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Research Article
Application of Crude Extract of *Hibiscus sabdariffa* as Stain for Bacterial Cells

Terdzungwe Thaddaeus Sar, Samuel Ozeh Ashiegieruwa and James Sesughter Orsar

Department of Microbiology, College of Biological Sciences, Joseph Sarwuan Tarka University, Makurdi 970101, Benue, Nigeria

Abstract

Background and Objective: Traditional synthetic bacterial strains are expensive, toxic and degrade the environment. Towards finding alternative substitutes, a study to investigate extracts from a common food and medicinal plant, *Hibiscus sabdariffa* for staining of bacterial cells was undertaken. The study evaluated the ability of the plant extracts to adequately stain bacterial cells and replace and/or complement and reduce the use of the costly, largely toxic, environmentally harmful synthetic dyes and stains. **Materials and Methods:** Aqueous, methanol and ethanol of *H. sabdariffa* were carried out and the physical and chemical properties were determined. The extracts were tested on four bacterial species: *Staphylococcus aureus*, *Escherichia coli*, *Streptococcus* spp. and *Bacillus cereus*. The stained cells were evaluated for clarity, definition and contrast. **Results:** Extracts of *H. sabdariffa* were acidic with an average pH of between 3.06 and 4.08 and were net negatively charged. It was observed that aqueous extracts of the plant stained better than the alcohol extracts. While the bacterial cells did not readily take up the plant stain by direct staining technique, negatively staining them gave sufficient contrast and clearly showed their morphologies. **Conclusion:** With further refinement, the pigments extracted from *H. sabdariffa*, could serve as low-budget stain alternatives that could be used to demonstrate bacterial shapes and morphologies, an especially useful in resource poor environments.

Key words: *Hibiscus sabdariffa*, stains, dyes, staining, bacterial cells, negative staining

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Corresponding Author: Terdzungwe Thaddaeus Sar, Department of Microbiology, College of Biological Sciences, Joseph Sarwuan Tarka University, Makurdi 970101, Benue, Nigeria Tel: +234 706 492 7606

Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Dyes, from which stains are made, may be either natural or synthetic products. Most dyes used to stain bacterial smears are synthetic derivatives of benzene and are usually referred to as aniline dyes[1]. Chemically synthesized dyes, besides being expensive, may also be harmful to humans and the environment. Some components may be carcinogenic or at least strongly allergic[2].

Microorganisms, especially bacteria need to be stained to increase visibility, accentuate morphological features and sometimes preserve them for further study[3]. Stains, therefore, impart colour to microbial cells to create contrast and make them more visible[4].

To achieve this, Ihuma *et al.*[5] used methanol extract of *Hibiscus sabdariffa* as a staining agent on three species of fungi: *Rhizopus stolonifer*, *Aspergillus niger* and *Penicillium notatum*. They found that the organisms were more visible than when treated with the traditional lactophenol in cotton blue fungal stain.

Hibiscus sabdariffa, also commonly known as rosella and red sorrel is a vascular flowering plant of the family Malvaceae. It is cultivated in Nigeria and tropical America for its juice and acidic, but edible calyces[6]. *Hibiscus sabdariffa* is also known to have therapeutic and laxative properties. As well as being antihypertensive, it is reported to lower cholesterol levels. Besides, it is known to exhibit high antioxidant activity, reduces hepatotoxicity and fever, is a diuretic and a remedy for scurvy[7].

In Nigeria, *Hibiscus sabdariffa* has two main uses; as a food vegetable and for the preparation of a local beverage drink called zobo, which has a characteristic deep purple colour. Attention to *Hibiscus sabdariffa* lately has mostly been on its nutritional benefits, with little focus on its potential as a biological staining agent.

According to Ihuma *et al.*[5] natural dyes are a potential source of cheaper stains, provide employment opportunities and are also ultimately more environmentally friendly. This study, therefore, investigated the effectiveness of extracts of *Hibiscus sabdariffa* as a biological staining agent for bacterial cells.

MATERIALS AND METHODS

Study area: The study was conducted in Makurdi, the Benue State Capital, Nigeria between May to September, 2020. Makurdi is in the middle belt Savannah Region of Nigeria. Makurdi has humid weather, with an average annual temperature of 35°C. Two major seasons predominate; the wet (rainy) season, which lasts from April to October and the dry season, from October to March. The soil is well-drained with a population estimated at about 456, 000, who are mostly traders, farmers and government workers[8].

Plant material and chemical used

Extraction and concentration of extract of *Hibiscus sabdariffa*: Fresh *H. sabdariffa* calyces were purchased from local markets, supplied by farmers in and around Makurdi town between the months of April to May, at the peak of the rainy season, when they are commonly available.

The calyces of *H. sabdariffa* were air-dried until crisp and pounded to rough powder using a pestle and mortar and further heated in a laboratory oven for 1 hr at 50°C and ground to finer powder using a blender, sieved with a fine sieve and stored in air-tight containers prior to extraction process according to the method of Ma'aruf *et al.*[9]. Hot aqueous, ethanol and methanol (absolute) Sohxlet extraction processes were carried out.

Hot extraction was carried out after Abubakar and Mainul Haque[10] as modified. Fifty grams of *H. sabdariffa* powder was soaked in 500 mL of hot (65°C) distilled water, shaken and left for 24 hrs. The solution was filtered using Whatman filter paper. The filtrate was evaporated in a 800 W hot air oven (Griffin and George LTD.-Great Britain) at 50°C until dry. The residue was scraped, ground to powder, weighed and stored in a dry, airtight container and stored at 4°C until needed.

Methanol (Absolute-Guanghua Chemicals-China) and ethanol (Absolute-Guanghua Chemicals-China) extracts of *Hibiscus sabdariffa* were obtained from 320 g of finely powdered calyces using a modified Soxhlet extraction method[11]. The fine powder was soaked in a respective alcohol solvent in a 500 mL beaker for 24 hrs to allow effective percolation of the extracting solvent before the extraction process. The soaked powder was then extracted in methanol and ethanol for 7 hrs using Soxhlet extractor, respectively. Both extracts were then concentrated for 5 hrs by transferring to a rotary evaporator set at 3000 rev min^{-1} at 65°C[11]. The respective pH readings were taken.

Preparation of oxidants and mordants: Ferric chloride (0.1 mL) was prepared by dissolving 1.62 g of ferric chloride salt in a beaker containing 100 mL of distilled water and allowed to dissolve completely.

Potassium permanganate (0.1 mL) was prepared by dissolving 0.58 of potassium permanganate solid in 100 mL of distilled water and stood for 24 hrs.

Sodium carbonate (0.1 mL) was also prepared by dissolving 0.53 g of sodium carbonate in 100 mL of distilled water and mixed by shaking.

Similarly, sodium hydroxide (0.1 mL) was prepared by dissolving 0.2 g of sodium hydroxide pellets in 100 mL of distilled water and shaken to mix.

Preparation of stain solutions: Stain stock solution was prepared as follows: 30 g of dried aqueous, methanol and ethanol extracts, prepared as previously described, were dissolved in 500 mL beaker containing 300 mL of distilled water, methanol and ethanol, respectively and stirred to dissolve and filtered using Whatman filter paper to remove undissolved solids[12].

The stain solution was divided into six portions in bottles labeled A-F. To the first bottle, labeled A, 10 mL iron solution (mordant) was added to 50 mL of the stain solution, giving a 1:5 ratio.

In the second bottle, B, the stain solution was ripened by oxidation with potassium permanganate and iron solution was added as mordant at a ratio of 1:1:5.

The stain solution was ripened by oxidation with sodium carbonate and iron solution was added as mordant at 1:1:5 in the third bottle labeled C.

The fourth bottle was labeled D and the stain solution ripened with potassium permanganate only, at a ratio of 1:5.

In the fifth bottle, E, the stain solution was ripened with sodium carbonate only at a 1:5 ratios.

Finally, the sixth bottle, F, had the stain solution only, without oxidant or mordant.

Determination of pH of stain solutions: The pH of prepared stain solutions was determined using a pH meter (Ohaus ST3100-USA) and calibrated at pH 4.0, 7.0 and 9.2, respectively.

Determination of stain net charge: The net charge of *Hibiscus sabdariffa* extracts was determined using gel electrophoresis. About 1% agarose gel buffer with Tris-Acetate EDTA (TAE) was employed. Migration was observed using a UV transilluminator (Fisher Scientific, USA).

Direct staining techniques: The extracted dyes were used on 24 hrs old bacterial cultures of *Staphylococcus aureus*, *Escherichia coli*, *Bacillus* spp. and *Streptococcus* spp., isolated locally, typed and stored in the stock culture collection (SCC) of the Department of Microbiology, Joseph Sarwuan, Tarka University, Makurdi, Benue State, Nigeria and which identities were further confirmed, before use, by biochemical tests including coagulase, catalase, indole, citrate, oxidase and urease.

Coagulase test: The slide test was performed. Rabbit plasma was dropped onto a clean glass slide. The test bacteria, emulsified in physiological saline were mixed with the plasma. The development of white precipitates showed the production of the enzyme coagulase.

Catalase test: Onto a clean glass slide was added a drop of H_2O_2 (Hydrogen Peroxide). A loop-full of bacteria to be tested was mixed into the drop. Effervescence indicated a positive test.

Indole test: Bacterial colonies were inoculated into tubes containing 2 mL tryptone water. These were then incubated at 37°C/24 hrs and tested for indole production using Kovac's reagent. Development of a cherry-red colour layer, confirmed indole production.

Citrate test: A suspension of the test bacteria was prepared in 0.25 mL physiological saline in a tube. A citrate tablet was added, the tube corked and incubated at 37°C/12 hrs. Development of a red colour indicated a positive test. A yellow-orange colour was a negative test.

Oxidase test: Oxidase reagent was dropped on Whatman filter paper in a Petri dish. Using a wire loop, a culture of bacteria was smeared onto the filter paper. Development of a dark-purple colour was considered a positive oxidase.

Urease test: To freshly prepared urea broth, the bacterial sample was inoculated using a wire loop and incubated for 24 hrs at 37°C. Development of a pink colour indicated a positive test.

Negative staining technique: A drop of each of the prepared aqueous, methanol and ethanol extracts stain solution was placed at the end of a clean glass slide. A loopful of bacteria was dispersed in the drop of stain solution. A second slide was used to spread the emulsified bacteria, producing a broad, even and thin smear. The smear was allowed to air-dry and viewed under the oil immersion objectives lens[13].

Microscopy: Microscopic observation of each slide was made under the ×100 oil immersion objective (KEN-A-VISION-Kansas, USA). The features of the various bacteria were recorded and compared with standard Gram-stained smears.

RESULTS

Physical properties of extracts of *Hibiscus sabdariffa*: The pH values and color of extracts were shown in Table 1. All extracts of *Hibiscus sabdariffa* were acidic with pH values between 3.28 and 3.77. Colors of extracts similarly ranged between mauve and red.

Migration of the negatively charged ions of *H. sabdariffa* extracts. With DNA, as control, between the wells of each extract, there was migration of the molecules of all the stain extracts from the negative towards the positive terminal, showing that they were negatively charged as shown in Fig. 1.

pH values and colors of aqueous, ethanol and methanol extracts of *H. sabdariffa*: Aqueous extract of *Hibiscus sabdariffa* were acidic after respective mixture with ferric chloride (mordant), potassium permanganate and sodium carbonates (oxidants) was shown in Table 2. The color of aqueous *Hibiscus sabdariffa* extracts treated with mordant and oxidants were reddish, except for extracts mixed with only sodium carbonate, which was violet with a pH of 4.08.

Ethanol extract of *Hibiscus sabdariffa* was acidic after treatment with ferric chloride (mordant), potassium permanganate (oxidant) and sodium carbonates (oxidant). The color of the ethanol extract of *Hibiscus sabdariffa* extracts treated with mordant and oxidants was red.

Similarly, the methanol extract of *Hibiscus sabdariffa* was acidic with slight differences after being mixed with ferric chloride (mordant), potassium permanganate (oxidant) and sodium carbonate (oxidant). The color of extracts treated with mordant and oxidants was red as shown in Table 2.

Aqueous extract of *H. sabdariffa* with sodium carbonate and sodium hydroxide: The aqueous extract of *Hibiscus sabdariffa* after being treated with sodium carbonate and sodium hydroxide, had the pH values raised to close to neutral for sodium carbonate and slightly alkaline for sodium hydroxide. The color at pH 6.19 for extract mixed with sodium carbonate, was violet and blue at pH 7.53 for mixture with sodium hydroxide.

Degree of staining of bacterial cells with extracts of *Hibiscus sabdariffa*: Table 3 shows the relative degree of staining of *Staphylococcus aureus*, *Escherichia coli*, *Streptococcus* spp. and *Bacillus cereus*, negatively stained with aqueous, ethanol and methanol extracts of *Hibiscus sabdariffa*. The results suggested that aqueous extracts achieved greater contrast of bacterial cells than ethanol and methanol extracts.

Table 1: Physical properties of extracts of *Hibiscus sabdariffa*

Extract/Distilled H_2O	pH	Color	Charge
Aqueous	3.77	Red	Negative
Methanol	3.28	Mauve	Negative
Ethanol	3.25	Mauve	Negative

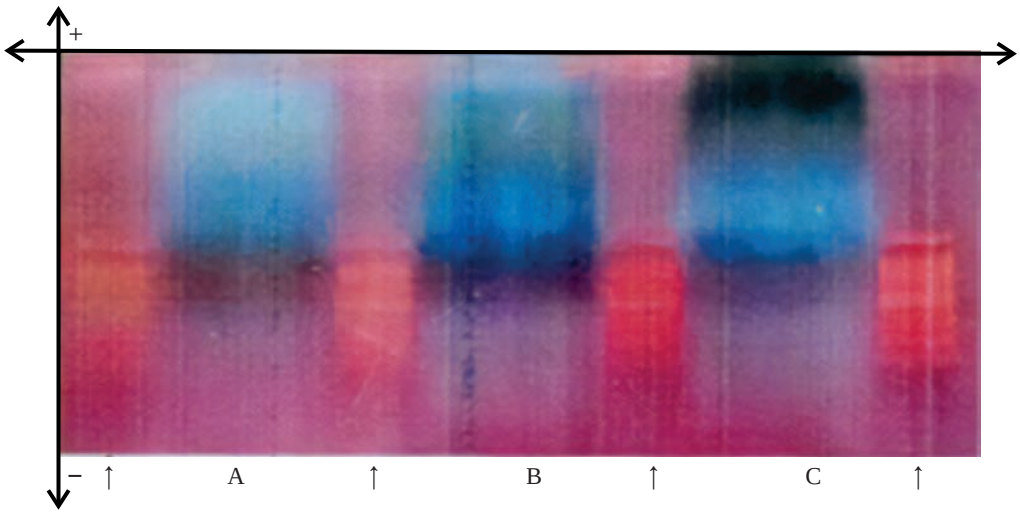

Fig. 1: Migration of charged particles of *H. sabdariffa* dye towards positive terminal
A: Aqueous extract, B: Ethanol extract, C: Methanol extract and ↑: DNA ladders (control)

Table 2: Colors and pH of extracts treated with oxidants, mordants and solvents

		Aqueous		Ethanol		Methanol	
Stain solution	Oxidant/Mordant	pH	Color	pH	Color	pH	Color
A	Na_2CO_3	4.08	Violet	3.45	Red	3.42	Red
B	$KMnO_4$	3.91	Red	3.34	Red	3.36	Red
C	No mordant/oxidant	3.77	Red	3.25	Red	3.28	Red
D	$FeCl_3$+Na_2CO_3	3.74	Red	3.24	Red	3.20	Red
E	$FeCl_3$+$KMnO_4$	3.53	Red	3.13	Red	3.17	Red
F	$FeCl_3$	3.38	Red	3.04	Red	3.06	Red

A-F: Stain solutions prepared with portions of oxidants/mordants

Table 3: Degrees of staining of bacterial cells by *H. sabdariffa* extracts

	Stained bacteria			
Extract solvent	*Staphylococcus aureus*	*Escherichia coli*	*Streptococcus* spp.	*Bacillus cereus*
Aqueous	++	++	++	++
Ethanol	+	++	+	+
Methanol	+	+	++	+

+: Slightly stained and ++: Moderately stained

DISCUSSION

Aqueous extract of *Hibiscus sabdariffa* with a pH value of 3.77 showed a clearer microscopic view of the morphologies, shapes, relative sizes and arrangements of the bacterial cells (*Staphylococcus aureus*, *Escherichia coli*, *Streptococcus* spp. and *Bacillus cereus*), than ethanol and methanol extracts which also clearly revealed the shapes, sizes and arrangements of *Escherichia coli* and *Streptococcus* spp. This was the objective of staining in the first place and was achieved. Though the cell surfaces of bacteria seemed to have repelled the stain, the stains still readily adhered to the slides leaving the bacterial cells relatively unstained and distinctly seen against the stained background.

Extracts of *Hibiscus sabdariffa* calyces appeared a deep reddish-purple after hot aqueous extraction and mauve with ethanol and methanol extraction. Anthocyanin, a compound of the flavonoid class is responsible for the color of this plant according to Rakic *et al.*[14]. The mauve color however might be due to bleaching by the alcohol solvents. The pH values of both aqueous and alcohol extracts observed in this study were also noted by Ihuma *et al.*[5], who showed that extracts of *Hibiscus sabdariffa* are acidic, with ethanol extracts being more acidic with average pH values of about 3.25. Ferric chloride increased the acidity of the stain solution by further lowering the pH values. According to Carleton *et al.*[15] natural dyes need to be ripened by oxidation either through natural means or by the addition of chemical oxidants. In this case, potassium permanganate and sodium carbonate were used. While oxidants slightly increased the pH of the stain solutions, they were not able to effectively stain bacterial cells. This could be that the ability to stain specific structures is determined by the pH values of stain, acidic structures stained by basic dyes and basic structures stained by acid dyes[16].

The various shades and changes in color of the stains might be due to the inherent instability of the anthocyanin content of *Hibiscus sabdariffa* according to Sipahli *et al.*[17]. Similarly, according to Khoo *et al.*[18], anthocyanin molecules change color depending on the pH of the environment they are in and may serve as pH indicators. Anthocyanin thus turns a reddish pink in acids of pH 1-6, reddish-purple in neutral solutions of pH 7 and green in alkaline or basic solutions with pH 8-14. The observed instability in maintaining color integrity and uniformity may decrease the desirability and use as a staining agent. It was observed that the prepared stain solution (pH 6.19-7.53) used to directly stain bacterial smears suffered non-adherence to the smears. Bacterial cells which are negatively charged and rich in nucleic acids have a higher affinity for basic dyes and will be more readily stained by solutions with neutral pH. Since extracts of *Hibiscus sabdariffa* were shown to be negatively charged and therefore acidic, the negatively charged molecules were probably repelled by the similarly charged bacterial cell walls.

In support of the foregoing, according to Jain *et al.*[1], stains are salts of bases and acids and are classified into acidic, basic, or neutral stains. Similarly, Katsikogianni and Missirlis[19] reported that bacteria are attracted to the positive ions of a stain. However, Thairu *et al.*[4] note that the pH of a dye may alter its staining effectiveness as a direct consequence of the relationship between the dye and the nature and degree of the charge of external cellular components, which change with the pH of the stain.

CONCLUSION

Extracts of readily available, low-priced and affordable *Hibiscus sabdariffa*, have been observed to be practically useful. *Hibiscus sabdariffa* can be applied as a negative staining agent, for basic cellular structures. In resource poor environments, the exploitation and use of extracts from *H. sabdariffa* as a staining agent could reduce costs and problems associated with over-dependence on toxic, expensive and environmentally harmful stains. However, it is suggested that further studies could focus on non-polar solvents for *H. sabdariffa* extraction, as well as greater refinement, which could help improve its staining properties.

SIGNIFICANCE STATEMENT

This study was carried out to investigate the suitability of unrefined pigments extracted from the calyces of a common, readily available plant, *H. sabdariffa*, for staining the cells of bacteria, an important step during their morphological study. It was found that aqueous extracts gave better visual contrast compared to alcohol extracts and could therefore be exploited in this form for this purpose. In the long run, as alternative to synthetic dyes, such a natural stain could help in protecting health and the environment.

REFERENCES

1. Jain, A., R. Jain and S. Jain, 2020. Basic Techniques in Biochemistry, Microbiology and Molecular Biology: Principles and Techniques. 1st Edn., Humana, New York, ISBN: 978-1-4939-9860-9, Pages: 282.
2. Bhuyan, R. and C.N. Saikia, 2005. Isolation of colour components from native dye-bearing plants in Northeastern India. Bioresour. Technol., 96: 363-372.
3. Willey, J.M., L. Sherwood, L.M. Prescott and C.J. Woolverton, 2008. Prescott, Harley, and Klein's Microbiology. 7th Edn., McGraw-Hill Higher Education, New York, ISBN: 9780072992915, Pages: 1088.
4. Thairu, Y., I.A. Nasir and Y. Usman, 2014. Laboratory perspective of gram staining and its significance in investigations of infectious diseases. Sub-Saharan Afr. J. Med., 1: 168-174.
5. Ihuma, J.O., G.H. Asenge, J.O.K. Abioye and S.K. Dick, 2012. Application of methanolic extracts from *Hisbiscus sabdariffa* Linn as a biological staining agent for some fungal species. Int. J. Plant Anim. Environ. Sci., 2: 254-259.
6. Morton, J.F., 2013. Fruits of Warm Climates. Echo Point Books and Media, Brattleboro, Vermont, ISBN-13: 9781626549760, Pages: 550.
7. McKay, D.L. and J.B. Blumberg, 2006. A review of the bioactivity and potential health benefits of chamomile tea (*Matricaria recutita* L.). Phytother. Res., 20: 519-530.
8. Akaakohol, M.A. and G.C. Aye, 2014. Diversification and farm household welfare in Makurdi, Benue State, Nigeria. Dev. Stud. Res., 1: 168-175.
9. Ma'aruf, S.Y., M.O. Mohammed, A.T. Muhammad, O.O. Okechi and R.I. Tsamiya *et al*., 2020. Staining property of alcoholic and aqueous *Hibiscus sabdariffa* extract in demonstration of selected bacteria in tissue sections of Wistar rats. Microbiol. Res. J. Int., 30: 26-33.
10. Abubakar, A.R. and Mainul Haque, 2020. Preparation of medicinal plants: Basic extraction and fractionation procedures for experimental purposes. J. Pharm. BioAllied Sci., 12: 1-10.
11. Redfern, J., M. Kinninmonth, D. Burdass and J. Verran, 2014. Using Soxhlet ethanol extraction to produce and test plant material (essential oils) for their antimicrobial properties. J. Microbiol. Biol. Educ., 15: 45-46.
12. Avwioro, O.G., P.C. Aloamaka, N.U. Ojianya, T. Oduola and E.O. Ekpo, 2005. Extracts of *Pterocarpus osun* as a histological stain for collagen fibres. Afr. J. Biotechnol., 4: 460-462.
13. Yashroy, R.C., 1990. Lamellar dispersion and phase separation of chloroplast membrane lipids by negative staining electron microscopy. J. Biosci., 15: 93-98.
14. Rakić, V.P., A.M. Ota, Š.F.M. Bornšek, M.N. Miljković, D.T. Sokolović and N.E.P. Ulrih, 2014. The colour and stability of cyanidin and cyanidin 3-O-β-glucopyranoside. Adv. Technol., 3: 5-9.
15. Carleton, H.M., R.A.B. Drury, 1967. Carleton's Histological Technique. 4th Edn., Oxford University Press, Oxford, England, ISBN: 9780192612052, Pages: 432.
16. Braide, W., C. Akobundu, R.N. Nwaoguikpe and L.C. Njiribaeko, 2011. The use of extracts from four local Nigerian plants for the staining of selected bacteria and moulds. Afr. J. Microbiol. Res., 5: 79-86.
17. Sipahli, S., V. Mohanlall and J.J. Mellem, 2017. Stability and degradation kinetics of crude anthocyanin extracts from *H. sabdariffa*. Food Sci. Technol., 37: 209-215.
18. Khoo, H.E., A. Azlan, S.T. Tang and S.M. Lim, 2017. Anthocyanidins and anthocyanins: Colored pigments as food, pharmaceutical ingredients, and the potential health benefits. Food Nutr. Res., Vol. 61. 10.1080/16546628.2017.1361779.
19. Katsikogianni, M. and Y.F. Missirlis, 2004. Concise review of mechanisms of bacterial adhesion to biomaterials and of techniques used in estimating bacteria-material interactions. Eur. Cells Mater., 8: 37-57.