Dye extract of calyces of *Hibiscus sabdariffa* has photodynamic antibacterial activity: A prospect for sunlight-driven fresh produce sanitation

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**Abstract**

Photodynamic sanitation of fresh produce could help reduce spoilage and disease transmissions where conventional methods of sanitation are not available, and sunlight is available for free. In this study, we evaluated the photostability and photodynamic antibacterial activity of the dye extracts of calyces of *Hibiscus sabdariffa*. The dye extracts were very photostable in water but bleached in acetate-HCl buffer (pH 4.6), phosphate buffer saline (pH 7.2), and tris base-HCl buffer (pH 8.6). The photostability correlated with the photodynamic antibacterial activity of the dye extracts. Both the methanol and water dye extracts at the concentration of 0.0625 mg/ml caused complete inactivation of *Bacillus subtilis* (reductions of 8.5 log CFU/ml) within 2 min either with the visible light exposure at 10 mW/cm² or in the dark without the light exposure. Reductions of 4.8 log CFU/ml and 2.2 log CFU/ml of *Escherichia coli* were observed when 1 mg/ml of methanol and water dye extracts were used, respectively, in water with the light exposure at 10 mW/cm² for 20 min. Discussions are included about the ease of the dye extractions of the calyces of *H. sabdariffa* even in water without the need of energy for heating and the suitability of the dye extracts for the fresh produce sanitation. Dye extract of calyces of *H. sabdariffa* has photodynamic and nonphotodynamic antibacterial activity which could be exploited for the development of a low-tech sunlight-driven fresh produce sanitation system that is cheap, sustainable, and environmentally friendly.

**KEYWORDS**
antibacterial, fresh produce, *Hibiscus sabdariffa*, photodynamic inactivation, Photodynamic sanitation, reactive oxygen species
methods of fresh produce sanitation are energy, chemical, and operational intensive and, therefore, the new quests for alternative methods such as sunlight-driven photodynamic sanitation that would be available, cheap, sustainable, environmentally friendly, and applicable in most rural areas where fresh produce are grown especially in developing countries (de Oliveira, Tosati, Tikekar, Monteiro, & Nitin, 2018; Luksiene, Kurilčik, Juršėnas, Radžiutė, & Būda, 2007; Luksiene, Buchovec, & Paskevičiute, 2010; Luksiene, Buchovec, & Viskelis, 2013; Majiya, Chowdhury, Stonehouse, & Millner, 2019).

The lack of postharvest sanitation of fresh produce has both public health and socioeconomic consequences (Aponiene, Paskevičiute, Reklaitis, & Luksiene, 2015; Buchovec et al., 2017; Buchovec, Luksevičiute, Marsalka, Reklaitis, & Luksiene, 2016; Buchovec, Paskevičiute, & Luksiene, 2010; Luksiene, Buchovec, Kairyte, Paskevičiute, & Viskelis, 2012). Many studies have shown links between foodborne outbreaks/diseases and consumption of contaminated fresh produce which are eaten uncooked (de Oliveira et al., 2018; Ghate et al., 2019; Maikai, Elisha, & Baba-Onoja, 2012; Olaimat & Holley, 2012; Thorn, Pendred, & Reynolds, 2017). However, the main impacts of foodborne diseases are seen in the developing countries because of unsafe water used for cultivating, cleaning, and processing of food, absence of food storage infrastructures, and inadequate/poorly enforced regulatory standards (Chigor et al., 2012; Chigor, Umoh, & Smith, 2010; WHO, 2015). There is little postharvest processing and sanitation of fresh produce in most of the sub-Saharan Africa and this contributed to about 55% and 72% of fresh produce grown to perish before they can be consumed (Adeoye, Odeleye, Babalola, & Afolayan, 2009; Idah, Ajisegiri, & Yisa, 2007; Olayemi, Adegbola, Bamishiaye, & Daura, 2010). Also, ordinarily, when weather and temperature are considered, the rate of fresh produce spoilage in sub-Saharan Africa would be 3–4 times than in the Europe. The daily average temperature in sub-Saharan Africa throughout the year is 30–35°C—this is an optimum temperature range for the growth and proliferation of most microorganisms including fresh produce spoilage and pathogenic bacteria.

Photodynamic effect is the generation of singlet-oxygen and other reactive oxygen species (ROS) by interaction of a photosensitizer, light of appropriate wavelength and molecular oxygen (Costa et al., 2013; Komagoe, Kato, Inoue, & Katsu, 2011; Lukšiene, 2005; Tavares et al., 2011). Photodynamic sanitation of foods is a process in which the generated singlet-oxygen and other ROS inactivate pathogens and reduces microbial load of the food to a level considered safe by the public health standards (Aponiene & Luksiene, 2015; Lukšiene, 2005; Lukšiene & Brovko, 2013). In developing countries including sub-Saharan Africa, there are high prospects of successfully establishing cheap, efficient, and sustainable low-tech photodynamic postharvest sanitation of fresh produce because food-grade photosensitizers can be sourced locally from the plant materials and wastes, and abundant sunlight can be used to irradiate the photosensitizers (Lukšienė & Zukauskas, 2009; Majiya et al., 2019).

Hibiscus sabdariffa (also commonly called Roselle) is an annual tropical and subtropical bushy shrub with red or pale green stems and red or pale yellow inflated edible calyces. It is native to west Africa but now grown in other tropical and subtropical countries especially Africa and Asia including Nigeria, Niger, Ghana, Angola, Sudan, Egypt, China, Thailand, Sri Lanka, Malaysia, West India, and also in the Mexico (Abou-Arab, Abu-Salem, & Abou-Arab, 2011; Da-Costa-Rocha, Bonnlaender, Sievers, Pischel, & Heinrich, 2014; Illori & Odukoya, 2005). The red pigmentation of the calyces is due to the high concentration of anthocyanins. Other chemical constituents of the calyces are organic acids, flavonoids, and other polyphenols. The facts that H. sabdariffa is not a staple crop, safe for usage as food and medicine, available in abundance, easy extraction of its dye, and easily grown in developing countries (Abou-Arab et al., 2011; Da-Costa-Rocha et al., 2014) make it a potential good candidate as a source of dye extract that can be used as a food-grade photosensitizer for the development of a low-tech sunlight-driven sanitation system for foods including fresh produce in those regions of the world that lacks the conventional systems of fresh produce sanitation.

In this work, we evaluated the dye extracts from the calyces of H. sabdariffa for potential usage as a food-grade photosensitizer. We investigated the photodynamic inactivation of model bacteria using the dye extracts.

2. MATERIALS AND METHODS

2.1. Calyces of H. sabdariffa

The dried pinkish red calyces of H. sabdariffa were purchased from the Kure Market, Minna, Niger State, Nigeria. The calyces were used as purchased for the extraction of the dye with no prior preparation and processing.

2.2. Solvents and other consumables

All the chemicals, solvents, and other consumables used were of analytical grade. All inorganic chemicals and salts were purchased from Sigma-Aldrich. Methanol and acetic acid were purchased from Acros Organics. LB broth and agar for culturing bacteria were purchased from Fisher Scientific: Janssen Pharmaceuticalaan. The distilled water used in this work was provided by the Thermo Scientific.

2.3. Light source and irradiation conditions

The light source used for the photostability and Photodynamic inactivation (PDI) experiments was a Slide Projector—Zett Royal II afs (Remote Control Leica Projection GmbH Type 508, Zett Gerate), which provides a cool bright light. A 420-nm light filter was inserted into the projector to cutoff the UV light. Fluence rate of irradiations were measured using a Digital Radiometer (Solar meter, model 9.6 Red light, Solar Tech Inc.). Visible light was used throughout the study and the fluence rates (radiant exposure) were 5–10 mW/cm² at 20–22°C under aerobic conditions. The solvents and buffers used...
for the photophysical characterization of the dye extracts and PDI were distilled water (dH₂O), methanol, Phosphate Buffer Saline (PBS; pH 7.2, 6.6, 7.6; 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, 137 mM NaCl, and 2.7 mM KCl), Acetate-HCl (AH) Buffer (pH 3.6, 4.6, 5.6; 0.1 M CH₃COOH and 0.1 M CH₃COOK), and Tris Base-HCl (TBH) Buffer (pH 8.6; 0.1 M Tris and 0.1 M HCl).

2.4 | Bacterial strains

The bacteria strains—E. coli Nissle 1917 (a model for Gram-negative bacteria) and B. subtilis DB 104 (a model for Gram-positive bacteria)—were from the laboratory of Prof. Ulrich Dobrindt, University of Munster, Germany.

2.5 | Extraction of the dye of calyces of H. sabdariffa

Methanol, 0.1% (v/v) acetic acid methanol, and distilled water were used as solvents for the extraction of dye of the calyces of H. sabdariffa. The methanol dye extractions of the calyces were carried out under the cold (room temperature 20°C) and hot (60°C) conditions. The water dye extractions were carried out under the cold (room temperature 20°C), hot (60°C), and boiling (100°C) conditions. Five grams of the dried calyces was suspended in 30 ml of the solvent in each of the extraction setup in 100-ml round-bottom flasks with continuous shaking at 500 rpm for 2 hr in the first extractions. The dye extracts were then decanted into separate Falcon bottles (50 ml) and 30 ml of the same solvents were added to the extraction flasks maintained under previous conditions for 1 hr for the second extractions. The dye extracts of the second extraction were also decanted into the separate Falcon bottles. The dye extracts were then centrifuged at 11, 500 g for 5 min. After the centrifugation, the supernatants were decanted into fresh clean round-bottom flasks (100 ml). The methanol dye extracts were then concentrated and dried using Rotavapor (BUCHI, Switzerland). The distilled water dye extracts were first frozen using liquid nitrogen and then lyophilized (Christ, Germany) to concentrate and dry. The dried samples were weighed, and the yields of the dye extracts were determined. The samples were then separately dispensed into smaller labeled bottles and stored in the dark under refrigeration for subsequent use.

2.6 | Photophysical–chemical characterization of the dye extracts of the calyces of H. sabdariffa

2.6.1 | Absorption spectra

The absorption spectra of the dye extracts (1 mg/ml) of the calyces of H. sabdariffa were determined in dH₂O, AH buffer (pH 3.6, 4.6, 5.6 and 6.6), PBS (pH 7.6), and TBH buffer (8.6) using Agilent 8453 UV-visible spectrophotometer (Agilent Technologies) and the wavelength range used was from 300 to 800 nm.

2.6.2 | pH stability in distilled water

The pH of different concentrations of the dye extract (CM) of the calyces of H. sabdariffa and the pH stability were determined and followed for 1 month using a digital pH meter (Seven Compact, Mettler Toledo, USA). The pH of the samples was read and recorded at the beginning of every week until the 4th week.

2.6.3 | Photostability and bleaching

Photostability and bleaching of the dye extract (CM) of the calyces of H. sabdariffa were determined in distilled water, Acetate Buffer (pH 4.6), PBS (pH 7.2), and Tris Base Buffer (pH 8.6) using Agilent 8453 UV-visible spectrophotometer (Agilent Technologies). The absorptions (300–800 nm) of the samples were read intermittently every 2 min of the irradiation at 5 mW/cm² until 20th minutes.

2.7 | Mass spectroscopy of the dye extracts of the calyces of H. sabdariffa

Mass spectroscopy of all the dye extracts was determined in methanol and water using MicroTof-ESI (Bruker Daltonics) and Orbitrap LTQ XL-ESI (Thermo Fisher Scientific) in order to assess the effect of the temperature conditions and solvents used for the extractions on the compositions of the extracts. Additionally, the fractionization and mass spectroscopy of the dye extract (CM) was done using 6110 HPLC-MS-ESI (Agilent Technologies). In all cases, both positive and negative ionization modes of the mass spectroscopy were acquired.

2.8 | Photodynamic inactivation of model bacteria using the dye extracts of the calyces of H. sabdariffa

Photodynamic inactivation (PDI) of the model bacteria (E. coli Nissle 1917 and B. subtilis DB 104) was investigated with different concentrations of the water and methanol dye extracts of the calyces of H. sabdariffa in distilled water (dH₂O), Acetate-HCl (AH) Buffer (pH 4.6), PBS (pH 7.2), and Tris Base-HCl (TBH) Buffer (pH 8.6), and at different times of light illumination. The concentrations of the dye extracts used were 0.0625 mg/ml to 1 mg/ml, light intensity was 10 mW/cm², and illumination times were 2–20 min. For each of the PDI light experiments (dye extract with light illumination), two control experiments were simultaneously setup; dark experiments which were carried out in the presence of dye extracts but without illumination, while “light only” experiments were exposed to light but in the absence of the dye extracts. All experiments were repeated three times. After PDI experiments, the solutions were plated for bacterial enumeration to determine the rate and extent of inactivation. Also, live/dead staining (BacLight Bacterial Viability Kit, Invitrogen) was used according to the manufacturer’s instructions to determine the viability of the bacteria after the PDI. Fluorescence
images were captured using 40× magnification objective with a Nikon Eclipse Ci microscope (Nikon Corporation Tokyo).

3 | RESULTS

3.1 | Effects of the temperature and solvents on the yields and pH of the dye extracts of the calyces of H. sabdariffa

The yields of dye extractions of the calyces of H. sabdariffa by different temperatures and solvents are shown in Table 1. When solvents are considered, more higher yields were observed in water dye extractions of the calyces (Table 1). As for the temperatures of the extractions, hot temperature gave higher yields compared with the cold temperature (Table 1). Although all the dye extracts irrespective of the solvents and temperatures of extractions are very soluble in water, the pH varies slightly in dH₂O at the same concentration (Table 1). The methanol dye extracts have lower pH when compared with the water dye extracts (Table 1).

3.2 | Photophysical characterization of the dye extracts of the calyces of H. sabdariffa

3.2.1 | Effect of buffers/solutions on the absorption spectra and colors of the dye extracts

The absorption spectra (Figure 1) of the dye extracts (1 mg/ml) of the calyces of H. sabdariffa were determined in distilled water (dH₂O), Acetate-HCl (AH) buffer (pH 3.6, 4.6, and 5.6), Phosphate Buffer Saline (PBS; pH 6.6 and 7.6), and Tris base-HCl (TBH) buffer (pH 8.6). The peak absorption for all the samples in dH₂O was at 520 nm (Figure 1a). However, the intensity of the absorptions differs, and samples extracted under cold conditions seem to have higher intensity compared with the samples extracted at hot and boiling conditions (Figure 1a). Also, although the absorption peaks for all samples were at 520 nm, the spectrum pattern of the sample extracted under boiling condition (BW) slightly differs with other samples (Figure 1a). The absorption spectra of the dye extract (CM) in different buffers and pH have different absorption peaks and colors (Figure 1b,c). The absorption peaks tend to move toward infrared as the pH increases (Figure 1b). The peaks for pH 3.6, 4.6, 5.6, 6.6, 7.6, and 8.6 were at 530, 538, 536, 559, 583, and 592 nm, respectively (Figure 1b). The changes in colors with different pH are characteristics of the dye anthocyanins. The pH stability of different concentrations of dye extract (CM) in dH₂O for 1 month is shown in Table 2. The dye extract is very stable in water (Table 2).

3.2.2 | Effects of buffers/solutions on the photostability and bleaching of the dye extracts

The absorption spectra (Figure 2) indicating photostability and bleaching rate of the dye extract (CM) of the calyces of H. sabdariffa were determined in distilled water, Acetate-HCL (AH) buffer (pH 4.6), Phosphate Buffer Saline (PBS; pH 7.2), and Tris Base-HCL (TBH) buffer (pH 8.6). The dye extract is very photostable in water and do not bleach (Figure 2a). However, the dye-extract bleaches in all the buffers used in this study (Figure 2b–d). The bleaching rate is faster in AH Buffer compared with other buffers under the same conditions of illumination with light (Figure 2b–d).

3.3 | Mass spectroscopy of the dye extracts of the calyces of H. sabdariffa

The mass spectroscopy of the dye extracts was determined in order to assess the effects of extraction temperatures and solvents on the compositions of the extracts. The first three peaks (for positive and negative ionization modes) with the highest intensities for each of the samples are shown in Table 3 (see Figure S1A–G). The peaks indicated that the temperature and solvent of extraction did not affect the compositions of the dye extracts (Table 3). However, boiling at 100°C might have affected the compositions slightly (Table 3). The mass spectroscopy of the fractions of the dye extract (CM) showed it is a complex compound with more than 40 compounds (see Figure S2A and B).

| Temperature and extraction solvent | Dye extract yield (mg) per 5 g of dried calyces | % yield of dye extracts | pH (1 mg/ml) |
|-----------------------------------|-----------------------------------------------|------------------------|-------------|
| CM                                | 1,407.2                                       | 28.14                  | 2.85        |
| HM                                | 1,554.4                                       | 31.09                  | 2.86        |
| CMA                               | 1,281                                         | 25.62                  | 2.76        |
| HMA                               | 1,402.5                                       | 28.05                  | 2.76        |
| CW                                | 1,775.8                                       | 35.52                  | 3.0         |
| HW                                | 1,987                                         | 39.74                  | 3.05        |
| BW                                | 1,514                                         | 30.28                  | 3.11        |

Abbreviations: BW, boiling distilled water dye extract; CM, cold methanol dye extract; CMA, cold acidified methanol dye extract; CW, cold distilled water dye extract; HM, hot methanol dye extract; HMA hot acidified methanol dye extract; HW, hot distilled water dye extract.
Photodynamic inactivation of *E. coli* and *B. subtilis* using the dye extracts of the calyces of *Hibiscus sabdariffa*

### 3.4.1 Effect of buffer/solution on the PDI of bacteria using the dye extracts

The PDI of *E. coli* and *B. subtilis* using the 1 mg/ml of dye extracts (CM and CW) was determined in distilled water (dH$_2$O), Acetate-HCl (AH) Buffer (pH 4.6), Phosphate Buffer Saline (PBS; pH 7.2), and Tris Base-HCl (TBH) Buffer (pH 8.6). The light experiments (dye extract with light illumination) and “light only” control experiments were illuminated at 10 mW/cm$^2$ for 20 min. The PDI of the model bacteria varies with different buffers/solutions used for the PDI (Figure 3). For both the dye extracts (CM and CW) used, the PDI was only clearly observed in dH$_2$O and more *B. subtilis* was inactivated compared with the *E. coli* under the same conditions (Figure 3). In dH$_2$O, both the PDI and non-PDI were
FIGURE 2  Photostability and bleaching rate of the dye extract (CM) of calyces of *Hibiscus sabdariffa* in different solutions and light illumination of 5 Mw/cm². (a) Absorption spectra of the photostability study of the dye extract in dH₂O. (b) Absorption spectra of the photostability study of the dye extract in Acetate-HCl buffer (pH 4.6). (c) Absorption spectra of the photostability study of the dye extract in Phosphate Buffer Saline (pH 7.2). (d) Absorption spectra of the photostability study of the dye extract in Tris Base-HCl buffer (pH 8.6). CM, cold methanol dye extract.

**TABLE 3**  Major peaks of the mass spectroscopy of the dye extracts of calyces of *Hibiscus sabdariffa*

| Dye extract | Positive | Negative |
|-------------|----------|----------|
|              | 1st      | 2nd      | 3rd      | 1st    | 2nd    | 3rd    |
| CM          | 118.080  | 203.051  | 245.024  | 189.0059 | 379.0166 | 407.0451 |
| HM          | 118.079  | 245.025  | 203.051  | 189.0048 | 379.0153 | 407.0465 |
| CMA         | 203.052  | 118.085  | 245.026  | 189.0062 | 379.0172 | 221.0316 |
| HMA         | 118.084  | 203.052  | 245.026  | 189.0066 | 379.0172 | 351.0578 |
| CW          | 118.086  | 597.146  | 219.027  | 189.0038 | 207.0145 | 351.0566 |
| HW          | 203.053  | 118.084  | 219.027  | 189.0079 | 379.0169 | 207.0182 |
| BW          | 203.052  | 383.1152 | 118.085  | 189.0051 | 210.9869 | 353.0872 |

Abbreviations: BW, boiling distilled water dye extract; CM, cold methanol dye extract; CMA, cold acidified methanol dye extract; CW, cold distilled water dye extract; HM, hot methanol dye extract; HMA, hot acidified methanol dye extract; HW, hot distilled water dye extract.
observed and could not be differentiated in *B. subtilis* as both the light experiments (dye extract with light illumination) and the dark experiments (dye extracts without light illumination) lead to the complete inactivation (limit of detection) equivalent to reductions of 8.5 log CFU/ml (Figure 3a,b). Also, inactivation (reductions of 1.5–8.5 log CFU/ml) including the controls were observed for *B. subtilis* in AH Buffer (pH 4.6; Figure 3a,b). As for the PDI in the remaining buffers, inactivation – most likely PDI was observed only when the dye extract (CM) was used for *B. subtilis* in PBS (pH 7.2) and TBH Buffer (pH 8.6) with the reductions of 1.7 log CFU/ml and 1.5 log CFU/ml, respectively (Figure 3a,b). As for the *E. coli*, PDI was only observed in dH2O with reductions of 4.8 log CFU/ml and 2.2 log CFU/ml for dye extracts CM and CW, respectively (Figure 3c,d).

### 3.4.2 Effect of the concentration and time of illumination on PDI using the dye extracts

The PDI of *E. coli* and *B. subtilis* using different concentrations of the dye extracts (CM and CW) and different time of illumination at 10 mW/cm² were determined in dH2O. We observed dose/concentration and illumination time-dependent inactivation (Figure 4). For the 20 min PDI of *E. coli*, reductions of 4.7, 1.2, 0.7, 0.6, and 0.4 log CFU/ml were observed for the 1, 0.5, 0.25, 0.125, and 0.0625 mg/ml of the dye extract (CM), respectively (Figure 4a). As for the *B. subtilis*, complete inactivation (reductions of 8.5 log CFU/ml) was observed for both the light experiments (dye extract with light illumination) and the dark experiments (dye extract without light illumination) for all the concentrations (0.0625–1 mg/ml) of the dye extract (CM) used (Figure 4b).
The illumination time-depended study of the *B. subtilis* also showed complete inactivation (reductions of 8.5 log CFU/ml) for both the light experiments (dye extract with light illumination) and the dark experiments (dye extract without light illumination) for all the time 2–20 min tested (Results not shown). However, we were able to follow and observed the illumination time (2–20 min)-depended PDI of *E. coli* for the dye extracts CM and CW (Figure 3c,d). For the PDI using the dye extract (CM), at times 4, 10, and 20 min of PDI, we observed reductions of 1, 1.3, and 4.8 log CFU/ml, respectively, of the *E. coli* (Figure 4c). However, using the dye extract (CW), at time 4, 10 and 20 min of PDI, we observed reductions of 0.5, 1, and 2.2 log CFU/ml, respectively, of the *E. coli* (Figure 4d). The fluorescence microscopy images of the live/dead staining of the PDI samples validated the extent of inactivation using the dye extract (CM) in water (Figures 5 and 6).

### 4 | DISCUSSION

#### 4.1 Extraction and photophysical characteristics of the dye extracts of calyces of *H. sabdariffa*: implications for applications

In this work, we extracted the dye from the calyces of *H. sabdariffa* using different solutions; distilled water, methanol and acidified
methanol, and different temperatures; cold, hot, and boiling temperatures. Most earlier reports suggested the use of acidified methanol/ethanol at cold and/or hot temperatures for the extractions of the dyes (Abou-Arab et al., 2011; Ilori & Odukoya, 2005). However, we also used water for the extraction of the dye of calyces of *H. sabdariffa* at cold, hot, and boiling temperatures because of the almost universal availability of water even in the remotest part of the world. For all the solvents and temperatures used, the % yield of the dried dye extracts ranges from 25 to 40 (Table 1). The water dye extractions had higher yield than the methanol and acidified methanol extractions (Table 1). However, at the same concentration (1 mg/ml) the water dye extracts were a little bit less acidic (pH 3.0) when compared with the methanol (pH 2.85) and acidified methanol (pH 2.76) extracts (Table 1).
These pH (2.76–3.0) can inactivate many of the bacteria especially the Gram-positive bacteria. The pH of the acidified methanol dye extract we observed agrees with the earlier reported (Abou-Arab et al., 2011). Using the same solvent but different temperatures for the dye extracts does not significantly affect the % yields and the pH. All these indicated the ease at which the dyes of calyces can be extracted even in cold water with no additional need for energy for heating thereby making it suitable for the aim of this project.

The absorption and mass spectra (except for the dye extract–BW) indicated that irrespective of the solvents and temperatures of extractions, the dye extracts are the same (Figure 1a and Table 3). The absorption peak for the dye extracts is the same which is 520 nm. This wavelength is within the visible range and it means sunlight (with spectrum of 300–1,400 nm and a peak at 500–600 nm; Jemli, Alouini, Sabbahi, & Gueddari, 2002; Loeb, Hofmann, & Kim, 2016) could be used to irradiate the dye for the photodynamic inactivation of fresh produce spoilage and pathogenic microorganisms. However, the absorption peaks and colors of the dye extracts were pH/buffer dependent (Figure 1b–c)—this is well-known characteristics of the anthocyanins especially the types that are found in the calyces of *H. sabdariffa* (Abou-Arab et al., 2011; Ilori & Odukoya, 2005).

The dye extract of the calyces of *H. sabdariffa* is very photostable and pH stable in water (Figure 2a and Table 2). This means that the dye extract has fulfilled one of the cardinal desired characteristics of a photosensitizer for the lack of photodegradation and bleaching in water (DeRosa & Crutchley, 2002). However, the dye extracts bleach in other buffers (acetate–HCl buffer pH 4.6, PBS pH 7.2, and tris base–HCl pH 8.6) used (Figure 2b–d). The bleaching may be pH and/or buffer type/compositions dependent. This may limit the application of the dye extracts as a photosensitizer to only environmental applications such as fresh produce sanitation where fresh water would be used as wash waters containing the dye extracts to photodynamically inactivate pathogenic and spoilage microorganisms. The dye extract may not be suitable for the PDI in biological samples and solutions/buffers of high salts contents. However, the non-PDI inactivation activity of the dye extracts could still be available or retained and could be exploited for the biological samples and solutions/buffers.

### 4.2 Dye extracts of the calyces of *H. sabdariffa* cause photodynamic and nonphotodynamic inactivation of *E. coli* and *B. subtilis*

The water and methanol dye extracts of the calyces of *H. sabdariffa* used in this work showed both photodynamic and nonphotodynamic inactivation of bacteria models (Figures 3 and 4). The photodynamic inactivation observed depends on the buffers/solutions of the PDI and was clearly observed in dH₂O. This follows and may be linked to the photostability of the dye extracts in dH₂O (Figure 2a) which allowed the molecules of dye at ground state to absorb the illuminated light and thereby become excited to singlet state then to triplet state which can then interact with the available molecular oxygen to generate singlet-oxygen and other reactive oxygen species which are cytotoxic and can inactivate microorganisms including bacteria in solution. Singlet-oxygen and other ROS oxidize and cause irreversible damage to proteins, lipids, nucleic acid, and other cellular components of microorganisms and ultimately inactivate them (Costa et al., 2013).

For the photosensitizers that are photostable including the dye extracts of the calyces of *H. sabdariffa* in water showed in this work (Figure 2a), the triplet state of their excited molecules interacts with the available molecular oxygen which ultimately lead to the production of the ROS. After the interaction with the oxygen, the molecules of the photosensitizers will go to the ground state to be excited again with the illuminated light and the process continues with the resultant production of ROS. However, for the photosensitizers that are not photostable including the dye extracts of calyces of *H. sabdariffa* in acetate–HCl buffer (pH 4.6), phosphate buffer saline (pH 7.2), and tris base–HCl buffer (pH 8.6) observed in this work (Figure 2b–d), at each interactions of the excited triplet state of the molecules of the photosensitizers with the molecular oxygen, the ROS produced especially the singlet oxygen, consumed the molecules of the photosensitizers that produces them thereby leading to the bleaching and photodegradation of the photosensitizers/dyes. This led to the steady reduction in the molecules of the photosensitizers which also corresponds to steady reduction in the ROS generation in the solution. The generation of the ROS in the solution would eventually stop within seconds to few minutes. The insufficient ROS production in solution, ROS produced degrading the molecules of photosensitizers that produces them instead of oxidizing the bacteria in the solution, and the eventual stoppage of the production of ROS maybe the plausible reasons for the lack or less bacteria PDI observed in this study when acetate–HCl buffer (pH 4.6), phosphate buffer saline (pH 7.2), and tris base–HCl buffer (pH 8.6) were used (Figure 3). We hypothesize that the polyphenols especially the anthocyanins in the dye extracts of the calyces of *H. sabdariffa* may be responsible for the PDI of bacteria observed in this work. To the best of our knowledge, the photodynamic antibacterial activity as shown in this work has never been reported before for the dye extracts of the calyces of *H. sabdariffa*.

Non-PDI was observed for *B. subtilis* in this study (Figures 3 and 4). This maybe attributable to the polyphenols especially the organic acids such as citric acid, hydroxyxycitric acid, hibiscus acid, malic acid, tartaric acid, oxalic acid, and ascorbic acid in the dye extracts (Da-Costa-Rocha et al., 2014). The dye extracts cause the water to be acidic (pH 2.85–3.85) and this, in addition to the antibacterial activity of other compounds present in the extracts, maybe responsible for the complete inactivation of *B. subtilis* for both the light experiments and dark control experiments in water observed in this work (Figures 3 and 4). *B. subtilis* used in this study was particularly sensitive to acetate–HCl buffer (pH 4.6) and the buffer with its low pH might have induced some molecules within the bacterium to act as photosensitizers even without the addition of the external photosensitizers to generate ROS which inactivated it (Figure 3a,b).
Like most antimicrobial agents, the PDI and non-PDI activity of the dye extracts of calyces of *H. sabdariffa* shown in this work are dye dose/concentration and illumination time dependent (Figure 4). Previous reports have shown that the PDI is proportionally related to the light dose/fluence rate (Costa et al., 2010; Majiya, 2017). The light intensity used for the PDI in this work is 10 mW/cm² which is just about 1% of the bright sunlight under clear sky conditions in sub-Saharan Africa. In this work, under the same conditions, more *B. subtilis*-a Gram-positive bacteria was inactivated compared with the *E. coli*-a Gram negative. This is a general trend for most of the antibacterial agents including the PDI. Gram-positive bacteria are easily inactivated by singlet-oxygen oxidation as compared with gram-negative bacteria due to differences in the structure of their cell membrane (Alves et al., 2009; Bourré et al., 2010; Carvalho et al., 2007; Costa et al., 2012; Komagoe et al., 2011; Maisch, Spannberger, Regensburger, Felgenträger, & Bäumler, 2012). Gram-negative bacteria have an additional outer membrane apart from the cytoplasmic (inner) membrane, giving them extra protection against antimicrobial agents including singlet-oxygen and other ROS produced during photosensitization.

**5 | CONCLUSION**

In summary, we observed that the photostability, photodynamic, and nonphotodynamic antibacterial activity of the water and methanol dye extracts of the calyces of *H. sabdariffa* were dependent on the solutions/buffers, dye dose/concentration, and illumination time. The dye extracts were very photostable in distilled water. However, the dye extracts bleached and photodegraded in acetate-HCl buffer (pH 4.6), PBS (pH 7.2), and tris base-HCl buffer (pH 8.6). This will limit the PDI use of the dyes to only environmental applications such as fresh produce sanitation in fresh water. The PDI of bacteria was clearly observed in distilled water because the dye extracts were photostable in the water. To the best of our knowledge, this study will be the first time that photodynamic antibacterial activity of the dye extracts of calyces of *H. sabdariffa* is reported. Rate and extent of the PDI and non-PDI was higher in *B. subtilis* compared with the *E. coli*. This study can lead way to the development of a low-tech sunlight-driven fresh produce sanitation system that could be used in developing countries especially the sub-Saharan African to reduce foodborne diseases/outbreaks and fresh produce spoilage/wastage.

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**CONFLICT OF INTEREST**

None.

**ETHICAL CONSIDERATION**

Ethics approval was not required for this research.

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SUPPORTING INFORMATION
Additional supporting information may be found online in the Supporting Information section.

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