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Can guava (*Psidium guajava*) leaf extracts develop an indigenous, simplified tool for a semi-quantitative assessment of iron in groundwater?

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ABSTRACT

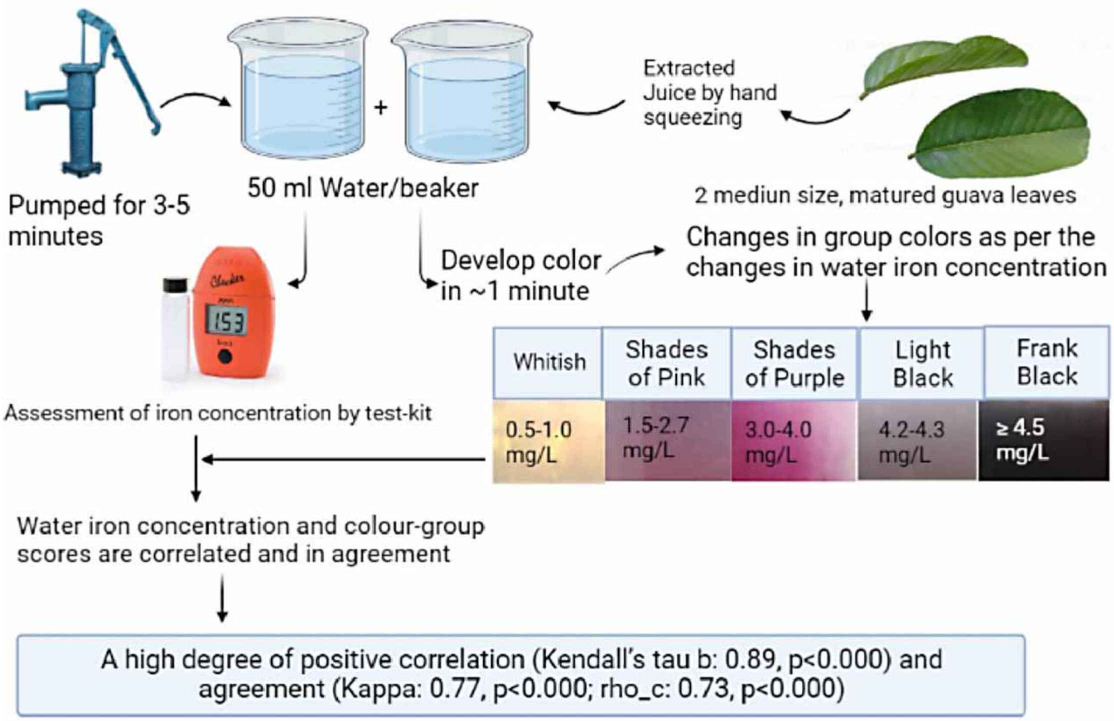
Iron present in the drinking groundwater is attributed to the low burden of iron deficiency (ID) in Bangladesh. The supplemental anemia prevention strategies involving iron need a cautious approach due to the excess load of iron and the side effects. The present pilot study examined the potential of the guava leaf extracts to use as a natural reagent for the assessment of iron in groundwater. Eighteen households with the drinking source of groundwater were randomly selected. Guava leaves were crushed and the shreds of the leaves were mixed with the water sample. Changes of water color were photographed. Five groups were identified – 'whitish', 'shades of pink', 'shades of purple', 'light black' and the 'frank black'. The iron concentration was measured by a test kit device. Each color group was assigned a number on the ordinal scale 1–5. Statistical correlation and agreements were performed between the methods. The positive correlation (Kendall's tau *b*: 0.89, $p < 0.000$) and the agreements (Kappa: 0.77, $p < 0.000$; rho_c: 0.73, $p < 0.000$) were observed. Guava leaf extracts may standardize an indigenous tool for a semi-quantitative measurement of groundwater iron content. Validation of the tool thus may aid in the design and evaluation of the iron supplementation and fortification programs.

Key words: anemia, guava leaf, groundwater iron, indigenous tool, iron supplementation

HIGHLIGHTS

- In a largely iron-replete population, iron supplementation programs need a cautious approach to limit side effects.
- Guava leaf extract by changing the color of the iron-rich groundwater concurs with the colorimetric iron testing.
- Guava leaf extract may standardize an indigenous tool for the measurement of groundwater iron content.
- Validation of the tool may aid the design of the anemia programs, promoting public health.

GRAPHICAL ABSTRACT



BACKGROUND AND RATIONALE

Iron in groundwater is present in high amounts in many parts of Bangladesh (British Geological Survey 2001), North-East Indian states (Sharma *et al.* 2021) and South and South East Asian countries (Karakochuk *et al.* 2015). In the 1970s, in the face of the widespread distribution of water-borne infections, the Government of Bangladesh instated groundwater for potable supplies instead of the surface water. Furthermore, to avoid the worm infestation, the groundwater (i.e. tube well water), the source of drinking water kept a low burden of the *Ascaris Lumbricoides* (Studies on the Etiology of Anaemia in Bangladesh: Effects of Helminths & Safe Water, Institute of Nutrition & Food Science University of Dacca 1982). Currently, groundwater is the ubiquitous source of drinking water in Bangladesh as 97% of the rural population relies on it for drinking (British Geological Survey 2001). Iron in the groundwater is associated with some operational issues, e.g. discoloration of foods while cooking and discoloration of cloths and teeth. However, recent studies have observed that the intake of iron from the drinking groundwater is positively associated with iron and hemoglobin status in Bangladeshi populations (Merrill *et al.* 2011; Rahman *et al.* 2016; Ahmed *et al.* 2018; Choudhury *et al.* 2021). On account of this, despite the burden of anemia being high in the Bangladeshi population, the prevalence of ID is low (National Micronutrient Survey 2011–2012). Physiologically, in an iron-replete state in the population, usage of iron supplement, such as micronutrient powder (MNP), might induce side effects, e.g. diarrhea, nausea and vomiting (Saito. 2014). Against this background, the anemia prevention programs in Bangladesh which use supplemental iron often suffer from suboptimum compliance and coverage (Sarma *et al.* 2020); and side effects are reported as the key reasons for the discontinuation and poor coverage (Mistry *et al.* 2015). Contribution of groundwater iron in relation to anemia prevention has been recognized in the national anemia control policy (National Anemia Consultation 2016). Estimation of the iron content in groundwater is recommended in the context of anemia control and micronutrient intervention for possible adjustment of the programs (Merrill *et al.* 2011; National Anemia Consultation 2016; Rahman *et al.* 2016).

Iron concentration in water is commonly estimated by using ferrozine-based assay, where acidified ferrozine (3-(2-pyridyl)-5,6-bis(4-phenylsulfonic acid)-1,2,4-triazine) or other reagents such as phenanthroline based reagents are added to water to develop color complex, which is estimated using a colorimeter (Jeitner 2014). However, the digital colorimeter for iron estimation is expensive and requires skilled operators. The cheaper and commonly used device, the semi-quantitative colorimetric test kit device employs a ferrozine reagent provided by the manufacturer in a small sachet (one-for-one test) which oxidizes the ferrous iron present in groundwater and indicates the level of iron by changing the color of the water samples into different shades of red. The color generated needs to match the given standardized color reference to indicate the iron concentration in the water sample. However, all the equipment for iron estimation in groundwater is imported. These are expensive and have a limited availability of the devices and/or reagents.

On an important note, the reagents used in iron assay might be detrimental to the environment and require a complicated procedure to dispose of spent reagents, so scientists are developing plant-based non-toxic reagents to estimate minerals in solutions (Armenta *et al.* 2008). Reagents prepared from guava leaf (Settheeworrarit *et al.* 2005; Grudpan *et al.* 2010) and green tea (Pinyou *et al.* 2010) have been developed to assay iron in water, and the system has been standardized for application in the pharmaceutical industry (Siriangkhawut *et al.* 2019). Nonetheless, these procedures require complex flow injection analytical procedures and have not been standardized for use in the field conditions.

Anecdotal experience in the rural Bangladesh settings suggests that after consuming guava, if the iron-rich groundwater is taken, the tongue turns into black discoloration. In some parts of India, there is a local wisdom of in-home assessment of iron in groundwater by getting the guava leaf extracts and mixing it with iron-rich water (HI-Aware ICIMOD UKAID/ICRD/CRDI 2017). Guava and/or guava leaf contain tannin, an organic component. Tannin is present in many types of plant material. Brester (1948) reported that the natural tannin is predominantly made up of the chemical compound gallotannin. It is a complex molecule with the structure of one glucose molecule bonded to five molecules of digallic acid. In water solution, the tannin molecule hydrolyzes to yield glucose and digallic acid. The anion resulting from the dissociation of tannic acid is $C_6H_2(OH)_3COOC_6H_2(OH)_2\ COO^{-1}$. This anion can combine with ferrous iron to form a ferrous-tannite compound (Hem 1960).

Further, tannic acid or other constituents present in guava leaf may combine with ferric iron to yield large high-colored molecules that behave as colloids. The black or blue-black or the lighter-colored compounds formed in solutions that contain ferric iron and tannic acid is a type of colloidal association between the tannic acid and ferric hydroxide molecules. At the pH values from about 4 to 5, the black compound is formed when the ferrous iron is oxidized rapidly to ferric hydroxide; however, the tannic acid mostly remains undissociated. At pH values higher than 5, the tannic acid is more extensively dissociated and the ferrous iron combines with the tannin anions. The ferrous-tannate compounds are oxidized and are slowly eliminated from the solution. As the ferrous-tannate compound oxidizes, ferric irons are released and the various shades of pinkish to black ferric-tannates are precipitated (Hem 1960).

At and above a certain level of pH (i.e. pH 5), a higher amount of ferrous iron in the water sample is associated with the higher formation and the precipitation of the ferric-tannate compound; and the higher is the intensity towards black discoloration of the water sample. Therefore, we conducted a small pilot study to observe the relation of the guava leaf-induced changes in the color of groundwater and a semi-quantitative assessment of the concentration of iron in the water sample.

METHODOLOGY

Study site

The study was conducted in Belkuchi (24.2917°N, 89.7000°E) of Sirajganj district – a North-Western sub-district of Bangladesh located ~125 km from the capital city Dhaka (Figure 1).

PROCEDURE

Eighteen ($n = 18$) tube wells were randomly chosen on the basis of the verbal consent of the owners. Prior to consent, the owner of the tube well was briefed about the purpose of the study. The water samples of the tube wells were tested with the finger-crushed guava leaf extracts and the color development was noted and photographed. The same water samples were tested for the level of iron by the iron test kit devices (HI 3834 Hanna Instruments. Hanna Inc. USA) and the concentration of iron was recorded.

Testing water samples with guava leaves

The water of the selected tube well was pumped for 3–5 min in order to reach the deeper aquifer which is representative of the actual status of iron in groundwater (Merrill *et al.* 2009). Further, pumping is likely to remove the ferric precipitations which might have built up at the inner surface of the well. An enumerator collected the sample water in a 50 mL glass beaker. Before collection of the water sample, the beaker was rinsed with distilled (i.e. deionized) water to remove any residue of iron. Two

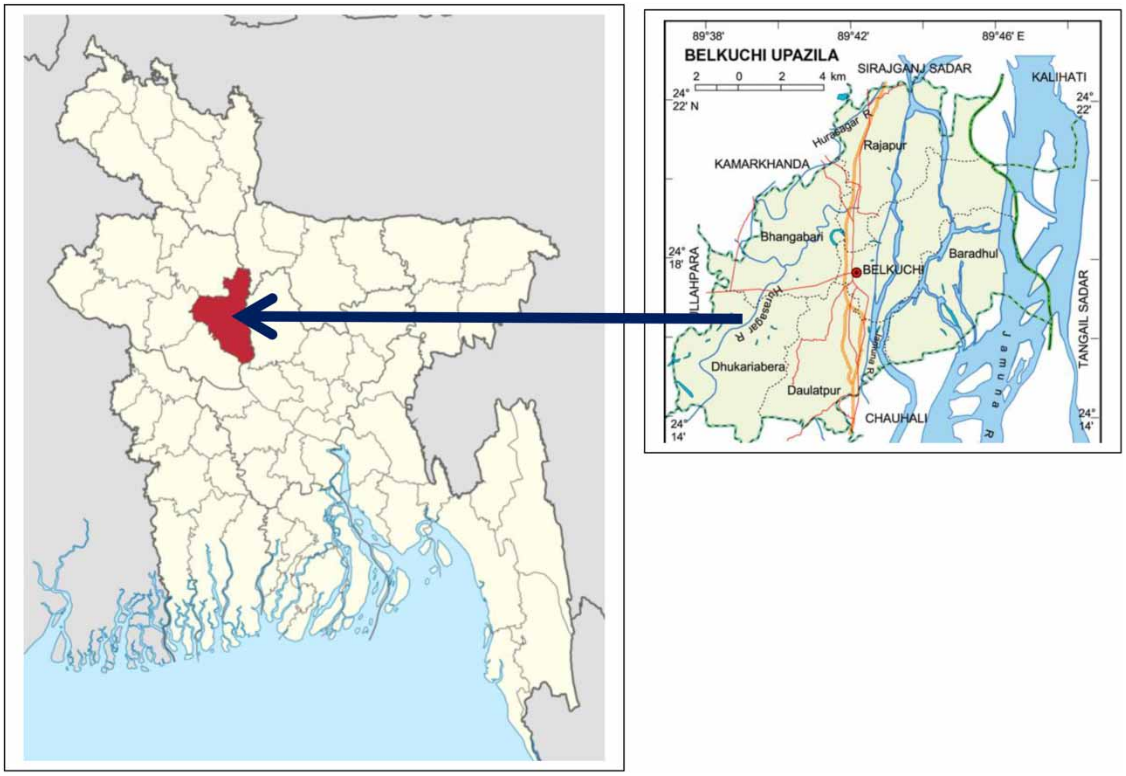


Figure 1 | Belkuchi, a north-western sub-district of Bangladesh (24.2917°N, 89.7000°E).

medium-sized, matured leaves of guava tree measuring approximately 3–4 inches in length were collected. The leaves were crushed and squeezed between the thumb and the fingers and the extracted juice along with the small shreds of the leaves were mixed with the water sample in the beaker. The leaf extracts were mixed thoroughly with the water sample with the fingers and the color started to change or remained largely colorless according to the concentration of iron in the sample. After a minute, the residue of the leaves was taken out by the fingers and discarded. A photograph of the water-filled beaker was taken.

Estimation of iron concentration of the water sample

Iron concentration of the tube-well water was estimated by the portable iron test kit devices (HI 3834 Hanna Instruments Hanna Inc. USA).

Principle

The test kit determines total iron levels in water via a colorimetric method. First, all ferric ions are reduced by sodium sulfite to ferrous ions. The reagent phenanthroline combines with ferrous ion to constitute an orange-colored solution. The intensity of the color of the solution determines the iron concentration.

At first, the tube well was pumped for 3 min. The test water sample was taken up to the 10 mL mark in the manufacturer-supplied test beaker. The supplied reagent (phenanthroline) was poured onto the sample water and thoroughly mixed. The reagent-mixed test water was poured into the test chamber of the test kit device. After 4 min, the resultant color of the test water was matched with the reference color supplied by the device. The matching indicated the concentration of iron in the test water sample. The test kit device provides the estimation of iron concentration up to the concentration of 5 mg/L. If the color of the test sample appeared to have exceeded the reference color equivalent to 5 mg/l, the test was repeated with a two-fold dilution of the test water sample. The final reading was adjusted by multiplying by two.

The pH of the water samples was estimated by the pH meter (HI 96107 Hanna Instruments Hanna Inc. USA).

Statistical analysis

The guava leaf extract mixed with groundwater samples was operationally classified into five subgroups depending on different concentrations of iron and the corresponding discernible identification of the color of the water samples. These are very low (iron <1 mg/L), low-to-moderate (iron 1.5–2.7 mg/L), medium (iron 3–4 mg/L), high (iron 4.2–4.3 mg/L) and higher (iron ≥4.5 mg/L). The corresponding color groups of the water samples were – 'Whitish', 'Shades of pinks', 'Shades of purples', 'Light black' and the 'Frank black', respectively (Figure 2). The individual water samples were distributed as per the color groups to the corresponding iron concentrations recorded (Table 1). The basis of these operational groupings is the study of Merrill *et al.* (2012).

A number of statistical methods were employed to examine the association and agreement between the guava leaf-induced changes in color groups of the groundwater samples vs. iron concentrations of the groundwater samples.

First, the Kendall's tau *b* correlation was calculated between the changes in the groundwater color groups and the corresponding actual iron concentration of the individual samples ($n = 18$) (Table 2).

Second, to assess the extent of the agreement by accounting for chance, we used the weighted kappa statistic (w) with prerecorded weights, which assessed the agreement of the measures estimated by the two methods while accounting for the possibility of the agreement occurring by chance (Dehghan *et al.* 2013) (Table 3).

Third, an absolute agreement, the Lin's concordance (Lin 1989, 2000) which constitutes both precision and accuracy of the changing group colors of the groundwater samples, i.e. the test method vs. the actual measured concentrations of iron, was performed (Figure 3).

RESULTS

Figure 2 depicts the changes in the color of the water samples with the treatment of guava leaf extracts (i.e. color groups) and the corresponding values of iron concentration as measured by the colorimetric test kit device.

The visual observation clearly shows that as the concentrations of iron in the water samples increased, the guava leaf-treated color of the water samples (i.e. color groups) changed from lighter to the darker shades. The color of the samples was consistently distinguished from the iron concentration of 0.5 mg/L to that of 4.3 mg/L. At the higher concentration levels of iron (from 4.5 to 9.0 mg/L), the demarcation of color was less marked. Depending on the distinction of the leaf extract treated water color, the following sub groups could be identified (Table 1).

Table 1 depicts the changes in the color groups of the guava leaf extract-treated groundwater sample and the corresponding concentration of iron in the water samples. As the color changes from light to the darker shades, there is a trend of higher concentration of iron in the water samples.

Table 2 clearly shows that there is a positive association between the guava leaf-treated color groups and the concentration of the groundwater iron. As color groups changed from the lighter to the darker shades, the water iron concentration increased significantly; Kendall's tau *b* coefficient 0.89; $p < 0.000$.

Table 3 shows the extent of the agreement of the iron content assessed by the guava leaf extract-treated color group method and the colorimetric measurement of iron concentration by accounting for a chance. The weighted kappa estimate was 0.77 ± 0.15, $p < 0.000$.

Figure 3 depicts the Lin's concordance coefficient (rho_c) of the absolute agreement between the test method of iron concentration by the color groups (guava leaf extract treated) and the standard/reference method of the colorimetric measured iron in groundwater.

Figure 3 depicts the Lin's concordance coefficient, the rho_c estimate was 0.727 ± 0.094 at the 95% CI 0.487–0.865 (Z-transform); $p < 0.000$.

DISCUSSION

The study examined an easy, inexpensive and field-friendly natural reagent method (test method) for the iron content assessment in groundwater samples against a reference method (i.e. commercial reagent). The guava leaf extracts method which imparts changes in color of the water sample to indicate the iron content was compared to the colorimetric test kit method by correlational association and a number of agreement tests.

The association between the methods was strong as evidenced by the magnitude of the Kendall's tau *b* coefficient which was large despite the small sample size. It suggests the strong effect of the changes of the color across the color groups on the

Figure 2 | Guava leaf-induced changes in color groups of the groundwater samples vs. actual iron concentrations of the groundwater samples. Interpretation of the color groups: Whitish: 0.5–1 mg/L; Shades of pink: 1.5–2.7 mg/L; Shades of purple: 3–4 mg/L; Light black: 4.2–4.3 mg/L; Frank black: ≥4.5 mg/L.

Table 1 | Provisional groups of color of the guava leaf extract treated groundwater samples and the corresponding semi-quantitative concentration of groundwater iron

	Color groups sorted by color type of the water samples	Sample identity[a]	$n = 18$	Concentration of groundwater iron (mg/L)	Groundwater iron classification (provisional)[b]
1	Whitish	Samples#1-2	2	0.5–1	Very low
2	Shades of pink	Samples#3-5	3	1.5–2.7	Low to moderate
3	Shades of purple	Samples#6-8	3	3–4	Medium
4	Light black	Samples#9-10	2	4.2–4.3	High & Higher
5	Frank black	Samples#11-18	8	≥4.5	

[a]Figure 2.
[b]BGS (2001), Merrill *et al.* (2012).

Table 2 | Kendall's tau association between the guava leaf-induced color groups of the groundwater samples and the corresponding actual groundwater iron concentration of the individual samples

n	Kendall's tau *b*	Kendall's score ± SE	*p*-value
18	0.89	117 ± 24.8	<0.000

Table 3 | Weighted kappa association between the guava leaf extract treated color groups and the corresponding actual groundwater iron concentration of the individual samples

Agreement (%)	Expected agreement (%)	Kappa	Standard error	*Z*	*p*-values
91.5	62.8	0.77	0.15	4.94	<0.000

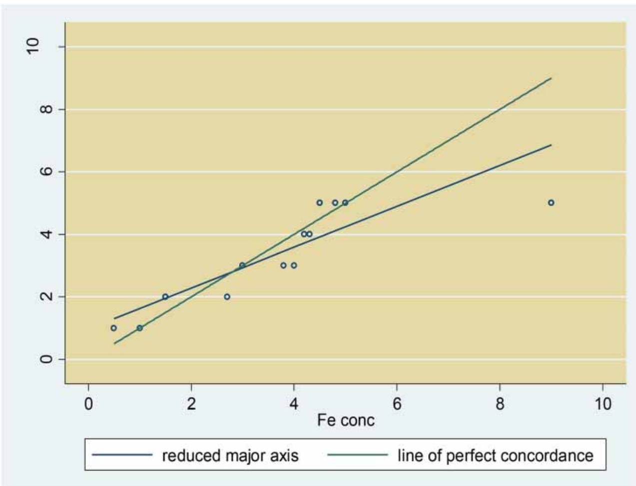


Figure 3 | Lin's concordance of the absolute agreement between the methods.

concentration of the groundwater iron, emphasizing the potency of the natural reagent of the guava leaf extracts in discerning the iron content in groundwater.

The weighted agreement of kappa is classified as 'good' (Altman 1991), complementing the actual agreement which well surpassed the expected agreement. The estimate of the concordance agreement rho_c suggests a fair degree of absolute agreement which is marginally short of being termed 'excellent' (Altman 1991). The possible reason for not reaching the 'excellent' grade could be the small number of sample size.

The pH of the samples was estimated between 6.4 and 6.8 (results not shown) which was higher than 5. As stated above, the pH values (observed in the present study) if exceeding >5 enabled quick dissociation of the tannins into anions, rapid oxidation of the ferrous iron and formation and precipitation of the ferric-tannate compounds, resulting in the prompt changes in the color of the guava leaf treated water samples. In Bangladesh, the predominant pH values of the groundwater samples are 6.0–7.5 (British Geological Survey 2001; Merrill *et al.* 2011). Hence, across the country, should the groundwater contain a fair-to-high amount of iron, the pattern of the guava leaf-treated color change of the iron-rich water samples is likely to be consistent with that observed in the present study.

The present pilot study, despite being small in scale, could disentangle the color changes according to the increasing concentration of the water iron. It roughly corresponded to the magnitude of groundwater iron concentration from 'very low' through 'medium' to 'high and higher'. This was supported by the statistical methods of the association and the extent of the agreements (Tables 2 and 3, Figure 3). A larger validation study has the potential to develop, standardize and fine tune the reference of the color scheme with the corresponding iron concentrations of the water samples. Since guava leaves are universally available in the country and because of the simplicity of the testing, introducing a reliable reference color scheme (guava leaf extract generated) might develop an indigenous tool for a semi-quantitative assessment of iron levels in groundwater. This may be an alternative to the imported iron test kit and the colorimetric devices and reagents which are expensive and not readily available in the country. This natural reagent might avoid the usage of the bio-hazardous chemicals and its plastic sachets, and thus can be environmentally friendly.

The groundwater iron content is fair-to-high in Bangladesh (British Geological Survey 2001; National Drinking Water Quality Survey 2009) and in some neighborhood countries. As stated elsewhere, there is an established association between groundwater iron and the population iron and hemoglobin status (Merrill *et al.* 2011; Rahman *et al.* 2016, 2019; Ahmed *et al.* 2018). The outcome of a larger validation study of the method has potential implications for the management of public health anemia. In areas with a high magnitude of groundwater iron (i.e. iron-replete populations), the use of iron supplementation/fortification in a blanket manner in the standard dose might evoke iron-induced side effects (Rahman *et al.* 2016, 2019). Iron dosing reduction might be useful in those settings.

The proposed indigenous, simple method through validation might identify the areas with different content of iron in groundwater. It may semi-quantitatively assess the intake of iron through water, rice and other staples and thus might indicate the body's iron status. This may inform the design and evaluation of the iron supplemental/fortified anemia control program for the overall benefits of the program and the beneficiaries. The method can be used to update the groundwater mapping of the country for the guidance of the iron supplemental programs. Further, the method could be used in relevant manufacturing industries to measure iron in the water systems as a monitoring of the iron-associated clogging of the appliances.

A limitation of the study is that it was conducted with a small number of tube-well water samples and limited to a single sub-district. The corresponding iron estimation was done by a colorimetric test kit device, which was not a gold standard. However, a similar test kit device (HACH Inc. USA) was validated (Merrill *et al.* 2009) in Bangladesh and it showed that the device had good agreement with 'gold standard' (i.e. Atomic Absorption Spectrophotometry). The shortcoming of the method to discern iron level at and above the level of 4.5 mg/L is a limitation. Nonetheless, the level of iron concentration falling on the 'high and higher' side of the water iron color spectrum adds little value. Since the iron content (≥4.5 mg/L) in groundwater is considered high, the management and decision on the anemia control program (i.e. adjustment of the program and/or iron-dosing) is unlikely to be difficult. Hence, further segregation of the color-grouping at the high end of the spectrum may not be essential.

In conclusion, guava leaf extracts can potentially develop a cheap, easy-to-use indigenous tool for a semi-quantitative assessment of iron levels in the groundwater samples. A larger validation study is required for further confirmation of the tool. The validated environmentally-friendly tool may help to optimize the design and evaluation of the anemia control programs of the country, thus potentially contributing to public health.

ETHICS APPROVAL

The study was conducted as a sub study under an efficacy trial of a new formulation of MNP (with a low dose of iron) in children of the areas with high levels of iron in groundwater. The trial received approval from the Research Ethical

Committee of Faculty of Biological Science, Dhaka University (Ref# 46 /Biol. Scs. /2017-2018), Bangladesh and Griffith University Human Research Ethics Committee, Australia (Ref# 2017/467).

AUTHOR'S CONTRIBUTION

S.R. conceived and designed the study. S.R. collected and analyzed the data and wrote the first draft of the manuscript. N.S., A.A.S., N.S. and S.S. critically reviewed the manuscript to finalize. All authors read and approved the final version of the manuscript.

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DATA AVAILABILITY STATEMENT

Data cannot be made publicly available; readers should contact the corresponding author for details.

CONFLICT OF INTEREST

The authors declare there is no conflict.

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