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RESEARCH ARTICLE

PHYTOCHEMICAL STUDY, ANTIOXIDANT AND ANTIMICROBIAL ACTIVITIES OF AQUEOUS EXTRACTS OF BRYOPHYLLUM PINNATUM AND KALANCHOE CRENATA

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ABSTRACT

Background: In underdeveloped countries, the emergence of microbial diseases is amplified by drug resistance, hence the perpetual search for alternative solutions to fight infectious diseases. *Bryophyllum pinnatum* and *Kalanchoe crenata* (Crassulaceae), are two plants used in traditional medicine for the treatment of various skin diseases in infants. The objective of this work is to study the aqueous extracts of fresh leaves of these two plants to know their composition in secondary metabolites and their antioxidant and antimicrobial potentialities. **Methods:** Phytochemical analysis was performed by Thin Layer Chromatography (TLC) while 2, 2-diphenyl-l-picrylhydrazyl (DPPH) method was used to evaluate the antioxidant activities of the studied extracts. Antimicrobial activities were evaluated by liquid and solid medium dilution methods on *Staphylococcus aureus* and *Trichophyton rubrum.* **Results:** This study revealed the presence of polyphenols, flavonoids, sterols and terpenes, followed by a low antioxidant activity of the aqueous extracts of both plants. Antimicrobial tests showed that both extracts were active on S. aureus. However, *B. pinnatum* was twice as active as *K. crenata*. On T rubrum, both extracts are not active because they did not show any inhibition of the germ. **Conclusion:** This study reveals the presence of natural substances with antimicrobial properties in the plants studied. It justifies the use of both plants in the treatment of Staphylococcal skin infections in infants.

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INTRODUCTION

Developing countries are the most vulnerable to the emergence and re-emergence of infectious diseases in the world. Thus, infectious diseases are a major public health concern there because of their frequency and severity (Traoré *et al*., 2012). Also, in recent decades, microbial drug resistance has evolved significantly (Mitscher *et al*., 1999) due to the misuse of antimicrobial agents (Barah, 2010). According to Buckwold *et al* (1979), 50% of antimicrobial agents, such as antibiotics, are used inappropriately. Faced with this observation, the search for new anti-infectious molecules must be intensified and medicinal plants are a privileged avenue.

Indeed, plants have always been a usual source of remedies in the form of traditional preparations (Djerrou, 2011). Also, herbal medicines are often well tolerated (Nahida *et al*., 2012).Currently, the World Health Organization (WHO) estimates that about 81% of humanity uses traditional herbal preparations for primary health care (Lam, 2000). It is to contribute to the search for new therapeutic molecules that our study focused on *Bryophyllum pinnatum* and *Kalanchoe crenata*, two plant species of the Crassulaceae family that show a great proximity in their traditional use on infectious pathologies. In ethnomedicine, these two plants are generally used against earaches, coughs, diarrhea, dysentery, abscesses, wounds, colds, eye diseases and ulcers (Theophil *et al*., 2006; Burkill, 1985).

The general objective of our investigations is to compare the therapeutic potential of plants. For this purpose, their antimicrobial activities, their antioxidant powers and their phytochemical compositions were studied.

MATERIALS AND METHODS

Material: The biological material consisted of fresh leaves of Bryophyllum pinnatum and Kalanchoe crenata whose samples were collected in September 2020 and identified at the National Floristic Center under the following herbarium numbers: B. pinnatum (Lam.) Oken: UCJ004314; K. crenata (Andrews) Haw: UCJ004320. These plants were tested against two microbial strains including Staphylococcus aureus (a bacterium) and Trichophyton rubrum (a fungus)

Study methods

Preparation of the aqueous extracts: The aqueous extract is obtained according to the method described by Zihiri *et al* (2003). Indeed, 100g of our fresh plant material were washed, wrung out, cut, crushed then shaken vigorously in two liters of distilled water, using a Mixer. The homogenate obtained was filtered once on a percal cloth and then twice successively on hydrophilic cotton. The filtrate obtained was evaporated in an oven at 50°C for three days (72h) to obtain a solid aqueous extract.

Qualitative phytochemical analysis of secondary metabolites

Identification of alkaloids: For the identification of alkaloids, the aqueous extracts were deposited in spot on a silica plate using a Pasteur pipette. The plate was then air-dried and placed in a tank containing methanol to migrate the compounds. After migration, the plate is left to dry. The orange coloration that appears indicates the presence of alkaloids in the extracts (Harborne, 1998).

RESEARCH OF POLYPHENOLS

The extract spots were deposited on the silica plate; then air dried. Then, the plate is placed in the elution tank containing methanol to make the compounds migrate. After migration, the plate is left to dry then impregnated with iron trichloride ($FeCl_3$). The blackish blue coloration indicates the presence of polyphenols in the extracts (Wagner, 1983).

Detection of flavonoids: The aqueous extracts were deposited in spot on a silica plate using a pasteur pipette. The plate was then air-dried and placed in a tank containing methanol for migration and then dried. For flavonoid research, Aluminium trichloride ($AlCl_3$) is used as a developer. The yellow-green staining indicates the presence of flavonoids in the extracts (Mangambu *et al*., 2014).

Identification of tannins: The presence of tannins was determined using the Stiasny reagent according to the method of Mibindzou (2004) with modifications. Two milligrams of each dry extract were weighed in a hemolysis tube. Then, two milliliters of ethanol was added to the extract and shaken. Subsequently, one milliliter of Stiasny's reagent was added to the tube and heated to 90°C in a water bath for 30 min. The test was positive when a red precipitate was obtained.

Detection of sterols and terpenes: The extracts were deposited in spot on a silica plate; then dried. The plate was then placed in methanol for migration. After drying, Sulfuric Vanillin was applied as a developer. Purple and blue staining indicates the presence of sterols and terpenes in the extracts (Mangambu *et al*., 2014).

Identification of anthraquinones: For the search of Anthraquinones, spots of extract were made on a silica plate. The plate is then air-dried and placed in an elution tank containing methanol. After migration, the plate was left to dry. Potassium hydroxide (KOH) and sodium hydroxide (NaOH) are used as developers. The appearance of pink-purple or red coloration indicates the presence of anthraquinones in the extracts (Mangambu *et al*., 2014).

Determination of antioxidant activity: The experimental protocol was that used by Benhammou *et al*. (2007). The DPPH solution was prepared by solubilizing ten milligrams of DPPH in 100 ml of methanol to obtain a 0.1 mg/mL solution. Four milligrams of extract was dissolved in four milliliters of methanol to obtain a concentrated stock extract solution to 1 mg/mL. After preparation of the stock solution, six tubes containing 100%, 50%, 25%, 12.5%, 6.25% and 3.12% concentrates of the stock solution respectively were prepared by making successive half dilutions by mixing 2ml of a preceding concentrate in two milliliters of methanol. After this series of half dilutions, two milliliters of DPPH solution was added to each tube and then incubated for 30 min in the dark at room temperature. After 30 min of incubation, absorbances at 517 nm are read against a control (blank) consisting of two milliliters of DPPH solution and two milliliters of methanol. Vitamin C (1 mg/mL) and quercetin (1 mg/mL) were used as reference molecules.

Study of the antimicrobial activities of plant extracts

Antibacterial activities

Preparation of the concentration ranges of the vegetable extracts: Concentration ranges from 100 to 1.56 mg/mL were prepared in a geometric sequence of reason 1/2 in a series of seven hemolysis tubes by the double dilution method. For this purpose, the first tube T1 contains two milliliters of sterile stock extract solution concentrated to 100 mg/mL and the other six tubes each contain one milliliter of sterile distilled water. One milliliter is then taken from the first tube and diluted in the second tube. Then, after shaking, one milliliter is also taken from the second tube to be diluted in the third tube; and so on, this operation is repeated to give in all tubes, these successive concentrations: 100; 50; 25; 12.5; 6.25; 3.125; 1.56 mg/mL (Bolou *et al*., 2011).

Preparation of Staphylococcus aureus inoculum and inoculation: A few young 18 h colonies of S. aureus are picked with a platinum loop and spiked in 10 ml of sterile distilled water. The turbidity of this suspension is adjusted to 0.5 Mac farland using an opacity control (Bio-Rad) (Bolou *et al*., 2011). This estimated 108 cfu/mL bacterial suspension is diluted 1:100 in sterile Mueller-Hinton broth prepared as a twofold concentrate (0.1 mL of bacterial suspension in 9.9 mL of broth) to give an estimated 106 cfu/mL bacterial inoculum. Then, one milliliter of the inoculum is added to each tube in the concentration range. The concentrations tested are finally as follows: 50; 25; 12.5; 6.25; 3.125; 1.56; 0.78 mg/mL.

A growth control tube (GC) containing 1mL of sterile distilled water and 1mL of inoculum, and a sterility control tube (SC) containing 1mL of sterile distilled water and 1mL of sterile Mueller-Hinton broth are prepared. The set of nine tubes thus prepared for each extract is incubated at 37°C for 24 hours (Kouadio *et al*., 2015).

Determination of the minimum inhibitory concentration (MIC): After 24 h of incubation, the bacterial growth resulting in turbidity in each tube is examined. The MIC of an extract against a given strain is the lowest concentration showing no visible growth of the germ.

Determination of the minimum bactericidal concentration (MBC): After plating the concentration range, the inoculum is counted to determine the BMC. A bactericidal control is performed by streaking on Mueller-Hinton agar, dilutions 100; 10-1; 10-2; 10-3; 10-4 of the inoculum, corresponding respectively to 100% 10% 1% 0.1% and 0.01% of survivors. This bactericidal control is incubated for 24 h and then stored in a cold room for the BMC reading. After the MIC reading, streak replicates from tubes without visible growths are incubated for 24 h at 37°C. After 24 h of incubation, these replicates are then compared to the bactericidal control. The BMC is the smallest concentration whose transplants show microbial growth less than or equal to 0.01% survivor (Kouadio *et al*., 2015).

Antifungal activities: To carry out the antifungal activity tests, we proceeded by the dilution method in solid medium.

Incorporation of plant extracts into the agar: The incorporation of plant extracts into the agar was performed using the double dilution method in tilted tubes. Each series has five tubes containing the plant extract incorporated into the culture medium and two control tubes, one without plant extract for germ growth control (TC), the other without plant extract or germ for sterility control (TS) of the working material (Ahon, 2014; Thès, 2001). The test tubes contain a decreasing concentration range of extract that varies from 50to 3.125 mg/mL describing a geometric sequence of reason 1/2.To perform the double dilution, 0.5 g of plant extract is homogenized in ten milliliters of liquefied Sabouraud agar, previously prepared in tube T1 (50 mg/mL). Half of the volume of this homogeneous mixture is transferred to the next tube (T2), containing five milliliters of Sabouraud agar and then this mixture is homogenized. This operation is repeated successively for the other tubes until tube 5 (T5), which should contain the lowest concentration (3.12 mg/mL). For this last tube, half of the volume of the mixture is rejected after homogenization. The tubes thus prepared are sterilized at 121 °C for 15 min in an autoclave and tilted at laboratory temperature to allow cooling and solidification of the agar (Zirihi *et al*., 2003; Bagré *et al*., 2011).

Preparation of Trichophyton rubrum inoculum and Incubation: The inoculum of *Trichophyton rubrum* is prepared from five-day-old colonies. A perfectly isolated colony is stirred rigorously in ten milliliters of sterile distilled water to give an inoculum estimated at 106 cfu/mL. A 1/10 dilution of this suspension is performed by introducing one milliliter into nine milliliters of sterile distilled water, to obtain a suspension corresponding to105 cfu/mL (Guede-Guina *et al*., 1997; Kra, 2001). After solidification of the agar and preparation of the inoculum, the tubes from T1 to T5 and TC are inoculated with ten microliters of the inoculum estimated at 105 cfu/mL, approximately 1000 microbial particles, except for the sterility control (SC) which receives ten microliters of sterile distilled water. The inoculated tubes are incubated at 30°C for six days.

Determination of the Minimum Inhibitory Concentration (MIC): At the end of the incubation time, the minimum concentration for which there is no visible growth of the germ is determined (Guédé-Guina *et al*., 1997; Zirihi *et al*., 2003).

Determination of the minimum fungal concentration (MFC): The minimum fungal concentration is the lowest concentration of extract in the tube that gives at least 99.99% inhibition compared to the growth control tube. It is determined by a sterility test of the tubes without visible growth of the germ. In fact, samples taken on the surface of the inhibitory concentrations are transferred from the MIC. These subcultures are streaked on a new agar without extracts, in Petri dishes and incubated for six days. The MFC will be the smallest concentration whose subculture shows no visible germ growth (Doughari *et al*., 2008; Ackah *et al*., 2016).

RESULTS

The aqueous extraction yields from the leaves of B. pinnatum and K. crenata are presented in Table 1. The extraction of *B. pinnatum* was done with a yield of 2.07% while that of *K. crenata* gave a yield of 1.57%. The results of the qualitative phytochemical analysis of the aqueous extracts of both plants are presented in Table 2. This analysis shows the presence of polyphenols, including flavonoids, and also traces of sterols and terpenes in both aqueous extracts. However, alkaloids, tannins and anthraquinones were not detected. The antioxidant activity test gave the results recorded in Figure 1.

Table 1. Aqueous extraction yields of the two plant species studied

Plant species	family	Yields (%)
K. crenata	Crassulaceae	1,57
B. pinnatum	Crassulaceae	2,07

Table 2. Phytochemical constituents of aqueous extracts of *B. pinnatum* and *K. crenata* revealed by TLC

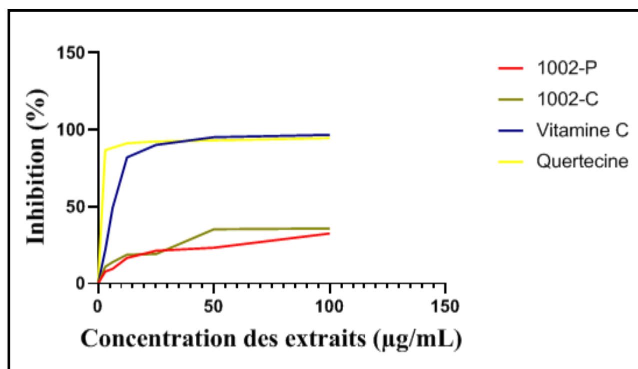
Group of compounds	*B. pinnatum*	*K. crenata*
Alcaloids	-	-
Stérols et terpenes	±	±
Polyphenols	+	+
Flavonoids	±	±
Tannins	-	-
anthraquinones	-	-

+: prsence of compounds ±: traces -: absence of compound

Table 3. Antimicrobial parameters of plant extracts

plant extracts	*Staphylococcus aureus*		*Trichophyton rubrum*	
	MIC	MBC	MIC	MFC
K. crenata	12,5 mg/mL	>50 mg/mL	>50mg/mL	>50mg/mL
B. pinnatum	6,25 mg/mL	25 mg/mL	>50 mg/mL	>50 mg/mL

Ascorbic acid and quercetin showed antioxidant activity with IC50s of 6 µg/mL and 1.7 µg/mL respectively. While for the aqueous extracts, the IC50 could not be determined. They are higher at all concentrations tested (IC50> 100 mg/mL).



Bryophyllum pinnatum (1002-P).*Kalanchoe crenata* (1002-C)

Figure 1. Evaluation curve of the antioxidant activity of the aqueous extracts of *B. pinnatum* (1002-P) and *K. crenata* (1002-C) against standard references (Vitamin C and Quercetin)

The antimicrobial activities of the extracts obtained from the leaves of *B. pinnatum* and *K. crenata* against the tested germs are presented in Table 3.Indeed, antibacterial tests showed that the aqueous extract of K. crenata inhibits the growth of S. aureus with a MIC of 12.5 mg/mL and its BMC is higher than the maximum concentration used (50 mg/mL). Concerning the activity of the aqueous extract of B. pinnatum on the same strain of staphylococcus, the MIC obtained is 6.25 mg/mL and the BMC is 25 mg/mL. Also, the antifungal tests revealed that both extracts were unable to inhibit the in vitro growth of T. rubrum up to the maximum concentration tested (50 mg/mL). Therefore, the MIC and MFC values are considered higher than 50 mg/mL.

DISCUSSION

Microbial resistance to several antibiotics is becoming a real public health problem. Given the increasing rate of antimicrobial resistance that is ravaging not only the African continent but the world as a whole, the search for new sources of antimicrobial molecules is becoming essential if we want to properly control bacterial infections. Our work consisted firstly in the phytochemical characterization of two Crassulaceae species (*B. pinnatum* and *K. crenata*). Then, their antioxidant and antimicrobial activities were evaluated. This study started with the preparation of aqueous extracts. The aqueous extraction of *B. pinnatum* has a higher yield (2.07%) than that of the aqueous extraction of *K. crenata* (1.57%). This means that *B. pinnatum* is richer in water-extractable metabolites. Indeed, these yields could be related to their biological activities. Also, it can be observed that this family of fatty plants, because of their high water content, is relatively low in compound concentration. Phytochemical analysis of plant secondary metabolites has identified the presence of two major groups of secondary metabolites, polyphenols and terpenes. However, in the work of Lewise (2007), the extract of *K. crenata* does not contain terpene compounds. Indeed, secondary metabolites being sometimes transient in plants, it is not surprising that we find traces of them while other authors do not. This difference is also justified by the conditions of extraction, harvesting and cultivation of the plant species studied. The different chemical compounds present in the two plant extracts have therapeutic properties.Flavonoids have antioxidant, anti-inflammatory and anti-tumor activities (Bahorun, 1997). Terpenoids are used as antiseptics and in certain fields such as cosmetics. These two extracts showed the same phytochemical composition. This could be explained by the fact that they are of the same family and also by the fact that their harvesting site is identical. Phenolic compounds are often endowed with antioxidant power. Therefore, we tried to evaluate the level of radical scavenging by the aqueous extracts of the two studied plants. The results obtained showed that these plant extracts had a very low antioxidant activity because they could not inhibit 50% of DPPH. These results are in agreement with those of Lewise (2007) who showed that the digest of *K. crenata* did not have a significant antiradical activity. The polyphenols detected in these extracts are certainly not sufficiently concentrated for more effect. Antimicrobial activity tests of both plant extracts showed that they are active on *S. aureus* with an MIC of 6.25 mg/mL for *B. pinnatum* and 12.5 mg/mL for *K. crenata*. However, B. pinnatum is more active. This extract shows bactericidal activity because the BMC/MIC ratio is equal to 4. This is also explained by the fact that the extract of *B. pinnatum* is more concentrated than that of *K. crenata*. Also, these results corroborate the work of Aibinu *et al*. (2007) on the same extract of *B. pinnatum* and gives a significantly better MIC. Furthermore, sterols, terpenes and flavonoids present in the aqueous extract of *B. pinnatum* may be the source of this activity as they are known to have antimicrobial properties. Concerning the antifungal activity on T. rubrum, both aqueous extracts were inactive. These results and the work of Aibinu *et al* (2007), showed that the aqueous extracts of *B. pinnatum* and *K. crenata* had no effect on the in vitro growth of *C. albicans*, allowing to deduce that these plants do not have antifungal power. It has been shown that the antimicrobial efficacy of extracts depends on their chemical compositions and the nature of the microorganism (Nikolić *et al*., 2014).

CONCLUSION

The work carried out allowed to confirm the presence of polyphenols and terpenes in the two plant extracts. These highlighted compounds are responsible for the antibacterial properties observed on the *staphylococcus* with the two extracts, but with a more important activity with *B pinnatum.* Also, it is necessary to remember that both plants have neither antifungal nor antioxidant power. Finally, this work has shown that the use of these plants in traditional medicine is justified. Indeed, they are used to heal wounds, coughs and staphylococcal syndromes in infants. They contain phytomolecules capable of serving as a basis for the development of new antibacterial agents.

- **Ethics:** In order to avoid any harvesting in the wild, at the risk of disrupting biodiversity, plant samples were grown at the Centre National de frisque and then harvested for this work.
- **Conflict of interest declaration:** The authors declare that there are no conflicts of interest related to this manuscript.

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List of abbreviations

AlCl3: Aluminium Trichloride
TLC: Thin Film Chromatography
MBC: Minimum Bactericidal Concentration

MFC: Minimum Fungicidal Concentration
MIC: Minimum Inhibitory Concentration
DPPH: 2, 2-diphenyl-l-picrylhydrazyl
FeCl3: Iron trichloride
KOH: Potassium hydroxide
NaOH: Soda
WHO: World Health Organization
TC: Growth control
TS: Sterility control
cfu: colony forming unit
UFHB: Felix Houphouët-Boigny University
UFR: Training and Research Unit

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