

Article Info

 Open Access

Citation: Aslam, M.M., Karanja, J.K., Bello, S.K., Zaynab, M., Fatima, M., Uddin, J.B.G., 2019. Antioxidant Capacity of *In-vitro* generated *Solanum tuberosum* L. Cultivar (Favorita). *Int. J. Nanotechnol. Allied Sci.*, 3(1): 19-29.

Received: March 13, 2019

Accepted: May 10, 2019

Online first: May 20, 2019

Published: May 31, 2019

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Antioxidant Capacity of *In-vitro* generated *Solanum tuberosum* L. Cultivar (Favorita)

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Abstract:

Plant-based diets are rich in antioxidants that scavenge harmful free radicals in order to reduce the risk of the development of oxidative stress-related diseases. *Solanum tuberosum* L. is endowed with a high content of antioxidants including phenolic acids and flavonoids, however, concentrations of these secondary metabolites in cultivars such as Favorita remain largely undefined. Thus, the total phenolic contents of Favorita were analyzed using HPLC, while percentage inhibition of DPPH and ABTS assays were performed to determine antioxidant activities. The major constituents of phenolic acid in the potato root methanol extract were vanillic acid and chlorogenic acid. Similarly, antioxidant activity ranged from 5.7% (% inhibition of DPPH) to 69.85% (% inhibition of ABTS) in comparison to the three available standards. Total phenolic content and antioxidant capacity revealed a significantly high linear correlation ($R^2 = 0.951$, $P = 0.01$), suggesting a direct involvement of phenolics in the antiradical activity. These results demonstrate that Favorita contains potent antioxidant capacity; however, this potency is dependent on the specific free radicals utilized. These findings would facilitate future research on potato micropropagation for improved agronomic traits as well as give information on its nutritional use as an antioxidant.

Keywords: Antioxidants capacity; *Solanum tuberosum* L. (Favorita); ABTS; DPPH; Total phenolic content; HPLC.

INTRODUCTION

Potato (*Solanum tuberosum* L.) belongs to the family Solanaceae. After wheat and rice, potato is recognized as an important crop globally owing to its high vigor to grow on marginal lands, nutritional content, high productivity, and economic value (Felcher *et al.*, 2012). It is a rich source of most essential nutrients as well as vitamins. Consumption of potato can be fresh, processed and the tubers are utilized as planting materials for propagation (Stewart and McDougall, 2012). It is a highly productive vegetable that can achieve global food security, climate change and other demographic factors notwithstanding (Roy *et al.*, 2015). According to FAO, the production of potato in Pakistan was 2.5 million metric tons in 2008 (Badoni and Chauhan, 2009). In developing countries, approximately 325 million metric tons yield is reported per year (Dowling, 2004).

Solanum tuberosum L. is vegetatively propagated asexually through tubers. Whilst potato seed quality is the premier interest of potato breeders, this has not been achieved; this is in part, because vegetative method of propagation is a source of viral infection transmitted from infected mother plant to next progeny as well as due to poor germination technology (Ah-Hen *et al.*, 2012). Germination technologies are affected by various biotic/abiotic factors that include temperature, light, water quality, soil mineral contents, seed quality, and disease-free seeds. Quality germination technology produces vegetables with appropriate content of antioxidants such as phenolics, flavonoids, catalases, Superoxide dismutase (SOD), and glutathione (GSH) that scavenge free radicals in cells to mitigate against oxidative stress in a bid to proffer a potent healing mechanism (Doliński and Olek, 2013). The rice chitinase gene expression in potato confers enhanced resistance against two major fungal diseases of potato in Pakistan (Zaynab *et al.*, 2017).

Plants and plant derived metabolites are believed as rich source of antibacterial, antifungal and antioxidant agents, with their fewer side effects (Al-Deen and Al-Jobory, 2018; Ali *et al.*, 2017; Amin and Edris, 2017; Hashmi *et al.*, 2018; Hussain *et al.*, 2016; Iqbal *et al.*, 2015; Kalim *et al.*, 2016). Elaborate research findings have demonstrated that plant-based diet protects against chronic oxidative stress-associated diseases such as hypertension, obesity, cataract, diabetes type 2, coronary heart disease, and cancer (Grassmann, 2005). Human diet dominated by vegetables (potatoes), fruits, and whole starchy foods lowers the risk of developing such diseases (Grassmann, 2005; Hanif *et al.*, 2005). Reactive oxygen species (ROS) arise from partially reduced form of oxygen. Although ROS have deleterious effect on nucleic acids, proteins and lipids; they play a crucial role in cell signaling (Ahmad *et al.*, 2012; Fang *et al.*, 2002; Fridovich, 1999; Pandey and Pandey-Rai, 2015). A common feature that characterizes cell damage is oxidative stress imbalance (Birben *et al.*, 2012; Fridovich, 1999). An intricate defense network including catalase (CAT), ascorbate peroxidase (APX), SOD, glutathione peroxidase (GPX) and non-enzymatic systems such as (GSH) and phenolic compounds has evolved in response to such damage (Vaghasiya *et al.*, 2011). Therefore, antioxidants are recognized as key constituents of immunity systems against cell damage caused by oxidative stress (Dzialo *et al.*, 2016; Mouffouk *et al.*, 2019). The anti-inflammatory properties of potato are attributed to proteins that inhibit protease enzyme, and antioxidants such as phenolic acids, carotenoids, or anthocyanins (Alkhatib, 2018; Kaspar *et al.*, 2011).

Solanum tuberosum L. is amenable to cell culture and has been continually improved through biotechnology (Barrell *et al.*, 2013). Availability of high quality tuber has been a long-time desired breeding objective over the traditional propagation characterized by disease infestation, which significantly decreases crop harvest (Washimkar and Shende, 2016).

Currently, diverse biotechnological techniques, including but not limited to, plant tissue culture are commercially viable approaches for crop improvement by generating abiotic stress-resistant and disease-free planting materials (Hussain *et al.*, 2012). Breeding effort in potato production is virtually low in comparison to other important food crops. Therefore, the current investigation was carried out on *Solanum tuberosum* L. cultivar (Favorita) with an objective to: (i) Measure antioxidant profiles of *in vitro* generated potato tubers, and (ii) Define the utility of the species for inclusion as a dietary antioxidant and for therapeutic application.

MATERIALS AND METHODS

Reagents and standards

The reagents and standards used were procured from Sigma, Sigma-Aldrich, USA and the list includes: ascorbic acid (AA), BHT (butylated hydroxytoluene), BHA (butylated hydroxyanisole), 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)/ABTS, DPPH (2, 2-diphenyl-1-picrylhydrazyl), and Dulbeco's phosphate buffered saline (PBS), different acids, methanol, absolute ethanol and acetonitrile (HPLC grade).

Plant material

Solanum tuberosum L. cultivar (Favorita) seeds were collected from research library of plant biotechnology, The University of Lahore, Pakistan. Dried root samples of the explants were used for subsequent biochemical analysis.

Surface sterilization of explants

The following steps were followed for sterilization of the explants (potato cultivar Favorita). Tap water was used to remove traces of soil from explants. The explants were dipped in 70% absolute ethanol (2 min), and washed again by using distilled water to remove all traces of ethanol. Explants were washed using washing detergent for 4 to 5 min and rinsed 2-3

times with distilled water to remove the detergent. Sodium hypochlorite (NaOCl) solution was used to treat explants for 15 min and rinsed with distilled water to remove any residues of sodium hypochlorite solution. Finally, the potato explants were treated with 1% tween 20 for ten minutes, then washed again 3 times by using distilled water to remove residues of tween 20. Sterile explants obtained were used for further experiments.

Culture conditions

Explants used for *in vitro* culture were grown on MS (Murashige and Skoog) medium containing 3% sucrose, 6 g/L agar was added at pH 5.7 to 5.8. Autoclaving was done at 121 °C, 15 lbs pressure for 20 min. appropriate phytohormones; BAP (benzyl-amino purine) and IAA (indole-3-acetic acid), were added before autoclaving as they are not sensitive to high temperatures up to 121°C. After autoclaving, the medium was dispensed into culture jars inside the laminar hood and then kept in sterilized environment. Plants were kept in the culture room at 25°C under a 16h/8h (light and dark) photoperiod with 6000 - 8000 lux light intensity, and 60 - 70% relative humidity (RH).

Antioxidants content analysis

Total phenolic content

Total phenolic content (TPC) in the root was determined using 0.5N of Folin-Ciocalteu reagent (Sigma Chemical Co., St. Louis, MO) following previous method (Barański *et al.*, 2014). Briefly, reaction mixture was prepared by using 100µl of Favorita root extract and 2.5ml of 0.5N Folin-Ciocalteu reagent. The mixture was kept in dark for 15 min; added 2 ml of 7.5% sodium carbonate, finally kept in the dark for approximately 30 min. UV spectrophotometer (model UV3000, 21029 Hamburg, Germany) was used to measure absorbance at 750 nm. Synthetic antioxidants: BHA, BHT and AA were used as standards for comparison.

Glutathione content

Both reduced sulphhydryl glutathione (GSH) and oxidized glutathione disulfide (GSSH) obtained from the potato root extract were determined as described by Rahman et al. (2006). Reaction between Ellman's reagent (5, 5-dithio bis (nitrobenzoic acid) or DTNB) and GSH produces chromophore TNB with optimal absorbance at 412 nm and GSSH (Wang and Jiao, 2001). The reaction mixture for total GSH measurement constituted 0.1 mL of extract containing 60mM KH₂PO₄, 0.6mM DTNB dissolved in 200 mM Tris-HCl at pH 8.0, 2.5 mM EDTA buffer at pH 7.5, and 0.2mM NADPH. The concentration of total GSH was measured at 412 nm absorbance level. GSH was determined in a similar reaction mixture as total glutathione. Finally, incubation of the mixture was done at 30 °C for 10 min and the concentration measured at 412 nm absorbance level. Standard curve generated with pure GSSH and GSH in the range of 0 - 100/µM was used to calculate total glutathione and GSH. GSSH was the difference between GSH and total glutathione.

Catalase content

Catalase content of Favorita root extract was analyzed using modified protocol described by Aebi (1974). Reaction mixture consisted of 2 mM H₂O₂ in a 10 mM phosphate buffer (pH 7.0) and enzyme extract (0.1 mL) in final volume (3mL). CAT activity was measured spectrophotometrically at 230 nm following H₂O₂ decreased absorbance by comparing with standard curve derived from known values of catalase. Catalase enzymatic activity was expressed as mg/100 g root tissue.

Determination of superoxide dismutase activity

Superoxide dismutase activity was evaluated as described by Kakkar et al. (1972). The mixture consisted of 0.1 ml potato root extract, 1.2 ml sodium pyrophosphate buffer (pH 8.3, 0.052 M), 100 µl phenazinemethosulphate, 0.3 ml nitroblue tetrazolium and 0.2 ml (750

µmol) NADH (to initiate reaction). After incubation at 30°C for 1.5 min, 100 µl of glacial acetic acid was added to stop the reaction. The mixture was shaken vigorously in 4.0 ml n-butanol. It was allowed to stand for 10 min; n-butanol layer was separated by centrifugation. The color intensity was measured at 560 nm and concentration of SOD (mg/100 g) sample was calculated by comparing with known SOD standard values.

Estimation of Thiobarbituric acid reactive substances

Thiobarbituric acid reactive substances (TBARS) obtained from Favorita root extract were analyzed using method proposed by Wijeratne et al. (2006). 200 µl of extract was added in eppendorf tube containing 200 µl 8.1% sodium dodecyl sulfate (SDS), 1.5 ml 0.8% TBA, and 1.5 ml 20% acetic acid which was added earlier. Mixture was centrifuged at 3000 rpm for 10 min and the supernatants obtained by filtering. Filtrates were kept in water bath for 45 min, and then cooled to room temperature. Absorbance of the resultant chromogens was read at 532 nm against the blank. 1,1,3,3-tetramethoxypropane, a precursor of malondialdehyde (MDA) was used to prepare standard curve which was used to obtain TBARS values in terms of MDA equivalents.

In vitro antioxidant assays

Antiradical activity on ABTS

The antiradical activity of potato shoot extract on ABTS was measured following the protocol proposed by Pandya-Jones et al. (2013) and Engreitz et al. (2013) with some modifications. Reaction mixture was composed of 0.5 mM ABTS⁺, oxidizing agent (MnO₂, manganese dioxide), in 0.1M phosphate buffer at pH 7 for (30 min) at ambient temperature in a dark chamber. Ethanol was used to dilute resultant ABTS⁺ solution to a concentration (0.7±2) at 734 nm absorbance levels. 200µl of Favorita shoot extractions were added to ABTS⁺ solution (3.9 mL). UV visible spectrophotometer

(Ce202, CECIL, Cambridge UK) was used to monitor absorbance at 734 nm. Antiradical activity on ABTS radicals was determined as % inhibition according to equation (Equation 1) (Camire *et al.*, 2009).

$$\% \text{ inhibition of ABTS} = \frac{\text{Abs.of control} - \text{Abs.of sample}}{\text{Abs of control}} \times 100 \quad \text{Equation 1}$$

Antiradical activity on DPPH

The antiradical activity of potato shoot extract on DPPH was evaluated following the modified protocol cited by Singh and Rajini (2004). Favorita shoot extract (0.1 mL) was added to 3.9 mL methanol, containing 25mg/mL DPPH. Final mixture was stirred vigorously and kept in a dark chamber for 30 min. Absorbance of the resulting solution at 517 nm was compared against blank with standards of BHT, BHA, and AA. The antiradical activity on DPPH was obtained as % inhibition according to equation (Equation 2).

$$\% \text{ inhibition of DPPH} = \frac{\text{Abs.of control} - \text{Abs.of sample}}{\text{Abs of control}} \times 100 \quad \text{Equation 2}$$

HPLC Analysis

High performance liquid chromatography (HPLC) system was used for sample analysis. Reverse phase HPLC RBAX ECLIPSE, XDB-C18 (5 μ m, 4.6 mm \times 150 mm) column (Agilent Technologies, USA) was used to separate phenolic compounds fitted with 4.6 mm \times 2.0 mm column. Mobile phase was composed of buffer A (10 mM formic acid + NH₄OH) at pH 3.5, buffer B composed of 100% methanol and ammonium formate. Solvent gradient was adjusted as: 100% buffer A for 0 - 60 sec, 15% buffer B for 60 – 75 sec, 45% buffer B for 15 - 30 min, and 100% buffer B for 30 - 45 min. Ultra-violet detection was set at 280 nm. 10 μ l sample was injected at 1.0 mL min⁻¹ rate. The mobile

phase was passed through 0.2 mm millipore membrane filters followed by degassing sonication in an ultrasonic bath at 37 °C. Samples were analyzed in duplicates. Identity of novel compounds in Favorita root extract (chlorogenic, vanillic acids, and quercetin) was determined by retention time and ultra-violet spectra with known standards.

Statistical analysis

Data presented in the current study indicate mean \pm standard deviation of three replicates. Spearman's rank correlation coefficients were generated using SPSS software (version 19.0) for the association between antioxidant capacity and TPC. Correlations were statistically significant at 5% level ($p < 0.05$).

RESULTS AND DISCUSSION

Evaluation of phenolics

Antioxidant levels and their corresponding antioxidant capacity in *Solanum tuberosum* cultivar (Favorita) were investigated in the current study. Phenolics constitute a potent class of antioxidants or free radical scavengers; thus, TPC in Favorita root methanol extracts was evaluated. 19.4 mg/100 g of TPC was obtained as depicted in Table 1. Lachman *et al.* (2009) reported 42.1 mg/100 g of potato tuber, while Al-Weshahy and Rao (2009) presented similar results at 48 mg/100 g dry weight of potato peel extracts. Lower value of potato phenolic concentration was realized by Singh and Rajini (2004), 3.94 mg/100 g dry weight. The differences in the obtained results are most likely as a result of different growth conditions, and cultivars investigated in distinct studies. Indeed, Delaplace *et al.* (2009) and Lachman *et al.* (2008) proposed that potato phenolic contents largely depend on genotypes, culturing, and environmental cues such as abiotic stress.

Table 1. Antioxidant profiles of potato extract. Data presented here represent mean \pm standard deviation of three replicates.

Antioxidants	Concentrations
Total phenolic content, TPC	19.40 \pm 0.2 mg/100 g
Superoxide dismutase, SOD	7.96 \pm 0.01 mg/100 g
Glutathione, GSH	6.58 \pm 0.1 mmol/g
Lipid Peroxidation, TBARS	21.92 \pm 0.4 mg/100 g
Catalase, CAT	73.56 \pm 0.2 mg/100 g

Determination of phenolic acids in explants root extract

Phenolic compounds are synthesized in plants to confer defense against pathogens; numerous findings have demonstrated that they exhibit health-promoting effects in humans (Deußer *et al.*, 2012). Phenolic acids in potato tubers exist either in free or conjugated form, chlorogenic acid being the dominant constituent (Andre *et al.*, 2007). Analysis of phenolic compounds is complex owing to their high

reactivity as well as robust diversity. However, novel techniques including ultraviolet-visible light (UV-Vis), HPLC combined with mass spectroscopy (MS), and nuclear magnetic resonance (NMR) have achieved considerable success in these analyses (Huang *et al.*, 2007). HPLC is preferable since it achieves high rate of versatility compared to other chromatographic systems; besides, it allows for separation of a myriad of bioactive compounds (Navarre *et al.*, 2011). Key phenolic acids from potato root extract were systematically determined by HPLC (Figure 1). Major peaks generated were compared with standards and corresponded to chlorogenic acid, quercetin, and vanillic acid. This observation conforms with earlier findings that have reported the presence of phenolics as well as chlorogenic acid as the dominant component of various potato tuber peels obtained from diverse cultivars (Islam *et al.*, 2002; Mattila and Kumpulainen, 2002; Padda and Picha, 2007).

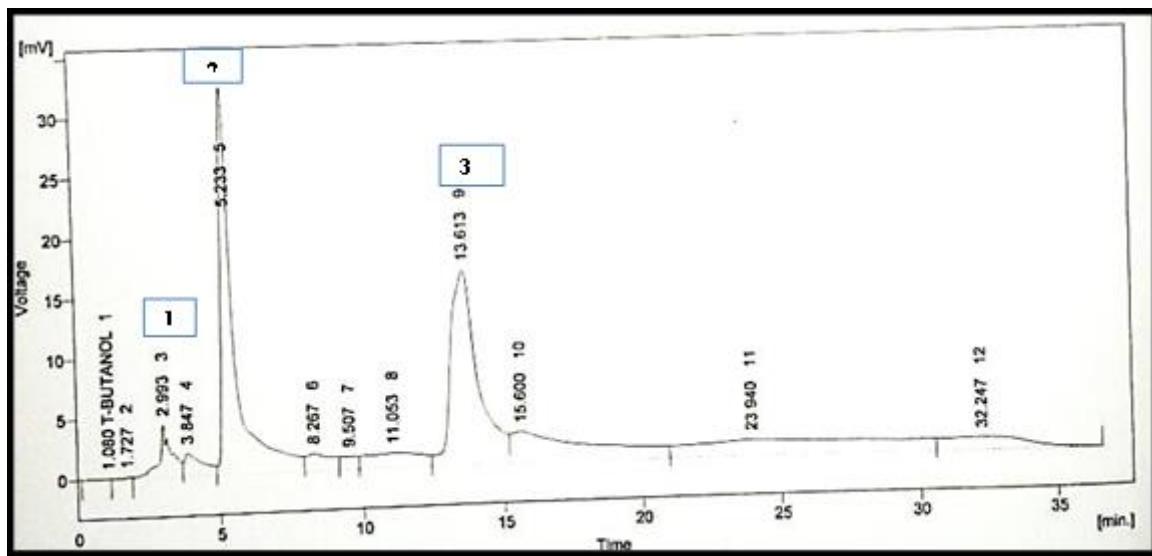


Fig. 1. HPLC profiles of potato extract. Peaks: 1-quercetin (2.9933 min); 2-chlorogenic acid (5.2335 min); 3-vanillic acid (13.6139 min). In the chromatogram, the first small peaks (1.7-1.9) are ignored as “noise” from the injection made, while the three larger peaks represent chemicals in the methanol extract obtained from potato root sample.

Potato shoot extract antioxidant capacity

Analysis of the antioxidant potential of methanol extracts, obtained from the potato shoot, against stable free radicals indicates more potent antioxidant capacity against ABTS than DPPH (Figure 2). These findings suggest that antioxidant activity is largely dependent on individual free radical utilized. Thus, more investigations are warranted to decipher more reliable determinants of antioxidant activity. In addition, the present study findings showing strong significant association between antioxidant capacity and TPC conforms to observations of Leo et al. (2008), who showed similar correlation, suggesting direct involvement of phenolic compounds in antiradical activity. Polyphenols exhibit multifaceted roles in plants as iron chelating agents, antioxidants, reducing agents as well as quenching singlet oxygen. They possess critical property as potent antioxidants, thus, in synergy with other endogenous compounds they offer cell protection against oxidative stress effects (Albishi et al., 2013).

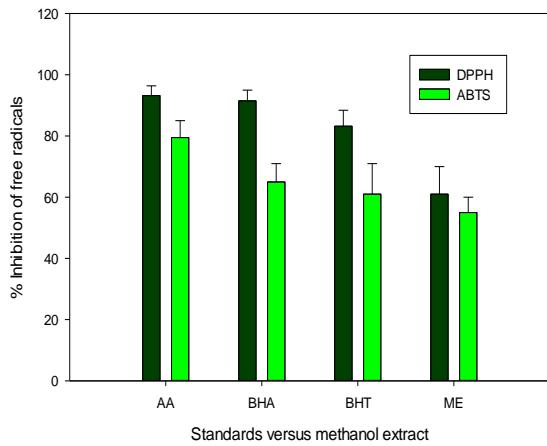


Fig. 2. Percentage (%age) inhibition of potato methanol extract on free radicals (DPPH and ABTS) in comparison to three standards (AA, BHA, and BHT). Data presented here represent mean \pm standard deviation of three replicates.

Glutathione (GSH), SOD, CAT, and lipid peroxidation

Free radical mediated damage such as lipid membrane peroxidation, measured in terms of TBARS, which is deleterious to cell constituents (Kanatt et al., 2005). Potato root extract capacity in retarding lipid peroxidation (evaluated in form of TBA number) was evaluated since the extract demonstrated a considerable antioxidant activity. It was found to be 21.92 mg/100g (Figure 1); and this observation mirrors previous findings presented by Kanatt et al. (2005) investigating effects of potato peel extract on the lipid peroxidation. Multiple reports show increased TBA values in response to lipid peroxidation (Amarowicz et al., 2004; Singh and Rajini, 2004). Altogether, these results depict that potato tubers contain potent bioactive metabolites that inhibit cell damage by retarding unfavorable metabolic processes such as lipid peroxidation.

Environmental stresses such as heat, excessive light, and drought, may disrupt normal cell metabolic activities, which in turn upsets free radical balance. Constitutive or induced levels of antioxidants not only ameliorate this imbalance by providing resistance to particular stresses, but also confer resistance against activated oxygen which is highly reactive and causes harmful oxidation of lipids, nucleic acids, and proteins (Bartwal et al., 2013). Thus, relative concentrations of CAT, SOD, and GSH of *in vitro* generated potato root extract are worth being examined as shown in Figure 1. Results of CAT and SOD reported in the current study are corroborated by observations of earlier studies evaluating different potato cultivars (Afify et al., 2012). GSH is the most widely characterized soluble thiol expressed by plant cells, and serves as an indispensable regulatory factor of intracellular redox homeostasis (Sabetta et al., 2017). In this study, GSH exhibited a considerable content comparable to those reported by a previous study (Ryant et al., 2008). Altogether, the present finding therefore highlights the indirect possibilities of enhanced

antioxidant capacity against oxidative challenge in potato plants. Interpretation proposed herein agrees with the findings of Smirnoff (2005), implicating antioxidant compounds in membrane protection against ROS, a physiological function involving synergistic interaction with other secondary metabolites.

CONCLUSION

Potato phenolic compounds exhibit potent antioxidant capacity that is largely dependent on specific free radical encountered. Favorita has robust potential for inclusion in human diet recommendations aiming to combat oxidative stress-associated effects because it displays antiradical activity that would guarantee cell production. Micro-propagation is one of the finest and fastest methods of plant multiplication through *in vitro* techniques of plant tissue culture as the plants often present high genetic integrity, quality and vigor. Collectively, these findings suggest that potato micro propagation through *in vitro* culture may enhance fast clonal multiplication of potato explants with superior vigor for defense against biotic and abiotic stressors.

AUTHOR'S CONTRIBUTIONS

Mehtab Muhammad Aslam conceived and designed the study. Karanja J.K was involved in statistical analyses and co-drafted the manuscript. S.K Bello critically reviewed the paper. All authors proofread and approved the final manuscript.

ACKNOWLEDGEMENT

This work is supported by The University of Lahore, Defense road campus, Pakistan. Special thanks to Dr. M.H. Qazi and Dr. Asif Saleem for their intellectual contribution and support to in the course of this research.

CONFLICT OF INTEREST

The authors have no conflict of interests to declare.

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