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LC-MS identification and preliminary pharmacological study of the aqueous and ethanol extracts from *Combretum glutinosum* Perr ex DC. (Combretaceae)

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ABSTRACT

Background: *Combretum glutinosum* is a plant whose leaves are consumed as a vegetable and used in traditional medicine for the treatment of microbial infections.
Objective: The present study was designed to identify the compounds in *C. glutinosum* leaves extracts, and evaluate its antimicrobial activity, antioxidant ability and its toxicity in *Artemia salina* larvae *in vitro*.
Methods: The aqueous and ethanol extracts obtained from the leaves of the plant as well as known compounds previously isolated and characterized from the leaves of *C. glutinosum* were tested on eleven different microbial strains. The antioxidant activity of the extracts was evaluated by the Ferric Reducing Antioxidant Power method and the larval toxicity on *Artemia salina* larvae was also detected. Phytochemical screening and HPLC-DAD-HRESI-MS analysis were performed on the extracts to characterize its chemical composition.
Results: When tested at a concentration of 20 $mg{\cdot}mL^{-1}$, the extracts of *C. glutinosum* leaves strongly inhibited the growth of the bacterial strains with an inhibition diameter ranging from 7.25 mm to 44 mm, superior to those of the positive controls (tetracycline at 30 $\mu g{\cdot}mL^{-1}$ and amikacin at 30 $\mu g{\cdot}mL^{-1}$), inhibition diameters from 15 mm to 33 mm. The evaluated larval toxicity demonstrated that it had no harmful effects on *Artemia salina* larvae. The extracts present a good antioxidant activity at a concentration of 0.17 and 1.33 mmol ascorbic acid (per gram of extract) for the aqueous and ethanol extracts, respectively. However, none of the compounds tested at 500 $\mu g{\cdot}mL^{-1}$ were able to show good activity on the 11 reference strains. Phytochemical analysis revealed the presence of alkaloids, polyphenols, steroids, triterpenoids, reducing compounds etc. in both extracts. The HPLC-DAD-HRESI-MS analyses revealed 18 compounds in the ethanol extract, from which 3 were identified, 15 compounds in the aqueous extract from which 5 could be identified.
Conclusion: The present work has shown that *C. glutinosum* extracts can be a good source of antimicrobial agents. They also possess the antioxidant property with absence of toxicity on *A. salina* larvae. A further bio-guided study could allow the identification and isolation of the active ingredients.

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1. Introduction

Traditional medicine as the sum of knowledge, skills and practices that are based, rationally or otherwise, on the theories, beliefs and experiences of a culture have long been used to maintain human health and to prevent, diagnose, treat and cure both physical and mental illness (WHO, 2000).

Today, the emergence of new diseases, the steady growth of bacterial resistance against existing drugs and the side effects of these synthetic products, make it an everyday challenge and imperative for chemists to search for (synthesis) new products with greater and more selective biological efficacy (Amimour, 2009). In developed countries, plants represent an important source for pharmacological research and drug development, not only because plant constituents are used directly as therapeutic agents, but also as raw materials for drug synthesis (Boughrara, 2016). Plants thus play a major role in the lives of humans and animals as they represent an inexhaustible source of secondary metabolites exploited by humans in perfume, food, cosmetic and pharmaceutical industries. In developing countries, they are the most widely used means of healing (Boughrara, 2016). Further, many patients are looking for a more traditional medicine using nature's products as a therapeutic source (Baudel, 2017). This is all the more important as the World Health Organization (WHO) estimates that more than 80% of the African population depends on traditional medicine for their medical security (WHO, 2002). It is clear that there is nowadays a growing demand to limit the use of medicinal inputs at the expense of medicinal plants (Walachowski, 2016).

This attachment to traditional medicine is not only linked to the lack of financial resources in developing countries but also to the emergence of new pathogens resistant to the antibiotics commonly used for the treatment of infections in humans and animals. It is therefore important, following the lead of developed countries, to develop medicinal plants for the search new active ingredients. This valorization according to WHO (WHO, 2013) consists of evaluating the safety and efficacy of plant-based medicines in order to improve the quality of care.

Of the thousands of plants available in the Beninese flora, *C. glutinosum*, a plant of the combretaceae family, caught our attention. *C. glutinosum* is widely used in traditional medicine to treat various ailments (Toklo et al., 2021a). The decoction of the leaves is recommended to treat anemia (Maydell, 1980; Kerharo and Adam, 1974), dysenteric amebiasis (Traore, 1983) and dysentery (Adjanohoun et al., 1989). In addition, the leaves are also used to treat sickle cell disease (Sall et al., 2017) and wounds (Maydell, 1980; Burkill, 1985; Kerharo and Adam, 1974; Malgras, 1992). Further, leaf decoction is used as a diuretic (Kerharo and Adam, 1974; Adjanohoun et al., 1989; Pousset, 2004) and to treat coughs (Maydell, 1980; Kerharo and Adam, 1974). The leaves are also used in baths to combat tetanus (Imperato, 1977) and to treat colds (Hallam, 1979; Maydell, 1980; Burkill, 1985). For the treatment of malaria, decoction and infusion of the leaves are used (Maydell, 1980; Kerharo and Adam, 1974; Malgras, 1992). The leafy branches are used in decoction to treat jaundice (Malgras, 1992; Adjanohoun et al., 1989), arterial hypertension, bilious fever, liver problems, oedema, urinary problems, etc. (Kerharo and Adam, 1974). Finally, the leaves are also used to combat tooth decay (N'diaye 1962; Hallam, 1979).

Combretum glutinosum exhibits several interesting pharmacological properties. Studies have shown that it possesses antifungal activities (Baba-Moussa et al., 1999) and anticancer activity against *Schistosoma mansoni* (Albagouri et al., 2014). Furthermore, it has demonstrated antioxidant properties (Amadou, 2004; Coulibaly, 2019), anti-inflammatory effects (Amadou, 2004), antiparasitic activities (Toklo et al., 2021b), and has shown beneficial effects in the treatment of malaria (Ouattara et al., 2006) and sickle cell disease (Sall et al., 2017).

In order to deepen the pharmacological knowledge on *C. glutinosum* known as an antibiotic used in traditional medicine in Benin and other countries (Adjanohoun et al., 1991; Konkon et al., 2017; Kerharo and Adams, 1974), the present study was undertaken to evaluate the antimicrobial activity of extracts of the plant including some compounds isolated from its leaves, some of which were identified by HPLC-DAD-HRESI-MS.

2. Materials and Methods

2.1. Plant collection

The leaves of *Combretum glutinosum* were collected in the north of Benin (Kandi) and then identified at the national herbarium of the University of Abomey-Calavi under the numbers AA YH 241 / HNB. They were dried in the laboratory for about two weeks at room temperature and outside under the sunlight, and then reduced to powder using an electric grinder (Flour MILLS NIGERIA, EI MOROR N° 1827).

2.2. Chemicals, Reagents and reference strains

Ethanol of HPLC grade was purchased from Fisher Scientific (Tournai, Belgium). Sulphuric acid, Fehling's solution (A and B), ferric chloride, ammoniac, chloroform, Shinoda, Mayer reagents, glacial acetic acid were got from Extrasynthèse (Genay, France). Sodium acetate was purchased from prolabo (France), dinitrobenzoic acid and acetic anhydride were purchased from Sigma-Aldrich (Germany). The eggs of *Artemia salina* Leach obtained from ArFiDel Sprl (Belgium) were used for toxicity assay. Reference strains like *Enterococcus faecalis* ATCC 29212, *Micrococcus luteus* ATCC 10240, *Staphylococcus aureus* ATCC 29213, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853 were obtained from the American Type Culture Collection. *Streptococcus oralis* NCTC 8029 was obtained from National Collection of Type Cultures. *Staphylococcus epidermidis* T22695, *Proteus mirabilus* A24974, *Proteus vulgaris* A25015, *Salmonella typhi* R30951401 and *Candida albicans* MHMR were obtained from Laboratory of Biology and Molecular Typing in Microbiology of Science and Technology faculty of Abomey–Calavi University.

2.3. Extraction and procedure for obtaining the compounds

2.3.1. Aqueous extract

The aqueous extract was obtained by boiling 200 g of the plant's leaves powder in 2 L of distilled water for 30 min. After decantation and filtration of the mixture, the filtrate obtained was evaporated under vacuum to obtain the dry extract (26.7 g), stored at 4°C and used for the various tests.

2.3.2. Ethanol extract

200 g of leaves powder was macerated under magnetic stirring for 72 h in 2 little of ethanol-water (70:30, v/v) at room temperature. After filtration, the resulting mixture was evaporated to dry under vacuum using rotary evaporator and the dry extract (33.6 g) was stored at 4°C.

2.3.3. Tested compounds

In view of the effect that ethanol extract had on the bacterial strains, compounds obtained from *C. glutinosum* leaves previously isolated and reported by (Toklo et al., 2021b), were tested on the different bacterial strains at the dose of 500 $\mu g \cdot mL^{-1}$. These were corymbosin, umuhengerin, 5-demethyl-sinensetin, (20S,24R)-ocotillone, lupeol, oleanolic acid, betulinic acid, β-sitosterol and β-sitosterol glucoside.

2.4. Phytochemical screening of C. glutinosum extracts

The phytoconstituents of the aqueous and ethanol extracts were determined by tube staining reactions and precipitation of the main groups of chemical compounds (Houghton et al., 1998; Sina et al., 2021). The different tests performed are summarized in Table 1.

Table 1
Summary of specific reactions for phytochemical screening.

Class of active ingredient	Specific reagents and reactions
Alkaloids	Mayer (potassium iodomercurate): yellow or louche precipitate in the tube
Catechic tannins	Stiasny reagent: pink precipitate
Gallic tannins	Saturation of sodium acetate + a few drops of 1% $FeCl_3$: blue or black color
Flavonoids	Shinoda (cyanidin reaction): staining
Flavones	Orange
Flavonols	Red
Flavonones	Violette
Anthocyanins	Hydrochloric acid then alkalinisation with diluted ammoniac: red coloration
Leucoanthocyanins	Shinoda (hydrochloric alcohol): cherry red coloration
Quinone derivatives	Born-Trager (reaction between quinone cycles in NH_3 medium) : pink to purplish-red coloration Born-Trager (reaction between quinone rings in NH_3 medium): pink to purplish red coloration
Steroids	Kedde (dinitrobenzoic acid 2% in ethanol + NaOH (1N) 1 :1): red-purple or red wine coloration
Triterpenoids	Liebermann-Buchard (acetic anhydride-sulphuric acid 50:1): violet, blue or green coloration
Saponosides	Foam Index (positive if MI >100)
Cyanogenic derivatives	Guignard (paper soaked in 5% picric acid): orange to brown coloration
Mucilage	Ethanol absolute: flaky precipitate
Reducing compounds	Hot Fehling liquor: brick-red precipitate
Anthracene derivatives	Chloroform + ammoniac: more or less intense red coloration

2.5. Qualitative analysis of extracts constituents using HPLC-DAD-HRESI-MS

2.5.1. LC-MS conditions

For compound identification, a spectrometer attached to an Ultimate 3000 HPLC system (Thermo Fisher, USA) consisting of an LC pump, a diode array detector (DAD) (detecting wavelength : 190-600 nm), an auto sampler (injection volume 10 µL) and a column oven (40°C) was used for analysis. Separations were performed using a Synergi MAX-RP 100A column (50 × 2 mm, 2.5 µm particle size) with an H_2O (containing +0.1% HCOOH) (A)/acetonitrile (containing +0.1% HCOOH) (B) gradient (OmniLab) (flow rate 500 µL·min^{-1}, injection volume 5 µL) was used. High resolution mass spectra were obtained with a QTOF spectrometer (Bruker, Germany) equipped with a HRESI source. The spectrum was run in positive mode (mass range: 100-1500, with a scan rate of 1.00 Hz), automatic gain control to provide high accuracy mass measurements and then a deviation of 0.40×10^{-6} using sodium formate as a standard. 4.5 kV is the sputtering voltage and the capillary temperature is 200°C. Nitrogen was used as the sheath gas (10 L·min^{-1}).

Samples were analyzed using a gradient programme as follows: 95% A isocratic for 1.5 min, sequentially followed by linear gradient to 100% B over 6 min, and 100% B isocratic for 2 min. The system then returned to its initial state (90% A) in 1 min, and was equilibrated for 1 min.

2.5.2. Extracts preparation

Aliquots of 5.00 µL of the 5 mg·mL^{-1} concentration test sample (dissolved in analytical methanol (OmniLab) and then filtered through a seringue-filter-membrane) were injected into the LC–DAD/MS Dionex Ultimate 3000 HPLC (Dionex Softron GmbH Germany).

2.5.3. Identification of peaks

HPLC-DAD-MS analysis was used to identify the constituents of each sample by comparing the UV, MS spectra and peak fragments with the isolated compounds of the plant genus reported in the literature using the Scifinder and MassBank of North America (MoNA) databases.

2.6. Evaluation of antimicrobial activity

2.6.1. Tested microorganisms and antibiogram

The sensitivity of the microorganisms to the extracts was studied by the agar diffusion technique following the method described by (Bauer et al., 1966).

Eleven reference strains were tested for antimicrobial activity including five Gram positive bacteria (*Enterococcus faecalis* ATCC 29212, *Micrococcus luteus* ATCC 10240, *Staphylococcus aureus* ATCC 29213, *Staphylococcus epidermidis* T22695, *Streptococcus oralis* NCTC 8029); five Gram negative bacteria (*Escherichia coli* ATCC 25922, *Proteus mirabilus* A24974, *Proteus vulgaris* A25015, *Pseudomonas aeruginosa* ATCC 27853, *Salmonella typhi* R30951401) and one fungal (*Candida albicans* MHMR).

A bacterial pre-culture (1 colony in 1 mL of MH broth) from the previous day was diluted to a turbidity of 0.5 on the McFarland scale (i.e. 10^8 CFU·mL^{-1}) and reduced to 10^6 CFU·mL^{-1}. Petri dishes containing the solid medium (solid MH) were inoculated with 1 mL of the inoculum. Wells of approximately 6 mm diameter with 4 wells per dish were made and impregnated with 30 μL of the extract solution (20 mgmL^{-1}). After 15 min of diffusion at room temperature, the Petri dishes were incubated at 37°C for 24 h. For each extract, the experiment was performed twice. Antimicrobial activity was determined by measuring the diameter of the zone of microbial growth inhibition produced around the wells after incubation using a ruler. A negative control (DMSO) prepared with 1% distilled water and positive controls consisting of modern antibiotics (tetracycline 30 µg; amikacin 30 µg) were also tested.

2.6.2. Determination of Minimum Inhibitory (MIC) and Bactericidal (MBC) Concentrations and then of Antimicrobial Power (AP)

The liquid macro-dilution method described by Chokki et al. (2020) was used. For its implementation, a range of extract concentrations from 20 mg·mL^{-1} to 0.15 mg.mL^{-1} was prepared in test tubes. Each tube containing 1 mL of the appropriate concentration of extract was inoculated with 1 mL of inoculum (10^6 CFU·mL^{-1}) prepared in Muller Hinton Broth. The whole set was incubated at for 24 h. The growth or not of the microorganisms was assessed visually.

Based on the results of the MIC test, all tubes showing no visible growth were identified. Each tube was plated onto a petri dish containing MH agar medium and incubated at 37°C for 24 h. The lowest concentration of the extract where no growth was observed was considered as the Minimum Bactericidal Concentration (MBC).

Antibacterial power (AP) is the ratio of the Minimum Bactericidal Concentration (MBC) and the Minimum Inhibitory Concentration (MIC). It provides information about the bactericidal and bacteriostatic character of a product (Marmonier, 1990; Okou et al., 2018).

$$AP = \frac{MBC}{MIC}$$

If the antibacterial power is less than or equal to four (AP ≤ 4), the tested product is bactericidal.

If the antibacterial power is higher than four (AP > 4), the tested product is bacteriostatic.

2.7. Evaluation of antioxidant activity

Antioxidant activity was carried out by Ferric Reducing Antioxidant Power (FRAP) method as described before (Hinneburg et al., 2006). To 1 mL of the test solution, 2.5 mL of phosphate buffer (0.2 M; pH 6.6) and 2.5 mL of the aqueous solution (1%) of potassium hexacyanoferrate [$K_3Fe(CN)_6$] were added and the mixture was incubated at 50°C in a water bath for 30 min.

After incubation and addition of 2.5 mL of trichloroacetic acid (10%), the whole mixture was then centrifuged for 10 min at 3000 r/min. To 2.5 mL of the supernatant was added 2.5 mL of distilled water and then 0.5 mL of an aqueous $FeCl_3$ solution (0.1%).

The absorbances were read at the wave length of 700 nm against a calibration curve obtained from ascorbic acid (0 $mg{\cdot}L^{-1}$-200 $mg{\cdot}L^{-1}$). The reducing power was expressed as ascorbic acid equivalents (EAA) per gram of dry extract and determined according to the formula:

$$C = \frac{Co \times D}{M \times Ci}$$

C = concentration of reducing compounds in mol per gram of EAA dry extract;

Co = concentration of the sample read; D = dilution factor of the stock solution;

Ci = concentration of stock solution; M = molar mass of ascorbic acid.

2.8. Toxicity test of extracts on Artemia salina larvae

The toxicity test is carried out to determine the lethal concentration of the test sample at which 50% of the *A. salina* larvae died from a graph showing the number of dead larvae as a function of different concentrations of the test sample. Sixteen larvae contained in 1 mL of seawater are seeded in a test tube for 24 hours in 1 mL of test extract at different concentrations (25; 12.5; 6.25; 3.12; 1.56; 0.78; 0.39; 0.19; 0.09; 0.04 $mg{\cdot}mL^{-1}$) with 2 replicates per concentration. Control tubes containing larval solutions only were made. After 24 hours, the number of dead larvae was recorded. The lethal concentration (LC_{50}) which expresses the toxic or non-toxic effect of the extracts (Ahomadegbe et al., 2018) was derived from the graph showing the number of dead larvae according to the different concentrations.

3. Results and Discussion

3.1. Quantitative analysis of major chemical groups of C. glutinosum extracts

The qualitative phytochemical investigation was carried out to determine the families of bioactive compounds present in the two extracts of *C. glutinosum,* which revealed classes of compounds summarized in Table 2.

Apart from the free anthracenics present in the ethanol extract and absent in the aqueous extract, all the other families of compounds identified in one extract are present in the other, such as alkaloids, tannins, flavonoids, anthocyanins, leuco-anthocyanins, quinone derivatives, steroids, triterpenoid, mucilage and reducing compounds. The only chemical group various (free anthracenics) could be related to the difference in polarity between these solvents or to the extraction method (maceration for 72 h for the ethanol extract). These results corroborate with those of several authors who identified different classes of secondary metabolites in leaves and extracts of *C. glutinosum* (Albagouri et al., 2014; Alowanou et al., 2019). Most of these secondary metabolites, are cited in the literature as being able to contribute to the control of microbial disease pathogens. This was demonstrated by the fact that anthracene derivatives isolated from endophytic fungi had strong antimicrobial activity against pathogenic multidrug resistant microorganisms (Debbab et al., 2012). Similarly, some anthracenes and their derivatives have shown good antimicrobial activities against intestinal bacteria (Kim et al., 2009a, 2009b). The antimicrobial activity of mucilage extracted from *Cordia myxa* fruits showed an inhibitory effect on Gram-negative bacteria, including *E. coli* and *K. pneumoniae*, isolated in urine (Jasiem et al., 2016). Several alkaloids (alkaloid extracts or compounds) have also shown better antimicrobial activity and appeared to be very effective against bacteria and fungi (Erdemoglu et al., 2007; Özçelik et al., 2011). The tannin extract of *Psidium Guajava* leaves was reported to inhibit the growth of *E. coli, P. aureginosa, S. aureus, A. niger* and *C. albicans* (Mailoa et al., 2014). Similarly, different tannin compounds isolated from *Terminalia citrina* showed that each tannin had antimicrobial activity against different microorganisms (Burapadaja et al., 1995). Finally, several studies have also examined the relationship between flavonoid compounds and antibacterial activity which are in close agreement (Cushnie et al., 2005). These and other secondary metabolites identified in the extracts may explain the different uses of this plant in traditional medicine including the antimicrobial properties which are highlighted in this work.

Table 2
Phytochemical composition of *C. glutinosum* extracts.

Major chemical groups	*C. glutinosum*	
	Aqueous extract	Ethanolic extract
Alkaloids	+	+
Catechin tannins	+	+
Gallic tannins	+	+
Flavonoids (flavone)	+	+
Anthocyanins	+	+
Leuco-anthocyanins	+	+
Quinone derivative	+	+
Steroids	+	+
Triterpenoid	+	+
Saponosides	-	-
Cyanogenic derivative	-	-
Mucilage	+	+
Coumarin	-	-
Reducing compound	+	+
Free anthracene	-	+
Combined anthracenes	-	-
O-heteroside	-	-
C-heteroside	-	-

Legend: - : absence; +: presence

3.2. Antibacterial activity of C. glutinosum extracts

Because of the numerous uses of *C. glutinosum* in traditional medicine and particularly in the fight against various microbial problems (Traore, 1983; Adjanohoun et al., 1989), a biological screening was carried out on bacterial and fungal strains with a view to valorising this plant. Aqueous and ethanol extracts generally used by the population in decoction and maceration respectively, were used in this study. The effect of the extracts on the bacterial and antifungal strains was tested, which made it possible to measure the diameters of the zones of inhibition of the extracts, as recorded in Table 3. It appears that the extracts of *C. glutinosum* have a very interesting antibacterial activity by presenting a wide spectrum of activity on almost all the microbial strains. Only the bacterial strains of *E. coli* and *S. typhi* out of the 10 strains tested developed a strong resistance to *C. glutinosum* extracts. The strongest antibacterial activity was observed on the ethanol extract which showed a higher inhibition diameter than the 2 modern antibiotics tested on 3 different microbial strains namely *S. aureus; P. aeruginosa* and *S. epidermidis* (Table 3). Furthermore, the extracts had no effect on the only yeast used in our study.

Table 3
Inhibition diameters (mm) of *C. glutinosum* extracts.

Microbial strains	Ethanolic extract (20 mg·mL^{-1})	Aqueous extract (20 m·.mL^{-1})	Tetracycline (30 µg·mL^{-1})	Amikacin (30 µg·mL^{-1})
Staphylococcus aureus	44.00 ± 9,89	26.00 ± 5.65	25.50 ± 0.70	25.00 ± 0.00
Pseudomonas aeruginosa	43.50 ± 3,53	15.00 ± 0.00	24.50 ± 0.70	26.50 ± 0.70
Proteus mirabilus	21.50 ± 2,12	17.50 ± 9.19	28.50 ± 2.12	-
Micrococcus luteus	13.00 ± 4,24	15.00 ± 1.41	25.00 ± 0.00	24.24 ± 1.41
Staphylococcus epidermidis	30.00 ± 8,48	13.00 ± 1.41	28.50 ± 2.12	28.00 ± 0.00
Proteus vulgaris	16.75 ± 3,88	11.00 ± 1.41	27.00 ± 0.00	25.25 ± 2.82
Streptococcus oralis	13.50 ± 2,12	19.00 ± 4.24	33.00 ± 0.00	-
Enterococcus faecalis	12.00 ± 0.00	7.25 ± 0.00	25.50 ± 0.70	25.50 ± 0.70
Escherichia coli	-	-	16.00 ± 0.00	23.00 ± 0.00
Candida albicans	-	-	15.00 ± 0.00	24.00 ± 0.00
Salmonella typhi	-	-	30.50 ± 0.70	-

Legend: - : inactive

Table 4
Minimum Inhibitory Concentration (mg.mL^{-1}), Bactericide (mg.mL^{-1}) and Antimicrobial Power (AP) of *C. glutinosum* extracts.

Microbial strains	Ethanolic extract			Aqueous extract		
	MIC	MBC	AP	MIC	MBC	AP
S. aureus	10	-	n.d	5	-	n.d
P. aeruginosa	0.31	0.31	1	20	10	0.5
P. mirabilus	20	10	0.5	20	10	0,5
M. luteus	2.5	10	4	20	20	1
S. epidermidis	1.25	-	n.d	5	20	4
P. vulgaris	2.5	20	8	20	-	n.d
S. oralis	5	10	2	10	10	1
E. faecalis	10	10	1	20	-	n.d

Legend: - : inactive ; n.d : not determined ; MIC : Minimum Inhibitory Concentration (mg.mL^{-1}) MBC : Minimum Bactericide Concentration (mg.mL^{-1}) ; AP : Antimicrobial Power

3.3. *Minimum inhibitory and bactericidal concentration of* C. glutinosum *extracts and compounds*

With the results obtained for the evaluation of the antibiogram, we determined the minimum inhibitory concentration (MIC) by the dilution method in liquid medium and the minimum bactericidal concentration (MBC) of the extracts having presented a MIC then, the antibacterial capacity to better appreciate the inhibitory effect of the extracts. It was found that the ethanol extract of *C. glutinosum* has a bacteriostatic power (AP > 4) on *P. vulgaris* (AP = 8) and on almost all the other strains, and the extracts with a quantifiable MBC have a bactericidal power (AP ≤ 4) (Table 4). These results are mostly superior to those obtained by (Yahaya et al., 2012) who evaluated the effect of aqueous and methanol extracts of *C. glutinosum* leaves and bark on four clinical strains (*Salmonella typhi, Pseudomonas aeruginosa, Staphylococcus aureus, Escherichia coli*). Our results also seem to be superior to those obtained by (Usman et al., 2017) who also evaluated the effect of flavonoid and saponin extracts from *C. glutinosum* roots on five different clinical strains (*Escherichia coli, Shigella dysenteriae, Bacillus subtilis, Corynae bacterium* and *Aspergillus niger*). These differences in results could be related to the solvents used for the extractions, especially since our best results are obtained with the ethanol extract. While mentioning that our results with the aqueous extract are also better, these two solvents (water and ethanol) available to the population can facilitate the best use of this plant in the process of its valorization.

3.4. *Identification of compounds of* C. glutinosum *extracts and their antimicrobial activities*

The aqueous and ethanol extracts of *C. glutinosum* were analyzed by HPLC coupled with diode array detectors and electrospray ionisation mass spectrometry (ESI-MS) in positive mode (Bilanda et al., 2020). Representative chromatograms of the different peaks (Fig. 1) allowed the identification of 18 compounds in the ethanol extract (Table 5) of which the structures of 3 putative compounds (combretene B; 2,3-(S)-hexahydroxydiphenoyl-D-glucose ; quadragularol A) could be determined (Fig. 2) and 15 compounds identified in the aqueous extract (Table 5) with the structures of 5 putative compounds determined (Combretol; lupeol; stigmast-4-en-3-one; methyl 23-deoxojessate; lupenone) (Fig. 2) on the basis of chromatogram data, UV spectra and then in comparison with previously isolated compounds of the genus *Combretum*. Although little information is available in the literature on the antimicrobial properties of several of these identified compounds, lupeol and lupenone were active at 100 mg.mL^{-1} against four microorganisms including one fungal strain (*Saccharomyces cerevisiae*) and three bacterial strains (*Staphylococcus aureus* ATCC 29213, *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853) (Mubiu et al., 2017). In contrast, when evaluated on various bacteria (*Bacillus subtilis* ATCC 6633, *Micrococcus luteus* ATCC 10240, *Staphylococcus aureus* ATCC 6538, *Escherichia coli* ATCC 94863, *Pseudomonas aeruginosa* ATCC 14028 and *Salmonella choleraesuis* ATCC 14028) and one fungal strain (*Candida albicans* ATCC 18804), lupenone showed a selective bacteriostatic effect only on *M. luteus* with a MIC of 50 μg·mL^{-1} (Eberson et al., 2020).

The extracts of *C. glutinosum*, for exhibiting good activities on bacterial strains, can be valorized not only in traditional medicine in the manufacture of improved traditional medicines with antibacterial properties but also can constitute a source of research for new antibacterial compounds in the fight against the resistances we witness nowadays. Compounds that could be identified from extracts in the present work are cited as having potential antimicrobial activity. This is the case of lupeol which inhibited the growth of bacterial strains including *Staphylococcus aureus* ATCC 29213, *Enterococcus faecalis* ATCC 29212, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853 (Wal et al., 2015). This compound was tested *in vitro* along with other compounds isolated from the plant. It was found that all 11 strains developed resistance to the 9 compounds tested at 500 µg.mL^{-1}. These results are in contradiction with those reported by Wal et al. (2015) who found the antibacterial effect of lupeol and betulinic acid on *Staphylococcus aureus* ATCC 29213, *Enterococcus faecalis* ATCC 29212, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853 at a lower concentration than the one we tested, i.e. 250 µg·mL^{-1}. Similarly, on other bacterial strains, Jessica et al. (2015) reported that oleanolic acid inhibited the growth of strains of *Mycobacterium tuberculosis* resistant to streptomycin, isoniazid, rifampicin and ethambutol; methicillin-resistant *Staphylococcus aureus; Bacillus subtilis; Enterococcus faecalis* and *Pseudomonas aeruginosa*. The differences in results obtained with the same compounds can be explained in part by mutations that occur in these strains to cause resistance. In any case, bio-guided studies are needed to identify and isolate the active ingredient or to understand whether the good activity of the extracts is due to a synergistic effect of the compounds.

Table 5
HPLC-DAD-HRESI-MS data of major putative compounds in ethanolic and aqueous extracts of C. glutinosum.

Extract	N°	RT (min)	$[M + H]^+$ m/z	Exact Mass	Molecular formula	Putatively identified compounds	Main fragments	Isolated previously from	References
Ethanolic extract	1	2.8	321.2623	321.1338	$C_{17}H_{21}O_6$	n.i	321.26 ; 295.23 ; 281.21 ; 265.21 ; 241.20 ; 223.19 ; 181.13 ; 158.04 ; 141.99 ; 126.02 ; 98.54		
	2	3.2	359.2055	359.207	$C_{18}H_{31}O_7$	n.i	359.20 ; 345.18 ; 314.27 ; 279.23 ; 228.61 ; 219.61 ; 144.03		
	3	3.3	389.2237	387.2171	$C_{23}H_{31}O_5$	n.i	389.22 ; 359.20 ; 345.18 ; 325.78 ; 314.27 ; 264.14 ; 255.13 ; 243.62 ; 234.61 ; 126.02		
	4	3.4	629.5235	627.52	$C_{37}H_{71}O_7$	n.i	629.52 ; 569.48 ; 547.49 ; 529.48 ; 469.44 ; 453.44 ; 423.42 ; 411.20 ; 389.22 ; 359.20 ; 329.18 ; 306.26 ; 253.21		
	5	3.5	359.2047	359.207	$C_{18}H_{31}O_7$	n.i	359.20 ; 329.18 ; 293.24 ; 251.22 ; 219.61		
	6	3.9	461.4008	461.3995	$C_{30}H_{53}O_3$	combretene B or isomer	461.40 ; 443.43 ; 429.41 ; 405.41 ; 355.28 ; 343.77 ; 313.31 ; 279.30 ; 1 77.21	*Combretum molle*	Bahar et al., 2004
	7	4.2	301.2198	301.2168	$C_{20}H_{29}O_2$	n.i	301.21 ; 279.23 ; 205.14 ; 149.06		
	8	4.4	483.0774	483.0775	$C_{20}H_{19}O_{14}$	2,3-(S)-hexahydroxydiphenoyl-D-glucose or isomer	483.07 ; 466.42 ; 452.38 ; 397.39 ; 375.34 ; 305.30 ; 301.21 ; 249.33 ; 207.23 ; 149.06	*Combretum glutlnosum*	Jossang et al., 1994
	9	4.5	311.3348	311.3314	$C_{21}H_{43}O$	n.i	311.33 ; 305.32 ; 283.33 ; 265.32 ; 191.23 ; 109.13		
	10	4.7	497.4833	497.3995	$C_{33}H_{53}O_3$	n.i	497.48 ; 477.45 ; 457.48 ; 439.46 ; 399.40 ; 355.37 ; 307.30 ; 191.23 ; 145.02		
	11	5.0	593.4232	593.4265	$C_{31}H_{61}O_{10}$	n.i	593.42 ; 455.46 ; 437.45 ; 421.44 ; 399.46 ; 367.37 ; 353.35 ; 321.32 ; 177.21		
	12	5.1	499.3782	499.3763	$C_{30}H_{52}O_4Na$	quadragularol A or isomer	499.37 ; 477.50 ; 473.25 ; 459.38; 445.50 ; 399.45 ; 347.32 ; 177.21	*Combretum quadrangu-lare*	Banskota et al., 2000a
	13	5.3	483.5024	481.4985	$C_{32}H_{65}O_2$	n.i	483.50 ; 461.50 ; 443.50 ; 425.48 ;		
	14	5.4	483.5037	481.4985	$C_{32}H_{65}O_2$	n.i	399.45 ; 335.34 ; 257.31 ; 191.23		
	15	5.5	481.4891	469.0982	$C_{20}H_{21}O_{13}$	n.i	481.48 ; 459.49 ; 441.48 ; 425.48 ; 349.35 ; 217.25 ; 143.14		
	16	5.7	621.4619	619.4574	$C_{37}H_{63}O_7$	n.i	621.46 ; 537.51 ; 487.51 ; 441.46 ;		
	17	5.9	621.4596	619.4574	$C_{37}H_{63}O_7$	n.i	421.44 ; 353.39 ; 269.35 ; 225.31 ;		
	18	6.0	397.4311	395.4253	$C_{27}H_{55}O$	n.i	397.43 ; 379.38 ; 347.38 ; 301.21 ; 145.02 ; 80.07		
Aqueous extract	1'	3.4	359.2573	359.2586	$C_{23}H_{35}O_3$	n.i	359.25 ; 343.32 ; 315.21 ; 295.30 ; 293.27 ; 279.26 ; 265.25 ; 239.22 ; 185.08 ; 158.07 ; 144.06 ; 126.04 ; 110.06		
	2'	3.6	389.1284	389.1236	$C_{20}H_{21}O_8$	combretol or isomer	389.12 ; 373.53; 371.16 ; 357.83 ; 279.27 ; 264.18 ; 252.67 ; 243.66 ; 234.65 ; 221.66 ; 167.08 ; 158.07 ; 144.06 ; 126.04 ;	*Combretum quadrangu-lare*	Toume et al., 2011
	3'	3.7	341.4896	337.347	$C_{23}H_{45}O$	n.i	341.48 ; 329.23 ; 321.36 ; 295.30 ; 259.20 ; 253.24 ; 237.20 ; 158.07 ; 144.06		
	4'	4.1	363.0722	363.0716	$C_{17}H_{15}O_9$	n.i	363.07 ; 357.40 ; 323.38 ; 313.36 ; 265.28		
	5'	4.2	611.5901	609.5822	$C_{39}H_{77}O_4$	n.i	611.59 ; 571.57 ; 529.55 ; 511.53 ; 469.51 ; 451.49 ; 385.53 ; 369.53 ; 355.33 ; 343.82 ; 334.81 ; 267.26 ; 144.06 ; 126.04		
	6'	4.3	627.5889	627.5928	$C_{39}H_{79}O_5$	n.i	627.58 ; 597.57 ; 569.56 ; 553.55 ; 527.53 ; 509.60 ; 485.51 ; 467.49 ; 451.49 ; 409.43 ; 363.33 ; 351.82 ; 342.81 ; 283.26 ; 144.06		
	7'	4.5	301.2650	299.2586	$C_{18}H_{35}O_3$	n.i	301.26 ; 279.27 ; 205.15 ; 149.09 ; 126.04 ; 90.57 ; 80.09		
	8'	4.7	507.5262	507.0047	$C_{20}H_{11}O_{16}$	n.i	507.52 ; 485.49 ; 463.48 ; 441.44 ; 419.44 ; 399.51 ; 375.39 ; 331.35 ; 307.38 ; 267.37 ; 219.25 ; 144.06		
	9'	5.5	427.3980	427.394	$C_{30}H_{51}O$	lupeol or isomer	427.39 ; 409.25; 385.06 ; 335.38 ; 291.32 ; 191.26 ; 132.02 ; 91.02	*Combretum micranthum*	Welch, 2010
	10'	5.8	529.6141	527.5403	$C_{34}H_{71}O_3$	n.i	529.61 ; 495.52 ; 485.55 ; 459.49 ; 405.49 ; 363.43 ; 301.26 ; 227.05 ; 145.04		
	11'	5.9	467.6698	467.3373	$C_{27}H_{47}O_6$	n.i	467.66 ; 452.48 ; 441.52 ; 430.46 ; 397.48 ; 377.45 ; 353.44 ; 328.44 ; 227.04 ; 145.04		
	12'	6.0	375.472	375.108	$C_{19}H_{19}O_8$	n.i	375.47 ; 353.44 ; 315.28 ; 301.26 ; 269.39 ; 243.23 ; 227.05 ; 171.17 ; 159.03 ; 145.04 ; 132.02 ; 102.71 ; 91.02		
	13'	6.1	413.3705	413.3783	$C_{29}H_{49}O$	stigmast-4-en-3-one or isomer	413.37 ; 393.45 ; 384.44 ; 279.27 ; 271.25 ; 227.05 ; 159.03 ; 149.09		Amako and Nnaji, 2016

(*continued on next page*)

Table 5 (*continued*)

Extract	N°	RT (min)	[M + H] $^+$ m/z	Exact Mass	Molecular formula	Putatively identified compounds	Main fragments	Isolated previously from	References
	14'	6.4	513.3951	513.3944	$C_{33}H_{53}O_4$	methyl 23-deoxojessoate or isomer	513.61 ; 495.86; 481.56 ; 453.47 ; 425.52 ; 413.43 ; 381.48 ; 375.48 ; 227.05 ; 145.04	*Combretum quadrangulare*	Banskota et al., 2000b
	15'	6.7	425.3780	425.3783	$C_{30}H_{49}O$	lupenone or isomer	425.37 ; 409.51 ; 396.06 ; 387.53 ; 383.35; 301.26 ; 281.32 ; 243.23 ; 227.05 ; 213.23 ; 145.04 ; 132.09	*Combretum glutinosum*	Balde et al., 2019

RT : Retention time; n.i : not-identified

3.5. Antioxidant activity by Ferric Reducing Antioxidant Power (FRAP) method

Antioxidant activity by the FRAP method is the ability of extracts to donate electrons to promote the reduction of Fe^{3+} ion to Fe^{2+} ions. The concentrations were determined using a calibration curve realized with ascorbic acid (Fig. 3) and the values were expressed in mmol of ascorbic acid equivalents/g extract (EAA). The higher the concentration, the greater the reducing power. The ethanol extract (1.33 EAA) was found to have a higher reducing power than the aqueous extract (0.17 EAA). This antioxidant activity of the extracts is in agreement with the results of Amadou (2004) and Coulibaly (2019) who, in qualitative tests with aqueous and alcoholic extracts of *C. glutinosum* trunk bark and root bark on TLC plate, obtained the reduction of DPPH indicated by the change from purple to yellow color on the TLC plate indicating the anti-radical capacity of the extracts. Similarly, the IC_{50} of the methanol extract of *C. glutinosum* leaves was 0.65 $\mu g \cdot mL^{-1}$, indicating the strong anti-radical capacity of the plant (Sall et al., 2017). This high antioxidant activity of the extracts can be explained on the one hand by the presence of different chemical groups found in the extracts including flavonoids, tannins etc. (Table 2), on the other hand, which confirms the good antimicrobial activity of the extracts and this, because various researches have shown that compounds with antioxidant activity can also have other biological properties such as antibacterial, antiviral etc. (Eloff et al., 2005). Thus, the high antioxidant potential of the extracts remains a great asset for the valorization of this plant, especially as it can facilitate the fight against any oxidative stress that may weaken the organism and make it more vulnerable in the case of attack against microbes.

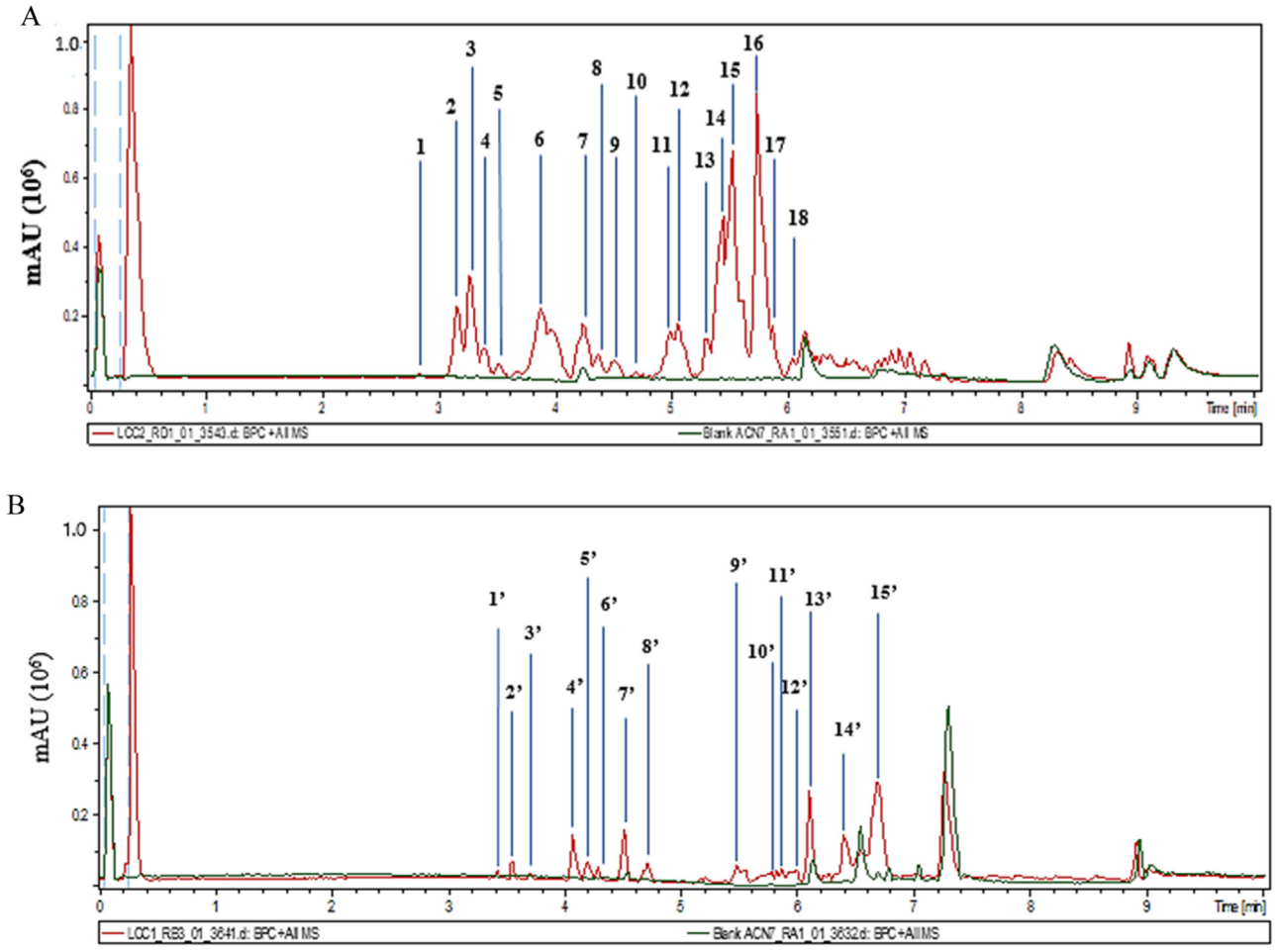
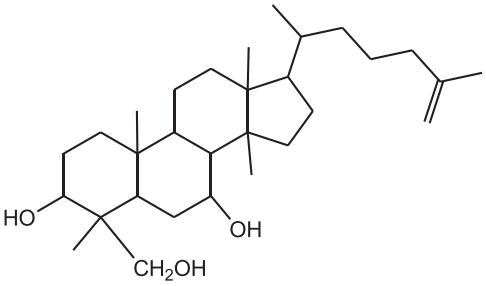
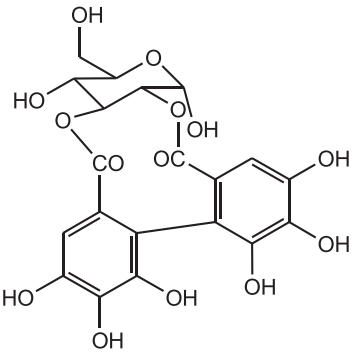


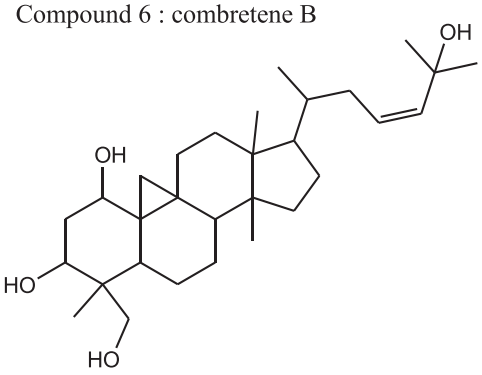
Fig. 1. Base peak chromatogram (BPC) profiles of ethanol (A) and aqueous (B) extracts of *C. glutinosum* showing the main compounds identified and labeled by numbers.



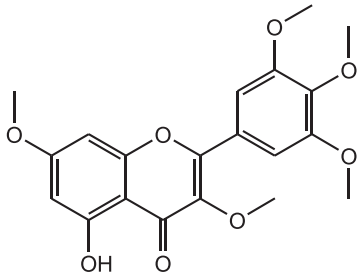
Compound 6 : combretene B



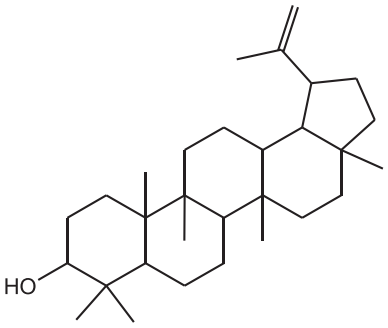
Compound 8 : 2,3-(S)-hexahydroxydiphenoyl-D-glucose



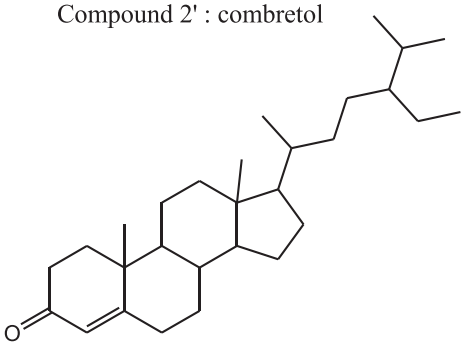
Compound 12 : quadragularol A



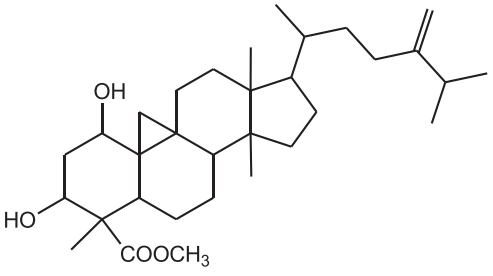
Compound 2' : combretol



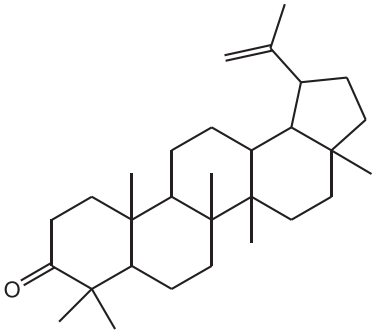
Compound 9' : lupeol



Compound 13' : stigmast-4-en-3-one



Compound 14' : methyl 23-deoxojessate



Compound 15' : lupenone

Fig. 2. Structure of putative compounds identified from ethanol (compound 6, 8 & 12) and aqueous (compound 2', 9', 13', 14' & 15') extracts C. glutinosum.

3.6. Larval toxicity study

The larval toxicity is tested based on the survival of shrimp larvae in seawater in the presence of the test solution. The lethal concentration (LC_{50}) for which half of the larvae could not survive was determined for each extract (Fig. 4). The analyses indicate that the LC_{50} of the decocted and ethanol extracts of *C. glutinosum* leaf is 0.59 mg·mL^{-1} and 1.49 mg·mL^{-1} respectively. These values being higher than 0.1 mg.mL^{-1} reveal that the extracts are not toxic to *Artemia salina* larvae according to the correspondence scale established by (Mousseur, 1995). In turn, the extracts are non-toxic to human nasopharyngeal carcinoma cells (9PS and 9KB cells), lung carcinoma cells (A549 cells) and colon carcinoma cells (HT-29 cells) (José et al., 2002). The absence of toxicity of the extracts on these larvae could therefore be an asset to val-

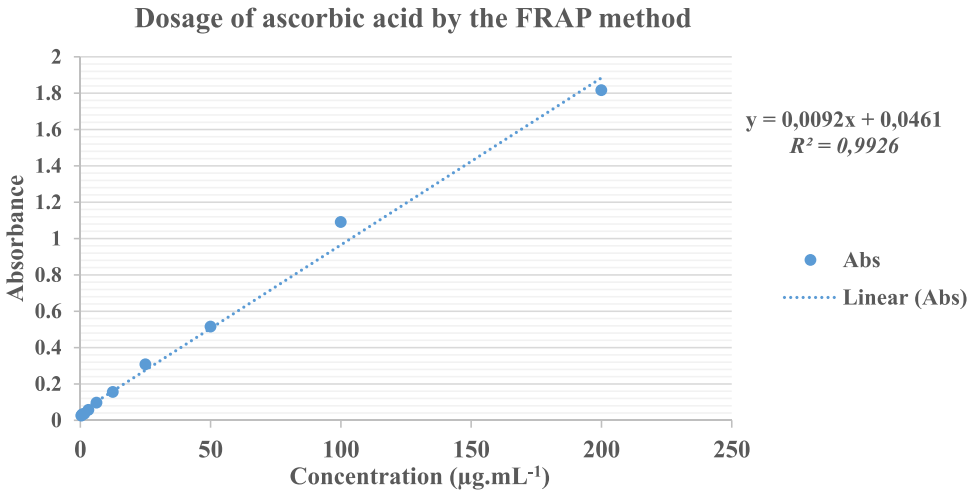


Fig. 3. Calibration curve of ascorbic acid by FRAP method.

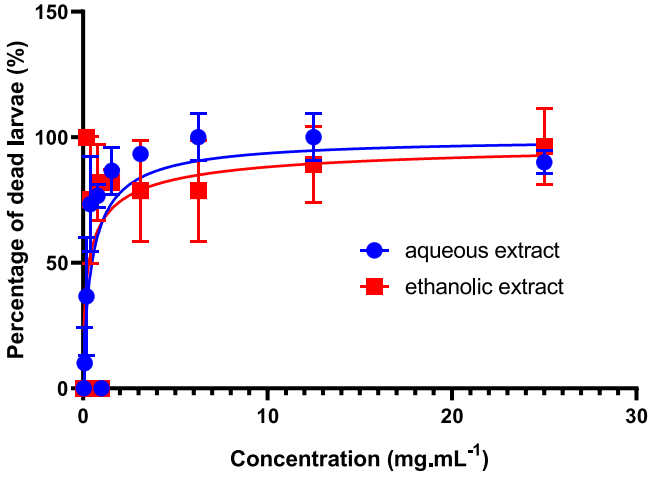


Fig. 4. Percentage of dead A. salina larvae as a function of concentration.

orize the extracts of the plant in traditional medicine. Moreover, previous studies have shown that the methanol extract of the leaves of the plant is non-cytotoxic on human cancer lines (A549, U373, Hs683, PC-3) (Balde et al., 2019) and the acute oral toxicity evaluated *in vivo* on Wistar strain rats also confirms the absence of toxicity of the plant at a single dose of 2000 mg·kg^{-1} body weight (Alowanou et al., 2015).

4. Conclusion

The present work was devoted to study of the antibacterial and antifungal effect of extracts and compounds of *C. glutinosum* leaves, a plant widely used in traditional medicine. The results reveal that the aqueous and ethanol extracts seem to present interesting antibacterial activities against 8 strains of the 11 microorganisms studied and this activity is sometimes much higher than that of reference antibiotics. At the same time, compounds previously isolated from the leaves of *C. glutinosum*, seem to be devoid of any antimicrobial activity on the 11 strains studied. When the chemical composition of the extracts was determined by HPLC-DAD-HRESI-MS, it turned out that several compounds remain unknown (unidentified) and may also be involved in the strong antimicrobial activity presented by the extracts. Synergy between these different chemical constituents may also be at the origin of the good activity obtained. Moreover, no toxic effects were observed on the extracts on *A. salina* larvae. Also, they present very good antioxidant activity *via* the reduction of ferric ion to ferrous ion. Our results therefore confirm the interest of *C. glutinosum* leaves used in traditional medicine and could therefore be used in the manufacture of antimicrobial phytomedicines. However, further studies are still needed to evaluate the safety of these extracts and to find the active fractions or principles by a bio-guided study.

Ethical Approval

Not applicable.

Data availability

The data that support the findings of this study will be available from the corresponding author Placide Mahougnan Toklo at placidetoklo@gmail.com.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT Authorship contribution statement

Placide Mahougnan Toklo conceived, designed and performed the experiments, analyzed and interpreted the data, wrote the manuscript. Mathias Amour Ahomadegbe: Performed the experiments and revised the article. Durand Dah-Nouvlessounon, Billy T. Tchegnitegni, Steven Collins Njonte Wouamba, Joseph Tchamgoue : Analyzed, interpreted the data and revised the article. Djidénou Ahoton, Mahoudo Fidéle Assogba: contributed to the testing and analysis of antioxidant activity and larval toxicity data. Jean-Bosco Jouda performed the acquisition of HPLC-DAD-HRESI-MS data and revised the article. Bruno Ndjakou Lenta YaBiNaPA project coordinator, revised the article. Simeon Fogue Kouam, Lamine Baba-Moussa, Eléonore C. Yayi-Ladekan and Joachim Djimon Gbenou supervised the research work and revised the article.

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