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Phytochemical screening and antioxidant activities of secondary metabolites from green and yellow cocoa (*Theobroma cacao L*) pod waste

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Abstract: Ethanol-water mixture (70/30: v/v) was used during maceration to extract secondary metabolites of green and yellow cocoa (*Theobroma cacao* L) pod waste. The successive fractionation of hydroalcoholic extracts with solvents of increasing polarity led to hexane, dichloromethane, ethyl acetate, ethanol and water fractions. Phytochemical screening of hydroalcoholic extracts and fractions showed the presence of alkaloids, polyphenols, flavonoids, anthocyanins, saponosides, tannins, quinones as well as terpenes and sterols. Generally, hydroalcoholic extract and fractions from yellow pod husks, with IC_{50} values varying from 2.969±0.040 to 8.272±0.030 mg/mL of extract and Trolox Equivalent Antioxidant Capacity varying from 22.621±0.021 to 100.008±0.137 µmol TE/ L of extract, were more active than those from green pod husks. Ethyl acetate fraction from yellow pod husks was more active than ascorbic acid, taken as standard reference antioxidant, with IC_{50} value of 2.042±0.020 mg/mL of extract in 2,2-Diphenyl-1-picryl hydrazyl free radical scavenging test and a Trolox Equivalent Antioxidant Capacity value of 96.163±0.059 µmol TE/L of extract in 2,2'-Azino-bis-3-ethylbenzothiazoline-6-sulfonic acid radical–cation scavenging test.

Keywords: *Theobroma cacao, Waste, Cocoa pod Husks, Phytochemical screening, antioxidant activity*

1. Introduction

Agriculture still occupies an important place in the lives of people around the world, particularly in terms of food security and jobs. Thus, *Theobroma cacao* L. (Sterculiaceae), cultivated in several tropical countries, is a major export product on a global scale (Ouattara *et al*., 2021). It is cultivated for its bean, widely used in food industries. In Côte d'Ivoire, cocoa sector contributes around a third to export revenues and around 20% to the formation of national wealth. Nearly a fifth of Ivorian population depends on its culture. Its production increases regularly due to growing global demand, leading to expansion of cultivated areas to detriment of forest destruction. These environmental constraints which face all producers, particularly the preservation of forest, forced actors to act on other levers, such as yield and industrialization of the sector. However, its production generates enormous quantities of waste estimated at 90%, of which pod husks represent approximately 60% (Campos-Vega *et al*., 2018). This large quantity of waste causes environmental pollution (Cho *et al*., 2020) and its

decomposition spreads diseases such as black rot of pods (Vriesmann *et al*., 2011), hence the need for its management which could improve the income of producers and contribute to poverty reduction. Thus, some ways of valorization of cocoa pod husks, without distinction of varieties, have been undertaken in chemical (Rachmawaty *et al*., 2018), physico-chemical (Vásquez *et al*., 2019), thermochemical (Adjin-Tetteh *et al*., 2018), biochemical (Giwa *et al*., 2020), food (Vriesmann *et al*., 2017), pharmaceutical (Sartini et Gemini, 2008), biofuels and biopolymers fields (Vásquez *et al*., 2019).

In other words, human kinds used natural plants in traditional medicine because of the presence of many natural compounds that have antioxidant activity (Saeed *et al*., 2012; Elmsellem *et al*., 2019). These compounds called "green" are categorized into vitamins (Vitamin C and E), polyphenols (flavonoids, phenolic acids, stilbenes, lignans), and terpenoid groups. Vitamins in plants act as primary antioxidant substances: Vitamin E acts as an essential lipid-soluble antioxidant, while vitamin C protects against oxidative stress-induced cellular damages (Abeyrathne *et al*., 2022; Jideani *et al*., 2021; Aourabi *et al*., 2021).

However, secondary metabolites from crushed cocoa pod husks remain insufficiently valued while biomolecules from several plants are known for their numerous biological properties (Mezni *et al*., 2022a) and antioxidant activities (Alkadi, 2021; Belwal *et al*., 2022; Adjémé *et al*., 2023; Ramos *et al*., 2023).

The present study aims to identify the families of secondary metabolites of hydroalcoholic extracts and fractions from yellow and green cocoa pod husks as well as to evaluate their antioxidant activity. To our best knowledge, there is no similar comparative study in literature.

2. Materials and methods

2.1. plant material

Yellow and green cocoa pods were collected in March 2021 in Logbakro (6°44'115" North and 5°12'091" West), in the center of Côte d'Ivoire, at the start of the rainy season (**Scheme 1**). The pods were grouped according to their colors then shelled to separate pod husks (waste from the cocoa pod) from seed and mucilage. Pod wastes were dried in the shade at room temperature (28 ±2°C) for 14 days and then crushed. After sieving ground materials through a 0.5 mm mesh sieve, powders obtained were conserved at 4°C.

2.2. Methods

2.2.1. Hydroalcoholic extracts

Hydroalcoholic extracts from green and yellow cocoa pod waste were prepared following the protocol of Kassi et *al.* (Kassi *et al*., 2014). The extractions were carried out in triplicate.

2.2.2. Fractionation of hydroalcoholic extracts

The method described by Bouamama et al. (Bouamama *et al*., 2006) was used for successive fractionation of hydroalcoholic extracts from cocoa pod waste (green and yellow) with solvents of increasing polarities to afford hexane (FHE), dichloromethane (FDM), ethyl acetate (FAE), ethanol (FET) and water (FAQ) fractions. The tests were carried out in triplicate.

2.2.3. Phytochemical screening of secondary metabolite families

Phytochemical screening method used is that described by Harbone (Harbone, 1998). The tests were carried out in triplicate.

Scheme 1. Some images of yellow and green pods and sieving ground materials

2.2.4. Antioxidant Assays of extracts and fractions

Antioxidant Assays of hydroalcoholic extracts and fractions were evaluated by the combination of methods of 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS). Antioxidant assays were repeated three times (Salhi *et al.*, 2019; Taibi *et al.*, 2023).

2.2.4.1. 2,2-Diphenyl-1-picryl hydrazyl free radical scavenging test

The antioxidant effect of each extract or fraction on DPPH was measured according to the protocol described by Sánchez-Moreno et al. (Sánchez-Moreno *et al.*, 1998). Ascorbic acid was used as a reference antioxidant. The study of variation in percentage inhibition as a function of concentration was used to determine inhibition concentration at 50% (IC_{50}). A lower IC_{50} value leads to a greater antioxidant activity of the extract or fraction.

2.2.4.2. 2,2'-Azino-bis-3-ethylbenzothiazoline-6-sulfonic acid radical–cation scavenging test

This test was carried out according to the method described by Miller et al. (Miller et al., 1993). Trolox was used as a reference antioxidant and antioxidant activity of extracts or fractions was expressed by Trolox Equivalent Antioxidant Capacity (TEAC) which corresponds to the concentration of Trolox (reference antioxidant). A smaller TEAC value leads to a smaller antioxidant activity of extract or fraction.

3. Results and discussion

3.1. Phytochemical screening

The results of phytochemical screening carried out on hydroalcoholic extracts (EHA) from yellow and green pods as well as on their hexane (FHE), dichloromethane (FDM), ethyl acetate (FAE), ethanol (FET) and water (FAQ) fractions are given in **Table 1**. The results of phytochemical screening of hydroalcoholic extracts and fractions from yellow and green cocoa pods show that the different families of secondary metabolites sought are variously present in all the extracts and fractions. All the families of secondary metabolites sought are present in hydroalcoholic extracts from yellow and green cocoa pods. Alkaloids are present in ethyl acetate and ethanol fractions from yellow pod while they are only present in hexane and ethyl acetate fractions from green pod. Polyphenols and flavonoids are present in all the fractions except hexane and dichloromethane fractions from green pod. Anthocyanins are absent from hexane, dichloromethane and, ethyl acetate fractions from yellow pod as well as from ethyl acetate fraction from green pod. Saponosides are only absent from dichloromethane fractions from yellow and green pods. Catechic tannins are only absent from hexane fraction of green pod while gallic tannins are absent from ethanolic and aqueous fractions from green pod. Quinones are absent from hexane fraction of yellow pod as well as from the two aqueous fractions. Terpenes and sterols are absent from ethanolic and aqueous fractions of yellow and green pods, respectively.

Table 1. Phytochemical screening of extracts and fractions from green and yellow cocoa pod husks

Secondary metabolites Families	Extracts and fractions											
	Yellow cocoa pod husks						Green cocoa pod husks					
	EHA	FHE	FDM	FAE	FET	FAQ	EHA	FHE	FDM	FAE	FET	FAQ
Alkaloids	+	-	-	+	+	-	+	+	-	+	-	-
Polyphenols	+	+	+	+	+	+	+	-	-	+	+	+
Flavonoids	+	+	+	+	+	+	+	-	-	+	+	+
Anthocyanins	+	-	-	-	+	+	+	+	+	-	+	+
Saponins	+	+	-	+	+	+	+	+	-	+	+	+
Catechical tannins	+	+	+	+	+	+	+	-	+	+	+	+
Gallic tannins	+	+	+	+	+	+	+	+	+	+	-	-
Quinones	+	-	+	+	+	-	+	+	+	+	+	-
Sterols and terpenes	+	+	+	+	-	+	+	+	+	+	+	-

Presence: +; Absence : -

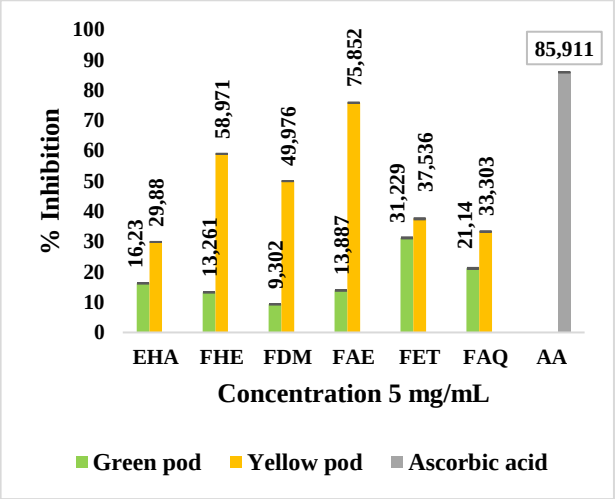
These results show that structures of secondary metabolites of families could be different in the two pods. They also show the influence of the color of a plant matrix organ on its chemical composition as recently reported in literature (Kouassi *et al.*, 2018). Generally, semi-polar and polar solvents are the richest in secondary metabolites families as reported in previous studies (Soumahoro *et al.*, 2020). Hydroalcoholic mixture extracted all the families of secondary metabolites sought in pods and it constitutes a good mixture for extracting secondary metabolites from plant matrices (Perva-Uzunalic *et al.*, 2006). Cocoa pods in this study contain all the families of secondary metabolites sought. These results are similar to those of dried cocoa pod shells collected in Nigeria (Olutayo *et al.*, 2022).

3.2. Antioxidant Assays

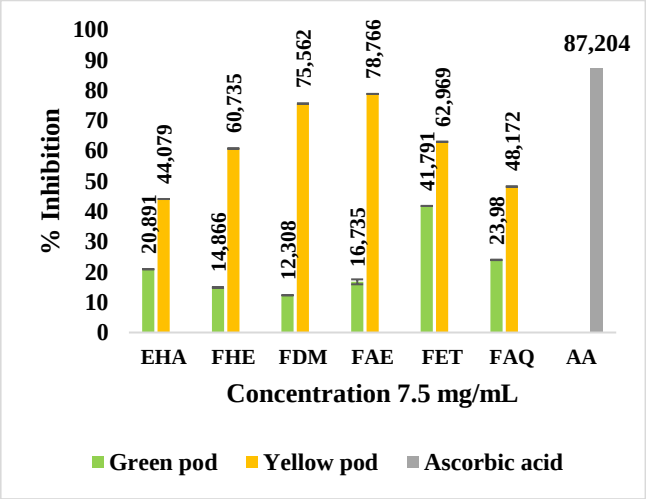
3.2.1. 2,2-Diphenyl-1-picryl hydrazyl free radical scavenging test

Percentages inhibition (PI), at different concentrations, of hydroalcoholic extracts and fractions from yellow and green cocoa pod waste as well as ascorbic acid (reference antioxidant) obtained during

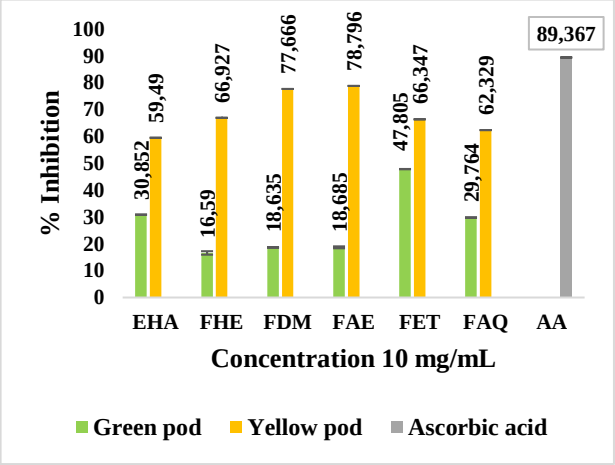
DPPH free radical scavenging test are recorded in **Figure 1**. The results in **Figure 1** show that percentages inhibition obtained with hydroalcoholic extracts and fractions increase with concentration, the best values being obtained at concentration of 20 mg/mL of extract or fraction. Similar results have been reported in literature (Adjémé et al., 2023). Generally, percentages inhibition of hydroalcoholic extract and fractions from yellow cocoa pod waste are higher than those from green cocoa pod waste. These results agree with those of phytochemical screening which show that yellow pod is richer in secondary metabolites families than green pod. These results also show influence of color and maturity of a plant organ on its chemical composition as reported with cashew apple (Kouassi et al., 2018), olives (Bengana, 2017) and argan fruit pulp (Harhar et al., 2019). Generally, ethyl acetate fraction from yellow pod presents the highest percentages inhibition values whatever concentration, followed by dichloromethane fraction. In addition, percentages inhibition of extracts and fractions are lower than those of ascorbic acid. To better understand antioxidant activity of extracts and fractions from cocoa pod waste, concentrations corresponding to 50% inhibition (IC_{50}) were determined from the PI curves (%) = f (concentration). The IC_{50} values of hydroalcoholic extracts and fractions from yellow and green cocoa pods are presented in **Figure 2**.



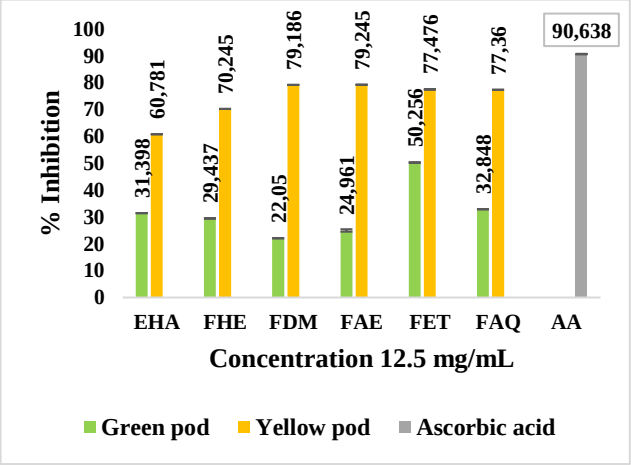
a. Percentage inhibition at 5 mg/mL



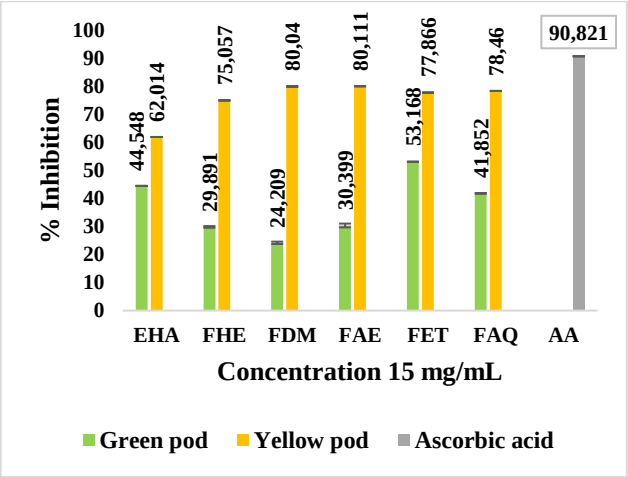
b. Percentage inhibition at 7.5 mg/mL



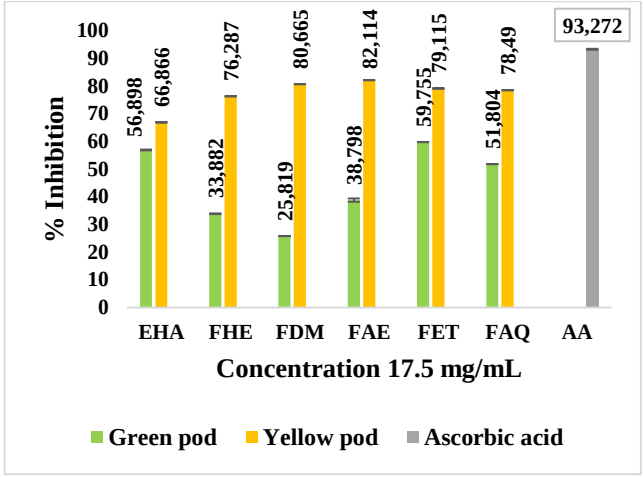
c. Percentage inhibition at 10 mg/mL



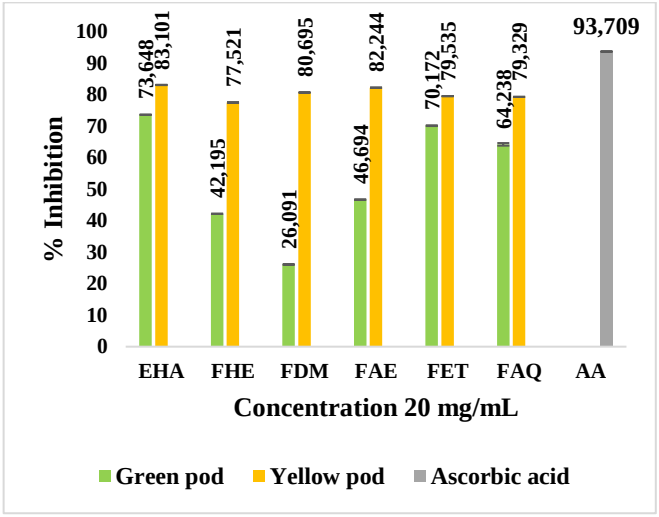
d. Percentage inhibition at 12.5 mg/mL



e. Percentage inhibition at 15 mg/mL



f. Percentage inhibition at 17.5 mg/mL



g. Percentage inhibition at 20 mg/mL

Figure 1. Percentages Inhibition of hydroalcoholic extracts and fractions from cocoa pod husks and ascorbic acid at different concentrations

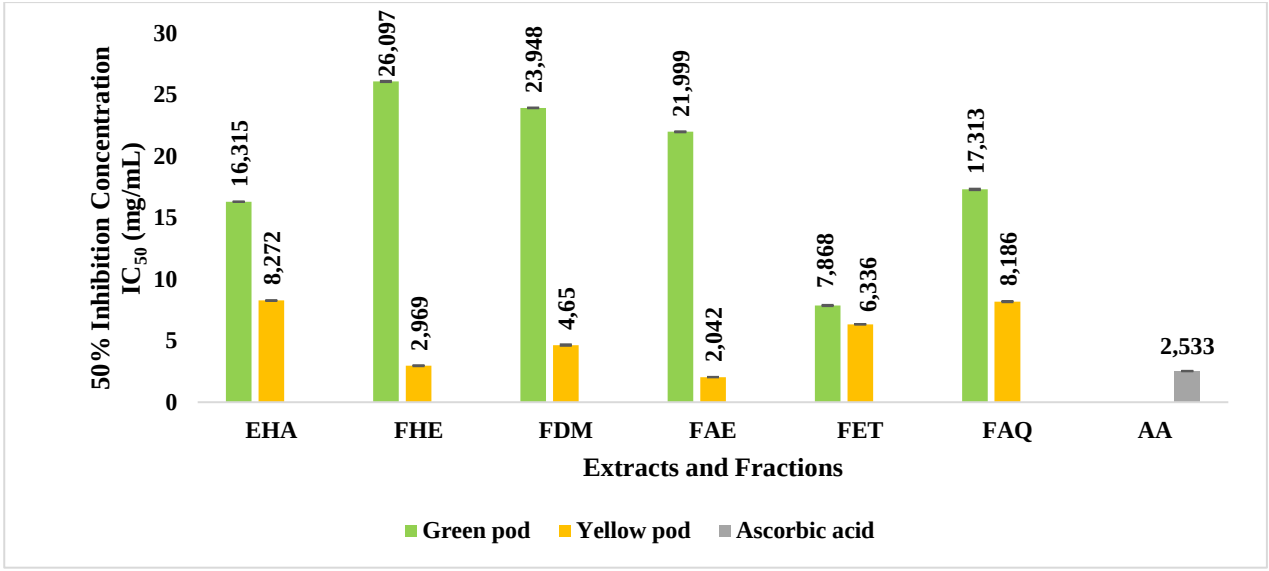


Figure2. IC_{50} values of hydroalcoholic extracts and fractions from yellow and green cocoa pod husks

The IC_{50} values of hydroalcoholic extracts are 8.272±0.030 and 16.315±0.020 mg/mL of extract for yellow and green pods, respectively. Those of fractions vary from 2.042±0.020 to 8.186±0.050 mg/mL and from 7.868±0.050 to 26.097±0.050 mg/mL of fraction extract for yellow and green pods, respectively. Extracts and fractions from yellow pod, with lower IC_{50} values, have higher antioxidant activities than those from green pod. In addition, the lowest antioxidant activities were obtained with hexane and dichloromethane fractions from green pod. These results agree with those of phytochemical screening where these two fractions did not contain polyphenols or flavonoids and confirm the important role of these two families of secondary metabolites in antioxidant activities of plant matrices (Alkadi, 2021; Shen *et al.*, 2022). The most important antioxidant activity of fractions is that of ethyl acetate fraction from yellow pod with IC_{50} = 2.042±0.020 mg/mL of fraction, lower than that of ascorbic acid (IC_{50} = 2.533 ±0.020 mg/mL of ascorbic acid). This fraction is therefore more active than reference molecule. Antioxidant effectiveness of ethyl acetate fraction from plant extracts has also been reported in literature (Afsar et al., 2018). Ethanol fraction from green pod (IC_{50} = 7.868±0.050 mg/mL of fraction extract) has the highest antioxidant activity of fractions from this pod. However, aqueous fraction from yellow pod, the least active of fractions from this pod, has an antioxidant activity close to that of the most active fraction from green pod. These results once again confirm the influence of the color of a plant matrix on its chemical composition as well as on its activity (Bengana, 2017; Kouassi *et al.*, 2018; Harhar *et al.*, 2019).

3.7.2. 2,2'-Azino-bis-3-ethylbenzothiazoline-6-sulfonic acid radical–cation scavenging test

Antioxidant activity of hydroalcoholic extracts and fractions by the $ABTS^{+\bullet}$ radical cation scavenging test was carried out using Trolox calibration line (**Figure 3**). The results obtained are presented in **Figure 4**.

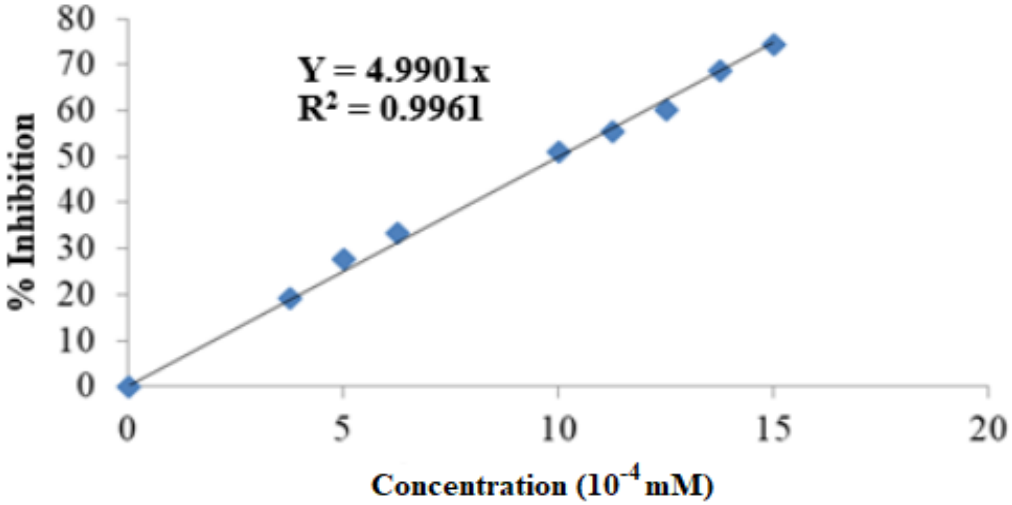


Figure 3. Trolox calibration curve for antioxidant activity assay

Figure 4 shows that hydroalcoholic extracts and fractions have antioxidant activities by $ABTS^{+\bullet}$ radical cation scavenging test. Similar to DPPH test results, Trolox Equivalent Antioxidant Capacity (TEAC) of hydroalcoholic extract from yellow cocoa pod (100.008 ± 0.137 µmol TE/L of extract) is approximately three (3) times greater than that from green pod (31.062 ± 0.005 µmol TE/L of extract). Concerning fractions, only dichloromethane (47.744 ± 0.059 µmol TE/L of fraction extract), ethyl acetate (96.163 ± 0.059 µmol TE/L of fraction extract) and water (44.330 ± 0.091 µmol TE/L of fraction extract) fractions from yellow pod have higher antioxidant activities than those from green pod. Ethyl acetate fraction from yellow pod has the highest antioxidant activity (96.163 ± 0.059 µmol TE/L of

fraction extract) followed by ethanol fraction from green pod (64.177 ± 0.032 µmol TE/L of fraction extract). The lowest antioxidant activity was obtained with hexane fraction from yellow pod (22.621 ± 0.021 µmol TE/L of fraction extract) and could reflect the low content of phenolic compounds and particularly in flavonoids in this fraction (Benslama *et al.*, 2021), (Kouadio *et al.*, 2020).

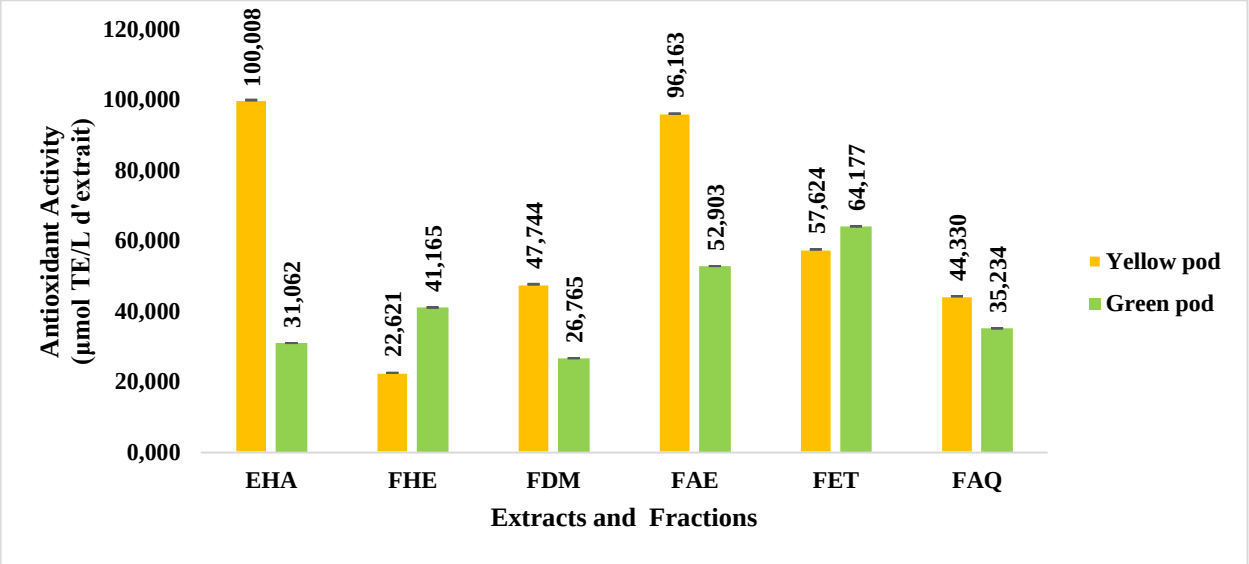


Figure 4. Antioxidant activity of extract and fractions from yellow and green cocoa pod husks by ABTS test

The results of antioxidant activities by DPPH test differ from those obtained by ABTS test for hexane and ethanol fractions. This difference could be linked to the nature of secondary metabolites present in these fractions (Sarr *et al.*, 2015) such as phenolic acids and proanthocyanidins (Tabart *et al.*, 2009a) as well as to the mechanism of action on DPPH and ABTS, and confirms the combination of responses from different complementary tests to provide an indication of antioxidant capacity of a sample（Tabart *et al.*, 2009b； Mezni *et al.*, 2022b）.

Conclusion

Green and yellow cocoa pod waste contains all the families of secondary metabolites sought in our study. Generally, hydroalcoholic extract and fractions from yellow cocoa pod have higher antioxidant activities than those from green pod. Ethyl acetate fraction from yellow pod has the greatest antioxidant activity in DPPH and ABTS tests and is more active than ascorbic acid used as a standard. Determination of contents of secondary metabolites families in this plant matrix is in progress.

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