Antioxidant, antibacterial, and antimutagenic activity of *Piper nigrum* seeds extracts

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**Abstract**

*Piper nigrum* is a widely used plant in traditional remedies and known for its numerous biological properties. However, fraction-based antioxidant activity and their antimutagenic potential are not yet fully investigated. Different extracts of the seeds *P. nigrum* were obtained by sequential extraction in different solvents. All extracts were evaluated for antibacterial and antioxidant activities using different methods. The most active fraction was analyzed for antimutagenic activity using the Ames *Salmonella typhimurium* test. The antibacterial activity against methicillin-resistant *Staphylococcus aureus* was found to be more prominent compared to ESBL producing *Klebsiella pneumoniae* isolates. The MIC values were found to be lower against MRSA than *K. pneumoniae*. The extract showing highest antioxidant activity (methanol extract) was further tested for antimutagenic activity both against direct and indirect-acting mutagens. A varying level of antimutagenic activity was shown by methanol extract at highest tested concentration (200 µg/plate). Alkaloids, phenols, and flavonoids were detected as major class of compounds in methanol extract. Gas chromatography-mass spectrometry (GC–MS) analysis showed the presence of various phytocompounds. Based on molecular docking of two major active phytocompounds (piperine and nol extract. Gas chromatography-mass spectrometry (GC–MS) analysis showed the presence of various phytocompounds. Based on molecular docking of two major active phytocompounds (piperine and copaene), they were found to interact at the minor groove of DNA. Molecular dynamics (MD) simulation revealed that both the ligands were quite stable with DNA under physiological conditions. The ability of phytocompounds to interact with DNA might be reducing the interaction of mutagens and could be one of the possible mechanism of anti-mutagenic activity of *P. nigrum* extract. This study highlights the antioxidant and antimutagenic potential of *Piper nigrum*. The role of phytocompounds present in the bioactive extract is needed to be explored further for herbal drug research.

**Keywords:**

*Piper nigrum*
Antioxidant
Antimutagenic property
Phytochemicals
GC–MS analysis
*Ames Salmonella typhimurium* test
Molecular docking

**1. Introduction**

Medicinal plants and plant derived products are known to play significant role in primary healthcare and in the management of various ailments since long times (Shakya, 2016). Bioactive plant extracts are the integral part of traditional system of medicine and often exhibit multiple biological activities including antioxidant and antimutagenic activities (Khan and Ahmad, 2020). This has led the researchers for the systemic evaluation of various biological properties of medicinal plants.

Reactive oxygen species (ROS) are known to be involved in the onset of cancer and other pathophysiological conditions (Görlich et al., 2015). The most susceptible target of free radicals are the lipids, proteins and DNA that causes the formation of malondialdehyde, 4-hydroxynonenal, carbonyl moieties, and DNA damage; leading to disturbance in genetic stability (Berlett and Stadtman, 1997). They may cause DNA-protein crosslinking, alterations in nitrogenous bases and strand breaking leading to DNA mutations (Coppedè and Migliore, 2015; Gandhi and Abramov, 2012). The
DNA damage caused by the different types of ROS is implicated in the complex process of mutagenesis and considered as primary step involved in the onset of different types of cancers. This has led to the discovery and characterisation of various antioxidant and antimutagenic agents (Jackson and Loeb, 2001). Several studies have demonstrated the role of medicinal plants in chemoprevention of ROS associated problems. Many plant-derived phenolic compounds such as flavonoids, anthocyanins, catechins, etc., among other secondary metabolites, have shown strong antioxidant and antimutagenic activities (Sloczyska et al., 2014). A direct correlation between phenolics and antioxidant activity of the plant has been reported earlier (Cai et al., 2004; Gil et al., 2000). Various mechanisms proposed for these phenolics against ROS action includes the reduction reaction, proton donation, metal chelation and acting as singlet oxygen and nitrogen quencher (Brewer, 2011). The precise mechanism of bioactive plant extracts and phytocompounds exhibiting antimutagenic activity is still unclear. It has been proposed that antimutagenic activity is due to the ability of phytocompounds to form a stable complex with mutagens and neutralizing them, secondly it can activate the cellular detoxification system and, thirdly by neutralizing ROS directly (acting as an antioxidant). Recent studies have also highlighted the role of plant extracts/phytocompounds as protective barrier between DNA and an attacking mutagen or carcinogen (Khan et al., 2018; Sloczyska et al., 2014).

Considering the damaging health effects of ROS and free radicals, there is need to systematically evaluate the rich diversity of Indian medicinal plants for potential broad-spectrum antimutagenic and antioxidant properties. Previously, we have reported the antioxidant and antimutagenic activities of several medicinal plant such as *Carum copticum*, *Murraya koenigii*, *Punica granatum*, *Psidium guajava*, *Piper cubeba*, *Syzygium cumini* (Khan et al., 2018; Zahin et al., 2018, 2017, 2013, 2010a, 2010b).

*Piper nigrum*, commonly known called as black pepper, is used as a spice, grown in tropical regions including Brazil, Indonesia, and India. *P. nigrum* and its active constituents are used in different traditional systems of medicine (Ayurvedic and Unani) as a stimulant, carminative, diuretic, anticholera, sialagogue, anti-asthmatic, etc. (Khare, 2007; Salehi et al., 2019). Scientific investigations revealed the antimicrobial, antioxidant (Zarai et al., 2013), anti-inflammatory (Tasleem et al., 2014), hepatoprotective (Bai et al., 2011), anti-depressant (Hritcu et al., 2015) and immune-modulatory (*Sunila and Kuttan*, 2004) activities of the black pepper. Piperine (1-peperonyl piperdine), and other major alkaloids of *P. nigrum* has been shown to possess numerous pharmacological properties (Mehghwal and Goswami, 2013; Stojanović-Radić et al., 2019).

Considering these aspects, there is need to identify the bioactive antioxidant fractions from natural sources capable preventing the mutagenicity induced by toxicants or mutagens. The detailed fraction-based antioxidant and antimutagenic studies on the seeds of *P. nigrum* plant is not available. Therefore, fraction-based extracts of *P. nigrum* (seeds) were evaluated using different in vitro antioxidant methods and characterized the most active fraction for its antimutagenic potential. The extract was phytochemically characterized using GC–MS analysis. The identified compounds were then studied for their interaction using computational tools to decipher the role of its major constituents i.e., piperine and copaene, in DNA protection.

2. Materials and methods

2.1. Bacterial strains and chemicals

The Ames tester strain i.e., *S. typhimurium* strains TA97a, TA100, TA102, and TA98 were obtained as a gift from Prof. B. N. Ames (University of California, Berkeley, California, USA). Benzo(a)-pyrene (B(a)P), 2-aminofluorene (2-AF), and DPPH were obtained from Sigma Chemical Company, USA. Rest of other chemicals, buffers, and reagents used were of analytical grade.

2.2. Collection of plant material and preparation of extracts

*Piper nigrum* (L.) seeds were obtained from authorized stockist of local market of Aligarh, UP, India. The material of plant was identified at Department of Botany, AMU, Aligarh (Voucher specimen number: MBD-20/06). The crude methanol extract was prepared by adding 500 g powdered seeds of *P. nigrum* in 2.5 l methanol and left for 5 days with intermittent shaking. The extract was filtered and evaporated using rotatory evaporator. Various fractions of the plant were obtained by dissolving 500 g seed powder in 2.5 l petroleum ether for 5 days. The extract was filtered and concentrated in rotatory evaporator. The concentrated extract was further reextracted sequentially in benzene followed by ethyl acetate, acetone, methanol, and ethanol in increasing order of polarity (Zahin et al., 2010b). All extracts were kept at 4 °C and reconstituted in DMSO for further experiments.

2.3. Antibacterial assays

2.3.1. Agar well diffusion assay

The preliminary antibacterial activity was tested using agar well diffusion assay (Ahmad and Beg, 2001). Test bacteria (10^5 CFU/ml) were spread on Muller-Hinton agar plates and wells (8 mm) were punched in plates. The wells were filled with 100 μl plant extract (100 mg/ml) and equal concentration of DMSO was used as solvent blank. The petri plates were incubated overnight at 37 °C. The zone of inhibition was measured and presented in millimetres (mm).

2.3.2. Assessment of minimum inhibitory concentration (MIC) of plant extracts

MIC of plant extracts were determined against clinical strains of bacteria by broth dilution assay using p-iodonitrotetrazolium violet as growth indicator dye (Eloff, 1998). In short, 2 ml broth (Muller-Hinton) was mixed with 2 ml extract and serially two-fold diluted. Bacteria from log phase were added as inoculum and incubated at 37 °C for 18 hrs. 800 μl indicator dye (0.02 mg/ml) was added and incubated at 37 °C for 30 min. The tubes were observed for colour change and the absence of bacterial growth was further confirmed by spreading the culture from tubes on nutrient agar plates.

2.4. Antioxidant assays

2.4.1. Assessment of total antioxidant capacity by phosphomolybdenum assay

Total antioxidant capacity of *P. nigrum* extracts was determined using the standard procedure (Prieto et al., 1999). Briefly, varying concentrations of plant extracts (25–200 g/l) was mixed with 1 ml reagent containing sodium phosphate (28 mM), ammonium molybdate (4 mM), and sulfuric acid (600 mM). The reaction mixture was boiled for one and half hour and absorbance was recorded at 695 nm. Total antioxidant capacity of the plant extracts is expressed in equivalents of ascorbic acid (μmol/g).

2.4.2. DPPH radical scavenging assay

DPPH radical scavenging assay was performed by the modified method of Gヤmfi et al. (1999) (Gヤmfi et al., 1999). Briefly, a total volume of 50 ml of varying concentrations of extract (25–200 g/l) was used in this assay. All samples were incubated for a half-hour at room temperature. The reduction of DPPH free radicals
was assessed by recording optical density (OD) at 517 nm. Butylated hydroxytoluene and ascorbic acid were used as positive controls and methanol was used as vehicle control. The % inhibition was calculated using equation-1:

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\% \text{ Inhibition} = \left( \frac{OD_c - OD_t}{OD_c} \right) \times 100
\]

where, OD\(_c\) and OD\(_t\) are absorbances of control and test samples.

2.4.3. FRAP assay (Fe\(^{3+}\) reducing power assay)

The reducing capacity of extracts was assessed using standard procedure with few modifications (Oyaizu, 1986). Briefly, the extract (750 µl) was mixed with 1% potassium ferricyanide and 2 M sodium phosphate (1.25 ml each) followed by acidification with 10% trichloroacetic acid (1.25 ml). Half millilitre FeCl\(_3\) (0.1%) was added and OD was recorded at 700 nm.

2.4.4. CUPRAC assay (cupric ions reducing ability)

The cupric ions reducing ability of extracts were determined using standard assay with minor modifications (Apak et al., 2007). Briefly, a varying concentrations of plant extracts (25–200 g/l) was mixed with 250 µl ethanolic neocuproine solution (7.5 mM), 250 µl CuCl\(_2\) solution (0.01 M), and 250 µl CH\(_3\)COONa buffer (1 M). The volume of reaction mixture was adjusted to 2 ml using double distilled water and incubated for half hour at room temperature. The OD was recorded at 450 nm.

2.5. Assay for anti-mutagenic activity

The anti-mutagenic assay was performed using standard Salmonella mutagenicity assay (Maron and Ames, 1983). Varying concentrations (25–200 g/l) of P. nigrum extracts were evaluated for anti-mutagenic potential using Salmonella typhimurium tester strains (TA97, TA100, TA102, and TA98) against indirect (B(a)P and 2-AF) and direct (Na\(_2\)S and MMS) acting mutagens. The detailed procedure is described earlier (Zahin et al., 2010a).

2.6. Detection of major phytochemical classes and estimation of total phenolic content

The major class of phytocompounds such as alkaloids, tannins, flavonoids, glycosides, phenols, and saponins were detected using previously described standard colour tests (Ahmad and Beg, 2001). Total phenolic content of P. nigrum seed extracts was assessed using Folin-Ciocalteau method (Spanos and Wrolstad, 1990). Briefly, 500 µl plant extract (of different concentration 25–200 g/l) was added to 2.5 ml Folin- Ciocalteau’s reagent (1/10th diluted) and then 2 ml Na\(_2\)CO\(_3\) (7.5%) was added. The reaction mixture was incubated for 15 min at 45°C. Total phenolic content was estimated and expressed as mg of gallic acid equivalent per gram of the dry weight of extract (mg GAE/g DW).

2.7. Gas chromatography-mass spectrometry (GC–MS)

The GC–MS analysis of P. nigrum extract was performed using GCD 1800 A (Hewlett Packard) coupled with HP-1 column. Detector and injector temperatures were 280 °C and 250 °C, respectively. Initial oven temperature was fixed between 100 °C and 250 °C at the rate of 10 °C/min, hold time at 250 °C was fixed for 3 min. Final temperature was set between 250 and 280 °C at the rate of 30 °C/min, hold time was 2 min at 280 °C. The dilutions were prepared in methanol and helium was used as carrier gas (Zahin et al., 2017). The identification of phytocompounds was performed using standard database (NST library).

2.8. Molecular docking

Piperine and copaene were detected as the most abundant compounds by GC–MS analysis in P. nigrum extract. Molecular docking of these detected compounds was performed using AutoDock vina to understand their binding site and mode with DNA (Trott and Olson, 2009). 3D structure of piperine [CID: 638024] and copaene [CID: 19725] were downloaded from PubChem. The ligand molecules were made flexible using AutoDockTools1.5.6 and coordinates were saved. 3D structure of B-DNA dodecamer (PDB ID: 1BNA) was downloaded from Protein Data Bank. To avoid hindrance in docking, water molecules in receptor were removed. The non-polar hydrogens were added and then Kollman charges were added. (Morris et al., 1998). The grid size was set to 24 × 28 × 42 Å with 1 Å spacing. The grid centre was x = 14.78, y = 20.974, z = 8.806. The analysis of docking results was done using PyMOL and Discovery Studio.

2.9. Molecular dynamics simulations

The interaction of piperine and copaene with DNA was further studied using molecular dynamics (MD) simulations. MD simulation was performed in Gromacs 2018.1 using amber99sb-ILDN force field (Berendsen et al., 1995; Hornak et al., 2006). DNA alone, DNA-piperine complex, and DNA-copaene complex were solvated in the triclinic box using TIP3P water model. 22 sodium ions were added to neutralize the structures. The topology of piperine and copaene were generated with antechamber in AmberTools19. The energy of systems was minimized using the steepest descent minimization of 5000 steps to remove the weak Van der Waals contacts. The systems were then equilibrated for equilibrated for NVT at 300 K and NPT at 1.0 bar for 1 ns each. The standard MD simulation of each system was carried out for 50 ns each. The trajectories were subjected to periodic boundary conditions (PBC) corrections before analysis. The binding energies of the system were calculated using MM-PBSA analysis (Kumari et al., 2014).

2.10. Statistical analysis

The statistical analysis of the data was done using one-way ANOVA by Tukey test in SPSS. The data presented is average of three experimental replicates.

3. Results

In this study, sequential fractionation of P. nigrum (seeds) was carried out to obtain different fractions of varying polarity. Benzene and petrol ether fractions yielded only 0.2% extract; but the yield was 7–10 times higher in acetone (2.0%), ethyl acetate (1.5%), ethanol (2%), and methanol (5.2%) fractions. The antibacterial activity was further evaluated by determining MIC. Benzene and petrol ether fractions yielded only 0.2% extract; but the yield was 7–10 times higher in acetone (2.0%), ethyl acetate (1.5%), ethanol (2%), and methanol (5.2%) fractions. The antibacterial activity of crude extract was tested against 5 methicillin-resistant S. aureus (MRSA) isolates and 6 ESBL producing K. pneumoniae isolates (supplementary Table 1). The zone of inhibition ranged from 10 to 14 mm against different MDR isolates. The antibacterial activity was further evaluated by determining MIC. A similar observation was recorded with lower MIC (0.8 mg/ml) against MRSA and relatively higher (1.6–3.2 mg/ml) against K. pneumoniae. Overall, methanolic and ethanolic fractions were inhibitory to greater number of test pathogens compared to other fractions.

The antioxidant activity was assessed at varying concentrations (25–200 g/l). All fractions of P. nigrum extract showed moderate to weak (≤50%) free radical scavenging activity as evident from DPPH assay (Fig. 1A). The fractions were found to be less active using potassium ferricyanide reduction assay (Fig. 1B). A fair
Reducing potential in methanol fraction was found using CUPRAC assay followed by ethanol and other fractions (Fig. 1C). Similarly, a concentration-dependent antioxidant activity was recorded using phosphomolybdenum assay (Table 1). At 200 g/l, methanol fraction exhibited highest antioxidant capacity (583.3 µmol) followed by ethanol fraction (465.9 µmol). Other fractions showed lower levels of antioxidant capacity. The most antioxidant fraction (methanolic fraction) was further tested for antimutagenic activity.

Since many plant extracts have been documented to be mutagenic in nature, therefore, we first determined the mutagenic potential of the methanolic extracts of *P. nigrum* at the selected concentrations. At all tested doses (25–200 µg/plate), methanol fraction exhibited insignificant mutagenicity and toxicity in *S. typhimurium* strains. The data presented in Figs. 2–5 revealed that the methanol fraction showed dose-dependent antimutagenic activity. NaN₃-induced mutagenicity against TA97α and TA100 was reduced by 66.8% and 68% at 200 µg/plate, respectively. However, antimutagenic response was insignificant for TA102 and TA98 strains (Fig. 2, Supplementary Table S2). Likewise, similar trend was recorded for MMS-induced mutagenicity in which there was 63.6% and 64.2% decrease in His⁺ revertants number for TA97α and TA100, respectively, (Fig. 3 and Supplementary Table S3). Further, there was also inhibition (60.2–68.4%) of mutagen induced His⁺ revertants in different tester strains. Results clearly demonstrated that the most antioxidant active fraction (methanol) of *P. nigrum* exhibited a fair antimutagenic activity.

The antioxidant and antimutagenic activities of plant extracts are due to presence of active phytoconstituents. Colour test analysis of all fractions showed the presence of phenolics and alkaloids as the major phytochemical classes (Supplement Table S6). Total phenolic content was maximum in petrol ether fraction (53.0 ± 2.3 mg/g) followed by ethanol (52.6 ± 3.1 mg/g) and methanol (41.5 ± 3.4 mg/g) fraction. Other fractions of *P. nigrum* exhibited relatively low levels of polyphenolic content that ranged from 30.6 to 40.8 mg/g of the dry extract (Supplement Table S7). The GC–MS analysis of *P. nigrum* confirmed the presence of 13 compounds. The predominant compounds were piperine (46.10%), adamantane (8.07%), and copaene (12.25%). Many other compounds were also present, but in lower amounts (0.65–5.72%), as enlisted in Table 2.

 Piperine and copaene were found to be the most abundant compounds detected in *Piper nigrum* methanol fraction. These two compounds were used to study their interaction with DNA by in silico methods. To decipher the binding site of phytocompounds (piperine and copaene), molecular docking was performed. Molecular docking revealed that piperine and copaene interacted at minor groove of DNA with binding energies as −7.6 and −5.3 kcal/mol, respectively. Piperine formed hydrogen bonds with dG10 of chain A, and dC15 and dG16 of chain B as shown in Fig. 6. Copaene was found to interact with dA15 via hydrophobic bond (Fig. 7).

Furthermore, molecular dynamics of the interaction of copaene and piperine with DNA was investigated by simulating the complexes for 50 ns under physiological conditions. The RMSD of DNA alone, DNA-copaene complex, and DNA-piperine complex respect to their respective initial structures is shown in Fig. 8A. The RMSD of DNA alone was found to be below 0.4 nm for the entire simulation period indicating it stability in aqueous environment. However, DNA-copaene complex was relatively more unstable for 25 ns, then the system became fairly stable after 25 ns. The data suggests that system for DNA-copaene complex got equilibrated and became stable after 25 ns. The RMSD curves of both the complexes were similar to DNA alone and the visual analysis of the trajectories confirms the stability of the both ligands with DNA. The RMSF of DNA alone, DNA-copaene complex, and DNA-piperine complex is shown in Fig. 8B. As evident from the data, the RMSF of nearly all nucleotides were found to be <0.2 nm and the fluctuation curve of both complexes were similar to that of DNA alone. The average RMSF of individual atoms of both ligands (copaene and piperine) was also determined Fig. 9A. The RMSF of

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**Table 1**

Antioxidant capacity of *P. nigrum* seed fractions expressed as ascorbic acid equivalents (µmol/g of extract) by phosphomolybdenum method.

| Concentration (g/l) | Antioxidant capacity (µmol/g of extract) |
|---------------------|-----------------------------------------|
|                     | Petrol ether | Benzene | Ethyl acetate | Acetone | Methanol | Ethanol |
| 25                  | 86.2 ± 4.6   | 88.9 ± 3.7 | 87.8 ± 6.5 | 89.6 ± 5.4 | 286.1 ± 9.9 | 215.2 ± 10.0 |
| 50                  | 158.0 ± 10.5 | 159.6 ± 8.8 | 161.0 ± 7.1 | 167.7 ± 7.1 | 378.3 ± 8.3 | 336.6 ± 9.5 |
| 100                 | 233.1 ± 7.4  | 236.4 ± 7.0 | 236.2 ± 7.9 | 242.1 ± 9.8 | 487.4 ± 16.1 | 418.8 ± 8.0 |
| 200                 | 295.1 ± 5.4  | 315.7 ± 5.7 | 312.4 ± 6.5 | 321.2 ± 5.7 | 583.3 ± 11.0 | 465.9 ± 11.8 |
| LSD at 5%           | 11.16        | 5.94     | 14.60        | 8.73     | 25.60     | 22.90   |

The above data are the mean of three experiments ± SD; Different letters in the column shows a significant difference in means.
both ligands showed some variations which indicates dynamical shift from their respective initial positions in complexes. Such dynamical shift in ligand position caused different interaction modes with the nearby nucleotides of DNA during MD simulation (Qais et al., 2021). To further verify the stability of DNA and its complexes with copaene and piperine, total energy and potential energy of the system s were calculated (supplementary Fig S1). The data clearly shows that the energies of any system (DNA or complexes) did not changed and remained in equilibrium and stable during entire simulation period (Fouedjou et al., 2021).

Radius of gyration (Rg) is also considered important in assessing the stability of biological macromolecules in MD simulation. The changes in Rg of simulated systems were calculated as function of time (Fig. 9B). The Rg of both the complexes were found to be similar to that of DNA alone. The solvent accessible surface area (SASA) of the systems with respect to time is presented Fig. 10A. The SASA of DNA alone, DNA-copaene complex, and DNA-piperine complex were found to be 47.416 ± 1.426, 49.515 ± 1.465, and 46.280 ± 2.019 nm², respectively. The data indicates that the complexes did not underwent any noticeable structural alterations.

The detailed insight regarding binding energies for the interaction of piperine and copaene with DNA was also explored using MM-PBSA analysis. As mentioned above, the RMSD of DNA-copaene complex showed some variations, while it became stable after 25 ns. Therefore, the MMPBSA analysis was performed after system got equilibrated i.e., from 30 to 50 ns. The MM-PBSA binding energies of 100 snapshots of 30–50 ns MD simulation is shown in Fig. 10B. van der Waals were found to be the largest contributor for the interaction of both ligands with DNA. Electrostatic energy (Elec) and solvent accessible surface area (SASA) energy were contributed positively but to the lower extents. However, polar solvation energy (PSE) impaired binding of both ligands with DNA. The overall binding energy (BE) for the interaction of copaene and piperine with DNA was found to be −17.021 and −32.495 kcal/mol, respectively.

4. Discussion

Antioxidant property is one of common attributes of food and medicinal plants, contributed by various phytocompounds of both secondary and primary metabolites. The role of antioxidants has been well recognized in protection against cellular damage caused both in infectious and chronic diseases. Therefore, identification of broad-spectrum antioxidant active plant extracts and its associated properties such as antimutagenic and DNA protective properties may be useful for in identification of effective plant derived formulation. Overall, methanolic extract was found to be more antibacterial against methicillin-resistant S. aureus (MRSA) than ESβL producing K. pneumoniae. Moreover, fraction-based antibacterial activity showed that methanolic and ethanolic fractions were inhibitory to greater number of test pathogens compared to other fractions. There is an alarming prevalence and spread of MDR...
pathogenic bacteria making a worldwide public health concern (Arciola et al., 2018). Development of AMR has led the scientist to focus on the discovery of antimicrobial from plant, especially that of ethnopharmacological origin. A report on antibacterial activity of *P. nigrum* has found the MIC range of 50–500 µg/ml against reference strains of *S. aureus*, *S. faecalis*, *B. cereus*, *P. aeruginosa*, *K. pneumoniae*, *S. typhi*, and *E. coli* (Karsha and Lakshmi, 2010). The variation in MIC values might be due to the methods used for extract preparation and nature of test pathogens as we tested against multi-drug resistant (MDR) strains.

The antioxidant activity showed that all fractions of *P. nigrum* showed moderate to weak free radical scavenging capacity by DPPH assay. On contrary, antioxidant activity by CUPRAC assay showed a fair reducing potential in methanol fraction compared to other fractions. The present finding is in agreement with previous report for higher antioxidant activity in methanolic fraction (Vardar-Ünlü et al., 2003). Variations in the antioxidant activity might also be due to the method adopted for the extract preparation. Using similar extraction methodology and in vitro antioxidant assay, many plant extracts exhibiting good antioxidant activity is reported from our laboratory (Zahin et al., 2018, 2017, 2013, 2010a, 2010b). The antioxidant active plant extracts are more likely to possess antimutagenic activity which could be attributed to their high antioxidant active phytocompounds, especially the phenolics (Makhafola et al., 2016). Many workers have reported the correlation between phenolic constituents of the plant extracts with their corresponding antioxidant and antimutagenic activity (Cano-Lamadrid et al., 2016; Makhafola et al., 2016; Neri-Numa et al., 2013).

The antimutagenic activity of the most antioxidant methanolic fraction was tested using *Salmonella typhimurium* tester strains. The methanol fraction was not mutagenic or toxic to *Salmonella typhimurium* strains. The antimutagenic activity against NaN₃ induced mutagenicity was more against TA97a and TA100, while the fraction showed an insignificant effect on TA98 and TA102 strains. The results showed that most antioxidant active fraction (methanol) of *P. nigrum* exhibited fair antimutagenic activity. The observed antimutagenic activity of methanolic fraction could be due to synergistic effect various bioactive constituents that scavenge mutagen generated ROS before reacting with DNA (Zahin et al., 2018). It has been increasingly recognized that compounds with antioxidant properties can scavenge ROS generated by the mutagens before they react with DNA (Lee et al., 2011). Additionally, the antimutagenic action of the methanolic fraction against promutagens, which require metabolic activation, suggests that it could be able to inhibit the enzymes involved in the biotransformation of promutagens into mutagenic compounds such as the cytochrome P450 family of enzymes (Carrière et al., 1992). Kaur et al. (2010) observed that phytoconstituent isolated from *Terminalia arjuna* decreased the mutagenic effect of 2-AF due to the inhibition of the metabolic activation of the promutagens into the mutagenic form (Kaur et al., 2010).

Phytochemical analysis using GC–MS revealed that piperine (46.10%) was most abundant followed by copaene (12.25%). Therefore, these two compounds were studied for their interaction with

![Fig. 3. Effect of *Piper nigrum* (methanol fraction) on the mutagenicity induced by methyl methanesulfonate (MMS). The asterisk above bars denotes the significantly different compared to the positive control using paired t-test. *P < 0.05; **P < 0.01 and ***P < 0.005.](image-url)
Fig. 4. Effect of *Piper nigrum* (methanol fraction) on the mutagenicity induced by 2-aminoflourene (2-AF). The asterisk above bars denotes the significantly different compared to the positive control using paired t-test. *P < 0.05; **P < 0.01 and ***P < 0.005.

Fig. 5. Effect of *Piper nigrum* (methanol fraction) on the mutagenicity induced by benzo(a)pyrene. The asterisk above bars denotes the significantly different compared to the positive control using paired t-test. *P < 0.05; **P < 0.01 and ***P < 0.005.
DNA using computational tools. *In silico* techniques have become a crucial tool in understanding the binding mode of small molecules (like drugs), and therefore also used for computational screening purposes. Using such tools, the binding of a ligand with receptor molecules can be studied in lesser time with more detailed insights (Elokely and Doerksen, 2013). Both these ligands (piperine and copaene) interacted at the minor groove of DNA. Previously, it has been reported that the molecules which bind at the minor groove of DNA contribute in enhanced stability of DNA (Chandran et al., 2012). Moreover, the binding energy of both compounds was found to be comparable with those of minor groove binders as reported earlier (Qais et al., 2017; Qais and Ahmad, 2018; Shahabadi and Maghsudi, 2014). It has been documented that the DNA binding capability of a compound may result in reducing the mutagenicity of mutagens (Buraka et al., 2014). The mutagens used in antimutagenic activities of this study directly interact with DNA. The ability of phytocompounds to interact with DNA might be reducing the interaction of mutagens and could be a possible mechanism of anti-mutagenic activity of *P. nigrum* extract. However, there is need to validate the *in silico* results of this study in biological systems.

The molecular dynamics of complexes of copaene and piperine with DNA were investigated by simulating the complexes under physiological conditions. The root mean square deviations (RMSD) analysis of such MD simulation is considered as direct indicator of the stability of the system. The RMSD of complexes were found to be <0.4 nm during after the system got equilibrated. This indicates the stability of the ligands with DNA in aqueous environment. Similarly, RMSD of DNA-piperine complex was also quite stable during simulation with slightly less fluctuation that DNA alone. The data

![Fig. 6. Molecular docked structure of piperine complexed with DNA. Figure represents minor groove binding of piperine with DNA dodecamer d(CGCGAATTCGCG)₂ (PDB ID:1BNA).](image-url)
suggests that system for DNA-copaene complex got equilibrated and became stable after 25 ns.

The dynamic behaviour of the nucleotides of DNA was explored by calculating root-mean-square fluctuations (RMSF) by averaging over all conformations of 50 ns MD simulation. RMSF values provide useful insight regarding the structural flexibility of DNA. The RMSF of most of the nucleotides was <0.2 nm and the fluctuation curve of both complexes were similar to that of DNA alone. However, the terminal nucleotides of both chain of DNA showed relatively more fluctuation which is owed to their hanging position.

The RMSF of atoms of both the ligands showed some variations that is due to the dynamical shift from their initial position in the binding site.

Radius of gyration (Rg) also is also considered as an indicator of the stability of the biomacromolecular complexes during MD simulation. There was a negligible alteration in RMSD of DNA alone, DNA-piperine complex, and DNA-copaene complex during simulation period. Similarly, negligible change in SASA of both complexes were recorded. A negligible fluctuation in their SASA and a minimal variation in SASA of both complexes compared to DNA confirms...
that interaction of ligands did not induced any noticeable structural alterations. The data further validate the stability of piperine and copaene with DNA.

Various binding energies involved in the interaction of both ligands with DNA was calculated using MM-PBSA analysis. In typical drug-DNA interactions, the non-covalent forces are the major contributors to the overall binding energy. Such non-covalent forces include van der Waals (vdW) interaction, electrostatic forces (Elec) interaction, hydrogen bond, and hydrophobic interactions. These interactions either contribute positively or negatively to overall binding energy of the ligand with DNA (Siddiqui et al., 2019). The negative values of these binding energies show the positive contribution in overall binding energy, while positive values are considered to be unfavourable for the overall binding. van der Waals energy was the largest contributor for the interaction of both ligands with DNA followed by electrostatic energy (Elec) and solvent accessible surface area (SASA) energy. The negative overall binding energy confirms that interaction of both ligands was energetically favourable. Therefore, it can be inferred that interaction of phytoconstituents of \textit{P. nigrum} extract with DNA formed stable complexes. Moreover, these stable complexes protected the DNA from chemical induced mutagenicity.

5. Conclusion

The findings revealed that the most active antioxidant fraction of \textit{P. nigrum} seed extract demonstrated fair antimutagenic activity. The methanolic and ethanolic fractions were inhibitory to greater number of test pathogens compared to other fractions. Its main active compounds (piperine and copaene) interacted with DNA and could be responsible for the above mentioned antimutagenic activity. The active extract and their major phytoconstituents should be explored further to understand the exact role of phytoconstituents and their therapeutic efficacy. Further isolation and characterization of major active phytoconstituents are required to understand their contribution in biological activities and exploitation in modern phytomedicine.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.sjbs.2021.05.030.
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