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Exploration of antibacterial and chemical potential of *Corchorus olitorus* Linn: a vegetable used in Beninese traditional pharmacopoeia

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Abstract

The use of plants as a primary remedy in health care has taken enormous proportions in the world and especially in Africa. The use of natural remedies for the relief of several diseases is expanding with the development of the potential of medicinal plants. Also, the therapeutic failures due to the multi-resistance of certain bacteria have led to the search for new solutions. The present study was initiated to evaluate the chemical activity and biological activity of leaves of *Corchorus olitorus* Linn by ethanolic extraction on multidrug-resistant enteropathogens. The ethanolic extract of the leaves showed interesting flavonoid and plolyphenol contents and was nontoxic at the concentration of 100mg/ml. The non-toxicity of the extract justified its use in culinary preparations and as a remedy in traditional medicine. The diffusion methods in agar medium and in liquid medium were used for the sensitivity test against the selected bacterial strains. The ethanol extract prepared showed no inhibitory activity on both the clinical strains of enteropathogens and on the reference strain *E. coli* ATCC 25922. The antibacterial effect of *Corchorus olitorus* Linn was reversed in this study. It was then revealed that extraction methods can be a major factor in the *in vitro* verification of the properties of plant extracts.

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Introduction

Herbal remedies have been known throughout history to be effective in the treatment of many diseases. Plants continue to demonstrate their effectiveness in the field of human health, especially in underdeveloped countries (Czygan 1993, Ody 1993). Modern drugs have shown their limitations especially antibiotics in the treatment of infectious diseases (Gupta *et al.*, 1998; Corazo *et al.*, 1999). The vegetable *Corchorus olitorius* Linn is a dicotyledonous plant species of the family Tiliaceae (Kiebre *et al.*, 2016). According to Loumerem and Alercia., 2016), *Corchorus olitorius* Linn is a plant native to India or the Indo-Burmese region. *Corchorus olitorius* Linn is currently widespread in all tropical regions, and is probably present in all tropical African countries (Bonnet, 2015). In several African countries and mainly in Benin, it is consumed in the form of sauce (Loumerem and Alercia, 2016). Leafy stems are also used in the treatment of heart problems in Congo, constipation in Tanzania, and seeds are used for purging in Nigeria (Bonnet, 2015). According to Mahmoud *et al.*, 2016 the leaves of *Corchorus olitorus* Linn possess antibacterial properties.

It is also used in Benin in the treatment of typhoid fever, according to Dougnon *et al.*, 2017. Given scientists' renewed interest in natural substances derived from the plant kingdom (Nitta *et al.*, 2002, Souza *et al.*, 2003), it is essential to promote African plants of known utility in order to confirm their effectiveness, but also to bring more or new hope in the fight against multidrug-resistant bacteria. Several substances in plants can be at the origin of their effectiveness in the treatment of diseases. Crude plant extracts are of interest as a potential source of bioactive natural molecules. They are being studied for their possible use as an alternative for the treatment of infectious diseases.

The present study will therefore focus on the search for bioactive compounds potentially present in active *Corchorus olitorus* Linn leaves that may explain its in-vivo efficacy in the treatment of infections related to pathogens of the digestive tract in Benin.

Materials and methods

The work was carried out at the Research Unit in Applied Microbiology and Pharmacology of Natural Substances at the University of Abomey-Calavi, Benin.

Sample collection and treatment

The plant material consisted of fresh *Corchorus olitorus* Linn's leaves. These leaves were harvested in Southern Benin in February 2017. They were authenticated by the National Herbarium of the University of Abomey-Calavi. The leaves were carefully washed in water containing bleach (1/100) and dried at room temperature for three weeks. The dried leaves were crushed and the resulting powders were sieved through a mesh of 0.2mm. They were then stored in clean containers at laboratory temperature. Fig. 1 shows the image of leaves of *Corchorus olitorus* Linn.

Fig. 1. Leaves of *Corchorus olitorus* Linn.

Extraction and preparation of extract

Ethanol extract

The extraction method used is an adaptation to the protocol used by Sanogo *et al.* (2006) and N'Guessan *et al.* (2007). It has the advantage of putting the powder correctly in contact with the solvent with continuous stirring.

A mass of 50g leaf powder was soaked in 500ml of ethanol 96° with continuous stirring for 72 hours. The mixture was filtered three times on hydrophilic cotton then once on Whatman No. 1. Fig. 2 show the filtrate obtained after stirring. Fig. 3 shows the extract powder obtained after drying at the temperature of 40°C in an oven. The dry mass obtained represents the ethanolic extract.

Fig. 2. Filtrate obtained after stirring.

Fig. 3. Extract powder obtained after filtration and drying.

Preparation of extract

Ethanolic extract of the leaves were taken up in distilled water in an amount of 100 mg per 1ml. The stock solutions and concentrated to 100mg/ml were prepared. They were then sterilized by autoclaving at 121°C for 15min. The sterility of the stock solutions of extracts was verified by plating aliquots of each solution on Mueller Hinton medium and incubated at 37°C for 24 hours. The absence of colonies on the medium Mueller Hinton after 24 hours confirmed the sterility of the whole extracts solutions.

Yield of extraction

The yield of the crude extract is defined as the ratio between the mass of the dry extract obtained and the mass of the treated plant material (Harborne, 1998). This efficiency was calculated with the equation:

$$R(\%) = \frac{Me}{Mv} \times 100$$

R (%): Yield in%

Me: Mass of the extract after solvent evaporation

Mv: mass of plant material used for extraction

Determination of Chemical substances (flavonoids and phenolic compounds)

Content of total polyphenols

The determination of total phenols was performed by a method adapted from (Singleton *et al.*, 1999) using the commercial Folin-Ciocalteu reagent Folin consisting of a mixture of phosphotungestique acid (H3PW12 O40) and phosphomolybdic acid (H3PMO12 O40) is reduced during the oxidation of phenols, a mixture of blue oxides of tungsten and molybdenum. The blue coloration produced, whose maximum absorption is at 760nm is proportional to the amount of polyphenols in the various extracts. The rate of total polyphenols in the different extracts was calculated from a linear calibration curve (y=ax+b), established with specific gallic acid as the reference standard concentrations.

Determination of phenolic component in samples

Each test sample was dissolved in ethanol so as to obtain a concentration of 10mg/ml and then diluted 1/100 with distilled water. A volume of 125µl of diluted solution was then mixed with 625µl of Folin-Ciocalteu reagent 10% (diluted 10th in distilled water) and incubated for 5min. 500µl of an aqueous solution of sodium carbonate (Na2CO3) at 75g/l were then added and mixed by vortexing and incubated for 2h. After incubation, the optical densities (OD) were read at 760nm using a spectrophotometer. Three readings were taken per sample. The reading was taken against a blank consisting of a mixture of 0.5ml of FCR and 1ml of Na_2CO_3. The total phenolic contents were determined using a calibration curve Gaelic acid (0-200mg/l).

The calibration curve of gallic acid

From an aqueous stock solution of gallic acid of 10mg/ml mass concentration, a standard range of aqueous test solutions was prepared. Using a micropipette, 125ml of each working solution were put into test tube and then 625µl of Folin-Ciocalteu reagent at 10% (diluted 1/10 with distilled water) is added. After 5 minutes of incubation, 500µl of sodium carbonate (Na_2CO_3)at 75mg/ml and 4.75ml of distilled water was added. The tubes werethen stirred and placed in the dark for 30 minutes at laboratory temperature.

Absorbance of each solution prepared was read using a spectrophotometer at a wavelength of 760nm against a blank prepared in the same manner except that it contains distilled water rather than gallic acid. The absorbance values for each concentration enabled to draw the calibration curve of gallic acid (Singleton *et al*., 1999).

The contents expressed as milligram gallic acid equivalent to 100 mg of extract or fraction (mg/EAG/100 mg of extract) were determined by the following formula:

$$C = \frac{c \times D}{Ci} \times 100 \text{ Avec}$$

C = total phenolics concentration in mg EAG/100mg dry extract
c = concentration of the sample read on the standard curve
D = sample dilution factor under assay
Ci = initial concentration of the sample solution to be assayed

Determination of flavonoids

Flavonoids contents were measured by a method adapted from Zhishen *et al.* (1999) and Kim *et al.* (2003) using aluminum trichloride ($AlCl_3$) as a reagent. The presence of a free space in the reagent $AlCl_3$ forms a dative bond with the lone pairs of the oxygen of the OH groups of flavonoids, producing a yellow colored complex, whose maximum absorbance is recorded at 415nm. The amounts of flavonoids in our extracts were calculated from the calibration curve of a standard flavonoid (rutin).

The calibration curve of rutin

A stock solution of rutin with mass concentration 10mg/ml was prepared in ethanol. From this stock solution, a standard range of aqueous working solution was prepared. A volume of 500µl of each working solution was placed in test tube completed by addition of 500µl of aluminum trichloride at 2% and 3ml of ethanol. The tubes were then slightly shaken and incubated in the dark for 10min at room temperature. Absorbance of each solution prepared was measured in the same spectrophotometer at a wavelength of 415nm against a blank. The absorbance values obtained have allowed drawing the calibration curve of rutin.

Determination of flavonoids in the extracts

500µl of $AlCl_3$solution (2%) were collected and 500µl of the sample was added. 3ml of ethanol to this mixture was added. Blank consisted of 500µl of $AlCl_3$ and 3.5ml of ethanol. The reading of absorbance was done on a spectrophotometer at 415nm after incubation for 10min.

Cytotoxicity testing

Hosts fresh water and brackish, brine shrimp (*Artemia salina*) survive extreme salinity levels (up to about 350g/l) prohibiting the development of any other animal body. These small shrimp larvae do not exceed 13mm. The cytotoxic effect of the extracts was assessed following an adaptation of the method described by Kawsar *et al*., (2008). The tests were conducted on the larvae hatch obtained by 10mg of *Artemia salina* eggs (ARTEMIO JBL GmbH D-67141 Neuhofem) with continuous stirring in 1 liter of sea water for 72 hours. To 1ml of each dilution in geometrical series reason½, extract prepared from a stock solution of 20mg. ml-1, was added 1ml of seawater containing 16 larvae. The number of surviving larvae was counted after 24 hours of incubation. The LC50 was determined from the regression line obtained from the representative curve of the number of surviving larvae on the basis of the concentration of the extracts. Each test was performed in duplicate. To interpret these results, correlation grids associating the degree of toxicity LC50 have been proposed (Moshi *et al*., 2004; Sparkling, 1995). LC50≥ 0.1mg/ml, the extract is not toxic. 0.1mg/ml> LC50 ≥ 0.050mg/ml, the extract has a mean toxicity. LC50 <0.01mg/ml, the extract shows strong toxicity.

Bacterial species

Bacterial species was composed of 09 clinical strains namely *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Pseudomonas oryzihabitans*, *Citrobacter freundii, Escherichia coli*,

Klebsiella rhinocleromatis, *Shigella flexneri*, *Klebsiella oxytoca* and *Salmonella cholereasius* and a reference strain *Escherichia coli* ATCC 25922. These bacterial strains were provided by the National Laboratory of Benin.

Antimicrobial activity test using the agar well diffusion method

Bacterial preculture (1 colony of 18h in 5ml of sterile distilled water) was diluted to obtain a turbidity of 0.5 McFarland (or 108 CFU/mL) and reduced to 106 CFU/ml in sterile distilled water. Each inoculum was plated with swabs on petri dishes containing Mueller Hinton agar (CA-SFM, 2012). Using the tip of sterile Pasteur pipette, wells of 6mm in diameter were drilled. Then using a sterile cone and a micropipette, 50µl of each sample was deposited in the previously dug wells. A well containing sterile distilled water was used as negative control. Antibiotic discs standards were also used to serve as positive controls. The Petri dishes were left for 1 hour at laboratory temperature to a pre-diffusion of the substances before being incubated at 37°C in an oven for 18 hours to 24 hours. After incubation period, the dishes were examined to note zones of inhibition (diameter measured in mm). All tests were performed in duplicate.

Determination of Minimum Inhibitory Concentration (MIC) by micro dilution and minimum bactericidal concentration (MBC).

A sample stock solution was prepared at a concentration of 100mg/ml in distilled water. 100µl of Mueller-Hinton Broth (MHB) was deposited in each well of the micro plate (1 to 8 wells). 100 µl of the extract stock solution was deposited into the first well. After homogenization by suction-discharge using a micropipette, 200µl of extract solution at 100mg/ml is obtained. 100µl of this new solution were collected and mixed with MHB contained in the 2nd well and continues this ½ dilution from well to well until 6th wells. Finally, 100µl of the bacterial suspension were added to each well. The 7th and 8th wells were respectively the positive control and the negative control and contain 100µl of MHB + 100µl of the bacterial suspension to a positive control and 100µl of MHB to the negative control.

The microplates were coated placed for 24 hours in an oven at 37°C. MICs were estimated with the naked compared to controls and each well was plated eye on the MH agar and placed at 37°C for 24 hours. The MBC corresponded to the lowest concentration of extract for which there was no bacterial colonies.

Processing and analysis of data

The file Managed data and encoded Were Recorded in an Excel database. The graphs have been realized with the Graph Pad Software. Descriptive statistics were performed using SPSS 20.

Results

Yield extraction and Sterility test

Table 2 shows the yield on extraction of the ethanolic extract of *Corchorus olitorus* Linn. Sterility tests revealed no contamination of the extract.

Table 1. Standard used for reading the results of susceptibility testing plant extracts.

Diameter of the inhibition's area (Δ)	Level of sensibility	Symbol
Δ < 7mm	Insensitive	-
7mm ≤ Δ < 8mm	Sensitive	+
8mm ≤ Δ < 9mm	Quite sensitive	++
Δ ≥ 9mm	Very sensitive	+++

Source: OMS., 2002; Tsirinirindravo et Andrianarisoa, 2009.

Table 2. Yield of extraction of the ethanolic extract of *Corchorus olitorus* Linn.

Ethanolic extract	*Corchorus olitorus* L.
Powder mass (g)	50g
Extract mass (g)	4,46g
yield (%)	8,92%

Total polyphenols and flavonoids content of extracts

Chemical test to determine polyphenol and flavonoids contents of the plant extract was used to determine the content of the ethanolic extracts of *Corchorus olitorus* in each composant. A variation in the polyphenol content of the plant extract was observed from the regression line obtained from the curve calibration of Gallic acid. Fig. 4 shows the calibration curve of Gallic acid. A variation of the content of flavonoid was observed from the equation of the

regression line obtained from the calibration curve of rutin. Fig. 5 shows the calibration curve of rutin. Fig. 6 shows polyphenols and flavonoids contents of ethanolic extract of *Corchorus olitorus*.

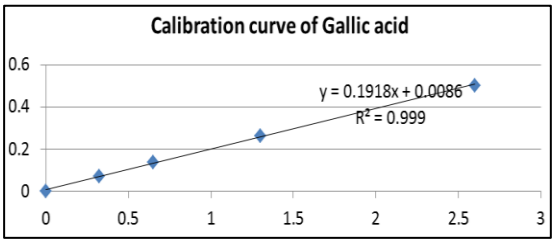

Fig. 4. Calibration curve of the Gaelic acid.

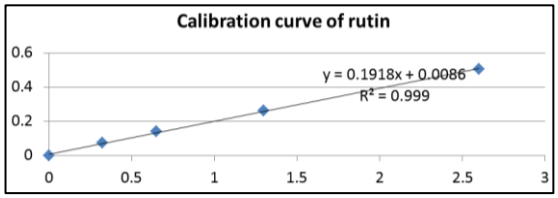

Fig. 5. Calibration curve of rutin.

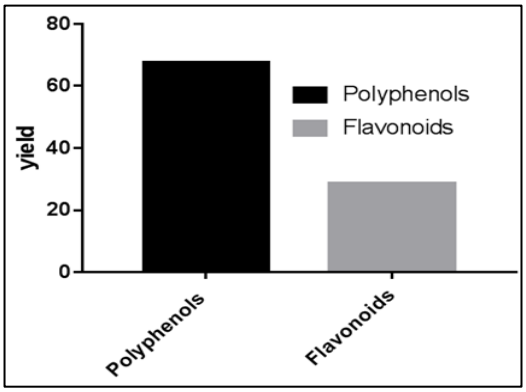

Fig. 6. Total polyphenols and flavonoids content of extracts

The polyphenol content is EAG microgram/extract 100milligramme and the content in flavonoids is ER microgram/100 mlliigramme. The ethanolic extract of *Corchorus olitorus* contains not negligible concentration of polyphenolic compounds and flavanoids. Fig. 5 shows that the extract is more concentrated in polyphenolic compounds than flavonoids.

Larval cytotoxicity of the extract

Artemia salina larvae were sensitive against the extract at a dose of 20mg/ml. Table 3 shows the correspondence table between the LC50 and toxicity.

Table 4 shows the average lethal concentrations (LC50) of the extract and regression coefficients. Change inLC50 was observed from the regression line obtained from the representative curve of the number of surviving larvae on the basis of the concentration of the extract show by Fig. 7. The extract has an LC50 greater than 0.1 mg/ml and therefore is non-cytotoxic with respect to larvae of *Artemia salina*.

Table 3. Correspondence between LC50 and toxicity.

LC50	Toxicity
LC50 _ 0.1 mg / ml	- (Non-toxic)
0.1 mg / ml> LC50 _ 0050 mg / ml	+ (Low toxicity)
0.050 mg / mL> LC50_ 0.01 mg / ml	++ (Moderatetoxicity)
LC50 <0.01 mg / ml	+ + + (High toxicity)

Table 4. Average lethal concentrations (LC50) of the extract and regression coefficient.

Extract	*Corchorus olitorus* Linn
LC50 mg/ml	1,673
R^2	0,958

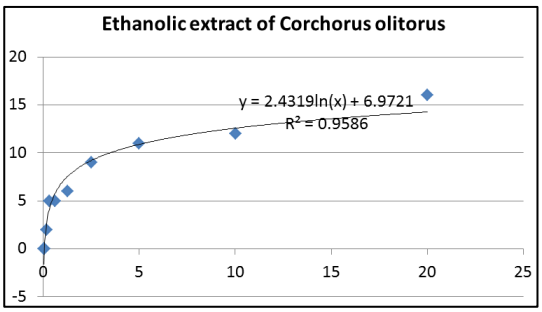

Fig. 7. Representative curve of the number of surviving larvae on the basis of the concentration of the extract.

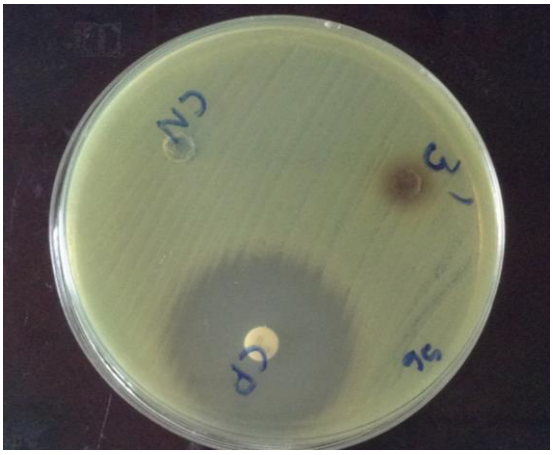

Fig. 8. Appearance of a petri dish containing the ethanolic extract of *Corchorus olitorus* Linn.

Antibacterial activity

Sensitivity test

The extract was prepared at a concentration of 100mg/ml. The tested strains showed the same insensitivity to the tested extract and no inhibition diameter was observed with the selected bacterial strains. The ethanolic extract of *Corchorus olitorus* Linn therefore had no antibacterial activity on the strains tested. Under these conditions no CIM and CMB could be calculated. Fig. 5 shows the appearance of a petri dish containing the ethanolic extract of *Corchorus olitorus* Linn. The extract is on position 3' and the conventional antibiotic use as positive control is on position "cp". Under these conditions no CIM and CMB could be calculated.

Discussion

Corchorus olitorus Linn is a leafy vegetable that goes into many culinary preparations in Benin. This study aimed to assess the biological potential of ethanolic extracts of leaves of *Corchorus olitorus* Linn commonly used in traditional medicine in southern Benin for the treatment of gastric disorders. The results of the study showed that leafy vegetables are relatively rich in phenolic compounds (total phenols flavonoids). Concentrations of polyphenols and flavonoids in the extract were determined by conventional methods. The presence of polyphenols and flavonoids in the ethanolic extract of *Corchorus olitorus* Linn in accordance with the study of Takin *et al.*, (2014) which showed that plants are naturally rich in phenolic components which are known as natural compounds with antioxidant properties. The high proportion of phenolic compounds denoted could be due to the use of ethanol for extraction. Ethanol is an organic solvent that allows the extraction of a large quantity of present bioactive molecules (Dorta *et al.*, 2012). Several works by authors such as

Popovici *et al.* (2009), Khan *et al.* (2012), have demonstrated the anti-radical property of phenolic compounds. *Corchorus olitorus* Linn possessing significant levels of phenolic compounds, could have this property. Furthermore, Bidié *et al.* (2011) proved that the functional groups present in the structures of polyphenols can easily give up an electron or a proton to neutralize free radicals. The leaf extract was inactive on all strains of enteropathogens tested. These results are contradictory with those of Adegoke *et al.* carried out in 2009 which obtained a very good antibacterial activity of the methanolic and aqueous extracts of *Corchorus olitorus* Linn on gram-negative strains of bacteria. This contradiction could be explained by the fact that methanol and water were able to extract bioactive molecules endowed with antibacterial activity. This contradiction could also be the result of the use of fertilizers and pesticides in market garden crops to increase the volume of crop production. Also, it has been proven by Bhebhe *et al.* in 2016 that leaves of *Corchorus olitorius* Linn are rich in many phenolics compounds that are preferably soluble in water. This assertion may justify the efficacy of leaf infusion in the treatment of typhoid fever and the inactivity of the ethanolic extract on the strain of *Salmonella cholereasius* tested in-vitro in this study. According to Durling *et al.* 2007 the hydroethanolic extract would be the one that allows better extraction of phenolic compounds. On the other side the inactivity of the extract could be justified by the fact that Gram-positive bacteria are more sensitive to ethanolic extracts in comparison to Gram-negative (Kamrani *et al.*, 2007). The work of Amira *et al.* carried out in 2018 showed a very good antibacterial activity of the plant on various bacterial strains. This observed difference could also be related to the specificity of the bacterial strains but also to the culture conditions of the species used in the different works carried out. It is undeniable that plants possess bioactive molecules that justify their use as an antibacterial agent (Benjamin, 1980, Adebayo and Adegoke, 2008), but extraction methods can sometimes be at the root of poor activities obtained in vitro. The non-toxicity of the extract of *Corchorus olitorus* Linn on the larvae of *Artemia salina* justifies its use in the preparation of food for human consumption for millennia without notification of intoxication.

Conclusion

The present study allowed appreciating the richness of the leaves of *Corchorus olitorus* Linn in phenolic compounds.

However, its effectiveness in treating infectious diseases such as typhoid fever could not be confirmed. It has emerged that extraction methods can sometimes favor or not the extraction of certain components of plants. *Corchorus olitorus* Lin being a sticky plant the etahnolic extract was the easiest to realize which did not allow discovering all the potentialities of the plant. It will be necessary to make other extracts with different solvents in order to explore all the potentialities of the plant and explain its effectiveness when used in-vivo.

Conflict of interest

No conflict of interested associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be done by the authors.

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