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In vitro qualitative analysis of natural-edible antioxidant remedies in reducing the oxidative stress mediated blood coagulation and preventing thrombosis in sickle cell disease

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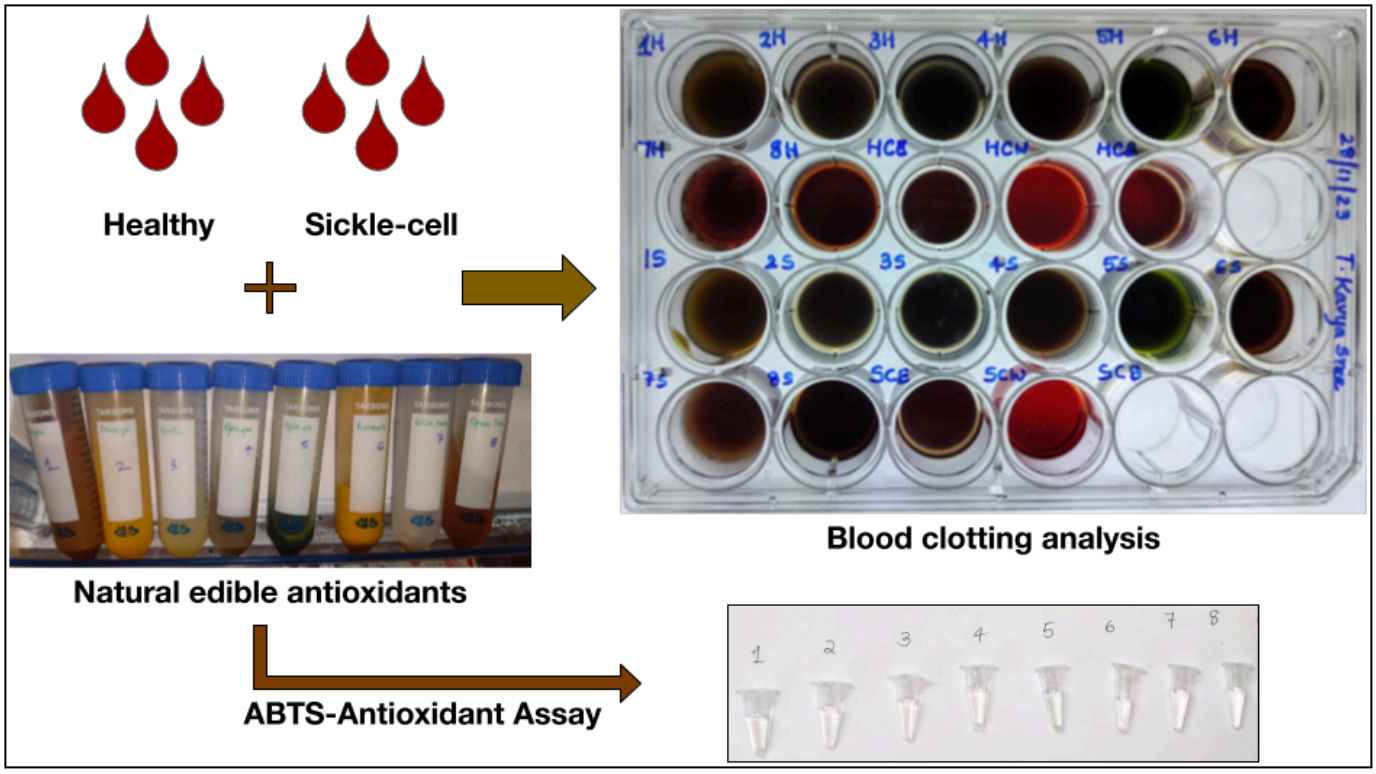
Oxidative stress caused by the deformed red blood cells often cause blood coagulations leading to thrombosis in patients with sickle cell disease (SCD). Although thrombosis is a temporary obstruction in the blood vessels, it may take up to one year to clear in some patients. Inflammation and pain are also associated with thrombosis. Typically patients are given blood thinners to clear thrombosis. However, in the case of SCD, the high frequency of thrombosis poses a threat for the blood thinner prescriptions. In this study, we screened a list of natural foods for their antioxidant potential in order to test their capability to reduce the blood coagulation in SCD patients. The natural extracts were added to the blood samples of healthy and SCD volunteers and incubated. We identified that apples were able to reduce the blood clotting in the SCD blood sample and were also found to contain high antioxidants. Our *in vitro* results suggest that the natural edibles such as apple can be regularly consumed by SCD patients to reduce their frequency of thrombosis.



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Sickle cell disease (SCD) is an inherited blood disorder caused by abnormal hemoglobin [1]. Sickle cell disease limits the oxygenating role of hemoglobin, resulting in the damaging or the 'sickling' of the red blood cells [2]. This disorder affects all parts of the human body and differs widely among individuals [3]. For many generations sickle cell disease has been a prevalent disorder in Africa. Reports show that the west African natives had several local names for this disease before it was discovered in America [4]. Studies indicate that approximately 1 in 12 African-Americans are heterozygous for the disorder, and approximately 1 in 500 Africans- Americans newborns are diagnosed annually with SCD [5]. Also, the life expectancy for SCD has doubled since the 1960s. Before that time, few patients lived to reach adulthood [6].

In sickle cell disease, the hemoglobin is abnormal, causing the red blood cells to be rigid and shaped like a 'c' or sickle, the shape from which the disease takes its name. Sickle cells can get stuck and block blood flow, causing pain and infections. Complications of sickle cell disease occur because the sickled cells block blood flow to specific organs. The worst complications include stroke, acute chest syndrome (a condition that lowers the level of oxygen in the blood), organ damage, other disabilities, and in some cases premature death [6]. The FDA approved the first gene therapies for sickle cell anemia. The two approved treatments, Casgevy and Lyfgenia will cost $2.2 million and $3.1 million [7]. Stem cell transplant is recommended only for people, usually children, who have significant symptoms and complications of sickle cell anemia [8].

While there is currently no definitive cure, ongoing research has yielded several treatment options to manage the condition like Hydroxyurea is an oral medicine that has been shown to prevent several complications of sickle cell disease and it should not take while the patient is pregnant [9]. Sickle cell anemia is a genetic blood disorder, resulting from a mutation in the hemoglobin gene, leading S. This causes red blood cells to assume a rigid, crescent shape, hindering their flexibility and leading to various complications, such as pain, anemia, and organ damage [10]. Because excess iron can damage your heart, liver and other organs, you might need treatment to reduce iron levels if you undergo regular transfusions [11]. In the current study we are aiming at understanding the effectiveness of antioxidants in managing blood clotting. For the study, antioxidant solutions obtained from the natural sources were used and tested with healthy person blood samples and sickle cell person blood samples. Blood samples were obtained by a skilled paramedic following the standard procedures of the clinic under the supervision of a physician as a part of research collaboration in this study. The SCD patient was informed and consent was obtained by the physician as a part of the records for the clinic. Patient details were kept confidential.

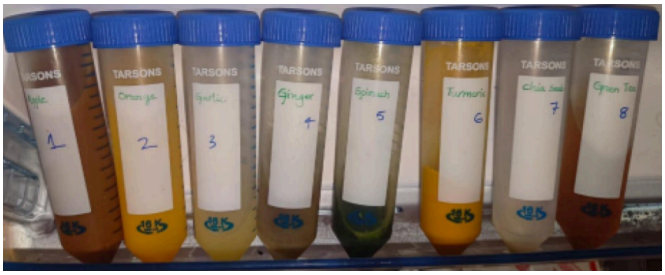

Figure 1. Solutions prepared from natural and edible sources of antioxidants.

Materials and Methods:

Preparation of antioxidant solutions: A total of 8 solutions were prepared using the apples, oranges, garlic cloves, ginger paste, spinach leaves, turmeric paste, chia seeds & green tea. As shown in Figure 1, all solutions were aqueous and no organic solvents were used in this study. EDTA solution (50 mM) and deionized water were used as controls. Apple and orange solutions were prepared by using ground pulp of the fruit mixed with deionized water and filtered. Garlic, ginger and spinach solutions were prepared by grinding them in the blender with some deionized water followed by filtration. Turmeric and green tea solutions were prepared by directly dissolving the powder in deionized water followed by filtration. Chia seeds were presoaked and were then ground and mixed with deionized water followed by filtration. Apparently the density of all 8 solutions were similar.

Antioxidant assay using ABTS: The antioxidant capacity of individual filtered antioxidant solutions was assessed using the widely employed ABTS (2,2′-azino-bis-(3-ethylbenzothi azoline-6-sulfonic) acid) assay [12-14]. ABTS is a common compound utilized for determining the total antioxidant capacity (TAC) in various substances, including plant extracts, food, and clinical fluids. To initiate the evaluation, hydrogen peroxide (H_2O_2) was added to the ABTS solution, which results in a color change from a colorless solution to a greenish blue. One µl of ABTS was taken per reaction in the presence of 150 µl of either antioxidant solution (Figure 1) or deionized water (control). Pictures were taken immediately after adding ABTS and 10 min post incubation at room temperature.

Anticoagulation assay: The 10 antioxidant solutions were added to a 24-well plate in duplicates such that in each set of solutions, 500 µl of blood from healthy volunteer and SCD patient can be added in order to test the coagulation within the same plate. The plate was incubated at room temperature for an hour. Pictures were taken before and after the incubation for comparison. EDTA solution (50 mM) and deionized water were used as controls using same volumes.

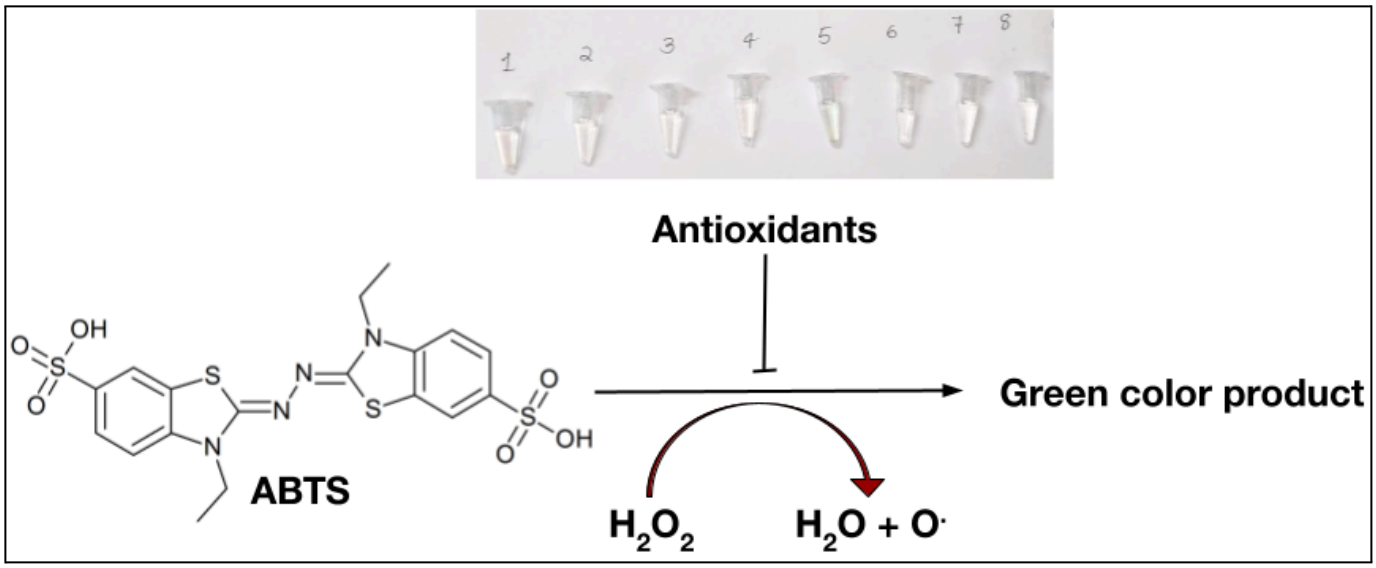

Figure 2. Antioxidant assay for 8 solutions.

In one well for healthy volunteer, only blood was added without adding any other ingredients mentioned above in order to calculate the time taken for the healthy volunteer's blood to naturally coagulate in the absence of any external factors such as the antioxidants, etc.

Results and Discussion:

All ten sample solutions displayed antioxidant properties: The ABTS-antioxidant assay revealed that all 10 samples tested in this study qualitatively yielded equal antioxidant activity. As shown in Figure 2, the ABTS in the presence of hydrogen peroxide can result in a green color product due to the partial oxidation by the unstable hydrogen peroxide which releases the free radical (also known as reactive oxygen species, ROS). Typically, the horseradish peroxidase (HRP) enzyme catalyzes this reaction to yield a deep green color. However, in this study, HRP was not used because the hydrogen peroxide is usually unstable i.e., produces some ROS that can participate in the partial oxidation of the ABTS. All 10 samples tested in this study are potent antioxidants as evidently seen in Figure 2 where all the tubes containing ABTS and hydrogen peroxide are apparently colorless.

One might argue that the addition of 1µl of ABTS to 150 µl sample may mask any color change compared to the control. In order to delineate this dilemma, we used equal volumes of the sample and the ABTS solutions in a 1:1 ratio of 150 µl each. Interestingly, yellow color was seen for samples 1 and 8, light yellow color for sample 6 and pale purple color for sample 4 that were persistent over a 24 hour incubation period at room temperature. One exception was the spinach sample which has the dominating green color originating from the sample even before adding the ABTS solution. It has been previously reported that this type of color change is normal [12, 13]. Hence we ruled out the possibility of color masking and concluded that all the samples used in this study contain antioxidant activities. These samples were further used to evaluate their anticoagulation activity against the healthy and SCD blood samples in a 24-well format.

Anticoagulation activity of the apple was higher than the other samples: Anticoagulation activity of all 10 samples was performed with both the healthy volunteer's blood and the SCD patient blood. As shown in Figure 3, the picture of the 24-well plate that was taken immediately after adding the blood shows reflected light rays to visualize the samples clearly. However, the picture of the same plate taken after a 24 hour incubation at room temperature shows the light rays passing through the samples from the bottom of the plate in order to clearly visualize any clotting. The normal clotting time for the healthy volunteer's blood without any additives was a few minutes. Unfortunately, clots were not that clearly visible in a few minutes in the 10 samples tested in this study. Hence a 24 hour incubation was chosen in order to rule out any possibility of time-dependent clotting in the presence of the antioxidants. The 24-well plate was manually observed to check for clotting after 24 hours incubation. As shown in Figure 4, the apple sample showed lower amount of clotting in both healthy volunteer's blood and the SCD patient blood suggesting that the antioxidant properties of apple sample probably controlled the ROS and in turn avoided any coagulation in the wells. Other than the apple sample, all the others displayed almost 70% to 90% clotting after the 24 hours incubation at room temperature.

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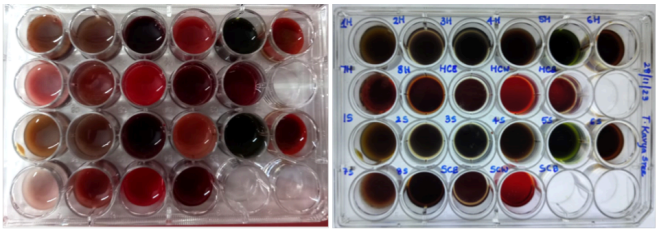

Figure 3. Anticoagulation activity evaluation of samples.

It is common knowledge that apples contain antioxidants such as quercetin, catechin, phloridzin and chlorogenic acid [15]. Additionally, they are a rich source of vitamins C, E and B6 [16]. A wealth of antioxidants present in apples explain the reason for significant decrease (50%) in the coagulation of blood from the SCD patient. Interestingly, the decrease in coagulation by apples in healthy volunteer's blood was only 20%. Followed by the apple sample, the spinach and chia seeds samples also display some decrease in the coagulation but not as significant as the apple sample. Moreover, in the Indian scenario spinach is usually consumed after cooking while apples can be consumed raw. The results suggest that people with SCD may have health benefits by consuming apples on a daily basis potentially to avoid thrombosis.

Conclusion and Future directions:

In the current study we confirmed that the apples, orange, garlic, ginger, spinach, turmeric, chia seeds and green tea samples contain antioxidants based on the ABTS assay. Further, the apple sample displayed highest anticoagulation activity against both the healthy volunteer's blood and SCD patient blood tested within the same assay. Based on these results we conclude that adding apples to the diet of the SCD patients may have significant health benefits. The observed reduction in clotting tendencies suggests that consumption of these foods may contribute to improved blood flow and circulation in individuals with SCD, thereby reducing the risk of vaso-occlusive crises and associated complications. Additionally, the high antioxidant content of these foods indicates their potential to mitigate oxidative stress and inflammation, which are key contributors to SCD pathophysiology. Further research is warranted to elucidate the specific mechanisms underlying these effects and to explore optimal dietary strategies for managing SCD complications. By incorporating these nutritious foods into their diet, individuals with SCD may enhance their overall well-being and quality of life.

However, it is important to acknowledge the limitations of this study, including the small sample size and the need for further research to elucidate specific mechanisms underlying the observed differential anticoagulation effects in this study.

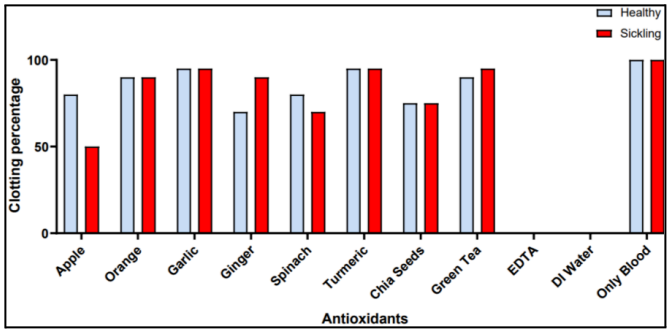

Figure 4. Qualitative analysis of anticoagulation activity of natural and edible remedies.

Future studies should explore the optimal dietary strategies for individuals with SCD, considering factors such as individual variability, dietary preferences, and cultural considerations. In future we plan to repeat this study using a large cohort of the blood samples. Additionally a full LC-MS profiling will also be added to the study in order to test whether any differences between the phytochemical profiles of native Indian vs. imported apples may affect the blood coagulation differently.

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Conflict of interest: The authors declare no conflict of interest in this study.

Author contributions: K.T. prepared all the antioxidant solutions; M.V. performed antioxidant assay; K.T. and M.V. performed anticoagulation assay; M.V. supervised K.T. and helped in data analysis; R.S.Y. is the principal investigator who designed the project, trained K.T. and M.V., secured required material for the project, provided the laboratory space and facilities needed. R.S.Y. edited and finalized the manuscript.

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Full figure legends:

Figure 1. Solutions prepared from natural and edible sources of antioxidants. The samples from left to right are apples, orange, garlic, ginger, spinach, turmeric, chia seeds and green tea. All the final solutions were filtered before use for the antioxidant and anticoagulation assays in this study.

Figure 2. Antioxidant assay for 8 solutions. All the 8 samples display colorless reactions in the tubes indicating that they quenched the ROS generated from the unstable hydrogen peroxide, suggesting their antioxidant potential.

Figure 3. Anticoagulation activity evaluation of samples. The image on the left was taken immediately after adding blood to the antioxidant samples and the image on the right was taken after 24 hours incubation at room temperature. The top 2 rows from left to right contain apple, orange, garlic, ginger, spinach, turmeric, chia seeds, green tea, EDTA, and deionized water with healthy volunteer's blood while the bottom 2 rows contain the same samples with SCD patient blood. The right most well in the second row contains healthy volunteer's blood only without any additives.

Figure 4. Qualitative analysis of anticoagulation activity of natural and edible remedies. As expected no coagulation was seen for the EDTA and deionized water samples but varying amounts of coagulation was seen for all the other 8 samples. The normal blood coagulation was taken as 100% to compare the varying amounts of coagulation in the 8 samples tested in this study. Apple displays a significant decrease in the coagulation followed by spinach and chia seeds.