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A comparative analysis of the secondary metabolites, polyphenol contents and antioxidant potential of ethanolic, hydro-ethanolic and aqueous extracts of *Annona muricata* Linn leaves in Abomey-Calavi, Benin

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Abstract

Annona muricata Linn commonly called corossol in BENIN is a plant of the family of Annonacceae known throughout the world through its virtues in traditional medicine. This study aimed to compare the phytochemical composition and the antioxidant activity between ethanolic, hydro-ethanolic, and aqueous extracts of *Annona muricata* leaves collected in Abomey-Calavi, Benin. Qualitative and quantitative phytochemical tests were used to detect the presence of bioactive molecules. Antioxidant activity was performed using the DPPH method and the FRAP reducing power method. The phytochemical screening showed the presence of flavonoids, tannins (catechic and gallic), alkaloid, anthocyanin, leuco anthocyanins, triterpenoids, steroids, mucilage, reducing compounds and coumarins in all three extracts. The ethanolic extract showed a higher antioxidant potential (IC_{50}= 0.01mg/ml) than the hydro-ethanolic (IC_{50}= 0.013mg/ml) and aqueous extracts (IC_{50}= 0.014mg/ml) by the DDPH method. The ethanolic extract showed a higher reducing power than the aqueous extract and the hydro-ethanolic by the FRAP test. This confirms the agreement in the results. However, the ethanolic extract of *Annona muricata* is less active than ascorbic acid, which is a reference antioxidant.

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Introduction

Herbal medicine remains a first-line solution in developing countries and especially for rural populations. So through the ages, man has been able to count on nature to meet basic needs (Lhuillier, 2007). The African continent has a rich heritage of plants with edible and therapeutic virtues. The use of traditional medicine varies from 70% in Benin to 90% in Burundi and Ethiopia (WHO, 2013).

According to the World Health Organization (WHO), medicinal plants are a good source for the discovery and development of new drug of new medicines. Therefore, medicinal plants should be studied to better understand their different properties, safety and efficacy (Nascimento *et al*., 2000). In Benin, of the 3,000 plant species inventoried in forest ecosystems, 172 are consumed by local populations as food plants and 814 as medicinal plants (Sinsin B., Owolabi L., 2001).

Among these plants that constitute the wealth of the Beninese forest is the corossol tree. Commonly referred to by the name of its fruit, the corossol "magic fruit". It is said to originate in tropical America and then spread to other tropical regions of the world: Europe, Asia, Africa (Gavamukulya *et al*., 2014; Pieme *et al*., 2014). The soursop tree with the scientific name *Annona muricata Linn* belongs to the large family of Annonaceae. All the organs of the soursop tree (pulp, stem, leaf, bark, root, seed) are used in herbal medicine (Padma *et al*., 1999).

Numerous scientific researches carried out in the plant have validated pharmacological activities including hepatoprotective (Padma *et al*., 1999), hypotensive (Nwokocha *et al*., 2012), anti-inflammatory (Abdul Wahab *et al*., 2018), insomnia (Yajid *et al*., 2018), antiviral, hypoglycemic and antidiabetic (Adewole et Caxton-Martins, 2009; Florence *et al*., 2014), antioxydant (George *et al*., 2015), insecticide (Leatemia et Isman, 2004), (Ishuwa *et al*., 2016). The soursop tree also possesses bioactive substances, antioxidants and rich in acetogenins that give it a potential biological activity and even an anti-cancerous property (Ana *et al*., 2016; Coria-Téllez *et al*., 2018; Widyastuti et Rahayu, 2017).

The leaves are used to prevent and overcome arthritis, asthma, bronchitis, biliary disorders, diabetes, heart disease, hypertension, intestinal worms, liver disorders, malaria, rheumatism, tumours and cancers (Gavamukulya *et al*., 2014; Pieme *et al*., 2014), (Orak *et al*., 2019).

Considering the therapeutic importance given to this plant, the present study aimed to make a comparative analysis of the phytochemical composition and the potential antiradical power of the ethanolic, hydroethanolic and aqueous extracts of the leaves of *Annona muricata* Linn harvested in Benin.

Material and methods

1.1. Vegetal material

The vegetal material consists of the leaves of *Annona muricata Linn,* harvested in October 2019 in the district of Toba in the commune of Abomey-Calavi. They have been certified at the National Herbarium of Benin under the number YH264/HNB. The leaves were dried at laboratory temperature (25°C-30°C) for 45 days. Once dried, they were powdered.

Preparation of extracts

The technique used is that of maceration. 50g of sample *Annona muricata* leaves powder were introduced into a 1000ml bottle containing 500ml of the extraction solvents (Ethanol, water and 50/50 ethanol-water). The vial was capped and left under continuous shaking for 72 hours. After filtration, the extracts were evaporated dry at 60°C using a Heidolph type rotavapor. The yield (R) of the extraction is calculated by the formula below.

$$R\ (\%) = \frac{\text{Mass of the extract}}{\text{Mass of plant product used}} \times 100$$

1.3. Determination of Secondary Metabolites

Phytochemical screening is based on the differential reactions (colouring and precipitation) of the main groups of chemical compounds contained in plants according to the method of (Houghton et Raman, 1998), taken up by (Assogba, *et al*., 2015).

Determination of phenolic compounds

Total Phenols

The determination of total polyphenols was carried out according to the method of Singleton and Rossi, 1965, taken up by Ahoton *et al.*, 2019 with some slight modifications. This assay is carried out using the Folin-Ciocalteu reagent and the content is expressed in milligrams of gallic acid equivalent per gram of extract.

Flavonoids

The total flavonoid content of plant extracts can be estimated by the aluminium trichloride ($AlCl_3$) method. Rutin is used as the reference compound to produce the calibration curve (Kim DO *et al.*, 2003). The content is expressed in milligram of Rutin equivalent per gram of extract.

Tannins

The method used to determine the content of condensed tannins is that of sulphuric vanillin (Broadhurst et Jones, 1978 and modified by (Heimler *et al.*, 2006). The condensed tannin content is expressed in milligrams of pyrogallol equivalent per gram of extract.

Anti- Radical Activity

The antiradical activity of the extracts was determined using two methods: the DPPH free radical scavenging method and the FRAP iron reduction method.

DPPH free radical scavenging test

For this test, samples were prepared by dissolution in distilled water (Ahoton & al., 2019). For each extract, a stock solution was prepared in distilled water at 200µg/mL. This solution is then diluted in a geometric series of 2 parts to different concentrations.

In dry, sterile test tubes, 1ml of the test extract solution is introduced, 1ml of DPPH solution (0.04mg/ml) is added. After vortex shaking, the tubes are placed in a dark place at laboratory temperature for 30 min. The absorbance measurement is performed at 517nm with the spectrophotometer (Biomate UV/VIS).

For each dilution, a blank is prepared consisting of 1ml of the test solution plus 1ml of ethanol. The positive control is represented by ascorbic acid (200µg/ml) and is treated under the same conditions as the test sample.

The results were expressed as the mean of three measurements ± standard deviation. The values of IC_{50} (the concentration of the substrate that causes 50% loss of DPPH activity), EC_{50} (the effective concentration of the substrate that causes 50% loss of DPPH activity and takes into account the concentration of DPPH in the reaction medium) and APR (antiradical potency) were determined (Parakash D *et al.*, 2007).

Iron Reduction Test (FRAP)

The extract ability to reduce Fe^{3+} was assessed (Oyaizu, 1986). 1ml of the extract was mixed with 2.5ml phosphate buffer (0.2 M, pH 6.6) and 2.5ml of 1% potassium ferricyanide [$K_3Fe(CN)_6$]. After a 20 min incubation at 50°C, 2.5ml of trichloroacetic acid (10%) were added to the mixture and centrifuged for 10 min (3000 r/t). A 2.5ml aliquot of the upper layer was mixed with 2.5ml of distilled water and 0.5FeCl3 (0.1%). The absorbance was read at 700nm. Iron (III) reducing activity was determined as ascorbic acid equivalents (mmol ascorbic acid/g extract). The values are presented as a mean of triplicate analyses.

Statistical Analysis

The data were entered and processed in Excel and Graph Pad 5 software for comparison of means, determination of the confidence index and Pearson correlation coefficients.

Results discussion

Extract yield

The Table 1 present the yield values for the different extracts.

The hydro-ethanol and aqueous extracts gave the highest yields; the ethanolic extract gave the lowest yield. Yield values differed from each other with a statically significant difference ($p<0.05$). In Brazil, high percentages were found (12.5%) for ethanol extract and for water extract (15%) (Justino *et al*, 2018).

Our percentages, although low, are higher than the yields obtained by Orak *et al* 2019 in Turkey, which were solvent dependent (for hexane (3.66%) dichloromethane (1,10%) and ethyl acetate (0.83%)) but lower than for methanolic extraction (12.07%) (Orak *et al.*, 2019). Ethanolic extraction obtained by Ojezele *et al.* (2016) in Nigeria gave a yield (4.9%) lower than our result (Ojezele *et al.*, 2016). Nga *et al.* (2018) in Cameroon obtained high yields for hydro-ethanolic and aqueous extractions respectively (18.07% and 11.87%) (Nga *et al.*, 2018), The quality of the solvent, the harvesting region of the organ used, the drying time, the granulometry of the grind, the percentage of ethanol in the hydro-ethanolic solvent, the ratio of solvent volume per mass of grind, the maceration time and the stirring speed are all factors that influence the yield (Koné *et al.*, 2017).

Table 1. Extraction yields.

Extracts	Mass of plant product used (g)	Mass of extract (g)	Yields (%)
Ethanolic	50	3,854	7,708
Hydro-ethanolic	50	4,418	8,836
Aqueous	50	4,162	8,324

3.2. Phytochemical screening

The phytochemical screening results of *Annona muricata* extracts are put in the table 2. The table 2 presents bioactive compounds such as alkaloids, flavonoids, reducing compounds, coumarins which are present in all three extracts, soponosides, anthracenes, and cardenolides are absent in all three extracts. The flavonoids identified are flavones. The same compounds are found in the ethanol extracts of the leaves of *Annona muricata* in Nigeria and Andonesia (Ojezele *et al.*, 2016), (Hasmila *et al.*, 2019). The methanolic extraction performed in Nigeria revealed the presence of the cardiocglycoside absent in our study (Ezealisiji et Belema, 2017). Cardiocglycosides were also found in hydroalcoholic and aqueous leaves extracts in Cameroon (Nga *et al.*, 2018). Other authors have highlighted these different metabolites in different organs of *Annona muricata* (Vijayameena *et al.*, 2013), (Ana *et al.*, 2016), (Onyegeme-Okerenta *et al.*, 2018). However, differences in the absence or presence of any of these metabolites in different studies could be explained by the solvent used, the identification methodology, the age of the plant, the nature of the soil, the climatic conditions in the country, the region of harvest within the same country. In vivo and in vitro studies have demonstrated the pharmacological activity of the leaves and other organs of the plant due to the presence of flavonoids, tannins, alkaloids and its richness in acetogenin. It has been shown to have antibacterial, antiviral, antifungal, hepatoprotective, anxiolytic and hypoglycemic activity (Florence *et al.*, 2014), (George *et al.*, 2015),(Saleem, 2017),(Ana *et al.*, 2016), (Hansra *et al.*, 2014),(Iyanda-Joel *et al.*, 2019). The anti-tumour properties revealed in breast and prostate tumours were linked to flavonoids and acetogenin richness in the leaves of Annona muricata (Gavamukulya *et al.*, 2014; Hansra *et al.*, 2014; Coria-Téllez *et al.*, 2018). These different bioactivities justify the popularity of using this plant in phytotherapy.

Table 2. Phytochemical composition of *Annona muricata Linn.*

Chemical compounds	Ethanolic	Hydro-ethanolic	Aqueous extract
Alkaloids	+	+	+
Tannins	+	+	+
Cathechic tannins	-	+	+
Gallic tannins	+	+	+
Flavonoïds	+ (flavone)	+ (flavone)	+ (flavone)
Anthocyanins	+	-	+
Leucoanthocyans	+	+	-
Quinone derivatives	-	+	+
Mucilage	-	+	+
Reducing compound	+	+	+
Saponins	-	-	-
Coumarins	+	+	+
Free anthracene	-	-	-
O-Heterosides	-	-	-
C-Heterosides	-	-	-
Triterpenoids	+	+	-
Steroids	+	+	-
Cardiac glycosides	-	-	-

(+) Presence; (-) Absence

3.3. Determination of phenolic compounds

Polyphenolic compounds dosed in leaf extracts act as antioxidants by inhibiting radicals induced by oxidative stress (Ezealisiji et Belema, 2017). Considering flavonoids, for example, the presence of

the flavone nucleus, the number, positions and types of substitutions influence radical scavenging and chelation activity (Heim *et al.*, 2002). In our work we were able to quantify three chemical families including flavonoids, polyphenols and tannins (fig. 2, 3, 4). Flavonoids in the ethanolic extract are about twice as high as in the other extracts, (fig. 3). but this value is lower than that obtained in Nigeria (365.6 (µg /mg QE) with methanolic extracts of *Annona muricata* leaves (Ezealisiji et Belema, 2017). As for polyphenols, they are 1.5 times more expressed in the hydroethanolic extract than in the aqueous and ethanolic extracts. The same remark was made in Cameroon, with the values of 22.52 ± 0.0011 and 16.94 ± 0.0015 (µg/mg GAE) respectively for the hydroethanolic and aqueous it should be noted that polyphenols are higher in our extracts than those of Cameroon (Nga *et al.*, 2018). The maceration time was 72h in our study versus 48 h in Cameroon. The aqueous extract presents relatively low contents compared to the other extracts (fig. 2). In India, the polyphenol content for a concentration of 100µg was 10.3 ± 0.06µg/mg GAE (George *et al.*, 2015) value lower than 75.142±0.917µg/ml observed in our study.

Fig. 1. Photography of the *Annona muricata Linn* tree with its fruit on the left and dried leaves powder on the right.

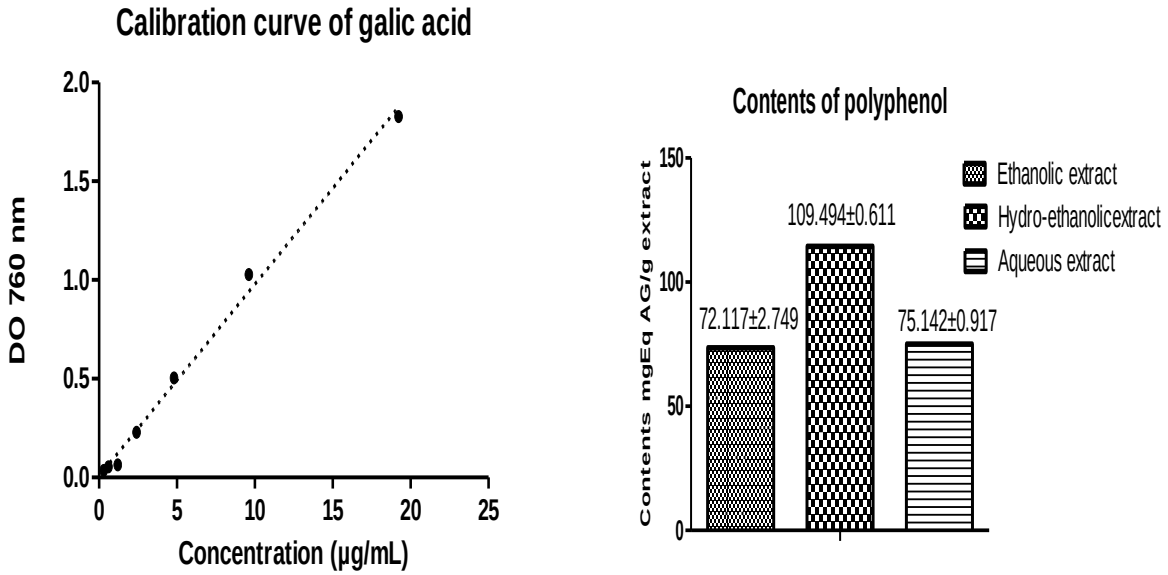

Fig. 2. Calibration curve for galic acid and total phenol contents.

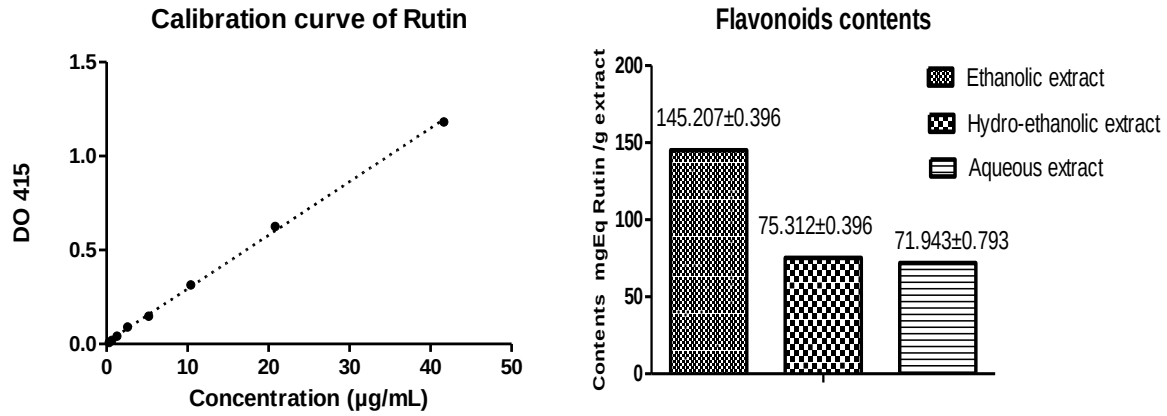

Fig. 3. Rutin calibration curve and flavonoid contents.

Antiradical activity

For this activity, we determined the concentration at which half of the DDPH (IC_{50}) was reduced by each extract. These IC_{50} values are shown in Table 3.

Table 3. DDPH and FRAP tests results.

	IC_{50} (µg/ml)	EC_{50}	ARP	FRAP (mmol ascorbic acid/g extract)
Aqueous extract	14.01	0.7	1.429	0.182
Hydro-ethanol extract	13.02	0.65	1.538	0.301
Ethanolic extract	10.01	0.5	2	1.126
Ascorbic acid	0.0074	3.7 e-3	8.378	/

Table 3 above shows the values of the concentrations that inhibited 50% of the free radicals in DPPH. These values are indicative of the ability of leaves extracts to scavenge free radicals.

The antioxidant activity of the three extracts was evaluated by two methods DPPH and FRAP for confirmatory purposes. The lower IC_{50}, show the more active extract (Heimler *et al.*, 2006). It thus appears from Table 3 that the ethanolic extract is more active than the hydroethanolic extract, which is also more active than the aqueous extract with a statistically significant difference ($P<0.05$) between the values.

Studies by Coria-Téllez *et al* (2016) in Mexico, by Orak *et al.* (2019) in Turkey, Hasmila *et al.* (2019) in Indonesia showed that ethanoic extract is more active respectively (IC_{50} = 70µg/ml and 0.136 ± 0.06mg/ml, 141.127µg/ml). However our extract is the most active.

The ethanolic and aqueous extracts of our study gave lower IC_{50} values than those found in Brazil respectively: 28.1±4.4µg/ml and 43.1±6.3µg/ml (Justino *et al.*, 2018), Similarly in Nigeria, the antioxidant activity sought in the ethanoic and aqueous extracts of *Annona muricata* showed that the ethanoic extract is more active 37.8mg/ml versus 79.95mg/ml. Our results corroborate the latter and indicate that the ethanoic extract is more active. However, it is less active than ascorbic acid (0.00749µg/ml).

The iron reduction test (FRAP) is a simple, rapid and reproducible test (Benzie et Strain, 1996). It is universal and can be applied to plants as well as organic and aqueous plasmas and extracts (Ahoton *et al.*, 2019). The reducing power of the extracts has shown that the ethanoic extract of Annona muricata has a power three times higher than the hydroethanoic extract and at least five times higher than the aqueous extract which confirms our results with DDPH.

The reducing power of the Annona muricata species is probably due to the presence of hydroxyl groups in phenolic compounds that can serve as electron donors. Previous studies have reported that the reducing power of a compound can be a significant indicator of its antioxidant activity (Jeong *et al.*, 2004). The ethanolic extract of the leaves showed a higher antioxidant capacity than the extract of seeds (Widyastuti et Rahayu, 2017).

It was proven that the ethanolic extract has the capacity to decrease the risk of chronic diseases caused by free radicals, in particular cancer and heart attacks also the capacity to reduce the peroxidation of the lipids of the liver (Justino *et al.*, 2018). This radical scavenging capacity could be due to the high flavonoid content in the various extracts. Comparing antiradical activities with total phenol, flavonoid, and tannin levels in the linear correlation model (Pearson test) (Table 4), non-significant correlation was found, with an estimated coefficient of determination, (r^2) (fig. 5), at $p < 0.05$.

Table 4. Correlation between antioxidant activity and content.

	Flavonoïd	Polyphenol	Tannin
IC_{50} (mg/mL)	0,7570773	0,3293843	-0,2418611
p-value	0,1214614	0,3353078	0,3790694

The flavonoids presenting the best correlation would be at the base of the antioxidant activity of the extracts. Other authors have shown that phenolic compounds such as flavonoids in plants are the main bioactive substances responsible for antioxidant activity (J *et al.*, 2003), (Ahmed *et al.*, 2015).

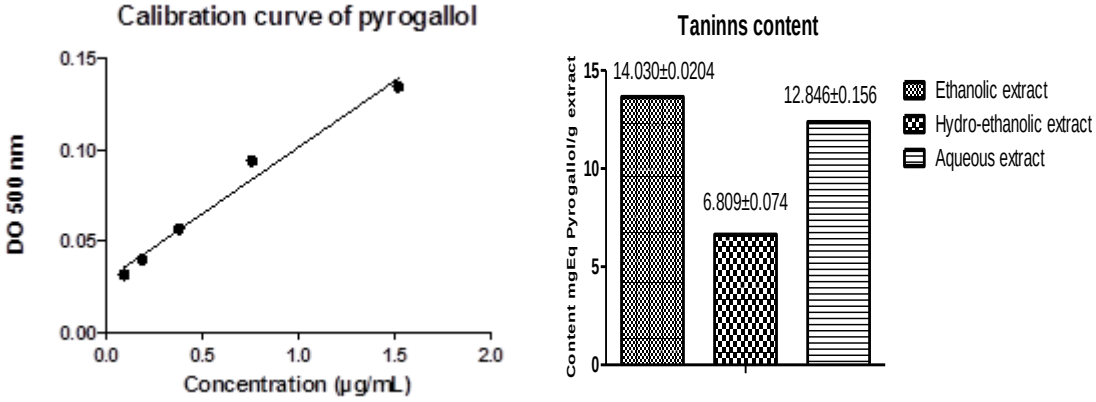

Fig. 4. Calibration curve for pyrogallol and tannin contents.

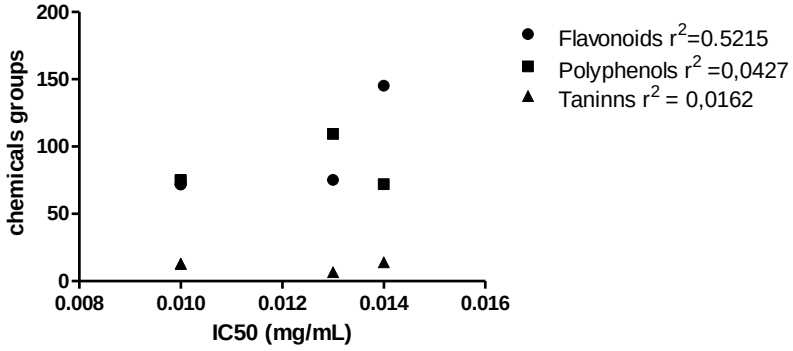

Fig. 5. Correlation between IC_{50} and three chemical groups.

Conclusion

Phytochemical screening of the three ethanolic, hydro-ethanolic, and aqueous extracts of *Annona muricata Linn* leaves have increased their wealth in secondary metabolites and phenolic compounds. The Pearson's correlation test allowed us to observe that flavonoids could be at the base of the antioxidant activity of the extracts. The ethanolic extract has a strong potential to trap free radicals even though it is low in total polyphenols. It is probably for this richness in secondary metabolites that traditional therapists advise the leaves and other organs of the plant in the fight against diseases, especially chronic degenerative diseases.

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