

ANUSALVE™

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1.0 Short Summary of ANUSALVE™.

About hemorrhoids:

Haemorrhoids are clusters of veins in the anus, just under the membrane that lines the lowest part of the rectum and anus. Symptoms of haemorrhoids include fissures, fistulae, abscesses, or irritation and itching. Haemorrhoids can bleed after a bowel movement. Internal haemorrhoids are those that occur inside the rectum. (This area lacks sensitive nerve endings; internal haemorrhoids are usually not painful).

External haemorrhoids are those that occur outside of the anal verge. (They are usually painful, and are often accompanied by pruritus and an itching, swelling, and burning sensation).

Medications for treating haemorrhoids are not efficient and they include: Local anaesthetics, Vasoconstrictors, Protectants, Astringents, Antiseptics and Steroids.

Our novel product:

ANUSALVE™ is a safe and highly effective combination of 2 herbs which work synergistically together to treat the symptoms of haemorrhoids

Active Constituents.

A specific combination of *Nigella sativa* and *Citrus limonum* extracts.

Indications.

- Mild to severe haemorrhoids.

Actions.

- Astringent and vein constrictor.
- Anti-inflammatory.
- Antiseptic.
- Soothing.

Mechanism.

Natural products on the market are generally only astringent herbs, which act on one aspect of the problem. This product is a synergistic combination which is highly and uniquely effective. It combines proven anti-inflammatory effects against the symptoms of irritation, pain and itching, proven treatment of local immune reactions and vein relaxation, combined with classical astringent and antiseptic properties to reduce the swelling and tissue damage.

Research.

Antaki has carried out its own research on *Nigella* which confirmed anti-inflammatory effects via controlling immune over responses (NO release), beside the ability to restore veins contraction. There is extensive research backing the anti-inflammatory and antiseptic action of this species combined with the astringent and healing properties of the special Citrus extract.

Proprietary Position

The herbal active ingredients and herbal combination is currently undergoing the patent process. The specific uses, plant parts, varieties and processing methods are unique and proprietary.

Safety and Regulatory Position.

These plants are extremely safe for internal consumption and external use. They are classed as foods and are permitted for open sale in all countries. In addition they are on the INCI list of plants regarded as safe and permitted for topical use. Antaki has carried out a 21 day patch test of these herbal extracts at a major hospital in Israel and found no side effects or irritation whatsoever.

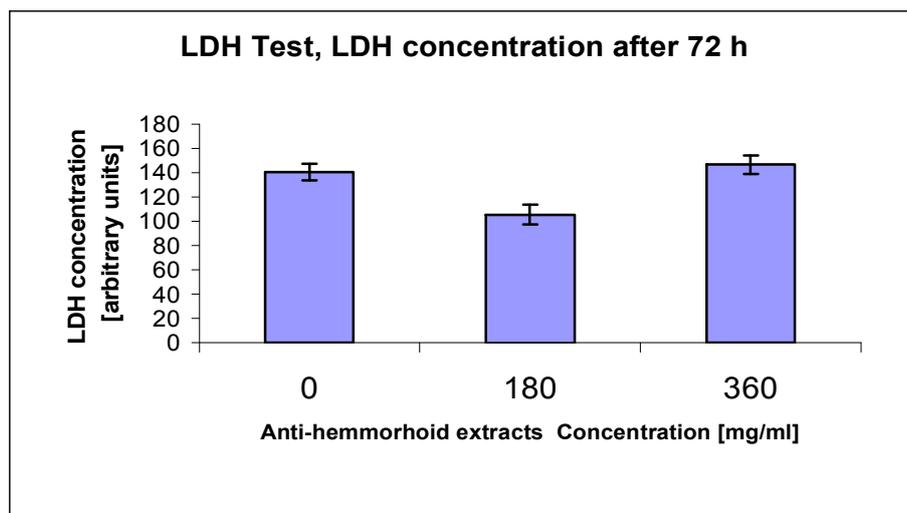
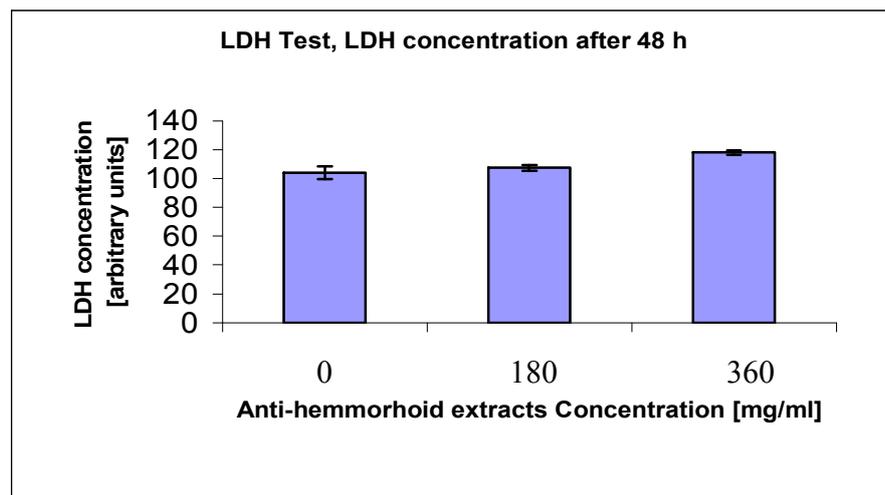
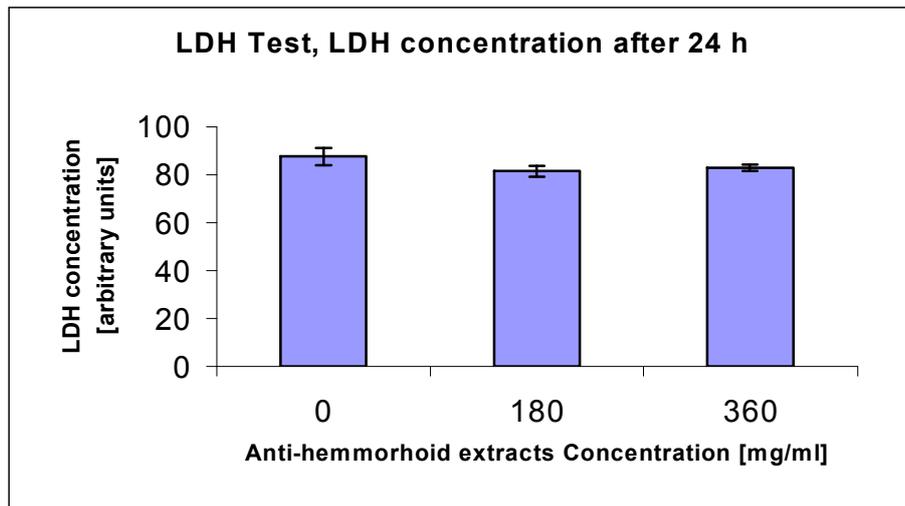
2.0 Preclinical Data.

2-a) Toxicity: LDH test

To assure the safety of the (ANUSALVE™), LDH release assay was performed. It was reported that LDH is released from the cells when plasma membrane integrity is destroyed by necrotic rupture. The integrity of the plasma membrane was determined by measuring LDH activity released into the culture medium. LDH activity was monitored following the oxidation of NADH as the decrease in absorbance at 334 nm. The reaction was carried out in a potassium phosphate buffer (40 mM K₂HPO₄, 10 mM KH₂PO₄, pH 7.5), containing 0.24 mM NADH and 0.62 mM pyruvate. The percentage of LDH released was defined as the ratio of LDH activity in the supernatant to the sum of LDH amount released plus the activity measured in the cell lysate.

Human Fibroblast cells were incubated with different amounts of extracts (ANUSALVE™) for 24h, 48h and 72h. In the end of the incubation time, the LDH activity was measured in the medium. Our results indicated that exposure of the cells to different amounts of the ANUSALVE™ extract (180 µg/ml and 360 µg/ml) did not affect LDH release from the cells as compared to controls (see the attached Figure). The basal release of LDH from control cells was not statistically different from treated cells. This assures plasma membrane integrity during incubation of the cells with the extract.

LDH test results:



The results show that cultured human fibroblast cells exposed to ANUSALVE™ extract did not exhibit any signs of toxicity nor was their growth diminished.

2. b. Anti-inflammatory effects

Nitric Oxide Determination (NO) – Test for inflammation detection in co-cultures of cells:

In vitro cell culture:

Cells: Human hepatoplastoma cell line HepG2 that retains differentiated parenchymal functions of normal hepatocytes and can be grown indefinitely, thus permitting long-term studies to be performed. The cells from HepG2 cell lines were grown in Dulbecco's modified Eagle's medium (DMEM) with a high glucose content (4.5 g/l) supplemented with 10% vol/vol inactivated fetal calf serum, 1% nonessential amino acids, 1% glutamine, 100 U/mL penicillin, and 10 µg/ml streptomycin. Human monocytes cell line THP1 and mouse macrophages cell line J774 were maintained in the same DMEM as for HepG2 cells. All three cell lines were maintained in a humidified atmosphere of 95% O₂ – 5% CO₂ at 37°C. The culture medium of the cell lines was changed twice a week. At 70 – 80% confluence, cells were trypsinized and plated in microtiter dishes. 24h after cell seeding, cells were exposed to various concentrations of the plant extracts in fresh serum-free medium.

In vitro cell culture: In the next phase of our experiments, co-cultures of hepatocytes and macrophages were created using three-dimensional foam structures (DegraPol-foam) as cell carriers (Saad et al., 2003). Both, hepatocytes and macrophages were found to adhere, proliferate and preserve their specific phenotype when cultured on DegraPol-foam. Cells from the hepatocyte cell line HepG2, from the macrophage cell line J774, and from the human monocyte cell line THP-1 were used. The viability of the cells was assessed by the trypan blue exclusion test and cells with more than 85% viability was used.

The *In vitro* test was performed as follows:

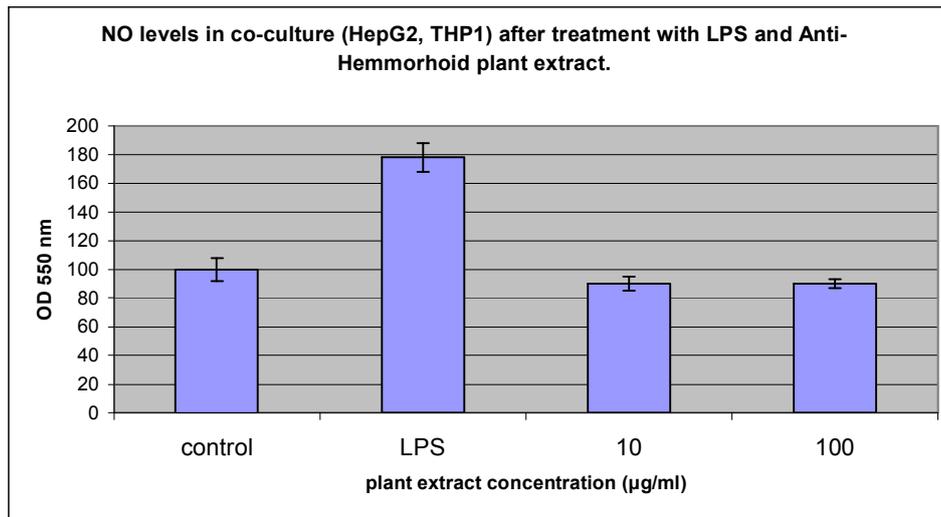
(1) Collagen type I coated DegraPol-foam discs of 14 mm diameter and 300 µm in thickness was placed in the bottom of each well of a 6-well tissue-culture plate. (2) Hepatocytes and macrophages/monocytes were seeded on DegraPol-foam at a density of 3×10^6 cells/cm² and 5×10^5 cells/cm² in 1 ml culture medium, respectively. (3) Co-cultures were maintained at 37°C and 5% CO₂ for 4h. (4) Five ml of fresh medium were added. (5) At 24h, the medium was exchanged with the same culture medium containing 10 µg Lipopolysaccharide/ml and various concentrations of potential plant extracts diluted with fresh medium. (6) After 24h and 48h of treatment, NO measurement was carried out as follow.

Nitric Oxide Determination (NO) – Test for inflammation detection in co-cultures of cells:

Nitrite determination was done on 50 µl aliquots of sample mixed with 200 µl of the Griess reagent (Ding et al., 1988). The absorbance was read at

540nm after 10 min of reaction and NO₂⁻ concentration was determined with reference to a standard curve using concentrations from 1 to 250 µM sodium nitrite in culture medium.

LPS-induced NO production by the Hepatocytes and/or monocytes from the THP-1 cell line.



The results shown above indicate a significant anti-inflammatory effect of ANUSALVE™ extract

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2. c. Anti-microbial test.

Disk Diffusion Method

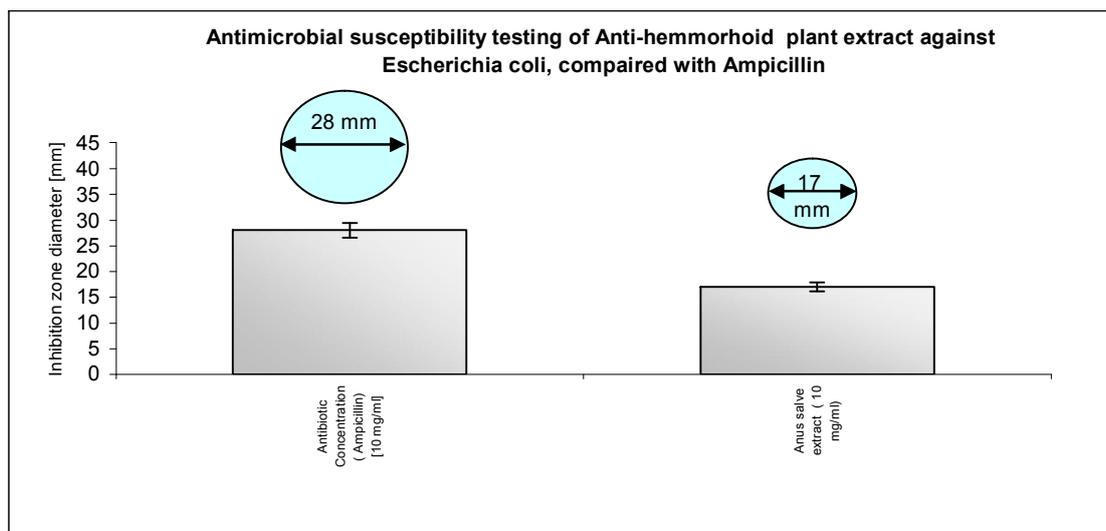
Application of Extracts on Sterile Disks: Disks of 6 mm diameter were prepared from Whatman filter paper no.1, placed in glass petridishes and autoclaved for 15 min. Twenty-five microliters of the required extract were added to each sterile disk, and disks were dried under a laminar flow sterile bench. The final content of each disk was 5 mg of extract.

Preparation of inocula:

Part of an isolated bacterial colony was transferred into a 5-ml Muller-Hinton broth (MHB) tube for aerobic bacteria, or into 25 ml reinforced clostridium medium (RCM) for *Propionibacterium acnes*, and the tube was incubated for 4-18 hours (for aerobic bacteria), or incubated anaerobically in Gaspak jars for 48 hours, at 37 °C. The growth turbidity in the broth was adjusted by further incubation or dilution with sterile physiological saline, after comparison with that of a MacFarland nephelometer tube no. 0.5 (10^8 cfu/ml) using a spectrophotometer at 625 nm (optical density 0.08-0.1). An inoculum of 10^6 cfu/ml of bacterial suspension was prepared by diluting 0.1 ml of the prepared bacterial broth culture with 9.9 ml sterile saline.

Susceptibility Test

Using a sterile cotton applicator, 10^8 cfu / ml of bacterial suspension was swabbed on the surface of Muller-Hinton agar (MHA) plates for aerobic bacteria, or blood agar (with sheep blood 5-7%) (BASB) medium plates. The selected extract discs (mg/disc) were then distributed evenly on the surface of the seeded agar plate. The specific reference antibiotics discs (10 mg/disc) were placed onto the agar plate beside the extract discs. Three replicate plates were used for each test. The MHA plates were incubated upside down at 37 °C for 18 hours. The BASB plates were incubated upside down anaerobically in Gaspak jars at 37 °C for 48 hours. The inhibition zone around each disc was then measured using transparent ruler.



Anti-hemorrhoid extract showed *In vitro* antibacterial activity against *Escherichia coli* 60% from the reference antibiotic (Ampicillin). (The extract inhibited the growth of the bacteria 60% compared with the reference antibiotic.) In this test we used the bacteria of *Escherichia coli* (Gram positive) nonpathogenic, using Disk Diffusion Method.

2. d. Vasoconstriction effect of ANUSALVE™.

The study was conducted by the Regional Research and Development Center – The Galilee Society, Israel, under the supervision of Dr. Omar Said

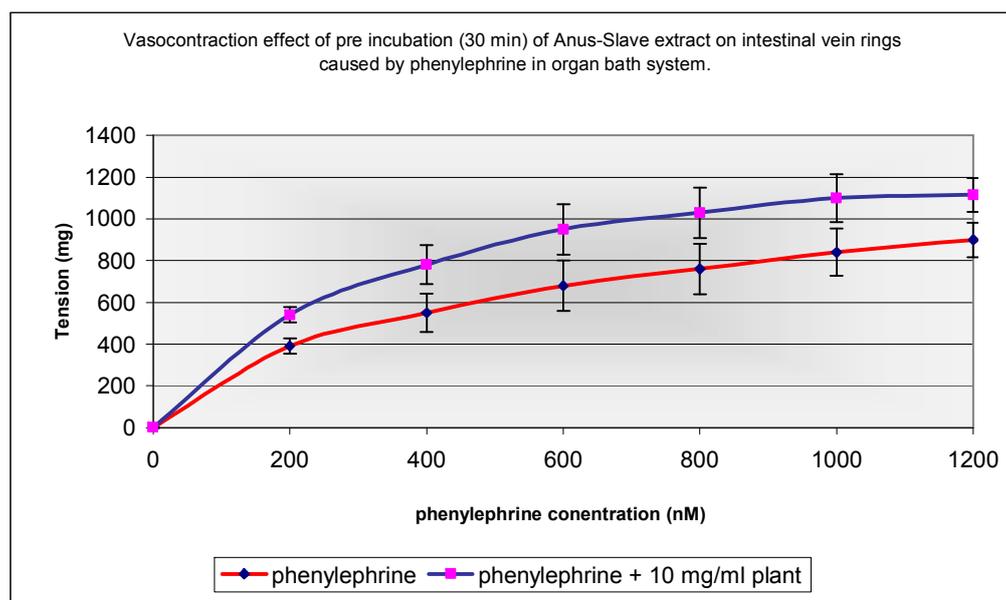
Each result represents an average of 6 repeated experiments

Blood vessels were harvested from Sprague-Dawley male rats. Anaesthesia was induced by subcutaneous injection of 10% chloral hydrate, intestine vein was dissected and rapidly immersed in Krebs-Henseleit solution and all conjunctive and adipose tissues were removed. Intestine vein rings were obtained and each ring was suspended by a fine steel wire and connected to isometric tension transducer

(Myograph F60). The transducer was connected to a Narco Trace 40 polygraph (Narco-Bio-Systems Inc., Texas, USA). The rings were maintained in 15 ml Krebs-Henseleit solution at 37°C bubbled with 95 % O₂ and 5% CO₂. A tension of 500 mg was maintained during an equilibrium period of 60 minutes during which the bathing medium was changed every 20 minutes. The rings were exposed to accumulative concentrations of phenylephrine with the presence and absence of 10mg/ml of ANUSALVE™ extract.

References:

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The results shown in the graph indicate a significant Vasoconstriction effect of ANUSALVE™ extract which can explain its ability to restore vein swelling during inflammatory conditions.

3.0 Clinical Data.

This study was carried by Antaki Center on 20 patients (Ages 25-58) in the northern region of Israel in the years 2002-3. The test period for patients ranged from one - three weeks.

Results: Ten patients (internal haemorrhoid) reported that the haemorrhoid symptoms disappeared after a week of using the cream.

Three patients (internal) reported that the haemorrhoid symptoms disappeared after two weeks.

Four patients felt significant improvements, however reported that the size of haemorrhoid minimized but did not disappear totally. (The four patients had external and internal haemorrhoid).

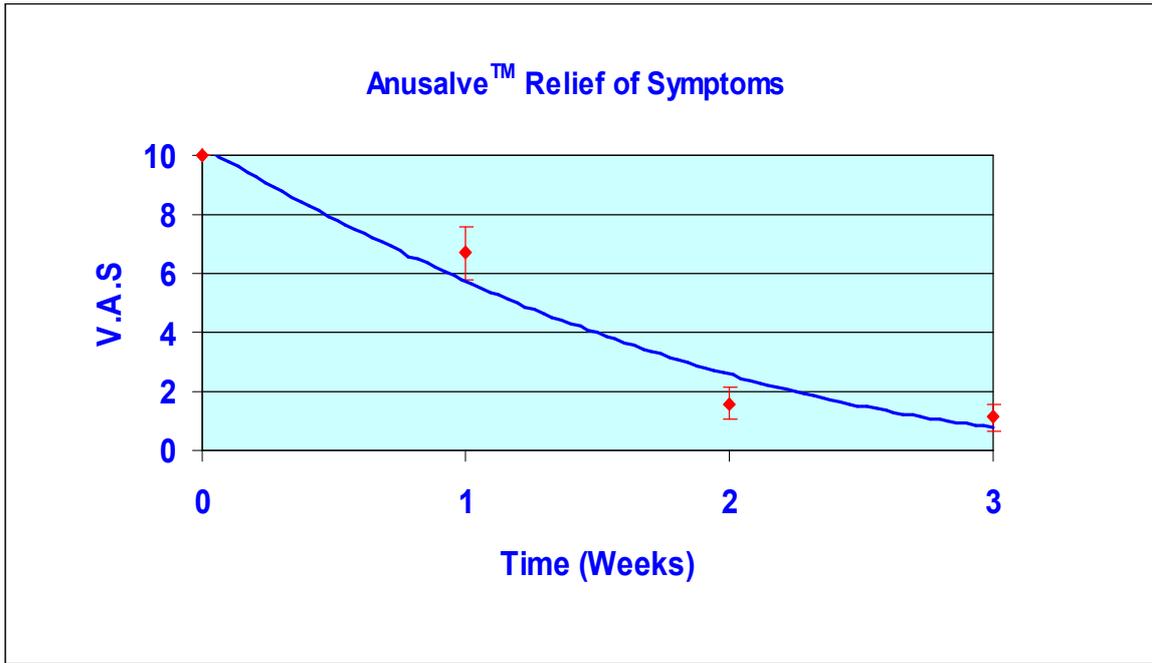
Three patients (External haemorrhoid) reported that the cream improved little bit their situation but they continued to suffer from the same symptoms before use.

Three patients supposed to be operated, however cancelled the operation after using the cream successfully.

Data.

(Scale: 0 - no improvement, 10 - complete recovery)

Patient No	Age/s ex	Severity before treatment	Improvement first week	Improvement second week	Improvement third week	Period of illness
1	38/M	Intermediate (4-5)	10	10	10	1 year
2	58M	Severe (0-2)	2	5	5	3 years
3	36F	Severe	5	7	9	8 months
4	45F	Intermediate	7	10	10	4 years
5	30M	Intermediate	10	10	10	9 months
6	43F	Intermediate	10	10	10	1 year
7	28M	Intermediate	7	10	10	10 months
8	33M	Severe	4	6	8	6 years
9	25M	Severe	2	4	4	4 years
10	37F	Intermediate	10	10	10	5 months
11	34M	Light (6-7)	10	10	10	2 years
12	29F	Intermediate	10	10	10	2 years
13	41F	Severe	2	4	5	7 years
14	44M	Severe	4	6	8	6 years
15	53M	Intermediate	10	10	10	3 years
16	49F	Intermediate	10	10	10	2 years
17	32F	Light	10	10	10	10 months
18	27M	Light	10	10	10	1 year
19	48M	Severe	5	6	8	3 years
20	35F	Intermediate	8	10	10	15 months



- Note: "10" Heavy symptoms, "0" No symptoms

4.0 Opinion and Survey; monographs

By Dr. Stephen Fulder, Phd.

User's direction:

apply in anal area twice a day.

INGREDIENTS	%
<i>Aqua</i>	To 100
<i>Isopropyl myristate</i>	9.0
<i>Cetyl alcohol</i>	6.0
<i>Nigella Sativa</i>	5.0
<i>Lanolin alcohol</i>	4.8
<i>Cera alba</i>	4.8
<i>Cetyl palmitate</i>	3.5
<i>Glyceryl stearate</i>	3.0
<i>Sodium lauryl sulfate</i>	0.5
<i>Citrus limonum</i>	1.5
<i>Phenoxyethanol</i>	0.7
<i>Imadazolidinyl urea</i>	0.2
BHT	0.15

Nigella sativa L. (Black Seed, Black Cumin) Seed oil.

Nigella, or black cumin, is well known as a spice and folk medicine throughout the Middle East and Asia. It bears no botanical or other relationship to cumin seed. It is highly valuable medicinal oil in India where it is known as Kalonji, and where it is used in against pain, inflammation, irritation, and internally to treat a variety of inflammatory diseases.

Constituents.

The major active components of the oil are quinones, including thymoquinone and its derivatives such as nigellone(I). In addition, the oil contains essential oil phenols including carvacrol, t-anethole, and 4-terpineol. The oil is also unusually rich in a variety of fatty acids and phospholipids(II).

Traditional Use.

Traditional herb books describe the plant as helping against inflammation, causing sweating and helping the menstruation process. The seeds are used to aid digestion, and to support the liver. In Indian medicine there is a mention of *Nigella sativa* but only as a complex mixture with other plants, in skin treatments such as eczema and pityriasis, which is a condition of flakiness of the skin, like dandruff (III). The oil has long been known in folk medicine to provide symptomatic relief for skin inflammations, which is why it can be bought today as soap and in various folk preparations of the seeds.

Research.

Black seeds are known to have a distinct anti-inflammatory effect, which would tend to make it symptomatic for all kinds of internal and external inflammations, such as eczema. The effect is not general, on all aspects of the immune response, but tends to be on macrophage rather than lymphocyte stimulation (IV). The anti-inflammatory active ingredients are known, and are regarded as thymoquinone (V), as well as derivatives and relatives of this compound (VI). These compounds are found in the oily fraction. The oil is similar to other omega 3 oils in its anti-inflammatory effect.

An interesting patent has been filed which does indicate that inflammatory skin conditions can be treated by ingesting large quantities of oil internally in a manner similar to for example evening primrose oil (VII). The anti-inflammatory effect is of obvious importance in the treatment of haemorrhoids. In addition, the seed oil has an antinociceptive (pain-relieving) action, which appears to be via kappa opioid pain receptors (VIII). Like many anti-inflammatory herbs, the anti-inflammatory action is accompanied by strong anti-oxidant effects in the oil phase. This too contributes to reduced irritation on local application (IX). Another area that has been researched is in cancer prevention. The methanol extract has been found to be cytotoxic to a number of types of tumour cells in tissue culture (X).

There are several studies showing that *Nigella* has an anticarcinogenic effect when given alongside, before or after chemical carcinogens (XI, XII). There is evidence too that *Nigella* has an attenuating effect on the toxic adverse effects of anticancer drugs, and therefore has been suggested as an adjuvant during cancer treatment (XIII). Besides this, *Nigella* has antibacterial effects, which may be helpful in the treatment of haemorrhoids, to ensure antiseptic action in the anal region (XIV). There are also useful antihepatotoxic and liver – supporting effects which can help overall health, if the oil is consumed internally (XV). An important new review of this plant has confirmed potent anti-inflammatory, anti-oxidant, hepatoprotective, antimicrobial and some immune supporting effects, a range similar to that found with sources of omega-3 oils (XVI).

Nigella Sativa monograph

The final product undergoes the following tests

Assay – Linoleic acid , additional internal peaks

Foreign matter EP (2.8.2)

Microbiology EP 5.14 4-B - Maximum 10^4

Relative density EP (2.2.5)

Ethanol EP (2.9.10)

Methanol and Propanol EP (2.8.16)

Analytical Procedures

The extract was analyzed by HPLC and Spectroscopy for some of the peaks

Reagents: Acetonitrile: Buffer Phosphate 9:1

Buffer phosphate: - Phosphoric acid Analytical grade: Water distilled 2: 1000. All reagents used – HPLC grade.

Sample Preparation: - 100 grams milled plant was extracted with 300 ml Ethyl alcohol 50% for 1.5 hours at a temperature of 70 °C. Then filtered through a fine filter. The residue was again extracted using another 300 ml Ethyl alcohol 50% and filtered.

The filtrates were mixed together for HPLC analysis.

Standard Preparation: Linoleic acid from Fluka 62230 - 3mg/ml

Chromatographic Conditions

HPLC type: HP 1090 Diode array

Column: Phenomenex Luna C18 ; 250 X 4.6 mm

Column Temperature: 40 °C

Mobile Phase: isocratic

Acetonitrile: Buffer Phosphate 9:1

Flow Rate: 1.0 ml/min

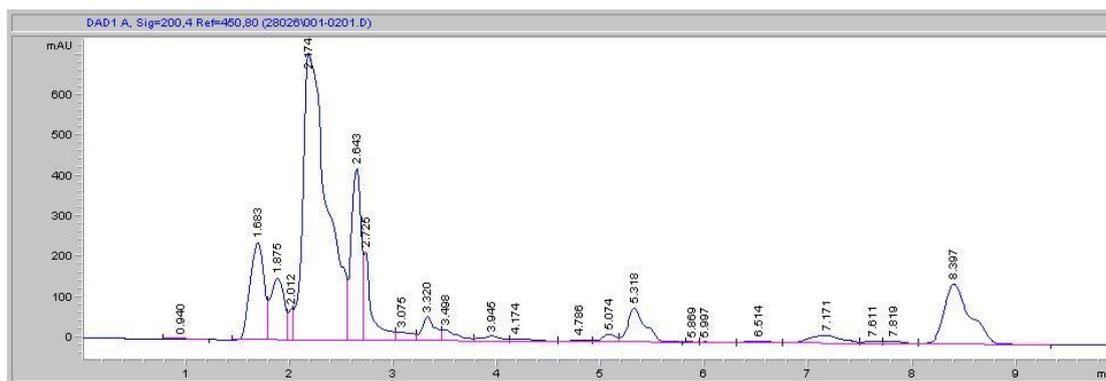
Detection: 210 nm and Diode array

Injection Volume: 5µl.

Run Time: 10 minutes

Peaks Identification: Retention time and UV/ Vis spectrum

Discussion of the Chromatogram(s)



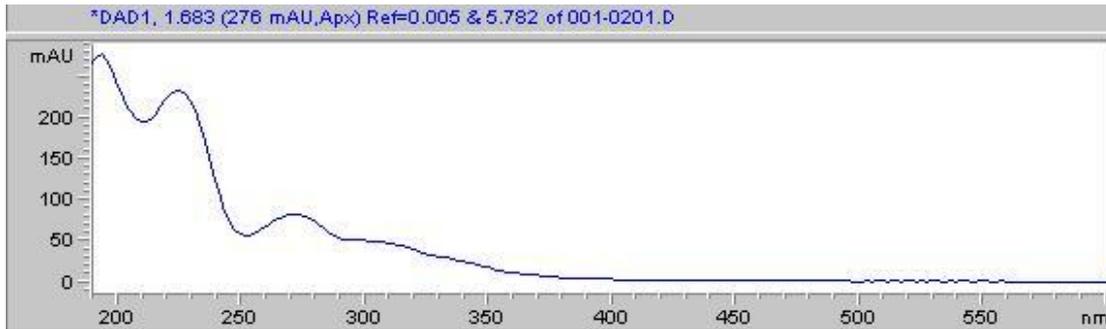
The peaks to be used as a finger prints are:

<u>Retention time</u>
1.68
2.17
3.94
8.38

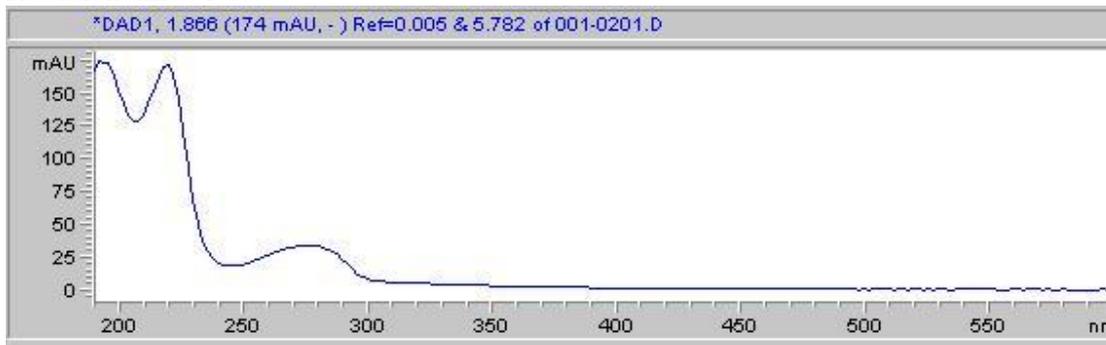
Spectrophotometric Assay.

UV Vis spectrums of main peaks

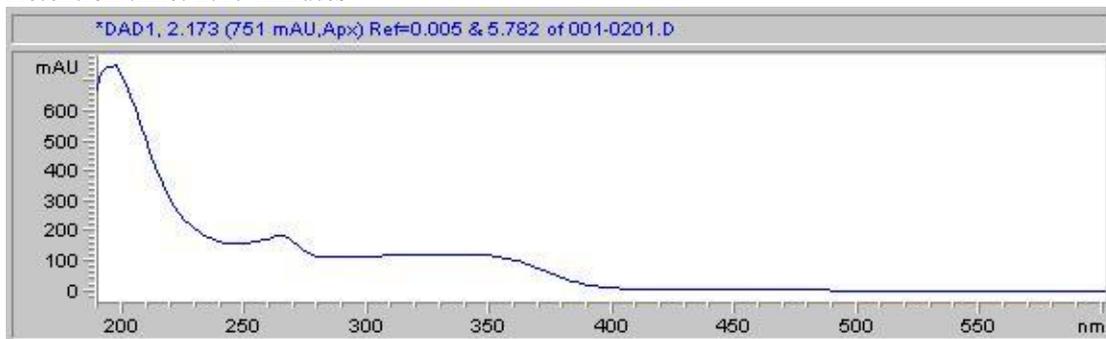
Retention time: 1.68 minutes



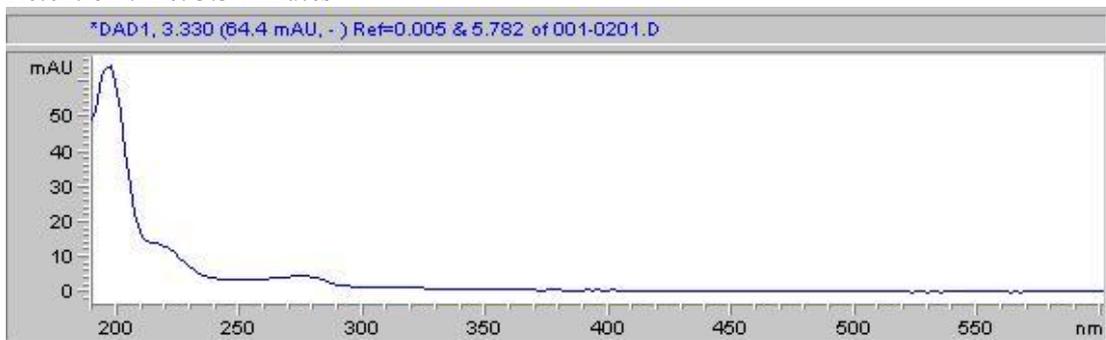
Retention time : 1.86



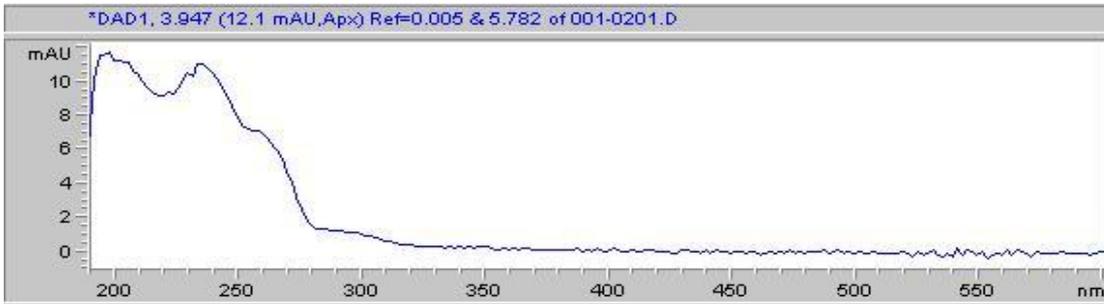
Retention time: 2.17 minutes



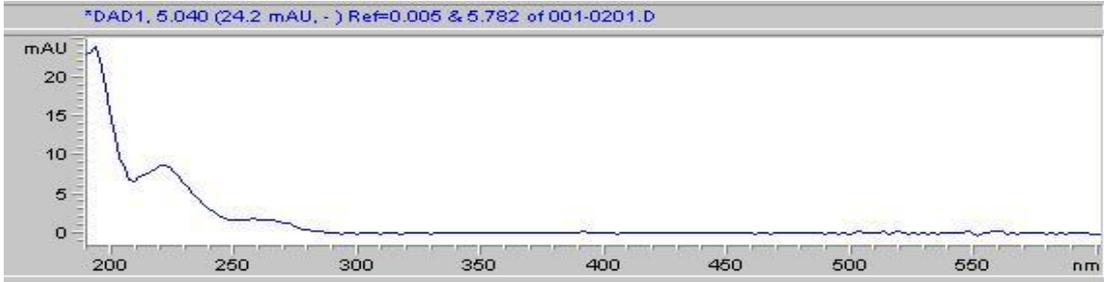
Retention time: 3.3 minutes



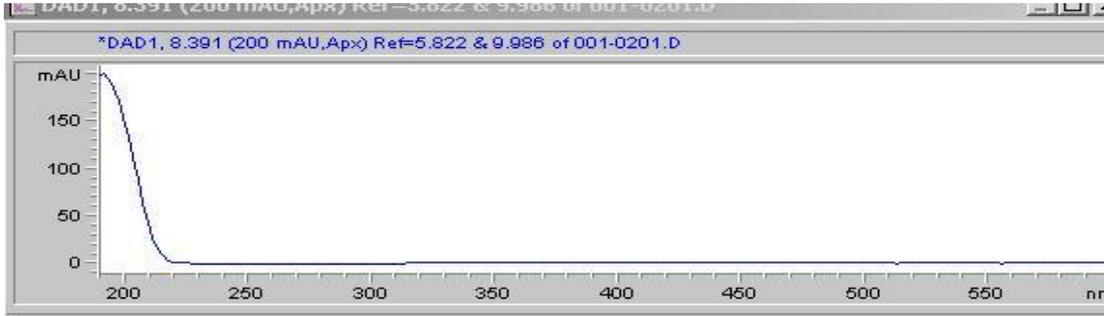
Retention time: 3.94 minutes



Retention time: 5.04 minutes

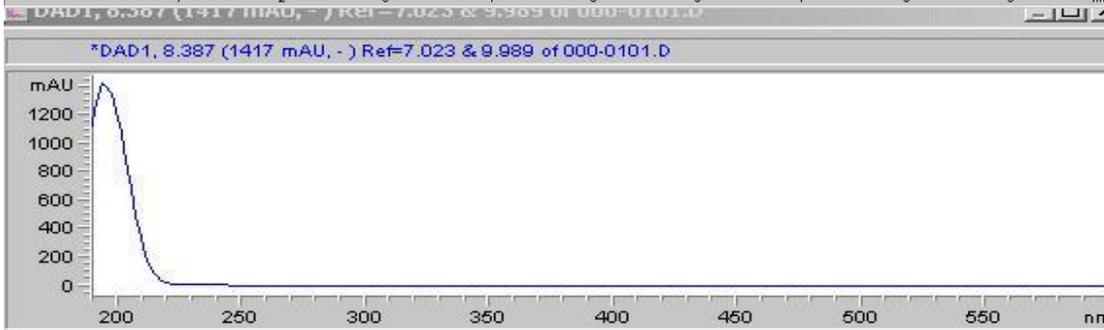
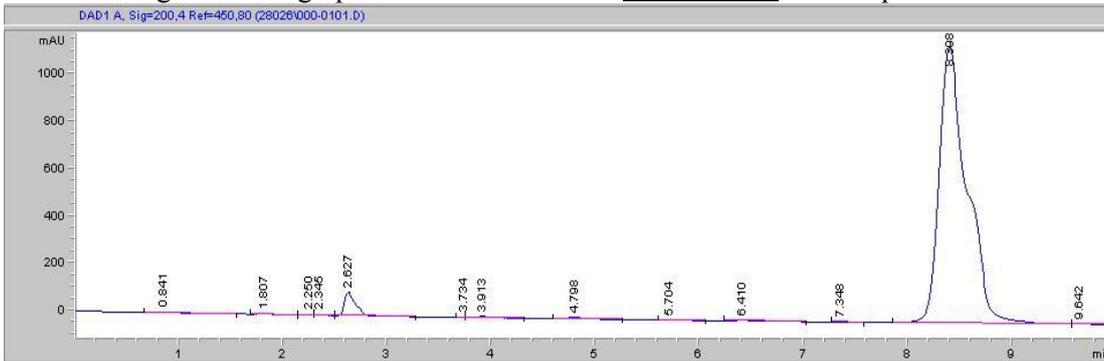


Retention time: 8.38 minutes



Further the peak at 8.38 minutes was identified as Linoleic acid

The following chromatographs shows the standard *Linoleic acid* and its spectrum.



Citrus medica, Citrus limonum (Citrus) Dried fruit extract.

This is a variety of dried lemon or lime, derived from Iran, and available throughout the Arabic countries. It can be regarded as similar to the lemon in biological and therapeutic effects. However the preparation used here of the dried fruit is equivalent to dried lemon peel, which is rather different to the traditional use of lemon juice, oil or rind of fresh lemon. Lemon is one of the most Universally used of medicinal foods.

Constituents.

The dried peel contains some essential oils of lemon, such as limonene, cineole, beta and gamma pinene, terpinene, and citral, and is rich in flavonoids, such as hesperidin, as well as phenolic fruit acids such as ascorbic and coumarinic acid. It also contains pectin.

Traditional Use.

Herbal sources list a very large number of uses of lemon, almost all of them internal. Internally it is used to reduce stomach and systemic acidity, and to help stimulate the liver and remove bile as well as small stones from the bile gland. It has a healing antiseptic property, which can stop bacterial growth. Externally, it has cosmetic uses, including assistance in removing spots and cellulitis, and can help in skin whitening. It is regarded as a soothing antioxidant and anti-inflammatory remedy that can be used in sunburn and itching. It has a strong astringent effect, which makes it useful in problems like acne and hemorrhoids. It can also combat oily skin, inflammation and external irritations.

Research.

Lemon and all the citrus family contain citrus flavonoids in the peels, which can have anti-inflammatory, cancer-preventive and antihistamine effects. These include, for example, stimulation by auraptene, a flavonoid of one of the orange species, of a number of immunological factors including macrophage and lymphocyte functions (XII). Such effects could in a general way reduce the inflammatory symptoms of any inflammatory skin condition, including haemorrhoids (XVIII). There is a study, for example of the treatment of psoriasis, a typical inflammatory skin condition involving irritation similar to haemorrhoids, by juice of grapefruits (XIX). However this is relatively distant from lemon. There is evidence that citrus peel, oil, etc. contains antioxidants that could explain some of the relief in such cases (XX). Lemon oil in particular seems to have a strong anti-oxidant effect (XXI). Citrus species in general all seem to have components that can have cytostatic or antiproliferative effects on cells in culture (XXII). *Citrus limonum* has antifungal and bacteriostatic effects which allow its use in skin treatment of fungal and other infections (XXIII).

Opinion.

The two herbs comprising this mixture are all well-known and safe herbs, in wide current use today. Their traditional usages indicate that both have anti-inflammatory effects and the ability to treat inflammation and irritation. Strong antioxidant effects are also present in both cases. The astringent effects of lemon complement well the anti-inflammatory effects of black seed. Thus the combination seems rational for the intended purpose.

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