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Research Article

Cytotoxicity and antioxidant potential of dichloromethane and ethyl acetate extracts of *Morinda morindoides* leaves

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Abstract



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The incidence of cancer and related mortality are on the rise worldwide, and this requires the search for new plant-based medicines. In this study, the antioxidant potential and cytotoxic activity against HCT-116 cell line were evaluated. Dichloromethane and ethyl acetate extracts of *M. morindoides* were prepared by maceration and evaluated for some biological properties. Cytotoxicity was assessed according to MTS method on the cancer line HCT-116 (colorectal carcinoma) using doxorubicin as reference. Antioxidant potential was carried out by DPPH and ABTS tests. Tri-phytochemistry was carried out from coloring and precipitation tests. On HCT-116 cell line, extracts demonstrated dose-response activity. The lowest IC50 value was obtained with the acetate extract with an IC50= 16.38 ± 0.2 µg/mL. IC50 values for antioxidant activities were evaluated at 39.70 ± 1.7 µg/mL and at 57.65 ± 4.3µg/mL by DPPH and ABTS test respectively. Finally, phytochemical screening showed presence of polyphenols, flavonoids and quinone. *M. morindoides* ethyl acetate extract showed the best cytotoxicity and antioxidant activities. Therefore, it should be an interesting source for discovery of new compounds for colorectal cancer treatment.

Keywords: cytotoxicity, cancer cells, antioxidant, *Morinda morindoides*.

INTRODUCTION

Colorectal cancer is the second leading cause of cancer-related deaths worldwide. In 2018, colorectal cancer was one of the most diagnosed cancers in the world, with 1.8 million cases and 881,000 deaths¹. The number of cases is estimated to reach 20 million by 2022². This pathology is characterized by an anarchic proliferation of abnormal cells resulting from persistent and unrepaired DNA damage³. Risk factors that may be associated with this pathology include a diet low in fiber and high in fat, age, lifestyle, family history, and carcinogens such as ionizing radiation or ultraviolet radiation. Activated oxygen species (AOS), which include free radicals and singlet oxygen, play an important role in altering the genetic material of cells with metastatic phase in the cancer process⁴.

Treatment for colorectal cancer includes chemotherapy and radiation therapy. However, these treatment options are complex and associated with side effects that are sometimes intolerable for the patient¹. In light of the above, it is necessary to search new anticancer compounds of plant origin that have minimal toxicity to normal cells and can also

neutralize oxidative stress. One of these approaches is to explore products derived from plants, which are perceived as natural products with less risk or hazard to humans and environment compared to synthetic products⁵.

Morinda morindoides, a climbing vine of the Rubiaceae family with opposite leaves and bumpy fruits⁶ is widely used in traditional medicine to treat certain pathologies. In Congo, the leaves are used in decoction to treat malaria, rheumatic pain⁷ and hemorrhoids⁸. The fruits are used locally to treat epilepsy. In Côte d'Ivoire, ZIHIRI *et al.* (2005), showed in their work that the ethanolic extract of this species had *in vitro* antiplasmodial activity on chloroquine-resistant FcB1/Colombia strains of *Plasmodium falciparum*⁵ the ethanolic extract of the leaves of this plant also showed good anti-vibrio cholera activity in a study conducted by Koffi (2013). With the overall objective of valorizing natural substances, the present work aims to evaluate the cytotoxic and antioxidant potential of *Morinda morindoides* leaves. Subsequently, the chemical compounds present in the extract will be determined.

MATERIAL

Cell lines and culture conditions

HCT-116 cancer cells were maintained in RPMI 1640 (Sigma-Aldrich R2405-500mL), supplemented with 10% fetal bovine serum + 1% (penicillin + streptomycin).

Cells were incubated at 37°C in 5% CO₂ and 95% air in a humidified incubator. HCT-116 human colorectal adenocarcinoma cells were provided by American Type Culture Collection (ATCC).

Plant material

The leaves of *Morinda morindoides* were collected in the city Daloa, Côte d'Ivoire, in February 2023 and identified at the national and floristic center with the herbarium number of the specimen was CNF N°17710. The leaves have been dried at room temperature. They were then ground into powder and stored in a refrigerator at 4°C.

Chemicals

Solvents such as ethyl acetate, ethanol, and cell culture media were purchased from Sigma-Aldrich (USA), and reagents DPPH (2,2-diphenyl-1-picryl-hydrazyl), ABTS (2,2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid), ascorbic acid, potassium persulfate and gallic acid were used for antioxidant assays, DRAGENDORFF and STIASNY reagents, ferric chloride etc. for secondary metabolites characterization.

METHODS

Extract preparation

Firstly, a quantity of 20 g of vegetable powder was macerated in ethyl acetate for 24 hours. Then, the macerate was filtered. Secondly, another 20 g of powdered sample of the plant was extracted with dichloromethane for 2 hours using a soxhlet apparatus. The two extracts were separately evaporated using a rotary evaporator. The dry extracts obtained were named ACE fe.Mm for ethyl acetate extract, DCM fe.Mm for dichloromethane extract. These extracts were stored in the refrigerator until use.

For serial dilutions preparation, 10 mg of extracts were weighed and dissolved in a mixture of DMSO/H₂O mQ (1:1) to obtain a stock solution of 10 mg/mL. Diluted solutions of extract in culture medium were prepared at 2X concentration (400 µg/mL; 80 µg/mL; 16 µg/mL; 3.2 µg/mL). Cells were treated with a range of concentrations of each extract: 200 µg/mL; 40 µg/mL; 8 µg/mL; 1.6 µg/mL (final concentration in the wells).

Cytotoxicity activity by MTS method

This method consists of evaluating cell viability after treatment with a product. Cells were seeded in 96-well plates and placed in an incubator set at 37°C with humidified CO₂ (5%) for 24 hours. The extracts were solubilized in 1% DMSO, which was also used as control. For untreated cells, 100 µL of medium was added to each well. Doxorubicin (1 mM), a reference anticancer agent, was used as a positive control. Except for the blank (MTS), 100 µL of each extract at the different concentrations (400 µg/mL; 80 µg/mL; 16 µg/mL; 3.2 µg/mL) were tested in triplicate by addition to wells already containing 100 µL cells of HCT-116 (20,000 cells/well).

Then, 100 µL of MTS/PBS mixture (1:9) was added to the plates and incubated for 1 hour (depending on the staining). The plates were removed and the absorbance of the plates was measured at 490 nm using a spectrophotometer. The absorbance values measured are directly proportional to the

number of live cells, and the results were expressed as a percentage of cell viability according to the formula %:

$$\%Viab = \frac{A_{\text{sample}} - A_{\text{white}}}{A_{\text{whitess}} - A_{\text{white}}} \times 100$$

%Viab: Percentage of cell viability

A_{Sample}: Absorbance of the treated cells tested (extracts)

A_{Witness}: Absorbance of the untreated cells

A_{White}: Absorbance of blank (MTS)

All experiments were performed in technical triplicates (3 wells/concentration) then in biological triplicates (n=3). Results are expressed as mean ± standard error of the mean. Data treatment was performed using GraphPad Prism (version 8). The sigmoid dose-response curve obtained from non-linear regression analyses (log of inhibitor versus normalized response) was used to determine the 50% inhibitory concentrations (IC₅₀)⁹.

Antioxidant activity

Two chemical tests were used in this study, the DPPH test and ABTS.

For DPPH test, stock solution was prepared by dissolving 0.024 g of DPPH in 100 mL of ethanol. The resulting solution was measured using a spectrophotometer at 517 nm. This solution has an absorbance of approximately 0.98 ± 0.021. Then, 1680 µL of the DPPH stock solution was mixed with 1600 µL of each extract at different concentrations ranging from 100 µg/mL to 3.12 µg/mL. The reaction mixture was vortexed and incubated for 30 min at room temperature in the dark. Absorbance was measured at 517 nm. The ascorbic acid control was prepared as an antioxidant reference and tested under the same conditions as the samples¹⁰.

For ABTS test, 0.038 g of ABTS was previously dissolved in 10 mL of water before adding 0.0067 g of potassium persulfate. The resulting mixture was stored in the dark at room temperature for 12 hours before use. It was subsequently diluted with ethanol to obtain an absorbance of 0.7 at 734 nm. The extracts were prepared in a concentration range from 200 µg/mL to 12.5 µg/mL. Antioxidant activity was measured by adding 2 mL of ABTS+• solution to each dilution. Gallic acid was used as a reference antioxidant, which was prepared and tested at the same concentrations as the extracts¹¹. Absorbance was read after 2 minutes on the spectrophotometer at 734 nm using ethanol as a blank.

Each test was performed in triplicate and the results were presented as the average of the three tests. The percentage inhibition (PI) of the DPPH and ABTS radical was calculated according to the equation described by¹⁰:

$$IP = \frac{OD_{\text{control}} - OD_{\text{sample}}}{OD_{\text{control}}} \times 100$$

IP: inhibition percentage of the DPPH or ABTS radical

OD_{control}: Optical density of the control (ethanolic solution of DPPH or ABTS)

OD_{sample}: Optical density of extracts tested

The IC₅₀ or 50% inhibitory concentration, is the concentration of test samples required to reduce the DPPH and ABTS radical by 50%. The IC₅₀ were determined graphically using the inhibition percentage curves as a function of the different concentrations tested, then using GraphaD version 8.1 software the histograms were constructed.

The lowest IC50 values indicate greater antioxidant activity of the tested product.

Qualitative characterization of secondary metabolites

This study will highlight the main chemical groups of pharmacological interest (alkaloids, phenolic compounds, terpenes). It will be done using qualitative methods as described by¹² and based on specific chemical reactions.

Testing for presence of alkaloids

The alkaloids were revealed using DRAGENDORFF reagents (potassium iodo-bismuthate reagent) and Valser-Mayer reagents. A volume of 6 mL of each solution was evaporated in a capsule. The residue was taken up in 6 mL of ethanol (60°). The resulting solution was divided into two test tubes. In the first tube, two drops of DRAGENDORFF were added, the appearance of a precipitate or an orange color indicates the presence of alkaloids. In the second tube, two drops of Valser-Mayer reagent were added, the appearance of a milky white precipitate indicates a positive reaction.

Testing for presence of polyphenols

One drop of a 2% alcoholic solution of ferric chloride was added to a volume of 2 mL of extracts. Ferric chloride, in the presence of polyphenolic derivatives, gives the appearance of a more or less dark blue-blackish or green color.

Testing for presence of flavonoids

A volume of 2 mL of each extract was evaporated to dryness in a capsule in a water bath. After cooling, the residue was taken up in five milliliters of half-dilute hydrochloric alcohol. The resulting solution was poured into a test tube. The successive addition of two to three shavings of magnesium and three drops of isoamyl alcohol. In the presence of flavonoids, an intense pink-orange or purple color appears.

Testing for presence of saponosides

This test is based on the property of aqueous solutions containing saponosides to foam when stirred. A volume of 15 mL of the extract was placed in a tube, the tube was shaken vertically for ten seconds and then allowed to stand for ten minutes. After this resting period, the height of the foam is measured. If the height is greater than 1 cm, saponosides are present.

Testing for presence of tannins

To the residue of five milliliters of the evaporated extract, 15 mL of STIASNY reagent (30% formalin, concentrated HCl:

1/0.5) was added. The whole was placed in a water bath at 80°C for 30 min. The observation of precipitates in large flakes after cooling indicates the presence of tannins.

Testing for presence of sterols

A volume of 5 mL of the extract was evaporated to dryness in a porcelain capsule in a sand bath. The residue was dissolved hot in 1 mL of acetic anhydride. The whole was inverted into a test tube to which 0.5 mL of concentrated sulfuric acid was added.

Testing for presence of quinones

Two (2) mL of the extract are evaporated to dryness in a sand bath in a porcelain capsule, then triturated with 5 mL of 1/5 dilute hydrochloric acid. The whole is placed in a boiling water bath for 30 minutes. After cooling, the solution is added to 20 mL of chloroform in a test tube. The chloroform phase is then saturated with 0.5 mL of half-strength ammonia.

Statistical analysis

All experimental measurements were performed in triplicate and tests were subjected to ANOVA using Statistica version 7.1 software. In case of unequal variance, a Tukey post ANOVA test was performed to determine the homogeneity groups.

RESULTS AND DISCUSSION

Results

Yield: The extraction carried out on 20 g of *M. morindoides* leaves powder allowed to determine the yields. The dichloromethane and ethyl acetate extracts of *M. morindoides* leaves gave a yield of 3.01% and 3.91%, respectively.

Cytotoxicity activity: Figure 1 shows two dose-response curves. Between 0.1 µg/mL and 5 µg/mL, cells viability is 100% for two extracts. From 5 µg/mL to 20 µg/mL, cell viability was approximately 50% for ACE fe.Mm extract. At the same percentage of viability (50%), the concentrations were 100 µg/mL to 150 µg/mL for DCM fe.Mm extract. 10% live cells are observed between 20 µg/mL and 200 µg/mL for ACE fe.Mm and at 200 µg/mL for DCM fe.Mm. At the level of 1% DMSO, the viability is 100% between 0.1 µg/mL and 90 µg/mL. This viability decreases to 60% between 100 and 200 µg/mL.

DCM fe.Mm extract showed toxicity to HCT-116 with an IC50 of 51.14 ± 0.01 µg/mL, while ACE fe.Mm extract showed toxicity with an IC50 of 16.38 ± 0.2 µg/mL (Figure 1). The ACE.fe.Mm extract seems to have a good dose-response on the cells.

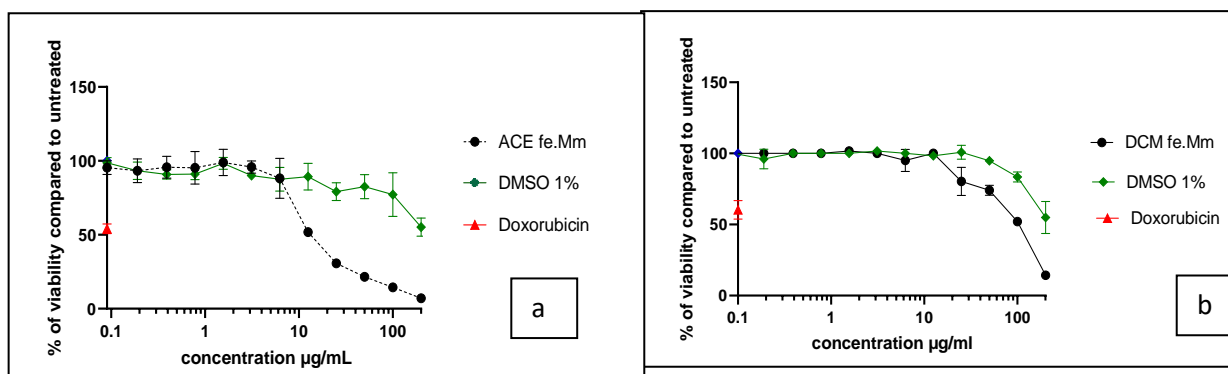


Figure 1: Dose-response activity of *M. morindoides* leaves extracts on HCT-116.

a) Dose-response activity of ACE fe.Mm extract -b) Dose-response activity of DCM fe.Mm extract.

Antioxidant activity

DPPH test

Figure 2 shows the percentage inhibition of the DPPH radical by vitamin C and the tested extracts in an increasing concentration range. An inhibition of about 92% was obtained by ascorbic acid at concentrations between 50 µg/mL and 100 µg/mL. While the maximum inhibition percentage obtained by the extracts is 59% for the dichloromethane extract and 62% for the ethyl acetate extract.

Therefore, to better understand the antioxidant power of the extracts studied, the IC50 values were determined. The IC50 values of ascorbic acid and the extracts are shown in Figure 3. The IC50 of ascorbic acid was 7.31 ±0.2 µg/mL, that of dichloromethane extract was 52.44 ±4.1 µg/mL and that of ethyl acetate was 39.70 ±1.7 µg/mL. The analyses show that there is a significant difference in the different values (p < 0.05).

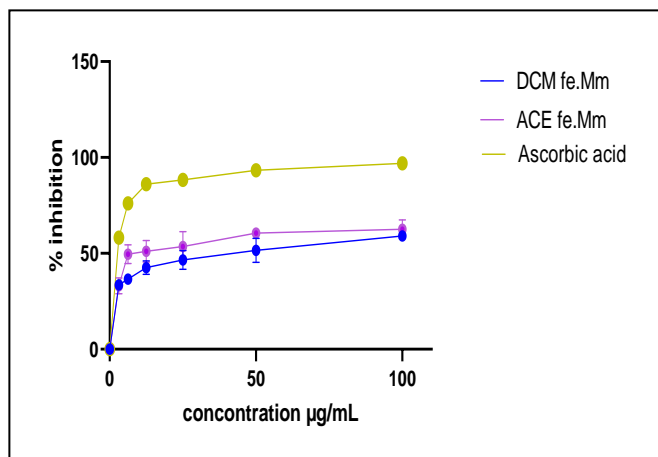


Figure 2: Evolution of percentages of radical inhibition by ascorbic acid, *M. morindoides* leaves extracts.

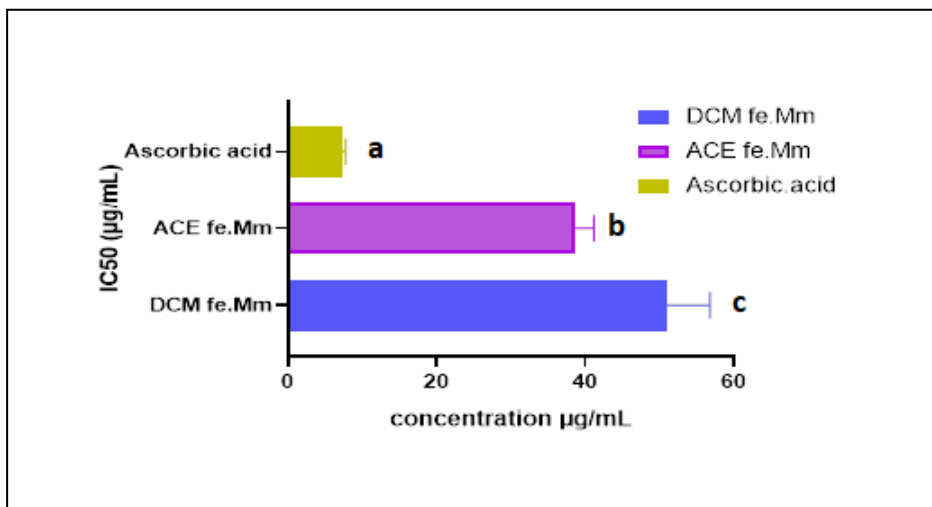


Figure 3: IC50 values of ascorbic acid and *M. morindoides* leaves extracts.

The letters a, b, c, mean that there is a significant difference between the extracts and the control.

ABTS+• test

Figure 4 shows the percentages of inhibition of the ABTS radical by gallic acid and tested extracts with an increasing concentration range from 12.5 µg/mL to 200 µg/mL. An inhibition of about 96% was obtained by gallic acid at concentrations between 100 µg/mL and 200 µg/mL with an IC50 value of 25.72 µg/mL. While the maximum inhibition

percentage obtained by the extracts is 86.76 µg/mL for the dichloromethane extract and 60.42 µg/mL for the ethyl acetate extract.

The IC50 values were plotted as histograms (Figure 5). Statistical analysis shows that there is a significant difference between these values (p < 0.05).

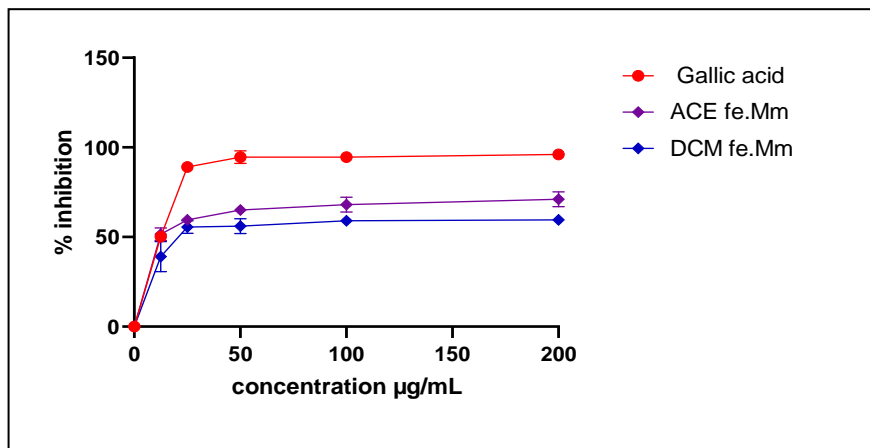


Figure 4: Evolution of percentages of radical inhibition by Gallic acid *M. morindoides* leaves extracts.

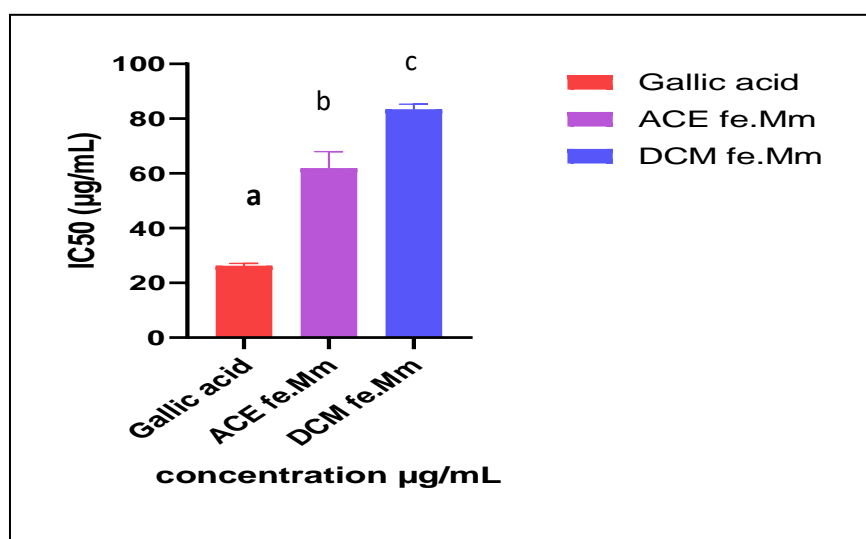


Figure 5: IC₅₀ values of ascorbic acid and *M morindoides* leaves extracts. The letters a, b, c, mean that there is a significant difference between the extracts and the control

Qualitative characterization of secondary metabolites

Phytochemical screening makes it possible to highlight the different chemical constituents of plant extracts. The result of this phytochemical screening is summarized in Table I. It indicates the presence or absence of a group of secondary

metabolites. It varies very little from one extract to another. But in general, the two extracts (dichloromethane and ethyl acetate) didn't contain alkaloids, saponosides, sterols & terpenes, and tannins but contained polyphenols, flavonoids, and quinones.

Table I: Phytochemical constituents of extracts.

Compounds	Ethyl acetate extract (ACE fe.Mm)	Dichloromethane extract (DCM fe.Mm)
Alkaloids	-	-
Flavonoids	+	+
Polyphenols	+	+
Saponosides	-	-
Sterols & terpenes	-	-
Tannins	-	-
Quinones	+	-

+: Presence; -: Absence

DISCUSSION

The use of medicinal plants as an approach to the prevention and treatment of cancer has been followed for many years. Numerous studies have been conducted and have revealed the anticancer activity of certain plants. In the present study, dichloromethane and acetate ethyl extracts of *M. morindoides* leaves were evaluated for their cytotoxic potential on the colorectal cancer cell line HCT-116. The most effective extract was the acetate extract with an IC₅₀=16.38 µg/mL. That could be due to a significant accumulation of chemical compounds responsible for anticancer activity in the acetate extract compared to that of dichloromethane extract. Many phytochemicals exert their cytotoxic effects by acting as cell cycle and apoptosis regulators as well as anti-inflammatory agents¹³. These results are better than those of who obtained¹ an IC₅₀ of 179 ± 0.81 µg/mL on the same HCT-116 cancer cell line using the methanolic extract of *Eclipta alba*. However, this result is less interesting to that of¹⁴ who obtained an IC₅₀ of 11.86 µg/mL with ethyl acetate extract of *Senna alata* tested on HCT-116 cells. Studies have shown that high levels of reactive oxygen species (ROS) promote an increased risk of developing cancer. Another study showed that patient's cancer was deficient in antioxidants compared to healthy individuals⁴. Therefore, in this study, we decided to also evaluate the oxidative activity of *M. morindoides*. DPPH free radical scavenging test showed that the extracts had an antiradical potential but much lower than ascorbic acid with IC₅₀ of 7.31 ± 0.2 µg/mL; 52.44 ± 4.1 µg/mL and 39.70 ± 1.7 µg/mL for ascorbic acid, dichloromethane extract and ethyl acetate respectively. Despite the fact that the antioxidant activity of the extracts is lower than that of the reference substance, they are still considerable. The difference in activity between the two extracts would be linked to the concentration of active ingredients.

Comparison of the IC₅₀ extracts with those of other ethyl acetate extracts revealed that the antioxidant potential of *M. morindoides* extracts is better than that of other ethyl acetate extracts of *Genista quadriflora* and lower than that of *Ipomoea staphylina*. In fact, and obtained by the same DPPH radical trapping test, the respective IC₅₀ was 61.84 µg/mL for *Genista quadriflora*¹⁵ and 35.84 µg/mL for *Ipomoea staphylina*¹⁶.

To confirm the results of the anti-radical efficacy of *M. morindoides* extracts obtained by the DPPH test, a second test based on the ability to trap protons by the ABTS + radical was performed. This test shows that the extracts also inhibit the ABTS radical. This confirms the antioxidant potential of these extracts. These results show that ethyl acetate extract being more polar than dichloromethane, concentrates more chemical constituents and has the capacity to donate hydrogen to a free radical¹⁷. These results are better than those obtained by Kouadio (2022), when evaluating the antioxidant activity of *Turraea heterophylla dichloromethane* and ethyl acetate extracts, reported IC₅₀ values of 14.50 ± 0.32 mg/mL and 13.69 ± 0.33 mg/mL, respectively.

This activity of *M. morindoides* could be due to the nature of the phytochemicals present in this plant. For this purpose, a qualitative characterization test was carried out to highlight the secondary metabolites found in this plant.

The results of the phytochemical screening carried out on the two extracts of *M. morindoides* for cytotoxic and antioxidant purposes showed almost no variation. Both extracts contain polyphenols and flavonoids. The ethyl acetate extract also contains quinones. These results are somewhat in the same direction as those of Koffi (2013) who, after working on the 70% aqueous and ethanolic extract of *M. morindoides* leaves, highlighted not only the presence of polyphenols and flavonoids but also the presence of tannin, alkaloids, quinones,

sterols and saponosides contained in the leaves of *M. morindoides*. The absence of these chemical families in the two extracts could be explained either by a different testing method¹⁷, or by climatic, agro-pedological and geographical factors¹⁸ or either the nature of the solvent.

The cytotoxic and antioxidant activity of two extracts could therefore be justified by the presence of these secondary metabolites. Indeed, flavonoids are known for their anti-tumor power against colorectal, stomach, lung, and breast and medulloblastoma cancer. They are capable of inducing cell death and inhibiting the growth of cells produced in colorectal cancer¹⁹ with their reducing power, flavonoids are also capable of scavenging free radicals²¹. According to Boua et al (2013), good activity is due to the presence of phenolic compounds. *M. morindoides* would therefore be a good candidate in the search for anticancer and antioxidant molecules.

CONCLUSION

This study demonstrated the potential antioxidant and cytotoxic activity of ethyl acetate and dichloromethane extracts of *Morinda morindoides* leaves. According to the results obtained in this study, phytochemicals as polyphenols could be the source at the origin these activities justifying its traditional use in the treatment of numerous diseases. The ethyl acetate extract showed the best free radical scavenging activity of DPPH and ABTS, and good cytotoxic activity against colorectal cancer line cells HCT116. These results could provide a solid scientific basis for the research of new anti-tumor compounds.

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