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Phytochemical Screening, Antioxidant and DNA Protection Study of Nigella Sativa Seed Extract

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Abstract: *Nigella sativa seed extracts were obtained using both methanol and water as solvents, employing cold maceration and Soxhlet extraction techniques. GC-MS analysis was performed to compare the phytochemical constituents in seed extract of Kalonji in chloroform with and without evaporation. The antioxidant activity was investigated using DPPH method. The protective effects of all extracts against oxidative DNA damage induced by photolyzed hydrogen peroxide were assessed using the pBR322 plasmid. In GC–MS analysis, constituents with high therapeutic potential—such as Dodecan-1-amine, thymoquinone, and p-cymene—were detected only in the original extract prepared without solvent evaporation. The results of GCMS indicated that bioactive components are lost during the evaporation or drying of the extract. The DPPH study confirmed the highest radical scavenging activity in methanolic seed extract prepared by Soxhlet method. The findings from the DNA damage assay further demonstrated the potential of Nigella sativa seed extracts in effectively preventing the DNA damage.*

Introduction

Medicinal plants have been widely studied for their therapeutic potential, primarily due to the presence of diverse phytochemical constituents[1-5] Each medicinal plant typically contains multiple bioactive compounds, making the understanding of its chemical composition essential before undertaking pharmacological or biological investigations. Identification, separation, and quantification of these phytochemicals are crucial steps in determining the compounds responsible for specific therapeutic effects. Gas chromatography–mass spectrometry (GC–MS) is a powerful analytical technique commonly used for the separation and identification of complex mixtures of phytochemical[6]. It enables qualitative and quantitative analysis without the need for standards, although its application is limited to thermally stable and volatile compounds[7].

In biological systems, reactive oxygen species (ROS) and free radicals are naturally generated and play a role in defense against pathogens. However, excessive production of these species can lead to oxidative stress, resulting in damage to cellular components such as DNA, proteins, and cell

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membranes[8]. To counteract this, the body relies on antioxidants, which neutralize free radicals and prevent chain reactions that cause cellular damage[9,10]. Dietary intake of antioxidants, especially from natural sources, is therefore essential in reducing the risk of various diseases.

Plants are recognized as rich sources of natural antioxidants, particularly due to the presence of phenolic compounds [11-14]. Increasing awareness of the adverse effects associated with synthetic antioxidants has led to growing interest in plant-based alternatives [15-17]. According to the World Health Organization, approximately 80% of the global population depends on medicinal plants for primary healthcare, highlighting their significance[18].

Nigella sativa, commonly known as black seed, is a well-known medicinal plant used in traditional systems such as Ayurveda and Unani medicine. It is also regarded as a significant therapeutic agent in Islamic medicine[19-21]. Extensive research has demonstrated its pharmacological potential, particularly its antioxidant properties[22]. The antioxidant activity of Nigella sativa is largely attributed to its bioactive constituents, which can be effectively evaluated using assays such as the DPPH (2,2-diphenyl-1-picrylhydrazyl) method. The DPPH assay is widely used due to its accuracy, simplicity, rapidity, and applicability to both hydrophilic and lipophilic antioxidant systems[23-24]

The present study focuses on the identification of phytochemicals present in Nigella sativa seeds using GC–MS analysis, optimization of extraction conditions to obtain antioxidant-rich extracts, and evaluation of their free radical scavenging activity. Additionally, the study aims to investigate the potential of these extracts in protecting against oxidative DNA damage.

Hypothesis

Nigella sativa seeds contain significant bioactive phytochemicals with strong antioxidant properties, and optimized extraction of these compounds will yield extracts capable of effectively scavenging free radicals and protecting against oxidative DNA damage.

Plant Material, Chemicals and Methods

- **Plant material:** Seeds of Nigella sativa purchased from local market of Mumbai city.
- **Chemicals:** DPPH(2,2- diphenyl-1-picrylhydrazyl) of Analytical grade, pBR322 from Thermo Fisher and methyl alcohol. Dipotassium hydrogen phosphate, potassium dihydrogen phosphate and H_2O_2 bought from SD Fine Chemicals Ltd.

Extract Preparation for DPPH and DNA Damage Assay

The dried seeds of N. sativa were used to prepare the aqueous and methanolic extracts using below methods.

Soxhlet Extraction

For Soxhlet extraction, 15.0 g of Nigella sativa seeds were initially soaked in 60 mL of solvent for 24 hours. An additional 50 mL of the same solvent was then used to load the sample into the Soxhlet apparatus. The extraction was carried out for a period of 6 hours, with ice-cold water circulated through the condenser using a pump. The solvent used for extraction was dried to get residue. The known weight of brownish residue was then dissolved in a known volume of solvent for further analysis.

- **Cold Extraction:** 15.0g seeds in 80ml solvent were soaked for 24 hours. The flask was agitated for 6 hours on a shaker at 240 rpm. Then flask was placed for 15hour in refrigerator at 277K and the solution was filtered. The known quantity of residue obtained after drying filtrate at 308K, dissolved in suitable solvent.

The extracts were stored at low temperature in refrigerator.

GC-MS Analysis

To study the effect of solvent evaporation on chemical constituent GC-MS analysis of two extracts of Nigella sativa seeds were carried out. The extracts were prepared by Soxhlet method. Two seed extracts were prepared in methanol. The original extract without evaporation obtained after Soxhlet extraction was directly analyzed by GCMS. The other extract used for GC-MS was prepared by dissolving the residue obtained after evaporating solvent. The analysis of these four samples was done at Centre for Advanced Research & Development Centre, Viva College, Virar, Dist. Thane. The samples were run on AGILENT GC system couple with a mass spectrometer. The fused silica DB-5 (Length = 30m, inner diameter = 0.25mm, film thickness = 0.25μm) was used. The carrier gas used to separate the

components was helium which was maintained at constant flow rate of 1 ml/min. The oven was maintained at 700C and after 2 minutes it was programmed at 200C/min. Final temperature was set at 2500C. 1µl of sample was used for injection. The constituents were identified on the basis of computer matching the data base of NIST11.L (National Institute of Standards and Technology) connected with the GC-MS system.

Determination of DPPH radical scavenging Activity

Radical scavenging activity was assessed using the DPPH method, chosen for its ease, speed, and prevalence in analyzing natural products. This method is originally developed by Blois[25]. The method used to assess DPPH$^{\cdot}$ radical scavenging activity was modified from previously established protocols. A solution of DPPH was prepared by dissolving 5mg of the compound in 100 mL methanol. Methanol was then used to adjust the initial absorbance of this reagent solution to the target range of 0.9–1.0 at a wavelength of 515 nm. A volume of 3 mL of methanolic DPPH solution was combined with 0.5 mL of aqueous seed extract in clean and labeled glass tubes. The control sample was prepared using 3 mL of the DPPH solution and 0.5 mL of methanol. The mixtures were then placed in a dark water bath at 30°C for 30 minutes. The decrease in absorbance at 515nm was measured using Spectrophotometer. The standard solution used was ascorbic acid (5 -100 µg/ml). The experiment was performed in triplicate. The equation used to calculate % inhibition is

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

DNA Damage Protection Study

DNA damage protection was assessed via an assay conducted according to Lee et al. [26], with minor modifications. Supercoiled pBR322 plasmid DNAwas used in present study. The p stands for plasmid and BR for Bolivar" and "Rodriguez." Hydroxyl radical generated from the photolysis of hydrogen peroxide, can react with almost all the components of DNA molecules, including purine and pyrimidine bases and the deoxyribose backbone[27,28]. Supercoiled pBR322 plasmid DNA (0.250 µg) was suspended in 100 mM potassium phosphate buffer (pH 7.4) and exposed to oxidative conditions by incubating with 60 mM hydrogen peroxide at 310K for 3 hours, with or without the addition of plant extracts (10.0 µg). The untreated controls consisted of plasmid DNA in buffer mixed with water or methanol.

Results and Discussion

- **GC-MS Analysis:** Effect of solvent evaporation on retention of active ingredients of *Nigella sativa* extract in methanol

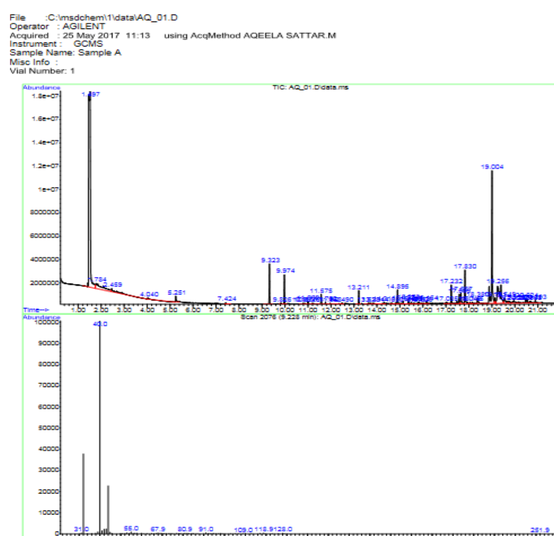


Figure 1: Chromatogram of extract prepared after air drying in methanol

Source: Viva center for advance research and development

Table 1: Phytochemical Constituents Identified in Methanol Extract after Evaporation

Sr. No.	% Peak Area	Nomenclature of the Compounds	Sr. No.	% Peak Area	Nomenclature of the Compounds
1	**0.11**	Hydroxylamine	25	**0.32**	(-)-cis-Myrtanylamine
2	**0.1**	2,2'-Bioxirane	26	**0.13**	2,4(1H,3H)-Pyrimidinedione, dihydr o-5-hydroxy-
3	**0.34**	o-Ethylhydroxylamine	27	**0.14**	cis-1,3-Bis(aminomethyl)cyclohexan
4	**0.22**	Carbonic acid,	28	**0.26**	Ethanamine, 2-phenoxy-
5	**0.6**	Phenol	29	**0.18**	Aminocaproic acid
6	**0.09**	Ethanamine, 2-phenoxy-	30	**0.26**	3-Butyn-1-ol
7	**2.73**	**Thymoquinone**	31	**0.25**	2,4(1H,3H)-Pyrimidinedione, dihydr o-5-hydroxy-
8	**0.13**	Urea, butyl-	32	**1.54**	Hexadecanoic acid, methyl ester
9	**1.97**	Thymol	33	**1.16**	Tridecanoic acid
10	**0.11**	Ethylene oxide	34	**1.20**	Bicyclo[5.3.0]decane (cis)
11	**0.10**	N,N'-Dimethylsulfamide	35	**3.0**	6,10,14,18,22-Tetracosapentaen-2-o l
12	**0.34**	Cystamine	36	**0.2**	Benzeneethanamine, 4-fluoro-
13	**0.22**	Phenol, 4-(2-aminoethyl)-	37	**0.25**	2,4(1H,3H)-Pyrimidinedione
14	**0.75**	Longifolene	38	**0.77**	1,2-Benzenediamine, 4-nitro-
15	**0.17**	Threo-2-methyl-3,4-dibromo-2-butanol	39	**13.49**	Cyclopentanol,
16	**0.31**	2,4(1H,3H)-Pyrimidinedione	40	**0.55**	Acetamide, N-ethyl-
17	**0.14**	Acetaldehyde	41	**7.02**	9,12-Octadecadienoic acid(Z,Z)-
18	**0.95**	Ethanone, 1-(2-hydroxy-4-methoxyphenyl)-	42	**1.33**	5-Hydroxy-2,4(1H,3H)-dihydro Pyrimidinethiodione.
19	**0.31**	5-Hydroxy-2,4(1H,3H)-Dihydropyrimidinedione	43	**0.49**	(-)-cis-Myrtanylamine
20	**0.15**	2,4(1H,3H)-Pyrimidinedione	44	**0.76**	Nonadecylamine
21	**0.36**	N,N'-Diacetylethylenediamine	45	**0.29**	2,4(1H,3H)-Pyrimidinedione,
22	**0.21**	2,2'-Thiodisuccinic acid	46	**1.04**	2(1-Cyano-3-methyl-2-butenylamino)-butanedinitrile
23	**1.43**	Pentanedioic acid, 2-chloro-, dimethyl ester	47	**0.42**	2,4(1H,3H)-Pyrimidinedione,
24	**0.20**	Heptanedioic acid, dimethyl ester	48	**0.32**	Nonadecylamine

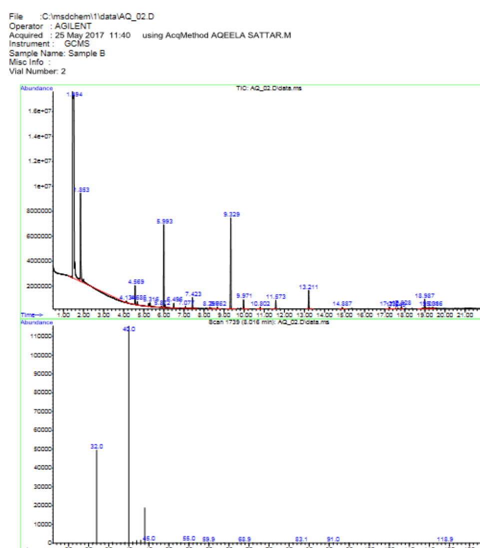


Figure 2: Chromatogram of Original Extract without Evaporation

Source: Viva center for advance research and development

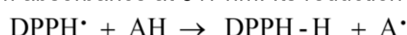
Table 2: Phytochemical Constituents Identified in Original Methanol Extract

Sr. No.	Peak Area (%)	Nomenclature of the Compounds
1	**67.20**	Hydroxylamine
2	**2.92**	Trichloromethane
3	**0.05**	Benzeneethanamine
4	**1.89**	4-Methyl-1(sec.butyl)bicyclo[3.1.0]2-hexene,
5	**0.35**	2,6,6-Trimethylbicyclo[3.1.1]2-heptene
6	**0.72**	β-Pinene
7	**0.09**	1-Propanamine, 3-chloro-
8	**8.02**	o-Cymene
9	**0.44**	γ-Terpinene
10	**0.21**	$H_2N(CH_2)_7NH_2$
11	**1.07**	.gamma.-Terpinene
12	**0.20**	Benzeneethanamine
13	**0.21**	Ethanamine, 2-phenoxy-
14	**9.90**	**Thymoquinone**
15	**0.95**	3-Methyl-4-isopropylphenol
16	**0.20**	Ethanamine, 2-phenoxy-
17	**0.92**	2(1-Cyano-3-methyl-2-butenylamino)-butane-1,4-dinitrile
18	**2.01**	Phenol, 4-methoxy-2,3,6-trimethyl-
19	**0.21**	Ethanamine, 2-phenoxy-
20	**0.25**	Acetamide, N-ethyl-
21	**0.12**	1-Dodecanamine
22	**0.26**	Cyclododecanol, 1-aminomethyl-
23	**1.30**	2-Furanmethanamine, tetrahydro-
24	**0.34**	(-)-cis-Myrtanylamine
25	**0.28**	2-(3-Methylguanidino)ethanol

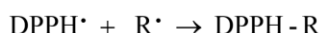
From table 1 and 2 it is evident that the amount of important bio active components such as Thymoquinone retained by the extract depends on method of preparation. The composition and abundance of important phytochemicals of Nigella sativa seed extract has varied significantly with evaporation of solvent. From the data obtained in table 1 and 2 it can be concluded that the bioactive components are lost during evaporation or drying of extract.

Antioxidant Activity

In methanolic solution, the stable free radical 2,2-Diphenyl-1-picrylhydrazyl ($DPPH^{\cdot}$) shows maximum absorbance at 517 nm. Its reduction reaction in presence of an antioxidant (AH) is



$$DPPH^{\bullet} + AH \rightarrow DPPH\text{-}H + A^{\bullet}$$



$$DPPH^{\bullet} + R^{\bullet} \rightarrow DPPH\text{-}R$$

DPPH assay measures antioxidant activity by the decolorization of $DPPH^{\cdot}$ (violet to yellow) at 517 nm, using ascorbic acid as a reference standard. Results (Fig. 1-4) demonstrate that *N. sativa* extracts exhibited significantly higher antioxidant activity. Soxhlet extraction yielded the most potent *N. sativa* methanolic extract, followed by cold extraction.

Methanolic extracts of N. sativa obtained through cold extraction demonstrated significant antioxidant activity (Figure 4).

Fig : 1 DPPH Radical Scavanging assay of standard Ascorbic acid, Aqueous & Methanolic extract of N. sativa

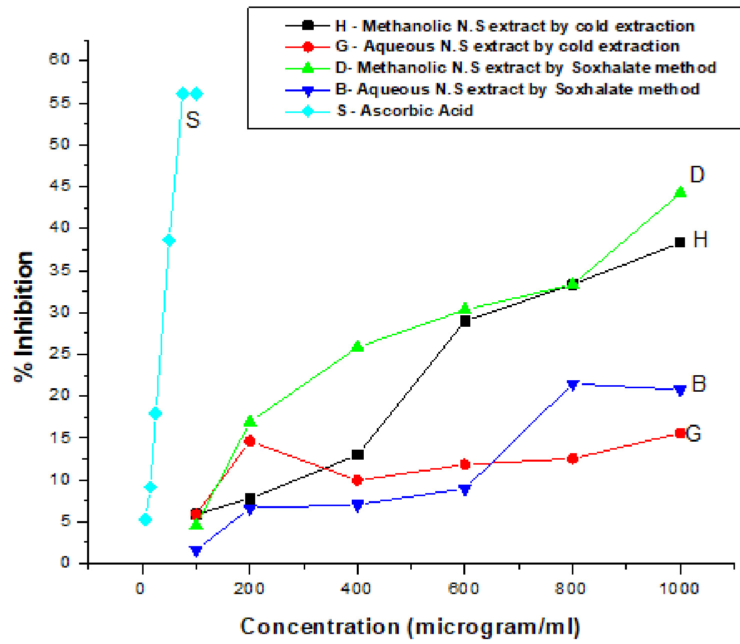


Fig :2 DPPH Radical Scavanging assay of standard Ascorbic acid, Aqueous & Methanolic extract of Fenugreek

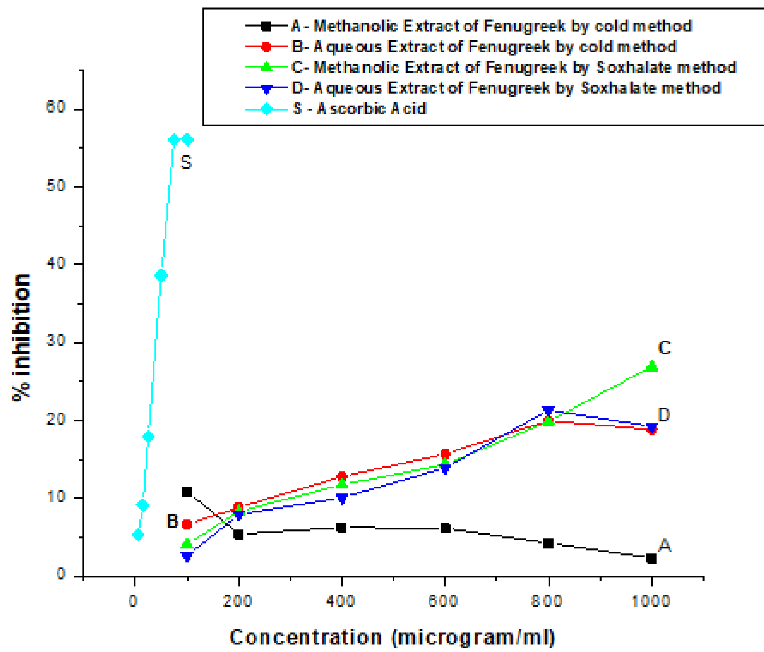


Fig : 3 DPPH Radical Scavanging assay of standard Ascorbic acid, Aqueous & Methanolic extract prepared by Soxhalate method of N. sativa and Fenugreek

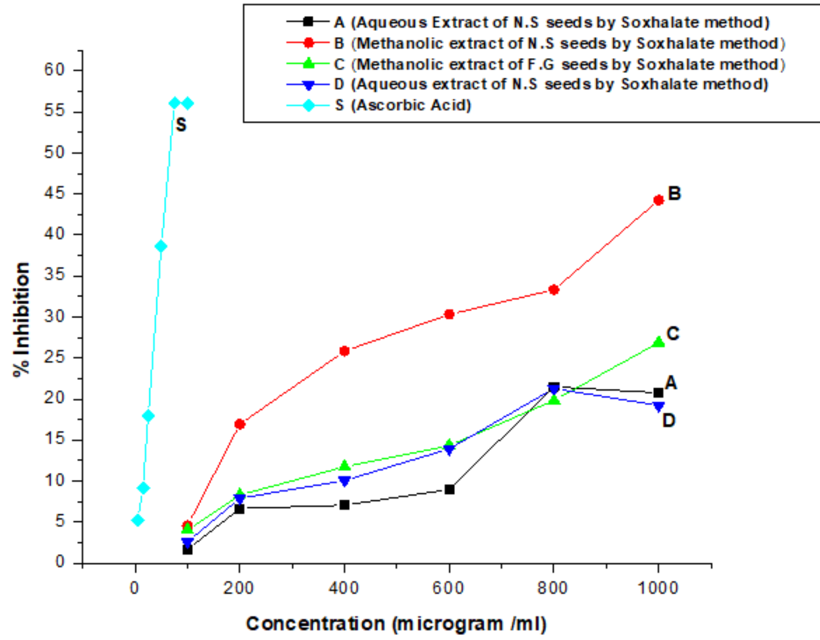
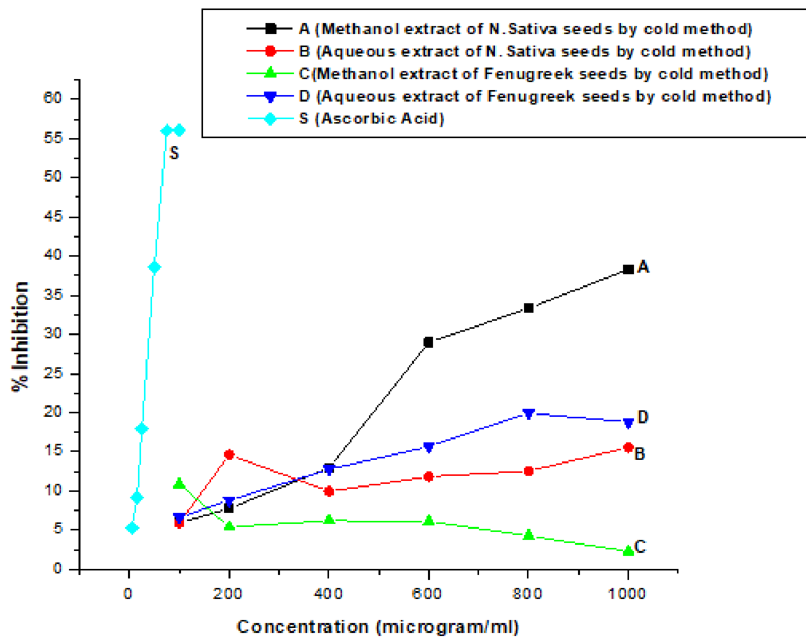


Fig :4 DPPH Radical Scavanging assay of standard Ascorbic acid, Aqueous & Methanolic extract of N. sativa and Fenugreek seeds by prepared cold extraction



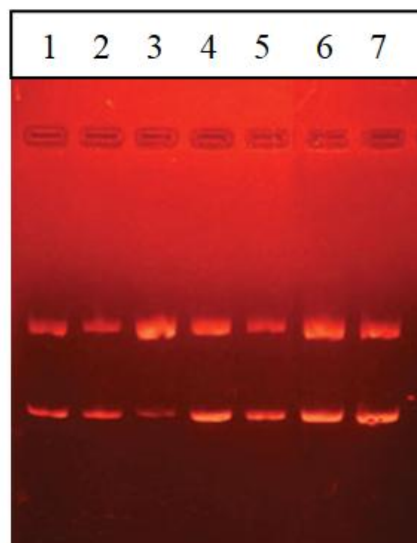
Source: Royal College Physical Chemistry Lab.

DNA Damage Protection

Figure 5 represents the gel electrophoresis results illustrating the impact of *N. sativa* seed extracts on DNA damage.

Fig 5: Effect of *N. sativa* seed extract on DNA damage

Lane Description:
Lane 1: pBR322 only,
Lane 2: pBR322+ Methanol
Lane 3: pBR322+ H_2O_2,
Lane 4: pBR322+ H_2O_2
+ Aqueous NS (Soxhalet),
Lane 5: pBR322+ H_2O_2
+ Methanolic NS (Soxhalet),
Lane 6: pBR322+ H_2O_2
+ Aqueous NS (cold),
Lane 7: pBR322+ H_2O_2
+ Methanolic NS (cold)



Source: University of Mumbai, Life Science Lab.

Fig. 5: Effect of N. sativa seed extract on DNA Damage

Treatment of pBR322 plasmid with hydrogen peroxide resulted in DNA strand breaking, leading to the conversion of its supercoiled structure into the open circular form (Fig. 5; Lane 3). Conversely, *N. sativa* extracts (prepared via Soxhlet and cold methods using aqueous and methanolic solvents) prevented this H_2O_2-induced damage (Fig. 5; Lanes 4-7). Notably, Soxhlet-extracted samples (Fig. 5; Lanes 4-5) exhibited superior DNA protection compared to cold-extracted counterparts (Fig. 5; Lanes 6-7).

Conclusion

The GC-MS study showed that quality of kalonji extract had improved with respect to bio active phytochemicals only if drying is avoided. Results from the DPPH assay demonstrated that Soxhlet-extracted *N. sativa* contained higher antioxidant levels than cold-extracted samples. Subsequent DNA damage analysis confirmed the robust antioxidant activity inherent to *N. sativa* seed extracts.

The analysis by DPPH, GCMS and DNA Assay require quantification of extracts, for which generally drying of solvent is done by different methods. Even if drying is done at room temperature, present work has revealed that medicinal properties of extract is lost by drying. Further research is required in this area to find method which will provide quantified extract rich in active ingredients.

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