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Assessment of the antibacterial effect of *Khaya senegalensis* on some Gram-negative bacteria

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Abstract

Background: The matter of antimicrobial resistance evokes the urgency to explore alternatives to the antibiotics traditionally used for microbial infections. This study aimed to elucidate the mechanism of action of the antibacterial effect of *Khaya senegalensis* liable for bacterial strains responsible for diarrheal infections.

Results: The data collected indicate that the bacterial strains tested (*Salmonella* Typhimurium ATCC 14028, *Escherichia coli* ATCC 25922, *Shigella* spp. and *Salmonella* spp.) were sensitive to the extracts of *Khaya senegalensis* (Desr.) A.Juss. (aqueous and hydro-ethanol) to varying degrees. The hydro-ethanolic extract was active on all strains with a MIC of 25 mg/mL coupled with a bactericidal effect. The aqueous extract was only active on the *Salmonella* spp. strain. Membrane permeability test data show that *Khaya senegalensis* extracts affect the bacterial strains tested by attacking the stability of their outer membrane. This potential indicated by the high percentage of membrane destabilization of the bacteria is significantly ($p < 0.05$) better than that of cefixime used as a reference.

Conclusion: This study revealed that *Khaya senegalensis* destroys Gram-bacteria by attacking the stability of their cytoplasmic membrane. These data provide for the first time the mode of action of *Khaya senegalensis* extracts concerning their antibacterial activity.

Keywords: *Khaya senegalensis*, Mode of action, Antibacterial activity, Gram-negative Bacteria

Background

Infectious diseases constitute a serious public ill health thanks to their frequency and gravity especially in developing countries (Bourgeois et al. 2016). Among these diseases, diarrheal diseases are the foremost fatal, especially in children from West Africa. In fact, these diarrheal diseases are liable for 1.8 million deaths annually worldwide where 90% are children under the age of five living in developing countries (Bryce et al. 2005). Diarrheal diseases represent the third reason of death from infectious diseases of all ages and the 5th cause of premature death worldwide (WHO 2014). According to WHO, in Benin, diarrheal diseases are one of the main causes of morbidity. Indeed, they have a direct impact on the costs associated with health care, including several factors such as consultation, medication and in some cases, hospitalization, which represents a burden on household spending (WSP-ESI-Benin 2012). The pathogens of diarrheal diseases are mainly bacteria (Djague et al. 2020).

Medical therapy is based on the use of conventional antibiotics and antidiarrheal drugs. The use of antimicrobials should not be done routinely. In fact, a clinical distinction is made between diarrheal episodes caused by enterotoxigenic *E. coli* and those caused by rotavirus or *Cryptosporidium* against which antimicrobial drugs

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are ineffective (WHO 2005). Moreover, this use of antimicrobials sometimes presents undesirable effects and favors the development of resistant bacteria. Nowadays, antimicrobial resistance constitutes a real public health problem for the effective management of infectious diseases (Nahrgang et al. 2018). The most of the bacteria responsible for diarrheal episodes develop resistance to the antibiotics used in modern therapy (Ahmed et al. 2000; Qu et al. 2016). In addition, the anti-diarrheal drugs used until now are often ineffective and do not succeed in preventing dehydration and improving nutritional status, which should be the main objectives of treatment (WHO 2005). Concerning drug treatments that have shown some effectiveness, namely the use of oral rehydration solutions (ORS) to reduce fluid and electrolyte losses in diarrheic patients, it should be noted that in rural and semi-urban areas, the lack of knowledge of patients regarding their application limits their use (WHO 2005). In addition, the lack of qualified health personnel in most rural African communities and the high cost of therapies in case of severity do not contribute to a better management of diarrheal disease (WHO 2005). In the search for alternative solutions, World Health Organization (WHO) had initiated a diarrheal disease control program based on traditional medicine practices and prevention approaches (WHO 2011). This approach is even more justified given that about 80% of the population of developing countries like Benin continue to use traditional medicine based on the use of medicinal plants for their primary health needs (WHO 2002).

Khaya senegalensis (Desr.) A.Juss. (*K. senegalensis*) is a medicinal plant from the Beninese flora widely used in traditional African medicine (Akoègninou et al. 2006). In West Africa it is a plant that benefits from a multiple use value mainly used for bacterial infections (Issa et al. 2018). In southern Benin, the practitioners of traditional medicine used *K. senegalensis* for the treatment of bacterial infections, particularly diarrheal infectious (Akoègninou et al. 2006). Literature data confirmed the antibacterial potential of the stem bark of the *K. senegalensis* on bacterial strains such as *Salmonella* Typhi, *Salmonella* Paratyphi and *Salmonella* Typhimurium (Abdallah et al. 2016; Katawa et al. 2018; Koudoro Yaya et al. 2018; Ugoh et al. 2014). These data suggest the antibacterial potential of the plant and justify its use in traditional medicine in the treatment of bacterial infections. However, despite the plurality of data available on the biological activities of this plant, particularly on its antimicrobial properties, it is clear that no study has so far explored the mechanism of action of its antibacterial activity. This study aimed to elucidate the mode of action of the antibacterial effect of aqueous and hydro-ethanolic extracts of *K. senegalensis* on Gram-negative bacteria involved in diarrheal infections.

Methods

Plant material

The plant material used in this study consists of fresh stem bark of *Khaya senegalensis* (Desr.) A.Juss. These samples were collected in Abomey-Calavi in July 2020 and certified at the National Herbarium of Benin at the University of Abomey Calavi under the identification number YH435/HNB.

Biological material

The strains of Gram-negative bacteria involved in the occurrence of diarrheal infections, obtained from the Research Unit in Applied Microbiology and Pharmacology of natural substances, University of Abomey-Calavi, Benin were the biological material used. The characteristics of these strains are summarized in Table 1.

Study methods

Phytochemical screening of the plant

This part of the study consisted of the qualitative identification of eleven major phytochemical classes in the powder of the stem bark of *K. senegalensis* according to the method described by Houghton and Raman (1998). These are tannins, gallic tannins, flavonoids, anthocyanins, leuco-anthocyanins, alkaloids, mucilages, reducing compounds, sterols, terpenes and saponosides.

Preparation of plant extracts

The bark of the plant stems collected, cleaned and dried at room temperature at the Research Unit of Applied Microbiology and Pharmacology of natural substances was ground to powder using the Retsch electric mill. Two types of extraction (aqueous and hydro-ethanolic) were obtained following the methodology used by Klotoé et al. (2020). Briefly, fifty (50) grams of powder were macerated in 500 ml of distilled water for the aqueous extract and in 500 ml of the mixture of water and ethanol at 50% v/v for the hydro-ethanolic extract. After 72 h of agitation at room temperature, the homogenate obtained was filtered three times on cotton wool and once on Wattman paper

Table 1 Bacterial species used for antibacterial tests

Bacterial species	Origin
Salmonella Typhimurium ATCC 14,028	Reference strain
Escherichia coli 25,922	Reference strain
Shigella spp.	Clinical strain
Salmonella spp.	Clinical strain

N° 1. Crude extract was obtained after evaporation at a temperature of 40 °C in an oven (oven).

Determination of bioactive molecules of the extracts

- **Determination of total polyphenols**

The method of Singleton et al. (1999) using the commercial Folin Ciocalteu Reagent (FCR) was adopted. Briefly, 50 µL of the extract was mixed with 250 µL of the FCR (10 times diluted in distilled water) and 750 µL of an aqueous solution of sodium carbonate Na_2CO_3 (7.5%). After 8 min of incubation, 950 µL of distilled water was added and mixed with the vortex and incubated for 2 h. Optical densities (OD) were read at 760 nm using a CECIL CE 2041 spectrophotometer. The reading was made against a blank consisting of a mixture of 250 µL of FCR, 750 µL of Na_2CO_3 and 1 mL of distilled water. Samples were prepared in triplicates. Gallic acid (0–200 µg/mL) was used as standard reference. The total polyphenols content was determined as mg of gallic acid equivalent/g of extract (mg GAE/g) from the equation of the linear calibration curve ($y = 0.0012x - 0.0388$ with $R^2 = 0.9988$).

- **Determination of total flavonoids**

Flavonoids contents were measured by the method using aluminium trichloride ($AlCl_3$) described by Zhishen et al. (1999) and used by Klotoé et al. (2020). 500 µL µL of $AlCl_3$ (2%), 500 µL of the extract and 3 mL of methanol were mixed thoroughly. The blank consists of 500 µL of $AlCl_3$ and 3.5 mL of methanol. Absorbance reading was done at the spectrophotometer at 415 nm after 10 min of incubation. Samples were prepared in triplicates. Rutin (0–1 mg/mL) was used as a reference standard. Total flavonoids content was determined as mg of rutin equivalent/g of extract (mgRuE/g) from the equation of the linear calibration curve ($y = 44.135x - 0.1893$ with $R^2 = 0.9909$).

In vitro Antibacterial activity of plant extracts

Three steps were followed for performing this antibacterial test.

- **Preparation of extracts and sterility test**

100 mg/mL of extract solution (aqueous and hydroethanolic) were prepared in distilled water. To verify the sterility of these prepared extract solutions an inoculation of aliquots of each solution was applied on Mueller Hinton medium according to the methodology described by Agbankpe et al. (2016).

- **Sensitivity test of bacterial strains to extracts**

A portion of pure 24-h colony from Mueller Hinton's medium from each strain was emulsified in 5 mL of physiological water to obtain a turbidity of 0.5 on the MAC Farland scale. Each inoculum was inoculated by swabbing onto plates containing Mueller Hinton agar (CA-SFM 2020). 50 µL of each extract (100 mg/mL) was placed in the 6 mm diameter wells. A negative control was prepared with sterile distilled water. Standard antibiotic discs (Ciprofloxacin, Cefixime and Gentamicin) were used as positive controls. For an hour, the Petri dishes were pre-incubated at room temperature for the pre-diffusion of the substances and then incubated at 37 °C in an oven for 18 h. The test was repeated three times. The incubated plates were examined to measure the zones of inhibition. The standard used for reading the results of the antibiogram tests is presented in Table 2 (Tsirinirindravo and Andrianarisoa 2010; WHO 2002).

- **Determination of the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of active extracts**

This part of the study following the method applied by Lègba et al. (2018). It consisted of the 96-well plate method. 100 µL of the initial extract solution at the concentration of 100 mg/mL, were added to 100 µL of Mueller–Hinton broth. A series of dilution from well to well was carried out and then 100 µL of various bacterial suspensions were added. Positive (100 µL of MH broth added to 100 µL of bacterial suspension) and negative controls (100 µL of MH broth added to 100 µL of the extracts) were prepared. The microplates were incubated at 37 °C for 24 h. All the wells of the MIC at the higher concentrations were then inoculated on a Mueller Hinton agar then the Petri box were placed at 37 °C for 24 h for the determination of the MBC. The antibiotic power (AP) of each extract was then calculated with the formula MBC/MIC. The antibacterial effect or power is judged to be bactericidal

Table 2 Standard used for reading the results of antibiogram tests of plant extracts

Inhibitory diameter (Δ)	Germ sensibility
$\Delta < 7$ mm	Resistant
7 mm $\leq \Delta < 8$ mm	Sensitive
8 mm $\leq \Delta < 9$ mm	Moderately sensitive
$\Delta \geq 9$ mm	Very sensitive

or bacteriostatic based on the AP = MBC/MIC. If $1 \leq AP \leq 2$, the effect is bactericidal and if $4 \leq AP \geq 16$, the effect is bacteriostatic.

Permeability test outer membrane of Gram-negative bacteria

This test is based on the evaluation of the destabilizing power of the membrane of bacteria by plant extracts. It was determined according to the method employed by Djague et al. (2020). In a 96-well microplate, the MIC and 2 MIC of the extract in triplicate were prepared by serial dilution. 100 µL of the suspension of the tested bacteria (1.5×10^6 CFU/mL) was added to all wells and the plate was incubated at 37°C for 24 h. Cefixime was used as a positive control. Muller Hinton broth and bacterial suspension served as negative control. The optical densities were read at 405 nm. The percentage of destabilization was calculated using the formula below:

$$\%D = (A_o - A_s)/A_o \times 100$$

$\%D$: Percentage of destabilization; A_o: Absorbance of the negative control; A_s: Absorbance of test samples.

Data analysis

The in vitro antibacterial test was repeated three times and the results were analyzed using Graph Pad 7 software. The quantitative variables were then presented as mean ± standard deviation. ANOVA analysis of variance was used to compare the percentage of bacterial membrane destabilization between samples. The Student's *t*-test was used to analyze the quantitative composition in total polyphenols and flavonoids of the extracts. The significance level was set at 5%.

Results

Phytochemical composition

Data collected for the phytochemical screening revealed the presence of tannins, flavonoids, leuco-anthocyanins, alkaloid, mucilage, reducing compounds and saponosides in the powder of stem bark of *K. senegalensis.*

Determination of total polyphenols and flavonoids content

The extracts present interesting content of polyphenols and flavonoids. Hydro-ethanolic extract exhibited significantly ($p < 0.05$) higher total polyphenol and flavonoid content compared to aqueous extract (Table 3).

Sensitivity test

Aqueous and Hydro-ethanolic extracts of *K. senegalensis* were tested against *E. coli* ATCC 25922, *Salmonella* Typhimirium ATCC 14028, *Shigella* spp. and *Salmonella* spp. Results of antibiogram showed variable sensitivity depending on the type of extract. Indeed, on all bacterial strains tested, *K. senegalensis* aqueous extract was active only on *Salmonella* spp. with an inhibition diameter of 14 ± 1 mm. The hydro-ethanolic extract was active on all strains tested with variable activity (Fig. 1). The best antibacterial activity for this hydro-ethanolic extract was obtained on *Salmonella* spp. (17 ± 2.51 mm as inhibition diameter).

Table 3 Total polyphenols and flavonoids of extracts of *Khaya senegalensis*

Extracts	Total polyphenols (mg GAE/g)	Total flavonoids (mgRuE/g)
Aqueous	32.37 ± 3.30	12.46 ± 0.44
Hydro-ethanolic	116.30* ± 0.46	51.1* ± 6.74

Legend: *Significant difference between the extracts

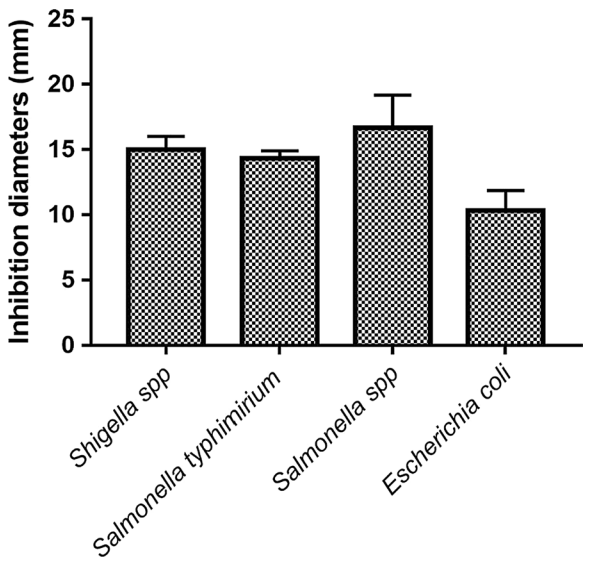

Fig. 1 Diameter of inhibition of the hydro-ethanolic extract of *khaya senegalensis* on the bacterial strains

MIC, MBC and AP of the extracts studied on the tested strains

The collected data related to MIC, MBC and AP are presented in Table 4. Analysis of these data indicates that the MIC was 25 mg/ml for all strains sensitive to both hydro-ethanolic and aqueous extracts. The same observation is made for the MBC. These observations were used to determine the antibiotic power of these active extracts (AP = 1).

Effects of extracts on outer membrane permeability of bacterial strains

Figure 2 presents the results of the permeability test of the outer membrane of bacteria strains. The analysis of

Table 4 Minimum inhibitory concentration, minimum bactericidal concentration and the antibiotic potency of the plant extracts

Extracts	Parameters	Bacterial strains			
		***Salmonella* Typhimurium ATCC 14,028**	***Escherichia coli* ATCC 25,922**	***Shigella* spp.**	***Salmonella* spp.**
Aqueous extract	CMI (mg/mL)	–	–	–	25
	CMB (mg/mL)	–	–	–	25
	AP	–	–	–	1
Hydro ethanolic extract	CMI (mg/mL)	25	25	25	25
	CMB (mg/mL)	25	25	25	25
	AP	1	1	1	1

AP antibiotic potency, *MIC* minimum inhibitory concentration, *MBC* minimum bactericidal concentration

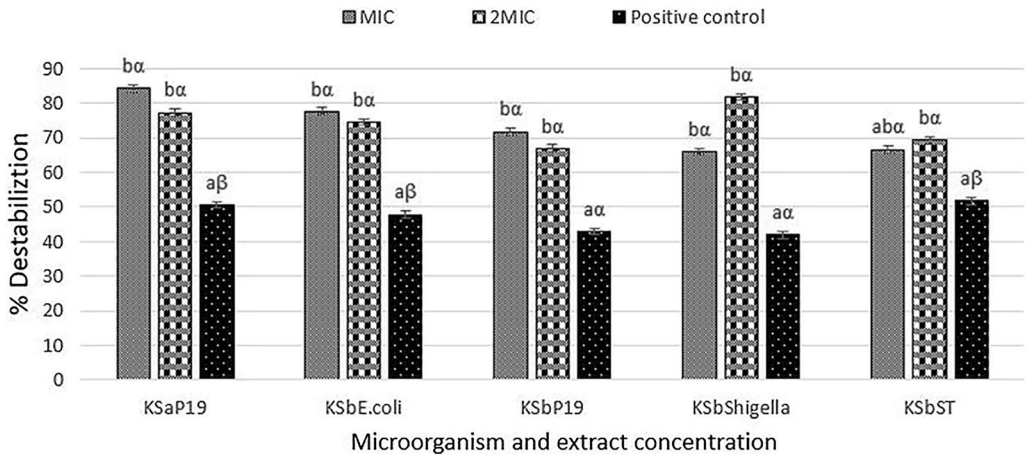

Fig. 2 Effect of destabilization of outer membranes of various bacteria by *Khaya senegalensis*. Legend: For same microorganism, bars carrying same letter (a, b) are not significantly different while for same concentration, bars carrying the same Greek alphabet (α, β) are not significantly different ($p > 0.05$), Waller Duncan. KSa: Aqueous extract; KSb: hydro-ethanolic extract

the data shows that the extracts studied complexed more divalent ions (increase of the destabilization percentage) than the positive control (Cefixime) at the concentrations tested (MIC and 2 MIC). However, no significant difference is to be reported between the effect obtained (percentage of destabilization) for MIC and 2 MIC ($p > 0.05$). The same trend is obtained regardless of the microorganism.

Discussion

This study aimed at providing scientific data on the mechanism of action of the antibacterial effect of *K. senegalensis* extracts. Data from the qualitative screening of *K. senegalensis* stem bark powder indicate the presence of tannins, flavonoids, leuco-anthocyanins, alkaloid, mucilage, reducing compounds and saponosides. In addition the extracts studied showed richness in total polyphenols and flavonoids. Similar observations were reported by Ugoh et al. (2014) in a study conducted in Nigeria. These observations are in agreement with the richness in polyphenolic compounds of plants of the same botanical family (Meliaceae) as *K. senegalensis* (John et al. 2014; Olatunji et al. 2021; Saleem et al. 2018). These data translating a richness in bioactive molecules of *K. senegalensis* could justified the use of the plant in the traditional treatment of diseases in several African pharmacopoeias (Abdel-Wareth et al. 2014). Furthermore, the difference observed between the polyphenolic compound content of the two extracts studied could be explained by the influence of the extraction solvent. Indeed, it is documented in the scientific literature that the extraction solvents have an influence on the quantity of bioactive molecules concentrated in the plant extracts (Babbar et al. 2014; Dirar et al. 2019; Do et al. 2014). In this study, the hydro-ethanol extract showed the best content of total polyphenols and flavonoids. This reflects that the mixed solvent (water–ethanol) concentrated more bioactive molecules than water. These data corroborate those reported by several authors who underlined the strong capacity of mixed solvents in the extraction of total polyphenols and

flavonoids (Bourgou et al. 2016; Do et al. 2014; Mohammedi and Atik 2011; Venkatesan et al. 2019; Vieito et al. 2018). Trabelsi et al. (2012) justify this extraction capacity of mixed solvents by the increased solubility of bioactive compounds in this type of solvent.

Data obtained from the in vitro antibacterial test indicate that *K. senegalensis* bark is endowed with antibacterial properties on the strains involved in the diarrheal infections tested in this study. From the two extracts tested, the hydro-ethanolic extract was active on all bacterial strains with a Minimum Inhibitory Concentration (MIC) of 25 mg/mL coupled with a bactericidal effect. This antibacterial potential of the *K. senegalensis* could be justify by the existence of bioactive compounds such as flavonoids, tannins, alkaloids identified in the stem bark of *K. senegalensis.* Indeed, these bioactive molecules are known for their antimicrobial power referring to several reports in the literature (Djague et al. 2020; Kabir et al. 2015; Scalbert 1991). Specifically, some data in the literature attribute the antimicrobial activity of medicinal plants mainly to their polyphenolic compounds (Ghimire et al. 2017; Miklasińska-Majdanik et al. 2018). Indeed, these secondary metabolites are known to be responsible for a variety of biological activities of medicinal plants. Among them, flavonoids are one of the most studied polyphenolic compounds for their antibacterial properties (Cushnie and Lamb 2005; Farhadi et al. 2019). Similarly, tannins are a group of polyphenolic compounds that are very active in antimicrobial activities (Maisetta et al. 2019). Other data in the literature report the antimicrobial potential of alkaloids (Cushnie et al. 2014; Othman et al. 2019). These data justify the correlation, reported by several authors, between the antibacterial activity of *K. senegalensis* and these bioactive compounds (Abdallah et al. 2016; Kubmarawa et al. 2009; Ugoh et al. 2014). However, the difference of the antibacterial activity of the extracts observed in this study could be explained by the difference of their polyphenolic compounds content. Indeed, the hydro-ethanolic extract (the most active on the inhibition of the studied bacterial strains) is richer in polyphenolic compounds than the aqueous extract. These data show that the intensity of the biological activity of medicinal plant extracts is proportional to their content of bioactive compounds (Wang et al. 2016).

However, the anti-salmonella power of *K. senegalensis* obtained in the present study is similar to that reported by Ugoh et al. (2014) on *Salmonella enterica* subsp. enterica serovar Typhi strain. On the other hand, the comparison of the data of the present study with those obtained by Abdallah et al. (2016) and Katawa et al. (2018) shows opposite results. Except for *Salmonella* spp., the aqueous extract was inactive on the other three tested strains (*E. coli*, *Salmonella* Typhymirium and *Shigella* spp.) while the study of Abdallah et al. (2016) inform, for the same type of extract (aqueous extract), an inhibition of *E. coli*, *Salmonella* spp. and *Shigella* spp. with an MIC varying between 12.5 and 25 mg/mL. Katawa et al. (2018), on the other hand, showed contrary to our data that the hydro-ethanolic extract of de *K. senegalensis* was not active on *Salmonella* Typhimirium strain but active on other *Salmonella* strains (*Salmonella* Typhi, *Salmonella enterica* and *Salmonella* Paratyphi). This discrepancy in results observed could be attributed to the influence of environmental factors on the composition of secondary metabolites in medicinal plants (Borges et al. 2017; Liu et al. 2016).

Several models were used to study the mode of action of antibacterial agents in relation to different bacterial targets (Pinto et al. 2017). In this study the permeability test of the outer membrane of bacteria was adopted to evaluate the mode of action of aqueous and hydro-ethanolic extracts of the stem bark of *K. senegalensis* against *Salmonella* Typhimurium ATCC 14028, *Escherichia coli* ATCC 25922, *Shigella* spp. and *Salmonella spp*; four strains involved in diarrheal infections. The data obtained highlight a remarkable potential for destabilizing the membrane of the bacterial strains of the extracts tested with a better effect compared to Cefixime used as a reference. These observations reflect that the extracts have a more enhanced mode of action on the destabilization of the outer membrane of the bacterial strains tested than Cefixime. Similar results were reported by Djague et al. (2020) for different extracts of *Garcinia kola* and *Alchornea cordifolia* and Polymyxin B used as a reference in their study and which has the same mode of action as cefixime. Moreover, with regard to their phytochemical composition, the mode of action of the antibacterial activity of the extracts tested on strains responsible for diarrheal infections can be attributed to phenolic compounds (flavonoids, tannins etc.). Indeed, these phenolic compounds are able to destabilize the external membrane of bacteria by complexing the divalent cations that stabilize them (Frirdich and Whitfield 2005; Vaara 1992). This antibacterial action promotes the bursting of the cytoplasmic membrane and alterations in Ionic homeostasis between the intracellular and extracellular compartments of Gram-negative bacteria (Trombetta et al. 2005). Thus, the membrane antigens responsible for the virulence of bacteria will be affected. These observations explain the destabilizing effect of *K. senegalensis* extracts for the bacterial membrane of the strains tested.

This study allowed to elucidate for the first time the mode of action of the antibacterial effect of *K. senegalensis* regarding its antibacterial activity, compared to the scientific literature currently available on the antibacterial properties of this plant. Such data evoke prospects

for precision-oriented research on the mechanism of action of antibacterial effects of medicinal plants. This would optimize the search for new bioactive molecules for the fight against antimicrobial resistance.

Conclusion

This study revealed a variation in the antibacterial activity of aqueous and hydro-ethanolic extracts of *K. senegalensis* on strains of *Salmonella* Typhimurium ATCC 14028, *Escherichia coli* ATCC 25922, *Shigella* spp. and *Salmonella* spp. involved in diarrheal infections. The hydro-ethanolic extract showed better activity compared to the aqueous extract. These tested extracts exert their antibacterial effects on the tested bacteria by attacking the stability of their cytoplasmic membrane. This study provided for the first time data on the mode of action of *K. senegalensis* extracts concerning their antibacterial activity.

Abbreviations

$AlCl_3$: Aluminium trichloride; Ao: Absorbance of the negative control; AP: Antibiotic Potency; ATCC: American Type Culture Collection; As: Absorbance of test samples; CA-SFM: Antibiotic Committee of the French Society of Microbiology; CFU: Colony format unit; FCR: Folin ciocalteu reagent; GAE: Gallic acid equivalent; HNB: National Herbarium of Benin; MBC: Minimum Bactericidal Concentration; MIC: Minimum inhibitory concentration; Na_2CO_3: Sodium carbonate; OD: Optical densities; RuE: Rutin equivalent; SPSS: Statistical Package for the Social Sciences; YH: Yedomonhan Hounnankpon (ID of the name of the curator of the National Herbarium of Benin); WHO: World Health Organization; WSP-ESI: Water Sanitation Program- Economic and Social Impacts.

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Authors' contributions

HE, DV, AE, AA, SK, SJ, B-ML wrote the protocol. HE, AE, AA, SK processed the samples. DV, HE, AE did the statistical analyses. HE, DV, AE wrote the draft of the manuscript. DV, SJ, B-ML reviewed the manuscript. All the authors have read and approved the manuscript.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Declarations

Ethics approval and Consent to participate

Not Applicable.

Consent for publication

Not Applicable.

Competing interests

The authors declare that they have no competing interests.

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