

Full Length Research Paper

Phytochemical screening and *in vitro* antifungal activities of extracts of leaves of *Morinda morindoides* (Morinda, Rubiaceae)

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The aim of the present study was to compare antifungal activity of various extracts (aqueous, ethanol, ethyl acetate and hexane extracts) of *Morinda morindoides* (Baker) Milne-Redh (Rubiaceae) and investigate their phytochemical screening in order to determine which components give potency to the extract that show highest antifungal activity. *M. morindoides* leaves were extracted in water (E_{taq}), ethanol (E_{eth}), ethyl acetate (E_{ace}) and hexane (E_{hex}) and each extract was tested against *Candida albicans* (yeast) and *Trichophyton rubrum* (mould). Agar dilution method at serial concentrations ranging from 125 to 1.95 mg/ml was used for the determination of the antifungal parameters- minimum inhibitory concentration (MIC) and concentration producing 50% inhibition (IC_{50}) for these strains. Phytochemical screening of the extracts of leaves of *M. morindoides* showed the presence of alkaloids, polyphenols, tannins, flavonoids, saponins, quinones and sterols in various concentrations. The ethyl acetate did not contain tannins and quinones. Polyphenols, saponins and quinones were absent in hexane extract. Compared to other extracts (E_{taq} , E_{eth} and E_{ace}) of *M. morindoides*, hexane extract showed the highest antifungal activity against *C. albicans* with the MIC of 31.25 mg/ml and IC_{50} of 6.17 ± 1.04 mg/ml. The same extract presents the strongest activity against *T. rubrum* with the MIC of 15.62 mg/ml and IC_{50} of 2.68 ± 1.19 mg/ml. Among the extracts of *M. morindoides* leaves, hexane extracts exerted best antifungal potency against strains which are mostly responsible for superficial mycoses. Thus, this extract may be used for the treatment of dermatomycoses.

Key words: *Morinda morindoides*, antifungal activity, hexane extract, dermatomycoses.

INTRODUCTION

Natural products have long been used for their therapeutic properties. The main sources of medicines were plant, animal and mineral products (Kao, 1980). Many efforts have been made to discover new antimicrobial compounds from various kinds of sources such as microorganisms, animals and plants. Their systematic screening may result in the discovery of new effective antimicrobial compounds. Among the remedies used, plant drugs constitute an important part. A number

of scientific investigations have highlighted the importance and contribution of many medicinal plants (Grayer and Harbone, 1994). In recent times, the rapid development of multiresistant bacterial and fungal strains of clinically important pathogens fetches the interest of scientist to develop newer broad spectrum antimicrobial agents (Weisser et al., 1966). The less availability and high cost of new generation antibiotics implies looking for the substances from alternative medicines with claimed antimicrobial activity. A number of herbs with significant antimicrobial activity have been reported in different traditional literatures (Balandrin et al., 1985). In Ivory Coast, traditional medicines are increasingly sought from

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tradipractitioners and herbalists for the treatment of various diseases. Among the plants used, *Morinda morindoides* is well known in the traditional medical practice of the west central part of Ivory Coast. It is commonly called "Zêlékelé" in the local language of 'Bété' and is used as an antimicrobial agent (Zirihé et al., 2005). The leaves of the plant are used in traditional medicine to treat diarrhoea. In the Democratic Republic of Congo, *M. morindoides* has long been used in villages and towns in the treatment of some parasitic diseases, and the leaf extracts of this plant are said to possess antiprotozoal activity particularly against *Entamoeba histolytica* and rheumatic pain (Cimanga et al., 2006). The decoction of the leaves is used for the treatment of malaria, intestinal worms, and amoebiasis (Kambu et al., 1990). In addition, aqueous, ethanol and ethyl acetate extracts of leaves of *M. morindoides* have revealed *in vitro* and *in vivo* some interesting results such as antimalarial (Zirihé et al., 2005), antifungal (Bagre et al., 2007), antibacterial (Moroh et al., 2008), anti-diarrheal (Meite et al., 2009) and antispasmodic (Cimanga et al., 2010) activities.

Phytochemical investigations of these extracts of *M. morindoides* showed the presence of saponins, flavonoids, terpenes, steroids, tannins, quinines and alkaloids (Tona et al., 1999; Bagre et al., 2007). In previous study, hexane extract of leaves of this plant was incorporated in soap which presents interesting antifungal activity (Toué et al., 2010). To the best of our knowledge, this extract was not investigated alone to his antifungal activity and phytochemical screening. The aims of this study were therefore to compare antifungal activity of hexane extract with those of the three other extracts of *M. morindoides* aforementioned against *Candida albicans* and *Trichophyton rubrum* and to identify which components are deeply involved in this activity.

MATERIALS AND METHODS

Plant material

The leaves of *M. morindoides* (Rubiaceae) were collected from Daloa (central west region of Ivory Coast) in June 2006. The plant was identified and authenticated by Pr Ake-Assi, of the Department of Botany, University of Cocody. A voucher specimen (no. 17710) of the plant was deposited in the herbarium of the National Floristique Center of the University of Cocody-Abidjan.

Preparation of extracts

The leaves of *M. morindoides* were cleaned of extraneous matter, air-dried at room temperature for 7 days and ground into a fine powder. From this powder, aqueous, ethanol and ethyl acetate extracts were obtained successively following the method described by Zirihé (2007), Bagre (2007), Meite (2009) and Tra-Bi (2010). Forty grams (40 g) of fine powder of *M. morindoides* was mixed with one liter of distilled water for 24 h with constant stirring at 80°C. The extract was filtered twice through cotton wool, then through Whatman filter paper (No. 1). The filtrate was evaporated to

dryness using under vacuum in a rotary evaporator (Buchi) at 60°C. We obtained around 4.52 g (11.31±0.32%) of brown powder denoted aqueous extract of *M. morindoides* (E_{laq}). This operation was repeated several times to prepare enough aqueous extract for the ethanol extract preparation. (25 g) of E_{laq} was dissolved in 500 ml of ethanol 96% (350 ml) and distilled water (150 ml) mixture and after thorough mixing, the supernatant was evaporated using a rotary evaporator to give 5.3 g (21.20±0.27%) of pale brown powder which was taken as ethanol extract of *M. morindoides* (E_{eth}). Several extractions were given sufficient quantity of this extract to produce ethyl acetate extract. This extract was obtained by dispersed 25 g of E_{eth} in 500 ml of ethyl acetate (250 ml) and distilled water (250 ml) mixture and mixed for 24 h with constant stirring. From the two phases formed, the supernatant was evaporated using a rotary evaporator, and the resulting black powder (4.6 g; 18.4±0.57%) was taken as the ethyl acetate extract of *M. morindoides* (E_{ace}). To obtain hexane extract of *M. morindoides*, dry powdered plant material (100 g) was extracted with 250 ml of hexane (Merck, Darmstadt, Germany) for 24 h using a Soxhlet extractor. The extract was filtered with Whatman filter paper no.1, and the filtrate was evaporated under vacuum in a rotary evaporator (Buchi) at 55°C. We obtained 9.24 g (9.24±0.18%) of greenish paste as hexane extract (E_{hex}). The different extracts of *M. morindoides* (E_{laq}, E_{eth}, E_{ace} and E_{hex}) obtained were used for the phytochemical screening and *in vitro* antifungal tests.

Phytochemical screening

Phytochemical screening of different extracts of *M. morindoides* was performed using standard procedures (Ayoola et al., 2008).

Test for alkaloids

0.5 g of extract was diluted into 10 ml with acid alcohol, boiled and filtered. To 5 ml of the filtrate was added 2 ml of dilute ammonia. 5 ml of chloroform was added and shaken gently to extract the alkaloidal base. The chloroform layer was extracted with 10 ml of acetic acid. This was divided into two portions. Mayer's reagent was added to one portion and Draggen dorff's reagent to the other. The formation of a cream (with Mayer's reagent) or reddish brown precipitate (with Draggen dorff's reagent) was regarded as positive for the presence of alkaloids.

Test for polyphenols and tannins

About 0.5 g of the extract was boiled into 10 ml of water in a test tube and then filtered. A few drops of 0.1% of ferric chloride was added and observed for brownish green or a blue-black coloration.

Test for terpenoids (Salkowski test)

To 0.5 g each of the extract was added 2 ml of chloroform. Concentrated H₂SO₄ (3 ml) was carefully added to form a layer. A reddish brown coloration of the interface indicates the presence of terpenoids.

Test for flavonoids

Three methods were used to test for flavonoids. First, dilute ammonia (5 ml) was added to a portion of an aqueous filtrate of the extract. Concentrated sulphuric acid (1 ml) was then added. A yellow coloration that disappears on standing indicates the

Table 1. Phytochemical constituents of different extracts (E_{taq} , E_{eth} , E_{ace} and E_{hex}) of *M. morindoides*.

Test	Extracts			
	E_{taq}	E_{eth}	E_{ace}	E_{hex}
Alkaloids	++	+++	+++	+++
Polyphenols	++	++	++	-
Tannins	++	+	-	++
Flavonoids	+++	+	++	++
Saponins	+++	+++	+	-
Quinones	+	+	-	-
Sterols	+	+	+	+++

Key: (-) Absent, (+) Present in low concentration, (++) Present in moderate concentration, (+++) Present in high concentration.

presence of flavonoids. Secondly, a few drops of 1% aluminium solution were added to a portion of the filtrate. A yellow coloration indicates the presence of flavonoids. Next, a portion of the extract was heated with 10 ml of ethyl acetate over a steam bath for 3 min. The mixture was filtered and 4 ml of the filtrate was shaken with 1 ml of dilute ammonia solution. A yellow coloration indicates the presence of flavonoids.

Test for saponins

To 0.5 g of extract was added 5 ml of distilled water in a test tube. The solution was shaken and observed for a stable persistent froth. The frothing was mixed with 3 drops of olive oil and shaken after which it was observed for the formation of an emulsion.

Test for quinines

0.5 g of the extract was boiled with 10 ml of sulphuric acid (H_2SO_4) and filtered while still hot. The filtrate was shaken with 5 ml of chloroform. The chloroform layer was pipette into another test tube and 1 ml of dilute ammonia was then added. The resulting solution was observed for colour changes.

Test for steroids and terpenoids

A quantity (9 ml) of ethanol was added to 1 g each of the extracts and refluxed for a few minute and filtered. Each of the filtrates was concentrated to 2.5 ml in a boiling water bath. Distilled water, 5 ml was added to each of the concentrated solution, each of the mixtures was allowed to stand for 1 h and the waxy matter was filtered off. Each of the filtrates was extracted with 2.5 ml of chloroform using a separating funnel. To each 0.5 ml of the chloroform extracts in a test tube was carefully added 1ml of concentrated sulphuric acid to form a lower layer. A reddish-brown interface showed the presence of steroids. To another 0.5 ml each of the chloroform extract was evaporated to dryness on a water bath and heated with 3 ml of concentrated sulphuric acid for 10 min on a water bath. A grey colour indicates the presence of terpenoids.

Test microorganisms

In vitro antifungal studies of extracts of *M. morindoides* were carried out on two fungi strains *C. albicans* (clinical isolate

n° 3076/PV/06.04.2000) and *T. rubrum* (clinical isolate n° 14301/D/29.12.2005) provided from the Mycology Laboratory of the Department of Medical Sciences of University of Cocody (Abidjan-Ivory Coast).

Antifungal assay

The antifungal activity was assessed according to the agar dilution method (Tona et al., 1998; N'guessan et al., 2007) on Sabouraud agar (Scharlau). The fungi cultures were inoculated in Sabouraud agar (Scharlau) and incubated for 48 h at $30.0 \pm 0.1^\circ\text{C}$. Each extract of *M. morindoides* was incorporated into growth medium to give serial two-fold dilutions. The resulting concentrations ranged from 125 to 1.95 mg/ml. A medium containing nutrient broth only seeded with the test organisms was served as control of growth. The counts of fungi cultures were adjusted to yield 10^5 to 10^6 ml^{-1} , respectively, using the standard McFarland counting method. The test microorganisms (0.1 ml) were inoculated with a sterile swab on the surface of appropriate solid medium in tubes. The cultures were incubated for 2 to 5 days at $30.0 \pm 0.1^\circ\text{C}$. All experiments were performed in triplicate. The antifungal parameters- minimum inhibitory concentration (MIC) and concentration producing 50% inhibition (IC_{50}) were determined after counting the colony of fungi of each series. The total score of colony of the control tube was considered as 100%. The MIC is defined as the lowest concentration that produced no visible fungal growth after the incubation time and the IC_{50} is defined as concentration for 50% of inhibition. Values of IC_{50} were determined on the survival curves of microorganisms strains established with Graph Pad software, U.S.A.

Statistical analysis

Data were analyzed by one-way ANOVA followed by Dennett's t-test using Instat® (Graph Pad software, U.S.A). At 95% confidence interval $p < 0.05$ was considered statistically significant.

RESULTS

Phytochemical screening of extracts of leaves of *M. morindoides* revealed the presence of plant secondary metabolites such as alkaloids, polyphenols, tannins, flavonoids, saponins, quinones and sterols (Table 1).

Table 2. Comparative antifungal activities of extracts of *Morinda morindoides* (E_{taq} , E_{eth} , E_{ace} , and E_{hex}) against *C. albicans* (mean \pm SEM, $n = 3$, $P < 0.05$).

Extract	Extracts concentrations (mg/ml)							
	0	1.95	3.9	7.81	15.62	31.25	62.5	125
E_{taq}	100 \pm 1.53	92 \pm 1.45**	84 \pm 1.16	70 \pm 0.88**	52 \pm 1.45**	17 \pm 0.58	0 \pm 0.00	0 \pm 0.00
E_{eth}	100 \pm 1.16	95 \pm 1.33**	83 \pm 1.76**	70 \pm 1.53	22 \pm 1.20	0 \pm 0.00	0 \pm 0.00	0 \pm 0.00
E_{ace}	100 \pm 1.00	91 \pm 0.88	84 \pm 1.53**	67 \pm 1.53**	33 \pm 0.88**	0 \pm 0.00	0 \pm 0.00	0 \pm 0.00
E_{hex}	100 \pm 1.16	87 \pm 0.88**	68 \pm 0.88**	37 \pm 1.16	12 \pm 0.00**	0 \pm 0.00	0 \pm 0.00	0 \pm 0.00

Values are expressed as mean \pm S.E.M ($n = 3$) ** $p < 0.05$, when compared to the control.

Table 3. Comparative antifungal activities of extracts of *Morinda morindoides* (E_{taq} , E_{eth} , E_{ace} , and E_{hex}) against *T. rubrum* (mean \pm SEM, $n = 3$, $P < 0.05$).

Extract	Extracts concentrations (mg/ml)							
	0	1.95	3.9	7.81	15.62	31.25	62.5	125
E_{taq}	100 \pm 1.16	85 \pm 1.45**	50 \pm 0.33**	33 \pm 1.20**	16 \pm 0.33	0 \pm 0.00	0 \pm 0.00	0 \pm 0.00
E_{eth}	100 \pm 1.73	60 \pm 1.16	40 \pm 0.33**	20 \pm 0.33**	3 \pm 0.58**	0 \pm 0.00	0 \pm 0.00	0 \pm 0.00
E_{ace}	100 \pm 1.16	51 \pm 1.53	40 \pm 0.58**	20 \pm 0.33	5 \pm 1.16**	0 \pm 0.00	0 \pm 0.00	0 \pm 0.00
E_{hex}	100 \pm 1.16	76 \pm 1.20	33 \pm 1.16**	11 \pm 0.58**	0 \pm 0.00	0 \pm 0.00	0 \pm 0.00	0 \pm 0.00

Values are expressed as mean \pm S.E.M ($n = 3$) ** $p < 0.05$, when compared to the control.

Except aqueous extract which contained alkaloids in moderate concentration the others extracts showed the presence of these secondary metabolites in high concentration. Polyphenols were present in moderate concentration in aqueous, ethanol and ethyl acetate extracts. They were absent in hexane extract. Tannins were present in moderate concentration in aqueous extract and absent in ethyl acetate extract. Ethanol and hexane extracts showed the presence of these metabolites in low concentration. Flavonoids were present in high concentration in aqueous extract and in low concentration in ethanol extract. Ethyl acetate and hexane extracts contained these components in moderate concentration. Saponins were present in high concentration in aqueous and ethanol extracts. They were present in low concentration in ethyl acetate extract and absent in hexane extract.

Aqueous and ethanol extracts contained quinines in low concentration while these metabolites were absent in ethyl acetate and hexane extracts. Sterols were present in low concentration in aqueous and ethanol extracts. They were present in moderate concentration in ethyl acetate extract and in high concentration in hexane extract. The *in vitro* antifungal activities of extracts of *M. morindoides* (E_{taq} , E_{eth} , E_{ace} , and E_{hex}) are shown in Tables 2 and 3. The values were used by the survival curves (Figures 1 and 2) for the determination of MIC and IC_{50} , and the data for these parameters are recorded in Table 4. All the survival curves are decreasing. Then all the extracts tested present antifungal activity against the two test fungi. Compared to water extract (E_{taq}), the three

other extracts (E_{eth} , E_{ace} and E_{hex}) of *M. morindoides* showed the highest antifungal activity against *C. albicans* with the MIC of 31.25 mg/ml. The hexane extracts of *M. morindoides* presented the strongest activity against *T. rubrum* with the MIC of 15.62 mg/ml. According to IC_{50} values, the hexane extract of *M. morindoides* exerted the best activity against the two fungi tested with the IC_{50} of 6.17 \pm 1.04 and 2.68 \pm 1.19 mg/ml, respectively for *C. albicans* and *T. rubrum*.

DISCUSSION

In this study some extracts of leaves of *M. morindoides* were investigate for their phytochemical constituents and their antifungal activity against two strains implicated in dermatomycoses. For the polar solvents, the phytochemical analyses showed that from aqueous extract to ethanol and ethyl acetate extracts, intensities of polyphenols and sterols were the same. Alkaloids were increase into ethanol and ethyl acetate extracts while intensities of tannins, flavonoids, saponins and quinones were reduce or nil in the same extracts. The results of phytochemical screening of the three extracts obtained with polar solvent in present study were in concordance with those of Bagre et al. (2007). Comparatively to the three extracts above, the non-polar solvent (hexane) more concentrated the sterols. Reports have shown that the extracts of *M. morindoides* have antibacterial and antifungal activities (Kambu et al., 1990; Cimanga et al., 2006; Bagre et al., 2006, 2007). The antifungal activity of

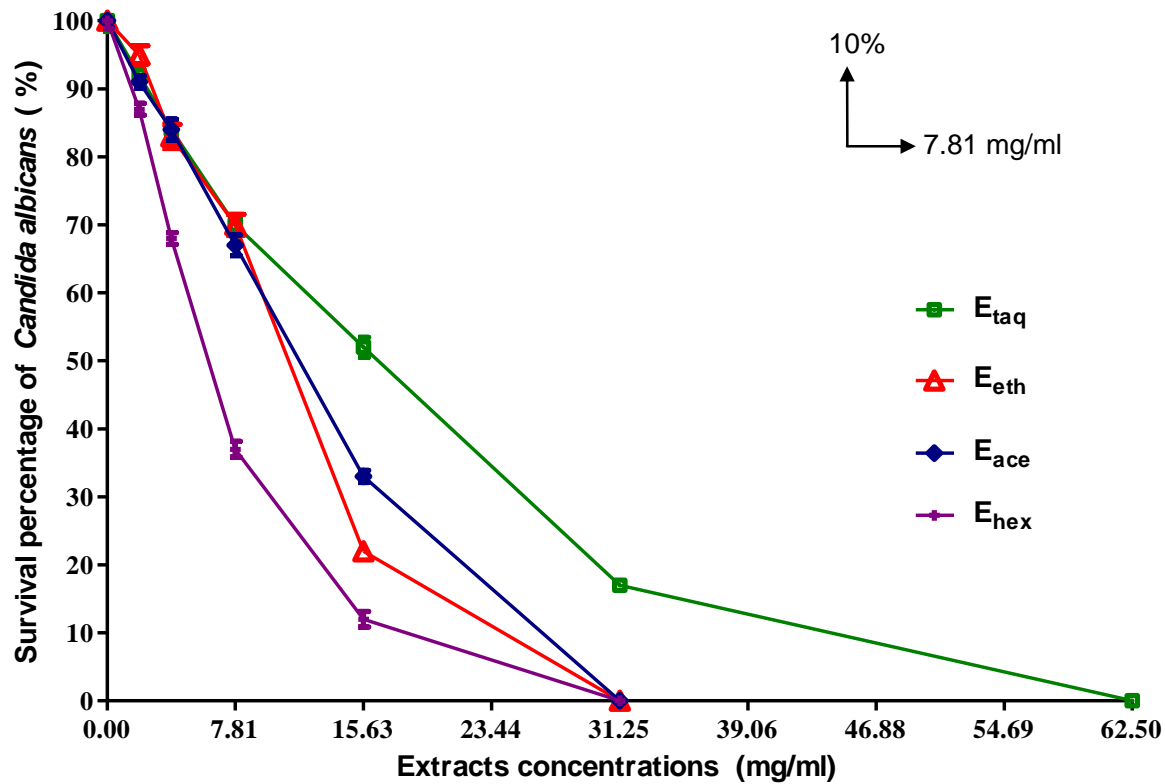


Figure 1. Survival curves of *C. albicans* towards different extracts of *M. morindoides*.

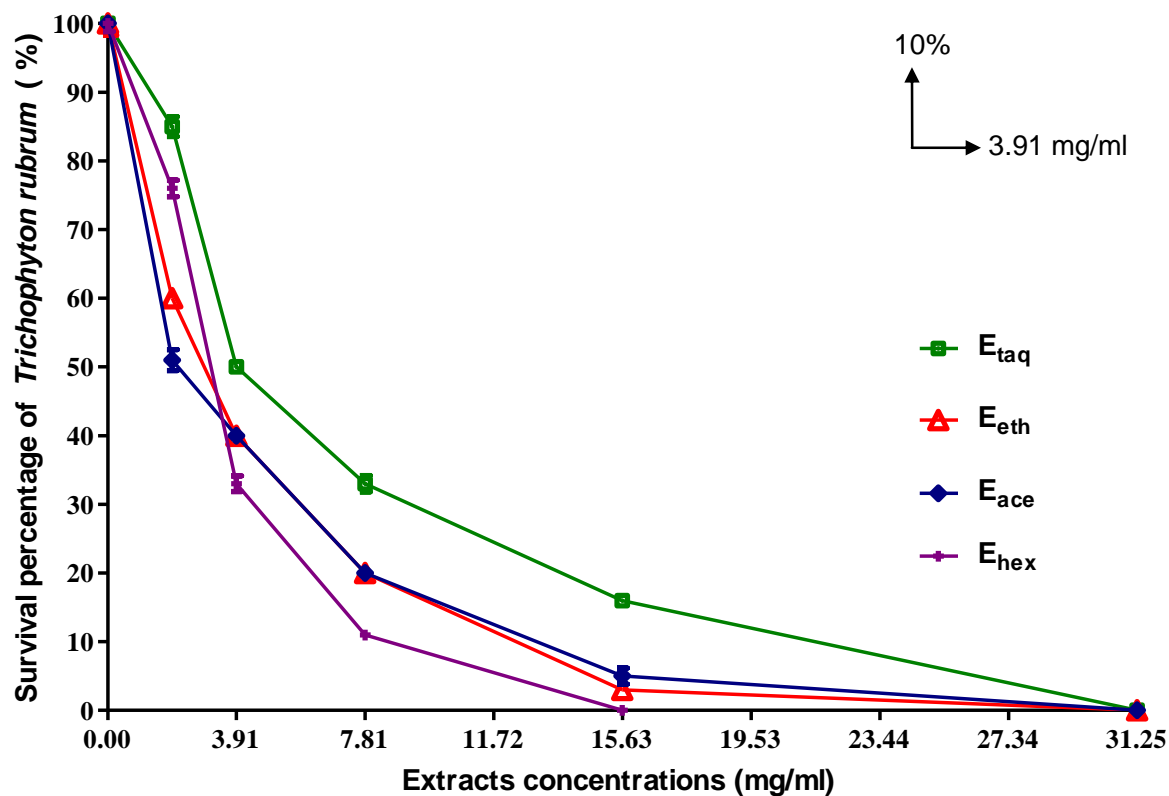


Figure 2. Survival curves of *T. rubrum* towards different extracts of *M. morindoides*.

Table 4. Antifungal parameters for extracts of *Morinda morindoides* (E_{taq} , E_{eth} , E_{ace} , and E_{hex}) against *C. albicans* and *T. rubrum*.

Extract	Antifungal parameter	Fungus	
		<i>C. albicans</i>	<i>T. rubrum</i>
E_{taq}	IC ₅₀ (mg/ml)	16.51±1.40	3.90±0.33
	MIC (mg/ml)	62.50	31.25
E_{eth}	IC ₅₀ (mg/ml)	11.06±0.98	2.92±0.75
	MIC (mg/ml)	31.25	31.25
E_{ace}	IC ₅₀ (mg/ml)	11.72±1.21	2.13±0.76
	MIC (mg/ml)	31.25	31.25
E_{hex}	IC ₅₀ (mg/ml)	6.17±1.04	2.68±1.19
	MIC (mg/ml)	31.25	15.62

these extracts may be due to some of their components such as alkaloids, flavonoids and sterols. The alkaloids, in a general way, play a major role in the biological structures. They are also recognized for their high antibacterial activity (Scazzocchio et al., 2001; Moroh et al., 2008). Phytochemical screening of aqueous extract of *M. morindoides* by Cimanga et al. (1995) yielded ten flavonoids which are likely to have played a major role in antimicrobial activity. Hexane extract possess the best antifungal activity and contain the high quantity of sterols. The sterols may be responsible of the best activity of this extract. This finding confirms the effectiveness of hexane extract of *M. morindoides* against fungi through previous study carried out by Toure et al. (2010).

Conclusion

The fungi strains used in this study are mostly responsible for cutaneous diseases. However, treatments of these infections are sometimes ineffective. This study has shown that the leaf extracts of *M. morindoides*, particularly the hexane extract exerted the best antifungal activity against *C. albicans* and *T. rubrum* which are the most frequent fungal species implicated in cutaneous diseases in Ivory Coast. Further research is needed to determine the toxicological profile of this more active extract (E_{hex}), examine and elucidate the particular compounds responsible for the observed activity.

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