Estimation of Antioxidant Potential of Indigenous Halari and French Poitu Donkey Milk by using the Total Antioxidant Capacity and Ferric Reducing Antioxidant Power Essay

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10.18805/ajdfr.DR-1501

ABSTRACT
Over the last decade, research has been conducted towards the uses and properties of donkey milk that has distinct chemical composition and consequently particularly nutritional properties. It is interesting to determine antioxidant potential of indigenous Halari and French Poitu donkey milk by using the total antioxidant capacity and ferric reducing antioxidant power assay. These methods are reliable, fast and with sound methodological infrastructures. The objective of the present research was to investigate the applicability of the trolox based total antioxidant capacity (TAC) and ferric reducing ability of plasma (FRAP) assay for identification of antioxidant potential of Indian Halari donkey milk and French Poitu donkey milk. The donkey milk is quite popular for its anti-aging properties in Western and European countries as cosmetic and nutraceutical preparations. However, in Indian context no such study is conducted on Halari donkey milk. The Indian Halari donkeys are untapped resource for their dairy potential. Our results showed that the whole milk of Halari donkey is at par with French Poitu donkey milk in terms of their antioxidant potential.

Key words: Antioxidant, Assay, Donkey, Indigenous, Milk.

INTRODUCTION
Nowadays, it is considered that donkey milk is so similar to human milk that it can fulfill the nutritive requirements of the infants, as well as it is rich in lactose, lysozyme, Omega-3 and -6 polyunsaturated fatty acids which are similar to human milk. It is considered as an excellent source of antioxidants. All the living entities consist of large number of antioxidants like micro and macro molecules, enzymes among other examples, which shows the antioxidant capacity of the system. These antioxidants play a key role in preventing the oxidative stress in the body and thus owe to health and anti-ageing effects. Milk and milk products are nutritious food items containing abundant essential nutrients such as oleic acid, conjugated linoleic acid, omega-3 fatty acids, vitamins, minerals and bioactive compounds such as antioxidants (Khan et al., 2019).

Antioxidant capacity assays are useful in measuring the overall antioxidant activity in foods. Antioxidant capacity assays can be categorized into hydrogen atom transfer based assays and electron transfer based assays. Zuluetta et al. (2009) reported that hydrogen atom transfer based assays measured antioxidant activity from amino acids in milk that can act as hydrogen donors. The estimation of nitric oxide free radicals, DPPH free radicals and the inhibition of oxidation of linolenic acid, total phenolic contents, flavonoid contents and total reducing attributes can be applied for the depiction of antioxidant capacity in milk and other dairy products (Chen et al., 2003).

The composition of donkey's milk is different from the milk of the other more popular milking species like, cow, buffalo, goat and sheep. Donkey milk contains less fat, proteins and other inorganic salts but having more lactose content than cow milk. Despite having more lactose content than cow milk, the average energy content is much lower. This lactose helps in stimulation of intestinal absorption of calcium which is important for development of bones and proper development of nervous system in infants (Schaafsma, 2003). Environmental factors also play an important role in gross composition of milk.

The pH of donkey milk is slightly higher than the cow and buffalo milk. The pH values, 7.14 to 7.22 (Salimei et al., 2004; Guo et al., 2007), do not vary and remain similar throughout the milking period as in mare's milk (Mariani et al., 2001) and this signifies that the pH value is not affected by breed or stage of lactation. Donkey milk contains lower casein and phosphate content than cow milk (Salimei et al., 2004). It was observed by circadian studies that, during night, milk lipids and lactose content tend to be at peak and protein
content is at its peak during daytime in donkey (Piccione et al., 2008). In the present study, total antioxidant capacity of donkey milk is determined by micro plate assay technique (antioxidant assay kit) and Fe$^{3+}$-reducing power (ferric reducing antioxidant power assay, FRAP).

**MATERIALS AND METHODS**

**Ethics statement**

All the animal procedures were performed according to the standard procedures of ICAR-National Research Centre on Equines.

**Sample preparation and sampling**

The milk samples were collected from the Indigenous Halari (n=2) and French Poitu (n=3) donkeys respectively from Equine Production Centre of ICAR-NRCE at Bikaner, Rajasthan, India. Indigenous species of the donkey represent the Indian breed (native breed of Gujarat, the unuptapped genetic resource of India), while Poitu donkey is of foreign origin for comparison. Samples were collected in mid-February, transported in chilled condition and then transferred into laboratory at -46°C at Biotechnology lab, ICAR-NRCE at Hisar, Haryana, India till the analysis.

**Determination of total antioxidant capacity (TAC)**

Total antioxidant capacity of donkey milk was determined by micro plate assay technique according to the manufacturer’s instructions (Total Antioxidant assay kit, Sigma). The basic principle is based on the formation of ferryl myoglobin radicals from metmyoglobin and hydrogen peroxide which oxidizes the 2,2′-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) to produce a radical cation ABTS$^+$ which is a soluble chromogen and green in colour. It can be determined spectrophotometrically at 405 nm. Briefly, the standard trolox solution was prepared for obtaining a standard curve. ABTS working solution was prepared by adding 25µl of 3% hydrogen peroxide in 10ml of ABTS substrate solution. In 96 well plates, 10µl of trolox standard and 20µl of myoglobin working solution were added. Then 10µl of different test samples were added. After that, 150µl of ABTS working solution was added to each well. The reaction plate was incubated for 5 minutes at room temperature. After 5 minutes, 100µl of stop solution was added to each well to stop the reaction. The absorbance of plate at 405nm using spectrophotometer was recorded. A standard curve was prepared using different concentrations of Trolox and the results were expressed as milli mole/litre (mM) of Trolox equivalents (TE) of sample.

**Ferric-reducing antioxidant power assay (FRAP assay)**

The FRAP assay was carried out according to the procedure of Benzie and Strain (1996) with slight modifications. Briefly, the FRAP reagent was prepared from acetate buffer (pH 3.6), 10 mmol TPTZ solution in 40 mmol HCl and 20 mmol iron (III) chloride solution in proportions of 10:1:1 (v/v), respectively. The FRAP reagent was prepared fresh daily and was warmed to 37°C in a water bath prior to use. Fifty microliters of sample were added to 1.5 ml of the FRAP reagent. The absorbance of the reaction mixtures were then recorded at 593 nm after 4 min. The standard curve was constructed using iron (II) sulfate solution (100–2000 µM) and the results were expressed as µmole/L (µM). All the measurements were taken in triplicate and the mean values were calculated.

**RESULTS AND DISCUSSION**

Donkey milk is a treasured product for nutraceutical properties in babies suffering from multiple allergies and cosmetic production; therefore, new donkey dairy farms are on the air in India now. Diminutive information is available for farmers on sustainable production of donkeys, farm management, milk production, including animal welfare, milk quality and processing. Targeted dissemination of information on appropriate animal management would assist donkey dairy farmers in overcoming these problems. Donkey is mainly used as pack animal. It is not recognized as dairy animal till now in India. Today, the number of donkey dairy farms has not only increased in European countries but also has increased in Turkey, USA and western world. However, in India, donkey milk usage is not very popular except few reports from southern part of the country. It is an important issue to explore the donkey milk for nutritional applications as well as others and to list donkey milk as eligible milk for consumption. In India, donkey was not studied much in details. To cater to the needs of future therapeutic applications of donkey milk, this research aimed to study the bioactivities of donkey milk of indigenous Halari donkeys by assessment of antioxidant activity in comparison to exotic donkeys. Donkey milk consists of vegetal oil which is used to confirm the human milk energy (Iacono et al., 1992). Donkey milk proteins are quite identical to the human milk proteins but less than cow’s milk (Salimei et al., 2004). In a study, it was seen that during lactation there was decrease in amount of proteins in human, (Salimei et al, 2004) donkey, mare (Mariani et al., 2001) and as well as in cow (Fox, 2003).

It was also observed that the total protein percentage decreases with respect to the stage of lactation. Non–protein nitrogen is also present in donkey milk which are less than human milk but greater than other animals’ milk (Uniacke-Lowe et al., 2010; Salimei, 2011). Casein and whey proteins are also present in donkey milk. The amount of whey protein remains constant during the lactation period, but amount of casein protein shows decreasing trend (Alabisio et al., 2005). Lysozyme is also present in donkey milk. It is antimicrobial in nature. Lysozyme content in donkey milk is higher as compared to the human, cow and other dairying animals (Vincenzetti et al. 2008) and (Stelwagen, 2003). There are two types of donkey milk lysozyme viz. LysA (14680) (Godovac-Zimmermann et al., 1988) and Lys B (14631) (Herrouin et al., 2000). The mineral and ash content of donkey milk is very close to human milk but lesser than cow’s milk. Donkey milk is highly rich in calcium and phosphorous content (Pohdori and Vincenzetti, 2007).

It is extremely important to determine the antioxidant capacity of milk and milk products, as oxidation can only occur in case of an imbalance between the presence of reactive oxidants and the antioxidant defense mechanism (Halliwell, 1996). Antioxidant potential of milk from different
animals including human was studied by different researchers around the globe. Saif Alyaqoubi et al., (2015) assessed the antioxidant capacity goat’s milk from three locations in Malaysia. It is a popular belief that donkey milk carries unique characteristics owing to its antioxidant properties, however no such report is available for Halari donkeys till date, to our knowledge it is the first report of antioxidant capacity evaluation of Halari donkey milk in India.

Donkey milk is famous for its cosmetic properties with a value towards anti-ageing effect it causes according to the much popular beliefs. The anti-ageing capacity is due to rich antioxidant values it holds. In order to determine the much-valued antioxidant properties of donkey milk, we investigated the comparative tests for Halari donkey milk and French Poitu donkey milk with two most popular tests viz. the TAC and FRAP assays. Total antioxidant capacity of donkey milk is determined by micro plate assay technique, by using Total Antioxidant Assay Kit, Sigma. This kit consists of 10x assay buffer, stop solution, myoglobin, trolox, ABTS, phosphate citrate buffer, hydrogen peroxide. The results (Fig 1) showed that total antioxidant capacity is quite high in whole milk of Indigenous Halari donkey as mM Trolox equivalent than the French Poitu donkey. However, it is equivalent to whole milk of French Poitu donkey on an average. However, in FRAP assay results, the Poitu Donkey milk showed higher value of antioxidant activity in terms of FRAP of 101.95 µmole/L compared to 74.62 µmole/L in indigenous Halari donkey milk (Fig 2).

It is important to mention here that, this is a preliminary study and the samples were frozen for longer period and transported from Bikaner to Hisar. There is likelihood of lower values reported in the present experiment than can be possible in fresh milk samples. Therefore, further analysis will be carried out with more number of samples, recorded lactation stages and fresh milk as well as different fractions of milk along with lyophilized milk powder; that may result into better understanding of donkey milk and its unique attributes. It will be further interesting to compare the donkey milk with other more popular milk, such as goat, camel and bovine milks to gain a proper insight into the composition and bioactivities of donkey milk and its properties as reported worldwide.

ACKNOWLEDGEMENT
Authors thank the Director, ICAR-National Research Centre on Equines, Hisar, India for providing necessary facilities to carry out the research work. We extend our gratitude to Indian Council of Agricultural Research (ICAR) for providing the financial support to conduct the research work.

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