

The NAC Protocol

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A natural fungal mitigation and immunomodulatory protocol

Introduction

Fungal infections and their toxins (called mycotoxins) can be a major contributor to inflammation in the body. As a result of that inflammation, it can cause or exacerbate various conditions, including autoimmune diseases.

Relevant links to science articles will be presented as we go, listed so you can click on the link for more information. In addition, a more detailed scientific review of the protocol compounds is available at the end of the document for researchers and clinicians alike.

Fungal infections and their mycotoxins are seriously under-addressed in symptom management and the treatment of disease. This natural protocol focuses on effective elimination of fungal infections and modulation of immune response to improve outcomes.

Common Fungal Infections

Common Fungal Infections include *Candida*, *Aspergillus* and *Cryptococcus* species. Until recently, these fungi have been considered commensal, or a common part of the biome and generally considered harmless.

More recent research indicates that their byproducts (or metabolites) can be carcinogenic, inflammatory and even mutagenic to DNA [1].

These byproducts, or mycotoxins, are commonly measured in the body and regular exposure occurs through both food contamination [2] and inhalation of spores [3] in the home and outside environments.

By reducing fungal infection levels and their mycotoxins, we can reduce the corresponding inflammatory response in the body and promote homeostasis for improved immunity.

A Natural Anti-Fungal Protocol

There are 3 components to this natural protocol. Those are N-Acetylcysteine (NAC), Oregano Oil (OO) and Black Seed Oil (BSO).

All 3 components work safely and effectively at reducing fungal infection and supporting the body's detoxification process. Part of the process of removing fungal infections in the body is dealing with the cell death of pathogens, which release toxins and byproducts that the body must eliminate through detoxification. This process occurs in the liver and kidneys. During this cleanup process, a **Jarisch-Herxheimer reaction** is common [4].

This reaction can include various symptoms like headaches, tiredness, gastrointestinal symptoms or

even cold like symptoms. This necessary discomfort is required to remove detrimental fungal infections.

Overall, it can take 2 to 4 months to see a significant improvement in health and well-being. Periods of detoxification symptoms will come and go as you progress. It is common to notice small improvements along the way, and it can be a motivator to complete the process.

The process takes longer because depending on the fungi in question, you may have infection in the lungs, gastrointestinal tract, upper respiratory system, spinal fluid, synovial fluid in the joints, various tissues and organs and even the brain itself.

Another thing that makes this process take longer is biofilms. What you normally call plaque on the teeth is an example of biofilms. 80% of the pathogens you are targeting live inside these biofilms. As a result they gain protection from the immune system.

Biofilms can take time to break down, but all 3 components of the protocol serve this purpose. The pathogens targeted by this protocol are both pathogenic fungi and bacteria. Both can be the cause of dysbiosis, or an imbalance in your gut biome, which can lead to various issues [5].

The NAC Protocol

Morning

- 600mg NAC
- Oregano Oil (40mg Carvacrol)
- Black Seed Oil (1 teaspoon)

Night

- 600mg NAC
- Oregano Oil (40mg Carvacrol)
- Black Seed Oil (1 teaspoon)

*with contributions from S. C.

Continue daily for a minimum of two months and count out 3 weeks with no die off symptoms prior to discontinuing.

Fungal die off symptoms may include: *Tiredness, exhaustion, muscle soreness, increased chest or nasal discharge, cold or flu like symptoms, cold sores, headaches, rash, acne, irritability, change in stool frequency, volume or color; increased urination, bloated stomach, cramps, increased gas.*

Choosing Your Supplements

Oregano Oil's bioactive ingredient is called **Carvacrol**. You want to purchase your OO in capsule form and purchase a product that has around 40mg of Carvacrol per dose. Read the bottle instructions to determine if 1 or 2 capsules will provide the necessary Carvacrol amount.

NAC can be found easily in 600mg per capsule dosages at various retailers and online shops.

Black Seed Oil should be purchased in the cold pressed, unfiltered oil form. A large 16 ounce bottle is available through **Horbaach** and **SVA Organics**. These products have been tested by users to be effective.

The morning and evening dose can be taken with or without food, but should be taken with food if you have a sensitive stomach. Increase water intake and fiber while on the protocol to ease detoxification.

#1 Rule: Listen To Your Body

Everybody is different, and various factors can influence how strong your herxheimer reactions are, including level of infection, age and general health. Consult with your doctor before starting.

When you start this natural protocol, die off symptoms may be immediate or may take up to a month to begin. When this detox begins, listen to your body. If at any time the symptoms become too overwhelming, take a few days off, rest and increase your water intake.

You can then continue at lower dosages by reducing the Oregano Oil or taking it once daily, reducing NAC to once daily, and gradually increasing amounts as symptoms improve to reach protocol amounts.

As you advance further, it can be beneficial to scale up your dosages slowly to continue making progress.

If you decide to increase amounts, a general guideline for maximum daily intake based on studies is 400mg for Carvacrol amount, and 1800mg daily for NAC. Black Seed Oil can be maintained at 2 to 4 teaspoons daily.

Once you reach a period where die off stops occurring for 3 to 4 weeks and you are feeling good, you can take a break for a month and see how you react. If any symptoms return quickly, you may need

a more long term solution.

To approach correction of potential long-term immune dysfunction related to commensal or invasive fungi, the following page details a maintenance protocol for regular long-term use.

The Maintenance Protocol

The Maintenance Protocol focuses on a more gentle antifungal approach combined with immune modulation to prevent overactive response (autoimmune).

To get technical for a moment, Niacin provides a needed boost in the NAD+ pool [7], which works with Pterostilbene as a SIRT1 activator [8] to promote homeostasis by countering oxidative stress, inflammation and mitochondrial dysfunction.

Pomegranate Extract serves to provide additional antifungal, anti-inflammatory and anti-oxidative support.

Morning

- 600mg NAC
- 500mg Slo Niacin (nicotinic acid)
- 100mg Pterostilbene
- 250mg Pomegranate Extract (40% Ellagic Acid)
- Black Seed Oil (1 teaspoon)

Night

- 500mg Slo Niacin
- 100mg Pterostilbene
- 250mg Pomegranate Extract
- Black Seed Oil (1 teaspoon)

After every 3 weeks on the maintenance protocol, take 1 week off. Continue to use black seed oil during the off cycle.

Safety & Adverse Reactions

Always consult with your doctor before starting this protocol and receive prior approval. They can appropriately address interactions with medications or any current health conditions you have.

In addition, the protocol may lower specific vitamins and minerals including zinc, iron and calcium. A **multi-vitamin** is recommended to address this.

The protocol is known to lower blood sugar, blood pressure and can have a blood thinning effect.

In addition, asthmatics on corticosteroids should consider additional caution with NAC due to potential spasmodic activity. Refer to the science section for more detailed information.

Black seed oil contains thujone derivatives, which may aggravate certain conditions that are prone to seizures.

Protocol User Feedback

We've shared this protocol online for over a year and a half as we gathered anecdotal reports from users on their experience.

In that time, we've received hundreds of positive anecdotal reports from users of all ages and backgrounds. Various inflammatory issues improved, aches, pains, flexibility issues, mood and well-being increased, various forms of dysbiosis were corrected (including irritable bowel disorder) and general health and well-being improvements were reported.

We hope you experience the wonderful improvements in health and vitality that many have reported. If it helps improve your quality of life, please consider sharing this important information with the people you care about.

We wish you the best in health and vitality.

Advice For Clinicians

Frequent fungal infections like vaginitis (yeast infection), autoimmune skin disorders including seborrheic dermatitis, oral thrush on the tongue, chronic sinus issues, tooth decay and gingivitis, or recurrent infection of the skin, nails or mucous membranes should prompt further diagnostic tests to rule out *Candidiasis*, mold and mycotoxin exposure and genetic predispositions to fungal infections.

Candidiasis generally manifests as frequent yeast infections, fungal nail infections, white or yellow tongue thrush, advanced tooth decay, autoimmune skin disorders and infection of the mucous membranes.

Candida infections can vary in severity, with most testing done for *Invasive Candidiasis*, which is a more serious late stage infection. *Candida* actively works to penetrate the epithelial barrier of the gut, and if successful can be detected using blood culture tests, however culture tests have been shown to be mostly unreliable.

To rule out *Invasive Candidiasis* using non-culture tests, The Fungitell test (Associates of Cape Cod, East Falmouth, MA), multiplex PCR assays and T2Candida nanodiagnostic panel can be used, with 75% to 98% sensitivity. The Fungitell test should be

confirmed by two consecutive tests (80% sensitivity) and does not detect *Cryptococcal* infection [9].

If there is no positivity for *Invasive Candidiasis* and the patient is not immunosuppressed or compromised, a pattern of recurring infection should prompt further investigation. Frequent vaginitis non-responsive to fluconazole treatment, positive sputum cultures for *Candida* or persistent oral thrush that is non-responsive to fluconazole or nystatin oral suspension should prompt genetic testing if dysbiosis can be ruled out.

Candida spp. are associated with a number of specific gene mutations that predispose to fungal infections, impair immune response and improve the likelihood of both chronic infection and disseminated *Candidiasis* [10, 11, 12, 13, 14, 15, 16, 17, 18].

These autosomal dominant traits have been classified under *Familial Candidiasis* [6]. Molecular genetics tests are available to confirm [19].

It is important to stress that if a patient shows positivity for *Familial Candidiasis*, they will need a long-term antifungal solution in addition to an immunomodulatory solution to prevent autoimmune response. The NAC Protocol meets these requirements without the known hepatotoxicity of Amphotericin B or Fluconazole.

The NAC Protocol can be considered as a natural treatment option whenever signs of recurrent fungal infections are presented. The review at the end of this document covers the protocol's anti-fungal, anti-inflammatory, hepatoprotective and restorative benefits.

Additionally, mold exposure at home or in the workplace should be an additional query. *Aspergillus* spp. and their mycotoxins (Gliotoxin, Aflatoxin, Ochratoxin) should be ruled out in cases of chronic inflammation, frequent headaches, chest tightening or asthma, hemoptysis, eye symptoms or lung nodules detected during x-ray or CT scan. Both *Cryptococcus* and *Aspergillus* are frequently misdiagnosed as lung carcinoma [20, 21].

Antibody testing with *Aspergillus*-specific IgG can be used if *Pulmonary Aspergillosis* is suspected. Commercially available tests to detect serum galactomannan (early detection) and 1, 3 β -D-glucan can be used as a non-culture based diagnostic.

Urinalysis detecting the primary mycotoxins (aflatoxin, gliotoxin, ochratoxin) can give a good baseline of exposure [22], especially in absence of known environmental exposure, and can indicate an active infection. Baselines would be lower from food contamination [23].

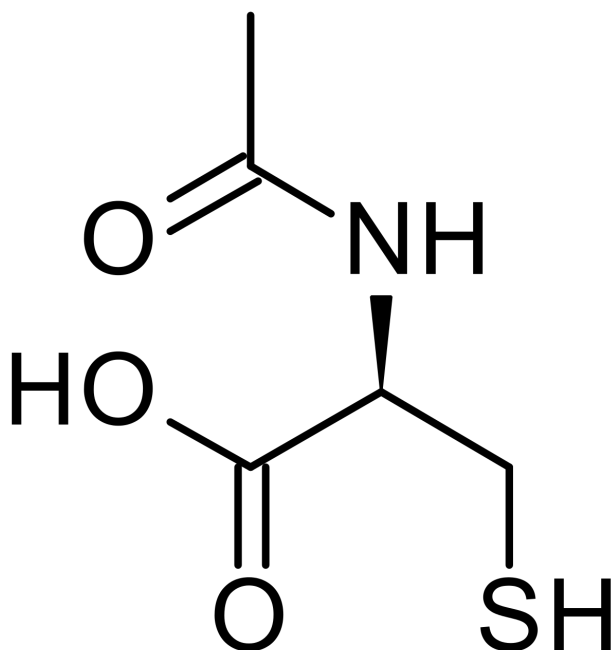


Figure 1: N-Acetyl Cysteine

Science Behind The NAC Protocol

N-Acetyl Cysteine

N-Acetyl Cysteine (NAC) is a derivative of amino acid L-cysteine, used clinically to treat acetaminophen overdose and associated hepatic injury. It's commonly used off label in the treatment of lung conditions, including COPD and cystic fibrosis [24]. The sulfhydryl grouping confers antioxidant effect, and NAC acts as a precursor to glutathione (GSH) production [25].

Primary Benefit And Methodology

N-Acetyl Cysteine (NAC) features a restorative and protective role in The NAC Protocol, both by ameliorating the genomic damage caused by fungal toxins and restoring excision and chemical repair of DNA.

The specific metabolites studied were Aflatoxin, Gliotoxin, Ochratoxin and Acetaldehyde.

Aflatoxin is a secondary metabolite of *Aspergillus*, specifically *A. flavus* and *A. parasiticus* [26]. Aflatoxin B1 (AFB1) is considered hepatotoxic, teratogenic and immunotoxic in humans [27].

Studies on a human epidermal cell line showed that concentrations of AFB1 >10 μ M are toxic to HaCaT cells and induce oxidative stress via ROS¹ and NO² generation [27].

Substantial damage to IMR32 neuronal cell lines was also observed, upregulating NOX2 and triggering DNA damage via downregulation of PARP1,

¹ Reactive Oxygen Species

² Nitric Oxide

BRCA2, and RAD51 [28].

Gliotoxin is also a toxic metabolite of *Aspergillus*, species *A. fumigatus*, and works via uptake of the disulfide bridge, which cycles between oxidized and reduced state, in turn generating ROS and destroying plasmid DNA [22]. Gliotoxin is also responsible for activating ROS-mediated apoptosis, and disrupting the integrity of the epithelial and endothelial barriers to enhance systemic fungal invasion [22].

Ochratoxin (OTA) is produced by multiple species of *Aspergillus* [29]. It is capable of inducing oxidative DNA damage and apoptosis, starting with glutathione depletion. Animal studies suggest that OTA-dependent oxidative stress is the precursor to cell lysing [30]. OTA concentrations were tested on a human renal proximal tubular epithelial cell line (HK-2), further confirming the role of oxidative stress in genotoxicity [31]. A study of OTA genotoxicity on porcine ovarian granulosa cells showed similar response to Aflatoxin, damaging repair related genes PARP1 and RAD51 [32].

Acetaldehyde is a metabolite of *Candida Albicans* resulting from glycolysis [32]. Both ROS and Ca²⁺ pathways are involved in Drp1 phosphorylation and mitochondrial fragmentation. Elevation of Drp1 phosphorylation was partly dependent on ROS-mediated activation of c-Jun-N-terminal kinase (JNK) and p38 mitogen-activated protein kinase (MAPK) [33]. Acetaldehyde chemically induced DNA adducts follow a dose-response relationship, with mutagenicity frequently occurring as aldehyde dehydrogenase reductions become overwhelmed [34, 35].

Guanine is the most frequently oxidized DNA base, causing transversions in DNA replication [36]. O6-methylguanine (O6mG) is a common mispairing, causing GC to AT transversion. Repair of O6mG to guanine is done by O6-alkylguanine-DNA alkyltransferase (AGT), which requires cysteine [37]. 8-Oxo-7,8-dihydroguanine (8-oxoG) is also frequently oxidized, causing GC to TA transversions [36].

A study on mice containing *Ataxia telangiectasia*, which show continuous oxidative stress, showed that Thiol-containing NAC counteracts 8-OH deoxyguanosine, a marker for DNA deletions and genome instability [38]. Further, NAC was also shown to restore O6mG, likely by preventing modification of essential thiol groups [39].

By modulating the intracellular redox state, NAC can directly reduce oxidative-mediated apoptosis and DNA damage, acting as a scavenger for ROS and maintaining reduced glutathione (GSH) production in the liver [40]. Increased levels of GSH from supplementary NAC intake act as a catalyst with Glutathione S-transferases (GSTs) to reduce AFB1 by metabolizing and excreting it [41].

NAC inhibits Gliotoxin-induced apoptosis by blocking activation of caspase-3-like proteases and also scavenging intracellular ROS [42]. With OTA it inhibits apoptosis by preventing glutathione depletion [30].

Finally, NAC binds to Acetaldehyde acting as a scavenger, attenuating ROS and further carcinogenic or genotoxic effect [43].

Anti-Biofilm Activity

The extracellular matrix of biofilms must be considered a target when eliminating fungal infections due to antimicrobial drug resistance and persistence of infections.

Biofilm formation by fungi and bacteria contribute to various pathogenic processes including gastrointestinal diseases, systemic autoimmune diseases, and neurodegenerative diseases [44]. Until more recently it was assumed that biofilms were formed exclusively by bacteria. Various pathogenic fungi can also form biofilms, including *Candida Albicans*, *Cryptococcus neoformans*, *Cryptococcus gatti*, *Aspergillus fumigatus* and *Saccharomyces cerevisiae*. The persistence of fungal infections is greatly enhanced by its ability to form biofilms [45, 46].

NAC is a powerful mucolytic antioxidant that efficiently inhibits and disrupts biofilms. *Pseudomonas aeruginosa* is an encapsulated bacterium that frequently causes infections in humans that are difficult to treat due to quick formation of biofilm.

At a concentration of 0.5 mg/ml NAC can detach mature *P. aeruginosa* biofilms, and at 10 mg/ml biofilms were completely disrupted [47]. A study on treatment of endodontic multi-species biofilms using NAC showed minimum inhibitory concentration (MIC) of 0.78–3.13 mg/ml. Multi-species culture consisted of *Actinomyces naeslundii*, *Lactobacillus salivarius*, *Streptococcus mutans*, and *Enterococcus faecalis* [48].

A study of NAC on *Candida Albicans* biofilm adhesion and disruption showed that NAC works effectively on mature biofilms (50-95% disruption) but less effectively on adhesion ($\geq 32.8\%$). The study also showed increased efficacy when combined with ketoconazole, an antifungal [49].

Cryptococcus Neoformans requires capsular polysaccharide for biofilm formation, which primarily consists of Glucurunoxylomannan (GXM) and is also a constituent of cryptococcal biofilm. These biofilms are composed of 80% GXM which provides a unique challenge [50]. The trans-cell wall vesicular transport system of *Cryptococcus* is dependent on laccase [51], which is susceptible to NAC via a superoxide reaction to copper, converting it to H₂O₂ [52]. This reaction in the laccase containing vesicle and corresponding membrane disruption appear to prevent

further virulence and tissue adhesion.

A study on wound biofilm formation treated with NAC showed interference with bacterial cellular redox states (NADH) and interference with ECM. Disruption of biofilms was primarily due to the molecular structure of NAC with acetyl and carboxyl groups [53].

Protocol Synergy

NAC has shown a synergistic effect with many antifungals, decreasing the MIC values significantly [54]. It is believed that this is due to better penetration through membranes and biofilms due to its mucolytic effect, hydrolyzing glycoproteins and lipids via disulfide bonds and decreasing viscosity [55].

Additional benefit is provided by correcting the imbalance between reactive oxygen species (ROS) and glutathione depletion, which offers a protective effect combined with antifungals. As an inhibitor to c-Jun N-terminal kinase (JNK) it can also reduce endothelial dysfunction, inflammation and invasion [56].

The antifungal activity of Carvacrol induces ROS [57] which is ameliorated by NAC, as it is commonly used in clinical settings to identify and test ROS inducers [58].

NAC plays a supportive role, including ROS scavenging, disulfide reduction and glutathione replenishment.

A recent study on NAC further investigated the method of action and proposed an alternative function for antioxidant activity, suggesting that NAC uptake and deacetylation decelerate and prolong Cys delivery, releasing hydrogen sulfide (H₂S), a product of Cys catabolism. A further product of H₂S, sulfate sulfur species, is also proposed to contribute to NAC's beneficial effects as a cytoprotective [25].

Safety Studies

NAC has a well-established safety profile, and its toxicity is rare. Elimination of NAC occurs through the renal system, with approximately 30% excreted through urine. In oral administration the most reported adverse effects are gastrointestinal symptoms such as nausea, vomiting or diarrhea [59].

Intravenous or oral inhalation can cause more serious adverse effects, including anaphylactoid reactions of flushing, itching, and angioedema, and systemic symptoms, such as bronchospasm and hypotension [60].

Oral dosages of 600mg and 1200mg daily showed no significant increase in adverse effects. Dosages as high as 3000mg daily resulted in minor gastrointestinal symptoms [61].

There was 1 fatal case of anaphylactoid reaction in a 40 year old woman with chronic asthma who

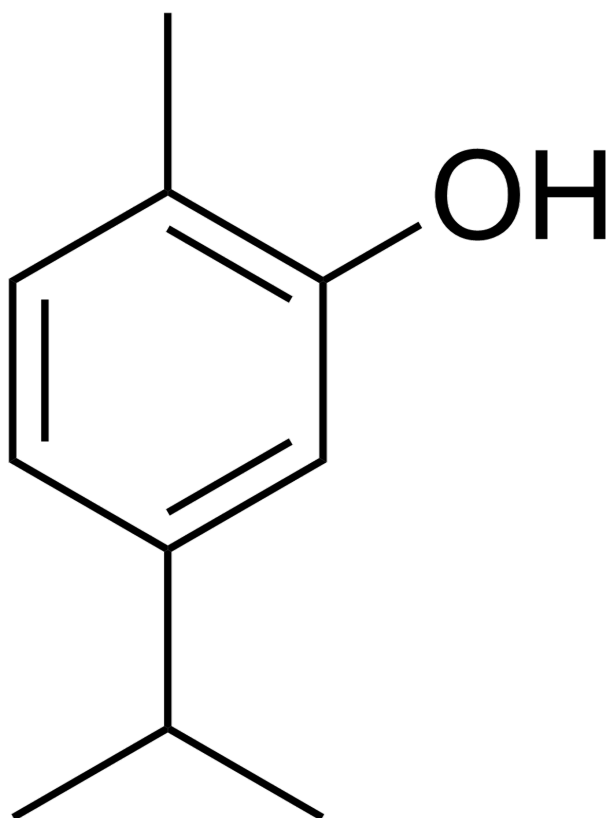


Figure 2: Carvacrol

received intravenous treatment [62]. A potential histamine response with asthmatic patients increases susceptibility to anaphylactoid reactions, and can potentially occur via oral administration [63]. Additionally, review of all available literature found no incidence of sulfa or sulfonamide allergy reactions with administration of NAC.

Reports of NAC preventing apoptosis have been a subject of debate. As an example, NAC can be beneficial to neuronal cells by preventing apoptosis caused by trophic factor deprivation [64] but in other cases can promote tumor progression by downregulating tumor antigen P53 [65]. Thymoquinone provides a counter to this with p53-mediated apoptosis [66], but more importantly is the action of *Origanum Vulgare* (Oregano) as it binds to sterols on the fungal membrane, specifically ergosterols and disrupts the permeability of the membrane leading to apoptosis. The two primary active compounds, Carvacrol [67] and Thymol [68] both contribute to this process.

Oregano Oil

Origanum Vulgare (Oregano) contains two active compounds in abundance, Carvacrol and Thymol. Carvacrol is the primary constituent, a p-menthane monoterpene derived from cymene that provides many benefits to the human body [69].

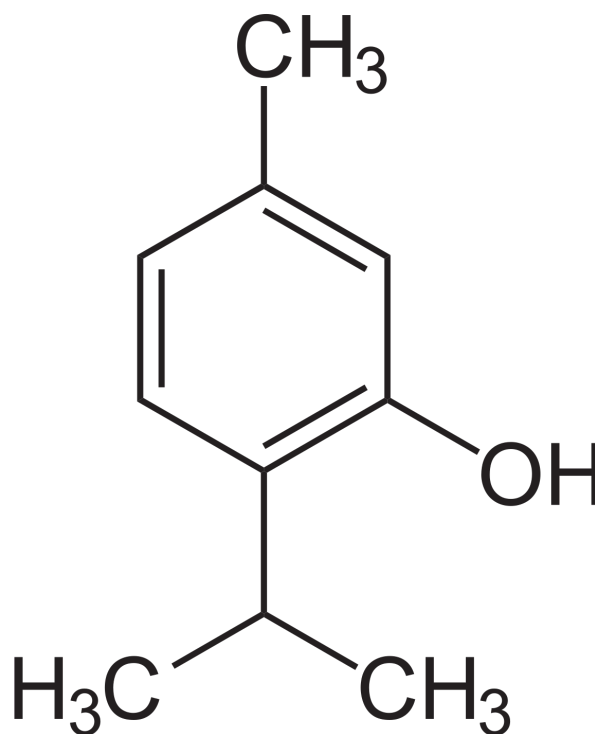


Figure 3: Thymol

Primary Benefit & Methodology

Oregano serves multiple purposes as part of The NAC Protocol, including antifungal, anti-inflammatory and immunomodulatory roles.

Several additional benefits are obtained by using Oregano over other natural antifungals. One instance is dysbiosis, where imbalances in the mycobiota can influence homeostasis and disease progression [70]. Carvacrol works effectively against pathogenic bacteria and fungi [67, 71, 72, 73] to ameliorate dysbiosis. In one study of mice with *C. difficile* infection, Oregano Oil positively altered the microbiome composition, as revealed by an increased abundance of beneficial bacteria and reduced the proportion of detrimental flora [74].

A similar study on weaned piglets found that Oregano Oil supplemented in chow (25 mg/kg) showed a lowered population of *Escherichia coli* in the jejunum, ileum, and colon. They found that Oregano Oil promotes intestinal barrier integrity by correcting dysbiosis and lowering inflammation by measuring mitogen-activated protein kinase (MAPK), protein kinase B (Akt), and nuclear factor κ B (NF- κ B) signaling pathways [75].

A study on oregano oil's effect on the intestinal barrier integrity of Hyland rabbits revealed that oregano essential oil increased significantly the gene expression of junctional adhesion molecule 2 (JAM2) and JAM3 in jejunum ($p < .05$), showing a direct improvement in intestinal barrier function [76].

A study on broiler chickens fed dietary oregano in their feed showed a reduction in *Campylobacter* spp. and *E. coli*, with a significant increase in *Lactobacillus* spp [77]. While another broiler study showed similar results, with *Lactobacilli* raised ($P < .001$) in ileum and cecum of all groups supplemented with Oregano [78].

These additional benefits to dysbiosis and intestinal barrier function were considered when choosing Oregano as the primary antifungal. Altered microbial composition, termed dysbiosis, has been implicated in mucosal barrier dysfunction and inflammatory responses. Restoring the epithelial barrier can potentially prevent autoimmune response and systemic infection [79].

Antibiofilm Activity

The two primary compounds of *Origanum Vulgare*, Carvacrol and Thymol [69], show both powerful inhibitory and disruptive activity against biofilms. Pathogenic fungi can make their own biofilms [80] or cohabitate in multi-species bacterial biofilms where they arrange micro colonies with distinct features [81].

Therefore it is important to address mixed biofilms to effectively treat fungal infections. In a study on *Staphylococcus aureus* and *Candida albicans* in single and mixed cultures, Carvacrol showed a strong decrease of cell count, biomass, metabolic activity, and vitality of established 24- and 48-h biofilms [82]. A synergy was also shown between Carvacrol and Thymol in a similar study on *Candida albicans* and *Staphylococcus epidermidis*, where this combination killed highly tolerant persister cells of mono-species and mixed-species biofilms and demonstrated less risk of resistance development [83]. Effectiveness against *Salmonella Enteritidis* biofilms also showed Carvacrol and Thymol as effective, showing inhibition of biofilm formation at sub-minimum inhibitory concentration and effectiveness against preformed biofilms [84].

Effectiveness was also found against biofilms produced by pathogenic fungi. In a study on oral candidiasis, carvacrol and thymol significantly reduced both mature biofilm biomass and metabolic activity [85]. A study on the antibiofilm and antifungal activity against *Cryptococcus neoformans* and *Cryptococcus laurentii* compared Oregano oil (Carvacrol), Cinnamon oil (Cinnamaldehyde), Lemongrass oil (Citral), Clove oil (Eugenol), Peppermint oil (Menthol) and Thyme oil (thymol). The top 2 compounds for antibiofilm activity were Thymol and Carvacrol, respectively [86].

Method of inhibitory action on biofilms was elucidated in a *Salmonella typhimurium* biofilm study. Proteomic analysis showed changes in the proteins DsbA

(thiol: disulfide interchange protein DsbA), LuxS (S-ribosylhomocysteine lyase), DksA (RNA polymerase binding transcription factor DksA), and SODs (superoxide dismutases) A, B and C showed inhibited synthesis [87].

Antifungal Activity

Origanum Vulgare (Oregano) and its primary constituents Carvacrol and Thymol have shown anti-oxidant, antiseptic, anticarcinogenic, anti-inflammatory, antidiabetic, immunomodulatory, antimicrobial, antispasmodic and antibacterial benefits. Effectiveness against a wide variety of pathogenic fungi and bacteria have been observed [88]. Carvacrol and Thymol are effective antifungal compounds that directly disrupt membrane integrity and ergosterol synthesis against *Candida* isolates [88].

Inhibitory activity against *Candida globosa*, *Candida albicans*, *Cryptococcus laurentii*, *Trichosporon asahii*, *Kodamaea ohmeri* and *Saccharomyces* using an Oregano ethanolic extract showed a MIC value of 1.56 mg/mL [89].

A study of Oregano against *Aspergillus flavus* and *Penicillium commune* as possible alternatives for food preservation showed a MIC of 4 mg/mL [90]. Effectiveness against *Aspergillus niger* and *Aspergillus flavus* was compared between oregano (*Origanum vulgare*), thyme (*Thymus vulgaris*) and clove (*Syzygium aromaticum*), with Oregano showing the highest inhibitory levels [91].

Tests of essential oils against heat resistant molds *Aspergillus fumigatus* and *Paecilomyces variotii* using citrus (*Citrus sinensis* L. Osbeck), laurel (*Laurus nobilis* L.), myrtle (*Myrtus communis* L.), oregano (*Origanum vulgare* L.), and savory (*Satureja thymbra* L.) showed Oregano as the most effective inhibitor of growth [92]. Another study on *Aspergillus niger*, *Aspergillus carbonarius*, and *Aspergillus wentii* showed inhibitory effect of 95.6%, 45.6%, and 100% at 2.5mL/100mL, respectively [93].

Effectiveness of essential oils tested against *Cryptococcus neoformans* and *Cryptococcus laurentii* showed Carvacrol and Thymol as most effective (16 and 32 $\mu\text{g/mL}$) as planktonic inhibitors, compared to Cinnamon oil (Cinnamaldehyde), Lemongrass oil (Citral), Clove oil (Eugenol), Peppermint oil (Menthol) and Thyme oil (Thymol) [86].

Protocol Synergy

There are a number of likely synergies between NAC, Oregano Oil and Black Seed Oil based on available studies.

Thymoquinone (TQ) is the active compound in Black Seed Oil (*Nigella Sativa*). In a study of oral candidiasis, TQ was tested against *Candida albicans*, *Candida tropicalis*, *Candida glabrata* and *Candida krusei*

strains and the synergistic antifungal activity of these strains in combination with nystatin. With TQ alone *C. albicans* was significantly inhibited at 7.5 µg/mL. Nystatin showed inhibition against *C. albicans* at 1.875 µg/mL, but when combined with TQ it lowered MIC to 0.234 µg/mL showing a strong synergy [94]. TQ has also shown a synergistic effect against multi-drug resistant bacteria and fungi when combined with antibiotics [95] or antifungal treatments [96]. We believe there will be a similar synergy between Carvacrol, Thymol, and Black Seed Oil compounds, decreasing inhibitory concentrations and increasing effectiveness against multi-drug resistant fungi.

Determining chemical composition of *N. sativa* shows many potential synergies. Some of the additional active compounds found by GC and GC/MS analysis were trans-anethole, p-cymene and limonene [97]. Carvacrol and p-cymene have shown synergy as compounds, reducing the minimum inhibitory concentration of Carvacrol [98]. Studies on Limonene-Carvacrol (Lim-Car) have also shown synergy in inhibitory concentrations [99].

Synergy between NAC and antifungals has been shown previously with Fluconazole and Caspofungin [100]. Data infers that the mucolytic activity of NAC combined with the antifungal activity of Oregano provide an effective treatment against eukaryotic and sessile forms of pathogenic fungi.

Immune Modulation

Oregano as part of The NAC Protocol acts as an immunomodulatory compound through several mechanisms. Both Carvacrol and Thymol play a role, with Thymol suppressing expression of iNOS and COX-2, blocking the phosphorylation of IκBα, NF-κB p65, ERK, JNK, and p38 MAPK [101]. Carvacrol showed similar effect against pro inflammatory IL-1b, COX-1 and COX-2, while upregulating IL-10 [102, 103] and demonstrating tissue healing ability against gastric ulcers and remodeling ability in a skin disease study. OEO significantly inhibited several inflammatory biomarkers, including monocyte chemoattractant protein 1 (MCP-1), vascular cell adhesion molecule 1 (VCAM-1), intracellular cell adhesion molecule 1 (ICAM-1), interferon gamma-induced protein 10 (IP-10), interferon-inducible T-cell alpha chemoattractant (I-TAC), and monokine induced by gamma interferon (MIG) [104, 105].

Oregano oil supports the immune system overall by also reducing mycotoxin burden via fungicidal action against susceptible pathogens like *Aspergillus* and *Candida*, as shown in **Oregano Oil Antifungal Activity** section

Additional immune modulatory benefit was demonstrated in **Oregano Oil Primary Benefit & Methodology** section, showing how Oregano can

ameliorate dysbiosis issues which disrupt homeostasis and promote disease progression.

Finally, the repair of the intestinal epithelial barrier by reducing inflammation and stabilizing dysbiosis promotes further immune enhancing effect. In all, Oregano is a powerful tool against pathogen-derived disruption of homeostasis and acute inflammatory response.

Safety Studies Oregano is one of the most widely studied natural antimicrobials, with animal studies in vitro and in vivo, as well as human clinical trials, approval as a food additive by the FDA, and used extensively as a food preservative to prevent spoilage.

A Phase I clinical study on the safety of Carvacrol studied 1mg/kg and 2mg/kg groups in a human trial for one month, finding all post-treatment measured parameters were within normal range. The results of this phase I study regarding carvacrol effects on healthy subjects, showed clinical safety and tolerability [106].

A Phase II clinical trial on the possible therapeutic effect of Carvacrol on asthmatic patients also showed no adverse outcomes [107].

Due to strong interest by the food industry for natural options for food preservatives, animal studies are also plentiful. A study on in vivo genotoxic effects produced in rats orally exposed to 81, 256 or 810 mg cavacrol/kg body weight (bw) at 0, 24 and 45 h found that carvacrol (81-810 mg/kg bw) did not induce in vivo genotoxicity or oxidative DNA damage in any of the tissues investigated [108].

Studies on Oregano Oil (OO) and Oregano Essential Oil (OEO) showed similar safety profiles. A study on OEO's oxidant effect (DPPH and ABTS assays) and cytotoxicity found OEO to be nontoxic [109]. A similar study on Wistar rats tested for genotoxicity over a 90 day trial, using 50, 100 and 200 mg/kg administered daily. Results obtained in the genotoxicity assays indicated lack of effect in micronucleus and standard comet assay under the conditions tested, showing no genotoxicity or oxidative damage to tissue [110].

The evidence currently suggests that Oregano Oil is safe for more long-term use, showing no indicators of oxidative damage, genotoxicity, mitochondrial dysfunction or morphological changes in healthy cells. Oregano Oil and its active compounds do show cytotoxicity against cancerous cells, however.

In a study on Acute myeloid leukemia cell lines (AML) carvacrol and thymol showed powerful synergy, inducing tumor cell death with low toxicity on normal cells. Cell death induced by the carvacrol and thymol combination is caspase-dependent in the HL60 cell line and caspase-independent in the other cell lines tested [111]. Furthermore, a study on

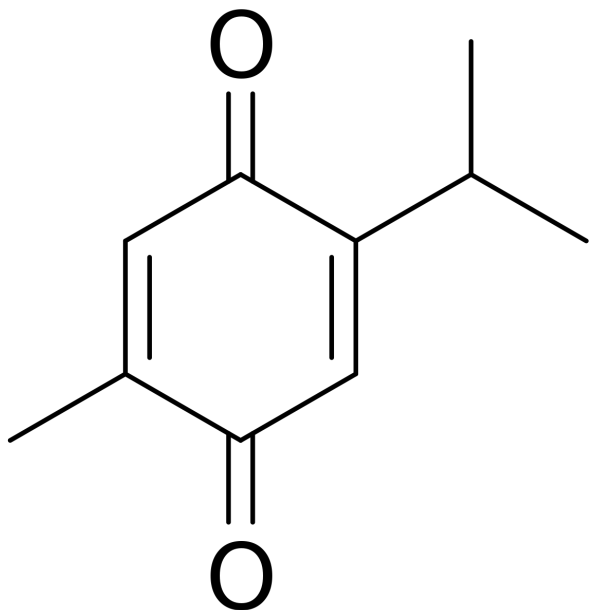


Figure 4: Thymoquinone

F1 DBA C57 Black hybrid mice studied OEO effect on Lewis carcinoma tumor engraftment. Mice were fed a low uptake dose of oregano essential oil with drinking water for three months, showing a tumor engraftment decreased by 1.8 times, its size decreased by 1.5 times, and the development of tumor was significantly suppressed. Interestingly, activity of antioxidant enzymes was found to increase after three months of essential oil uptake (by 1.5–3 times) as compared to the control group [112].

Oregano oil as part of The NAC Protocol is recommended at 40mg Carvacrol twice daily, or 80mg total intake per day. Safe levels were tested up to 600mg per day in the above referenced Phase I trial (2mg/kg), and up to 800mg/kg daily in animal trials showed no cytotoxic effects.

Black Seed Oil

Thymoquinone, derived naturally from *Nigella Sativa*, is a natural compound with widespread protective effects, including anti-oxidative, anti-inflammatory, immunomodulatory, anti-cancer, and anti-microbial [113].

Primary Benefit & Methodology

Black seed oil is typically produced using a cold press process, extracting the active compounds from the *Nigella Sativa* seed. A GC-MS analysis revealed more than 30 active compounds, including thymoquinone, fenchone, p-cymene, trans-anethole, limonene, carvone, carvacrol, longifolene and many additional active compounds [114].

The effect of Black seed oil (BSO) as part of The

NAC Protocol is multi-purpose, serving as hepatoprotective, a potentiator for antifungal activity, a biofilm disruptor, immune modulator, and a restorative which can increase t-cell count and differentiation [115, 98, 116, 117, 118].

A study of doxorubicin-induced cardiotoxicity in rats using 10mg/kg daily TQ in drinking water showed amelioration of induced cardiotoxicity. TQ proved to be a potent superoxide radical scavenger, with scavenging power being as effective as superoxide dismutase against superoxide [119]. A reduction of TQ in the liver to dihydrothymoquinone is part of this antioxidant mechanism, and combined they appear to mediate this protective action [120] and also act as effective OH radical scavenging agents [121]. TQ is known as a scavenger for hydroxyl and carbon centered radicals. It also shortens ROS-facilitated stress by yielding glutathionylated-dihydrothymoquinone via non-enzymatic reaction [122].

Antioxidant and Anti-inflammatory actions of TQ are the primary mechanisms that protect hepatocytes from injury. Myeloperoxidase activity in the liver tissue is an aggravating factor by increasing lipid peroxidation and free radical formation [123].

The hepatoprotective role of BSO is crucial as part of The NAC Protocol.

Antibiofilm Activity

As part of The NAC Protocol BSO acts primarily as hepatoprotective, immune modulatory and an antifungal potentiator. Anti-Biofilm activity is also robust due to the abundant monoterpenes and sesquiterpenes [114]. Minimum biofilm inhibitory concentration (MBIC) for Thymoquinone ranges from 25-100 $\mu\text{g}/\text{mL}$, with *Candida Albicans* being highly susceptible using in vitro assays [124]. *Staphylococcus aureus* and *Staphylococcus epidermidis* minimum biofilm inhibition concentration (BIC50) was reached with 22 and 60 $\mu\text{g}/\text{ml}$, respectively. TQ also prevented cell adhesion [125]. *N. sativa* oil (BSO) showed highest microbial activity when compared to aqueous and methanolic extracts. BSO was also shown to reduce preformed biofilms of multi-drug resistant MRSA 1294, MRSA 1295 and MRSE 1297 effectively [126].

The complexity of bioactive ingredients plays a major role. In a study testing BSO against *Listeria monocytogenes*, a common food contaminant, 30 ligands were tested. α -longipinene was selected based on in silico docking studies. Further in vitro studies demonstrated the anti-biofilm activity of α -longipinene [127]. The complexity of terpenes in the volatile oil likely contributes to its broad spectrum effectiveness. This complexity leads to many potential synergies. p-cymene, a major constituent of BSO based on GC-MS analysis [114] has shown to

have a synergistic effect with γ -terpinene, carvacrol and other active compounds in BSO to increase anti-biofilm activity [128].

Studies on the individual active compounds in BSO show several unique anti-biofilm qualities. Limonene interferes with *C. albicans* biofilm adhesion, while trans-Anethole shows synergy with biofilm inhibition against *S. aureus* [129, 130].

Antifungal Activity

Nigella sativa has been studied extensively for its pharmacological benefits, but antifungal research is limited. In a study of *N. sativa* in a methanolic extract, it was found effective against 20 different strains of *Candida* [131].

A further study on candidiasis of mice using an aqueous extract of *N. sativa* (6.6 mL/kg) showed significant inhibitory effect, only 24 hours after inoculation. A 5-fold decrease in *Candida* in kidneys, 8-fold in liver and 11-fold in spleen was observed [132]. Inhibitory effect on *Aspergillus parasiticus* (CBS 921.7) and *Aspergillus flavus* (SQU 21) was also demonstrated (1-3mg/100ml) using *N. sativa* oil (BSO) with potential metabolic effects on biosynthesis pathways for aflatoxin [133].

Investigating the composition of *N. sativa* volatile oil yields several active compounds, including thymoquinone, p-cymene, α -thujene, limonene, trans-anethole, fenchone and carvacrol [114].

Thymol, thymoquinone (TQ) and thymohydroquinone (THQ), all constituents of *N. sativa*, were tested against 30 pathogens acquired from patients at a concentration of 1mg/mL. 100% inhibition was demonstrated against eight dermatophyte, five yeast and five mold isolates. TQ was found to be the strongest antifungal compound against dermatophytes and yeasts. Thymol was the most effective against molds [134].

A study on human infection by *Fusarium solani*, a filamental fungi from the *Nectriaceae* family, was performed comparing Thymoquinone to Amphotericin B. A 10 day inhibition test using 1mg/mL was performed. TQ demonstrated 100% inhibition by day 10, however Amphotericin B only inhibited 72.4% of growth in the same time range [135].

P-cymene has shown to be effective against drug-resistant forms of *Candida*, showing synergy when combined with Thymol [136].

Trans-anethole also has strong antifungal properties. Fennel is known as a strong antifungal, which is composed primarily of trans-anethole [137]. Trans-anethole has demonstrated effect with other drugs as it exhibits synergistic activity against several fungi [136].

Fenchone was also shown to inhibit fungal growth (32-64 μ g/mL) testing against *Candida albicans* ATCC-

76645 and LM-05, *Candida tropicalis* ATCC-13803 and LM-20, and *Candida Krusei* ATCC-6258 [138]. Limonene was also shown effective against *C. tropicalis* (20-40 μ L/mL) using potato dextrose broth [139].

Protocol Synergy

N. sativa oil (BSO) has shown synergy with both antifungals and antibacterials as a potentiator [95, 96]. Active compounds in BSO have also shown direct synergy with Carvacrol, including p-cymene and limonene [98, 99]. Carvacrol and Thymol, the primary active compounds in Oregano are also featured in BSO [114]. BSO has been found effective against multi-drug resistant *S. aureus*, *P. aeruginosa* and *C. albicans*, and Carvacrol performs similarly [140, 141].

Carvacrol and Thymol concentrations in BSO are in lower concentrations [114] but when adding Oregano, which contains higher levels of Carvacrol and Thymol, two distinct methods of action are present [69]. Thymoquinone has been observed to disrupt *C. albicans* cell wall synthesis, disintegrate the cytoplasm and act as a pro oxidant inducing oxidative stress via ROS generation [142, 143]. Differentially, Oregano disrupts the cell membrane by interrupting ergosterol synthesis [88].

N-Acetylcysteine (NAC) was studied on chronic wound biofilms using mice with a 20 day maturation period. *Pseudomonas*, *Staphylococcus*, *Acinetobacter*, and *Enterobacter* were identified in the wound biofilm. NAC demonstrated effectiveness in disrupting the extracellular matrix of the biofilm, penetrating the bacterial cell membrane, inducing oxidative stress and disrupting protein synthesis [53].

We believe the mechanism of NAC combined with the antifungal compounds in *N. sativa* and Oregano provides a specific advantage when treating fungal and bacterial infections. This combination is crucial with up to 80% of the targeted pathogens residing in biofilms [144].

Immune Modulation

Nigella Sativa has been used in Middle Eastern folk medicine since biblical times, with modern research showing *N. sativa* has effect on respiratory problems, dyspepsia, metabolic syndrome, *diabetes mellitus*, inflammatory diseases, and various types of cancer [145, 146].

The primary purpose of Black Seed Oil (BSO) as part of The NAC Protocol is both hepatoprotective and immunomodulatory. BSO has potent antioxidant effect over several pathways, modulating NF- κ B, inhibiting iron-dependent lipid peroxidation, elevation in total thiol content and (GSH) level, radical scavenging, increasing the activity of quinone reductase, catalase, superoxide dismutase (SOD) and glutathione transferase (GST) and inhibiting COX/LOX

[147, 148].

As an anti-inflammatory, Thymoquinone (TQ) inhibits JNK, ERK and P38 phosphorylation and PI3K/mTOR signaling activation. In addition, BSO was shown to decrease lipid profiles (TG, TC, LDL, VLDL), liver enzymes (AST and ALT), hs-CRP inflammatory marker, IL-6 and TNF- α [149, 150, 151].

As an immunomodulator, BSO can directly improve immune response to fighting infection. A study of immunostimulation on a murine macrophage cell line showed that *N. sativa* ethanolic extract directly increased macrophage count in a cell proliferation assay, showing up to an 138% increase (25 μ g/ml) [152]. An additional study using ethanolic extract on blood derived, splenic and peritoneal macrophages showed a remarkable increase in phagocytic activity [153].

Immunostimulatory effect has also been demonstrated with peripheral blood mononuclear cells (PBMCs), LPS-induced doubling in phagocytic activity and upregulation of p-I κ B α and p-NF- κ B p65 [154, 155].

N. sativa can also enhance survivability in CD8-Positive T Cells by enhancing cytokine interferon- γ (IFN γ) production. A study on the immunomodulatory effect of BSO on rheumatoid arthritis found a positive Modulation of T lymphocytes as well [156, 157].

BSO strengthens immune response, T Cell proliferation and function, supporting the body's response against infection.

Safety Studies

N. sativa preparations have shown to provide gastro-protective, neuroprotective, anti-cancer, anti-diabetic, cardioprotective, bone regenerative and anti-arthritis effect [158, 159, 160, 161, 162, 163].

Several acute and subchronic toxicity tests have been carried out on *N. sativa*. Acute oral administration (LD50) was measured in mice (2.4g/kg) with signs of toxicity being difficulty in respiration and hypoactivity. Acute and sub-acute toxicity was measured in Sprague Dawley rats showing LD50 of 2000mg/kg, with sub-acute dosage of 500mg/kg showing a decrease in AST enzymes. No lethality was observed in all dosage groups (100, 500, 1000 and 2000 mg/kg). Analysis of liver and kidney observed no adverse morphology and BSO was considered safe and non-toxic [164, 165].

A Phase I human clinical trial on the safety of Thymoquinone (TQ) with Patients with Advanced Refractory Malignant Disease. 21 patients received 1 to 20 weeks treatment (median 3.7 weeks) with no side effects reported. No maximum tolerated dose was identified (75mg/day to 2600mg/day) [166].

An additional randomised, double-blinded

placebo-controlled Phase I human clinical trial was carried out on 70 individuals for a period of 90 days. Blood and serum collection tests were performed. Liver function parameters included alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP). Lipid profiles included Total cholesterol (TC), Low density lipoprotein (LDL), High density lipoprotein (HDL), Very low-density lipoprotein (VLDL) and Triglycerides (TG). Renal function markers (creatinine) were also tested. Recruited participants did not exhibit any clinical signs of toxicity or adverse effects. Liver toxicity and kidney function markers showed no change, however, lipid profiles showed significant decrease but were within safe limits. Change in TC, TG, LDL, VLDL and HDL were 12.1%, 19.66%, 16.33%, 12.76%, 8.21% and 15.27%, respectively [167].

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