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Evaluation of antioxidant and antimutagenic potential of *Justicia adhatoda* leaves extract

Arvinder Kaur, Davinder Kaur and Saroj Arora*

Department of Botanical and Environmental Sciences, Guru Nanak Dev University, Amritsar-143005, Punjab, India.

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In this study, the ethanolic extract of *Justicia adhatoda* (Acanthaceae) leaves was prepared by successive extraction procedure in increasing polarity order. Moreover, there are no antimutagenic evaluation reports found. In the present study our aim was to determine the antioxidant and antimutagenic potential of different fractions of ethanolic extract of *J. adhatoda.* Ultra high performance liquid chromatography (UHPLC) analysis revealed the presence of polyphenolic compounds and flavonoids which might be responsible for bioprotective activity. Among the five fractions (hexane, chloroform, ethyl acetate, n-butanol and aqueous), n-butanol and ethyl acetate exhibited significant antioxidant activity with minimum IC_{50} value (< 105.33 µg/ml) whereas, hexane, chloroform and aqueous fractions exhibits excellent antimutagenic potential against 2-aminofluorine for *S. typhimurium* TA98 and TA100 strains in the presence of S9 mix. These results indicate that these fractions need further research into its potential chemoprevention effects.

Key words: *Justicia adhatoda*, antioxidant, antimutagenic, ultra-high performance liquid chromatography (UHPLC), IC_{50} value.

INTRODUCTION

Reactive oxygen species (ROS), including superoxide anion radical, hydrogen peroxide, hydroxyl radicals and reactive nitrogen species (RNS) cause damage to DNA by oxidation, methylation and deamination (Wiseman and Halliwell, 1996). This may lead to the occurrence of various dreadful diseases like cancer. Moreover, the accumulation of ROS has been postulated to be implicated in the aging process (Beckman and Ames, 1998). An abundance of data indicates that human diet plays an important role in the cause and prevention of various types of cancers and cardiovascular diseases (Doll and Peto, 1981; Ames et al., 1995; Willett, 1995; Surh, 2003). Natural plant products have been a rich source of conventional medicine for the treatment of many forms of cancer (Cragg and Newman, 2005). Thus, many investigators have mapped out a variety of naturally occurring phytochemicals with antioxidant properties (Ramos et al., 2003; Gonzalez-Avila et al., 2003; Jayaprakasha et al., 2007; Singh et al., 2009). Some of these antioxidants are being identified as anticarcinogens (Ames, 1983). Antioxidants are substances that have ability to scavenge reactive oxygen species (ROS) and

*Corresponding author. E-mail: sarojarora.gndu@gmail.com. Tel: +91-183-2451048, +91-9417285485. Fax: +91-183-2258819, 2258820.

delay or prevent the oxidation of cellular substrates. Due to serious side effects associated with chemotherapies and radiotherapies, an alternative medication from natural sources has been observed (Lee, 2010). These natural plant products act by protecting or even reverting several types of human cancer and degenerative diseases (Chanarat, 1992; Feig et al., 1994; Kohlmeier et al., 1995; Yen and Chan, 1995).

Justicia adhatoda is a well-known plant used in Ayurvedic and Unani medicine (Claeson et al., 2000). A wide range of phytochemical constituents such as vasicine, vasicinone etc have been isolated from *J. adhatoda* which possess activities like antitussive, abortifacient, antimicrobial, cardiovascular protection, anticholinesterase, anti-inflammatory and other important activities (Singh et al., 2011).

There are very few reports in literature which indicate the role of this plant in radical scavenging capacity for the DPPH radical (Srinivasan et al., 2013). Moreover, this plant has also not explained the phenolic composition and antimutagenic potential of the fractions/extract. Therefore, the present study was undertaken to evaluate the antioxidant and antimutagenic potential by using different assays.

MATERIALS AND METHODS

Bacterial strains and chemicals

The *Salmonella* Typhimurium strains TA98 and TA100 were kindly provided by Prof. B.N. Ames, University of California, Berkeley, USA. Dextrose anhydrous purified was purchased from E. Merck (India) limited, Mumbai; nicotinamide adenine dinucleotide phosphate sodium salt, calcium chloride (fused) and ammonium chloride were purchased from Loba chemie Pvt. Ltd.; potassium dihydrogen orthophosphate, monobasic, bacteriological agar, sodium chloride, Luria broth, disodium hydrogen phosphate dehydrate and magnesium sulphate heptahydrate extrapure were purchased from Himedia Loboratories Pvt. Ltd. 2-Nitrofluorene (2-NF), sodium azide (NaN_3) and 2-aminoanthracene (2-AA) were purchased from Sigma-Aldrich and dissolved in dimethyl-sulfoxide (DMSO). For the UPLC analysis, different standard polyphenolic compounds (gallic acid, catechin, umbelliferone etc) with purity ≥90% were procured from Sigma-Aldrich. All the other reagents used to prepare buffers and media were of analytical grade.

Sample preparation

Leaves of *J. adhatoda* were collected from Bibi Kaulan Botanical garden, Guru Nanak Dev University, Amritsar. The plant was identified and submitted in herbarium where a voucher of specimen (Accession no.7034 dated 27th April 2014) in Department of Botanical and Environmental Sciences, GNDU, Amritsar. The fresh leaves of *J. adhatoda* were washed with tap water and then dried at room temperature. Dried leaves were crushed and extracted as per the procedure given in Figure 1.

Phytochemical analysis

All the fractions/extract were analysed for the presence of phenolic content, and estimated by the method of Yu et al. (2002). In this method, the phenolic compounds in the extract undergo reaction with phosphomolybdic acid in the presence of Folin-Ciocalteu reagent and give a blue coloured complex in alkaline medium. The total phenolic content of the extracts is measured in terms of gallic acid equivalents (GAE) which was expressed in terms of content as mg GAE/g dried weight of extract. Stock solution of extract that is, 1000 µg/ml was prepared. 500 µl of Folin-Ciocalteu was added to 100 µl of extract, followed by the addition of 1.5 ml of 20% of sodium carbonate. The final volume was made to 5 ml with distilled water after 2 h of incubation at room temperature. The absorbance was measured at 765 nm using spectrophotometer (Systronics PC based double beam 2202). Similarly, the total flavonoid content was determined using method as per the procedure given by Kim et al. (2003). An aliquot (1 ml) of extract solution was mixed with 4 ml of water and 0.3 ml of $NaNO_2$ (5%). After the incubation of 5 min, 0.3 ml of $AlCl_3$ was added and it was again incubated for the next 6 min. The incubation was followed by addition of 2 ml of NaOH. The final volume was made to 10 ml by addition of water. Absorbance was recorded at 510 nm. Total flavonoid content (TFC) was expressed as mg Rutin equivalent/g dried weight of extract/fractions.

Ultra performance liquid chromatography (UPLC)

All the six extracts/fractions of *J. adhatoda* were subjected to UPLC in order to identify the presence of various polyphenolic compounds like gallic acid, catechin, chlorogenic acid, umbelliferone and so on. For UPLC analysis, the dried extracts/fractions were dissolved in HPLC grade methanol (1.0 mg/ml), filtered through sterile 0.22 µm Millipore filter and subjected to qualitative and quantitative analysis by using Nexera UHPLC (Shimadzu) system.

Preparation of standard phenol solution

The standard phenolic stock solutions was prepared by dissolving 1 mg of each standard compounds like gallic acid, catechin, chlorogenic acid, epicatechin, caffeic acid, umbelliferone, coumaric acid, rutin, ellagic acid, tert-butyl hydroquinone, quercetin and kaempferol in 1 ml of methanol.

Apparatus and chromatographic conditions

The UPLC analysis was performed on Nexera UHPLC (Shimadzu) system. The system was equipped with LC-30 AD quaternary gradient pump, SPD-M20 A diode array detector (DAD), CBM-20 A Communication Bus Module, CTO-10 AS VP column oven, DGU-10 A_5 prominence degasser, and SIL-30 AC Nexera auto sampler. Detection wavelength used was 280 nm. The column used was an Enable C-18 column (150×4.6×5 µm particle size) equipped with a 0.2 µm filter. The flow rate for all the samples was 1 ml/min, the column oven temperature was 27°C and the full loop injection volume was 10 µl.

Evaluation of Antioxidant capacity

The antioxidant capacity of the various fractions/extract (Hexane, chloroform, ethyl acetate, n-butanol, aqueous and ethanolic extract) of *J. adhatoda* was estimated using different *in vitro* assays viz. 2-2-diphenyl 1-picryl hydrazyl (DPPH) radical scavenging potential, Fe^{3+}–Fe^{2+} transformation ability, reduction in cupric ions (Cu^{2+}), superoxide anion scavenging and $ABTS^{+}$ scavenging ability.

The hydrogen donating or radical scavenging ability of six fractions of *J. adhatoda* was measured by reduction in stable DPPH

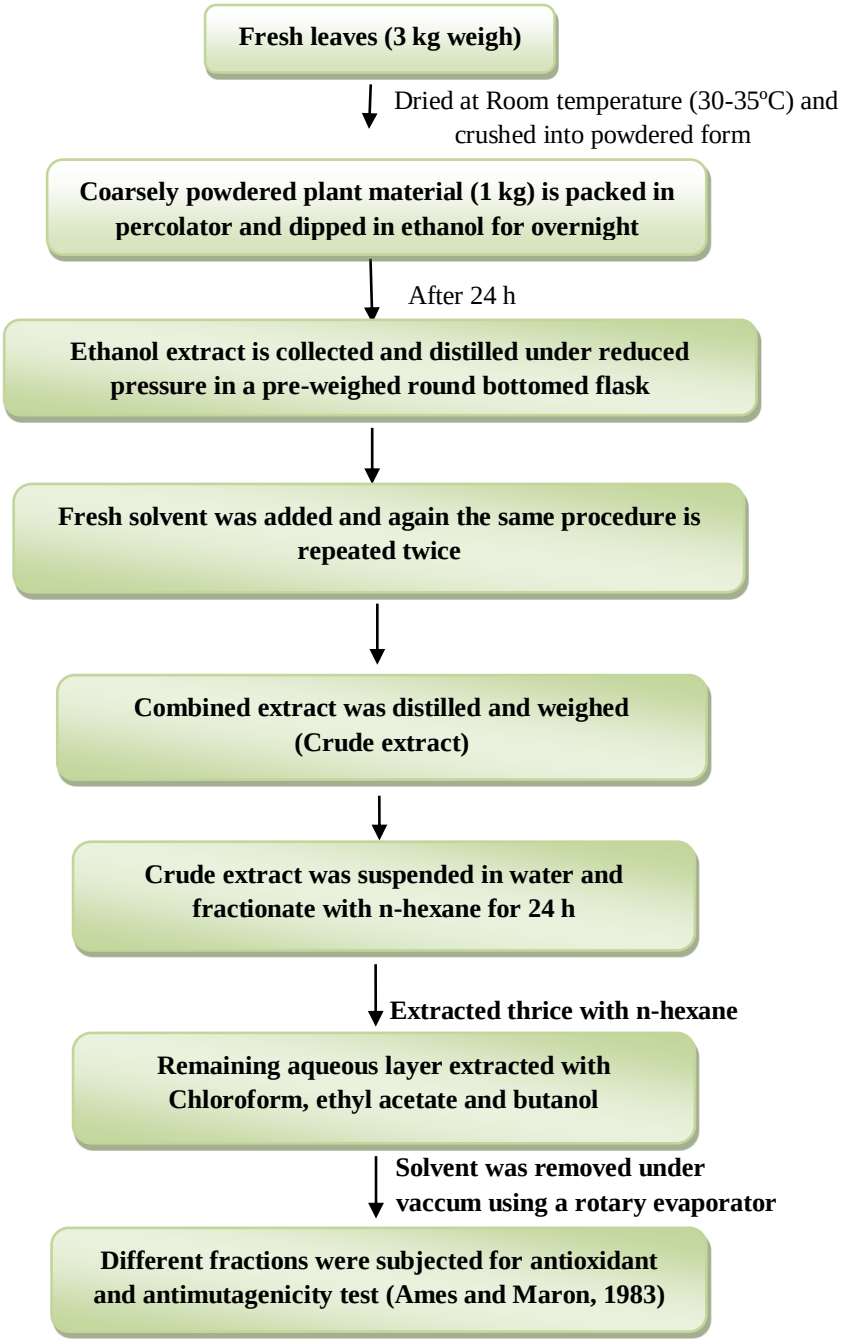

Figure 1. Schematic diagram for preparation of crude extract of *Justicia adhatoda* by alcoholic extraction method.

radicals spectrophotometrically as described by Dudonne et al. (2009) with minor modifications. Briefly, 0.1 mM DPPH solution in methanol was prepared and 2 ml of this solution was added to 300 µl extract solution at different concentration (0-1000 µg/ml). The absorbance was measured at 517 nm after 30 min incubation. The change in colored product from purple to yellow with increase in extract concentration was read spectrometrically at 517 nm. Rutin was used as the reference compound. Radical scavenging activity was expressed as the percentage free radical scavenging by the sample and calculated by using the following formula:

$$\text{Inhibition (\%)} = \frac{A_{Control} - A_{Sample}}{A_{Control}} \times 100$$

Where $A_{Control}$ = absorbance of the control and A_{Sample} = absorbance in the presence of sample.

The reduction potential of fractions was measured by ability to reduce ferricyanide ion that is, $[Fe(CN)6]^{3-}$ to ferrocyanide ion that is $[Fe(CN)6]^{4-}$ by using protocol as mentioned in Kannan et al.

(2010) with some modifications. The extract (0.75 ml) at various concentrations (0, 20, 40, 60, 80, 100, 200, 400, 600, 800, 1000 µg/ml) was mixed with 0.75 ml of phosphate buffer (0.2 M, pH 6.6) and 0.75 ml of potassium hexacyanoferrate ($K_3Fe(CN)_6$) (1%, w/v), followed by incubation at 50°C in a water bath for 20 min. The reaction was stopped by adding 0.75 ml of trichloroacetic acid (TCA) solution (10%) and then centrifuged at 800 g for 10 min. 1.5 ml of the supernatant was mixed with 1.5 ml of distilled water and 0.1 ml of ferric chloride ($FeCl_3$) solution (0.1%, w/v) was added and kept at room temperature for 10 min. The absorbance was read at 700 nm. The higher absorbance of the reaction mixture indicates greater reducing power.

$$\text{Inhibition (\%)} = \frac{A_{Control} - A_{Sample}}{A_{Control}} \times 100$$

Where, $A_{Control}$ = absorbance of the control and A_{Sample} = absorbance in the presence of sample.

The cupric ions (Cu^{2+}) reducing ability of *J. adhatoda* extracts was measured by the method of Apak et al. (2007) with slight modification as described by Gulcin (2010). In this assay, Cu (II)-Nc complex is reduced to the highly coloured Cu (I)-Nc chelate by the oxidation of given antioxidant. In this assay, Copper (II) chloride, Neocuproine and Ammonium acetate (NH_4Ac) buffer solutions (1 ml each) were mixed.

The extract solution (x ml) and H_2O (1.1-x) ml were added to the initial mixture so as to make the final volume 4.1 ml. The tubes were stoppered and after 30 min, the absorbance at 450 nm was recorded against a reagent blank. Rutin was taken as standard which act as a positive control. Increased absorbance of the reaction mixture indicates increased reduction ability.

The reducing activity on superoxide anion (O_2^{-*}) was measured by modified PMS-NADH system explained by Li et al. (2014). The superoxide anions were generated non-enzymatically in a phenazine methosulphate-NADH system, and assayed by development of blue coloured formazan dye upon reduction of nitro blue tetrazolium. 1 ml of NBT solution (144 µM in 100 mM phosphate buffer , pH 7.4), 1 ml of reduced NADH (677 µM in100 mM phosphate buffer, pH 7.4) and 1 ml of sample extract were mixed and the reaction was started with adding 1 ml of PMS solution (60 µM PMS in100 mM phosphate buffer, pH 7.4). The reaction mixture was incubated at 25°C for 5 min and the absorbance of coloured complex was measured at 560 nm. The inhibition percentage was calculated using the formula:

$$\text{Inhibition (\%)} = \frac{A_{control} - A_{sample}}{A_{control}} \times 100$$

Where $A_{control}$ = absorbance of the control and A_{sample} = absorbance in the presence of sample

The method of Re et al. (1999) was adopted for ABTS radical cation assay with slight modifications. The stock solutions included 7 mM ABTS solution and 140 mM potassium persulfate solution. Both solutions were added in such a proportion so as to make final concentration of 2.45 mM $ABTS^+$ solution and allowed them to react for 12-16 h at 30°C in the dark. The solution was then diluted by mixing $ABTS^+$ solution with methanol to obtain an absorbance of 0.706 ± 0.001 units at 734 nm using the spectrophotometer. Plant extracts (0.1 ml) were allowed to react with 1 ml of the ABTS solution and the absorbance was taken at 734 nm using the spectrophotometer. The $ABTS^+$ scavenging capacity of the extract was calculated as:

$$\text{ABTS radical scavenging activity (\%)} = \frac{A_{control} - A_{sample}}{A_{control}} \times 100$$

Where, $A_{control}$ = absorbance of the control and A_{sample} = absorbance in the presence of sample.

In vitro antimutagenic assay (Ames assay)

The *Salmonella* histidine point mutation assay of Maron and Ames (1983) was used to test the antimutagenic activity of the extracts/fractions, with some modifications as described by Aqil et al. (2008). The Ames tests and S9 mix protocol (Maron and Ames, 1983) were performed on both bacterial strains (TA98 and TA100) to determine the effect of *J. adhatoda* extracts on 2-Aminofluorene (2-AF), sodium azide (NaN_3) and 4-nitro-o-phenylediamine (NPD) induced mutagenicity. The samples were dissolved in DMSO while making the different concentrations namely 100, 250, 500, 1000 and 2500 µg/0.1 ml.

In brief, 0.5 ml of S9 mixture or phosphate buffer was distributed in sterilized capped tubes in an ice bath, then 0.1 ml of mutagen, 0.1 ml of plant extract and 0.1 ml of bacterial culture were added. After mixing it gently; 2 ml of top agar (0.6% agar, 0.5% NaCl, 0.5 mM L-histidine and 0.5 mM D-biotine) were added to each tube and poured immediately on the minimal agar plates. The procedure for the pre-incubation was similar with co-incubation, except that bacterial strain+ extract+mutagen+S9 mix were incubated for 30 min prior to the addition of top agar. The plates were incubated at 37°C for 48 h and the revertant colonies were counted on protocol colony counter.

Among the three mutagens, NaN_3 and NPD are direct-acting mutagens which affect the genetic material directly, leading to the structural damage, on the other hand 2-AF act on DNA in an indirectly manner. The inhibition rate of mutagenicity (%) was calculated by using equation from Ong et al. (1986)

$$\text{Inhibiton rate (\%)} = \frac{x - y}{x - z} \times 100$$

Where x, is the number of revertants induced by mutagen alone (Positive control), y is the number of revertants induced by mutagen in the presence of extract (co-incubation or pre-incubation) and z is the number of revertents in the presence of extract alone (Negative control).

Statistical analysis

The results were expressed as mean ± Standard error (SE) of three independent experiments. The one-way ANOVA test was used to analyze the result and $P<0.05$ was considered significant.

RESULTS AND DISCUSSION

Phenolic compounds known to possess high antioxidant activity are commonly found in fruits, vegetables and herbs (Mustafa et al., 2010). Results of the study show the plant extracts are rich in phenolic compounds which varied from 27.267 to 182.6 mg GAE/g (Table 1). The total phenolic contents of ethyl acetate and n-butanol fraction of *J. adhatoda* were found to be maximum at 182.6 and 105.26 mg GAE/g ($y=0.001x+0.037$; $R^2=0.999$) respectively. Meanwhile, total flavonoid content of ethyl

Table 1. Total phenolic and flavonoid content of fractions of *Justicia adhatoda.*

Fraction	Total phenolic content (TPC) (mg Gallic acid equivalent/g)	Total flavonoid content (TFC) (mg rutin equivalent/g)
Aqueous	27.267	15.6
n-butanol	105.267	95.6
Chloroform	69.267	118.6
Ethyl acetate	182.6	299.6
Hexane	93.6	740.6

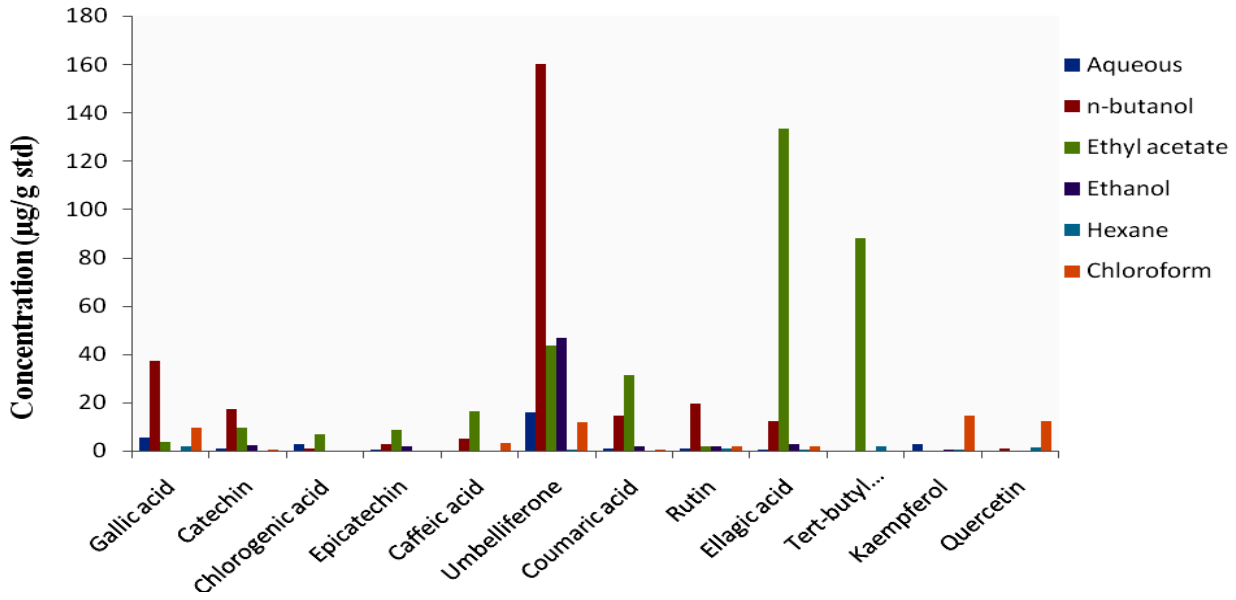

Figure 2. UHPLC analysis showing the concentration of polyphenolic compounds in the different fractions/extract of *Justicia adhatoda.*

acetate and n-butanol fraction were 299.6 and 95.6 mg RE/g (0.001x+0.045; R^2=0.993) respectively. Many studies reveal that antioxidant activity of phenolic compounds are due to their redox properties, which allow them to act as reducing agents, singlet oxygen quencher, hydrogen donators and chelating agents of metal ions (Rice-Evans et al., 1995; Mustafa et al., 2010). These phytochemical compounds are known to provide support for bioactive properties of plant, and thus they are responsible for the antioxidant properties of *J. adhatoda.* A significant relationship between antioxidant potential and total phenolic content was found, indicating that phenolic compounds might be the major contributors to the antioxidant potential.

All the extracts/fractions were further examined for their specific phenolic composition by the UHPLC to evaluate the presence of phenolic acids and flavonoids (Figure 2). The quantity of phenolic compounds ranged from 1.92 to 37.63 µg/g of gallic acid, 0.6 to 17.4 µg/g of catechin, 0.4 to 133.1 µg/g of ellagic acid, 0.765 to 160.1 µg/g and so on (Figure 3).

The DPPH scavenging activities of different fractions/ extract of leaves of *J. adhatoda* are shown in Figure 4a. The ethyl acetate and n-butanol fraction showed highest DPPH radical scavenging activity of 91.86 and 90.44%, respectively, whereas other fractions/extract leaves exhibited comparatively less inhibition. This revealed that both fractions have the highest free radical scavenging activity probably due to presence of high polyphenolic compound. A similar kind of studies conducted by Rao et al. (2013) demonstrated the significant DPPH scavenging activity of methanolic extract of *J. adhatoda* with an IC_{50} value 105.33 µg/ml.

Figure 4b depicts the reducing power of five extracts/fractions of *J. adhatoda* leaves in comparison to standard compound (Rutin). It was found that extract/ fractions have tendency to reduce Fe (III) to Fe (II). It was noted that among the different extract and fractions, ethyl acetate and butanol fraction of leaves exhibited the maximum reducing power of 84.36 and 50.99%,

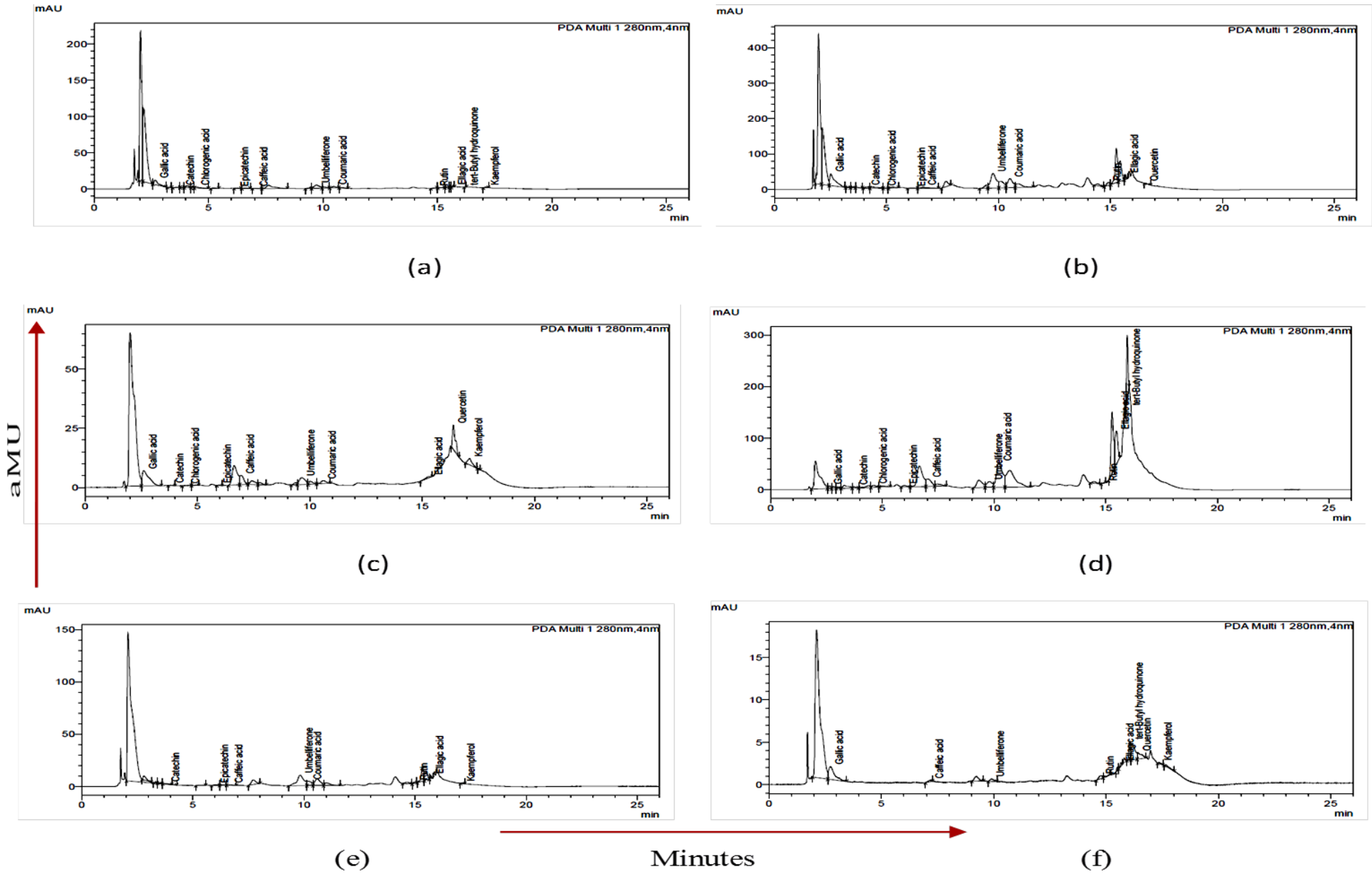

Figure 3. UHPLC chromatogram of different fractions of *Justicia adhatoda.* **(a)** Aqueou. **(b)** n-butanol. **(c)** Chloroform. **(d)** Ethanolic. **(e)** Ethyl acetate. **(f)** Hexane.

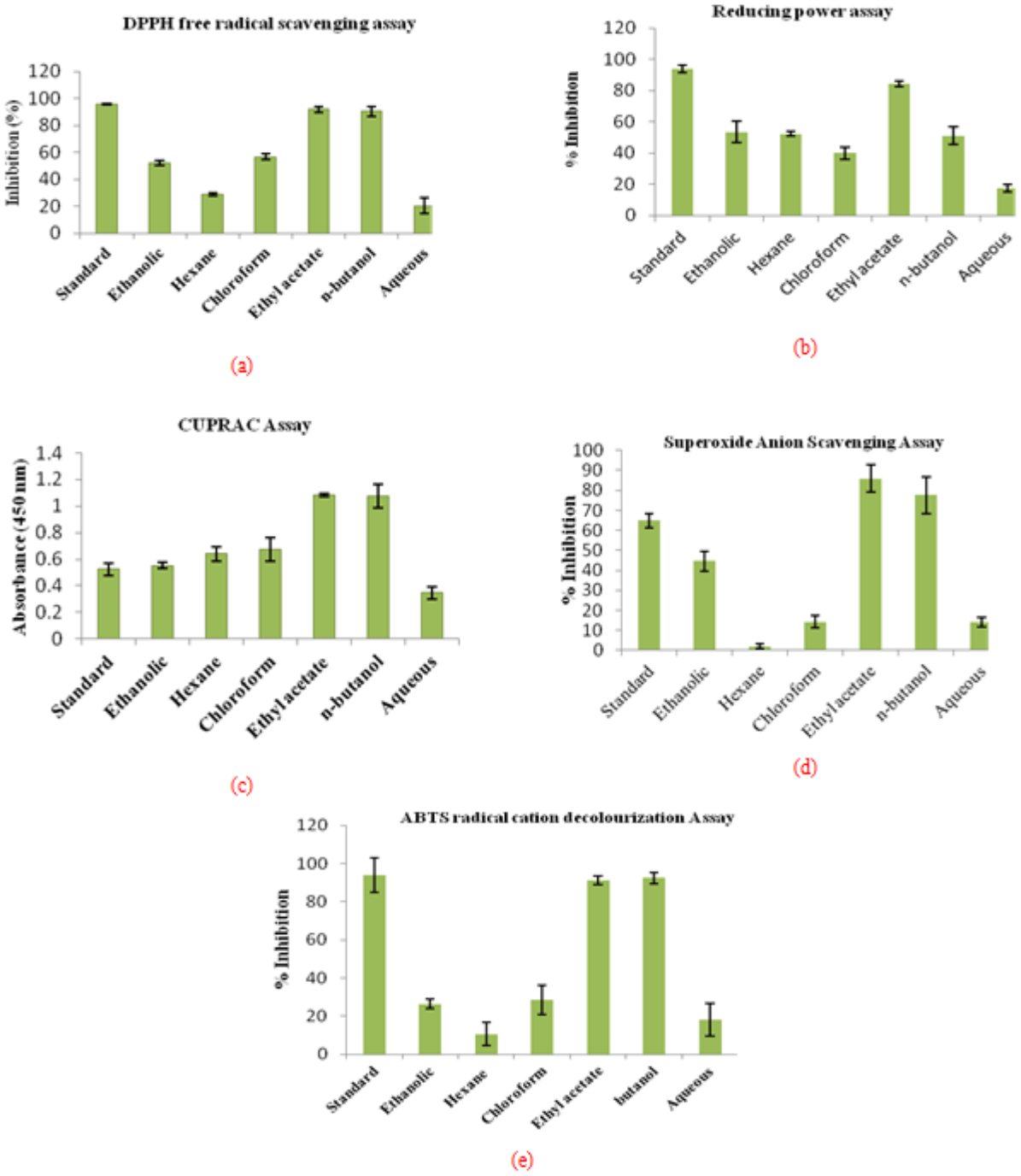

Figure 4. Inhibitory effect of five extracts/fractions of *Justicia adhatoda* at 200 µg/ml concentration by (a) DPPH assay; (b) reducing power assay; (c) Cuprac assay; (d) superoxide anion scavenging assay and (e) $ABTS^{+}$ radical cation decolorization assay develop and apply natural antioxidant so that they can scavenge the free radicals without retarding the function of biological system.

respectively.

In CUPRAC assay, Cu^{2+} gets reduced to blue coloured Cu^{+} chelate by antioxidants which showed maximum absorbance at 450 nm. In this assay, a higher absorbance indicates higher antioxidant activity. Figure 4c shows the maximum absorbance of 1.083 and 1.076 nm of ethyl acetate and n-butanol fraction at 200 µg/ml concentration respectively whereas, standard (Gallic acid) used shows less absorbance at same concentration.

The potential to scavenge O_2^{-*} radical from PMS-NADH coupling system by the fractions of *J. adhatoda* was evaluated by superoxide anion radical scavenging assay. The decrease in absorbance at 560 nm with increase in fraction concentration indicated the consumption of superoxide anion in the reaction mixture. The results as shown in Figure 4d indicate that among the six fractions/extracts, n-butanol and ethyl acetate fractions were more effective in scavenging the superoxide radicals with 77.51 and 85.82%, inhibition respectively. However, at the same concentration, the standard (rutin) showed 64.84% scavenging ability. These free radicals are found to be very hazardous for the health. To overcome the dire effect of these free radicals various synthetic antioxidants like butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) are used as a daily supplements. But the recent studies have reported that these synthetic antioxidants provoke tumors in the stomach of rodents (Grice, 1988).

Our results clearly indicate that the n-butanol and ethyl acetate fractions were efficiently active against free radicals in a concentration-dependant manner. Figure 4e represents the $ABTS^+$ scavenging ability of different extracts/fractions of *J. adhatoda* leaves in comparison to standard (Gallic acid). It was found that ethyl acetate and n-butanol exhibited maximum inhibitory percentage of 91.29 and 92.48%, respectively, whereas Gallic acid scavenged 93.86% radicals at the same concentration. The ABTS radical cation (ABTS+.) is formed due to the reaction between potassium persulfate and 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and it exhibited maximum absorbance at 700 nm. However, hydrogen donating property of phenolic compounds converts these colored species to colourless entities by causing their reduction.

Antimutagenic assay

The antimutagenic activity of different fractions of *J. adhatoda* was determined by using *S.* Typhimurium strains (TA98 and TA100) in the absence or presence of metabolic activation. Among the six fractions (Hexane, chloroform, ethyl acetate, n-butanol, aqueous and ethanolic extract), ethyl acetate and n-butanol fractions were able to prevent frame shift and base pair substitution mutation in the presence of metabolic activation in TA98 and TA100 strain respectively (Słoczyńska et al., 2014).

On the other hand, the fractions had a weak inhibitory effect on direct-acting mutagens (NPD and NaN_3) in the absence of metabolic activation (Figures 5 and 7). It was interpreted from the results that the fractions were found to be more effective and exhibited significant percent inhibition in case of pre-incubation mode with metabolic activation as compared to co-incubation without metabolic activation. In TA98 strain, among the six fractions tested, aqueous, chloroform and hexane showed 96.42, 96.03 and 96.42%, inhibition respectively at highest concentration (2500 µg/0.1ml per plate) in pre-incubation with metabolic activation mode of experimen-tation (Figure 6) whereas, the same fractions showed 41.62, 62.7 and 31.56% inhibition of mutagenicity of sodium azide at the same experimental conditions without metabolic activation.

However, the extent of inhibition with TA100 strain was slightly less as compared to TA98 strain. In TA100 strain, all the six fractions showed decrease in the number of revertants colonies against indirect acting 2-AF mutagen with percent inhibition ranges from 90.89% to 99.29% at 2500 µg/0.1 ml in pre-incubation (with metabolic activation) as shown in Figure 8. All the six fractions were found to inhibit the mutagenicity produced by 2-AF (with metabolic activation) but the results were not significant with the direct acting mutagens.

From the results obtained in the antioxidant and antimutagenic assay, it was seen that the fractions/ extract which show significant antioxidant activity did not show antimutagenic potential and vice-versa. Słoczyńska et al. (2014) reported that some antimutagenic compound do not possess antioxidant properties of their own but can be converted into derivative that show high antioxidant activity. Such phenomenon was well demonstrated by Parvathy et al. (2010) in which very high antimutagenic activity was observed in amino acid conjugates of curcumin than the curcumin itself.

Conclusion

The fractions obtained from *J. adhatoda* acquire excellent antioxidant and antimutagenic activities. It was seen that fractions are strong and effective enough as a scavenger of free radical, superoxide radical and hydrogen peroxide radicals. Furthermore, most of the fractions showed significant antimutagenic activity with metabolic activation. The observed bioactive activity may be due to group of phenolic compounds present in different fractions.

Conflict of interests

The authors did not declare any conflict of interest.

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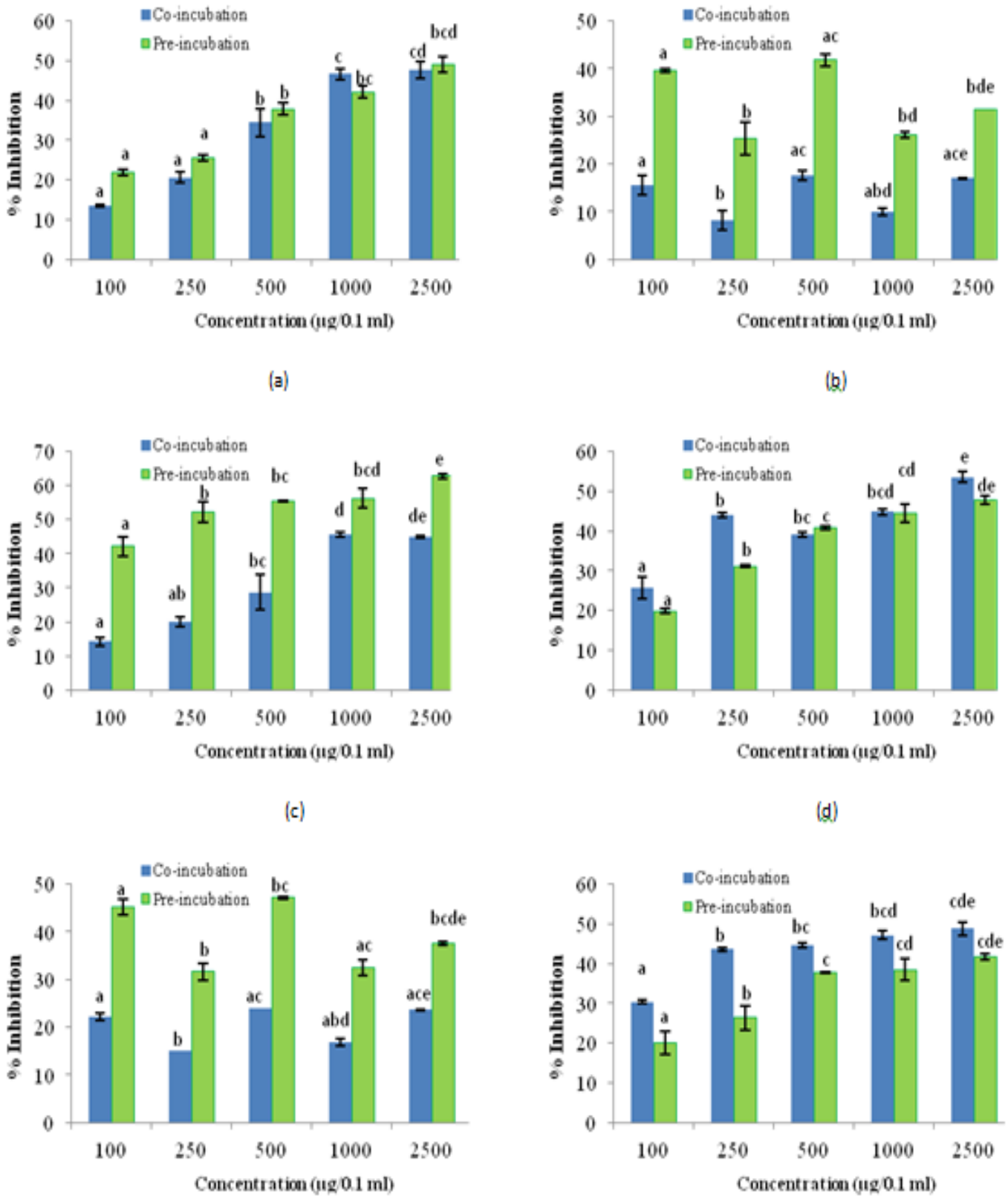

Figure 5. Antimutagenic potential of: (a) Ethanolic extract; (b) hexane fraction; (c) chloroform fraction; (d) ethyl acetate fraction; (e) n-butanol fraction and (f) aqueous fraction on *Salmonella typhimurium* strain TA98 without S9 against sodium azide. Data shown are mean ± SE of experiment performed in triplicate. Means followed by same letters are not significantly different using HSD multiple comparison test. The results were found to be statistically significant at $p \leq 0.05$.

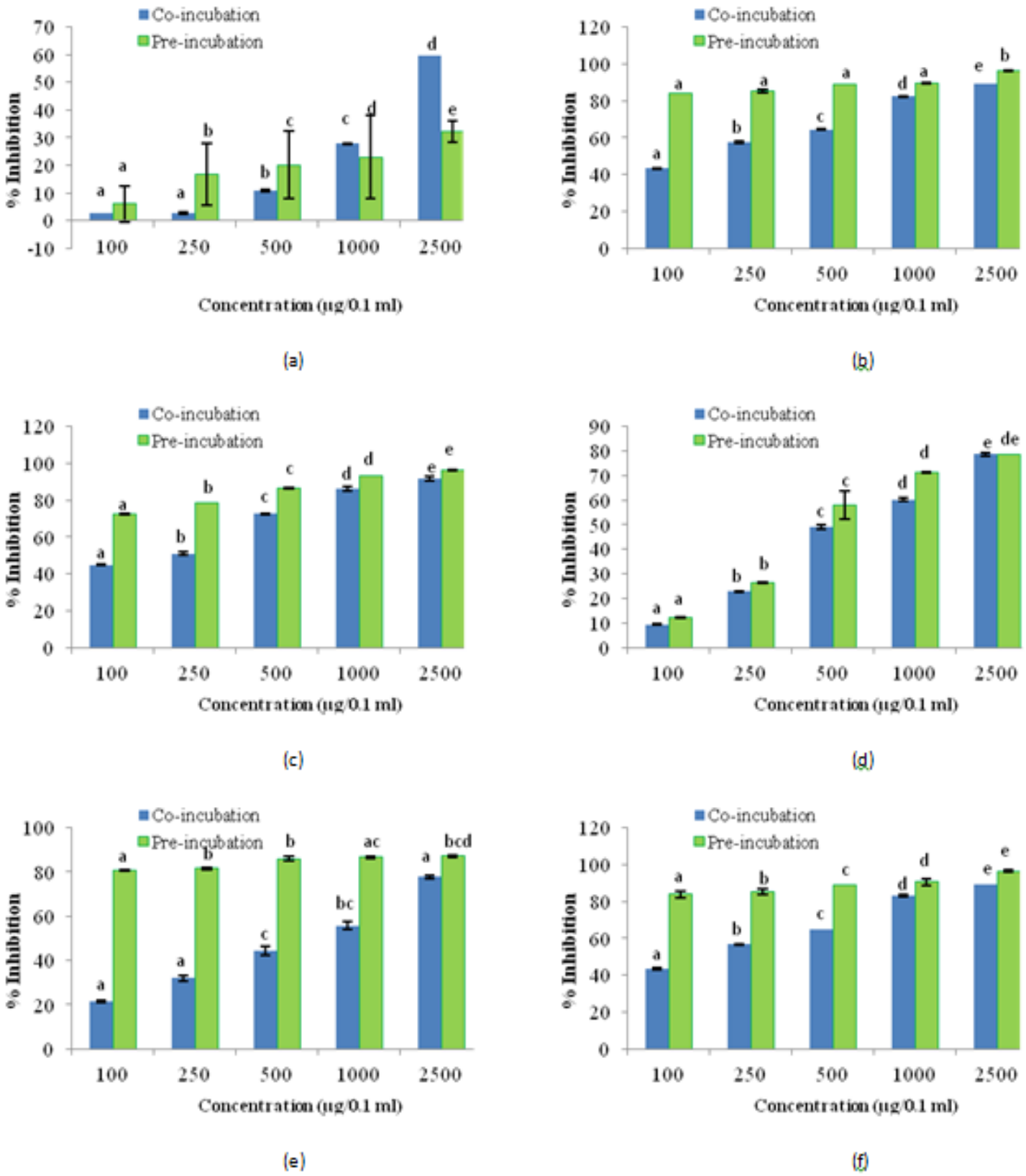

Figure 6. Antimutagenic potential of: (a) Ethanolic extract; (b) hexane fraction; (c) chloroform fraction; (d) ethyl acetate fraction; (e) n-butanol fraction and (f) aqueous fraction on *Salmonella typhimurium typhimurium* strain TA98 with S9 against 2-aminofluorene (2-AF). Data shown are Mean ± SE of experiment performed in triplicate. Means followed by same letters are not significantly different using HSD multiple comparison test. The results were found to be statistically significant at $p \leq 0.05$.

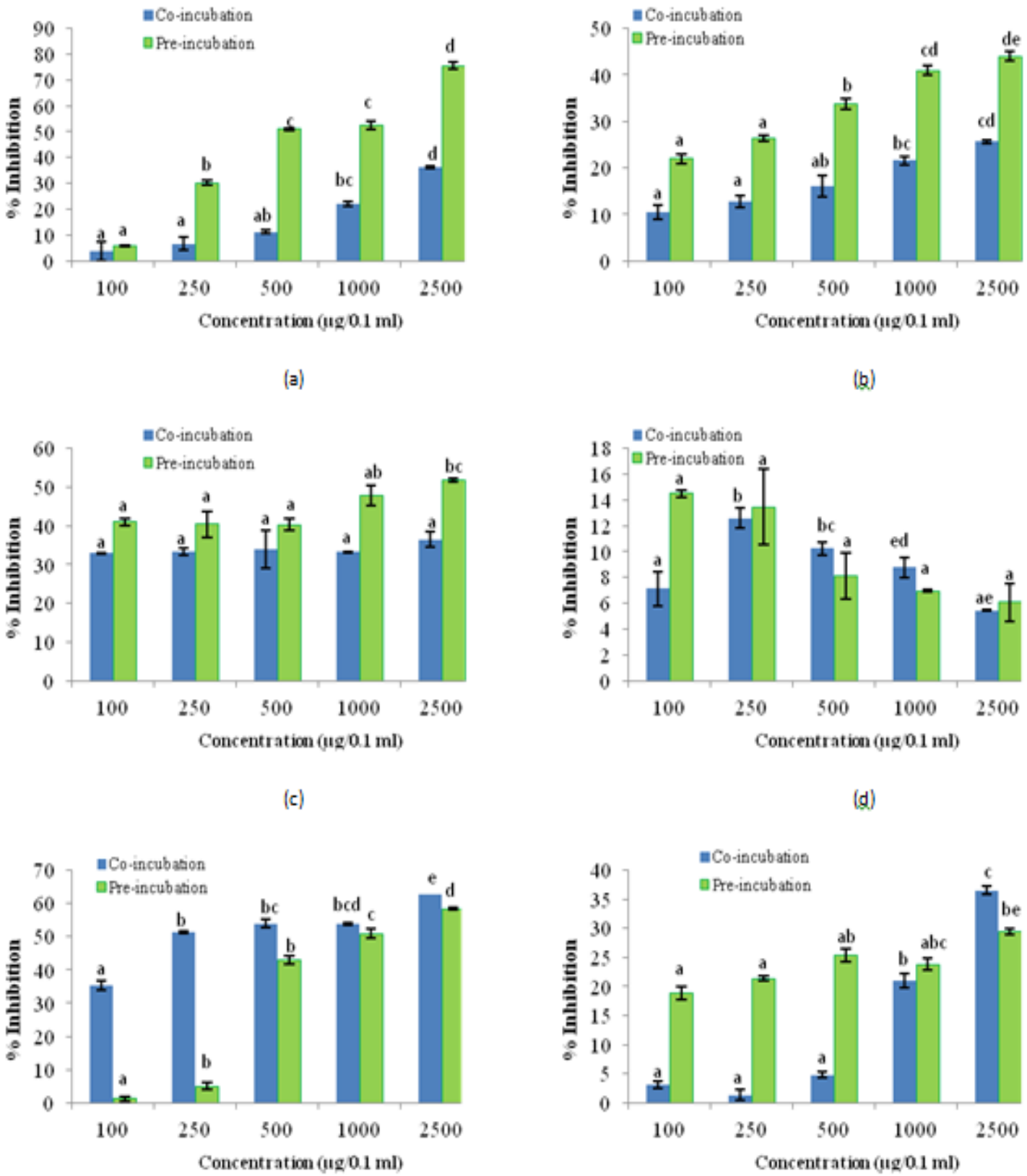

Figure 7. Antimutagenic potential of: (a) Ethanolic extract; (b) hexane fraction; (c) chloroform fraction; (d) ethyl acetate fraction; (e) n-butanol fraction and (f) aqueous fraction on *Salmonella typhimurium typhimurium* strain TA100 without S9 against 4-nitro-o-phenylenediamine (NPD). Data shown are Mean ± SE of experiment performed in triplicate. Means followed by same letters are not significantly different using HSD multiple comparison test. The results were found to be statistically significant at $p \leq 0.05$.

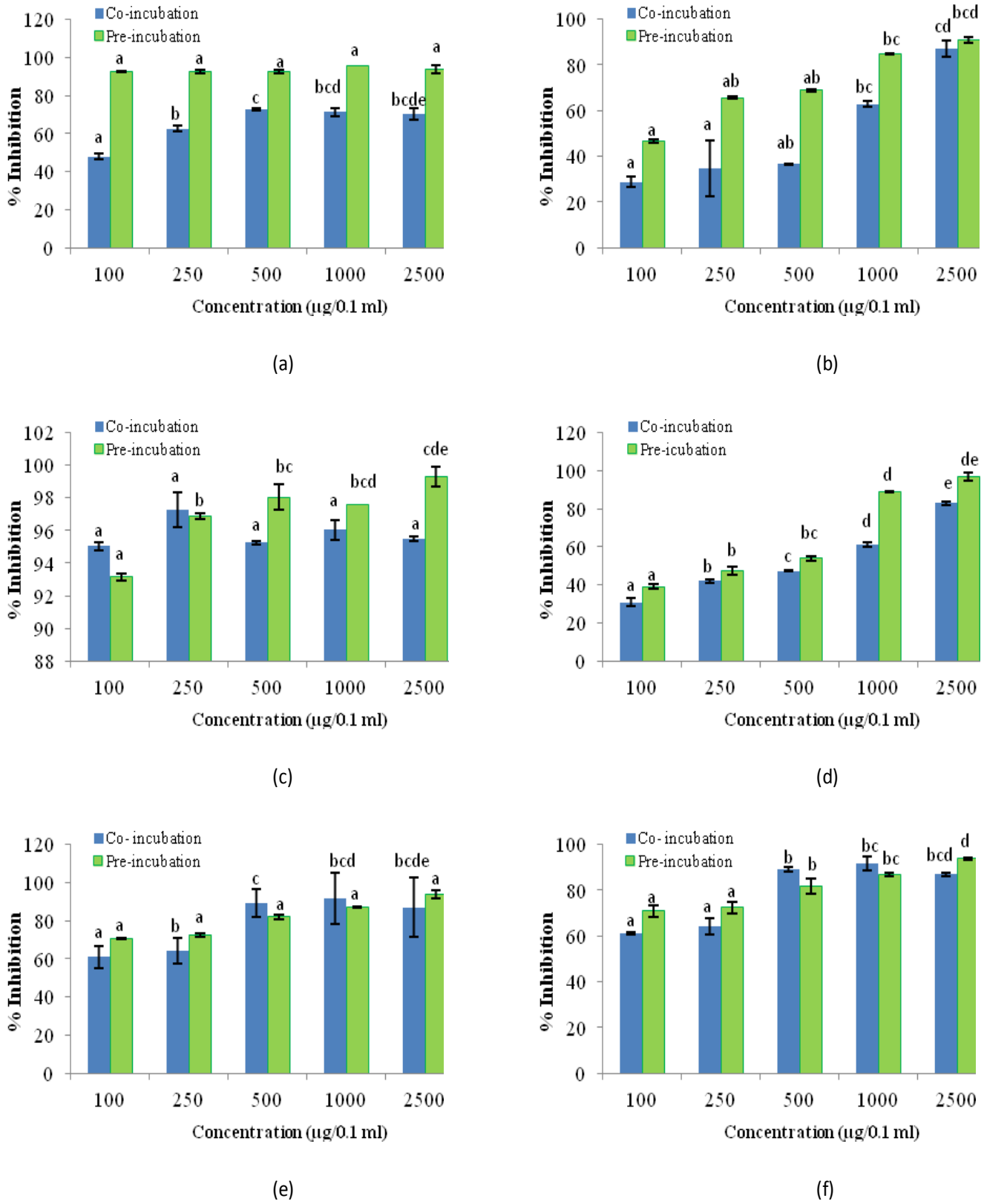

Figure 8. Antimutagenic potential of: (a) Ethanolic extract; (b) hexane fraction; (c) chloroform fraction; (d) ethyl acetate fraction; (e) n-butanol fraction and (f) Aqueous fraction on *Salmonella typhimurium typhimurium* strain TA100 with S9 against 2-aminofluorene (2-AF). Data shown are Mean ± SE of experiment performed in triplicate. Means followed by same letters are not significantly different using HSD Multiple comparison test. The results were found to be statistically significant at $p \leq 0.05$.

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