Results
The strategy adapted for screening, selection, fractionation and bioactivity
determination of selected plant extracts is presented in flowchart (MF2). The
antibacterial activity against drug resistant bacteria, antioxidant activity and
antimutagenic properties of most active fractions are carried out and described below.

4.1. Characteristics of *Staphylococcus aureus* and *Klebsiella pneumoniae* clinical
strains

The clinical strains of *Staphylococcus aureus* and *Klebsiella pneumoniae*, obtained
from J.N. Medical College Hospital, Aligarh Muslim University, Aligarh. These
strains were subjected to biochemical characterization, antibiotic resistance behaviour
and β-lactamase production and the data are presented in table 1 and 2. The *S. aureus*
strains (SA-01, SA-07, SA-12, SA-18 and SA-27) were resistant to methicillin and
other antibiotics. These strains were designated as methicillin resistant *S. aureus*
(MRSA) based on detection of mecA gene by RT-PCR in the laboratory of Prof. Niel
Woodford, London, UK. However, *S. aureus* (SA-22) strain was found sensitive to
the tested antibiotics. Similarly, *Klebsiella pneumoniae* strains were found resistant to
multiple antibiotics. Interestingly, all strains were extended spectrum β-lactamases
(ESβL) producers (Table 2).

4.2. Antibacterial activity of medicinal plant extracts

A total of 25 traditionally used Indian medicinal plants belonging to 23 genera of 15
different families were collected. Their scientific and vernacular names, parts used
and ethnobotanical data are given in table M3. The yield of the extract has been
demonstrated in table 3. These plants were tested for antibacterial activity against
both reference and multidrug resistant strains as described below.

4.2.1. Antibacterial activity against reference strains

Methanolic extracts of all the plants were screened for their antibacterial activity
against Gram positive and Gram negative bacteria including *Staphylococcus aureus*
(SA-22), *Bacillus subtilis* (MTCC 121), *Pseudomonas aeruginosa* (PA01),
*Escherichia coli* (UP 2566) and *Salmonella typhimurium* (MTCC 98). The tested plant
extracts showed varying level of antibacterial activity. The antibacterial activity in
terms of zone diameter of growth inhibition was ranged from 11 to 34 mm. Several
plants extract demonstrated broad spectrum activity and inhibited all the tested bacterial strains, *S. aureus* (SA-22) being the most sensitive to plant extracts. The sensitivity pattern of the reference strains to plant extracts varied in the decreasing order of *S. aureus* > *B. subtilis* > *E. coli* > *S. typhimurium* > *P. aeruginosa* (Table 3).

On the basis of this screening plants were distributed into three bioactive groups (i) plants with most promising broad spectrum activity (inhibited ≥ 80% test bacteria). (ii) Plants with potential broad spectrum activity (inhibited ≥ 60% test bacteria). (iii) Plants with poor broad spectrum activity (inhibited < 60% test bacteria) as depicted in table 4. Methanolic extracts of twelve plants namely *Allium sativum*, *Capsicum frutescens*, *Carum copticum*, *Cinnamomum zeylanicum*, *Coriandrum sativum*, *Murraya koenigii, Piper cubeba*, *Piper nigrum*, *Psidium guajava*, *Punica granatum*, *Syzygium aromaticum* and *Zingiber officinale* inhibited the growth of all test bacteria and designated as most promising medicinal plants. However, three plants extract; *Cuminum cyminum, Myristica fragrans* and *Myristica fragrans* showed almost similar activity by inhibiting 80% test bacteria (Table 4).

4.2.2. Antibacterial activity against multidrug resistant strains

All plant extracts were evaluated for their activity against two important problematic groups of multidrug resistant bacteria (MRSA and ESβL producing *K. pneumoniae*). The results presented in the table 5 have indicated that active plant extracts are almost equally effective both against reference strains and multidrug resistant bacterial strains. Moreover, strain to strain variation was also evident in their activity.

Based upon their broad spectrum antibacterial activity against reference as well as drug resistant strains, nine plants belonging to most promising bioactive group were selected for further study which includes *C. copticum* (fruits), *C. zeylanicum* (bark), *M. koenigii* (leaves), *P. cubeba* (fruits), *P. nigrum* (seeds), *P. guajava* (leaves), *P. granatum* (peel), *S. aromaticum* (buds) and *Z. officinale* (rhizomes). Potency of the above plant extracts was determined in terms of their minimum inhibitory concentration (MIC) against drug resistant pathogenic bacteria. The MIC values ranged from 0.4 to ≥3.2 mg/ml against MRSA and ESβL producing *K. pneumoniae* and are represented in table 6.
Results

Based on the potency, only seven plants were further selected for fractionation in organic solvents in order of their increasing polarity viz. petrol ether, benzene, ethyl acetate, acetone, methanol and ethanol to locate the most promising broad spectrum antibacterial fraction.

The respective fractions of seven plants namely C. copticum, M. koenigii, P. cubeba, P. nigrum, P. guajava, P. granatum and Z. officinale demonstrated varying level of antibacterial activity against MRSA and ESβL producing K. pneumonia ranging from 10-34 mm (Table 7-13). The minimum inhibitory concentrations of selected plant fractions exhibiting broad spectrum activity in disc diffusion assay were determined and presented in table 14. The MIC values of plant extracts ranged from 0.4-3.2 mg/ml for P. granatum, 0.4->3.2 mg/ml (C. copticum), 0.8->3.2 mg/ml (M. koenigii), 1.6->3.2 mg/ml (Z. officinale) and (P. guajava) and ≥3.2 mg/ml (P. cubeba and P. nigrum). Based on inhibition of zone size and MIC values, the methanol fraction was found to be the most active fraction in C. copticum, P. nigrum, P. guajava and P. granatum. While benzene, ethyl acetate and ethanol were the most active fractions for M. koenigii, Z. officinale and P. cubeba, respectively. Overall promising antibacterial activity and potency was obtained in the extracts of P. granatum followed by C. copticum, M. koenigii, Z. officinale, P. guajava, P. cubeba and P. nigrum.

4.3. Antioxidant activity of medicinal plant extracts

All plant extracts from 25 medicinal plants under study were subjected to antioxidant scrutiny by four different antioxidant methods viz. DPPH free radical scavenging activity, reducing power activity by FRAP and CUPRAC assays and total antioxidant capacity by phosphomolybdenum method. The methanol plant extracts were tested at concentrations ranging from 12.5-400 μg/ml. The standards curves graphs of antioxidant activity by phosphomolybdenum method and total phenolic content of medicinal plants are represented in fig 1.

Of these, seventeen plants extract demonstrated strong DPPH scavenging activity (>70% decolorization) at 400 μg/ml concentration. The order of activity among these plants was maximum for P. granatum followed by Z. officinale> S. aromaticum> P. guajava> C. copticum> M. fragrans> A. cepa> C. cyminum> L. nobilis> C. sativum> M. koenigii> F. vulgare> F. asafoetida> C. longa> M. fragrans (seed)> C.
• (Rfsufts frutescens) C. zeylanicum. The values were comparable to commercial standards ascorbic acid (94.2%) and BHT (92.0%). The remaining extracts showed good (50-70% decolorization) to weak free radical scavenging (<50% decolorization) activity. The concentration dependent free radical scavenging activity by DPPH assay (% decolorization) was recorded in all the plant extracts with tested concentrations of 12.5-400 μg/ml as depicted in table 15 and fig 2.

Similarly by ferric reducing antioxidant power (FRAP) method, except six plants namely A. sativum, A. subulatum, P. nigrum, P. somniferum, S. indicum and T. foenumgraecum, all plant extracts demonstrated powerful ferric ions (Fe³⁺) reducing ability (absorbance ≥ 1.0). In this assay, the higher absorbance values correspond with higher reducing ability. However, low ferric ion reducing ability was shown by the above six plant extracts when compared to standards (ascorbic acid and BHT). The reducing power of all plant extracts increased with increasing concentration of the extracts (concentration tested from 12.5 to 400 μg/ml) as shown in table 16 and fig 3.

On the other hand, when these extracts were tested by copper ion reducing antioxidant capacity (CUPRAC) method, eighteen plant extracts expressed their activity in order of P. granatum > C. copticum > P. guajava > S. aromaticum > Z. officinale > C. cymimum > M. fragrans (seed) > A. cepa > M. fragrans (aril) > L. nobilis > F. asafoetida > N. sativa > B. juncea > C. longa > F. vulgare > P. cubeba > C. frutescens > M. koenigii (absorbance ≥ 1.0) while other plant extracts showed low reducing antioxidant activity. Moreover, the reducing power of all the plant extracts increased with increasing concentration of samples (i.e. from 12.5 to 400 μg/ml) as depicted in table 17 and fig 4. The reducing power activity was expressed relative to standards, ascorbic acid and BHT.

The total antioxidant capacity was calculated by phosphomolybdenum method, where all the plant extracts showed response in a concentration dependent manner and results were expressed as equivalents of ascorbic acid (μmol/g) as presented in table 18. The fifteen plant extracts namely P. guajava followed by S. aromaticum, M. fragrans (aril), P. granatum, M. fragrans (seed), A. cepa, C. copticum, C. cymimum, C. sativum, C. longa, L. nobilis, A. subulatum, Z. officinale, T. foenumgraecum and
*M. koenigii* demonstrated high total antioxidant capacity (≥1000 μmoles/g) at the concentration of 400 μg/ml compared to other plant extracts.

The comparative analysis of medicinal plants extracts determined by all the four methods described above suggested that *A. cepa*, *C. copticum*, *C. cymminum*, *C. longa*, *L. nobilis*, *M. koenigii*, *M. fragrans* (aril), *M. fragrans* (seed), *P. guajava*, *P. granatum*, *S. aromaticum*, *Z. officinale* possessed broad spectrum antioxidant activity as evident from their respective activities. Although other plant extracts like *C. frutescens*, *C. zeylanicum*, *C. sativum*, *F. vulgare* and *F. asafoetida* showed fair antioxidant activity by two or more methods.

4.3.1. Fraction based antioxidant activity of selected medicinal plants

On the basis of broad spectrum antioxidant activity of medicinal plant extracts, five plants namely *Carum copticum*, *Punica granatum*, *Murraya koenigii*, *Zingiber officinale* and *Psidium guajava* were selected for fraction based antioxidant activity determination. On the other hand, two plants viz. *Piper cubeba* and *Piper nigrum* possessing relatively weak antioxidant behaviour were also selected in order to locate the possible antioxidant fraction for comparative analysis.

4.3.2. Antioxidant activity of *Carum copticum* (fruit) fractions

The yield of sequentially extracted fractions of *C. copticum* in different solvents was 0.4% (petrol ether), 0.2% (benzene), 0.8% (ethyl acetate), 1.2% (acetone), 2.0% (methanol) and 1.0% (ethanol) respectively. These fractions were tested for antioxidant properties in order to identify best active fraction.

The *C. copticum* fractions showed a varying level of free radical scavenging activity (Fig 5a). The methanol fraction was found to be the most active free radical scavenger exhibited 90.2% scavenging of DPPH at 100 μg/ml concentration compared to ascorbic acid (90.3%) and BHT (86.5%). Likewise, the ethanol fraction showed 57.1% scavenging activity at 100 μg/ml concentration. Petrol ether, benzene, ethyl acetate and acetone exhibited relatively less activity ranging from 3.8% to 20%.

As shown in fig 5b, these fractions had effective reducing power using the potassium ferricyanide reduction method when compared to the standards (ascorbic acid and BHT). Similarly, the antioxidant activity by CUPRAC assay indicated the highest
reducing power potential in methanol fraction followed by ethanol and acetone fractions (Fig 5c). While other remaining fractions displayed less reducing power activity. The results are comparable to ascorbic acid and BHT.

The *C. copticum* fractions when tested by phosphomolybdenum method exhibited concentration dependent antioxidant capacity with respect to ascorbic acid equivalents (Table 19). At 100 μg/ml concentration, methanol fraction showed maximum antioxidant capacity (2087.7 μmol) followed by ethanol fraction (1514.7 μmol). The activity of other fractions is in order of acetone > petrol ether > ethyl acetate > benzene. However, there was no significant increase in total antioxidant capacity at the tested concentrations (25, 50 and 100 μg/ml) among petrol ether, ethyl acetate and benzene fractions.

4.3.3. Antioxidant activity of *Punica granatum* (peel) fractions

The percent yield of sequentially extracted fractions of *Punica granatum* in different solvents *viz* petrol ether, benzene, ethyl acetate, acetone, methanol and ethanol was 0.2, 0.16, 1.2, 5.2, 10.2 and 6.1 respectively. A concentration dependent response is evident in the fractions (methanol, acetone and ethanol) at tested concentrations of 10-80 μg/ml (Fig 6a). These fractions almost completely inhibited DPPH absorption at 80 μg/ml concentration like methanol fraction (90.53%), acetone (86.4%) and ethanol fraction inhibited 83.2% absorption of DPPH. Positive controls, ascorbic acid and BHT inhibited 91.1% and 85.6% DPPH absorption respectively. Ethyl acetate fraction was relatively less efficient as radical scavengers with an inhibition of only 16.2%. Petrol ether and benzene fractions also showed remarkably lower degrees of radical scavenging activity.

These fractions also had effective reducing power using the potassium ferricyanide reduction method when compared to the standards. Similarly, the antioxidant activity by CUPRAC assays indicated the highest reducing power potential in methanol fraction followed by ethanol, acetone and ethyl acetate fractions (Fig 6b and 6c). The results are comparable to ascorbic acid and BHT.

Moreover, total antioxidant activity by phosphomolybdenum method exhibited concentration dependent antioxidant capacity with respect to ascorbic acid equivalents.
(Table 20). At highest tested concentration (80 µg/ml), the methanol fraction showed maximum antioxidant capacity (5067.7 µmol) followed by ethanol (3323.0 µmol), and acetone (2481.6 µmol) fractions. However, ethyl acetate, petrol ether and benzene fractions displayed relatively less total antioxidant capacity at the tested concentrations of 10-80 µg/ml.

4.3.4. Antioxidant activity of *Murraya koenigii* (leaf) fractions

*Murraya koenigii* yielded 1.0, 1.2, 1.2, 1.8, 2.2 and 0.8 percent of the extract when fractionated in petrol ether, benzene, ethyl acetate, acetone, methanol and ethanol respectively. The free radical scavenging activity of *M. koenigii* leaf fractions by DPPH method exhibited a concentration dependent response in all the fractions (Fig 7a). The benzene fraction was found to be the most active free radical scavenger (88.3% decreases at a concentration of 100 µg/ml) followed by ethyl acetate (79.5%) and petrol ether (78.7%) fractions. While positive controls (ascorbic acid and BHT) inhibited 93.1% and 86.5% DPPH absorption respectively. Moreover acetone, methanol, and ethanol fractions showed decolorization of 66.1%, 50.7% and 53.0% respectively.

Similarly, the antioxidant activity by FRAP and CUPRAC assays indicated the highest reducing power potential in benzene fraction followed by petrol ether and ethyl acetate as shown in fig 7b and 7c. The results are comparable to ascorbic acid and BHT. The total antioxidant activity of above fractions by phosphomolybdenum method exhibited concentration dependent antioxidant capacity with respect to ascorbic acid equivalents (Table 21). The benzene fraction showed maximum antioxidant capacity (3510.4 µmol) at 100 µg/ml followed by ethyl acetate (1982.3 µmol), petrol ether (1967.2 µmol) and acetone (1783.0 µmol) fractions. However, methanol and ethanol fractions displayed relatively less total antioxidant capacity at the tested concentrations of 12.5-100 µg/ml.

4.3.5. Antioxidant activity of *Zingiber officinale* (rhizome) fractions

The percent yield of sequentially extracted fractions of *Zingiber officinale* in different solvents *viz* petrol ether, benzene, ethyl acetate, acetone, methanol and ethanol was 0.5, 0.5, 2.5, 3.1, 1.7 and 1.5 respectively. The free radical scavenging activity of the
fractions of *Z. officinale* (rhizome) was measured as decolorizing activity following the trapping of the unpaired electron of 1,1-diphenyl-2-picrylhydrazyl (DPPH) as shown in fig 8a. A concentration dependent response is apparent in all the fractions at tested concentrations of 10-80 μg/ml. The ethyl acetate fraction showed remarkably higher degrees of radical scavenging activity of 88.3% while acetone was the second most DPPH radical scavenger (84.0%) followed by methanol and ethanol exhibiting almost 83.0% of DPPH radical scavenging activity. Positive controls (ascorbic acid and BHT) showed decolorization of 91.1% and 85.6%. Furthermore, benzene and petrol ether fraction was comparatively less efficient as radical scavengers with the decolorization of 68.6% and 63.1% respectively.

It is evident from fig 8b, the ethyl acetate and acetone fractions had effective reducing power using the potassium ferricyanide reduction method when compared to the standards followed by other fractions. Similarly, the antioxidant activity by CUPRAC assays indicated the highest reducing power potential in ethyl acetate fraction followed by acetone and methanol fractions. The results are comparable to ascorbic acid and BHT (Fig 8b and 8c).

These fractions when tested for total antioxidant activity by phosphomolybdenum method exhibited concentration dependent antioxidant capacity with respect to ascorbic acid equivalents (Table 22). At highest tested concentration of 80 μg/ml, the ethyl acetate fraction showed maximum antioxidant capacity (4944.1 μmol) followed by acetone (2731.2 μmol), and petrol ether (2148.7 μmol) fractions. However, benzene, methanol and ethanol fractions displayed relatively less total antioxidant capacity at the tested concentrations of 10-80 μg/ml.

### 4.3.6. Antioxidant activity of *Psidium guajava* (leaf) fractions

*Psidium guajava* leaves were fractionated into different solvents. It yielded 0.4% extract in petrol fraction, 0.6% in benzene, 1.0% in ethyl acetate, 1.3% each in acetone and ethanol fractions. The maximum yield was found in methanol fraction (4.2%).

By DPPH assay, a concentration dependent response is evident in all the fractions (methanol, acetone and ethanol) at tested concentrations of 10-80 μg/ml as illustrated...
in fig 9a. The highest free radical scavenging was exhibited by methanol fraction (85.8%) followed by acetone (80.8%) and ethanol fraction (77.9%). Positive controls (ascorbic acid and BHT) inhibited 91.1% and 85.6% DPPH absorption respectively. Interestingly ethyl acetate fraction also demonstrated relatively good DPPH scavenging with an inhibition of 73.5%. Petrol ether and benzene fractions showed lower degrees of radical scavenging activity (51.9% and 60.8%).

*P. guajava* (leaf) fractions showed strong antioxidant activity when tested in FRAP and CUPRAC assays. The reducing power of some of these fractions was comparable to the standards, ascorbic acid and BHT (Fig 9b and 9c). The highest reducing power potential was exhibited by methanol fraction followed by ethanol and acetone fractions. The other fractions demonstrated relatively lower activity as compared to the standards.

The *P. guajava* fractions by phosphomolybdenum method exhibited concentration dependent antioxidant capacity with respect to ascorbic acid equivalents (Table 23). At highest concentration of 80 µg/ml, the methanol fraction showed maximum antioxidant capacity (4175.1 µmol) followed by ethanol fraction (1733.4 µmol). However, other fractions displayed relatively less activity.

4.3.7. Antioxidant activity of *Piper nigrum* (seed) fractions

Sequential fractionation was carried out for the *Piper nigrum* seeds. Petrol ether and benzene fractions yielded only 0.2% extracts, however yield was 7-10 times higher for the ethyl acetate (1.5%), acetone (2.0%) and ethanol (2%). Similar to other plants methanol fraction yielded maximum extracts (5.2%).

The antioxidant activity was determined at higher concentrations (25-200 µg/ml) compared to other plants. All the fractions of *P. nigrum* showed poor (< 50.0%) free radical scavenging activity by DPPH assay (Fig 10a). In the same way, these fractions had ineffective reducing power using the potassium ferricyanide reduction method. But the antioxidant activity by CUPRAC assay indicated the moderate reducing power potential in methanol fraction followed by ethanol and other fractions (Fig 10b and 10c).

Likewise, total antioxidant activity by phosphomolybdenum method exhibited
concentration dependent antioxidant capacity with respect to ascorbic acid equivalents (Table 24). At highest tested concentration (200 µg/ml), the methanol fraction showed antioxidant capacity (583.3 µmol) followed by ethanol (465.9 µmol). However, other fractions displayed relatively very low total antioxidant capacity at the tested concentrations of 25-200 µg/ml.

4.3.8. Antioxidant activity of *Piper cubeba* (fruit) fractions

The percent yield of sequentially extracted fractions of *Piper cubeba* in different solvents viz. petrol ether, benzene, ethyl acetate, acetone, methanol and ethanol was 0.2, 0.4, 1.9, 2.7, 4.5 and 3.1 respectively. The free radical scavenging activity of the fruits fractions of *P. cubeba* by DPPH method revealed that all the fractions were relatively less efficient as radical scavengers except ethanol fraction that inhibited 63.4% absorption of DPPH (Fig 11a).

As can be seen from fig 11b, these fractions had low reducing power using the potassium ferricyanide reduction (FRAP) and CUPRAC method when compared to the standards. The antioxidant activity by assays indicated the moderate reducing power potential in ethanol fraction while other fractions represented low activity (Fig 11b and 11c).

Total antioxidant activity by phosphomolybdenum method exhibited concentration dependent antioxidant capacity with respect to ascorbic acid equivalents (Table 25). At highest tested concentration (200 µg/ml), the ethanol fraction showed maximum antioxidant capacity (521.7 µmol) followed by acetone (421.3 µmol) fractions. However, other fractions displayed relatively less total antioxidant capacity at the tested concentrations of 25-200 µg/ml.

4.4. Antimutagenic activity of antioxidant active fractions of tested medicinal plant extracts

On the basis of antioxidant activity of various fractions of seven plants, most active antioxidant fraction of each plant was selected to evaluate their antimutagenic potentials. Two fractions from *P. nigrum* and *P. cubeba* which revealed relatively poor activity had also been subjected to antimutagenic evaluation to assess the possible correlation between antioxidant and antimutagenic properties.
Based on promising antioxidant activity, the methanol fraction of *C. copticum* was selected and evaluated for its antimutagenic activity by Ames *Salmonella* assay against direct acting mutagens sodium azide (NaN₃) and methyl methanesulfonate (MMS) and in the presence of +S9 fraction against 2-aminoflourene (2-AF) and benzo(a)pyrene (B(a)P). The methanol fraction at tested concentrations (25, 50 and 100 µg/plate) by plate-incorporation method could not show any sign of mutagenicity and toxicity to *Salmonella typhimurium* strains, either alone or in the presence of S9 mix. The data presented in table 26 and 27 revealed that the antimutagenic activity of methanol fraction was found to be dose dependent. At a dose of 100 µg/plate, antimutagenic response was significant at (P < 0.05) against NaN₃ induced mutagenicity in TA97a with a percent mutagenicity decrease of 73.9 followed by TA100 (70.4%), TA102 (56.0%) and TA98 (54.4%) strains (Table 26 and Fig 12). The linear regression analysis between extract dose and antimutagenic response against respective test mutagen showed correlation with respect to dose dependent response in TA97a (R² = 0.99), TA98 (R² = 0.99), TA100 (R² = 0.98) and TA102 (R² = 0.99).

Similar trend of activity was obtained with MMS induced mutagenicity where percent decrease in number of His⁺ revertants was significant at (P < 0.005) for TA97a (83.1) followed by TA102 (75.1), TA100 (74.6), TA98 (58.9) as depicted in table 27 and fig 13. Linear correlation between extract dose and antimutagenic response was highly significant in the strain TA97a and TA98 (R² = 0.99) followed by TA100 (R² = 0.96) and TA102 (R² = 0.93). The antimutagenicity of *C. copticum* methanol fraction has also been demonstrated against indirect acting mutagens benzo(a)pyrene and 2-aminoflourene. The methanol fraction at 100 µg/plate was found to be inhibitory for B(a)P induced mutation at (P < 0.005) as evident from the data presented in table 28 and fig 14. The dose dependent response of the antimutagenic behaviour was ranged from 51.0% to 64.5%. The order of antimutagenic sensitivity detection by tester strains with significant regression values was found to be in order of *S. typhimurium* TA100 (R² = 0.91) > TA98 (R² = 0.93) > TA102 (R² = 0.99) > TA97a (R² = 0.99). Likewise,
antimutagenic activity has been shown by methanol fraction against 2-AF which ranged from 55.4% to 68.5%. The significant reduction (P < 0.005) in number of revertants was recorded for TA98 followed by TA100, TA97a and TA102 (Table 29 and Fig 15). Further, the linear regression analysis between extract dose and antimutagenic response showed strong correlation in TA97a ($R^2=0.99$) followed by TA98 ($R^2=0.99$), TA102 ($R^2=0.98$) and TA100 ($R^2=0.96$).

4.4.2. Antimutagenic activity of Punica granatum

Methanol fraction of P. granatum peels was evaluated for its antimutagenic activity against all four tested mutagens viz. (NaN₃, MMS, 2-AF and B(a)P) respectively. In a series of experiments preceding the antimutagenicity studies, it was ascertained that the different concentrations of methanol fraction (10, 20, 40 and 80 µg/plate) added to the Ames tester strains do not influence their viability and the mutation frequencies did not change significantly when compared to spontaneous ones.

The data of P. granatum methanol fraction on direct acting mutagens NaN₃ and MMS are presented in table 30 and 31. At the concentration of 80 µg/plate, the pomegranate exhibited maximum percent antimutagenicity in Salmonella tester strains, TA100 (84.5) followed by TA97a (80.4), TA98 (76.8) and TA102 (66.8) against NaN₃ induced mutagenicity (Table 30 and Fig 16). The results were statistically significant (P ≤ 0.05) whilst relationship between concentration and antimutagenic response was dose dependent in the strain TA97a ($R^2=0.93$), TA98 ($R^2=0.95$), TA100 ($R^2=0.99$) and TA102 ($R^2=0.88$). Likewise, the inhibition percent of MMS induced mutagenicity was recorded as 91.9% in TA100, 90.5% in TA102, 86.6% in TA97a and 76.6% in TA98. The antimutagenic effect of methanol fraction was found to be concentration-dependent ($R^2=0.98-0.99$) as depicted in table 31 and fig 17.

The antimutagenic behavior of P. granatum methanol fraction against benzo(a)pyrene and 2-aminoflourene is presented in table 32 and 33. The dose dependent antimutagenic response was highly significant (P < 0.001) with percent inhibition of mutagenicity ranged from 81.2% to 87.2% (Table 32 and Fig 18). All the strains demonstrated reduction in the revertants in a dose dependant manner with the regression values of 0.97 to 0.99. Similar trend of antimutagenic activity against 2-AF was also shown by P. granatum. The significant reduction (P < 0.001) in number of
revertants was recorded by TA100 (88.9%) followed by TA102 (86.0%), TA97a (83.8%) and TA98 (82.3%) as shown in Table 33 and Fig 19. Further, the linear regression analysis between extract dose and antimutagenic response showed strong correlation in TA100 ($R^2=0.99$) followed by TA98 ($R^2=0.99$), TA97a ($R^2=0.99$) and TA102 ($R^2=0.96$).

4.4.3. Antimutagenic activity of *Murraya koenigii*

The benzene fraction of *M. koenigii* at tested concentrations (12.5, 25, 50 and 100 µg/plate) was found non-mutagenic as well as non-toxic to *Salmonella typhimurium* strains, either alone or in the presence of S9 mix. Moreover, the antimutagenic activity of benzene fraction was found to be dose dependent (Table 34 and 35). At a dose of 100 µg/plate, antimutagenic response was significant at ($P < 0.05$) against TA97a with a decrease in mutagenicity by 84.9% followed by TA100 (84.4%), TA98 (73.2%) and TA102 (72.2%) against NaN₃ induced mutagenicity (Table 34 and Fig 20). The linear regression analysis between extract dose and antimutagenic response against respective test mutagen showed strong correlation ranging from 0.95-1.0.

Likewise, the decrease in number of His⁺ revertants was significant at $P < 0.005$ for TA102 (86.0%) followed by TA100 (83.6%), TA97a (80.0%), TA98 (74.1%) against MMS induced mutagenicity as depicted in table 35 and fig 21. Linear correlation between extract dose and antimutagenic response was highly significant in the strain TA102 and TA97a ($R^2=0.99$) followed by TA 100 ($R^2=0.98$) and TA98 ($R^2=0.96$).

The antimutagenicity of *M. koenigii* benzene fraction has also been demonstrated against indirect acting mutagens benzo(a)pyrene and 2-aminofluorene. At 100 µg/plate, the benzene fraction was found antimutagenic for B(a)P induced mutation at ($P < 0.001$) as evident from the data presented in table 36 and fig 22. The antimutagenic response was dose dependent ($R^2 \geq 0.98$) and it ranged from 80.1% to 86.0%. Similarly, trend in antimutagenicity has been shown against 2-AF as presented in table 37 and fig 23. Further, the linear regression analysis between extract dose and antimutagenic response showed strong correlation ranging from 0.99 to 1.0 against respective tester strains.
4.4.4. Antimutagenic activity of Zingiber officinale

Ethyl acetate fraction of *Z. officinale* (rhizome) was selected due to its high antioxidant potential. In a series of experiments preceding the antimutagenicity studies, it was ascertained that the different concentrations of ethyl acetate fraction (10, 20, 40 and 80 μg/plate) added to the Ames tester strains do not influence their viability and the mutation frequencies did not change significantly when compared to spontaneous revertants.

The data of *Z. officinale* ethyl acetate fraction on direct acting mutagens NaN₃ and MMS are presented in table 38 and 39. *Z. officinale* at concentration of 80 μg/plate exhibited maximum antimutagenicity in TA100 (86.9%) followed by TA97a (86.7%), TA98 (75.7%) and TA102 (70.4%) tester strains against NaN₃ induced mutagenicity (Table 38 and Fig 24). The results were statistically significant at \(P < 0.05\) except TA102 whilst linear relationship between extract dose and antimutagenic activity was dose dependent \((R^2=0.99)\).

Likewise, the inhibition percent of MMS induced mutagenicity was recorded as 96.7% in TA100, 91.5% in TA102, 86.7% in TA97a and 62.1% in TA98 (Table 39 and Fig 25). The significant antimutagenic effect \((P < 0.001)\) of methanol fraction was found to be concentration-dependent as evident from the regression analysis between extract dose and antimutagenic response against respective test mutagen in TA102 \((R^2=1.0)\) and TA97a \((R^2=1.0)\) followed by TA100 \((R^2=0.98)\) and TA98 \((R^2=0.91)\).

Similarly, *Z. officinale* ethyl acetate fraction was also evaluated for its antimutagenic behaviour against benzo(a)pyrene and 2-aminoflourene that infers mutagenicity by microsomal activation. Extract of *Zingiber officinale* significantly \((P < 0.005)\) inhibited mutagenicity ranging from 83.8% to 88.2% (Table 40 and Fig 26). All the strains demonstrated reduction in the revertants in a dose dependent manner with the regression values ranged from 0.97 to 0.99. Similar trend of antimutagenic activity against 2-AF was also shown by *Z. officinale*. The significant reduction \((P < 0.005)\) in number of revertants was recorded in TA100 (88.2%) followed by TA102 (86.3%), TA97a (84.5%) and TA98 (82.5%) as indicated in table 41 and fig 27. Further, the linear regression analysis between extract dose and antimutagenic response showed strong correlation in TA102 \((R^2=1.0)\) followed by TA97a \((R^2 = 0.99)\), TA98
4.4.5. Antimutagenic activity of *Psidium guajava*

Methanol fraction of *P. guajava* leaves (10, 20, 40 and 80 µg/plate), did not change mutation frequencies significantly when compared to spontaneous revertants indicated that they are non-mutagenic in nature.

The data of methanol fraction on direct acting mutagens NaN₃ and MMS are presented in table 42 and 43. *P. guajava* at 80 µg/plate concentration exhibited maximum antimutagenicity in TA100 (84.8%) followed by TA97a (80.7%), TA98 (76.1%) and TA102 (71.3%) tester strains against NaN₃ induced mutagenicity (Table 42 and Fig 28). The results were statistically significant at (P ≤ 0.05) though linear relationship between extract dose and antimutagenic response was strong in the strain TA97a (R²=1.0), TA100 (R²=0.99), TA98 (R²=0.97) and TA102 (R²=0.97). Similarly, the inhibition percent of MMS induced mutagenicity was recorded as 86.2% in TA102, 82.9% in TA100, 77.2% in TA97a and 73.9% in TA98 (Table 43 and Fig 29). The antimutagenic effect of methanol fraction was significant (P < 0.005) and found to be concentration dependent as evident from the regression analysis between extract dose and antimutagenic response against respective test mutagen in TA97a (R²=0.99) followed by TA100 (R²=0.98), TA102 (R²=0.96) and TA98 (R²=0.96).

The *P. guajava* methanol fraction when tested against benzo(a)pyrene and 2-aminoflourene in the presence of S9 revealed a dose dependent antimutagenic response (P < 0.001) with percent inhibition of mutagenicity ranged from 80.6% to 85.9% (Table 44 and Fig 30). All the strains demonstrated reduction in the revertants in a dose dependent manner with the regression values ranged from 0.98 to 1.0. Similar trend of antimutagenic activity against 2-AF was shown by *P. guajava* methanol fraction. The significant reduction (P ≤ 0.005) in number of revertants was recorded by TA102 (85.8%) followed by TA100 (85.1%), TA98 (84.0%) and TA97a (82.9%) as indicated in table 45 and fig 31. Further, the linear regression analysis between extract dose and antimutagenic response showed strong correlation in TA100 (R²=1.0) followed by TA97a, TA98 and TA102 (R²=0.99).
4.4.6. Antimutagenic activity of *Piper nigrum*

The methanol fraction of *P. nigrum* seeds at tested concentrations (25, 50, 100 and 200 µg/plate) could not show any sign of mutagenicity and toxicity to *Salmonella typhimurium* strains, either alone or in the presence of S9 mix. The data presented in table 46 and 47 revealed that the methanol fraction was antimutagenic and found to be dose dependent. At a dose of 200 µg/plate, antimutagenic response against NaN₃ induced mutagenicity was significant at (P < 0.05) against TA97a and TA100 with a decrease in mutagenicity 66.8% and 68% respectively. While antimutagenic response was non-significant for TA98 (61.5%) and TA102 (58.8%) strains (Table 46 and Fig 32). The linear correlation between extract and antimutagenic activity against respective test mutagens was dose dependent (R² = 0.99 to 1.0). Similar trend was obtained with MMS induced mutagenicity where percent decrease in number of His⁺ revertants was significant (P < 0.005) for TA97a (56.1%), TA100 (63.6%) and TA102 (64.2%) as depicted in table 47 and fig 33. Extracts showed 55.9% inhibition of mutagenicity against TA98, although it was not significant. Linear correlation between extract dose and antimutagenic response was dose dependent (R² = 0.95 to 0.99).

Moreover, the methanol fraction (200 µg/plate) inhibited mutagenicity induced by B(a)P and 2-AF (P < 0.005 and P < 0.05, respectively) as evident from the data presented in table 48 and 49; fig 34 and 35. Further they inhibited 60.2-68.4% His⁺ revertants induced by mutagens in different tester strains. *P. nigrum* showed linear correlation between tested concentrations and antimutagenic activity (R² = 0.99 to 1.0) irrespective of strains and mutagens.

4.4.7. Antimutagenic activity of *Piper cubeba*

Antimutagenicity of *P. cubeba* (ethanol fraction) against mutagenicity induced by NaN₃ and MMS are presented in table 50 and 51. Different concentrations (25-200 µg/plate) of the plant extract were taken for antimutagenicity assay. At the concentration of 200 µg/plate, the extracts exhibited 64.5% antimutagenicity in TA100 followed by TA97a (60.7%), TA102 (51.5%) and TA98 (46.2%) tester strains against NaN₃ induced mutagenicity (Table 50 and Fig 36). The results were statistically significant only for TA100 whilst linear relationship between extract dose
and antimutagenic response was ($R^2 = 0.98$ to 0.99) for respective strains. Likewise, *Piper cubeba* inhibited MMS induced mutagenicity by 64.9% in TA102, 60.4% (TA100), 55.1% (TA97a) and 50% (TA98). The antimutagenic effect of ethanol fraction was concentration dependent ($R^2 = 0.92$ to 0.98) as depicted in table 51 and fig 37.

Further, the response was dose dependent against B(a)P was highly significant ($P < 0.005$) with percent inhibition of mutagenicity ranged from 59.5% to 66.2% (Table 52 and Fig 38). All the strains demonstrated reduction in the revertants in a dose dependent manner with the regression values ranged from 0.95 to 0.99. Similar trend of antimutagenic activity was shown against 2-AF where the significant reduction ($P \leq 0.05$) in number of revertants was recorded for TA102 (65.0%) followed by TA100 (61.9%), TA97a (56.9%) and TA98 (56.4%) with regression values ($R^2 = 0.96$ to 0.99) as depicted in table 53 and fig 39.

4.4.8. Antimutagenic activity of pure phytocompounds (Punicalagin and Ellagic acid)

On the basis of antimutagenicity testing of seven plants against four mutagens, *Punica granatum*, showed best activity was selected for further studies. Phytochemical profiling of *Punica* (methanol extract) showed the presence of mainly punicalagins and some other compounds in traces. To evaluate the active principle, we further tested this ellagitannins (punicalagins A and B) and ellagic acid (another compound present in *Punica*) for their antimutagenic potential by Ames test against NaN₃, MMS, 2-AF and B(a)P induced mutagenicity. Punicalagins and ellagic acid at 50, 100, 250 and 500 µM/plate concentrations by plate-incorporation assay showed no sign of mutagenicity and toxicity to *Salmonella typhimurium* strains, either alone or in the presence of S9 mix (Table 54 and 55).

Ellagic acid at a dose of 500 µM/plate showed significant ($P < 0.005$) antimutagenic activity against TA97a with a decrease in mutagenicity by 72.1% followed by TA100 (65.9%), TA98 (64.2%) and TA102 (62.3%) against NaN₃ induced mutagenicity (Table 54 and Fig 40). Similar trend of activity was obtained with MMS induced mutagenicity where decrease in number of His⁺ revertants was significant at ($P < 0.005$) for TA102 (73.7%) followed by TA98 (69.0%), TA97a (66.5%) and TA100.
Results

(65.3%), as depicted in table 55 and fig 41. The antimutagenicity of ellagic acid against indirect acting mutagens benzo(a)pyrene and 2-aminoflourene was significant at P < 0.005 and presented in table 56 and 57; fig 42 and 43. Ellagic acid showed dose dependent antimutagenic behaviour against both mutagens (B(a)P and 2-AF) ranging from 78.6% to 88.9% (Table 54-57). The regression analysis between extract dose and antimutagenic response was significant (R²= 0.93-0.99), as presented in tables 54-57.

Likewise, at a dose of 500 μM/plate of punicalagin, antimutagenic response was significant at (P < 0.05) against TA97a with a percent mutagenicity decrease of 74.4% followed by TA100 (74.3%), TA98 (65.3%) and TA102 (59.8%) strains against NaN₃ induced mutagenicity (Table 58 and Fig 44). Punicalagins inhibited MMS induced mutagenicity by 75.0% in TA102, 72.1% in TA97a, 70.9% in TA98 and 66.0% in TA100 (Table 59 and Fig 45).

Punicalagin also showed significant (P < 0.005) inhibition of mutagenicity when tested in the presence of S9 mix. At 500 μM/plate concentration, punicalagins inhibited mutagenicity in the range of 76.7% to 85.0% against B(a)P (Table 60 and Fig 46) and 2-AF (Table 61 and Fig 47). Further, the linear regression analysis between extract dose and antimutagenic response was significant (R²=0.91-0.99), indicated in tables 58-61.

4.5. Phytochemical analysis of plant extracts

Total phenolic contents were determined by Folin-Ciocalteu method and expressed as gallic acid equivalent in all the plant extracts. Methanolic plant extracts tested in this study contained total phenolics in the range of 24.95 to 329.92 mg GAE /g of dry extract (Table 15). Phenolic contents in the extracts of A. cepa, C. frutescens, C. copticum, C. zeylanicum, C. longa, M. koenigii, M. fragrans, P. guajava, P. granatum, S. aromaticum and Z. officinale was found more than 15% on dry weight basis. Major groups of phytocompounds were also determined in active fractions by colour test and infrared spectroscopy (IR) analysis (Table 62 and Fig 48-54).

4.5.1. Phytochemical analysis of Carum copticum

Phytochemical analysis of Carum copticum extracts revealed the presence of alkaloids
and phenolics as major groups of compounds. The total phenolic content (mg GAE/g) of extract and various fractions determined by the Folin-Ciocalteu method showed highest polyphenolic content (119.2±0.3) in methanol fraction followed by petrol ether (90.0±1.7), benzene (49.5±0.2), acetone (48.3±0.6), ethyl acetate (45.9±0.3) and ethanol (43.3±1.2) fractions (Table 63).

The HPLC analysis of methanol fraction of *C. copticum* revealed the presence of various compounds at different wavelengths and different retention times. The retention time of thymol in aqueous phosphoric system was found to be 49.5 min and constitutes second largest peak in methanol fraction (Fig 55). However, the other largest peak could not be identified in HPLC due to unavailability of reference compounds. A total of 4 components were identified by gas chromatography–mass spectrometry (GC-MS) using direct similarity search for *C. copticum*. The major compound identified was thymol (95.14%) as evident from the GC-MS spectra (Table 64 and Fig 56). Other compounds present were linolelaidic acid methyl ester (1.54%), cis, cis-linoleic acid (2.55%), 3-nitrophthalic acid (0.77%) respectively. These numbers may be extended with the help of chemometric techniques.

### 4.5.2. Phytochemical analysis of *Punica granatum*

Phytochemical analysis of pomegranate (peel) revealed the presence of phenolics as major group of compounds. The total phenolic content of various fractions (mg/g of dry extract) was determined as gallic acid equivalent by the Folin-Ciocalteu method. Methanol fraction of peel contained 468.3±5.5 mg GAE / g of dry extracts followed by the ethanol (414.6±5.9), acetone (219.3±1.1) and ethyl acetate (20.3±0.7) fractions as depicted in table 63.

The plant fractions which displayed fair to good antioxidant activity were subjected to HPLC followed by LC-MS analysis. Interestingly, HPLC analysis of acetone and methanol fractions confirmed the presence of punicalagins A and B as well as ellagic acid; however the relative abundance of ellagic acid was more in methanol fraction as compared to acetone fraction (Fig 57). The retention times of punicalagins A and B and ellagic acid in aqueous phosphoric system were found to be 28.5 min, 30.5 min and 37.5 min when compared with the required standards. Other minor peaks in HPLC could not be identified due to non availability of suitable standards.
The presence of various polyphenols can be seen in all the fractions characterized by MS analysis. In addition to punicalagins and ellagic acid, punicalin, gallagic acid and few other phenolics are present in appreciable amount (Table 65). LC-MS spectra by direct infusion of *Punica* fractions shows the presence of punicalagins ($M - H\ m/z\ 1083$), punicalin ($M - H\ m/z\ 781$), corilagin ($M - H\ m/z\ 633$), gallagic acid ($M - H\ m/z\ 601$), 2,3-(S)-HHDP-D-glucose ($M - H\ m/z\ 433$), ellagic acid ($M - H\ m/z\ 301$). The other peaks of major compounds identified are presented in table 65 and fig 58.

### 4.5.3. Phytochemical analysis of *Murraya koenigii*

Phytochemical analysis of fractions revealed the presence of alkaloids, phenolics and glycosides as major group of compounds. The total phenolic content (mg/g) of *Murraya koenigii* various fractions showed highest polyphenolic content ($187.1\pm6.3$) in benzene fraction followed by petrol ether ($146.4\pm6.3$), ethyl acetate ($113.9\pm3.0$), acetone ($113.9\pm2.7$), ethanol ($110.3\pm2.0$) and methanol ($103.2\pm3.1$) fractions as depicted in table 63.

A total of 21 chemical components were identified in leaf extract by GC-MS analysis. These numbers may be extended with the help of chemometric techniques. The major compounds identified were caryophyllene (14.8%) followed by 3-undecen-5-yne (Z) (9.52%), phytol (9.17%), 2-methyl-3H-phenanthro [3,4-D]imida (8.90%), caryophyllene oxide (6.61%), propylparaben (6.11%) and D-limonene (6.01%). The remaining compounds were present in percentages of 1.06 to 5.72 as depicted in table 66 and fig 59.

### 4.5.4. Phytochemical analysis of *Zingiber officinale*

Different fractions revealed alkaloids and phenolics as major groups of compounds. Ethyl acetate fraction contained the highest amount of phenolics ($136.3\pm4.8$ mg GAE/g) of extract. Total phenolic was also higher in the acetone ($120.9\pm3.7$) followed by methanol ($101.2\pm3.9$), ethanol ($85.0\pm2.1$), benzene ($73.6\pm3.0$) and petrol ether ($69.3\pm5.8$) fractions (Table 63).

The GC-MS analysis of *Zingiber officinale* ethyl acetate fraction revealed 12 chemical components which may be extended with the help of chemometric techniques. The major compounds identified were 3,6-dimethyl-2,3,3a,4,5,7a-
hexahydro (23.69%), 1,3-cyclohexadiene,5-(1,5-dimethyl (14.48%), gingerol (13.54%), benzene,1-(1,5-dimethyl-4-hexenyl (10.60%), cyclohexene,1-methyl-4-(5-methyl (10.40%), 1,3-cyclohexadiene,5-(1,5-dimethyl (8.07%). The other compounds were present in low percentages ranging from 0.67-4.27 (Table 67 and Fig 60).

4.5.5. Phytochemical analysis of *Psidium guajava*

Phytochemical analysis of fractions revealed the presence of alkaloids, phenolics and glycosides as major group of compounds. The total phenolic content (mg/g) of *Psidium guajava* various fractions showed highest polyphenolic content (261.4±8.5) in methanol fraction followed by the ethanol (146.7±2.2), ethyl acetate (99.6±2.4), acetone (84.2±2.4), benzene (43.8±2.3) and petrol ether (41.2±1.9) fractions (Table 63).

Ten different compounds were identified in GC-MS. *Psidium guajava* leaves extract contained 4-methylthiazole (14.29%), 13-tetradecenal (11.62%), 2-nonanone, 9-hydroxy (10.48%), 2-butyne, 1, 4- dichloro- (7.10%). The remaining compounds (1.3-5.1% of total) have been shown in table 68 and fig 61.

4.5.6. Phytochemical analysis of *Piper nigrum*

Phytochemical analysis of fractions revealed the presence of alkaloids and phenolics as major group of compounds. The total phenolic content (mg/g) of *Piper nigrum* was maximum (53.0±2.3) in petrol ether fraction followed by ethanol (52.6±3.1) and methanol (41.5±3.4). The other fractions exhibited low polyphenolic content of (30.6-40.8) mg/g of dry extract (Table 63).

GC-MS analysis confirmed the presence of 13 components in *Piper nigrum*. These numbers may be extended with the help of chemometric techniques. *Piper nigrum* contained mainly piperine (46.10%), copaene (12.25%) and adamantane (8.07%). The other components were present in low amount (0.65-5.72%) as depicted in table 69 and fig 62.

4.5.7. Phytochemical analysis of *Piper cubeba*

Phytochemical analysis of fractions revealed the presence of alkaloids and phenolics as major group of compounds. The total phenolic content equivalent to gallic acid
(mg/g of dry extract) showed polyphenolic content of 50.1±3.9 mg in ethanol fraction followed by the other fractions which ranged from 34.7 to 37.7 mg/g of dry extract (Table 63).

The GC-MS analysis of *Piper cubeba* ethanol fraction revealed 15 chemical components which may be extended with the help of chemometric techniques. The major compounds identified were copaene (13.47%), naphthalene, 1,2,3,5,6,8a-hexahydro (10.36%) and ledol (6.25). The a-cubebene and other compounds were present in low percentages ranging from 0.18-4.54 (Table 70 and Fig 63).