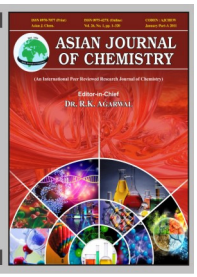
Asian Journal of Chemistry; Vol. 26, No. 4 (2014), 1241-1245

ASIAN JOURNAL OF CHEMISTRY

http://dx.doi.org/10.14233/ajchem.2014.16556



# Estimation of Antioxidant Power in Various Extracts of *Euphorbia helioscopia L.* with Five Different *in vitro* Antioxidant Models

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*Received*: 21 October 2013; *Accepted*: 23 December 2013; *Published online*: 15 February 2014; AJC-14737

Reactive oxygen species and reactive nitrogen species are generated within living body as a result of redox reactions and these may damage biomolecules *i.e.* lipids, proteins and DNA that cause number of pathological conditions. Plants are rich source of antioxidants that can combat oxidative stress-induced diseases. Various *in vitro* methods have been designed to assess antioxidant activity of medicinal plants. The aim of current study was to evaluate antioxidant activity of *Euphorbia helioscopia* extracts and latex employing five *in vitro* antioxidant assays (DPPH, TAC, FRAP, FTC and BCL). The data showed promising antioxidant activity in plant latex (highest) and L.MT (second highest) in all the methods. The other extracts did also show appreciable antioxidant activity but significantly lower than latex and L.MT. Significant positive Pearson correlation (at 0.01 level, two tailed) was also found among all the methods. It is concluded that the plants rich in antioxidants could be helpful in combating diseases.

**Keywords: Antioxidant, Total antioxidant capacity, Ferric reducing antioxidant power, β-Carotene-linoleic acid.**

## INTRODUCTION

Oxidation and reduction are biochemical reactions that normally occur within living cells and free radicals such as reactive oxygen species (ROS) like superoxide anion, hydrogen peroxide, hydroxyl radical and reactive nitrogen species (RNS) like nitrogen dioxide, nitric oxide are generated as a result of these chemical reactions[1]. The living system is equipped with different antioxidant enzymes such as catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx) that play pivotal role in scavenging the free radicals[2]. ROS and RNS may damage biomolecules (*e.g.* lipids, proteins, amino acids, DNA)[3] that lead to many pathological conditions such as nephrotoxicity, liver cirrhosis, parkinsonism[4,5] cancers and atherosclerosis[6-8], alzheimer disease[9], diabetes[10] and rheumatoid arthritis[11,12]. Plants are rich source of antioxidants[13,14]. Various *in vitro* antioxidant methods can be adopted to assess the antioxidant activity in plants. Generally these *in vitro* methods can be divided into two major groups, first category is of electron transfer reactions (E.T.R.), which includes α-diphenyl-β-picryl-hydrazyl radical scavenging assay (DPPH), trolox equivalent antioxidant capacity (TEAC), ferric reducing antioxidant power (FRAP), nitric oxide radical scavenging assay, total phenol assay and superoxide anion radical scavenging assay and second group is of hydrogen atom transfer reactions (H.T.R.), that includes β-carotene bleaching assay, oxygen radical absorbance capacity (ORAC) and total radical trapping antioxidant potential (TRAP)[15].

The current study was undertaken to evaluate the antioxidant activity of medicinal plant *Euphorbia helioscopia L.* using five *in vitro* models.

## EXPERIMENTAL

The plant was collected from local fields around the city of Lahore-Pakistan in February 2012 and dried under shade. It was authenticated by a taxonomist of Government College University, Lahore and voucher specimen (1501) was deposited to university herbarium. Stem and leaves were separated and ground to fine powder for extraction.

**Extraction methods:** Two extraction methods were employed. One was cold extraction and second was hot extraction using soxhlet apparatus. 1) Cold extraction method *i.e.* maceration was done with water and ethanol. Powder was socked in solvent in 1: 4 ratio for three consecutive days with occasional shaking and solvent was replaced with fresh one after every 24 h. 2) Sequential Soxhlet extraction was carried out with organic solvents, petroleum ether, chloroform and methanol. The solvents were used in increasing order of polarity.

**Solvents:** Organic solvents [petroleum ether (Sigma Aldrich), ethanol (BDH Laboratory), chloroform and methanol (Merck)], sulphuric acid, sodium phosphate, ferric chloride, ferrous chloride, ferric sulphate, ammonium molybdate, ammonium thiocyanate, hydrochloric acid, aluminium chloride, ammonium thiocyanate, linoleic acid, tween 40, lead acetate, acetic acid and ammonia were obtained from Merck (Pvt.) Ltd.(Germany) and DPPH (1,1-diphenyl-2-picryl hydrazyl), TPTZ (2,4,6-tripyridyl-*s*-triazine), ascorbic acid, BHA (butylated hydroxyl anisol) were of Sigma Aldrich chemical company Ltd. (USA).

Spectrophotometer UV-1700 Pharma Spec (Shimadzu), water bath having thermostat (Polyscience) were used in the study.

### β-Carotene-linoleic acid (BCL) assay

**Principle:** Oxidation reaction took place in an aqueous emulsion of linoleic acid and β-carotene when it was heated in water bath at 50 °C for removal of chloroform (used for dissolving β-carotene) from emulsion[16]. In this reaction hydrogen atom separates from active methylene group of linoleic acid and pentadienyl free radical is generated that attacks unsaturated β-carotene molecules[17]. β-Carotene naturally gives orange colour in emulsion, that undergoes degradation and colour intensity decreased or fade out with duration of reaction. Antioxidants neutralize free radicals generated from linoleic acid and delay the β-carotene degradation.

Spectrophotometer was used to measure the colour intensity of β-carotene emulsion.

**Description of assay:** β-Carotene (2 mg in 10 mL chloroform), Tween 40 (20 mg), linoleic acid (200 mg) were mixed together in round bottom flask. Chloroform was removed at 40 °C on rotary evaporator under vacuum. Emulsion was immediately diluted with 10 mL of triple distilled water and vigorously shake for 1 min. Oxygenated water (40 mL) was added to this emulsion before use in further process. This is stock emulsion. 0.2 mL of samples and reference standard/positive control butylated hydroxyl-anisole (BHA) (100 µg/mL each) were transferred to test tubes and 4 mL aliquots of this stock emulsion were added to these test tubes. All the solutions (samples & standard) were made in ethanol. Blank/negative control was prepared with same method; it contained ethanol in place of sample/BHA. Optical density (OD) of all the samples and reference standard was measured by spectrophotometer at 470 nm immediately (t = 0) and at 15 min interval upto 120 min. All the tubes were incubated in water bath at 50 °C during the assay. The test was carried out in triplicate. The antioxidant activity (AA) was measured with following formula:

$$\% \text{ AA} = 100[1-(A_o - A_t)/(A°_o - A°_t)]$$

where $A_o$ and $A°_o$ are the OD measured at zero time of the incubation for test sample and reference standard, respectively. $A_t$ and $A°_t$ are the OD measured in the test sample and reference standard, respectively, after incubation for 120 min[18].

**DPPH method:** DPPH radical scavenging activity was determined according to method described by Willams[19]. DPPH (0.1 mM) solution and samples solutions of different concentrations (0.01-1 mM) were prepared in methanol. BHA (dissolved in methanol) was used as reference standard/positive control. Test samples (0.5 mL) and BHA (0.5 mL) were mixed with 3.5 mL of DPPH solution in test tubes separately and kept at room temperature (28 ± 2 °C) for 30 min. Blank was prepared similarly, it included methanol (0.5 mL) in place of sample or reference standard. After incubation period, absorbance was read at 517 nm against blank. Each sample was tested in triplicate. Radical scavenging activity was calculated with following equation:

$$\% \text{ Scavenging effect} = [A_{blank}-A_{sample}/A_{blank}] \times 100$$

where $A_{blank}$ is OD value of control (all reagents except tested sample or standard), $A_{sample}$ is OD value of tested samples. $IC_{50}$ was calculated from graph plotted between % scavenging effect and concentrations of test samples.

**Ferric reducing antioxidant power (FRAP) test:** To perform FRAP test, the method of Benzie and Strain[20] was used with slight modification. Stock solutions and working solutions were prepared as under.

**Stock solutions:** 1) Acetate buffer 300 mMol, pH 3.6 (mixed 3.1 g $C_2H_3NaO_2.3H_2O$ with 16 mL $C_2H_4O_2$). 2) TPTZ solution 10 mMol prepared in 40 mMol HCl. 3) $FeCl_3.6H_2O$ solution 20 mMol

Working solution (FRAP reagent) prepared freshly by mixing TPTZ solution 5 mL, $FeCl_3.6H_2O$ solution 5 mL and acetate buffer 50 mL.

The test samples solutions were prepared in methanol (500 µMol/mL). Aqueous solution of ascorbic acid (500 µMol/mL) was used as standard. Blank contained all reagents except sample/standard. FRAP reagent 3 mL was mixed with 200 µL aliquotes of samples and incubated in dark for 0.5 h at 37 °C. OD was measured at 593 nm. Aqueous solutions of Fe (II) in concentrations range from 100 to 1000 µM were used for calibration. The test was repeated three times for each sample.

**Determination of total antioxidant capacity (TAC) by phosphomolybdenum complex method:** The method described by Prieto *et al.*[21] was adopted to find total antioxidant capacity of samples. According to this method, samples were prepared in methanol from all the extracts to be tested at concentration 500 µg/mL. Reagent solution was prepared by mixing 0.6 M sulphuric acid, 4 mM ammonium molybdate and 28 mM sodium phosphate. The blank/control contained 4 mL of reagent solution only. 1 mL of test samples were mixed with 4 mL of reagent solution in capped vials. These vials were placed in water bath at 95 ºC for 90 min. After incubation, vials were allowed to cool and then absorbance was measured at 695 nm against blank/control. The antioxidant activity was expressed relative to that of BHA. The assay was repeated three times.

**Ferric thiocyanate (FTC) assay:** Lipid peroxidation inhibition potential was measured by following the method described by Mistuda *et al.*[22].

### Principle

**Background:** Linoleic acid and arachidonic acid (unsaturated fatty acids) are embedded in cell membrane and more sensitive towards attack by free radicals that cause lipid peroxidation. In this quantitative assay linoleic acid is used as model tool to measure the lipid peroxidation level caused by free radicals.

The basic principle of this assay is to estimate the total amount of peroxides generated during lipid peroxidation, these peroxides react with ferrous chloride and form ferric ions. These ferric ions combine with ammonium thiocyanate and a ferric thiocyanate complex is formed whose colour intensity is measured at 500 nm.

**Description of assay:** In a screw cap bottle, a mixture containing, 10 mL of 0.05 M phosphate buffer (pH 7.0), 0.1 mL of test sample (0.5 mg/mL in absolute ethanol), 4 mL of 2.5 % linoleic acid in absolute ethanol and 5.9 mL of distilled water, was transferred and then incubated in dark oven at 40 ºC for overnight. 0.1 mL of this incubated mixture was taken in the test tube and 9.7 mL of 75 % ethanol and 0.1 mL of 0.02 M ferrous chloride in 3.5 % HCl were added to it. After 3 min, the addition of ferrous chloride, 0.1 mL of 30 % ammonium thiocyanate was added. Red colour appeared and its absorption was measured at 500 nm. BHA (0.5 mg/mL) with all the reagents was used as positive control/reference standard and mixture without test sample or BHA was used as negative control/blank. The test was done in triplicate.

**Statistical analysis:** Data were analyzed using SPSS version 17. The results are presented as mean ± SD. Analysis of variance (ANOVA) followed by Tukey's post-hoc test were applied to determine the statistical significance, P value < 0.05 was considered significant. Correlation among all antioxidant assays was concluded with Pearson correlation. Correlation was significant at 0.01 level (2-tailed).

## RESULTS AND DISCUSSION

Basically the antioxidant capability of the plant extracts is dependent on compositions, hydrophilic or hydrophobic nature of antioxidants, the solvent used for extraction, activity extraction method, temperature and other conditions of test procedure. Thus, it becomes mandatory to use various methods to evaluate antioxidant activity of plant extracts. This also helps in defining the mechanism of actions of antioxidants[23].

In this study, aqueous (AQ), ethanol (ET), petroleum ether (PE), chloroform (CH) and methanol (MT) extracts of leaves (L) and stem (S) parts of *Euphorbia helioscopia* and its latex were tested to evaluate their antioxidant activity with five *in vitro* antioxidant models. BHA and ascorbic acid were used as reference standard. Four E.T.R. a) DPPH, b) FRAP, c) TAC, d) FTC and one H.T.R. *i.e*. BCL assay were used. E.T.R measure the antioxidant potential of compounds by estimating their redox power. H.T.R. are mostly kinetics based wherein these reactions antioxidant and oxidant compete for peroxyl radicals that generate with heat with passage of time from azo compounds.

DPPH, TAC, FRAP, FTC (lipid peroxidation inhibition) results are summarized in Table-1.

L.MT showed highest % scavenging effect *i.e*. 91.583 ± 0.520 in DPPH assay. This observation is in agreement with the findings of Uzair *et al.*[24]. Plant latex had second maximum % scavenging effect (71.476 ± 0.502). Compared with reference standard (BHA), the activity of L.MT was significantly higher whereas that of latex was significantly lower ($p < 0.05$).

Under DPPH assay, $IC_{50}$ values of all the extracts were calculated from graphs plotted between concentrations and remaining DPPH (100- % scavenging effect). $IC_{50}$ has inverse relation with activity *i.e*. lowest $IC_{50}$ indicates highest antioxidant activity and *vice versa*. Plant latex showed 0.022 ± 0.001 mg/mL $IC_{50}$ that was lowest among all tested extracts and L.MT showed second lowest $IC_{50}$ *i.e*. 0.075 ± 0.0005 mg/mL. There was no significant difference between BHA (0.015 ± 0.049 mg/mL) and plant latex $IC_{50}$. Rest of the extracts showed significantly higher ($p < 0.05$) values as compared to BHA value. The % scavenging effect of rest of the extracts can be ranked in the descending order as follows: L.AQ > L.PE > S.ET > S.MT > S.AQ > L.CH > S.CH > S.PE > L.ET and all these values are significantly lower than BHA reference standard.

FRAP value was calculated from $Fe_2SO_4$ µM/L standard curve within concentration range of 100-1000 µM/L. linear regression equation Y = 0.7207 X-0.0173, $R^2 = 0.9996$ was used to calculate samples FRAP value. Ascorbic acid was used as reference standard, it has FRAP value 405.66 ± 0.58 $Fe_2SO_4$ µM/L. Plant latex has maximum FRAP value (220 ± 0.50 $Fe_2SO_4$ µM/L) and L.MT has second highest FRAP value (213 ± 0.50 $Fe_2SO_4$ µM/L). There is no significant difference between first and second highest FRAP values but these are significantly lower than reference standard (ascorbic acid) used.

TABLE-1

| Samples | DPPH | | FRAP | TAC | Lipid peroxidation inhibition (%) |
|---|---|---|---|---|---|
| | % Scavanging effect | $IC_{50}$ (mg/mL) | $Fe_2SO_4$ (µM/L) | % Scavanging effect | |
| L.AQ | 67.506 ± 1.792* | 0.100 ± 0.005* | 31.500 ± 0.50* | 0.1537 ± 0.002* | 50.28 ± 0.26* |
| S.AQ | 17.475 ± 0.502* | 0.120 ± 0.005* | 36.167 ± 0.29* | 0.1317 ± 0.008* | 35.22 ± 0.38* |
| L.ET | 4.447 ± 0.508* | 0.120 ± 0.005* | 121.500 ± 0.50* | 0.3850 ± 0.001* | 20.41 ± 0.52* |
| S.ET | 26.362 ± 0.554* | 0.105 ± 0.0005* | 106.830 ± 0.29* | 0.2853 ± 0.004* | 30.34 ± 0.29* |
| L.PE | 47.608 ± 0.534* | 0.165 ± 0.0006* | 35.830 ± 0.29* | 0.8443 ± 0.005* | 49.68 ± 0.28* |
| S.PE | 8.565 ± 0.513* | 0.195 ± 0.0006* | 25.167 ± 0.76* | 0.5243 ± 0.005* | 50.28 ± 0.24* |
| L.CH | 17.463 ± 0.504* | 0.089 ± 0.0006* | 60.167 ± 0.76* | 0.6840 ± 0.003* | 46.99 ± 0.01* |
| S.CH | 16.00 ± 1.00* | 0.084 ± 0.0005* | 134.00 ± 0.54* | 0.5033 ± 0.006* | 44.53 ± 0.50* |
| L.MT | 91.583 ± 0.520* | 0.075 ± 0.0005* | 213.00 ± 0.50* | 0.9957 ± 0.002 | 66.55 ± 0.77* |
| S.MT | 21.579 ± 0.519* | 0.079 ± 0.001* | 53.167 ± 0.29* | 0.2710 ± 0.008* | 31.14 ± 0.31* |
| LATEX | 71.476 ± 0.502* | 0.022 ± 0.001 | 220.00 ± 0.50* | 1.1667 ± 0.06* | 75.66 ± 0.56* |
| BHA | 83.616 ± 0.539 | 0.015 ± 0.049 | - | 0.9600 ± 0.01 | 70.96 ± 0.06 |
| Ascorbic acid | - | - | 405.66 ± 0.58 | - | - |

L = leaves, S = stem, AQ = aqueous, ET= ethanol, PE = petroleum ether, CH = chloroform, MT = methanol, values are given as mean ± standard deviation. *P < 0.05 when compared with respective standards.

TABLE-2
% ANTIOXIDANT ACTIVITY CALCULATED WITH β-CAROTENE LINOLEATE BLEACHING ASSAY

| Samples | β-Carotene linoleate bleaching assay | | | | | | | | | Antioxidant activity (%) |
|---|---|---|---|---|---|---|---|---|---|---|
| | 0 min | 15 min | 30 min | 45 min | 60 min | 75 min | 90 min | 105 min | 120 min | |
| L.AQ | 0.863 | 0.610 | 0.600 | 0.590 | 0.585 | 0.525 | 0.500 | 0.440 | 0.420 | 48.778 ± 0.192* |
| S. AQ | 0.863 | 0.632 | 0.621 | 0.620 | 0.619 | 0.539 | 0.521 | 0.480 | 0.430 | 49.884 ± 0.100* |
| L.ET | 0.863 | 0.549 | 0.547 | 0.546 | 0.545 | 0.537 | 0.499 | 0.465 | 0.427 | 49.653 ± 0.300* |
| S.ET | 0.863 | 0.545 | 0.540 | 0.538 | 0.536 | 0.535 | 0.498 | 0.462 | 0.430 | 49.884 ± 0.100* |
| L.PE | 0.863 | 0.553 | 0.552 | 0.551 | 0.550 | 0.539 | 0.501 | 0.482 | 0.435 | 50.407 ± 0.002* |
| S.PE | 0.863 | 0.565 | 0.564 | 0.562 | 0.560 | 0.520 | 0.485 | 0.458 | 0.440 | 50.995 ± 0.008* |
| L.CH | 0.863 | 0.512 | 0.511 | 0.510 | 0.508 | 0.498 | 0.488 | 0.468 | 0.439 | 50.912 ± 0.08* |
| S.CH | 0.863 | 0.510 | 0.509 | 0.508 | 0.507 | 0.499 | 0.488 | 0.473 | 0.435 | 50.604 ± 0.342* |
| L.MT | 0.863 | 0.652 | 0.651 | 0.650 | 0.649 | 0.600 | 0.585 | 0.575 | 0.560 | 64.926 ± 0.064* |
| S.MT | 0.863 | 0.620 | 0.618 | 0.617 | 0.616 | 0.590 | 0.550 | 0.499 | 0.430 | 49.884 ± 0.100* |
| LATEX | 0.863 | 0.780 | 0.778 | 0.770 | 0.760 | 0.758 | 0.755 | 0.730 | 0.690 | 79.969 ± 0.026* |
| Control | 0.863 | 0.361 | 0.314 | 0.314 | 0.320 | 0.301 | 0.300 | 0.298 | 0.295 | 34.181 ± 0.002 |
| BHA | 0.863 | 0.762 | 0.750 | 0.740 | 0.692 | 0.544 | 0.520 | 0.491 | 0.455 | 52.742 ± 0.250 |

L = leaves, S = stem, AQ = aqueous, ET= ethanol, PE = petroleum ether, CH = chloroform; MT = methanol, % Antioxidant Activity values are given as mean ± standard deviation. *P < 0.05 when compared with respective standards.

Total antioxidant capacity and ferric thiocyanatge assays results are identical to $IC_{50}$ values of DPPH. This means, similar to DPPH, the maximum and second maximum antioxidant effect of latex and L.MT in TAC test were 1.1667 ± 0.06 % and 0.9957 ± 0.002 %, respectively. Likewise FTC values for latex (75.66 ± 0.56 %) and L.MT (66.55 ± 0.77 %) also show the similar trend. The remaining samples have activity significantly lower than reference standard, latex and L.MT in both the tests.

Ferric thiocyanate (FTC) assay inhibits the lipid peroxidation which is the one of the most important causes of cardiovascular diseases and cancer. Lipid peroxidation comprise series of free radicals producing chain events that damage the biological system and cause oxidative stress induced diseases[25].

β-Carotene-linoleic acid assay results are presented in Table-2. The data showed exactly the same trend as indicated by the four tests mentioned in Table-1, ranking latex at the top and L.MT in second position. However it is worth mentioning that the activities of latex and L.MT are significantly higher than the reference standard BHA.

Table-3 showed Pearson correlation coefficient values of all the extracts for *in vitro* antioxidant activity. Though significant correlation (at 0.01 level, two tailed) was found amongst all the extracts but strong correlation can be observed between TAC and LPI (r = 0.817), DPPH and LPI (r = 0.814), FRAP and BCL (r = 0.803).

Polyphenolic flavonoids occur ubiquitously in medicinal plants and these are claimed to possess antioxidant activities[26,27]. Previously we have reported that an appreciable amount of flavonoids is present in various extracts (aqueous, ethanol, petroleum ether, chloroform and methanol) of *Euphorbia helioscopia* leaves and stem and the highest flavonoids percentage was found in L.MT extract[28]. Flavonoids are considered to play a significant role in improving and maintaining good health in humans because of effectively scavenging the reactive oxygen species that are deleterious for health[29,30].

In our findings, the antioxidant activity of latex was slightly higher than L.MT. The maximum antioxidant activity of latex may be due to high titer of flavonoids which subsequently are partitioned into different extracts in sequential extraction by organic solvents. There exists a strong evidence in literature that flavonoids have proven wide range of activities against bacteria, virus and beneficial effects in treating cancer, atherosclerosis, rheumatoid arthritis, liver cirrhosis and many other conditions that are caused by oxidative stress.

TABLE-3
CORRELATION AMONG ANTIOXIDANT ACTIVITY MEASURING MODELS

| | Correlations | | |
|---|---|---|---|
| | Pearson correlation | Sig. (2-tailed) | N |
| | DPPH | | |
| DPPH | 1 | | 33 |
| TAC | 0.619** | 0 | 33 |
| FRAP | 0.519** | 0.002 | 33 |
| LPI | 0.814** | 0 | 33 |
| BCL | 0.562** | 0 | 33 |
| | TAC | | |
| DPPH | 0.619** | 0 | 33 |
| TAC | 1 | | 33 |
| FRAP | 0.656** | 0 | 33 |
| LPI | 0.817** | 0 | 33 |
| BCL | 0.726** | 0 | 33 |
| | FRAP | | |
| DPPH | 0.519** | 0.002 | 33 |
| TAC | 0.656** | 0 | 33 |
| FRAP | 1 | | 33 |
| LPI | 0.493** | 0.004 | 33 |
| BCL | 0.803** | 0 | 33 |
| | LPI | | |
| DPPH | 0.814** | 0 | 33 |
| TAC | 0.817** | 0 | 33 |
| FRAP | 0.493** | 0.004 | 33 |
| LPI | 1 | | 33 |
| BCL | 0.694** | 0 | 33 |
| | BCL | | |
| DPPH | 0.562** | 0 | 33 |
| TAC | 0.726** | 0 | 33 |
| FRAP | 0.803** | 0 | 33 |
| LPI | 0.694** | 0 | 33 |
| BCL | 1 | | 33 |

**Correlation is significant at 0.01 level (2-tailed).

## Conclusion

It is concluded from the current study that plant latex and leave methanol extract of *Euphorbia helioscopia* had most promising antioxidant activity against number of free radicals. Hence the folklore use of *Euphorbia helioscopia* in conditions induced by oxidative stress seems to be justified. Further studies incorporating extracts (or active antioxidant principles) as adjuvant to therapies for various oxidative stress induced diseases might have added effect and provide protection against toxicity at the same time.

## ACKNOWLEDGEMENTS

Financial support from Higher Education Commission (HEC) of Pakistan is gratefully acknowledged.

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