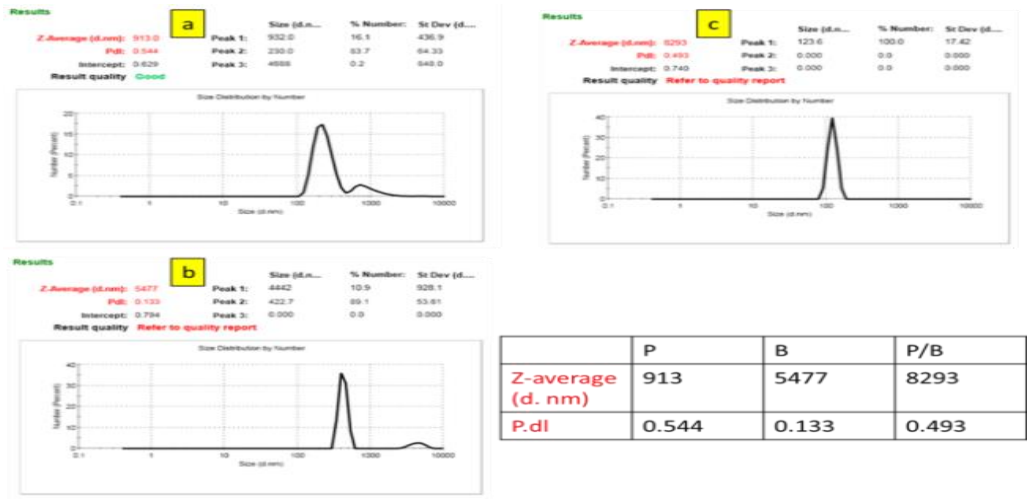
2.2. Particle size analyzer (P.S)

Emulsion Particle size has a relationship with emusion stability and structure. Actually particle size is directly proportional to emulsion physical stability. As particle size decreases emulsion become more stable. Conversely, the faster increase in particle size, the more unstable emulsion system becomes. The emulsion particles must be small enough to allow a film forming around emulsion droplets in the dispersed phase. Fig. 2 a-c showed the particle size stability after 2 months storage of prepared emulsions Peppermint, black see and peppermint/Black seed oil emulsions. The size of prepared peppermint emulsions around 13 nm and black seed around 5477 nm while the co-emulsions around 8913 nm. The increment in particle size may be attributed to many reasons as: phase inversion, flocculation, coalescence, creaming, Ostwald ripening, and cracking.

Menthol, menthol, neo menthol and mentone counted as main components of peppermint oil while black seed oil containing thymoquionne molecule. These mono tyrpin has quinone groups, this structure may interact with each other increase precipitation rate by increasing storage time. While Fig 3 a-c Fig. 2 a-c showed the particle size stability after 2 months storage of prepared emulsions Peppermint, Frankincense and peppermint/frankincense oil emulsions. The sizes of the prepared emulsions were 913, 332 and 372.8 nm for each peppermit, frankincense and peppermint/frankincense subsequently. The

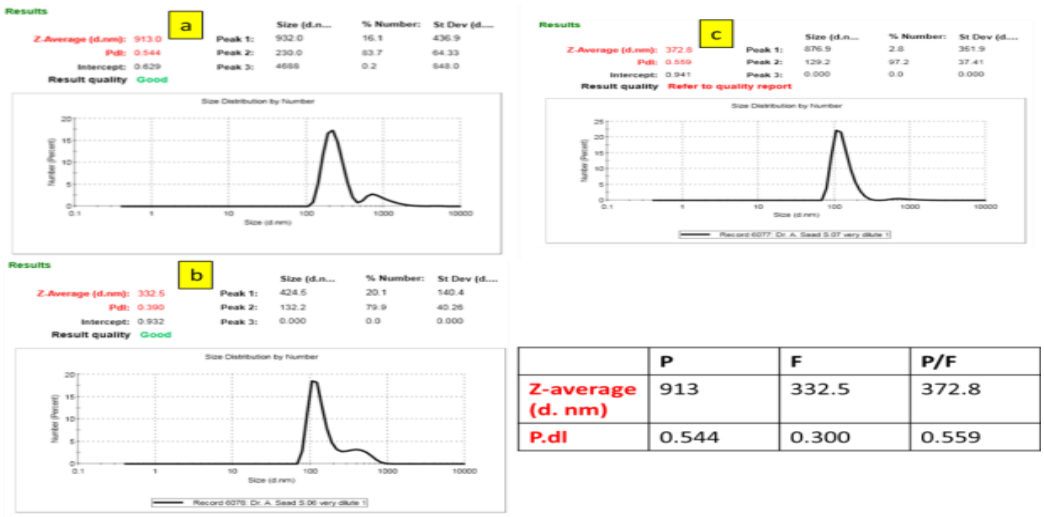
increment in particle size may be attributed to the previous reasons. Frankincense considered as pentaterpin, the higher molecular weight of frankincense showed more reduction in emulsion size after 2 month storage. However, stability of all emulsion oils affected by storage time (2 months), P/F co emulsion is least emulsion affected by storage duration. This may attributed to pentaterpin structure.

Zeta potential is the electrostatic attraction or repulsion forces between emulsion particles and it considered as important indicator for dispersion stability. As zeta potential values increases either negative or positive emulsion particle aggregation decreases. It may attribute to electrostatic interaction between emulsion particles and bioactive compound and with the biological environment.



	P	B	P/B
Z-average (d. nm)	913	5477	8293
P.dI	0.544	0.133	0.493

Figure 2: Zeta sizer of : a) P, b) B, and c)P/B, where Peppermint (P), Black seed (B)**.**



	P	F	P/F
Z-average (d. nm)	913	332.5	372.8
P.dI	0.544	0.300	0.559

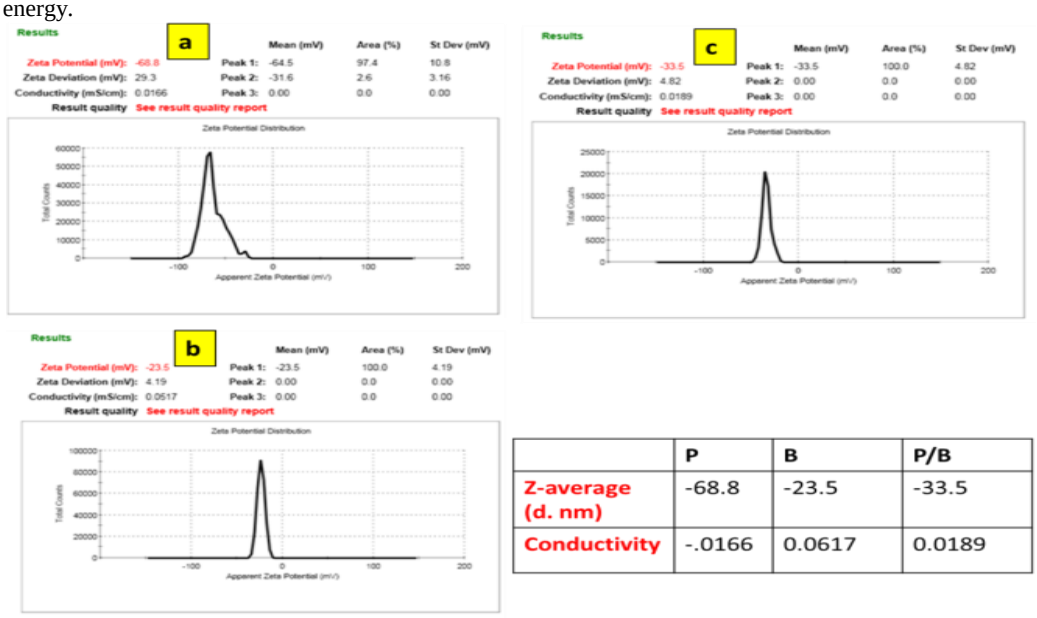
Figure 3: Zeta seizer of: a) P, b) F and c)P/F, where Peppermint (P) and Frankincense (F)

To measure Zeta potential, sample diluted and measured at predefined conditions correspondent to formulation properties at a constant temperature. Fig. 4 and Fig. 5 showed the Zeta potential of emulsion under investigations. Fig. 4 a-c showed Zeta potential of P, B and P/F emulsion oils. Obtained results were -68, -23 and -33 for the following emulsion Peppermint, Black seed and Peppermint/Black seed subsequently. its observed that potential energy of the emulsion of peppermint and its co-emulsion with black seed was higher than -25 while black seed is lower than -25.

It is well-established that when the zeta potential has an absolute value greater than or equal to 25 mV, the repulsive forces dominate over the attractive London forces, resulting in well-dispersed emulsion particles and a deflocculated system. Conversely, when the absolute value of the zeta potential is less than or equal to 25 mV, the attractive forces surpass the repulsive forces, causing the particles to bind together and flocculate.

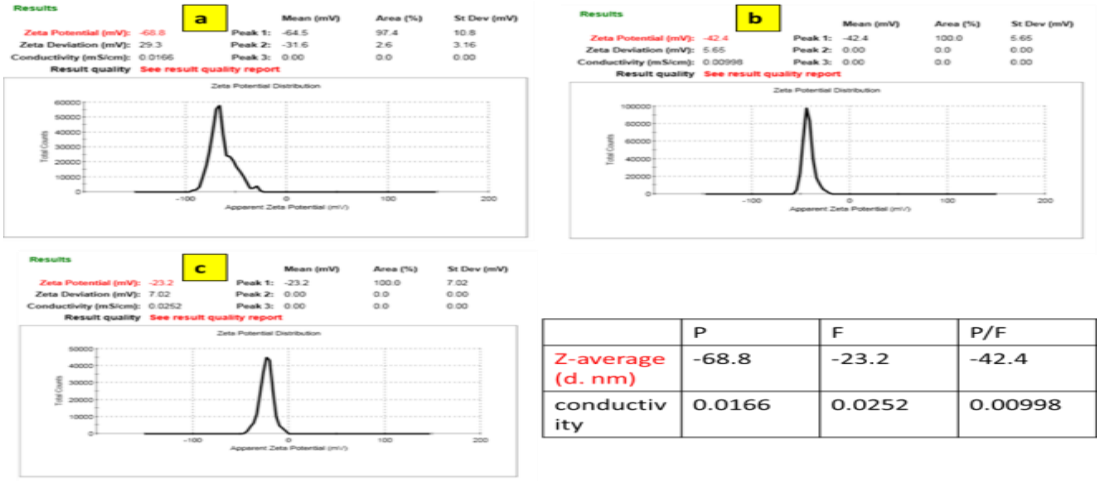
Fig. 5 a-c showed Zeta potential of P, F and P/F emulsion oils. Obtained results were -68, -23 and -42 for the following emulsion Peppermint, Frankincense and Peppermint/Frankincense subsequently. It's observed that potential energy of the emulsion of peppermint and its co-emulsion with frankincense was higher than -25 while frankincense is lower than -25.

The potential energies of single peppermint and its mixture with either black seed or frankincense showed ≥ 25 mV as an absolute value, the repulsive forces exceed the attractive London forces. Then, particles are dispersed and the system is deflocculated. It's worth to notice that co- emulsion P/F system is more dispersed and the system than P/B because it higher potential energy.



	P	B	P/B
Z-average (d. nm)	-68.8	-23.5	-33.5
Conductivity	-.0166	0.0617	0.0189

Figure 4: Zeta potential of : a) P, b) B and c)P/B, where Peppermint (P), Black seed (B)



	P	F	P/F
Z-average (d. nm)	-68.8	-23.2	-42.4
conductivity	0.0166	0.0252	0.00998

Figure 5: Zeta sizer of a) P, b) F, and c)P/F, where Peppermint (P) and Frankincense (F)

2.3. Fourier transmittance Infrared- Attenuated reflectance (FTIR-ATR)

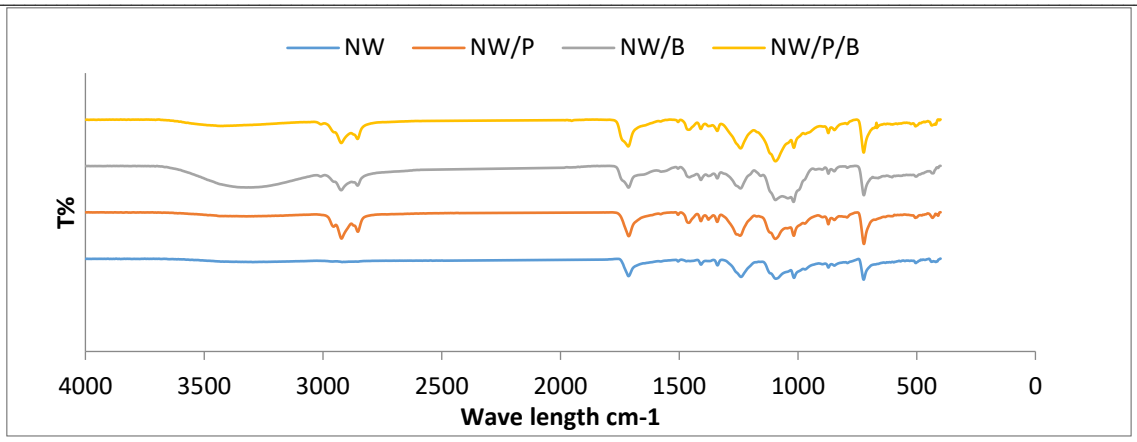


Figure 6: FTIT-ATR of NW, NW/P, NW/B and NW/P/B. where Nonwoven (NW), Peppermint (P), Black seed (B).

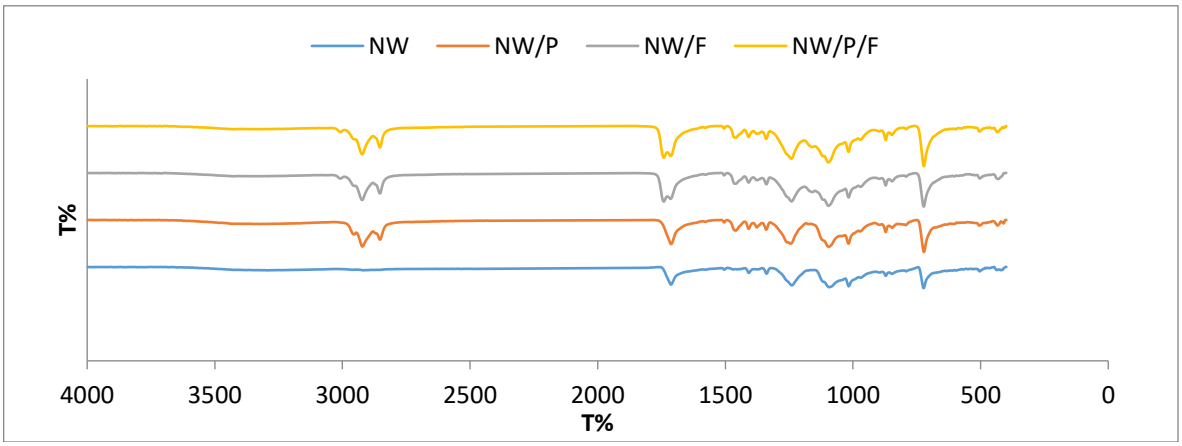


Figure 7: FTIR/AT of: a) NW, b) NW/P, c) NW/P/B and d) NW/P/F Where: Nonwoven (NW), Peppermint (P) and Frankincense (F).

The chemical interaction between nonwoven and/or emulsion and presented co-emulsion oils were displayed at FTIR-ATR (fig 6and7). Nonwoven samples treated with pepperemint, black seed and their co-emulsion were presented at Fig. 6. Polyester fibers (PE) or condensed phthalic acids are usually characterized by specific peaks as follows: Peaks in 1709, 1407, and 1339 cm^{-1} belong to PE function carboxylic groups, aromatic rings, and alkane chains (Fig. 6, 7).

In the FTIR-ATR spectrum of peppermint emulsion oil (P), the band observed at 3398 cm^{-1} corresponds to the OH groups, derived from the alcohol components present in the P emulsion oil. Additional characteristic peaks were identified at 2958–2869 cm^{-1}, 1712 cm^{-1}, 1459 cm^{-1}, 1370 cm^{-1}, 1246 cm^{-1}, and 1046 cm^{-1}. These peaks are associated with aliphatic -CH stretching, C=O stretching, aliphatic C-C stretching, and -C-O asymmetric and symmetric stretching, respectively, as evident in the FTIR spectrum of the P emulsion oil (Fig. 7) [18].

The notable characteristic peaks for Black seed emulsion oil (B) were at 2854, 2923 and 3009 cm^{-1} are belonging to (C-H in-CH2), (C-H in-CH2), (C-H in HC=CH) respectively. These represent the dominance of carbon chains in the fatty acids. In general, more than 98% of B oil is fatty acids [19].

The NW/P/B FTIR-ATR chart (Fig. 6) showed existing of either NW peaks and P and B oil emulsion peaks with no shift at their position or intensity. This attributed to physical interaction between NW and the two emulsion oils.

In the FTIR spectrum of the F emulsion oil, characteristic bands were observed at 2925 cm^{-1} and 2848 cm^{-1}, corresponding to the asymmetric and symmetric stretching vibrations of the methylene (CH_2) group, respectively. The peak at 1736 cm^{-1} is attributed to the ester carbonyl functional group of triglycerides. Additional peaks include 1454 cm^{-1}, associated with bending vibrations of CH_2 and CH_3 aliphatic groups; 1360 cm^{-1}, representing CH_2 bending vibrations; and 968 cm^{-1}, linked to bending vibrations of CH functional groups in isolated trans-olefins [20].

The NW/P/F chart Fig.7 showed existing of either NW peaks and P and F emulsion oils peaks with no shift at their position or intensity. This attributed to physical interaction between NW and the two emulsion oils.

2.4. Scanning Electron Microscope (SEM)

The morphology treated by both methods samples was investigated using SEM. Fig 8 shows SEM images of pristine NW dressing and NW-treated emulsion oil and their mixtures. Fig 8a showed NW fabrics, it appeared homogenous smooth and cylindrical shapes. While treated with either single emulsion oils, Peppermint (Fig 8b) or its mixture with black seed oil (Fig

8c) or frankincense (Fig 8d) showed non-homogeneity and emulsion depositions. This may be attributed to a reaction between emulsions.

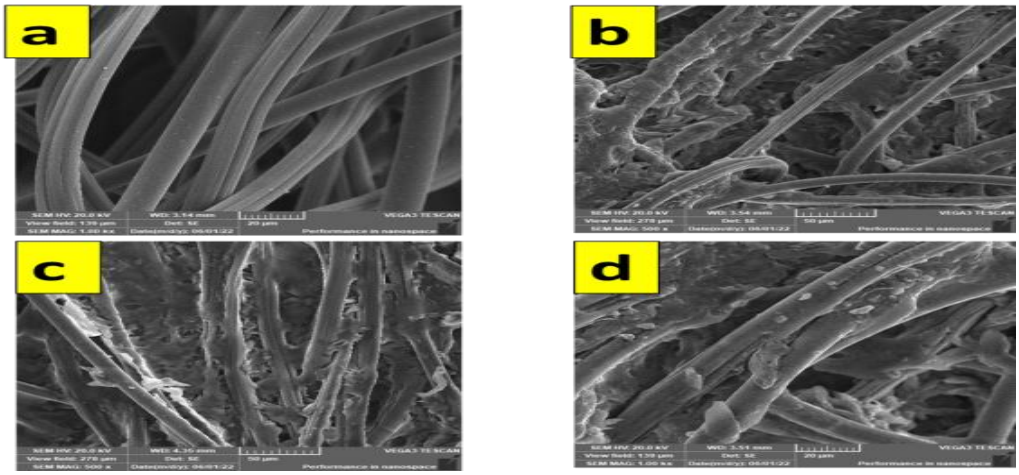


Figure 8: Scanning Electron microscope of: a) NWF, b) NWF/P, c) NWF/P/B and d) NWF/P/F

2.5. Burn healing evaluations

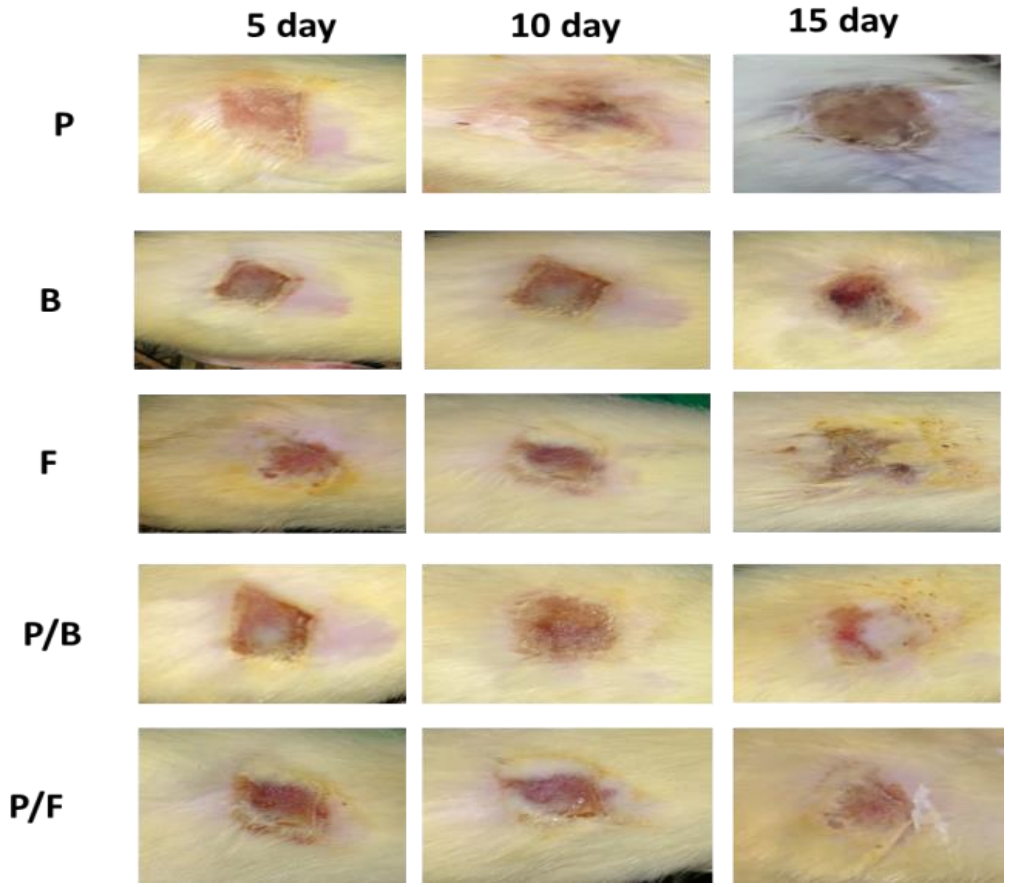


Figure 9: Digital images of burn healing and reduction of burn size.

This study evaluated burn healing over 15 days, measuring burn closure percent of wound contraction areas. Albino rats were burned at the back with 2*2 cm and treated with NW and NW dressings containing individual oils and their co-emulsion. As known, burns are classified into first, second, and third-degree. In the current study, rats burned in second-degree burns affect only the papillary layer (i.e. the upper dermis) or reticular layer (i.e. the deeper dermis) with white or yellow color, blistering of the skin, and a moist appearance.

Burn areas macroscopic photos captured at different durations 5, 10 and 15 days were displayed at Fig. 9. Edema, necrosis, and exudates were observed at day 1 for all wounded groups. In addition, wound edges started to appear clearly and surrounded by edema. Crust formations were observed on the skin in all single emulsion oils P, B, and F. At day 10, more healing occurred. On the 15th day, the crust layer formed for single oils emulsion while it fell off for mixed emulsion oils. It is worth mentioning that there is a change in color in all samples except P/F mixed emulsion oil.

The wound contraction ratio was assessed as the percentage reduction in wound size on days 5, 10, and 15 days. As illustrated in Fig. 9. The % of the burn area treated with single oils P, B, and F ranged from 70% to 50% in the period between 5 to 10 days. The % of wound area in rats treated with mixed oil emulsions P/B completely healing with rust, while P/F completely healing with rust fall off at 15 days, P/F showed superior complete healing with original skin color compared with other groups.

2.6. Histopathology evaluations

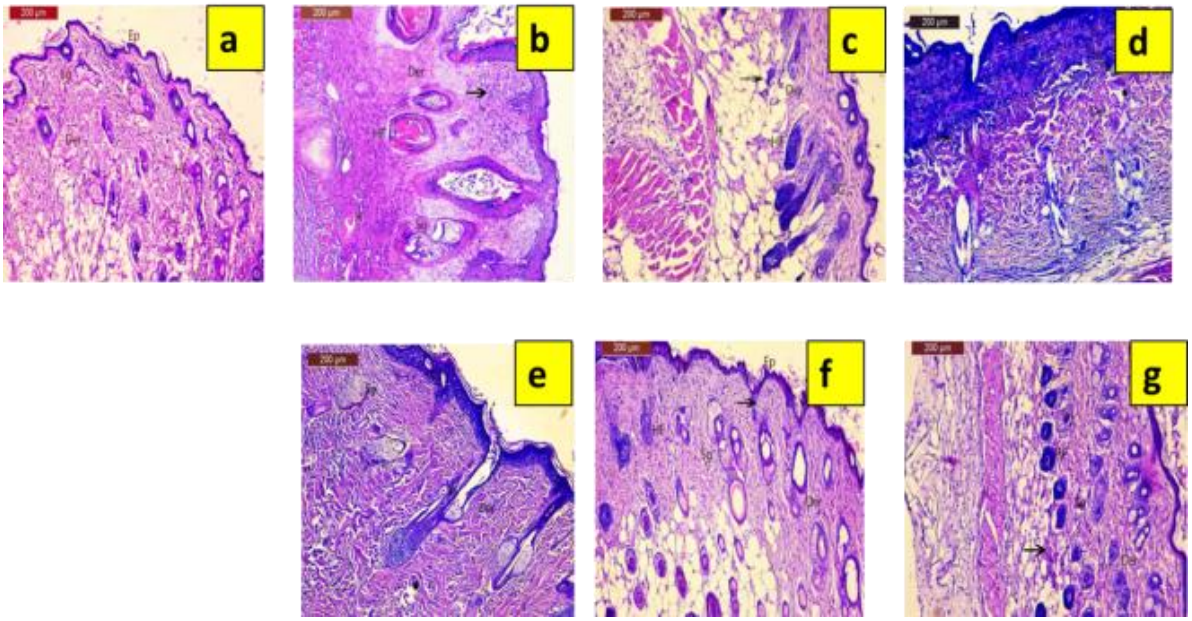


Figure 10: Histopathology evaluations of healed burns: a) control, b) positive, c) P, d) B e) F, f) P/B and g) P/F.

A photomicrograph of a rat skin section from the control group (Fig. 10a) displays a normal epidermis (Ep), dermis (Der), numerous hair follicles (HF), and sebaceous glands (Sg). In contrast, Fig. 10b shows a skin section with epithelium of irregular thickness, coagulative necrosis affecting the entire epidermis (Ep), degenerated hair follicles (HF), and sebaceous glands (Sg). Additionally, inflammatory cell infiltration in the epidermis and dermis (arrow) and hemorrhages (H) around the blood vessels were observed.

Figures 10c and 10d correspond to single emulsion oils P, B, and F, respectively. In Fig. 10c, a section of rat skin treated with P oil shows moderate structural improvement in the epidermis (Ep) and dermis (Der), reduced epidermal thickness, and sebaceous glands (Sg) and hair follicles (HF) with mild inflammatory cell infiltration (arrow). Figs. 10d and 10e represent sections treated with B and F oils, showing a thicker epidermal layer compared to P oil, with sebaceous glands (Sg), hair follicles (HF), and dermis (Der) intact and minimal inflammatory cell infiltration (arrow).

Figures 10f and 10g depict sections treated with mixed emulsion oils P/B and P/F, respectively. Fig. 10f (P/B) reveals an almost normal structure of the epidermis (Ep) and dermis (Der), with sebaceous glands (Sg), hair follicles (HF), and minimal inflammatory cell infiltration (arrow). Similarly, Fig. 10g (P/F) shows a normal epidermis (Ep) and dermis (Der), sebaceous glands (Sg), and hair follicles (HF), along with minimal inflammatory cell infiltration (arrow). Additionally, P/F treatment exhibited more granulation tissue formation compared to P/B and all single emulsion oils.

2.7. Antibacterial activity

Antibacterial activities of dressing loaded with oils emulsion has been published [21, 22]. Textile used as substrate for different active material as biopolymer, antibiotics, nano-metals, oils and emulsions for immobilizing bio active materials [23-28]. Prepared samples were measured and displayed in Figure 11. The antibacterial assessment used is zone of inhibition. All treated nonwoven samples with oils emulsion (Fig11 b-c) showed antibacterial activity against *E. Coli and S. Aureus* compared with pristine nonwoven dressing (Fig11 a). Logically, a blank dressing lacks active components such as those present in emulsion oils. As a result, the bacteria under investigation can survive and proliferate. In contrast, dressings treated with oils emulsion contain active pharmaceutical ingredients derived from *N. sativa* seeds, including thymoquinone, thymol, limonene, carvacrol, p-cymene, alpha-pinene, 4-terpineol, longifolene, and t-anethole benzene. Peppermint oil contributes additional bioactive compounds such as saponins, flavonoids, indazole-type alkaloids, cardiac glycosides, vitamins, and essential minerals like calcium, phosphorus, and iron. Co-emulsion of peppermint (Fig 11c) and black seed showed superior results against *E. Coil* as a gram negative bacteria, while Co-emulsion of peppermint and frankincense showed superior results against *S. Aureus as a* gram positive bacteria (Fig 11d). This may be attributed to a synergistic effect between active ingredients between both oils emulsions.

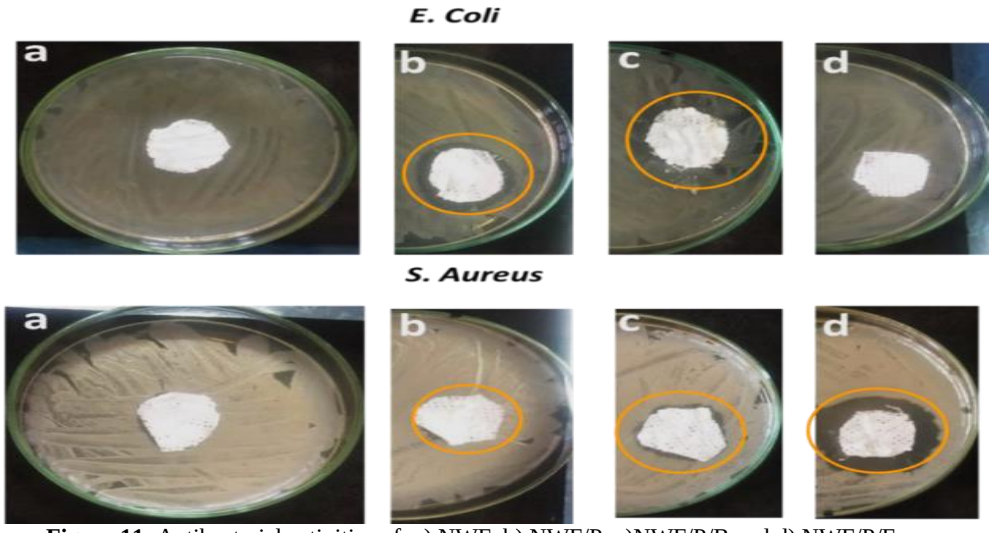


Figure 11: Antibacterial activities of: a) NWF, b) NWF/P, c)NWF/P/B and d) NWF/P/F.

Table 1: Inhibition zone (mm) after 24 hours of treated samples against E. coli and S. aureus

Sample	E. coli	S. aureus
a	0	0
b	12	11
c	13	12
d	0	15

Where: a) control, b) positive, c) P, d) B, e) F, f) P/B and g) P/F

3. Experimental

3.1. Nano-Emulsion Preparation

To formulate oil-in-water (O/W) emulsions, a solution of 2% Tween 80 (v/v) and Carbopol was prepared by dissolving them in double-distilled water at ambient temperature. The essential oils were added to the previous solution in a 1/1 ratio. The mixture was stirred for 10 minutes using magnetic stirrer to achieve a uniform liquid. Subsequently, distilled water was incrementally added drop wise at varying rates of addition. The initial crude emulsion was produced by shearing the mixture for 3 minutes at 7100 rpm using a high-speed shearing device (Sigma-Aldrich, Missouri, USA). The same procedure was repeated to prepare frankincense and black seed oils nano-emulsions. Both emulsions were further processed using an HG-15D high-pressure homogenizer (Daihan Scientific, Wonju, South Korea) to improve stability. The emulsions were then cooled in an ice water bath for 30 minutes. For Co-emulsion oils (Peppermint/ Frankincense and Peppermint/Black seed), the pH was adjusted to 7 using either 0.5 mol/L citric acid or 0.5 mol/L sodium hydroxide solution. The prepared samples were stored in tightly sealed vials for further analysis examination [29].

3.2. Preparation of spray formula

The oil spray formulation was packaged using the TS-800 Trigger Spray system from Calmar Dispensing Systems (Watchung, N.J., USA). This device is designed to spray an 8-inch diameter pattern from a distance of 8 inches when set to the spray mode. In stream mode, the spray device produces a 2-inch diameter pattern when sprayed from the same distance.

3.3. TEM measurements of the nanoemulsion's droplet size

The droplet morphology of the nano-emulsion oil was analyzed using transmission electron microscopy (TEM, Akishima, Tokyo, Japan). Samples were applied to 200-mesh formvar-coated copper grids and negatively stained with 50 µL of 1.5% (w/v) phosphotungstic acid for 10 minutes at room temperature. After a one-minute adsorption period and removal of excess liquid, the grids were examined using TEM equipped with a 20 m aperture. Images were captured using a 200 kV TEM after allowing adequate air drying of the samples [30, 31].

3.4. Particle size and Zeta potential

Particle size distribution (PS) and zeta potential (ZP) were measured using a Malvern Zetasizer analyzer (Ltd. GB) with a temperature of 25°C and humidity of 85%. The freshly prepared Nanoemulsion was diluted 50 times for the experiment. The limits for the instrument are 0.3 nm to 8.0 µm [32].

3.5. Burn model for animals

All animal studies were carried out in accordance with the National Institutes of Health (NIH) Guidelines for the handling and use of animals. The experiment was given the go-ahead by the National Research Center Committee for the Care and Use of Animals. In this study, Wistar albino rat models (males and females) weighing between 180 and 200 gm and 6 weeks of age were divided into five groups: untreated, Peppermint, black seed, frankincense, black seed with peppermint, and frankincense with peppermint. They were also exposed to adequate water and food, relative humidity of 60%, temperature of 25 °C, and 12 h L / 12 h D light cycles. The scald burn therapy was administered in accordance with a tried-and-true technique to cause a partial-thickness burn injury. The injection of urethane rendered the animal unconscious. After being touched by a 1 x 1 cm rod of aluminum that had been heated to 150 degrees Celsius, the skin experienced partial-thickness scald burns [33].

3.6. Assessment of the burn healing area

The wound healing capacity of the nanoemulsion oils was evaluated. Rats were anesthetized with pentobarbital prior to inducing the burn wounds. A wound was created on the anterior-dorsal region of each rat, with an approximate thickness and surface area of 500 mm². The test formulations were applied topically to the wounds once daily for 15 consecutive days. The primary parameters for assessing burn healing were the percentage of burn contraction and the time required for complete healing. To track changes in the wound size, the burn area was outlined on butter paper every three days. Image J software was utilized to measure the percentage of burn contraction by analysing the total burn area and the wound closure area. The following variables were used to determine how quickly the burn healed

Burn size - reduction (%) = A0– A0Ax x100

Where A burn area at day 0, Ax = burn area at day 15 of the experiment [33].

3.7. Histopathological evaluation

Skin specimens were collected from rats and preserved in a 10% buffered formalin solution for 24 hours. The samples were then dehydrated using a graded series of ethanol concentrations, cleared with xylol, and embedded in paraffin. Thin sections of the skin about 5 µm in thickness were sliced and mounted onto glass slides. These paraffin sections were stained using Hematoxylin and Eosin to screen the histological structures.

3.8. Antibacterial activity

The prepared samples were evaluated against Staphylococcus aureus ATCC 6538-P (Gram-positive), Escherichia coli ATCC 25933 (Gram-negative), Candida albicans ATCC 10231 (yeast), and Aspergillus niger NRRL-A326 (fungus), provided by the Microbiology Department, NRC, Egypt. Nutrient agar was poured into Petri dishes and allowed to solidify. The bacterial and fungal cultures were evenly spread across three separate plates. The prepared samples were carefully placed onto the solidified agar gel. The plates were then incubated at 37°C for 24 hours to assess the zones of inhibition [34].

Conclusion

Peppermint, black seed and frankincense individual and co-emulsions were sonically prepared and examined via TEM, particle size analyser, and Zeta potential techniques. The obtained emulsions showed nano-diameter sizes and stable properties against time. Produced dressing containing nano-emulsions showed superior burn healing besides more granulation tissue in their co-emulsion state especially the P/F co-emulsion. Additionally, the co-emulsions showed superior antibacterial properties.

Conflicts of interest

The authors declare that they have no conflict of interest.

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