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Effect Of Extraction Techniques On Antioxidant Activity, DNA Protection Potential And Antimicrobial Properties of N. sativa (Kalonji) seed extract

[1]Gayatri Barabde , [2]Aqeela A. S. Qureshi
[1,2]*Department of Chemistry, The Institute of Science, University of Mumbai , India.*
Corresponding Author:Gayatri Barabde

----------*ABSTRACT*----------
In the present study, the extracts of Nigella sativa seeds were prepared by Soxhalate, Hot and Cold extraction methods using water and methanol as solvent. The free radical scavenging activity of all extracts were investigated by DPPH (2,2-diphenyl-1-picrylhydrazyl). The super coiled pBR322 plasmid was used to study the protection of DNA against H_2O_2 - induced oxidative damage. Antimicrobial activity of each extract was determined by Well Diffusion method. The results of DPPH and DNA damage assay revealed that methanol is better solvent than water and the Soxhalate method and Hot extraction methods are effective techniques to get antioxidant rich extract. Methanolic extract prepared by Soxhalate method showed activity against E. coli. The extract obtained by cold extraction technique showed positive antibacterial activity and least antioxidant activity.

KEYWORDS *- Antimicrobial activity, Antioxidants, DNA Damage , DPPH*

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I. INTRODUCTION

Nigella sativa (N.S.) is an annual herbaceous plant from Ranunculaceae family. The black seeds of this plant known as kalonji or black cumin have aromatic odor and taste. N.S seeds are the common drugs used in Ayurvedic medicine and Tibbe-Nabvi (Prophet's Medicine) throughout the world. Prophet Muhammad once stated that the black seed has the cure for every illness except death[1]. N. sativa seeds are used in the treatment of diseases such as asthma, bronchitis [2], inflammatory diseases [3] and antifungal [4].

The medicinal properties of N. sativa is due to its ability to prevent cellular damage caused by oxidative stress. The antioxidant properties of black seeds were investigated by many researchers [5, 6, 7] .The research has reported that the antioxidants can prevent damage to organism caused by free radical induced oxidative stress [8]. An antioxidant is defined as a compound capable of preventing the biological oxidative damage [9]. It has been shown that antioxidants play an important role in prevention of DNA damage[10] . The natural antioxidants are more preferred than synthetic antioxidants due to low cost and less risk of side effects.

The researchers have also reported that the black cumin seeds possess antibacterial activities against multi-drug resistant bacteria [11, 12] .The past research has revealed that the essential oils of spices have antimicrobial activity against food borne pathogens [13]. They also show antimicrobial and antioxidant activities [14,15,16]. The constituents of medicinal plants, herb and spices have been shown to possess antimicrobial activity and act as a source of antimicrobial agents against food pathogens [17].

The extraction techniques play a crucial role in enhancing the medicinal property of extract. Hence the aim of present study was to prepare antioxidant rich extract from N.sativa seeds by different extraction methods and to assess the antioxidant, antimicrobial and DNA protective activities of extract.

II. MATERIAL AND METHODS

Plant material

The seeds of N. sativa were purchased from local market from Thane, Maharashtra, India.

Chemicals

DPPH extra pure purchased from SRL Pvt. Ltd. Agarose from Sigma chemicals and Ethidium bromide (EtBr) from HiMedia. Dipotassium hydrogen phosphate, potassium dihydrogen phosphate and hydrogen peroxide were purchased from SD Fine Chemicals Ltd. pBR322 was procured from Thermo Fisher. 0.25% Bromophenol blue, Xylene Cyanol FF 0.25%, Glycerol 30% , Tris base, Glacial acetic acid, EDTA , Methanol, GYEA (Glucose Yeast Extract Agar). All reagents and chemical used were of Analytical grade. The bacterial and fungal cultures Staphylococcus aureus, Escherichia coli, Bacillus cereus, Pseudomonas aeruginosa,

Aspergillus spp., Candida albicans were selected for testing antimicrobial activity.The cultures were procured from Department of Microbiology, Royal College, Mira Road.

Preparation of Extract

The seeds of N. sativa were dried at room temperature. Their aqueous and methanolic extracts were prepared by three methods described below.

Soxhlet Extraction

15g of seeds were soaked for overnight in 60ml solvent. The seeds were loaded in Soxhalate extractor using apx 50ml solvent. The extraction was carried out for 6 hours. Using pump ice cold water was circulated through condenser. The extract collected was dried and weighed . The extraction was carried out twice using water and methanol as solvent. The brownish residue obtained was dissolved in known quantity of methanol.

Cold Extraction

15g seeds were soaked in 80ml water for 24 hours. The solution was mixed in a shaker at 240 rpm for 6 hours. After shaking, mixture was stored in refrigerator at 4^0C for 15 hour. The cold solution was filtered using Whatmann filter paper. The filtrate was dried at 35^0C in water bath and extract was prepared by dissolving known quantity of residue in methanol. The procedure was repeated using methanol as solvent.

Hot Extraction

The seeds which were soaked in solvent overnight , were boiled gradually for 15 minutes. Then solution was kept in water bath at 50^0C for 6 hours. The solution was filtered using Whatmann filter paper. The extract was dried in thermostat at 35^0C and known weight of residue was dissolved in methanol. The method was followed twice to prepare aqueous and methanolic hot extract.

All the extracts were stored in glass bottles covered with metal foil and kept in refrigerator.

DPPH (1, 1-diphenyl-2- picrylhydrazyl) radical scavenging assay

Free radical scavenging activity of the extracts were measured using the stable free radical DPPH by slightly modified method of Miliauskas et.al [18,19]. The DPPH solution (0.005%) was prepared in methanol and initial absorbance was adjusted between 0.9 – 1.0 at 515nm by adding methanol. 0.5 ml of each solution of different concentrations (100 -1000µg/ml) of the aqueous N. sativa seed extract was added to 3 ml of DPPH solution. The control was prepared by mixing 3ml DPPH with 0.5ml methanol. Each solution was mixed and after 30 minutes the absorbance at 515nm was measured using Spectrophotometer. The solution of ascorbic acid (5 -100 µg/ml) was used as standard. The experiment was performed in triplicate. The % inhibition was calculated using equation

$$\%\ Inhibition = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

DNA Damage Inhibition Efficiency

The ability of each extract to prevent DNA damage was tested by the method of Lee et al., with slight modification[20]. The study was done by photolyzing pBR322 plasmid DNA in the presence of H_2O_2 and performing agarose gel electrophoresis with the irradiated DNA. The plasmid (0.25 µg) in 100 mM potassium phosphate buffer (pH 7.4) was incubated with H_2O_2 (60 mM) in the presence and absence of plant extract (10 µg). The tubes were incubated for three hours at 37°C. pBR322 plasmid DNA in 100 mM potassium phosphate buffer (pH 7.4) along with water/methanol was kept as a control.

Antimicrobial Assay

Agar well diffusion method was followed to determine the antimicrobial activity. Wells were made in nutrient agar plate using four bacterial cultures Staphyllococcus aureus, Escherichia coli, Bacillus cereus and Pseudomonas aeruginosa and two fungal cultures Aspergillus spp. and Candida albicans. The aqueous extracts were prepared of concentration 1000 µg/ml and 10,000 µg/ml . 50µl of extracts were added along with control using micropipette into the wells and allowed to diffuse in refrigerator for 10 minutes. The plates were incubated at 37^0C for 24 hours. The plate of Aspergillus spp., was incubated at room temperature for 48 hours. The diameter of inhibition zone (mm) was measured. The analysis was done in duplicate for each extract.

III. RESULTS AND DISCUSSION

DPPH radical scavenging activity

A stable free radical DPPH can accept an electron/hydrogen to become a stable diamagnetic molecule [21]. The deep violet methanolic solution of stable free radical 2,2- Diphenyl-1-picrylhydrazyl (DPPH˙) shows maximum absorbance at 517nm . The antioxidants donate proton to DPPH radical thereby decreasing absorbance. The scavenging of radical is noticeable through a colour change from violet to yellow. Hence, DPPH is extensively used as a substrate to evaluate the antioxidative activity.

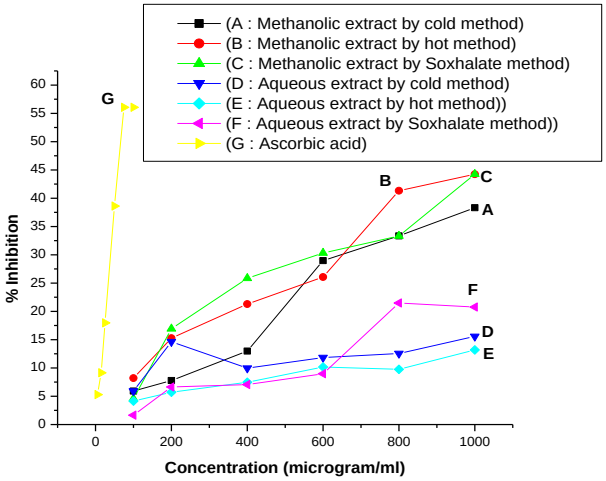

Figure : 1 DPPH Scavenging assay of Aqueous and Methanolic extract of N. sativa seeds

Fig. 1 shows the antioxidant activity of all the extracts in comparison with the ascorbic acid. The results indicate that all the extract reduces the radicals to the corresponding hydrazine. The antioxidant activities exhibited by methanolic extract are more than aqueous extract. The methanolic soxhalate extract shows significant scavenging property at lower concentrations but at higher concentration both methanolic extract prepared by Soxhalate method and hot extraction method shows similar antioxidant activity.

DNA Damage Assay

The results obtained through gel electrophoresis for the effect of extracts of N. sativa seeds on DNA damage are shown in Fig.2. The incubation of pBR322 in the presence of H_2O_2 resulted in strand breakage by converting the supercoiled form into circular form (Fig.2 : Lane 3) .The extracts prepared in both the solvents and by all the methods at concentration (10 µg/mL) reversed the damage of DNA which has been induced by H_2O_2 (Fig. 2; Lane 4- Lane 9). However lane 5 & 7 has shown better antioxidant activity compared to lane 8 and 9.

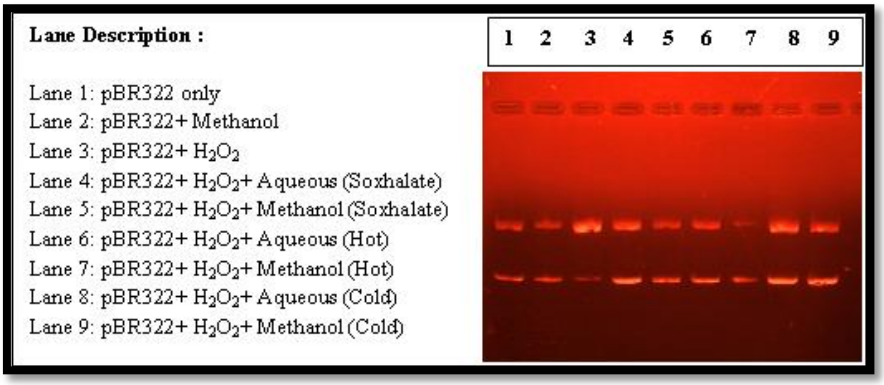

Figure.2. Effect of N.sativa extract on DNA damage

Antimicrobial Activity

In the present study, the antimicrobial activities of all six extracts were examined against four bacterial strains and two fungal strains. Results obtained by agar well diffusion technique, as a qualitative method, are summarized in Table 1.

Table 1 : Antimicrobial Activity of N. sativa extracts by Well diffusion method

N. sativa Extracts Concentration (1000ppm)		Zone of Inhibition (mm)					
		Bacterial species				Fungal species	
		S. aureus	B. cereus	Pseudomonas aeruginosa	E. coli	Aspergillus spp.	C. albicans
Method	Solvent						
Soxhalate	Methanol	-	-	-	10	-	-
Hot	Methanol	-	-	-	-	-	-
Cold	Methanol	-	10	-	-	-	-
Soxhalate	Water	-	-	-	-	-	-
Hot	Water	-	-	-	-	-	-
Cold	Water	21	-	-	-	12	-
10000 ppm							-
Soxhalate	Methanol	-	-	-	10	-	-
Hot	Methanol	-	-	-	-	-	-
Cold	Methanol	-	10	-	-	-	-
Soxhalate	Water	-	-	-	-	-	-
Hot	Water	-	-	-	-	-	-
Cold	Water	21	-	-	-	-	-

The methanolic Soxhalate extract and cold methanolic extract showed activity against E.coli and B.cereus respectively at both concentrations. The water extract prepared by cold extraction technique showed activity at 1000 ppm against S.aureus and Aspergillus spp. whereas at 10,000ppm it showed activity only against S.aureus. Candida albicans and Pseudomonas aeruginosa was found to be resistant to all extracts.

IV. CONCLUSION

The results of DPPH and DNA damage assay revealed that all extracts of N. sativa possess antioxidant property. The methanolic extract prepared by Soxhalate and hot method had similar scavenging potential which was confirmed by DNA damage assay. Candida albican and Pseudomonas aeruginosa was found to be resistant to all extracts. Only cold aqueous extract and methanolic extract showed activity against S. aureus and B. cereus respectively. The antimicrobial activity remained almost same with increase in concentration of seed extracts. The Soxhalate and hot extraction method using methanol as solvent was found to be more effective over cold extraction. The results of this work will help in optimization of extraction technique.

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