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An *in-vitro* study of evaluation of anti-oxidant property of *Annona squamosa* Leaf extract on NaF induced oxidative stress in sheep RBC

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Abstract
Oxidative stress is one of the foremost cellular damages occurring in many of the disease conditions. Among the different organelles, the erythrocytes are more prone to free radical damage. *Annona squamosa* contain high antioxidant activity as it is a rich source of carotenoids, polyphenolic acids and flavonoids. In the current study, the antioxidant action of *Annona squamosa* in sodium fluoride-induced oxidative stress in sheep RBC was studied. RBCs are more susceptible to oxidative stress because their membrane contains more polyunsaturated fatty acids. Sodium fluoride (NaF) was used to induce oxidative stress in red blood cells by incubating them for 24 hours, ascorbic acid and *annona squamosa* were used to protect them from oxidative stress. The altered amounts of SOD, GSH and MDA in RBC were used to assess oxidative stress. Treatment with ascorbic acid and *Annona squamosa* extract reverted the proportions of SOD, GSH and MDA to normal. Finally, ascorbic acid and Annona squamosa can alleviate the oxidative stress caused by sodium fluoride (NaF) in erythrocytes.

Keywords: Anti-oxidant, *Annona squamosa* Leaf, NaF

Introduction
Under normal circumstances, numerous antioxidative defence components can scavenge oxygen species that are reactive (ROS), a consequence of the metabolic progression. Oxidative stress is characterised as an imbalance among ROS and antioxidants [1]. According to Sies, oxidative damage is a major imbalance among oxidation and antioxidants, "a disruption in the prooxidant-antioxidant equilibrium in favour of the former, potentially triggering the damage" [2]. Fluoride is known to impair antioxidant enzymes, which promotes the formation of ROS [3, 4]. Fluoride occurs naturally in a variety of forms and its composites are frequently used. Fluorine is not ubiquitous in nature. Fluorine in drinking water is totally ionic, therefore it immediately, thoroughly and discretely crosses the intestinal mucosa, interfering with the key energy processes of the living system. Fluoride has a great preventive effect by reducing tooth cavities in modest doses, but in larger levels it produces dental and skeletal fluorosis [5]. According to some research, fluoride causes an increase in the generation of oxygen species that are reactive and may reduce the biotic activity of antioxidant enzymes as an example glutathione peroxidase (GSH-PX), superoxide dismutase (SOD) and catalase (CAT) [6]. Yet, the exact mechanism by which the whole-body impacts are created remains unknown.
NaF has been shown to modify ROS generation levels and antioxidative characteristics in the mouse kidney [7]. Fluoride revelation has been shown to cause oxidative damage in the liver, kidney, testicle, spleen, brain, heart and cecal tonsil, as well as diminish the expression of SOD, CAT, GSH-PX and GST in broiler, fish, rabbit, and rat liver [8-11]. Subsequently administration of fluoride for 70 days, [12] discovered that it can generate oxidative damage in the liver of female mice. Although there have been publications on the association among fluoride and oxidative damage, systematic limited investigated on the mechanism at the molecular level of NaF induced hepatic oxidative damage in mice have been conducted. Increased oxygen species that are reactive and malondialdehyde activity were associated with NaF-induced oxidative stress, as reflected in reduced mRNA expression levels and activities of SOD, CAT, GSH-PX and GST [7].

The presence of anti-oxidants in natural products has sparked intense attention in the pharmaceutical industry due to their exceptional significance in mitigating the damaging effects of ROS. *Annona squamosa*, also identified as sithapal, custard apple,sugar apple and sweet apres, belongs to the Annonaceae family, which has roughly genera-135 and species-2300 [13, 14]. Leaf poultice is used to treat boils and ulcers, while leaf infusion has been shown to be effective in reducing children prolapse. A cataplasm (plaster) produced from crushed leaves and salt is used to extricate guinea worms [15]. Leaves are used in medication of cuba to reduce uric acid concentrations. Diarrhoea and dysentery were treated with leaves, bark and unripe fruit [16]. Wide-ranging phytochemical analysis on diverse elements of the A. squamosa plant have revealed the existence of a wide range of phytochemicals and components, including essential oils, diterpenes (DITs), cyclopeptides (CPs), alkaloids (ALKs) and annonaceous acetogenins (ACGs). The ethanolic extract of *A. squamosa* demonstrated significant anti-oxidative activity, as well as moderate superoxide radical scavenging activity besides antilipid peroxidation ability [17]. *A. squamosa* extracts from various sections have been found to exhibit strong antioxidant activity [18, 19].

Materials and Methods

Leaf extract preparation

The leaves of *Annona squamosa* were freshly collected, shade dried, pounded to powder and stowed in an air tight flask. 100 ml of acetone was used to dissolve 10 grams of powder, which was then left on the Soxhlet equipment for 48 hours. For the filtering, Whatmann Filter Paper No. 1 was employed and it was maintained for evaporation. The extract was made by dissolving in 100 mg/ml of DMSO. (Fig-1)

Preparation of 10% RBC suspension

Blood (2 ml) is drawn from the sheep's jugular vein and placed in an EDTA vial. Centrifuged at 5 °C for 12 minutes at 1100 rpm. Plasma is washed in PBS for three to four times until clear supernatant is formed. 1800 µl of cold PBS were added to 200 µl of RBC pellet to make a 10% RBC solution and stored for future use at -20 °C. (Fig-1)

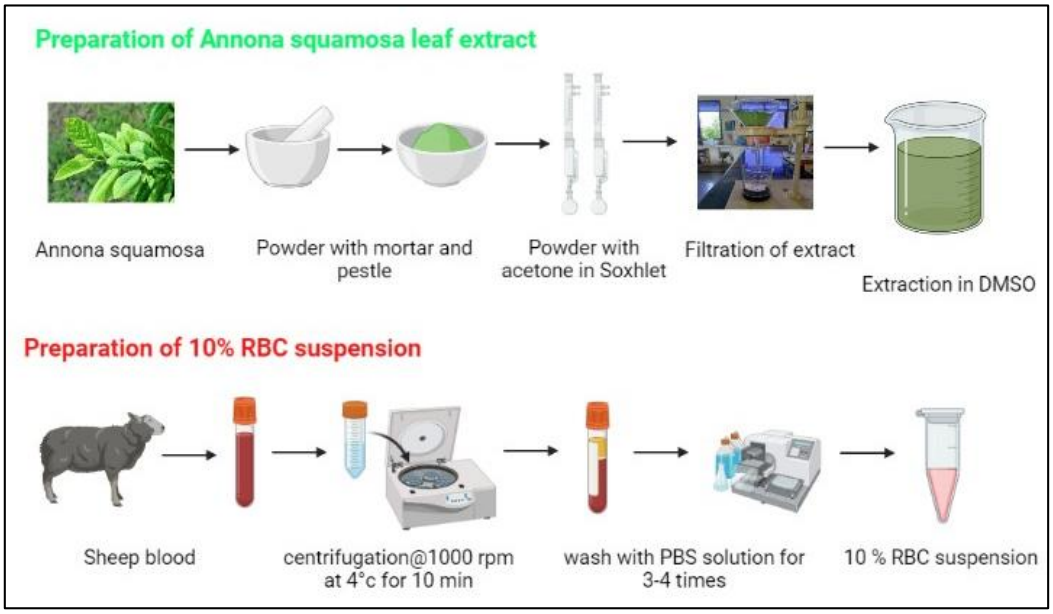

Fig 1: Pictorial representation of leaf extract and RBC suspension preparation.

Phytochemicals analysis

Phytochemicals analysis of leaf extract such as flavonoids, saponnins, glycoside and alkaloids are estimated by Lead acetate test, Froth test, Legal's test and Dragendroff's test.

Induction of oxidative stress

The 10% RBC suspension was alienated into separate sets and subjected with different concentrations of Sodium Fluoride, Ascorbic acid, *Annona squamosa* leaf extract and incubated on orbital shaker for 24 hours as per experimental design.

Antioxidant enzymes: TBARS [20], Protein concertation [21], GSH [22] and SOD [22] properties were measured.

Table 1: Experimental Design

Group	Treatment
I	RBC control (no treatment)
II	RBC + NaF (50 µM)
III	RBC + NaF (50 µM) +ascorbic acid (100 µg)
IV	RBC + NaF (50 µM) + *Annona squamosa* (100 µg)

Statistical analysis: The results of the experiments were provided as mean ± SE values. For the analysis of statistics, Software version 5.0 of Graph Pad Prism was utilised, which included a analysis of variance in one way following Tukey's multiple comparison test. At $p<0.05$, the findings were deemed significant.

Results

The leaf extract showed a positive for Alkaloids, Flavanoids, Saponnins and Glycoside. The total protein was reduced in toxic group II to 6.91±0.68 from 8.81±0.35 as in the control group. The tested group III and IV showed significant reduction in total protein with 6.35±0.62 and 6.99±0.81. The activity of SOD (U/mg protein) and the activity of GSH (µg/mg protein) in Group 2: NaF-treated were substantially reduced ($p<0.01$) than in the RBC group 1. The GSH levels in NaF treated RBC was 90.86±68.71 µg GSH/mg of protein in 50µM and it was increased in the *Annona squamosa* treated RBC with 128.0±10.57 µg GSH/mg of protein. However non-significant levels of GSH also found similar with group III

ascorbic acid 101.3±7.11 µg GSH/mg of protein. The levels of SOD in control group and toxic group were 10.29±1.1 SOD units/mg protein and 6.5±0.9 SOD units /mg protein correspondingly. In variance the SOD levels in treatment group *Annona squamosa* were significantly increased to 16.62±1.5 SOD units /mg protein. The SOD levels were normalized by the plant extract with significant protection was noticed. But there was no appreciable distinction between groups IV and III in terms of values. The activity of TBARS (nM/mg protein) in NaF-treated group 2 (601.7±4.08) exposed a substantial (p<0.001) surge in the RBC group 1(415.5±3.42), whereas *Annona squamosa* treatment group IV and ascorbic acid treatment group III displayed a substantial decrease in TBARS (p<0.001 and p<0.01, respectively) with a value of 481.4±3.03, 518.0±4.34. (Graphical representation are presented in Fig 2).

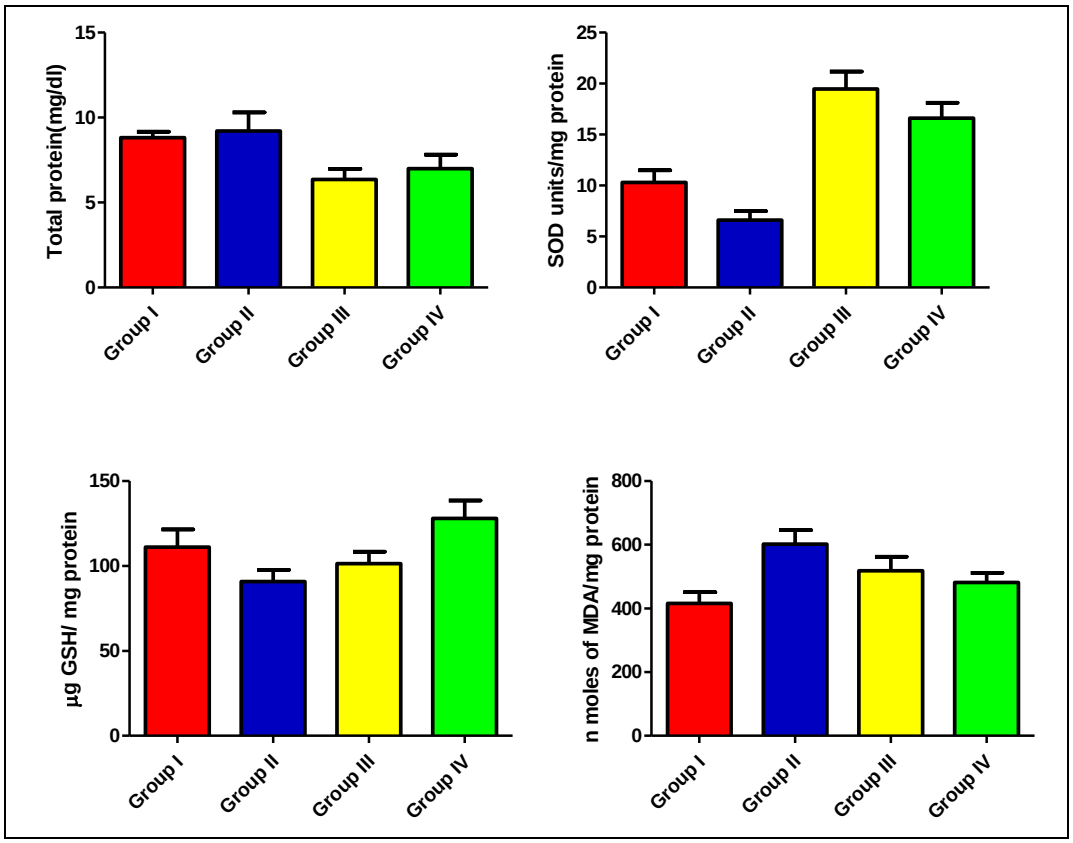

Fig 2: Graphical representation of Total protein, SOD, GSH and MDA.

Discussion

A significant imbalance between the creation of ROS and the mechanism that defends against them is referred to as oxidative stress [23, 24] which principals to source "a disturbance in the pro-oxidant-antioxidant balance in favour of the former, leading to potential damage". Finally, the lipids are degraded to generate MDA [25], indicating an increase in lipid peroxidation in addition to a drop in levels of intracellular glutathione and a consequent drop-in activity of SOD throughout the stress [26]. An augmented level of MDA can consequently aid as a mark of oxidative stress. The study observed the triggering of MDA values significantly in the NaF group and subsequently reduction in ascorbic acid group and *Annona squamosa* group. Because of its various phyto constituents, the squamosa leaves extract has a very active oxidative metabolism, which produce ROS. It is also abundant in antioxidant enzymes like GSH, which may scavenge free radicals and is crucial for preserving cellular integrity. GSH is a crucial part of the cellular antioxidant defence mechanism. It works by contributing one hydrogen atom immediately and eliminating the free radicals. SOD guards against ROS by dismutation of superoxide anion free radicals via catalysis into molecular oxygen (O_2) and hydrogen peroxide (H_2O_2) [27]. Following in the NaF group, GSH levels dropped and the *Annona squamosa* treatment effectively mitigated the levels in GSH. Under physiological circumstances, an anti-oxidant defense system comprising enzymatic SOD and non-enzymatic GSH processes restores oxidative stress-induced redox imbalance. The findings of the current research established a substantial surge in levels of malondialdehyde associated with substantial reduction of GSH and anti-oxidant enzyme SOD in group NaF, which was then dramatically reversed in the group of *Annona squamosa*. The current study's verdicts are reliable with those of Zaghloul [28].

Conclusion: The anti-oxidant potential of Annona squamosa (100µg) has demonstrated significant preventative effects against oxidative damage caused by sodium fluoride.

Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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