Scientific Research Publishing

Advances in Microbiology, 2020, 10, 478-491
https://www.scirp.org/journal/aim
ISSN Online: 2165-3410
ISSN Print: 2165-3402

Comparison of Antioxidant and Antimicrobial Activities of Acetone and Water Extracts of *Theobroma cacao* Beans

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How to cite this paper: Dogbey, B.F., Ibrahim, S. and Abobe, J.A.E. (2020) Comparison of Antioxidant and Antimicrobial Activities of Acetone and Water Extracts of *Theobroma cacao* Beans. *Advances in Microbiology*, **10**, 478-491.
https://doi.org/10.4236/aim.2020.109036

Received: August 12, 2020
Accepted: September 26, 2020
Published: September 29, 2020

Abstract

This present study compared antioxidant and antimicrobial activities of acetone and water extracts of *Theobroma cacao* beans against *Escherichia coli*. Total phenolic content (TPC) in both extracts was estimated by the Folin-Denis reagent. The present study showed that the 70% (v/v) acetone extract had a higher extraction yield and TPC (37% and 109 mg TAE g^{-1} dry weight) than the water extract (33% and 76 mg TAE g^{-1} dry weight). The antioxidant activities of both extracts were estimated by the DPPH Scavenging Assay. The extract obtained using 70% (v/v) acetone showed higher antioxidant activity (54%) compared to the antioxidant activity obtained using water (34%). Antimicrobial activities of acetone and water extracts from *Theobroma cacao* were measured against *Escherichia coli* and were screened by agar well diffusion method and further confirmed with the disc diffusion method. The bacterial growth was measured in Mueller Hinton agar. The extracts inhibited the growth of the *Escherichia coli* cultured, and the acetone extracts showed antimicrobial capacity comparable or equivalent, as seen in commercial ampicillin.

Keywords

Theobroma cacao, *Escherichia coli*, Antioxidant Activity, Total Phenolic Content, DPPH Scavenging Activity

1. Introduction

Enteropathogenic *Escherichia coli* (*E. coli*) continue to put human health at risk worldwide. *E. coli* is the common bacteria that reside in the intestines of humans and animals. This bacterium is acquired by consuming contaminated food. Individual-to-person transmissions occur when the individuals infected do not wash

their hands after using the toilet [1]. *Escherichia coli* are critical causes of infectious diarrhoea, specifically amongst infantile populations [2]. It can cause infection at a dosage as low as 10 - 100 cfu/g [3] [4]. The World Health Organization (WHO) reported that annually, diarrhoea killed 1.9 million people, including 760,000 children under the age of five [5]. It is the third cause of death in children below five years in Ghana, killing over 10,000 every year [6].

E. coli infectious diseases are mainly treated to mitigate symptoms by administering antibiotic agents that contain beta-groups, aminoglycoside, and fosfomycin [7]. However, there have been reported resistance of *E. coli* towards ampicillin, amikacin, aminoglycosides, cephalosporins, fosfomycin, and fluoroquinolones [8] [9] [10]. Studies have shown that bacterium forms a gene capable of overcoming antibiotics used widely and available on the market [11]. Therefore, alternative antimicrobial agents are needed to address this problem. For these reasons, International organizations like the World Health Organization have supported studies on the prevention and treatment of diarrhoeal diseases utilizing traditional medical strategies with high effectiveness and lower toxicity [12].

In the last decade, medicinal plants have proven to demonstrate antimicrobial potency against various human pathogens due to the presence of polyphenols [13]. Cocoa beans (seeds of *Theobroma cacao*) are known to be rich in polyphenols [14], and extracts could be a potential source of agents for antibacterial activities. Polyphenols measure the most category of antioxidants in unfermented cocoa beans, and that, they account for roughly 2% w/w of the bean and its products contain the main categories of phenolic resin compounds like flavanols (37%) proanthocyanidins (58%), and anthocyanins (4%) dimers which play a necessary role in conserving human health [15].

Lately, some antioxidant components of cocoa liquor prepared from cocoa beans have been characterized as procyanidins oligomers and flavan-3-ols [16]. Tannins are soluble polyphenolic metabolites of relatively high molecular weight and usually complex with some proteins and carbohydrates [17]. Tannins are bitter and have an astringent property that causes the drying and pucker taste in the mouth when the unripe fruit is taken [18]. Several health benefits have been attributed to tannins, including antimicrobial, antiviral, antimutagenic, and antidiabetic properties [19]. Also, *Terminalia citrina* Roxb (*Combretaceae*), commonly used as a traditional diarrhoea medicine in Thailand, shows antimicrobial activity of tannins, including corilagin, 1,3,6-tri-o-galloyl-β-D-glucopyranose, 1,2,3,4,6-penta-o-galloyl-β-D-glucopyranose, and chebulagic acid [20]. Leaf extract of *Galliandra portonricensis*, the herbal drug for gastrointestinal disorders in Southern Nigeria, contains tannins with antimicrobial effect against *E. coli*, *Staphylococcus aureus,* and *Streptococcus faecalis* [21]. Condensed tannins from cocoa extract at 0.25 g/L are reported to repress the germination of basidiospores of *Crinipellis perniciosa* [22]. Condensed tannins from strawberry also inhibited the growth of *Botrytis cinereal* [23]. The antimicrobial activity of tannins was attributed to the ester linkage of phenolic acids with a polyol, usually glucose [24]. Understanding the mechanism(s) involved in increasing the tannin component's

antimicrobial activity and evaluating the synergistic/antagonistic effects of these components will improve the ability to add tannins to the food system. More work was required to investigate the mode of action of tannins against micro-organisms, particularly the relationship between structure and behaviour of each tannin component. 70% (v/v) ethanol-water extracts of cocoa showed antibacterial activity against *Bacillus subtilis* and was used in the treatment of intestinal ailments by the ancient Aztec culture [25]. Cocoa's antioxidants are readily absorbed by the human body and are more stable and efficient than any other food [26]. Also, cocoa yields different antibacterial activity when extracted with solvents with different polarities. A large number of studies utilized several different solvents, including water, 70% (v/v) ethanol, 70% (v/v) methanol, acetonitrile, and diethyl ether [27] for extracting plant materials. Industries mostly used 70% (v/v) ethanol, but its antibacterial material poses health risks specifically in the liver due to its residual concentration and ethanol exposure [27]. Water is a protic solvent that possesses a robust swelling ability that contributes to its ability to generating higher residual phenolic compounds from plant materials [28]. Generally, a cocoa decoction is prepared in boiling water in the local setting.

Thus, the objective of this study was to determine the antioxidant and antimicrobial capacities of both water and acetone extracts of *Theobroma cacao* beans against *Escherichia coli* to develop new antimicrobials that are naturally derived, therapeutically affirmed and cost-effective to produce that can guarantee a consumer desire for natural food products.

2. Materials and Methods

2.1. Sample Collection, Preparation, and Extraction

Non-fermented cocoa (*Theobroma cacao* L.) beans (cv. Forastero) were purchased from a farmer at Subriso (6˚5'0" North, 1˚15'0" West), a community in the Ashanti Region of Ghana. The beans were harvested in February 2019. The samples were collected at a level of ripeness between 3.3˚ Brix and 5.6˚ Brix. The harvested pods were broken to recover the beans. The beans were freeze-dried and milled with Moulinex domestic blender. The blending machine was stopped intermittently after every 20 seconds of milling.

2.2. Extraction

2.2.1. Using Aqueous Acetone

Bioactive rich extracts were obtained by placing 1 g of the cocoa powder into three different Duran bottles, and 20 ml of 70% (v/v) acetone was added. The mixture in the bottle was placed on Universal Mechanical Table Shaker 709 at 180 rpm for 20 minutes. The resulting mixture was then transferred into centrifuge tubes and centrifuged at 4000 rpm using L530 Centrifuge instrument for 5 minutes. The supernatant was further filtered with Whatman No. 1 filter paper and volume of filtrate recorded with a measuring cylinder. Then, it was transferred into 50 ml falcon tubes and stored at −4˚C for further analysis. The solid

residue was collected and allowed to dry at 70˚C in an oven for 24 hours and weight determined using an electronic balance.

2.2.2. Using Water

1 g of the cocoa powder was put into three different Duran bottles, and 20 ml of distilled water at room temperature was added. The bottle containing the mixture was placed in a water bath at 70˚C for 60 minutes. The resulting mixture was then taken through similar processes as in the aqueous acetone extract.

The extraction yields of both aqueous and acetone solvents treatments were determined as described by Zhang *et al.* [29] with slight modifications. The extracts were lyophilised using Telstar lyoquest laboratory freeze-dryer to obtain dried extracts. The extraction yield was expressed as a percentage of the crude extract's weight to the raw material according to the formula:

$$\text{Extraction yield}(\%) = \frac{\text{Weight of the freeze} - \text{dried extract}}{\text{Weight of the original sample}} \times 100$$

2.3. Total Phenolic Content

The total soluble polyphenolic contents of the aqueous and acetone extracts of cocoa beans were determined by the Folin-Denis method as described by King and Heath and Allen *et al.* [30] [31] with some modifications. Tannic acid was used as standard and the calibration curve ($y = 0.09269x + 0.01080$, $R^2 = 0.9960$ (**Figure 1**)) was plotted by mixing 1 ml aliquots of 0, 1, 2, and 4 mg/ml tannic acid solutions with 2.5 ml of Folin-Denis reagent and 10 ml of 17% sodium carbonate solution. The mixtures were stored in the dark for 30 minutes. The absorbance for both aqueous and acetone extracts was measured with Spectrumlab 23_A spectrophotometer at 760 nm. The experiments were carried out in triplicates and the results expressed in terms of mg of tannic acid equivalents (TAE) per 1g dry weight of sample.

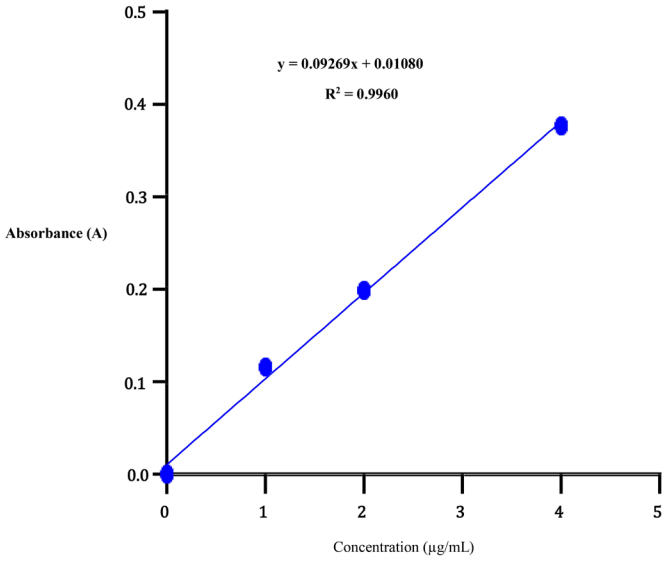


Figure 1. Tannic acid standard curve.

2.4. Identification and Quantification of Tannins by High Performance Liquid Chromatography (HPLC)

Separation, identification and quantification of tannins were done using A Cecil-Adept*Binary Pump HPLC with a CE 4300 WaveQuest DAD fast scanning detector. The separation was done on Zorbax C18 column (250 mm × 4.6 mm and 5 µm particle size) from Agilent operated at temperature 30˚C. The mobile phase consisted of HPLC water as eluent A and pure methanol as eluent B. The flow rate was set at 1 ml/min. and absorbance readings were taken at wavelength of 280 nm and the results were projected on a screen as a chromatogram. Identification of tannins was done based on retention times and UV spectra of standard.

2.5. DPPH Scavenging Activity

The free radical scavenging activity of both aqueous and acetone extracts was evaluated by 1,1-diphenyl-2-picryl-hydrazyl (DPPH) according to the method previously reported by Chu *et al.* [32] with slight modifications. 1 ml of both extracts at different concentrations (50 µg/ml, 100 µg/ml, 200 µg/ml, 400 µg/ml, 800 µg/ml) was taken in a test tube and 3 ml of methanol solution of DPPH was added. The mixtures were shaken vigorously and stored in the dark for 30 minutes for the reaction to be completed. The absorbance of the acetone and aqueous solutions were measured at 517 nm using a spectrophotometer (Spectrumlab 23_A) against methanol and distilled water as blank respectively. The samples were assayed in triplicates and compared with ascorbic acid as reference standard. 3 ml of DPPH solution was used as a negative control against a methanol and water blank. The capability of scavenging the DPPH radical was calculated by using the formula:

$$\text{DPPH scavenging effect}(\%) = 1 - \frac{\text{Absorbance of sample at 517nm}}{\text{Absorbance of control at 517nm}} \times 100$$

A linear regression analysis was carried out for calculating the inhibitory concentration of sample required to scavenge DPPH radical by 50% (IC_{50} value).

2.6. Antimicrobial Activity

The effect of the tannins extract on *E. coli* was assayed by agar well diffusion method and further confirmed by Disc diffusion method.

2.6.1. Sterilization of Equipment and Media Used

The work bench was disinfected with absolute ethanol. The prepared media, distilled water and pipette tips were autoclaved at 121˚C for sterilizing for 15 minutes.

2.6.2. Preparation of the Extracts

Both of the lyophilized cocoa bean extracts were dissolved in water and DMSO solutions to concentrations of 5, 50 and 100 mg/ml.

2.6.3. Preparation of Mueller Hinton Agar

19 g of commercially available Mueller Hinton agar was dissolved in 500 ml of distilled water. It was brought to boil to completely dissolve by continuous stirring and was sterilized by autoclaving at 15 lbs pressure for 15 minutes at 121˚C. The media was left to cool and stored at 4˚C.

2.6.4. Preparation of *E. coli* Culture

9 ml of an already prepared nutrient broth was taken into a sterilized test tube. 1 ml of *E. coli* culture was added and incubated at 37˚C for 24 hours.

2.6.5. Well Diffusion Method

The antibacterial activities of both extracts were determined by agar well diffusion technique described by Cheesbrough [33]. 25 ml of MHA (Mueller Hinton Agar) was poured into 100 mm sterile plates and allowed to cool. The MHA plates were seeded with 100 µl of *E. coli* isolate. The *E. coli* inoculum was spread evenly over the plates with a sterile metal spreader. Using a standard sterile cork borer of 6 mm diameter, four uniform wells were made on the surface of the agar seeded with *E. coli* culture. 100 µl of extracts were introduced into the wells using a micropipette. 1 of the wells in each plate was filled with 250 mg of commercial ampicillin
(2S,5R,6R)-6-([(2R)-2-amino-2-phenylacetyl]amino)-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2- carboxylic acid as a control. The plates were kept for 30 minutes at room temperature to allow diffusion of the extracts. The plates were then incubated at a temperature of 37˚C for 24 hours. The diameter of the zone of inhibition (>6 mm) at three different angles was measured with a pair of callipers after the incubation period. The mean of those measurements was taken. Tests were performed in duplicate.

2.6.6. Disc Diffusion Method

25 ml of MHA was poured into 100 mm sterile plates and allowed to cool. The MHA plates were seeded with 100 µl of *E.* coli isolate. The *E. coli* inoculum was spread evenly over the plate with a sterile metal spreader. Filter paper discs (Whatman no. 1) of approximately 6 mm in diameter were soaked in 15 µl of both extracts and placed on the previously prepared agar plates. Each disc was pressed down to ensure complete contact with the agar surface and distributed evenly so that they are not closer than 24 mm from each other, center to center. The agar plates were then incubated at 37˚C for 24 hours. The diameters of the complete inhibition zones were measured, including the diameter of the disc where commercial ampicillin was used as control.

3. Statistical Analysis

The experimental results were expressed as mean + standard deviation (SD) of three replicates. IC_{50} values (concentration at which 50% inhibition achieved) were obtained from a linear regression analysis. Data were analysed with statistical software GraphPad Prism for windows (version 8.1.2).

4. Results and Discussion

The mean moisture content of three batches of unfermented cocoa bean samples by oven drying (**Table 1**) was 50.35 + 2.03 g/100g, which is in agreement with [34]. Maximum care and protocols were observed to obtain quality results. Samples were freeze-dried to maintain the active ingredient, polyphenols and to guarantee the quality of the extracts. Freeze drying produces a dried product that is free from biological and chemical activities [35]. Freeze drying maintained the highest total polyphenol content compared with drying by exposure to hot air and sun drying [36]. The mean percent moisture content determined by the freeze-drying method was 86.26 g/100g of sample as shown in **Table 1**. This value was in agreement with what has been reported in literature [35] as moisture content of unfermented cocoa varies between 82 - 87 g/100g of sample.

The results for extraction yields and total phenolic contents of both aqueous and acetone extract are presented in **Table 1**. This study was aimed for maximum extraction. The yields of polyphenolic extracts and the resulting biological activity of plant matter are greatly influenced by the nature of the extracting solvent due to the presence of different bioactive compounds of different chemical properties and polarities that may or may not be soluble in a given solvent [37]. Organic solvents appear more efficient since phenolic compounds are more soluble in polar organic solvents due to the presence of a hydroxyl group, it has been proposed that acetone-water mixtures provided better outcomes in the production of procyanidins and phenols relative to other solvents. Some authors [38] defended the use of 70% (v/v) acetone in competition with 50% (v/v) methanol with reference to Bate-Smith [39] that 70% (v/v) acetone could extract certain types of condensed tannins with ease which was also confirmed by several researchers [40] [41] [42]. Thus, polarity differences coupled with safety reasons informed the choice of the two solvents used in the study.

The total phenolic contents were quantified for both extracts (**Table 1**). The water and 70% (v/v) acetone extracts contained 75.70 $mg{\cdot}g^{-1}$ and 108.97 $mg{\cdot}g^{-1}$. Few authors have reported that the total phenolic content of cocoa beans range between 67 and 149 and from 101 to 144 $mg{\cdot}g^{-1}$ for freshly harvested and two days fermented cocoa beans [43]. The least polar solvent (acetone) extracted significantly ($p < 0.05$) higher amount of phenolics (TP = 108.97 mg TAE-1 dry weight) than the most polar solvent (TP = 75.70 mg TAE-1 dry weight). The yield of cocoa extract using acetone solvent was insignificantly different ($p > 0.05$) from water, 37.39%, and 33.49%, respectively. The extraction yield was consistent with what has been reported previously [44] [45], who stated that the amount of phenolic compounds in organic extracts was higher than in water extracts. However, the approach suffers from the limitation that cocoa beans were not defatted prior to extraction. The fat in the beans interfered with the solubilizing activity of the solvent, and the polyphenolic content in recovered was considerably reduced [46]. Moreover, from a toxicological point of view, water is safer than acetone and other organic solvents [47]. A good correlation between

Table 1. Composition and Properties of cocoa beans.

Cocoa bean Extract by	Composition and Properties of cocoa beans						
	Moisture Content (%)	Dry Matter Content (g/100g water)	Extraction yield (%)	Total Phenolics (mg TAE g^{-1} dry weight)	Total tannin (mg TAE g^{-1} dry weight)	DPPH Scavenging Activity (%)	IC_{50} (mg/ml)
Water	86.26 ± 0.89	13.74 ± 0.89	33.49 ± 0.16	75.70 ± 4.12	12.52 ± 1.90	53.92	1.3517
70% (v/v) Acetone			37.39 ± 1.18	108.97 ± 12.55	94.99 ± 7.85	34.31	0.6966

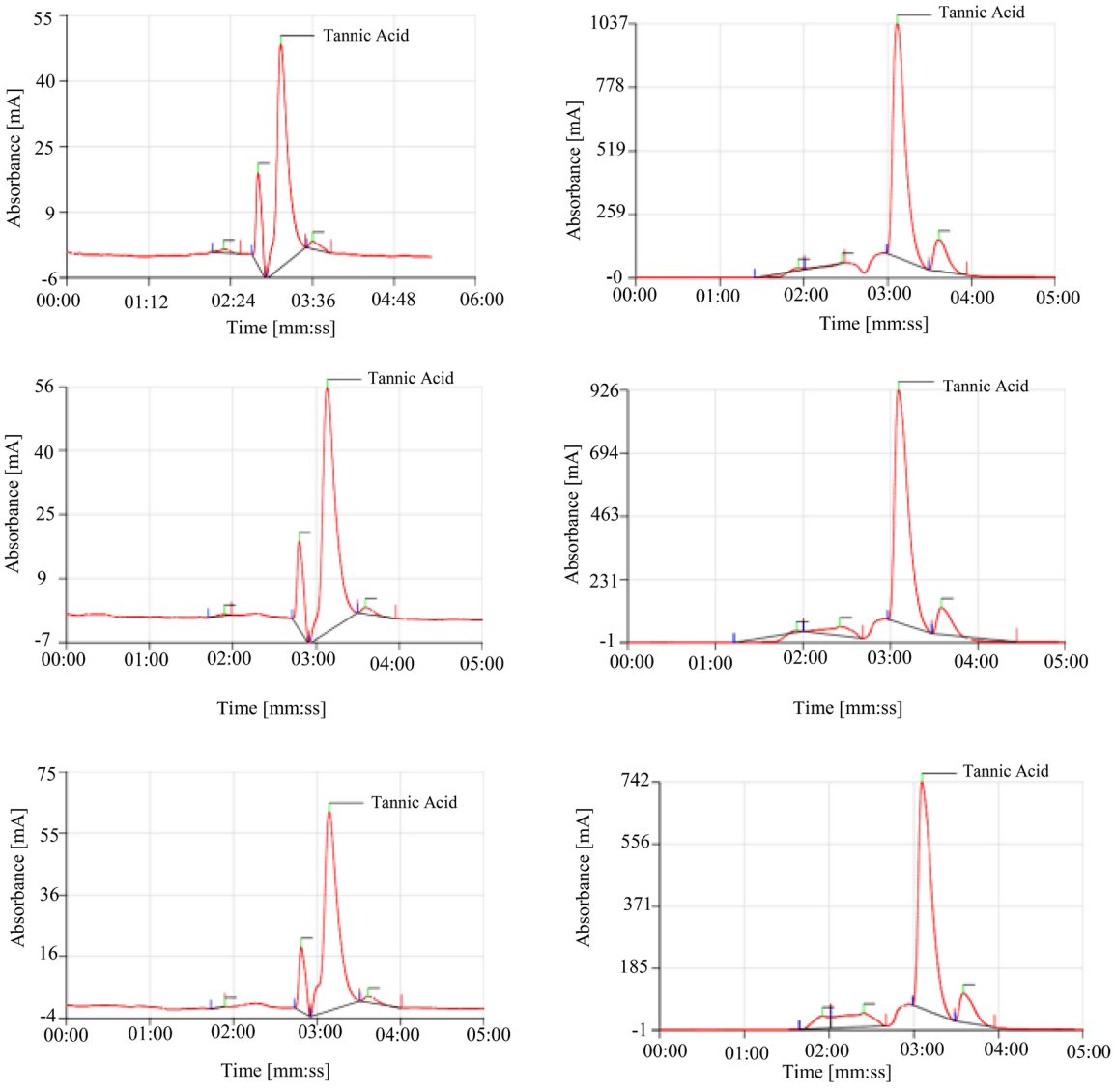
total phenolics and antioxidant capacity in cocoa beans and their products have been demonstrated [48]. Although total phenolic content is a useful indicator of potential nutritional benefit, the tannin content was also determined by HPLC as shown in **Table 1** since herbs with tannins as their main components are naturally astringent and are used to treat intestinal disorders such as dysentery and diarrhoea [49]. A typical HPLC profile for triplicate replications of acetone and water extracts is shown in **Figure 2**. It was not possible to assign identities to these peaks as standards were not readily available.

Phenolic compounds have redox properties, which allow them to act as antioxidants. The DPPH assay measures the antioxidant activity of water-soluble phenolics. **Figure 3** shows the dose-response curve of DPPH radical scavenging activity of aqueous and acetone extracts of cocoa beans compared with ascorbic acid. It was observed that the acetone extract had higher activity than water. At a concentration of 400 ug/ml, the scavenging activity of acetone extract reached 42.16%, but at the same concentration, that of water extract was 4.90%. A high antioxidant activity could also be due to other compounds besides phenolics, which are also soluble in water and acetone. Although the DPPH radical scavenging capacities of both extracts were significantly lower than that of the ascorbic acid, it was evident that the extracts possess some antioxidant abilities.

The lower the IC_{50} value of a compound, the higher its antioxidant activity [50]. Thus, the acetone extract of the cocoa beans possesses a stronger ability to scavenge DPPH radical as compared to water extract. Although the DPPH scavenging assay is a widely used method for measuring antioxidant activity, it is pertinent to use different assays in an efficient medium, instead of relying on a single assay to assess and compare the antioxidant activity.

The cocoa's antibacterial activity showed that extracts demonstrated an antimicrobial effect against the test isolate with higher activity in acetone extract than aqueous extract. The cocoa beans used in this study were unfermented and lyophilised to obtain a higher antibacterial capacity. The maximum zone of inhibition was shown by acetone extracts, while water extracts were shown by a lower zone of inhibition. This may be due to the better solubility of the active components in the organic solvent than water, which leads to better efficacy of the acetone extracts. It suggests that the active component was more soluble in

acetone than in water, increasing its sensitivity to cultured E. coli. The result obtained from this work also indicated that *E. coli* was sensitive to both extracts. This was further confirmed by the disc diffusion method (**Figure 4**). These results were probably achieved because *E. coli* is a gram-negative bacterium that has an outer membrane that can be penetrated by polyphenols to mediate cell response [7]. The results obtained also showed that the acetone extract reconstituted in DMSO showed inhibition zone diameter of 8, 5, and 6 mm at 100, 50 and 5 mg/ml, respectively, with 100 mg/ml showing equivalent or comparable antimicrobial capacity as seen in the commercial ampicillin. At both 50 and 100 mg/ml of water extract reconstituted in DMSO produced significant zones of inhibition against Escherichia coli because water is a solvent that can maintain a high quantity of essential compounds contained in natural materials [51].



(a) Chromatogram of acetone extract (1 in 100 dilution) (n=3)

(b) Chromatogram of water extract (n=3)

Figure 2. Chromatogram of extracts (triplicates).

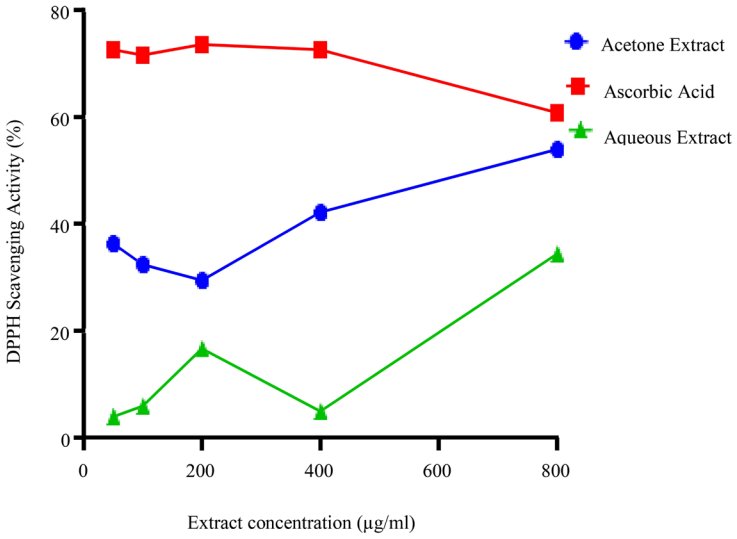
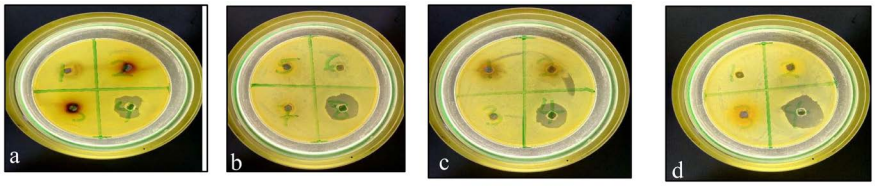
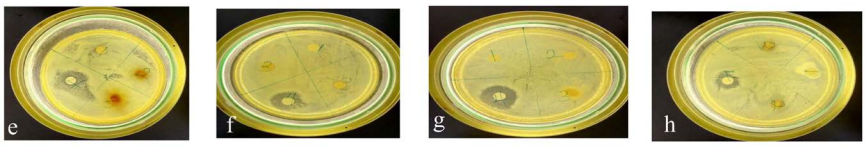


Figure 3. DPPH Scavenging Activity of cocoa bean extracts in different solvents.



Extracts of cocoa bean produce zones of inhibition at different concentrations with disc diffusion method



Extracts of cocoa bean produce zones of inhibition at different concentrations with disc diffusion method

Figure 4. Antibacterial activity of cocoa extracts. (a) Results of 70% (v/v) acetone extract reconstituted in DMSO against *E. coli* bacteria at 3 concentrations as well as the control grown in MHA. (b) Results of water extract reconstituted in DMSO against *E. coli* bacteria at 3 concentrations as well as the control grown in MHA. (c) Results of 70%(v/v) acetone extract reconstituted in water against *E. coli* bacteria at 3 concentrations as well as the control grown in MHA. (d) Results of water extract against *E.* coli bacteria at 3 concentrations as well as the control grown in MHA. (e) Results of 70% (v/v) acetone extract reconstituted in DMSO against *E. coli* bacteria at 3 concentrations as well as the control grown in MHA. (f) Results of water extract reconstituted in DMSO against *E. coli* bacteria at 3 concentrations as well as the control grown in MHA. (g) Results of 70%(v/v) acetone extract reconstituted in water against E. *coli* bacteria at 3 concentration as well as the control grown in MHA. (h) Results of water extract against *E. coli* bacteria at 3 concentrations as well as the control grown in MHA.

5. Conclusion

The present study has shown that 70% **(v/v)** acetone cocoa beans extract exhibited a higher extraction yield, phenolic and total tannin content, antimicrobial and antioxidant activity than cocoa beans water extract. This study also empha-

sizes the use of cocoa bean extracts as nutraceuticals to treat diseases caused by Escherichia coli. Nonetheless, pharmacological investigations are still needed to elucidate more fully the effect of other active compounds present in cocoa polyphenols and its associative effect.

Acknowledgements

We thank Mr. Anthony Abutiate, Mr. Vincent Torve, and Miss Angela Andoh for their expert technical assistance, critical review of methods, discussions, and suggestions.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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