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In-Vitro Analysis and Marker Based Standardization of *Nigella sativa* Seed Extract For Its Antioxidant Activity.

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ABSTRACT

The present research work was carried out to assess anti-oxidative potential of *Nigella sativa* seed extract by various in-vitro methods. It is common ingredient of Indian kitchen spices. The in vitro antioxidant activities were evaluated at concentration range 8-200 µg/mlby using various standard methods such as DPPH, Xanthine oxidase inhibitory activity, Ascorbate iron induced lipidperoxidtion, Inhibition of pyrogallol red by peroxynitrite, Non enzymatic activity and superoxide radical scavenging assay. *Nigella sativa* seed extract exhibited a very good antioxidant activity with concentration range 16-80µg/ml at IC_{50} 51.9 µg/ml value against peroxidation. Among the seven different performed in-vitro antioxidant assays, *Nigella sativa* extract showed potent antioxidant property against lipid peroxidation. Along with biological evaluation analytical standardization was carried out by using HPTLC and thymoquinone as marker compound.

Keywords: In-vitro, Nigella sativa, antioxidant.

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INTRODUCTION

It is widely recognized that the reactive oxygen species (ROS), or free radicals, are;molecular specieswith an odd, unpaired electron in an atomic orbital.(1)Examples include the superoxide radical anion (O_2), hydrogen peroxide (H_2O_2) and hydroxyl radicals (HO.), as well as organic molecules such as alkoxyl and peroxyl radicals that are continuously produced either from normal essential metabolic processes in the human body or from external sources such as exposure to X-rays, ozone, cigarette smoking, air pollutants, and industrial chemicals.(2) At low or moderate concentrations, ROS and reactive nitrogen species are necessary for the maturation process of cellular structures and cellular signaling pathway.(3) When produced in excess, free radicals generate oxidative stress.An excess of oxidative stress can lead to the oxidation of lipids and proteins, which is associated with changes in their structure and functions and DNA damage leading to a variety of chronic health problems, such as cancer, aging, Parkinson's disease, Alzheimer's disease and amyotrophic lateral sclerosis.(4)To prevent the damage, body has a defense system of antioxidants. Antioxidants are molecules which can safely interact with free radicals and terminate the chain reaction before vital molecules are damaged.At present toxicity of synthetic antioxidants such as ascorbic acid, butylatedhydroxy toluene, butylated hydroxyl anisole are documented.(5) Therefore, there is glowing interest in finding naturally occurring antioxidant due to their high impact on human health and disease prevention. Dietary antioxidant intake may be an important policy for inhibiting or delaying the oxidation of susceptible cellular substrates.(6)Now day'sphenolic, flavonoid, polyphenolic compounds have gained attention for their high anti oxidative activity.

Nigella sativa.Linn. belonging to family Ranunculaceae, (black cumene seeds) grows in the Mediterranean region and in western asian countries including India, as herb of 20-30 cm height.It is a common ingredient of Indian kitchen spices. Chemically, the seeds are rich in 30% fixed oil and 0.4-0.45% (w/w) volatile oil, including 4–24% thymoquinone (TQ) and 46% of many monoterpenes such as r-cymene and a-piene. Many researchers using an HPLC technique, were able to identify four major compounds; thymoquinone (TQ), dithymoquinone (DTQ), thymohydroquinone (THQ), and thymol (THY), in the oil of *Nigella sativa* seed (NSO)(7)

Much of the biological activity of N. sativa has been shown to be due to TQ, which is now considered the active component of its essential oil.(8-13) The beneficial medicinal effects of NSO and TQ have been attributed to their radical scavenging (anti-oxidative) activity, anti-proliferative and cytotoxic effects, antidiabetic activity, TQ is considered as potent anti-oxidant anti-cancer and anti-mutagenic agent.

Though the herbal bioactives are proved to be very effective against many aliments, they are prone to adulteration and substitution. This makes standardization of any herbal product a mandatory event. Standardization of herbal formulations in terms of quality of raw materials, manufacturing practices and composition is important to ensure quality and optimum levels of active principles for their bio-potency.

In our present research work, we have collaborated biological (extensive in-vitro antioxidant study) in and analytical (maker based standardization) evaluation of *Nigella sativa* seed extract to result in a comprehended commercial product.(7 and 14)

COLLECTION OF PLANT MATERIAL

Nigella sativa seed extract was gifted by Amsar Goa Pvt Ltd.

CHEMICALS AND REAGENTS

1,1-Diphenyl-2-picrylhydrazyl(DPPH), 2,2'-azinobis-(3-ethylbenzothiazoline)-6-sulphonic acid (ABTS), Allopurinol, Bovine Brain Extract (BBE), Bovine Serum Albumin (BSA), were procured from Sigma Chemical Co. (USA). Xanthine oxidase, Nitrobluetetrazoliumchloride (NBT) and Xanthine were procured from Himedia Ltd. Mumbai, India. Folin-Ciocalteusolution, Pyrogallol Red, Potassium dihydrogen phosphate and thiobarbituric acid, dipotassium hydrogen phosphate, trichloroacetic acid, anhydrous sodium carbonate, ascorbic acid, potassium persulfate, ascorbate, ethylene di-aminetetraacetic acid (EDTA), were purchased from S.D.Fine Chemicals, Mumbai, India. Standard thymoquinone was procured from Sigma Chemical Co. (USA).

All other chemicals and solvents used were of analytical grade.

INSTRUMENTS

The instruments used for the study were UV spectrophotometer (Jasco, V-630), laboratory centrifuge (Remi motors, R4C) and, Digital pH meter (Equip-tronics, EQ-610), Spectrofluorometry (Qubit 3.0), HPTLC (Camag).

IN-VITRO ANTIOXIDANT ASSAY

SCAVENGING OF 2, 2-DIPHENYL-1-PICRYLHYDRAZYL

Stock solution of DPPH was prepared by dissolving 150 mg of DPPH in 500 ml methanol. Working solution was prepared by taking different concentration of extract with DPPH individually. The change in deep violet color of solution was then analyzed at 520 nm spectrophotometrically. Reference standard compound being used was ascorbic acid and experiment was done in triplicate. The IC_{50} value of the sample, which is the concentration of sample required to inhibit 50% of the DPPH free radical was calculated. Higher free radical activity will be indicatedlower absorbance of the reaction mixture. The percent DPPH scavenging effect was calculated by using following equation. DPPH scavenging effect (%) or Percent inhibition was $A_0 * A_1 / A_0 \times 100$. Where A_0 was the Absorbance of control reaction and A_1 was the Absorbance in presence of test or standard sample[15-17].

ABTS RADICAL SCAVENGING ASSAY

ABTS solution (2mM) and potassium persulphate solution (17mM) were prepared separately, mixed and allowed to stand in dark for about 12-16 hrs. This results in ABTS cation radical formation. Different concentrations of extract solution were treated with above ABTS cation solution. Absorbance was recorded at 750nm[16].

ASCORBATE IRON INDUCED LIPID PEROXIDATION

Bovine Serum Albumin (BSA) suspension in phosphate buffer (pH 7.4) was prepared by ultra-sonication. Ferric Chloride solution (1mM), Extract Solutions, phosphate buffer (pH 7.4) and Ascorbate solution (1mM) were mixed together and incubated at 37^0C for 1 hr. After incubation, trichloroacetic acid(10%) was mixed and centrifuged at 1800 rpm for 10 min. 4 ml of supernatant liquid is treated with thiobarbituric acid (0.67%), boiled at 100^0 C for 20 min. Rapidly cooled and measured the absorbance at 532nm.

XANTHINE OXIDASE ASSAY

The inhibitory activity of nigella sativa seed extract was evaluated at different concentrations. The absorbance was read at the 295 nm by spectrophotometrically. The assay mixture consisted of 1 ml of test sample with 2 ml of xanthine and incubated at 37^0C for 15 min, followed by addition of 0.2 ml xanthine oxidase. Another reaction mixture was prepared (control) having water/DMSO instead of test compounds in order to have maximum uric acid formation. The resulting solutions were incubated at 25^0C for 30 min. Xanthine oxidase inhibitory activity was expressed as the percent inhibition of xanthine oxidase, calculated as (1-At/Ab)*100 where At-Absorbance of test solution and Ab-Absorbance of blank and value were compare with the standard Allopurinol [15-17].

SUPER OXIDE ANION RADICAL SCAVENGING ACTIVITY

Measurement of superoxide anion scavenging activity of extractwas based on the reduction of nitrobluetetrazolium (NBT). In this experiments, reaction mixture consisted of 3 mL of Tris-HCl buffer (16 mM, pH 8.0) containing 1 mL of NBT (50 mM) solution, 1 mL hypoxanthine (3 mM),and 0.5ml xanthine oxidase with various concentration of extract .The reaction started by adding 1 mL of phenazinemethosulphate (PMS) solution (10 mM) to the mixture. The reaction mixture was incubated at 25 ^{o}C for 30 min. The absorbance was readed at 560 nm by spectrophotometer was measured against blank samples. L-Ascorbic acid was used as a

control. Decreased absorbance of the reaction mixture indicated increased superoxide anion scavenging activity. The percentage inhibition of superoxide anion generation was calculated using the following formula.

$$\% \text{ Inhibition} = [(A_o - A_1) / A_o]/100$$

Where A_o=absorbance of the control, and A_1= absorbance of extract and standards.

BLEACHING OF PYROGALLOL RED BY PEROXYNITRITE

Peroxynitrite solution was prepared by reacting hydrogen peroxide, Nitric acid, and sodium nitrite (2M each) followed by addition of 4 M sodium hydroxide in frozen conditions (at -70^0 C). Pyrogallol red solution (100µm) was prepared using 100mM phosphate buffer (pH 7.4). Peroxynitrite solution was then reacted with different concentration of extract solution and Pyrogallol solution with immediate vortexing for 15 min. Absorbance was measured at 540.

NON ENZYMATIC ASSAY

The absorbance was measured by spectrofluorometry after two week incubation of the sample solution with bovine serum albumin (1 mg/ml), 200mol/L glucose, 0.2mol/L Phosphate Buffer solution and 0.01 % sodium azide. After two weeks the absorbance was recorded at 450 nm and 350nm filter [15-17].

STANDARDISATION OF EXTRACT BY HPTLC

The sample solutions were spotted in the form of bands of width 6 mm with a Camagmicrolitre syringe on precoated silica gel aluminium plate $60F_{254}$ (20 cm × 10 cm with 250 µm thickness; E. Merck, Darmstadt, Germany, supplied by Anchrom Technologists, Mumbai) using a CamagLinomat V (Switzerland). The plates were pre-washed by methanol and activated at 60°C for 5 min prior to chromatography. The slit dimension was kept at 5mm × 0.45 mm and 10 mm/s scanning speed was employed. The slit bandwidth was set at 20 nm, each track was scanned thrice and baseline correction was used. The mobile phase consisted of*n*-hexane-ethyl acetate and 15 ml of mobile phase was used per chromatography. Linear ascending development was carried out in 20 cm x 10 cm twin trough glass chamber (Camag, Muttenz, Switzerland) saturated with the mobile phase. The optimized chamber saturation time for mobile phase was 30 min at room temperature (25°C ± 2) at relative humidity of 60% ± 5. The length of chromatogram run was 8 cm. Subsequent to the scanning, TLC plates were dried in a current of air with the help of an air dryer. Densitometric scanning was performed with Camag TLC scanner III in the reflectance-absorbance mode at 282 nm (λ_{max} of thymoquinone) and operated by Win CATS software (1.3.0 Camag). Concentrations of the compound chromatographed were determined from the intensity of diffusely reflected light. Evaluation was carried out by comparing peak areas with linear regression.

Standard stock solutions were prepared by dissolving the reference standard in methanol to obtain a concentration of 1mg/ml for thymoquinone. The concentrations of thymoquonone reference standards used for calibration were 0.5, 1.0, 1.5, 2.0, 2.5, 3.0 mg/spot in methanol, respectively. Sample solution of extract was of concentration 1mg/ml prepared in alcohol. The peaks in HPTLC fingerprints were identified by comparing the retention times in the chromatograms of extract sample with the reference standard Thymoquinone peak.

RESULTS AND DISCUSSION

IN VITRO ANTIOXIDANT ASSAY

SCAVENGING OF 2, 2-DIPHENYL-1-PICRYLHYDRAZYL

DPPH• (2,2-diphenyl-1- picrylhydrazyl) is a stable free radical, due to the delocalization of the spare electron on the whole molecule. The delocalization on the DPPH• molecule determines the occurrence of a purple color, with an absorption band with a maximum around 520nm. When DPPH• reacts with a hydrogen donor, the reduced (molecular) form (DPPH) is generated, accompanied by the disappearance of the violet

color. Therefore, the absorbance linearly depends on antioxidant potential.*Nigella sativa* seed extract scavenged DPPH radicals at IC_{50} value 55.47µg/ml.

ABTS RADICAL SCAVENGING ASSAY

The potassium persulfate radical can oxidize ABTS (2,2-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) to generate a radical cation, ABTS+•,Chromophore that is blue or green in color and can be measured by absorbance at 405nm. Antioxidants react with ABTS+• by donationg e- and thus diminish the concentration intensity of cation radical. The concentration of antioxidant in the test sample is inversely proportional to the ABTS+• radical formation and 750nm absorbance. The % radical-scavenging activity of extract was determined using the formula

[(Acontrol–Atest)/ Acontrol] X 100,

Where A control is the absorbance of the control (ABTS+• solution without test sample) and Atest is the absorbance of the test sample (ABTS+• solution with extract).Ascorbic acid was used as standard antioxidants for comparison. All the tests were carried out in triplicate.

Extract of exhibited ABTS scavenging activity at IC50 values 88.76µg/ml with concentration range 40µg/ml-200µg/ml.

ASCORBATE IRON INDUCED LIPID PEROXIDATION

The end products of lipid peroxidation is malondialdehyde (MDA), may be mutagenic and carcinogenic. Malondialdehyde is produced in oxidative degeneration. Malondialdehyde is accepted as indicator in lipid peroxidation thus the level of MDA need to be checked in lipid peroxidation system.

Nigella sativa seed extract exhibited a good antioxidant activity with concentration range 16-80µg/ml at IC_{50} 51.9 µg/ml value against peroxidation of bovine brain extract, by ascorbate ions thus it can protect cell membrane against lipid peroxidation.

XANTHINE OXIDASE ASSAY

Xanthine oxidase is a flavoprotein, which catalyses the oxidation of hypoxanthine to xanthine and generates superoxide and uric acid. Xanthine oxidase-derived superoxide anion has been linked to various degenerative and metabollic disorders. Xanthine oxidase activity was determined by formation of uric acid from xanthine. Extract does show significant inhibition of xanthine oxidase from concentration rang15.6µg/ml-250µg/ml at IC_{50} 117.3 µg/ml.

SUPER OXIDE ANION RADICAL SCAVENGING ACTIVITY

Generated superoxide radicals can be measured by the ability to reduce NBT. The decrease of absorbance at 560 nm with antioxidants indicates the consumption of superoxide anion in the reaction mixture.

The abilities of the plant extract and the reference compound to quench superoxide radicals from reaction mixture is reflected against the absorbance at 560 nm. Extract inhibited the superoxide radicals at IC_{50} value 107.86µg/ml.

BLEACHING OF PYROGALLOL RED BY PEROXYNITRITE

Peroxynitrite ($ONOO^{\bullet}$) is a cytotoxicant with strong oxidizing properties. Peroxynitrite radical is a relatively stable species compared with other free radicals but once protonated gives highly reactive peroxynitrous acid (ONOOH).

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Pyrogallol red is dye which gets bleached by peroxynitrite. *Nigella sativa* seed extract is reacted with the free radical peroxinitrite and inhibit bleaching action with least IC_{50} 63.79µg/ml with concentration range 8µg/ml-128µg/ml.This results in decrease in the intensity of red color dye.

NON ENZYMATIC ASSAY

Non enzymatic glycation is the result of the typically covalent bonding of a sugar molecule, to a protein or lipid molecule, without any control action on enzyme. Non enzymatic reaction is an advanced glycation end products (AGEs) are proteins or lipids that become glycated as a consequences of glycated products. *Nigella sativa* seed extract inhibited glycoxidation at concentration range 40-200µg/ml with IC_{50} 109.09 µg/ml.

STANDARDISATION OF EXTRACT

The method employed for analysis of extract was able to give a well resolved paek of reference standard thymoquinone .Extract of Nigella sativa seed of was quantitatively determined using the developed HPTLC method. Each sample was analysed in triplicate to determine the mean content of thymoquinone. The amount obtained after analysis in extract was (10.8%).This indicates that there no loses of bioactive compounds of extract.

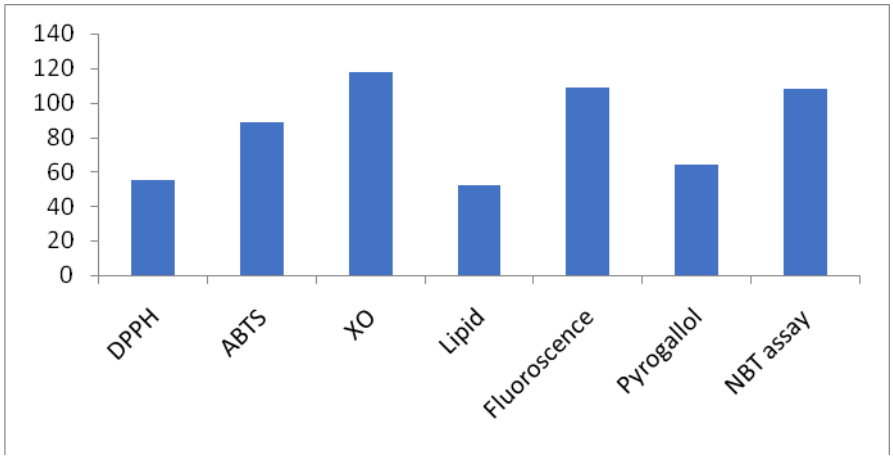

Fig 1: IC_{50} in µg/ml of standardized nigella extract

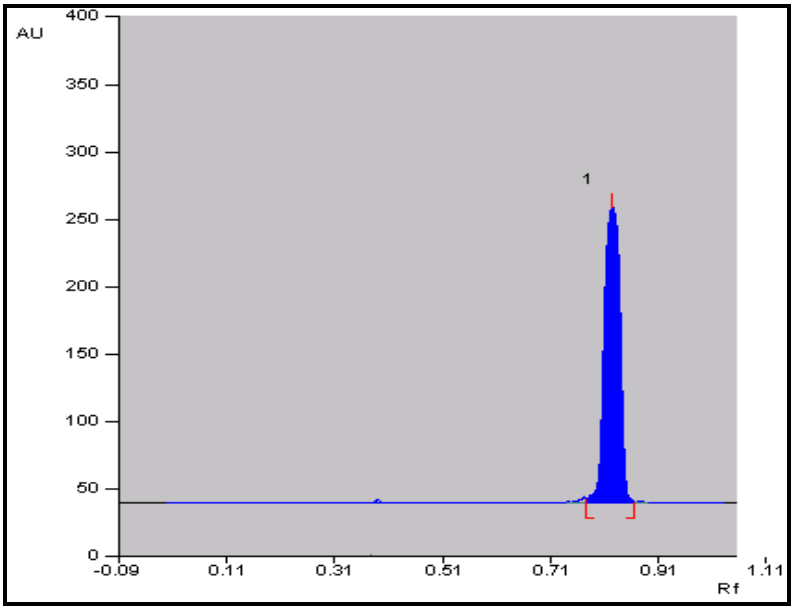

Fig 2: Chromatogram of isolated thymoquinone from the Nigella extract

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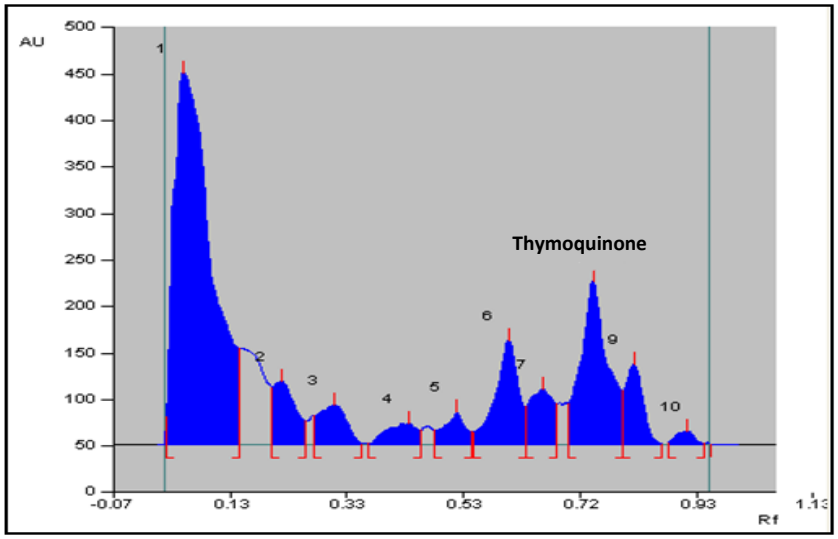

Fig 3: Chromatogram of standradized Nigella extractby thymoquinone

CONCLUSION

Nigella sativa seed extract was evaluated for its antioxidant properties by large of in vitro methods. Different methods help to lead the mechanism of action of any antioxidant. In our study it was observed that *Nigella sativa* seed extract(NSSE) has potential to inhibit anti-oxidative action. In our study it was observed that NSSE inhibited lipid peroxidation at least IC_{50} value 51.9 µg/ml and also it showed least inhibition of xanthine oxidase at IC_{50} 117.3 µg/ml.

Thus we would comment that NSSE has potential to inhibit lipid peroxidation. Geographical changes bring changes in phytochemical content of almost all plant [18]. Adulteration substitution and extraction procedure are also the reason to lower the efficiency of the plant extract. Thus, to ensure the potency of any extract it is necessary to standardized the extract with validated analytical method by using biological marker compounds.

This is the reason we attempted to standardize our commercial extract by thymoquinone as marker compound using validated HPTLC method. The *Nigella sativa* seed extract used showed 10.8% of thymoquinone.

In totality we can conclude that the commercial extract studied has excellent antioxidant effect with 10.8% TQ content.

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