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Original Article

Scientific Validation of Traditional Detoxification Process and Evaluation of its Impact on Anti-Microbial Potency, Phytochemical and Heavy Metals in *Nigella sativa*

Kalkatanur Ganesan Vanitha[1], Anbu Madheshwar Rajha Viknesh[1], Tahani Awad Alahmadi[2], Saleh H. Salmen[3], Natesan Sudhakar[4,*]

[1]Department of Microbiology, Muthayammal College of Arts and Science, Namakkal, Tamil Nadu, INDIA.
[2]Department of Pediatrics, College of Medicine and King Khalid University Hospital, King Saud University, Medical City, Riyadh, SAUDI ARABIA.
[3]Department of Botany and Microbiology, College of Science, King Saud University, Riyadh, SAUDI ARABIA.
[4]Department of Microbiology, Muthayammal College of Arts and Science, Namakkal, Tamil Nadu, INDIA.

ABSTRACT

Background: Medicinal plants are the best source for a variety of drugs. In traditional practices, the detoxification method is practiced to reduce toxicity of herbs, because low toxicity is one of the characteristics of phytomedicine which have a positive impact on pharmaceutical drugs. **Aim:** In the present study scientific validation attempt has been made to reduce toxicity by performing traditional practices called Sodhana/detoxification. In our study anti-microbial, phytochemical, heavy metal content and stability were evaluated for the detoxified seeds. Seeds of *Nigella sativa* were subjected to various detoxification methods like roasting, lime and calcium chloride treatment then ground to powder and extracted with 90% ethanol. **Materials and Methods:** The anti-microbial study was performed against multidrug-resistant bacterial strains *Escherichia coli, Pseudomonas aeroginosa, Staphylococcus aureus* and tested for their antifungal activity against *Aspergillus niger, Penicillium crysoginum* and *Malassezia furfur*. Metals present in the extract are analyzed by using plasma-optical emission spectrometry and Mass spectrum analysis was done for compound analysis. **Results:** Our experimental results reveal that Lime treated ethanol extract shows strong inhibitory activity against all the tested microbes than the unprocessed *N. sativa* extract except *E.coli*. Data reveals that the treatment of seeds has no impact on their major phytochemical; however, changes in metal concentration were recorded. The frequency of reduction by descending order was Lime≥$CaCl_2$≥ roasted. Moreover, the Stability of the extract followed by 12-month storage at room temperature showed significant anti-microbial activity like fresh extract. UPLC-MS/MS spectrum reveals that lime treated contained high levels of fatty acids. **Conclusion:** The present study concludes that, after detoxification, the heavy metal content was found to be decreased. Lime treatment is identified as a better method that successfully reduces the toxic elements and possesses high levels of fatty acids and phytochemicals with potent anti-microbial activity and revealed significant improvement in its anti-microbial potency.

Keywords: *Nigella sativa*, Lime treatment, Detoxification, Anti-microbial activity, Metal analysis, Heavy Metal Toxicity, Stability.

Correspondence:
Dr. Natesan Sudhakar
Department of Microbiology, Muthayammal College of Arts and Science, Namakkal, Tamil Nadu, INDIA.
Email: sudhakar.phd@gmail.com

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INTRODUCTION

Scientists are challenged to develop fresh concepts for alternative and revolutionary drugs as technology advances.[1] In ancient medical systems like Unani and Ayurveda, *Nigella sativa* (*N. sativa*) (Ranunculaceae family) is one of the most often utilized medicinal herb globally.[2] The seeds of *N. sativa* are pickling, aromatic, spicy and bitter.[3] Additionally, it is a good source of many vitamins and minerals, including Cu, P, Zn and Fe. The chemical components of *N. sativa* include thymoquinone, flavonoids, anthocyanins, alkaloids and significant fatty acids, especially linoleic and oleic acid. Additionally, the seeds contain carotene, which the liver transforms into vitamin A. In recent years, *N. sativa* seeds and their oil have been widely investigated and reported to exhibit several pharmacological effects such as analgesic, anti-inflammatory anti-bacterial and anti-cancer. An external application of the oil acts as a local anaesthetic and antiseptic. Roughly roasted black seeds are consumed to stop the vomiting.[4] The harmful toxic compounds are reduced and their suitability for the intended actions is improved by Kalaskar 2018.[5] with various detoxification techniques, including washing,

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crushing, boiling, frying, heating, or dipping in specified Liquids (media).[6,7]

In human life, heavy or trace metals may exhibit more detrimental impacts than positive impacts. Therefore, the consumption of herbal items made from medicinal plants that have been contaminated with heavy metals may be harmful to human health. Herbs may be contaminated during growing, harvesting and processing. Sources of heavy metal contamination in herbs could be linked to water used during irrigation, polluted soil, fertilizers and pesticides, industrial emissions, transportation and harvesting and storage processes.[8-10] However, toxicants have a significant negative impact on all living things.[11] Some metals and their constituents may cause cancer.[12,13] The International Agency for Research on Cancer (IARC) categorized arsenic, chromium, cadmium and nickel, as well as their compounds, as carcinogenic in 2012 (IARC).[1] A few numbers of metals, such as lead, cadmium and mercury, are deadly even in minute quantities.

Detoxification is an ancient practice that has been used for centuries to reduce the toxicity of herbals. In this study, we will be focusing on how traditional detoxification processes can help to reduce heavy metal toxicity. We will be looking at the scientific evidence behind these processes by evaluating anti-microbial potency, bioactive phytocompounds and how purification helps in reducing the heavy metals present. We will also be looking at the impact of the changes on the stability of the processed seeds. According to prior research, determining a new product's storage life is crucial for spotting potential storage issues and learning about ideal storage conditions to maintain the product's quality.[14] Bioactivity-based standardization is a simple approach for measuring the self-life potency of herbal drugs.[15]

The results of this study will be used to determine the best practices for handling the detoxification process and storing the processed seeds to ensure their safety and effectiveness.

MATERIALS AND METHODS

Processing of raw *N. sativa* seed

N. sativa seeds are obtained from Tamil Nadu Agricultural University (TNAU), Coimbatore, India. They were cleaned in distilled water to remove any dust and then dried in the shade and ground to a fine powder with homogenizer.

Purification Process of *N. sativa* seeds

Roasting: 50 g of *N. sativa* seeds were roasted in an oven at 100°C for 5 min, after which they were finely powdered.

Lime and calcium chloride-treatment

150 mL of distilled water was used to dissolve 30 g of each of the calcium carbonate (limestone) and calcium chloride ($CaCl_2$) (AR grade). 25 g of *N. sativa* seeds were steeped for 3.5 days in 100 mL of solution in a separate beaker. Seed is separated from the solution using Whatman filter paper, rinsed with distilled water and then allowed to dry at room temperature. Extraction Method unprocessed plain and processed (roasted, lime and calcium chloride) *N. sativa* powder is used (25 g of pulverized) for extraction with 150 mL of solvent took place over 7 hr. The solvent was vaporized and the extract was concentrated in a vacuum in a rotary evaporator (40°C, 110 RPM). Finally, the extract (about 12 g) was collected.

Microbial Cultures

All the microbial cultures used in our study were purchased from the Microbial Type Culture Collection (MTCC), Chandigarh-160036, INDIA. The purchased bacterial strains include *Staphylococcus aureus* (MTCC96), *Escherichia coli* (MTCC1687), *Pseudomonas aeruginosa* (MTCC424) and the multi-drug-resistant cultures of *Escherichia coli* (MTCC443) and *Pseudomonas aeruginosa* (MTCC2453). The fungal cultures purchased include *Malassezia furfur* (MTCC1765), *Aspergillus niger* (MTCC514) and *Penicillium crysoginum* (MTCC161).

Anti- Microbial Efficacy

By using the well diffusion technique, the anti-microbial activity of *N. sativa* powder and the obtained unprocessed *N. sativa* powder extracted with 90% ethanol, n-hexane, hydro extract.[16,17] Based on the results, 90% ethanol is selected for extraction of *N. sativa* seeds processed with plain, roasted, lime and calcium chloride treated were analyzed for their anti-microbial efficacy by well diffusion method.[18]

Inoculum preparation

Bacterial colonies were inoculated in Mueller Hinton broth and incubated for 24 hr at 37° C to prepare the inoculums for antibacterial susceptibility testing. Five or six loops full of fungal spores are transferred to sterile Mueller Hinton broth and incubated at 25°C for 24 hr.[19]

Disc Used for Agar Diffusion

HIMEDIA Anti-bacterial antibiotic disc (narrow-spectrum) Clindamycin (CD) 10 mcg, Erythromycin (E) 10 mcg and broad-spectrum antibiotics Tetracycline (TE) 10 mcg, Chloramphenicol (C) 10 mcg were used for the study. Anti-fungal antibiotics such as Nystatin (NS) 50 mcg, Fluconazole (FLC) 10 mcg, Ketoconazole (KT) 10 mcg, Amphotericin-B (AP) 100 mcg were used.[20]

Agar Diffusion Method

The well diffusion and disc diffusion methods are the most well-known and fundamental techniques for anti-microbial study. It is common practice to utilize the Agar well diffusion technique to assess the anti-microbial properties of herbal extracts.[21,22] The agar plate surface is inoculated using a process similar to the disk-diffusion protocol, which involves distributing a volume of

the microbial inoculum over the entire agar surface. After that, the well was aseptically punched with a diameter of 6 mm, and then a volume 20-100 µL of the unprocessed and processed *N. sativa* extracts was added to the well. Then, depending on the test microorganism, agar plates are incubated under the proper conditions. The diameters of the inhibitory growth zones are measured. The inhibition zone of bacterial cultures was compared with narrow and broad-spectrum antibiotic readymade discs and fungal cultures were compared with cutaneous and subcutaneous readymade antibiotic discs.[21]

Phytochemical analysis

Extracts prepared from detoxified *N. sativa* seeds were subjected to qualitative phytochemical analysis for quinines, anthraquinones, Phenol, tannin, flavonoids, triterpenoids, protein, alkaloids, saponins, glycosides and sugar tests using established methodologies.[23,24]

ICP/OES- Analytical Procedure

0.5 g of dried, unprocessed and processed *N. sativa* samples were digested with HNO_3 acid in a confined atmosphere.[25] The volume was filled by adding the filtered digested samples, 100 mL of deionized water and 5% HNO_3. We used Perkin-Elmer 3100 Inductively Coupled Plasma-Optical Emission Spectrometer (ICP/OES) (Norwalk, USA) equipped with an autosampler AS 91 and a Gem Cone nebulizer on a cyclonic spray chamber. The digested samples, blanks and reference materials were examined using ICP-OES. The measurements of element concentrations were confirmed using the certified values of the pertinent minerals in the reference samples. By analyzing Standard Reference Materials (SRM), the relevant analytical technique, ICP/OES, was confirmed (NIST 1570a). Element concentrations in samples mineralized by the mixture of HNO_3 and HBF_4 in a closed system were assessed using microwave radiation. We performed two digestions on each sample. Each diluted digestion solution was subjected to two trace element tests by ICP/OES, yielding a total of four analytical results for each sample that are agreed within a 5% uncertainty range.

UPLC-MS/MS analyses

Following UPLC separation, high-resolution mass was evaluated for metabolites. Chromatographic separations were carried out using an HSS T3 column and an Acquity UPLC system from Waters. The solvent system used acetonitrile 5% and water 95% while applying a gradient over time at a flow rate of 0.2 mL/min and a column temperature of 25°C. A total of 30 min were allotted for the run, with an injection volume of 5 µL. The AMDIS software was used to help with neighbouring peak deconvolution and background subtraction after UPLC-MS files were converted to NetCDF (network Common Data Form) file format using the File Converter function in the Bruker Daltoniks software. The UV-vis spectra of the metabolites (220-600 nm), retention periods in comparison to outside standards, mass spectra and comparisons to our internal database, the phytochemical dictionary of natural products database and reference material were used to characterize the metabolites.

Stability check of the stored sample

Following by 12-month storage of samples, a well diffusion technique was used to assess the anti-microbial efficacy of detoxified seed extracts compared with fresh extracts anti-microbial potency. The differences in the zone of inhibition among the samples were analyzed by one-way ANOVA.

RESULTS

Our results reveal significant improvement in anti-microbial efficacy by lime-treated ethanol extract against bacterial and fungal pathogens except against *E.coli*. The maximum antibacterial effect was observed in lime-treated extract which showed the inhibition zone recorded as 20≥24≥24 mm against *E. coli, P. aeruginosa* and *S. aureus* followed by unprocessed *N. sativa* ethanol extract. *E.coli* was highly sensitive to Calcium chloride-treated extract (25 mm) (Table 1, Figure 1a). A significant antifungal effect was identified on lime-treated extract which showed the inhibition zone was recorded as 21≥25≥22 mm against *A. niger, P. crysoginum* and *M. furfur* followed by unprocessed *N. sativa* ethanol extract. (Table 2, Figure 1b).

The analysis of 26 elements by ICP-OES reveals that comparison between unprocessed and detoxification processed *N. sativa* seeds ethanol extracts show considerable changes in concentration of major elements between the treatments (Table 3).

The levels of minerals in the roasted, calcium chloride and lime-treated ethanol extracts were compared to those in the unprocessed *N. sativa* seed extract and the lime treatment was shown to be the optimum method for processing the seed sample to minimize metal and mineral. Samples that have been roasted/ treated with calcium chloride resemble the content of unprocessed samples. However, samples that had been treated by lime revealed a considerable decrease in hazardous metals (Table 4). (Figure 2B) UPLC-MS/MS results of unprocessed *N. sativa* powder dissolved in water.

In our study, both in unprocessed and processed extracts found six major compounds, such as Thymoquinone, 11,14,17-eicosatrienoic acid, Linoleic acid, 8,11-octa decadienoic acid, Alpha-tocopherol and Oleic acid were predominantly found (Table 5).

N. sativa herbal extract retains its anti-microbial properties under specified storage conditions after a 12-month interval (Figure 5B).

Table 1: **Anti-microbial efficacy of unprocessed detoxified *N. sativa* seed ethanol extract against bacterial pathogens.**

Sl. No.		Product names	*E. coli* (MDR)	*P. aeruginosa* (MDR)	*S. aureus*
1.		Unprocessed *N. sativa* etanol extract	18±1	20±1.5	20±1
2.	Detoxified *N. sativa* ethanol extract	Roasted	14±1	9±1	16±1.5
3.		Calcium chloride treated	25±1.5	14±1	14±2
4.		Lime treated	20±1	24±1.5	24±1
5.	Negative control	Ethanol	0	0	0
6.		Calcium chloride supernatant	0	0	0
7.		Lime supernatant	0	0	0
8.		Water	0	0	0
9.	Antibiotics	Clindamycin 10 µg (Positive control)	0	0	22±2
10.		Erythromycin 10 µg (Positive control)	0	0	17±1.5
11.		Chloramphenicol 10 µg (Positive control)	0	0	10±1
12.		Tetracycline 10 µg (Positive control)	0	0	20±1

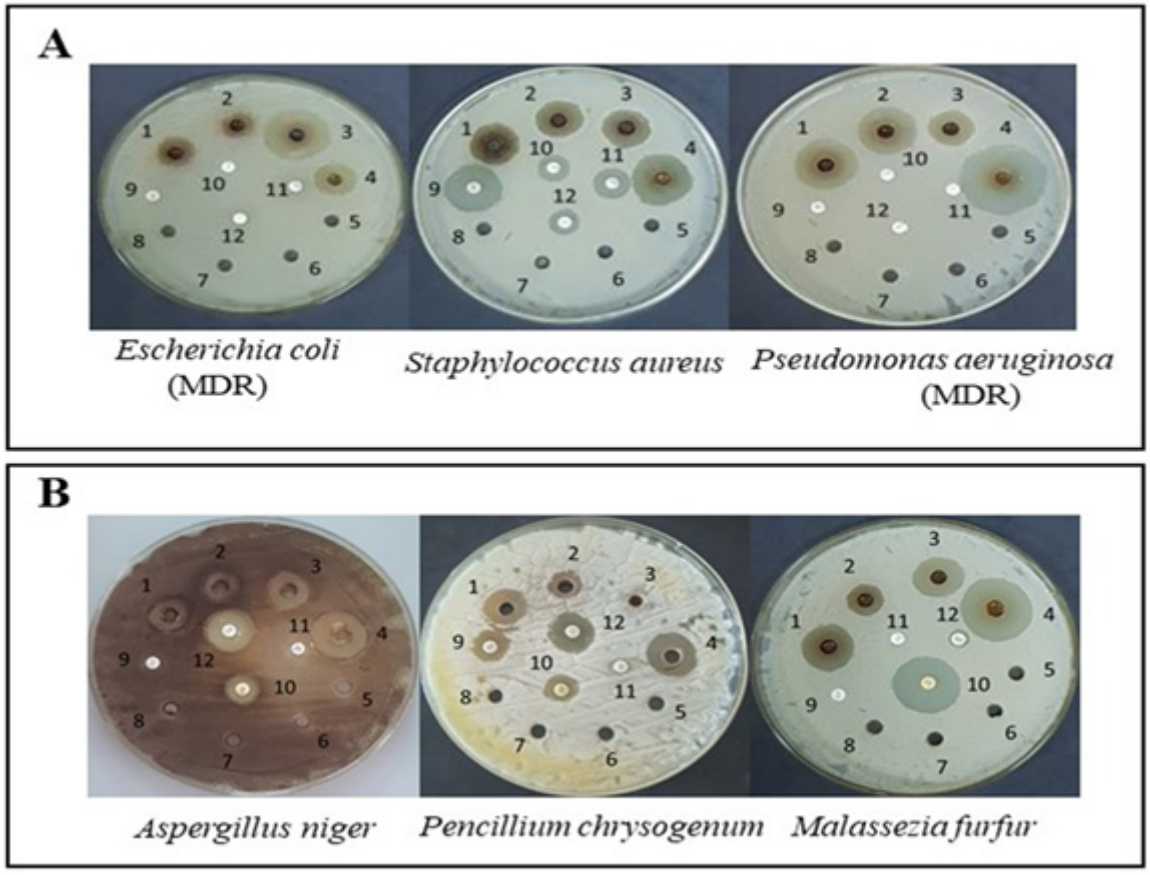


Figure 1: Anti-microbial efficacy of unprocessed and detoxification processed *N. sativa* seed ethanol extract against bacterial (A) and fungal (B) pathogens.

Table 2: Anti-microbial efficacy of unprocessed and detoxified *N. sativa* seed ethanol extract against fungal pathogens.

Sl. No.		Product names	*A. niger*	*P. chrysogenum*	*M. furfur*
1.		Unprocessed *N. sativa* etanol extract	18±1	20±1	21±1
2.	Detoxified *N. sativa* ethanol extract	Roasted	9±2	10±1.5	18±1
3.		Calcium chloride treated	15±1.5	0	16±1
4.		Lime treated	21±1	25±1.5	22±1.5
5.	Negative control	Ethanol	0	0	0
6.		Calcium chloride supernatant	0	0	0
7.		Lime supernatant	0	0	0
8.		Water	0	0	0
9.	Antibiotics	Ketoconazole 10 mcg (Positive control).	8±1	15±1	0
10.		Ampotericine-B 100 units (Positive control).	10±1	10±2	27±1
11.		Fluconazole 10 mcg (Positive control).	0	0	0
12.		Nystatin 50 mcg (Positive control).	18±1	20±1.5	8±1

DISCUSSION

Anti-Microbial Efficacy

Plant secondary metabolites are broad spectrum in nature.[26] Many studies have previously stated that different types of solvents are used to extract *N. sativa* seeds that exert antibacterial activity.[27] Likewise, *N. sativa* extracts in ethanol and n-hexane solvent produced remarkable dose-dependent antibacterial activity against the tested strains *E.coli, Staphylococcus epidermidis.*[28] Similarly, our results reveal that *N. sativa* ethanol extract is effective in inhibiting the growth of bacteria while aqueous and hexane extracts were found to be less effective as anti-microbial agents.[17] Moreover in the pharmaceutical drug market, *N. sativa* oil is sold as an effective anti-microbial agent. *N. sativa* seed oil has been shown by Bakal *et al.*[27] to have broad-spectrum antibacterial and antifungal action. However, our results reveal that among unprocessed samples, *N. sativa* powder is more effective than *N. sativa* oil against both bacterial and fungal pathogens tested. A previous study reveals that the medicinal herb *N. sativa* exhibited anti-microbial properties against bacterial strains that were resistant to commonly used synthetic antibiotic compounds. Vancomycin-resistant *S. aureus*[29] and MRSA strains were reported to be susceptible to ethanol extract of *N. sativa* by Hannan *et al.*[30] Similarly, our findings also reveal that ethanol extract is superior to other solvents and oil. Previous literature reveals *N. sativa* exerts significant antifungal activity against *Aspergillus niger* which was comparable to the antifungal drug Amphotericin-B. Moreover, reported that ethanol extract of the *N. sativa* seeds recorded potent inhibition of *Aspergillus flavus* than the standard drug Amphotericin-B.[31]

Preliminary phytochemical Analysis

The presence of compounds like Quinones and Phenolics is frequently reported in *N. sativa.*[32] One of the most abundant and widespread classes of plant metabolites, Phenolic compounds exhibit biological properties like anti-apoptosis, anti-ageing, anti-carcinogen, anti-inflammation, anti-atherosclerosis, cardiovascular protection, improvement of endothelial function, inhibition of angiogenesis and inhibition of cell proliferation activities.[33] Thymoquinone an active constituent of *N. sativa* seeds is pharmacologically active quinine which possesses several pharmacological properties including analgesic and anti-inflammatory actions.[34] The preliminary phytochemical screening of the unprocessed *N. sativa* ethanolic extract showed positive on steroids, Quinone, Anthraquinone, Phenols, Triterpenoids, Proteins, Saponins, Alkaloids, Flavonoids, Glycosides and Tannins. Moreover, our qualitative phytochemical investigation of detoxification processed samples revealed that moreover, all the extractions irrespective of treatment have identical chemical signatures.

ICP-OES Evaluation

Unprocessed N. sativa seed powder and N. sativa seed ethanol extract

Totally twelve non-essential and fourteen essential heavy metals were identified in *N. sativa* seeds. The trace elements Rubidium (Rb), Thallium (Th), Mercury (Hg), Lithium (Li), Selenium (Se), Lead (Pb) and Silver (Ag) have also been identified in *N. sativa* seeds (Figure 2A). Unprocessed seeds have higher than acceptable levels of trace minerals and non-essential heavy metals. Moreover, unprocessed seeds show more minerals, whereas the level of some metals and minerals like Calcium (Ca), Aluminium (Al), Manganese (Mn), Strontium (Sr), Copper (Cu),

Table 3: Mineral composition of unprocessed *N. sativa* seed powder and ethanol extract evaluated by ICP-OES.

Sl. No.	Parameters ppm	Permitted level / STD Limit	Unprocessed *N. sativa* seeds powder water extract	Unprocessed *N. sativa* seeds ethanol extract	% of reduction
1	Calcium (Ca)	Not Fixed	5700	4253	25.38
2	Magnesium (Mg)	Not Fixed	2342	2148	8.2
3	Sodium (Na)	Not Fixed	367.4	354.3	3.5
4	Potassium (K)	Not Fixed	5400	5385.1	0.27
5	Phosphorus (P)	Not Fixed	5284	5254	0.56
6	Chloride (Cl-)	Not Fixed	433	410	5.3
7	Aluminium (Al)	<10	95	84	11.57
8	Iron (Fe)	<150	77.8	75.4	3.08
9	Zinc (Zn)	<27.4	41.7	41.2	1.1
10	Manganese (Mn)	<200	44.3	40.2	9.2
11	Strontium (Sr)	7	28.7	21.5	25.08
12	Copper (Cu)	<15	16.2	15.4	4.9
13	Nickel (Ni)	Not Fixed	15.6	12.4	20.51
14	Barium (Ba)	2	14	10	28.5
15	Selenium (Se)	<0.4	0.5	0.3	40
16	Lead (Pb)	<2.0	0.05	0.02	60
17	Cobalt (Co)	<0.48	0.08	0.08	0
18	Chromium (Cr)	<2.0	0.2	0.2	0
19	Cadmium (Cd)	0.3	0.1	0.1	0
20	Silver (Ag)	Not Fixed	0.03	0.03	0
21	Arsenic (As)	3	0.04	0.04	0
22	Rubidium (Rb)	Not Fixed	1.1	0.5	54.54
23	Thallium (Th)	Not Fixed	0.9	0.85	5.5
24	Mercury (Hg)	1	<0.01	<0.01	0
25	Uranium (U)	Not Fixed	<0.1	<0.01	0
26	Lithium (Li)	Not Fixed	0.52	0.51	1.9

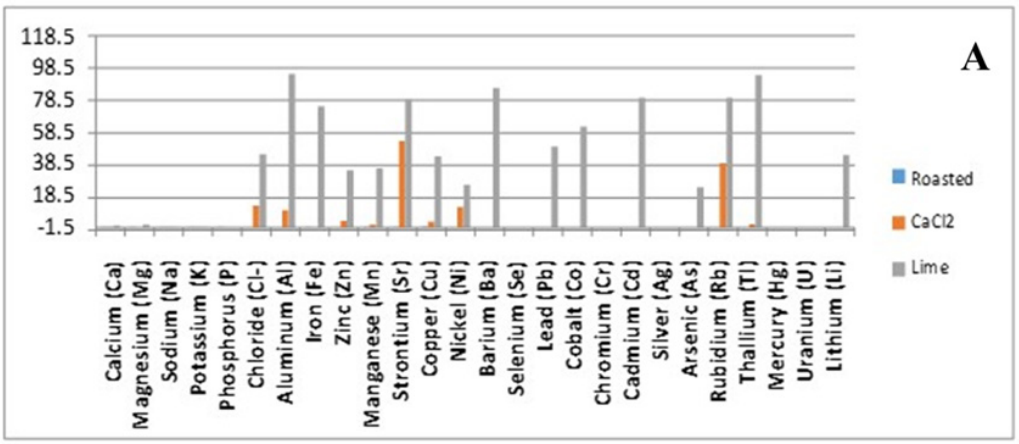
Nickel (Ni), Barium (Ba), Selenium (Se), Lead (Pb), Rubidium (Rb) and Thallium (Th) were reduced moderately during ethanol extraction (Table 3). All the macro, micro, trace and heavy metals found to be quantitatively varied among detoxification treatments (Table 3). This is one of the important scientific studies which support the ancient literature's description on the detoxification process. Our ICP-OES results of untreated *N. sativa* are similar to earlier studies by Iqbal *et al.*[35] and Sultan *et al.*[36] However, until now no study has been done in the aspect of evaluating element analysis of detoxified samples.

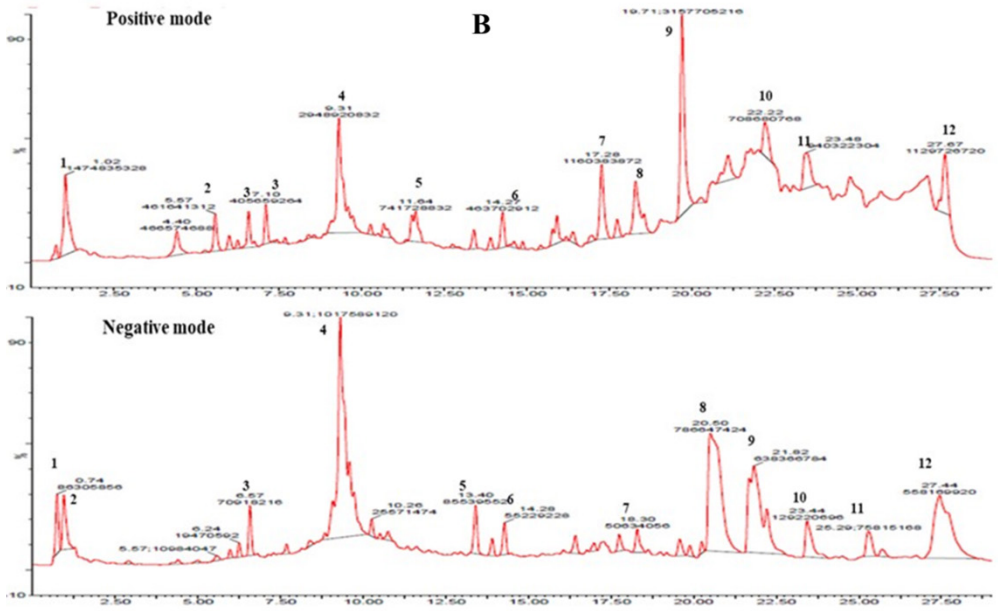
Processed *N. sativa* seeds ethanol extract ICP-OES study

Reduction in most of the investigated parameters has revealed that both roasting and $CaCl_2$ treatment are less efficient than lime treatment. In lime treatment minerals were reduced up to 50% and non-essential metals were reduced up to 60%. The chloride content of a raw sample is 410 ppm and reduced to 224 ppm in lime treatment and the percentage of reduction was 45.3% followed by $CaCl_2$ treated sample at 13.6%. The concentration of Aluminium 84 ppm and lime treated showed 4.3 ppm denotes the maximum reduction of metal recorded as 94% followed by $CaCl_2$ treatment 10.7%. Fe content of the unprocessed sample was 75.4 ppm and lime treated showed 19 ppm about 75% reduction. The level of Cd in unprocessed samples was 0.1 ppm and 0.02 ppm in lime treated (reduction 80%) likewise barium concentration was 10 ppm and 86% reduced in lime treatment and estimated as 1.4 ppm. The untreated sample Strontium (Sr) was reduced 79% by lime treatment and 53% in $CaCl_2$. Rubidium was 0.5 ppm and reduced to 0.1 ppm (80% reduction) followed by 40% in $CaCl_2$ treatment. The amount of thallium in an untreated sample was 0.85 ppm which is reduced up to 94.1% in lime treatment which is recorded as 0.05 ppm. Likewise, cobalt is reduced up to

Table 4: Efficacy of detoxification process in reduction of macro and micro minerals in *N. sativa* seeds evaluated by ICP-OES.

Sl. No.	Parameters PPM	STD Limit	Unprocessed *N. sativa* ethanol extract	Roasted *N. sativa* ethanol extract	% of Reduction Roast vs. unprocessed	$CaCl_2$ *N. sativa* ethanol extract	% of Reduction $CaCl_2$ vs. unprocessed	Lime *N. sativa* ethanol extract	% of Reduction Lime vs. unprocessed
1	Calcium (Ca)	Not Fixed	4253	4250	0.07	4250	0.07	4200	1.2
2	Magnesium (Mg)	Not Fixed	2148	2145	0.14	2148	0.0	2106	1.95
3	Sodium (Na)	Not Fixed	354.3	355	0.19	354.7	0.11	354.7	0.11
4	Potassium (K)	Not Fixed	5385	5384	0.02	5384	0.01	5384	0.02
5	Phosphorus (P)	Not Fixed	5254	5252	0.03	5254	0.0	5254	0.0
6	Chloride (Cl-)	Not Fixed	410	410	0.0	354	13.6	224	45.3
7	Aluminium (Al)	<10	84	84	0.0	75	10.7	4.3	94.8
8	Iron (Fe)	<150	75.4	75.2	0.2	75.4	0.0	19	75
9	Zinc (Zn)	<27.4	41.2	41.2	0.0	39.5	4.1	26.5	35.6
10	Manganese (Mn)	<200	40.2	40	0.5	39.4	2.0	25.4	36.8
11	Strontium (Sr)	7	21.5	21.4	0.46	10	53.4	4.5	79
12	Copper (Cu)	<15	15.4	15.3	0.6	14.8	3.8	8.6	44.1
13	Nickel (Ni)	Not Fixed	12.4	12.4	0.0	10.8	12.9	9.1	26.6
14	Barium (Ba)	2	10	10	0.0	10	0.0	1.4	86
15	Selenium (Se)	<0.4	0.3	0.3	0.0	0.3	0.0	0.3	0.0
16	Lead (Pb)	<2.0	0.02	0.02	0.0	0.02	0.0	0.01	50
17	Cobalt (Co)	<0.48	0.08	0.08	0.0	0.08	0.0	0.03	62.5
18	Chromium (Cr)	<2.0	0.2	0.2	0.0	0.2	0.0	0.2	0.0
19	Cadmium (Cd)	0.3	0.1	0.1	0.0	0.1	0.0	0.02	80
20	Silver (Ag)	Not Fixed	0.03	0.03	0.0	0.03	0.0	0.03	0.0
21	Arsenic (As)	3	0.04	0.04	0.0	0.04	0.0	0.03	25
22	Rubidium (Rb)	Not Fixed	0.5	0.5	0.0	0.3	40	0.1	80
23	Thallium (Tl)	Not Fixed	0.85	0.85	0.0	0.83	2.3	0.05	94.1
24	Mercury (Hg)	1	<0.01	<0.01	0.0	<0.01	0.0	<0.01	0.0
25	Uranium (U)	Not Fixed	<0.01	<0.01	0.0	<0.01	0.0	<0.01	0.0
26	Lithium (Li)	Not Fixed	0.51	0.51	0.0	0.51	0.0	0.28	45





Positive Mode- 1. Thymoquinone, 2.11,14,17-Eicosatrienic acid, 3. Linolenic acid, 4.8,11-octadecadienoic acid, 5.kaempferol, 6. Alpha-Tocopherol, 7. Sigmasterol , 8.Nigellicimine, 9. Oleic acid, 10. Longifolene, 11. Nigellicine, 12. Palmitic acid.

Negative Mode- 1. Thymoquinone, 2. Cholesterol, 3. Alpha-Tocopherol, 4. 8,11,Octodecadienoic acid, 5. Linolenic acid, 6.

Figure 2: (A) metals reduced in processed samples and (B) UPLC-MS/MS results of unprocessed N. Sativa powder dissolved in water

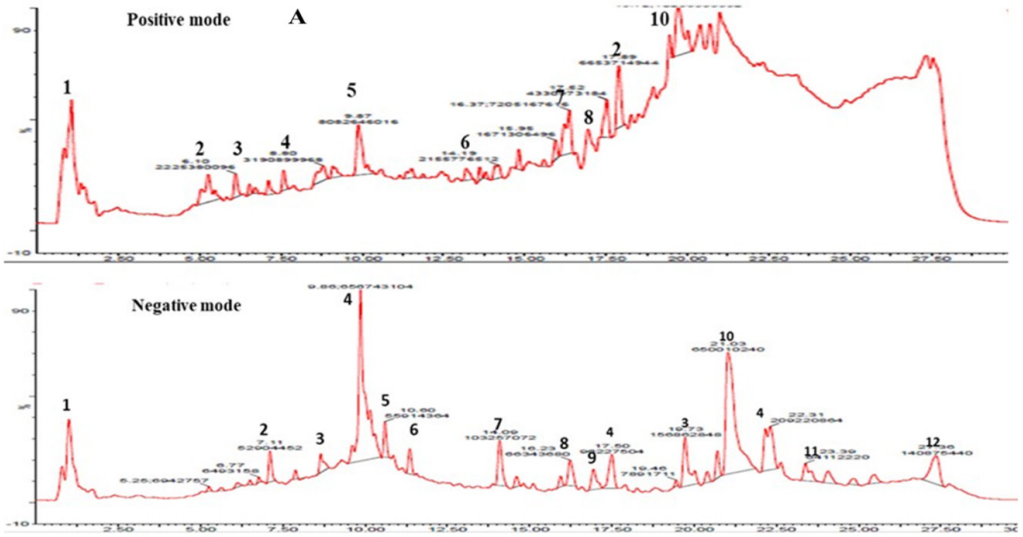
62.5%, Lead 50%, copper 45% and lithium is reduced to 45% in lime-treated *N. sativa* seeds.

Macro minerals

In $CaCl_2$ and Roasted ethanol extract, the levels of macro minerals i.e., Calcium, Magnesium, Potassium, Sodium, Phosphorus and Chloride were comparable to the unprocessed *N. sativa* seed ethanol extract. There was a significant decrease in macro minerals such as chloride (45.3%), magnesium (1.95%) and calcium (1.2) only in the sample with lime treated.

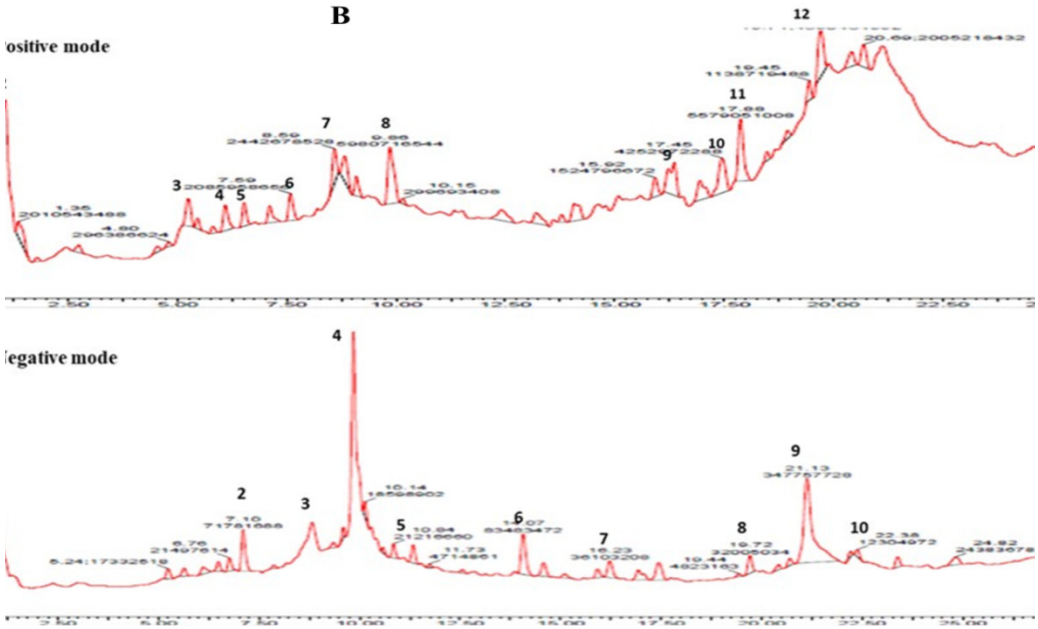
Trace minerals

The World Health Organization (WHO) and the Food and Agriculture Organization (FAO) jointly set the following permitted levels of heavy metals in medicinal plants: Fe, Cu, Cd, Pb, Cr, Co, Ni and Zn at 20, 150, 0.3, 10, 2, 0.48, 2.14 and 27.4 ppm, respectively Sungur *et al.*[37,38] There was a significant decrease in trace minerals such as Copper, Iron, Zinc, Manganese, Cobalt, Chromium, Nickel and Selenium only in the sample that had been treated with lime. Compared to Özcan and Akbulut (2008)[39] study, our study showed considerable change in trace metals. Results of the lime treatment showed that Fe (75%) Co (62%), Cu (44%), Mn (37%), Zn (35%) and Ni (27%) decreased in the



Positive Mode- 1. Thymoquinone, 2. Linolenic acid, 3. Thymohydroquinone, 4.Apigenin, 5. Alpha-Tocopherol, 6. Diterpene, 7. Nigellicine, 8.Nigellicimine, 9. Longifolene, 10. Oleic acid.

Negative Mode- 1. Thymoquinone, 2. Cholesterol, 3. Alpha-Tocopherol, 4. 8,11,Octodecadienoic acid, 5. Linolenic acid, 6. Nigellicimine, 7. Rutin, 8. kaempferol,, 9. Longifolene, 10. Oleic acid, 11. Aromadendrene, 12. 11,14,17 Eicosatrienoic acid.



Mode- 1. Thymoquinone, 2. Thymohydroquinone, 3. 11,14,17-Eicosatrienic acid, 4. 8,11-octadecadienoic acid, 5.kaemp
-Tocopherol, 7. Linolenic acid, 8. Nigellicine, 9. Longifolene, 10.Diterpene, 11. Oleic acid, 12. Nigellicimine.

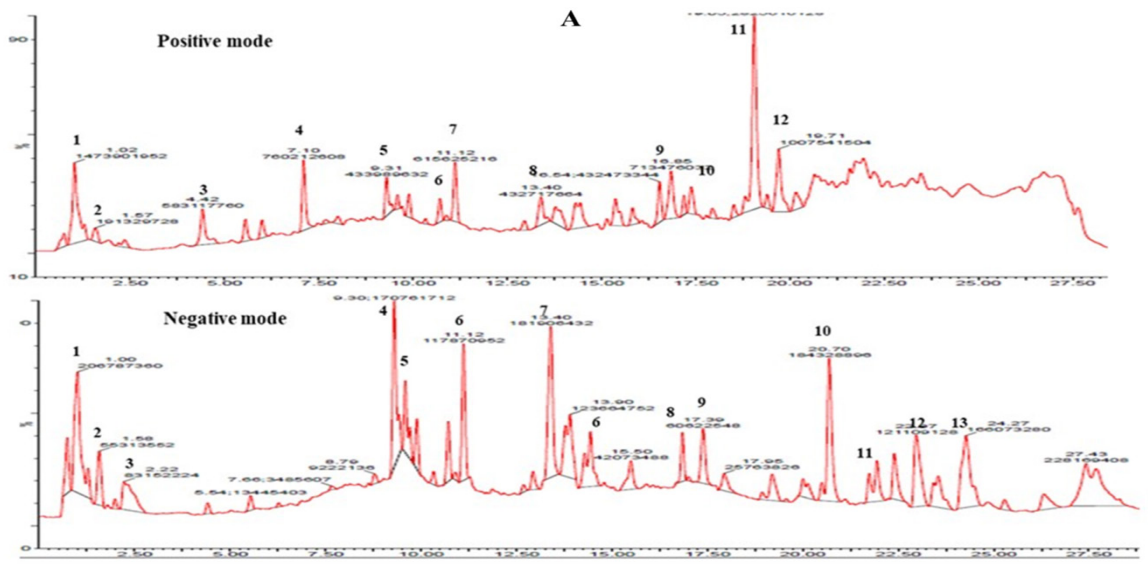
e Mode- 1. Thymoquinone, 2. Cholesterol, 3. Alpha-Tocopherol, 4. Linolenic acid, 5. 8,11,Octodecadienoic acid, 6. Nigel
8. Longifolene, 9. Oleic acid, 10. Aromadendrene.

Figure 3: UPLC-MS/MS results of unprocessed *N. sativa* seeds ethanol extract (A). UPLC-MS/MS results of calcium choloride treated *N. sativa* seeds ethanol extract (B).

trace mineral level. No decline in selenium and chromium was observed because extremely low concentrations were recorded. In the calcium chloride-treated sample, only nickel was decreased by about 12.9%. A more recent study by Cheikh-Rouhou *et al.*[40] analyzed several elements in seeds collected from Tunisia and the average concentrations (mg/kg, dry weight) observed were: K, 783; Mg, 235; Ca, 572; Na, 21; P, 50; Fe, 9; Cu, 1.7; Zn, 8; and Mn,4.4 mg/kg.

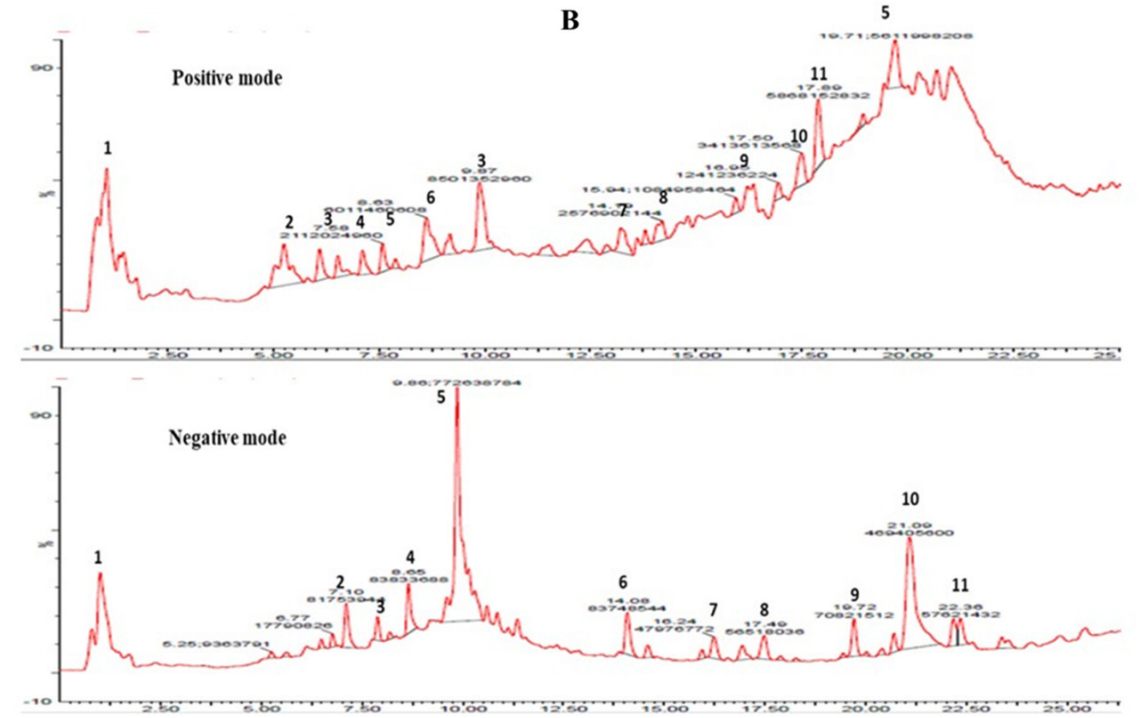
Non-Essential Heavy Metals

Non-essential heavy metals include Barium, Strontium, Aluminium, Lead, Cadmium, Silver, Arsenic, Rubidium, Thallium, Mercury, Uranium and Lithium, chromium have



Positive Mode- 1. Thymoquinone, 2. Thymohydroquinone, 3. 8,11-octadecadienoic acid, 4. 11,14,17-Eicosatrienic acid, 5.kaempferol, 6. Alpha-Tocopherol, 7. Linolenic acid, 8. Nigellicine, 9. Longifolene, 10.Diterpene, 11. Oleic acid, 12. Nigellicimine.

Negative Mode- 1. Thymoquinone, 2. Cholesterol, 3. Nigellicimine, 4. Linolenic acid, 5. 11,14,17 Eicosatrienoic acid, 6. Alpha-Tocopherol, 7. 8,11, Octodecadienoic acid, 8. Longifolene, 9. Diterpene, 10. Oleic acid, 11. Rutin, 12. Aromadendrene, 13. Nigellicine.



Positive Mode- 1. Thymoquinone, 2.11,14,17-Eicosatrienic acid, 3. Linolenic acid, 4.8,11-octadecadienoic acid, 5.kaempferol, 6. Alpha-Tocopherol, 7.Sigmasterol , 8.Nigellicimine, 9. Longifolene, 10. Nigellicine, 11. Oleic acid.

Negative Mode- 1. Thymoquinone, 2. Cholesterol, 3. Alpha-Tocopherol, 4. Linolenic acid, 5. 8,11,Octodecadienoic acid, 6. Nigellicimine, 7. Rutin, 8. Longifolene, 9. Oleic acid, 10. Aromadendrene, 11. 11,14,17 Eicosatrienoic acid.

Figure 4: UPLC-MS/MS results of lime treated *N. sativa* seeds ethanol extract (A). UPLC-MS/MS results of roasted *N. sativa* seeds ethanol extract (B).

been the most common heavy metals that induce poisoning to damage the vital organs of humans. According to Kim *et al.*[41-43] anthropogenic activities are contributing to an increase in the presence of heavy metals in food samples. The recommended acceptable levels of Aluminium in the human body are 5-10 mg/kg. Our experimental results reveal that samples treated with lime have 4.3 ppm, a 95% decrease in Aluminium. Barium reduction in the sample after lime treatment was 86%, which meets the barium

Table 5: Detection of unprocessed and detoxification processed *N. sativa* seeds bioactive compounds by UPLC-MS/MS.

(M+H) $^{+}$/ (M+H)$^{-}$	Phytochemical compound Names	Phytochemical Nature	Unprocessed Wate r Extract	Unprocessed Ethanol Extract t	Roaste d Ethan ol Extract t	$CaCl_2$ treated Ethanol Extract	Lime treated Ethanol Extract
321.3(320.5)	11,14,17-Eicosatrienoic acid.	Fatty acids	+	+	+	+	+++
293(292.5)	Linolenic acid	Fatty acids	++++	++++	++++	++++	++++
295.2(294.5)	8,11-Octa decadienoic acid	Fatty acids	+	+	+	+	+++
282.5(282.3)	Oleic acid	Fatty acids	+++	+++	++	++	++++
256.4	Palmitic acid	Fatty acid	+	+	-	-	-
165(164.2)	Thymoquinone	Terpenes	++	++	++	+++	+++
165.6(166.22)	Thymohydroquinone	Terpenes	-	+	-	++	+
204.3	Longifolene	Terpenes	+	+	+	+	+
287.6(288.25)	Aromadendrene	Terpenes	+	+	++	+	+
320.5	Diterpene	Terpenes	-	+	-	+	+
773	Kaempferol	Flavonoid	+	+	+	+	+
270	Apigenin	Flavonoids	-	+	-	-	-
219.2	Nigellamines	Alkaloids	+	+	+	+	+
204.9(204.3)	Nigellicine	Alkaloids	+	+	+	+	+
413.6(412.69)	Stigmasterol	Sterols	++	-	+	-	-
386.65	Cholesterol	Sterols	+	+	+	+	+
430.71	Alpha-Tocopherol	Vitamine-E	+	++	+	+	+++
610 (610.5)	Rutin	Phenols	+	+	+	+	+

permissible limit.[44] Thallium is reduced by around 94%, cadmium is reduced by about 80% and lead 50%[8] was detected. Only samples treated with lime show a significant heavy metal reduction. Very little silver metal was found. Low levels of Mercury and Uranium were detected (Table 4). The calcium chloride treated sample reveals a reduction in three metals, Sr-53%, Rb-40% and Al-11%. In roasted *N. sativa* seeds, the detection of non-essential heavy metal levels reveals no reduction in metal levels. However, the sample that has been treated with lime contained 0.03 ppm of arsenic (a 25% reduction), which is below the permissible limit.[45]

The concentration of Aluminium, Zinc, Strontium, Copper, Barium and Selenium were found to be higher than that of accepted permissible concentrations in raw samples. However, by lime treatment, it was recorded that more than 75% reduction of Aluminium, Strontium, Barium, Rubidium, Cadmium and Thallium were observed. A high level of Aluminium effect on bone marrow leads to the formation of abnormal red blood cells besides its effect parathyroid gland, Liver stenosis and nephrotic syndrome, osteoporosis and osteomalacia.[46,9] Taking very high doses of zinc is likely unsafe and might cause stomach pain, vomiting and many other problems.[10] High levels of radioactive strontium can cause impaired bone growth, anaemia and cancer.[47] Copper high levels may result in liver damage and gastrointestinal symptoms.[48] High levels of barium may induce gastrointestinal effects, cardiac dysrhythmias, abnormal blood pressure, muscle weakness and paralysis.[49] Symptoms of selenium toxicity include nausea; vomiting; nail discoloration, brittleness and loss; hair loss; fatigue; irritability; and foul breath odor.[50] However, samples that had been treated with lime revealed a considerable decrease in all the above hazardous metals.

UPLC-MS/MS

The yield of the bioactive compounds from plant materials may be greatly influenced by the sample treatment process.[51,52] Therefore, to understand the biochemical changes that take place due to the detoxification of *N. sativa* seeds, we compared all the unprocessed and processed seeds by UPLC MS/MS analysis. The identified compounds are summarized along with their retention time, ESI (M+H)+/(M+H)- and MS/MS, m/z base ions (Table 5). Our UPLC-MS/MS results of unprocessed *N. sativa* seed (water extracted) revealed fifteen compounds of which, three abundant compounds identified were Thymoquinone, Linoleic acid, and Oleic acid (Figure 2B). Whereas, in ethanol extracted 17 compounds were identified of which four major compounds identified are Thymoquinone, Linoleic acid, Alpha-Tocopherol and Oleic acid (Figure 3A). However, the detoxified *N. sativa* seed

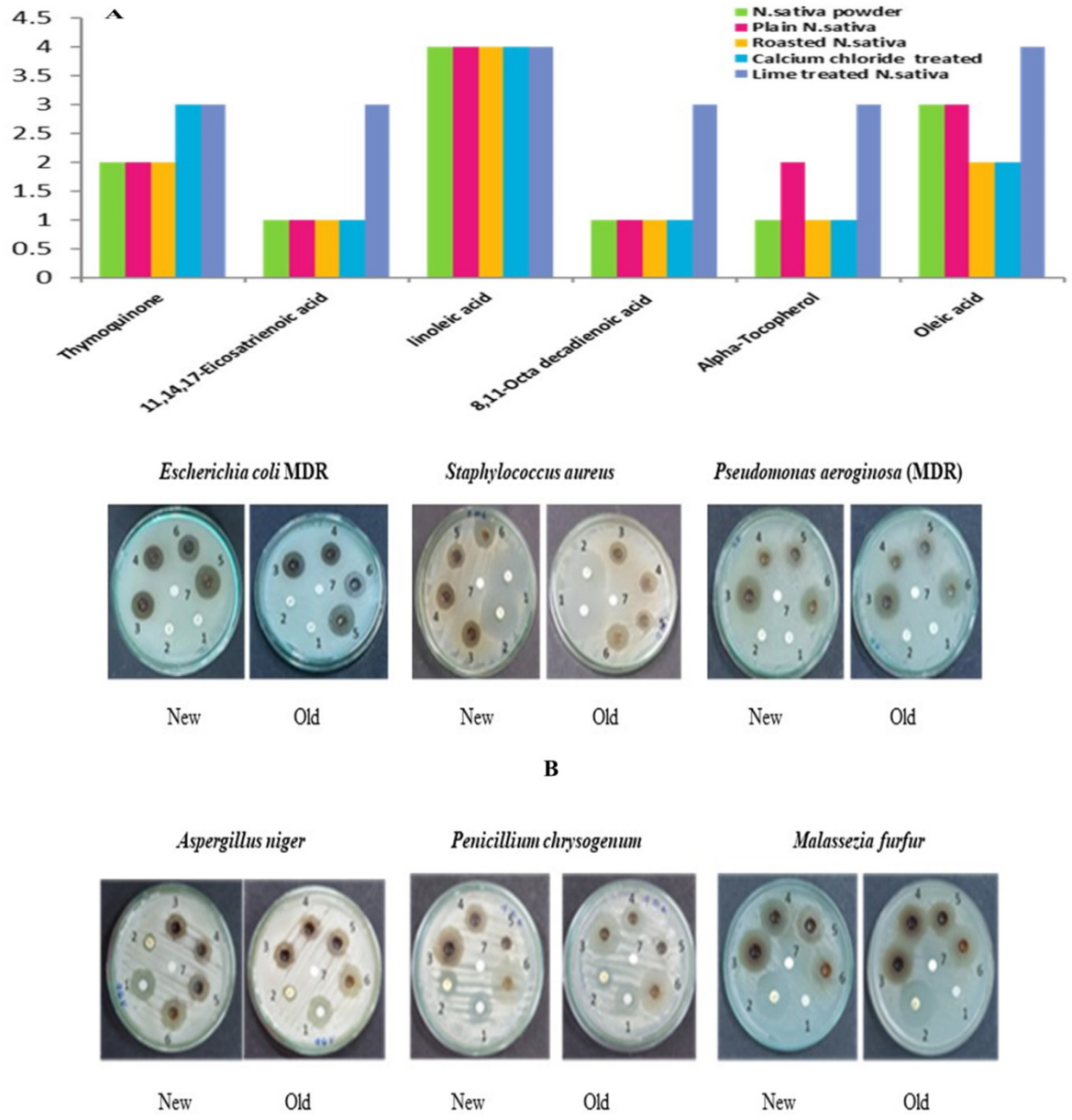


Figure 5: Major peak obtained from UPLC-MS/MS analysis of detoxified *N. sativa* (A). Stability potency of the extract was evaulated by an Anti-microbial study (B).

ethanol extracts of $CaCl_2$ and lime treated show 15 compounds. Calcium chloride treated *N. sativa* seeds ethanol extract have 2 major compounds Thymoquinone and Linolenic acid (Figure 3B) whereas Lime treated show six major compounds, Linoleic acid, Oleic acid, Thymoquinone, 11,14,17-eicosatrienoic acid, 8,11-octa decadienoic acid and Alpha-tocopherol (Figure 4A). All the compounds in lime treatment were found to be significantly higher compared to other samples (Figure 5A). The roasted *N. sativa* ethanol extract shows 14 compounds, of which Linoleic acid is found to be the major compound (Figure 5B).

A previous study reported that *N. sativa* was found to have Linoleic acid, Oleic acid, Margaric acid, cis-11, 14-eicosadienoic acid and Stearic acid were identified as important fatty acids. Our results showed the presence of 5 fatty acids (detoxification processed of *N. sativa* ethanol extracts) namely 11,14,17-Eicosatrienoic acid, Linolenic acid, 8,11- Octa decadienoic acid, Oleic acid and Palmitic acid. Articles published by Abdel-Fattah *et al.*[34] Yadav *et al.*[52] El-Najjar *et al.*[53] showed Thymoquinone (TQ) and its derivatives, such as Carvacrol, 4- terpineol, pinene, Thymol, t-anethol, Thymohydroquinone (THQ), Dithymoquinone, p-cymene, Sesquiterpene, Longifolene and several other compounds constitute the Terpenes and Terpenoids family, which is the major chemical group in *N. sativa*. Our UPLC-MS/MS analysis results revealed the presence of five Terpenes namely Thymoquinone, Longifolene, Aromadendrene, Thymohydroquinone, and Diterpene.

Also, two Flavonoid compounds were detected in our extracts, namely Kaempferol and Apigenin. Our study reveals one Phenolic compound "Rutin" in detoxification-processed extracts.[54] The same results were shown by Toma *et al.*,[55] in a previous study where Polyphenol and Flavonoid compounds Rutin, Kaempferol and Apigenin were identified from *N. sativa* seeds analyzed using HPLC-UV-MS. Moreover, two alkaloid compounds namely Nigellamines and Nigellicine, and two sterol compounds in detoxication processed extracts, namely Stigmasterol and Cholesterol were identified in our study. Similar results were reported in literature, that *N. sativa* contains several Sterols in *N. sativa* oil.[56] The oil also contains a smaller percentage of D7-stigmasterol, D7-avenasterol, Campesterol and Cholesterol. Alpha-Tocopherol also called as Vitamin E, is detected in our extracts. Previous literature by Kiralan *et al.*[57] revealed that *N. sativa* contained Alpha-tocopherol content which is the highest with quantity ranging from 8.57 to 34.23 ppm.

Thymoquinone is abundantly present in calcium chloride-treated and lime-treated seed extracts and moderate among other extracts. It is the main active compound in *N. sativa* seed, has the ability to stimulate pigment cells to darken the skin tone via neurotransmitter action and also act as antioxidant, anti-inflammatory, anti-microbial and anticoagulant activity.[58] Only lime-treated extract showed the presence of a high quantity of 11,14,17-eicosatrienoic acid, 8,11-octa decadienoic acid and Alpha-tocopherol. However, in other ethanol extracts, these compounds are detected in negligible quantity (Table 5). Jin *et al.*[59] detected 11,14,17-Eicosatrienoic Acid (ETA) which is an omega 3, polyunsaturated fatty acid, an essential fatty acid and it acts as an anti-ageing, reduces UV-induced skin damage. Panchabhai *et al.*[60] reported the presence of 8,11, Octadecadienoic acid, which is an unsaturated fatty acid and has the properties of anti-microbial activity, anti-inflammatory, skincare activity, anti-sinusitis and anti-respiratory tract infections. Tavano *et al*[61] reported the presence of Alpha-Tocopherol, which is a Tocopherol-Vitamin E used to treat anti-dandruff therapy, anti-inflammatory and skin care therapy (de tan activity).

Linoleic acid is abundantly present in all unprocessed and processed *N. sativa* ethanol extracts. Linoleic acid is categorized to be a polyunsaturated omega-6 fatty acid and it may employed as an anti-microbial, anti-diabetic, used to treat skin eczema and wound healing activity.[62] Oleic acid is abundantly detected in water extract of *N. sativa* powder and lime-treated extract, whereas moderately identified in roasted and calcium chloride-treated samples. A study by Nivetha and Prasanna[63] revealed that Oleic acid is a mono-unsaturated fatty acid and has the property of flavor, anti-carcinogenic and used to treat dermatitis. Moreover, Lime-treated ethanol extract contained high levels of fatty acids such as Linoleic acid, and oleic acid. So, our study revealed that lime-treated ethanol extract may be used as a promising healing source for dermatitis treatment.

Stability Test

Agarwal and Paridhavi[64] reported that the length of time the medicine retains its distinctive qualities is determined in *N. sativa* extracts and found to depend on the storage condition and processing method described by Thakur *et al.*[65] A herbal anti-microbial drug's biological potency is measured in a way that whether it is consistent with its anti-microbial activity.[66] Therefore, Stability testing was done to evaluate how our distinct detoxified (Roasted, Calcium chloride and Lime) *N. sativa* herbal extract retained its anti-microbial properties under specified storage conditions after 12-month interval (Figure 5B). Test of 1 year stored Plain, roasted, $CaCl_2$ and Lime treated samples reveal similar antibacterial activity against *E.coli MDR, Staphylococcus aureus, Pseudomonas aeroginosa* MDR culture's and antifungal activity against *A. niger, P. crysoginum* and *M. furfur* strains. The entire product's test results reveal negligible reduction of efficacy after 12 months. Moreover, 1-year-old samples exhibited almost close to fresh sample's effect. In addition, there is no significant difference observed between the detoxification processed samples.

CONCLUSION

Our research has concluded that with the exception of *E. coli*, lime treatment exhibits high inhibitory action against all of the examined microorganisms. Our research data represent seed treatment with a solution of Calcium chloride/lime/roasting results in the reduction of metal and minerals. Conclusively, the treatment of seed with lime was found to be an effective approach on the reduction of metals and sustaining major phenol, fatty acids, and flavonoids with promising anti-microbial property.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

$CaCl_2$: Calcium chloride; **N. sativa:** *Nigella sativa*; **UPLC:** Ultra-performance liquid chromatography: **MS:** Mass spectrometry; **IARC:** International Agency for Research on Cancer; **ETA:** Eicosatrienoic acid; **TNAU:** Tamil Nadu Agricultural University; **MTCC:** Microbial Type Culture Collection; **Rb:** Rubidium; **Th:** Thallium; **Hg:** Mercury; **Li:** Lithium; **Se:** Selenium.

SUMMARY

The utilization of medicinal plants for drug development necessitates stringent safety measures to mitigate potential toxicity. In this study, traditional detoxification methods, including roasting, lime, and calcium chloride treatments, were applied to Nigella sativa seeds to reduce toxicity while preserving therapeutic properties. The detoxified seeds were subjected to antimicrobial assays against multidrug-resistant bacterial strains and fungal pathogens, revealing enhanced inhibitory activity post-treatment, particularly with lime treatment. Metal content analysis demonstrated varying degrees of reduction, with lime treatment exhibiting the most pronounced effect. Stability assessments over 12 months highlighted sustained antimicrobial potency. Notably, UPLC-MS/MS analysis identified elevated levels of fatty acids in lime-treated extracts, correlating with enhanced antimicrobial activity. These findings underscore lime treatment as an effective detoxification strategy, yielding safer N. sativa extracts enriched with bioactive compounds, thus offering promising prospects for pharmaceutical applications.

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