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EVALUATION OF THE ANTIOXIDANT POTENTIALS OF TEN LEAFY VEGETABLES EXTRACTS COMMONLY CONSUMED BY THE GHANAIAN POPULATION

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ABSTRACT

Ten traditional leafy vegetables commonly consumed by Ghanaians have been evaluated for their antioxidant potential based on their polyphenolic and flavonoid contents. Among the plants studied the methanol extracts of *Ocimum basilicum* (akokobesa), and *Amaranthus incurvatus* (aleefo) exhibited the highest phenolic content of 16.4 mg GAE/g_{dw} and 11.3mg_{dw} GAE/g respectively. The highest phenolic content for water extracts were seen in *Manihot esculenta* (cassava; 9.29mg GAE/g_{dw}) and *Hibiscus sabdariffa* (shuuré; 7.28mg GAE/g $_{dw}$) and C esculanta (7.11 mg GAE/g_{dw}). The methanol extracts of *H. sabdariffa* (Shuuré*)*, *Vernonia amygdalina* (bitter leaves), *Manihot esculenta* (cassava leaves) and *Ocimum basilicum* (akokobesa) recorded the highest flavonoid content (FC) of 99.14 µg QE/g_{dw}, 70.20µg QE/g_{dw}, 70.08µg QE/g_{dw} and 63.37µg QE/g_{dw} respectively. For the FC of the aqueous extracts the order was; *A. incurvatus > H. sabdariffa > Talinum triangulare> Colocasia esculenta > M. esculenta > V. amygdalina> O. basilicum > Solanum macrocarpon > Launaea taraxacifolia > Corchorus olitorius*. A good positive correlation r^2= 0.663 was observed between polyphenolic content and antioxidant values for the aqueous extracts, however, no correlation was found between flavonoids, polyphenolics and total antioxidants. The study indicates that the leafy vegetables consumed by Ghanaians are potentially rich sources of dietary polyphenolic compounds and antioxidants, and might contribute important health and nutraceutical benefits to consumers.

KEYWORDS

Phytochemicals, antioxidants, free-radicals, health benefits, Leafy vegetables.

INTRODUCTION

While the vast majority of complex life on earth requires oxygen for existence, a paradox of metabolism is that, oxygen is a highly reactive molecule that damages living organisms by producing reactive oxygen species [1-3]. Consequently, organisms contain a complex network of enzymes and antioxidant metabolites that work together to prevent oxidative damage to cellular components such as DNA, proteins and lipids [2, 4, 5]. In general, antioxidant systems either prevent these reactive species from being formed, or remove them before they can damage vital components of the cells. The properties underlying the activities of these antioxidants towards free radicals and their scavenging effects relate particularly to their abilities to donate electrons or hydrogen atoms and to their relative propensities to undergo oxidation. They may also act through forming radical adducts which can undergo bimolecular decay. Generation of oxygen radicals such as superoxide and hydroxyl and non-radical species like hydrogen peroxide and singlet oxygen is associated with cellular metabolic injury, accelerated aging, cardiovascular diseases, neurodegenerative diseases and inflammation [3, 6]

Reactive oxygen species also do have useful functions in cells such as redox signaling; hence the function of antioxidant system is not to remove oxidants entirely, but to keep them at an optimum level. Humans and all animals have complex antioxidant defence systems but they are not perfect and oxidative damage will occur [7-9]

Much of the protective effects of fruits and vegetables have been attributed to their phytochemical content and compelling evidence exist to show that diets rich in fruits and vegetables can lower the risk of coronary heart disease, stroke, cancer and enhanced life expectancy [10-13]. Fruits and leafy vegetables are capable of reducing these disease risks because they have low fat content and good are sources of vitamins, minerals and fiber. They are also rich sources of potentially bioactive compounds known as phytochemicals. [14]. These secondary metabolites are produced in the plants to play countless supplemental functions in their life cycle including protection against UV radiation or physiological damage by pathogens [15]. Recent findings indicate that these secondary plant metabolites can also protect humans against diseases. Most phytochemicals have antioxidant activity and can protect cells against oxidative damage and reduce the risk of developing certain type of cancers. Since fruits and vegetables are high in antioxidants, a diet rich in these substances should help alleviate oxidative stress, retard the development of chronic diseases and slow aging.

Free radicals impact negatively on health by causing several diseases including cancer, hypertension, heart diseases and diabetes [3, 16]. These free radicals are generated during normal body metabolism. Though the human body produces its own antioxidants such as ubiquinone (Co-enzyme Q) and glutathione, the amounts may be augmented by external sources. Exogenous intake of antioxidants can help the body scavenge free radicals effectively. Numerous epidemiological studies have confirmed significant relationship between high intake of flavonoids and decrease in the risk of developing cardiovascular diseases [11, 17, 18] and other degenerative diseases.

The importance of Ghanaian indigenous vegetables as rich sources of proteins, minerals and vitamins has long been recognized. However, data on their antioxidant capacities, flavonoid and phenolic contents is scant. Therefore, the aim of this study was to assess ten traditional leafy vegetables commonly consumed by Ghanaians for their antioxidant potentials based on their polyphenolic and flavonoid contents.

MATERIALS AND METHODS

CHEMICALS

Folin-Ciocalteu phenol reagent (FCR), Gallic acid, 2, 2-diphenyl-1-picrylhydrazyl hydrate ($DPPH^{\bullet}$), quercetin and ascorbic acid (vitamin C) were from Sigma. $AlCl_3$, Na_2CO_3, CH_3COOK, were from Merck (Darmstadt, Germany). Ethanol and methanol were from Jansen Chimica (Beerse, Belgium). All chemicals were of analytical grade.

EXTRACTION PROCESS

Fresh vegetable leaves obtained from farmers and authenticated at the Ghana Herbarium were cut into pieces, frozen under liquid nitrogen and freeze dried (Tokyo Rikakikai Co. Ltd., Tokyo Japan). Lyophilized samples were pulverized, vacuumized, and stored at 4ºC till required. 2.5 grams of each pulverized sample were extracted in 40 ml of 96% methanol (cold percolation) for 24 hours. The extracts were centrifuged at 8500g for 10 min and supernatants recovered. Additional 20 ml of methanol was used to re-extract the plant residue and the supernatants pooled. The procedure was repeated using distilled water instead of methanol as solvent. Solid to solvent ratio of 1: 24 was a modification of the 1:3 and 1: 10 ratios as reported previously [19, 20]. The MeOH (MEL) and aqueous (WEL) extracts of leaf samples water stored in 50ml polypropylene tubes at 4 ˚C until needed.

EXTRACTION YIELD (EXTRACTABLE SOLVENTS)

Extraction yields were established for each solvent by evaporating off two (2) ml of each extract to dryness and measuring the solid residue to establish the amount of extractable solids (extraction yield). Each determination was performed in triplicate.

PHENOLIC CONTENT

The polyphenolic contents (PC) were measured by the Folin-Ciocalteu method using Gallic acid as standard [21] with modifications. Briefly, 50µl each of MEL and WEL were mixed with 3ml of distilled water (dH_2O) and 250µl of FCR. The mixtures were allowed to stand for 5 min, and then 750µl of 20% Na_2CO_3 was added. After incubation of the resulting reaction mixtures for 30 min at room temperature absorbance values were measured at 760nm using a UV-VIS Spectrophotometer (Shimadzu, 1201, Japan). All determinations were performed in triplicate. A calibration curve was prepared using serial dilutions of 5mg/ml, 10mg/ml, 15mg/ml, 20mg/ml and 25mg/l from a stock solution of 1000 mg/ml gallic acid dissolved in methanol. 50µl each of these solutions was treated like the samples and a calibration linear regression equation $y = 0.399x + 0.037$ ($r^2 = 0.999$) developed. [y = absorbance and x = gallic acid conc. (mg/ml)].

The polyphenolic content in each extract were calculated from the calibration curve and final results were recalculated and expressed as gallic acid equivalents per gram of dry leafy vegetable sample (mg GAE/g_{dw}) using the following equation:

$$c = \frac{c_p \cdot V_o}{m_u \cdot Dm} \cdot 100 \text{ [mg GAE/g}_{dw}\text{]} \qquad (1)$$

Where: c = concentration of total polyphenol calculated on dry basis; c_p = concentration total polyphenol in mg GAE/L; v_o = volume of solvent (L); m_u = mass of sample used and D_m = dry matter content.

FLAVONOID CONTENT

The aluminum chloride colorimetric assay method [22] was employed to evaluate total flavonoid content (TFC) in the samples using quercetin as standard. 500µl of extracts were mixed with 1500µl of 99.9% ethanol (EtOH), 100µl of 1 M potassium acetate, 100µl of 10% aluminum chloride and 3000µl of distilled water. The resulting mixtures were incubated for 30 minutes at room temperature and corresponding absorbance measured at 415 nm. All determinations were carried out in triplicates. A standard calibration curve was constructed using quercetin standard solutions of 12.5µg/ml, 25µg/ml, 50µg/ml, 75µg/ml and 100µg/ml. 500µl of each standard was treated in the same manner as the samples above and calibration linear regression equation $y = 0.062x + 0.011$ ($r^2 = 0.999$) generated. Flavonoid content of each extract were determined from the curve and the final results recalculated and expressed as microgram quercetin equivalent per gram of dry leafy vegetable sample (µg QE)/g_{dw}).

DPPH FREE RADICAL SCAVENGING ASSAY

The free radical scavenging activity was determined using 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay as described elsewhere [23]. 200µl of extracts were each added to 3800µl of 0.004% DPPH in methanol. After 60 min of incubation at room temperature in the dark, the absorbance was measured at 517 nm. A blank sample containing only methanol was used to zero the spectrophotometer. Ascorbic acid (Vitamin C) was used for comparison. Each experiment was performed in triplicate. Radical scavenging activity (I %) was calculated as follows:

$$I\% = [(Abs0 - Abs1) / Abs0] \cdot 100 \qquad (2)$$

Abs_0 = absorbance of 0.004% DPPH without analyte.
Abs_1 = absorbance of 0.004% DPPH plus the test compound.

RESULTS AND DISCUSSION

Table 1 shows the local and scientific names of the ten leafy vegetables used in the study and extraction yields in the two solvents used (expressed as w/w percentage) are presented in figure 1. With the exception of *Manihot esculenta* that yielded similar amounts of extractable solids in both solvents, higher yields were observed in the aqueous extracts. The extraction yields ranged from 41 to 84% for water and 29 to 46% for methanol. From the results, *Hibiscus sabdariffa* (shuuré) gave the highest extractable solid yield of 84% compared with the others. Our results *compare favourably* with the results of a similar study conducted previously [24] and probably suggest the presence of more hydrophilic components in these vegetables.

Local name	Scientific name
1. Aleefo	*Amaranthus incurvatus*
2. Ayoyo/Ademe	*Corchorus olitorius*
3. Shuure	*Hibiscus sabdariffa*
4. Kontomire (cocoyam)	*Colocasia esculenta*
5. Akokobesa	*Ocimum basilicum*
6. Gboma	*Solanum macrocarpon*
7. Bokorbokor	*Talinum triangulare*
8. Cassava	*Manihot esculenta*
9. Dandelion	*Launaea taraxacifolia*
10. Bitter leaves	*Vernonia amygdalina*

Table 1: Local and scientific names of leafy vegetables used in study

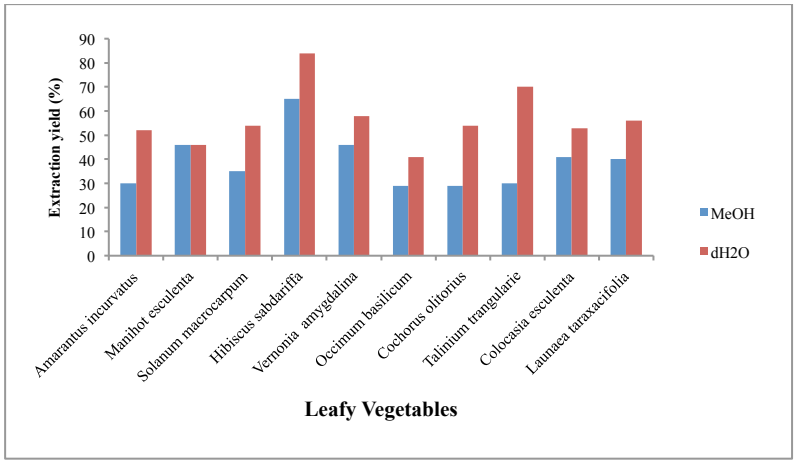

Fig 1: Extraction yields of leafy vegetables in methanol and distilled water

Figure 2 shows the profile of polyphenolic content (PC) of the vegetables studied. The highest PC of 16.4mg GAE/g_{dw} was observed in the methanolic extract of *O. basilicum* with a corresponding value of 4.6mg GAE/g_{dw} for its aqueous extract. This was followed by *A. incurvatus* (11.3mg GAE/g_{dw}), *T. triangulare* (12.73mg GAE/g_{dw}), *C. olitorius* (10.3mg GAE/g_{dw}), *C. esculenta* (10.2mg GAE/g_{dw}) and *M. esculenta* (9.9mg GAE/g_{dw}). Their corresponding aqueous PC values were 4.6mg GAE/g_{dw}, 3.3mg GAE/g_{dw}, 3.39mg GAE/g_{dw}, 7.1mg GAE/g_{dw} and 9.3mg GAE/g_{dw} respectively.

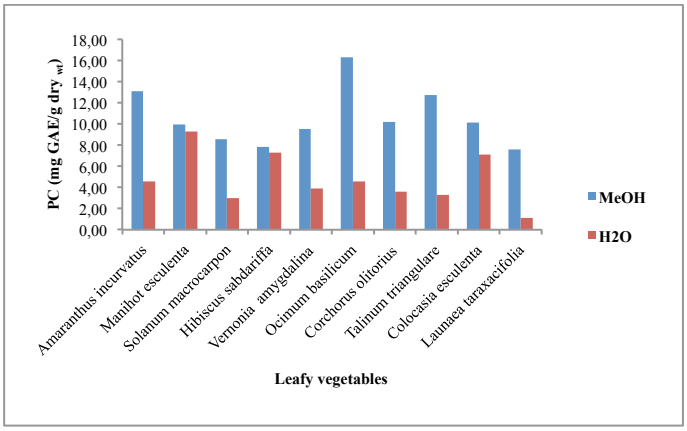

Fig 2: Polyphenolic content of methanolic and aqueous extracts of leafy vegetables

The PC values measured in the methanolic extracts of *V. amygdalina* (9.64mg GAE/g), *S. macrocarpon* (8.5mg GAE/g_{dw}) and *H. sabdariffa* (7.9g GAE/g_{dw}) were also significant. Of all the vegetables the highest PC in the aqueous extracts was found in *M. esculenta* (9.3mg GAE/g_{dw}) followed by *H. sabdariffa* (7.3mg GAE/g_{dw}), *C. esculanta* (7.11mg GAE/g_{dw}), *V. amygdalina* (3.9mg GAE/g_{dw}) and *C. olitorius* (3.6mg GAE/g_{dw}). In both methanolic and aqueous extract, *L. taraxacifolia* had the least phenolic content of 7.6mg GAE/g_{dw} and 1.2mg GAE/g_{dw} respectively.

By and large, relatively high polyphenolic contents were recorded in all the methanolic extracts of the ten leafy vegetables used in the study, while only three namely, *M. esculenta*, *H. sabdariffa* and *C. esculenta* showed high PC in the aqueous extracts. Comparing our methanol extract data to similar work on polyphenol and flavonoid content in the same vegetables consumed by Nigerians [25], wide specific differences were noted. All the polyphenolic contents of the vegetables sourced from Nigeria far exceeded that from Ghana. The unit of measurement of the Nigerian data was mg Quercetin/g of extract while our results are expressed in mg GAE/g_{dw}, nevertheless it may be worth doing this comparison. One reason for this observation may be that while the researchers in Nigeria employed 70% methanol for their extractions, we used 96% methanol. It has been proven that to improve extraction yields of polyphenols from plant biomass, it is necessary to add 30-40% water to the organic solvent, with a caution not to exceed 50% water or risk compromising the extraction yield [26-28]. Jokić *et al,* have [29] established that there is a significant influence of solvent (ethanol-water composition), temperature and extraction time on the kinetics and extraction yield of total polyphenols from soybeans. They authors optimised a 50% aqueous ethanol solution, 80°C and 20min extraction for maximum extraction yields of polyphenols from soybeans. As can be see, temperature has far reaching role in extraction yields for most extraction processes and might have contributed to the large disparity of results seen.

The relatively low content of polyphenolic compounds in our water extracts could be attributed in part to increased activity of polyphenol oxidase (PPO) enzyme which degrades phenolic substances in aqueous media but remains inactive in alcohol milieu [26].

In regards to flavonoid content (FC) the methanolic extracts also yielded higher values compared to their aqueous counterparts. The highest FC for the methanolic extracts was recorded in *H. sabdariffa* with FC of 99.14 µg QE/g_{dw}, followed by *V. amygdalina* 70.2 µg QE/g_{dw}, *M. esculenta* (70.08 µg QE/g_{dw}). The methanol extracts of *O. basilicum and C. esculenta* yielded FC values of 63.37µg QE/g_{dw} and 53.57µg QE/g_{dw} respectively. *Solanum macrocarpon*, *A. incurvatus* and *T. triangulare* yielded almost equal values of 46.01 µg QE/g_{dw}, 45.93 µg QE/g_{dw} and 44.67 µg QE/g_{dw} respectively. The lowest methanol extract FC values of 41.73 µg QE/g_{dw} and 26.92 µg QE/g_{dw} were recorded in *L. taraxacifolia* and *C. olitorius* respectively. Of all the aqueous extracts FC values, *A. incurvatus* afforded the highest value of 80.98 µg QE/g_{dw} which was nearly twice the value of its methanol counterpart. Again *H. sabdariffa* demonstrated a high FC content of 63.71µg QE/g_{dw}. *S. macrocarpon, L. taraxacifolia and C. olitorius* furnished the lowest aqueous extracts FC values of 8.85 µg QE/g_{dw}, 8.49 µg QE/g_{dw} and 2.69 µg QE/g_{dw} respectively. Again Data from Nigeria [25] point to a relatively higher flavonoid content in the same vegetables sourced from Nigeria. The presence of a number of hydroxyl groups in polyphenols (e.g. flavonoids especially the glycosides) make them more hydrophilic and generally more soluble in water-methanol solutions than in pure methanol or water alone [30] and could partly be responsible for the very high differences in our results. Geographical locations may also contribute albeit minimally to the differences in results even though the deviations appear to be very wide apart.

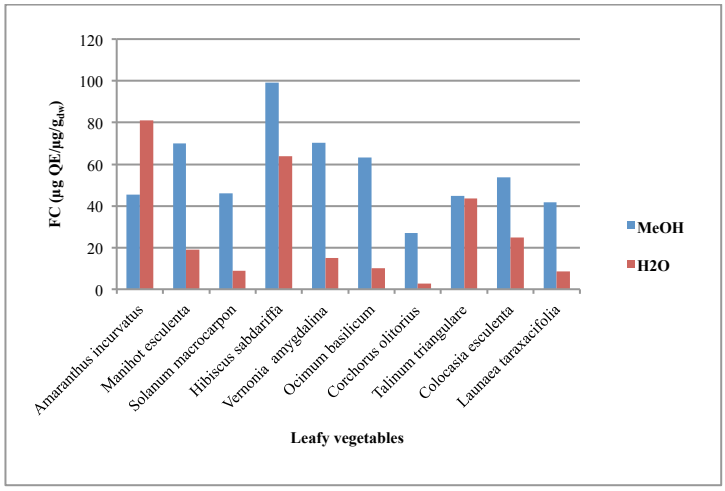

Fig 3: Flavonoids content of methanolic and aqueous extracts of leafy vegetables

The DPPH$^{\bullet}$ scavenging activities were measured at an effective concentration of 14.5µg/ml for all samples including the standard (vitamin C) and the results are displayed in figure 4. Relatively higher free radical scavenging activities were observed for all the methanolic extracts except for *M. esculenta* where the water extract registered a higher activity than its methanolic extracts. The highest scavenging activity of the methanolic extract of 80.5% was registered for *C. olitorius* whilst the lowest methanol extract of 4.3% was seen in *T. triangulare*. Aqueous extracts of *M. esculenta, C. olitorius, C. esculenta* and *O. basilicum* also had scavenging activities of 54.5%, 42.32%, 32.4% and 22.6% respectively. It is noteworthy to state that the antioxidant activities measured in this experiment may not be discrete for polyphenolic compounds as extract clean-up procedures were not incorporated into the protocol to exclude ascorbic acid and other interfering constituents. In this regard,

operating at a concentration of 14.5µg/ml for these crude extracts compared with pure vitamin C (at the same mass concentration) make the antioxidant values measured significantly high as most of the crude extract values exceeded that of the standard substance (Vitamin C) which scavenged a solitary 20.3%.

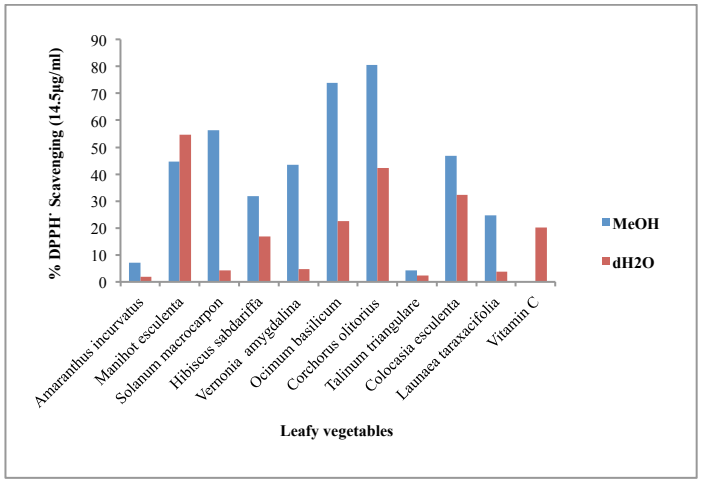

Fig 4: Free radical scavenging capacities of leafy vegetables extracts

As this is only an *in vitro* study, data on the bioaccessibility and the bioavailability of these antioxidants are not available. Undoubtedly, the method of extraction employed experimentally is indiscriminate and relatively harsh consequently more constituents are likely to be extracted from the vegetables than would be by the human digestive system. Prior work shows that only a fraction of the antioxidant potential measured using conventional extraction is extractable when simulated digestive fluids are used [15]. Several factors such as food source and chemical interactions with other phytochemicals and biomolecules present in the food interfere with the bioavailability of antioxidants. Thus this study might just inform of the presence and levels of antioxidants laden in the vegetables but stops short of stating the fraction of these antioxidants that may be available to consumers.
Despite the low extraction yields obtained for the methanolic extracts, methanol appeared to be more efficient at extracting the antioxidants present in the vegetables. Polyphenolic compounds presents both hydrophilic and lipophilic constituents therefore alcohol-water solvent composition will an ideal or optimum requirement to extract them from source plants [4].

A trend analysis using Pearson's correlation procedure revealed no relation between total phenolic content of MeOH extracts and free radical scavenging activity (plot not shown). However, A significant and positive high Pearson's correlations between PC and DPPH assay ($r^2 = 0.66$) was observed for all plants aqueous extracts ($P < 0.05$) fig. 5.

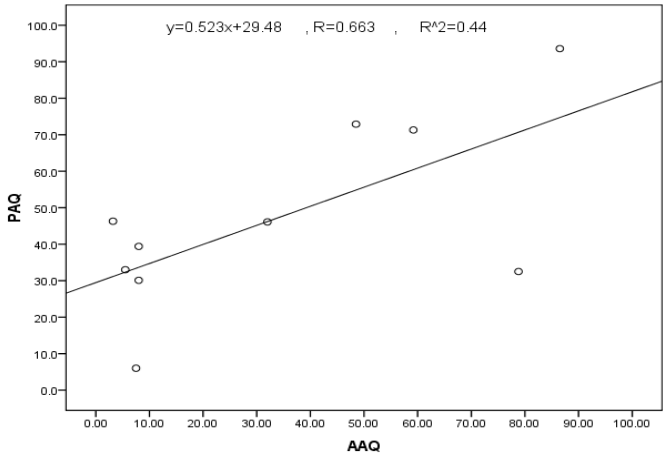

Figure5: Relationship between antioxidant activity and polyphenolic compounds of aqueous extracts.

Our result suggests that phenolic compounds were the main contributor of antioxidant activity in the aqueous extracts of the plants and are consistent with findings of other research groups who have reported a fair correlation between total phenolic content and antioxidant /free radical scavenging activity [20, 31].

We did not find a correlation between the total flavonoid and antioxidant/free radical scavenging activity for both methanolic and aqueous extracts analyzed, as was found by other authors [31, 32].

CONCLUSION

We have demonstrated that the extracts of some commonly consumed vegetables contain significant amounts of polyphenolic and flavonoid compounds capable of directly quenching DPPH$^{\bullet}$ free radicals. According to this study, a significant and linear relationship was found between the antioxidant activity and the phenolic content of the aqueous extracts with *M. esculenta* (9.29mg GAE/g_{dw}) followed by *H. sabdariffa* (7.28mg GAE/g_{dw}) and *C. esculenta* (7.11mg GAE/g_{dw}) displaying the highest concentrations. This indicates that phenolic compounds could be major contributors to antioxidant activity. We could not however correlate free radical scavenging activity with flavonoid content. Our comparative studies of absolute methanol extraction with traditional extraction (water) techniques indicate better recoveries for methanol within a 95% confidence interval. These antioxidant activities have been established in an *in vitro* system and is only indicative of a potential health benefits inherent in the samples, these results remain important as a first step in screening antioxidant activity of locally consumed vegetables. Short of bioaccessibility and bioavailability data it can be concluded that water extracts of the vegetables in the way in which they are consumed as foodstuff in Ghana can be used as an accessible source of natural antioxidants with consequent health benefits.

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