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HPLC-DAD-ESI-MS profile, antibacterial activity, and modulation of the activity of antibiotics by *Carica papaya* L. against *Escherichia coli* serotypes

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ABSTRACT

Background: Diarrhea is currently the second leading cause of death among children under five years old in the world and therefore, remains a global public health problem. Considering that most cases of diarrhea are due to gastrointestinal infections, and medicinal plants have been traditionally used to treat such conditions, this work aimed to evaluate the chemical composition, antibacterial activity, and antibiotic-enhancing properties of *C. papaya* against *Escherichia coli*, EPEC (Enteropathogenic *E. coli*), and ETEC (Enterotoxigenic *E. coli*) serotypes.

Methods: The chemical profile of the aqueous extract obtained from *C. papaya* leaves was determined by HPLC-DAD-ESI-MS. The antibacterial and antibiotic-enhancing activities were evaluated through the determination of the MIC, following the microdilution method.

Results: A total of 17 compounds were identified by HPLC-DAD-ESI-MS. However, the extract of *C. papaya* did not present an intrinsic antibacterial effect, in addition to showing no modulatory effect when associated with antibiotics.

Conclusions: Despite the presence of bioactive compounds, the aqueous extract *C. papaya* does not present antibacterial or antibiotic-enhancing effects against the ETEC and EPEC strains of *E. coli.* Nevertheless, further research is required to determine if its isolated components are potential drug candidates for the therapy of infections caused by these strains.

1. Introduction

According to the World Health Organization (WHO, 2017), diarrheal diseases are the second leading cause of death among children under five years worldwide. Despite being treatable and preventable (*i.e.*, through good hygiene practices), such diseases remain an alarming global public health problem.

Evidence has indicated that diarrheal diseases mostly affect communities with higher social vulnerabilities, who therefore have low access to education and basic sanitation . In addition, due to the lack of access to medicines and health products, many of these individuals resort to medicinal plants and other alternative treatments (Almeida et al., 1995).

In the early 1990s, the WHO reported that between 65% and 80% of the population in developing countries depended on medicinal plants to treat a series of health problems, including diarrhea (Calixto, 2005).

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Table 1
Minimum Inhibitory Concentration (MIC) *C. papaya* and antibiotics against EPEC and ETEC.

Treatment	EPEC MIC (μg/mL)	ETEC MIC (μg/mL)
C. Papaya	1024	1024
Metronidazole	1024	1024
Sulfamethoxazole + Trimethopim	0,630	1260
Ciprofloxacin	0,250	0,250

EPEC: Enterotoxigenic *E. coli.* EPEC: Enteropathogenic *E. coli.* Data are expressed as triplicate mean.

Table 2
Minimum Inhibitory Concentration (MIC) of antibiotics in association with *C. papaya* extract against EPEC and *ETEC*.

Treatment	EPEC MIC (μg/mL)	ETEC MIC (μg/mL)
Metronidazole	1024	1024
Metronidazole + *C. Papaya*	1024	1024
Sulfamethoxazole + Trimethopim	0,630	1260
Sulfamethoxazole + Trimethopim + *C. Papaya*	0,630	1260
Ciprofloxacin	0,250	0,250
Ciprofloxacin + *C. Papaya*	0,250	0,250

Data are expressed as mean ± SD. Two-way ANOVA using the geometric average of the triplicates as the central data and the standard deviation of the average. A Bonferroni post hoc test was then performed, where $p < 0.05$ was considered significant.

Thus, significant interest has arisen with regard to the search for natural products with antidiarrheal activity (Mishra et al., 2016). In this context, researchers have investigated the biological activity of extracts by analysing their ability to act as antispasmodic or antimicrobial agents against enteric pathogenic microorganisms (Palombo, 2006).

C. papaya (Caricaceae), commonly known as papaya, is a typical plant of tropical and subtropical regions (Ostrosky et al., 2008). In Brazil, where papaya is popularly known as "mamão", its fruits, bark, and leaves are used in the treatment of warts, callus, constipation, amenorrhea, eczema, and tumors. Studies carried out by De Brito Júnior and Ferreira (2015) showed that papain, a proteolytic enzyme found in the latex of papaya, presents anti-inflammatory, bactericidal, and bacteriostatic effects, indicating that this plant has medicinal properties (Brito Júnior and Ferreira, 2015).

Therefore, this work aimed to evaluate the chemical composition, antibacterial activity, and antibiotic-enhancing properties of *C. papaya* against *E. coli*, EPEC (Enteropathogenic *E. coli*), and ETEC (Enterotoxigenic *E. coli*) serotypes.

Material and methods

Plant material

The leaves of the *C. papaya* were collected in a rural area (*Fazenda Timbaúba*) of the municipality of Cedro, PE, Northeast Brazil (latitude: 07 ° 48.26742 'S, longitude: 39° 10.26135′ W; 535.336 m altitude). An exsiccate was prepared and registered at the herbarium of the Regional University of Cariri (*Herbário Dárdano de Andrade Lima*, URCA), under registry code HCDAL 8652. The collections were carried out in April in the morning, and the samples were cleaned and stored under refrigeration at 18 °C.

Extraction procedure

The aqueous extract was prepared by infusion using 200 g of *C. papaya* leaves and 3 L of water. Of note, the proportion of 10 g of leaves to150 mL of water was intended to be equivalent to an usual preparation of a cup of tea. The water was heated to 100 °C and then the fire was turned off. Afterwards, the leaves were added to the water and the container was covered until the infusion reached room temperature. Then, the infusion was frozen and taken to the freeze dryer (−60 °C) until complete drying. The extract was prepared at the Regional University of Cariri (URCA), while the chemical analyses were carried out at the Federal University of Paraiba (UFPB).

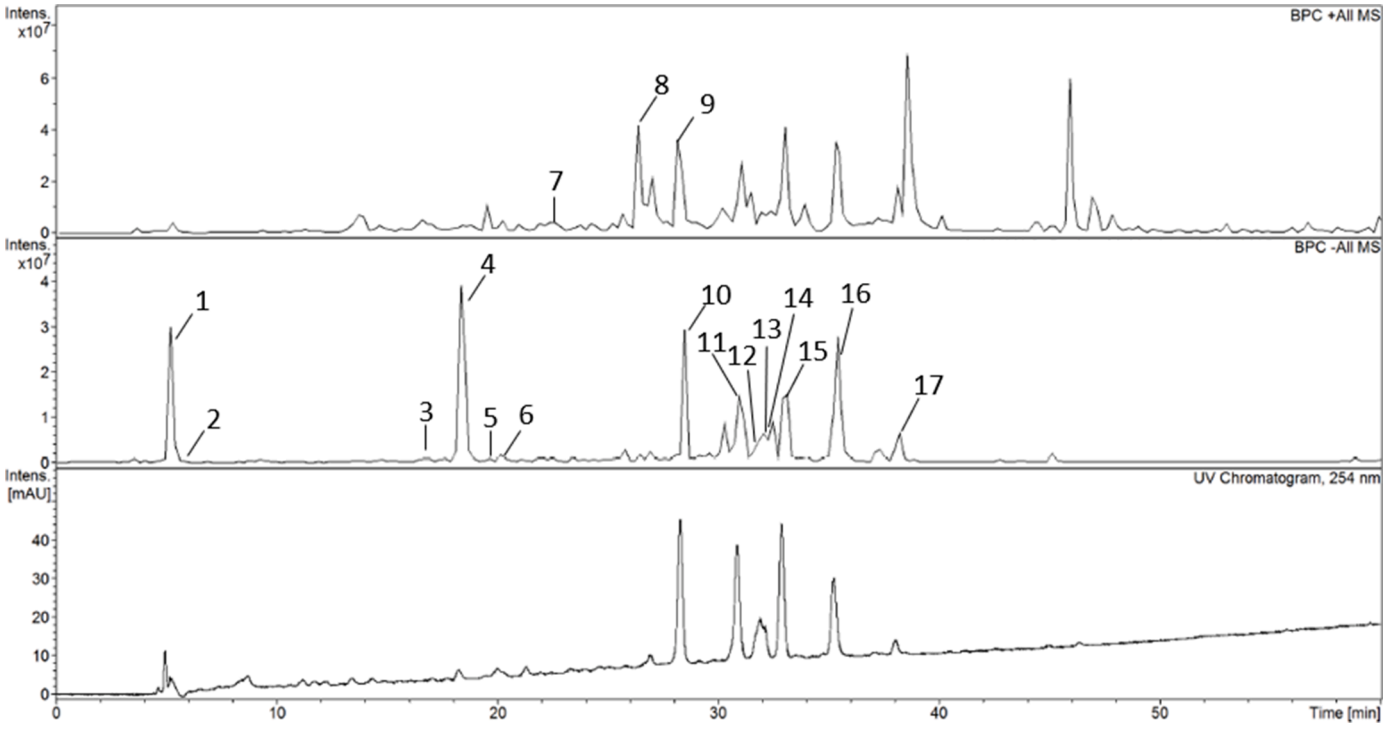

Fig. 1. Base peak chromatograms (BPC) in positive ion mode (A), in negative ion mode (B) and UV chromatogram at 254 nm (C) aqueous extract of the fresh leaves of *C. papaya* by HPLC-DAD-ESI-MS^n.

Table 3
Characterization of the compounds tentatively identified by HPLC-DAD-ESI-MS^n in *C. papaya.s.*

Peak No.	t_R (min.)	UV (nm)	*m/z* [*M* − *H*]$^-$/ [*M* + *H*]$^+$	Molecular formula	Calcd.	error (ppm)	MS^2/MS^3	Tentative assignment	Ref.
	5.2	–	341.1092/-	$C_{12}H_{21}O_{11}$	341.1089	−0.7	MS^2[341]: 179(100); 161(18); 143 (14)	Sucrose	Li, 2016
	5.9	–	191.0206/-	$C_6H_8O_7$	191.0197	−4.6	MS^2[191]: 173 (27); 129 (97); 111 (100); 85 (5);	Citric acid	Li, 2016
	16.9	–	315.0715/-	$C_{13}H_{15}O_9$	315.0722	2.1	MS^2[315]: 281(13.75); 153(100);	Protocatechuic acid glucoside	Rivera-Pastrana et al., 2010
	18.4	–	408.0417/-	$C_{14}H_{18}NO_9S_2$	408.0428	2.7	MS^2[408]: 275(37); 259(100) /MS^3 [408→259]: 139 (100); 97 (25);	Glucotropaeolin	Gogna, Hamide and Dorai, 2015 ; Seigler et al., 2002
	19.6	–	373.0928/-	$C_{19}H_{18}O_8$	373.0917	−2.7	MS^2[373]: 355 (50); 343 (100); 299 (7.22); 194 (9);	Syringic-caffeic acid ester	Zunjar et al., 2015
	20.4	–	385.1145/-	$C_{17}H_{22}O_{10}$	385.1129	−4.1	MS^2[385]: 367 (7); 223 (100); 205 (17);	Sinapic acid hexoside	Zunjar et al., 2015
	22.8	291; 325	-/475.3547	$C_{28}H_{47}N_2O_4$	475.3530	−3.5	MS^2 [475]: 238 (100); 220 (26)	Dehydrocarpaine II	Li, 2016
	26.4	293; 327	-/477.3674	$C_{28}H_{49}N_2O_4$	477.3686	2.7	MS^2 [477]: 459 (43); 240 (100); 238 (67); 222 (19)	Dehydrocarpaine I	Li, 2016
	28.2	294; 328	-/479.3843	$C_{28}H_{51}N_2O_4$	479.3808	7.4	MS^2 [479]: 461 (12); 240 (100); 222 (18)	Carpaine	Zunjar et al., 2016; Tang, 1979
	28.5	295; 328	295.0463/-	$C_{13}H_{11}O_8$	295.0459	−1.2	MS^2[295]: 179 (100); 195 (12); 133 (47); 115 (5)	Caffeoyl malate	Zunjar et al., 2015 ; Rivera-Pastrana et al., 2010
	30.9	253; 354	755.2028/-	$C_{33}H_{39}O_{20}$	755.2027	1.6	MS^2 [755]: 609 (27); 591 (45); 489 (26); 343 (16); 301 (44); 300 (100) /MS^3 [755→300]: 271 (100); 255 (64); 179 (7);152 (6)	Quercetin-3-O-(2G-α-L-rhamnosyl) -rutinoside	Van et al., 2019; Chen et al., 2015
	31.8	237; 311	163.0407/-	$C_9H_7O_3$	163.0401	−3.6	MS^2 [163]: 119(100); /MS^3 [163→119]: 93 (100);	Coumaric acid	Gogna et al., 2015.
	32.1	242; 320	279.0507/-	$C_{13}H_{11}O_7$	279.0510	1.3	MS^2 [279]: 163(100); 133 (67); 119 (12); 115 (4)	p-Coumaroyl malic acid	Zunjar et al., 2015. ; Rivera-Pastrana et al., 2010.
	32.3	243; 282; 327	193.0504/-	$C_{10}H_{10}O_4$	193.0506	1.0	MS^2 [193]: 178(62); 149(68); 134 (100) /MS^3 [193→134]: 106 (100);	Ferulic acid	Gogna et al., 2015.
	32.9	264; 342	739.2079/-	$C_{33}H_{39}O_{19}$	739.2091	1.7	MS^2 [739]: 593 (19); 575 (100); 473 (3); 393 (13); 323 (13); 285 (29); 284 (42)	Kaempferol-3-O-(2G-α-L-rhamnosyl) -rutinoside	Van et al., 2019; Chen et al., 2015.
	35.4	247; 356	609.1452/-	$C_{27}H_{29}O_{16}$	609.1461	1.5	MS^2 [609]: 301 (100); 300 (43.01) /MS^3 [609→301]: 271 (100); 179 (60); 255 (59); 152 (2)	Quercetin-3-O-rutinoside (rutin)	Van et al., 2019; Chen et al., 2015.
	38.2	245; 351	593.1503/-	$C_{27}H_{29}O_{15}$	593.1512	1.5	MS^2 [593]: 285 (100); 284 (17.48); 255 (4.79); 227 (2.19)	Kaempferol-3-O-rutinoside	Van et al., 2019; Chen et al., 2015.

Chemical and reagents

The following reagents were used in the antibacterial tests: Heart Infusion Agar (HIA) medium (Difco Laboratories Ltda); (+)-α-pinene (Sigma Aldrich, St. Louis, MO, USA); dimethyl Sulfoxide (DMSO, purity 99,9%, Synth ACS); and resazurin sodium (Sigma-Aldrich St. Louis, MO, USA).

Solvents of analytical and high-performance liquid chromatography (HPLC) grade were purchased from Vetec (Rio de Janeiro, Brazil), Quimex (São Paulo, Brazil), Qhemis (São Paulo, Brazil), Sigma–Aldrich and Tedia (Rio de Janeiro, Brazil) and used without further purification. Ultrapure Milli-Q water was obtained with a Millipore device (Milford, MA, USA) and used in the HPLC system. Coumaric acid (98%), Ferulic acid (99%) and rutin (94%) were purchased from Sigma–Aldrich and used as standard compounds in the HPLC-DAD-ESI-MS analysis.

Microorganisms and cell culture conditions

The serotypes EPEC and ETEC of *E. coli* were provided by the professor Dr. Rodrigo Otávio Silveira Silva of the Federal University of Minas Gerais - UFMG. They were stored under refrigeration (8 °C) in test tubes containing solid HIA medium (Difco Laboratories Ltda) at the Laboratory of Microbiology and Molecular Biology (LMMB) of the Regional University of Cariri (URCA).

Bacterial cultures were then seeded in Petri dishes containing solid HIA and grown at 37 °C for 24 h. Then, an aliquot of the microbial culture was removed with an inoculation loop and transferred to test tubes containing sterile saline solution (0.9% NaCl). Then, the turbidity of the solution was compared to the McFarland scale corresponding to 1 × 10^8 CFU / mL. The test was carried out in triplicate.

Preparation of drugs and reagents

After weighing, 10 mg of the extract was dissolved in 1 ml of dimethyl sulfoxide (DMSO, Synth ACS) and diluted in sterile distilled water to 1024 μg /mL for testing. Resazurin sodium reagent (Sigma-Aldrich) waterwas used as a colorimetric indicator of bacterial growth, following the oxidation–reduction method as previously reported (Sales et al., 2014; Salvat et al., 2001).

The antibiotics trimethoprim/sulfamethoxazole, metronidazole, ciprofloxacin, clindamycin, amikacin, and gentamicin were diluted in sterile water to the initial concentration of 1024 μg / mL.

Minimum inhibitory concentration (MIC) determination

The Minimum Inhibitory Concentration (MIC) was defined as the

lowest concentration capable of preventing bacterial growth in the wells on a microdilution plate as detected macroscopically. The MIC was determined by the broth microdilution method as previously established (CLSI, 2012). Briefly, the bacterial strains were seeded using 3 Petri dishes containing HIA for each strain. After 24 h, an aliquot of each bacterial culture was collected to obtain an inoculum with a final concentration of 10^5 CFU / mL. For each bacterial strain, test tubes were filled with 1350 µL of 10% Brain Heart Infusion (BHI) medium and 150 µL of inoculum. Then, 100 µL of this solution was distributed in the wells on 96-well plates. After this, 100 µL of the extract (at 1024 µg /mL) was added to the first well of the plate and serially diluted to achieve concentrations ranging from 512 to 0.5 µg /mL. The plates were then placed in the oven for 24 h at 37 °C. Following the incubation period, 20 µL of resazurin was added to each well and after 1 h, the color of the solution was observed. Growth and sterility controls were used throughout this test.

Analysis of antibiotic activity modulation by microdilution

The MIC was determined through the broth microdilution method in sterile 96-well plates. To this end, test tubes were filled with a solution containing 1163 µL of BHI solution, 150 µL of inoculum, and 187 µL of the extract, totalling 1500 µL. Afterwards, 100 µL of this solution was distributed in each well on the plate. Control wells were added with 100 µL of a solution prepared with 1350 µL of BHI and 150 µL of inoculum. Subsequently, 100 µL of the antibiotic solution (at 1024 µg/mL), was added to the first well of the plate, followed by with serial dilutions to achieve antibiotic concentrations ranging from 512 µg/mL to 0.5 µg/mL.

Analytical conditions and chemical characterization

From the lyophilized aqueous extract of *C. papaya,* 1 mg was dissolved in 1.0 mL of MeOH: H_2O (50:50) under an ultrasound water bath (Q5.9/40 - Eco-Sonics, Indaiatuba, SP, Brazil), filtered using a PVDF filter of 0, 45 µm (Nova Analítica, São Paulo, SP, Brazil), and analyzed by HPLC-DAD-ESI-MS. HPLC-DAS-ESI-MS analysis was carried out using a UFLC (Shimadzu Corp., Kyoto, Japan) containing two LC-20AD solvent pumps, SIL-20AHT auto-sampler, an SPD-M20A diode array detector (with a cell path length of 10 mm and volume of 10 µL and recorded between 200 and 800 nm), CBM-20A system controller, coupled with an ESI-Ion-Trap mass spectrometer (AmaZon X - Bruker, Billerica, MA, USA) or with an ESI-TOF (microTOF II - Bruker, Billerica, MA, USA). The HPLC experiments were performed using a C_{18} column (Kromasil 250 mm × 4.6 mm 5 µm, Bohus, Sweden) with the following elution gradient: solvent A = H_2O with formic acid (0.1% v/v); Solvent B = MeOH; he elution profile consisted in an exploratory gradient of 5% B, which was increased to 100% B over 60 min, with an injection volume of 20 µL and flow of 0.6 mL/min. The parameters of analysis of the ESI were as follows: capillary 4.5 kV, ESI in positive and negative mode, offset of the final plate 500 V, nebulizer 50 psi, dry gas (N_2) with the flow of 8 mL/min, and temperature of 300 °C. CID fragmentation was carried out in auto MS / MS mode using the advanced resolution mode for MS and MS / MS mode. The spectra (m/z 50–3000) were recorded every 2 s.

Results and discussion

Antibacterial and antibiotic modifying activity

The evaluation of the MIC revealed that the aqueous extract of *C. papaya* did not inhibit bacterial growth at concentrations below 1024 µg/mL (Table 1), which indicates a clinically irrelevant antibacterial activity against both serotypes of *E. coli* investigated in this study.

Using the disk-diffusion methodology, Suresh et al. (2008) demonstrated that the aqueous extract of *C. papaya* has antimicrobial activity against *Bacillus subtilis, E. coli, Klebsiella pneumoniae, Pseudomonas aeruginosa,* and *Staphylococcus aureus.* In addition, the study reported the presence of alkaloids, anthraquinones, flavonoids, saponins, steroids, tannins, and triterpenoids in the chemical composition of the extract, which may justify the reported biological activity.

Corroborating with the observations of Suresh et al. (2008), a qualitative analysis of the phytochemical constituents obtained positive results for alkaloids, saponins, tannins, and terpenoids for crude aqueous extracts obtained from the leaves, stem, and root of the same botanical species. It has been reported that the pawpaw has antibacterial activity against *E. coli*, an important bacterial component of the intestinal microbiota of animals. While most *E. coli* serotypes may be devoid of virulence factors, some strains may, during the evolutionary process, acquire different sets of genes that confer them the ability to cause disease, which contributes to the great pathogenic versatility of this species.

Studies evaluating the antibacterial properties of *C. papaya* extracts have obtained variable outcomes, which may be due to a series of factors, such as seasonal variation, type of extract, and method of analysis. Khan et al. (2012) reported that the warm aqueous extract of the mature bark presented an antibacterial activity that is comparable in magnitude to that observed for the antibiotic Tetracycline (MIC = 500 µg/mL). While the aqueous extract inhibited the growth of *S. aureus* (ATCC292), *E. coli* (ATCC 2522) was inhibited by the ethanolic extract. A study by Lohidas et al. (2015) demonstrated that the aqueous extract of *C. papaya* showed inhibitory activity against *E. coli*, which is corroborated by the findings of Nicolas et al. (2021). Research carried out by Gupta et al. (2017) also indicated an antimicrobial activity of the aqueous extract of leaves. However, only high concentrations of the extract showed significant differences from the control group. Arumugam et al. (2014) and Wilberforce Olivia (2017) evaluated the aqueous and ethanolic extracts of the pawpaw against *E. coli*, showing that the ethanolic extract has stronger activity. Evaluating the activity of an extract obtained with isopropanol against different bacterial strains, Igwe (2015) observed that *S. aureus* presented the highest sensitivity, followed by *E. coli, P. mirabilis*, and *S. faecalis*. Finally, Callixte et al. (2020) observed that, in comparison with the aqueous extract, the methanolic extract showed stronger antimicrobial activity against *S. aureus, E. coli,* and *Candida albicans*.

The type of solvent employed during the extraction has a direct impact on the phytochemical profile of the extract since each extract has a particular capacity for solubilizing the phytoconstituents according to their polarity (Marjorie, 1999). Consequently, the variable phytochemical profile of the extracts can justify the differences in the biological activities reported by the above-mentioned studies. Here, it is hypothesized that the lack of significant activity of the extract, despite the presence of known bioactive compounds may be due to the presence of constituents that may impair the access of active principles to their bacterial targets (Jigna et al., 2006).

The present in vitro study investigated the effectiveness of *C. papaya* leaf extract against pathogenic *E. coli* serotypes EPEC and ETEC, which are distinguished by the presence of virulence factors responsible for different clinical conditions. Of note, this study has a pioneer character as it searches for bioactive substances against pathogenic strains of *E. coli* since the current literature only reports the activity of the genus *Carica* against ATCC and MDR strains of *E. coli.* It was demonstrated that the association of the extract with antibiotics such as metronidazole, sulfamethoxazole + trimethoprim, and ciprofloxacin did not enhance their antibacterial activity against pathogenic *E. coli* serotypes EPEC and ETEC (Table 2) .

With regard to the extraction and chemical analysis, 16.61 g of lyophilized aqueous extract was obtained from the raw botanical material (200 g of leaves). The 17 compounds identified in the extract were tentatively assigned by the interpretation of their fragmentation patterns obtained from mass spectra (MS^2 and MS^3 experiments) (Fig. 1). Data provided by reference standards and literature information was also employed for the comprehensive evaluation of the samples. The retention times and mass spectrum data along with peak assignments for

compounds identified using positive and negative ionization are described in Table 3.

The alkaloid carpaine, which has been classicaly described in the genus, was detected and tentatively assigned. Carpaine (10), with the precursor ion at *m/z* 479 $[M + H]^+$ was assigned relying on the MS and MS/MS spectra that showed ion at *m/z* 240 representing the half of structure (Zunjar et al., 2016). Similarly, the precursor ions *m/z* 477 and *m/z* 475 with your products ions *m/z* 240 and *m/z* 238 were identified with dehydrocarpaine I and II, respectively (Tang, 1979).

Hydroxycinnamic ester and its derivatives were observed in peaks 2, 3, 5, 6, 9, 12, 13, and 14. These compounds were previously identified in some parts of this species (Zunjar et al., 2015; Rivera-Pastrana et al., 2010; Gogna et al., 2015). In this study, it was possible to assigne the compounds protocatechuic acid glucoside (*m/z* 315, 3), syringic-caffeic acid ester (*m/z* 373, 5), sinapic acid hexoside (*m/z* 385, 6), caffeoyl malate (*m/z* 295, 9), coumaric acid (*m/z* 163, 12), p-coumaroyl malic acid (*m/z* 279, 13) and ferulic acid (*m/z* 193, 13) (Zunjar et al., 2015; Rivera-Pastrana et al., 2010; Gogna et al., 2015). Furthermore, the compound glucotropaeolin (4) with the precursor ion at *m/z* 408 $[M - H]^-$ was assigned based on MS/MS spectrum and literature data of cyanogenic glycosides isolated from *C. papaya* (Seigler et al., 2002). Sucrose (*m/z* 341, 1) and citric acid (*m/z* 191, 2) were tentatively identified (Li et al., 2016).

The identification of glycosylated flavonoids was facilitated by the analysis of fragmentation pathways of ions in the negative modes and the observation of glycosidic residues (rhamnosyl (146 Da), glucosyl (162 Da), and rutinosyl (308 Da)) were cleaved sequentially and generated characteristic aglycone fragments compared to the available literature. Among these compounds, two compounds were identified as kaempferol glycoside (15 and 17) (Chen et al., 2015) and another two were identified as glycosides of quercetin (11 and 16) (Chen et al., 2015). In addition, kaempferol and quercetin derivatives were observed based on the main ion fragments produced in the MS/MS experiments, appearing at *m/z* 284 and 285 for kaempferol derivatives and *m/z* 300 and 301 for quercetin derivatives; these pairs of ion fragments corresponded to the respective homolytic and heterolytic cleavage of the glycosidic bonds in these compounds (Abreu et al., 2019).

Conclusion

Despite the presence of bioactive compounds, the aqueous extract *C. papaya* does not present antibacterial or antibiotic-enhancing effects against the ETEC and EPEC strains of *E. Coli.* Nevertheless, further research is required to determine if its isolated components are potential drug candidates for the therapy of infections caused by these strains, which remain important public health problems.

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All authors of the manuscript state that there is no conflict of interest.

Author agreement

We the undersigned declare that this manuscript is original, has not been published before and is not currently being considered for publication elsewhere. We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. We further confirm that the order of authors listed in the manuscript has been approved by all of us. We understand that the Corresponding Author is the sole contact for the Editorial process. He is responsible for communicating with the other authors about progress, submissions of revisions and final approval of proofs

On behalf all other authors, I affirm that all agree with this submission.

Author contributions

Conceptualization, C.N.F.L.G. and N.F.L.S.; methodology, D.A.C.B.; software, L.S.A. and M.A.S.A.; investigation, R.H.S.C..; resources, J.F.T. and M.S.S.; writing—original draft preparation, J.P.R.S.; supervision, H. D.M.C.; project administration,H.D.M.C.

Declaration of Competing Interest

The authors declare that they have no known competing financia-linterestsor personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

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