Exon

Exon 2(3): 255-266
ISSN: 3048-7706 (Online)
exonjournal.com

Original Research

Unveiling Urease Inhibitory Properties and Phytochemical Screening for the Therapeutic Potential of Extracts and Fractions of *Khaya grandifoliola* C.DC. and *Entada africana* Guill. et Perr

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ABSTRACT

Urease is an enzyme that plays a significant role in the pathogenesis *of Helicobacter pylori* responsible for numerous health issues, among which chronic gastritis, peptic ulcer disease, gastric neoplasia and even stomach cancer. Therefore, targeting urease represents a key strategy to attenuate gastritis induced by *H. pylori. Khaya grandifoliola* (KG) and *Entada africana* (EA) were prepared using methylene chloride/methanol (1:1, v/v). After that, the extracts were fractionated using a gradient of solvent and the anti-urease activities of crude extracts and fractions was evaluated. Mechanism of inhibition of urease by the most active fractions was determined using kinetic method. Polyphenols and flavonoids contents were determined. Extracts and fractions of KG and EA exhibited a significant inhibition of urease activity. The half inhibitory concentration (IC50) of KG (31.61 ± 0.23 µg/mL) and EA (35.70 ± 0.14 µg/mL) showed no significantly difference ($P > 0.05$) to the reference Thiourea (30.37 ± 0.30 µg/mL). In addition, the kinetic study revealed that the methanolic fraction (FMeOH) of KG and EA strongly inhibited the activity of urease with IC50 values of 31.60 ± 1.25 and 29.00 ± 1.05 µg/mL respectively. These fractions exhibited mixed-type inhibition with Kic and Kia values of 9.96 and 91.15 µg/mL for KG and 13.24 and 226.59 µg/mL for EA respectively. Both extracts contain phenolic compounds. Overall, this study indicated a high anti-urease activity of the methanolic fraction of KG and EA. Each fraction exerts a mixed inhibition on urease activity.

KEYWORDS: *Khaya grandifoliola, Entada Africana, anti-urease activity, Jack Bean urease, mixed inhibition.*

OPEN ACCESS

Editor Dr. Namrata Gupta, UIBT, Chandigarh University, India

Reviewers Dr. Ashwani Kumar, UIBT, Chandigarh University, India

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The authors declare no conflict of interest.

Received 23 June 2025
Accepted 09 September 2025
Published 13 September 2025

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Introduction

Urease inhibitors have been received much attention in recent years as potential treatment against infections involving bacterial urease producers and, solution to the problem of ammonia volatilization into the atmosphere upon the use of urea-based fertilizers. Urea is also a product of the metabolism of nitrogen compounds in humans and animals. In the presence of urease (urea aminohydrolase, EC 3.5.15), urea is hydrolyzed to ammonia and carbon dioxide. The ammonia formed after hydrolysis has a favorable effect on the growth and development of plants. Also, the exceptional ability of Helicobacter pylori to resist to the stomach acidity is dependent on ammonia production. Thus, urease is of great interest in health and agriculture (1). This paper focuses on the health significance of this enzyme.

Urease is involved in the pathogenesis of many clinical conditions, including *H. pylori* infection, urinary tract infections (UTIs), infection-induced urinary stones (IUS), gastroesophageal reflux disease (GERD), and periodontal disease. *H. pylori* is a pathogenic Gram-negative bacterium that specifically colonizes the human gastric mucosa and is considered as a fundamental causative agent of gastric ulcers (2). H. pylori strains require urease to colonize the stomach niche of human (3). The urease enzyme in the cytoplasm and/or on the surface of *H. pylori* is a major virulence factor in the human population (4). So, the control of urease activity is important in the field of health (5,6).

Urease inhibitors have been studied as anti-ulcer drugs (7). Inhibitors such as hydroxamic acids and phosphazenes have been applied in medicine and have been shown to be potent on urease activity. Current treatment regimens available for gastric ulcer and *H. pylori* infection use a combination of multiple antimicrobial agents and proton pump inhibitors. However, this combination approach leads to patient noncompliance during long term therapy and, management of *H. pylori* induced gastric ulcer is a burning issue that necessitates alternative treatment options. Moreover, many side effects have been observed and effective and safe inhibitors of urease are currently the subject of extensive research (8–11).

Khaya grandifoliola (KG) and *Entada Africana* (EA) are two African medicinal plants with a rich history of use in traditional medicine. These plants have been used to treat malaria, diarrhea, skin diseases, liver related affections, and many other diseases conditions (12,13). Previous studies have reported the presence of phenolic compounds in the plants added to antimicrobial, antioxidant and anti-inflammatory activities (14,15). But little attention has been paid to the anti-urease activity of these plants. Since many studies have shown significant enzymes inhibitory activities of phenolic and flavonoid compounds (16–18), it was hypothesized that the methanolic extracts and fractions of KG and EA have potent urease inhibitory properties, thanks to their polyphenolic content.

Therefore, the aim of the present study was to determine the urease inhibitory activity properties of the extracts and fractions of KG and EA on Jack Bean urease.

Materials and Methods

Chemicals

All reagents used in this study were of analytical grade and purchased from Sigma Chemicals Company (Hamburg, Germany) and Prolabo (Paris, France); Thiourea

Plant collection and preparation of extracts

Stem bark of *K. grandifoliola* (Meliaceae), and *E. africana* (Mimosaceae) were collected in Foumban (West Cameroon). The identification of each plant was made by comparison to a specimen deposited at the National Herbarium, Yaounde, Cameroon under the references 2334/YA/HNC, and 52661/YA/HNC respectively. Two hundred grams of powder of each plant were macerated in methylene chloride/methanol (1:1, v/v) for 48 h and the mixture was filtered using a Whatman No.1 filter paper. The filtrate was concentrated using a rotavapor by evaporation the solvent and dried in an oven using a convection air oven at 40°C to obtain a residue that constituted the crude extract.

Five fractions of KG and EA namely methylene chloride (FC), methylene chloride/methanol (95/5; v/v) (F5), methylene chloride/ methanol (90/10; v/v) (F10), methylene chloride/methanol (75/25; v/v) (F25), and methanolic fraction (FMeOH) were prepared using flash chromatography.

Phytochemical Screening, determination of total phenolic and flavonoids

Determination of Total Phenolic Content

The assay was performed according to the Folin-Ciocalteu method as described by (20). Briefly, 200 µL of the plant extract and 1 mL of Folin Ciocalteu's reagent (1/10) were mixed to 800 µL of Na_2CO_3 (75 g/L). The mixture was incubated at 25°C for 2 h, the absorbance at 765 nm and the total polyphenols content determined using the calibration curve established with Gallic acid. The results were expressed as milligrams of Gallic acid equivalent (GAEs) per gram of extract.

Determination of Flavonoid content

The procedure was performed according to the method described by (20). Briefly, 50 µL of the plant extract was added to 1 mL of $AlCl_3$ with ethanol (20%). The reaction mixture was incubated at 25°C for 1 h and the absorbance read at 420 nm. The flavonoid content was determined using the calibration curve established and the results expressed as milligrams of Quercetin equivalent (QEs) per gram of extract.

Urease inhibition activity assay

The urease assay was performed according to the method described by Shabana et al., 2010. The assay mixture, containing 0.2 mL of Jack-Bean urease solution (2.0mg/mL), 1.2 mL of 100 mM phosphate buffer pH 7.0 and 0.1 mL of each plant extract dissolved in DMSO at the final concentrations of 0.1; 1; 10 and 100 ¬µg/mL, was incubated for 5 min at 37°C. DMSO was put in the negative control tube. After pre-incubation, 0.5 mL (0.05 mM) of urea was added and the mixture incubated as above. After that, 0.5 mL of phenol reagent (1% w/v phenol and 0.005% w/v sodium nitroprusside) and 0.5 mL of alkaline reagent (1% w/v NaOH and 0.075% hypochloride) were added. The resulted mixture was incubated (37°C, 50 min) again and the absorbance recorded at 630 nm. Activity of uninhibited urease was chosen as the control activity of 100%. Thiourea was used as the standard inhibitor of urease and tested at the same concentrations as the plant samples. The percentage of inhibition was calculated by using the following equation (1).

$$\mathrm{I}\ (\%) = \left(100\ - \frac{\text{OD sample}}{\text{OD control}}\right) \times 100\ (1)$$

Where I (%) is the percentage of inhibition of the enzyme, OD sample is the absorbance of the tested sample (plant extract or positive control in the solvent) in the presence of enzyme, OD control is the absorbance of the solvent in the presence of enzyme. Data

are expressed as mean ± standard error (SD).

The concentration of extract that inhibited the hydrolysis of substrate by 50% (IC50) was determined using GraphPad Prism 8.0.2 software.

Determination of the type of urease inhibition by the most active fractions

Kinetic studies were performed according to the method of (21,22). The inhibitory assay was conducted as above described. But, for this experiment, four concentrations of substrate (urea) 0.05; 0.2; 0.8 and 1 mM and three concentrations of the both FMeOH fraction 1; 10 and 20 µg/mL were used. Graphs were plotted using the GraphPad Prism 8.0.2 program, slopes, intercepts, and their standard errors were obtained by linear regression analysis using the same program. Each point in the constructed graphs represents the mean of three experiments. The Kic and Kia values were calculated from the slopes of each line in the Lineweaver–Burk plot versus different concentrations tested fractions.

Determination of the mechanism of inhibition of active fractions (23)

Assay of residual activity (RA)

The test mixture containing 0.1 mL of Jack-Bean urease at the pre-determined concentration; 1.3 mL of phosphate buffer (50 mM; pH 7.8); 0.1 mL of a solution either of active fractions (at final concentrations of 10 or 100 µg/mL) or of DMSO (negative control) was pre-incubated at 37°C for different times: 0; 5; 10; 20; 40; 60; 80; 100 and 120 min. After pre-incubation, 0.5 mL of urea (0.05 mM) was added to each tube and the mixture was incubated at 37°C for 30min and the ammonia produced was assayed. Having the percentages of inhibition at different times, the percentage of residual urease activity was then calculated using the following formula (2):

% RA =100-I (%) (2)

% AR= percentage of residual urease activity

I (%) = percentage of inhibition calculated according to the equation (1)

Inactivation of urease by thiosulphinates (TS)

The urease solution (2.0 mg/mL) and the plant fraction were mixed in the ratio 1:1. The concentrations of TS used in the incubation mixtures were in the range of 1.5–1000 µM. After incubation, 0.5 mL aliquots of the mixture at different time intervals were transferred into the standard assay mixtures (25 mL) for urease residual activity determination.

Spectroscopic assays of SH groups with 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) in thiosulphinates modified urease

Urease is a metalloenzyme that relies on the presence of thiol groups in its active site for proper enzymatic function. These thiol groups are involved in forming disulfide bonds and maintaining the enzyme's structural integrity and catalytic activity. When thiol groups are modified or inhibited, the enzyme's ability to catalyze its reaction is compromised, leading to reduced urease activity. Urease [(2 mg/mL) in (50 mM phosphate buffer, pH 7.8, 2 mM EDTA)] and the inhibitor (plant extract) solutions were mixed at a ratio for 1:1. Upon incubation, the urease activity was assayed under standard conditions, and 2.5 mL of incubation mixture was transferred to a cuvette (light path 5 cm) and mixed with 2.5 mL 0.15 mM DTNB (prepared in 50 mM phosphate buffer, pH 7.8). The absorbance of mixtures was measured at 412 nm in continuous mode for 15 min as per Ellman, 1959. Before these measurements, the control measurements of absorbance of the mixtures used were performed: urease and DTNB–

plant extract in the proportions corresponding to the final reaction mixture at 412 nm into a continuous mode for 15 min. The recorded control absorbance was subtracted from the absorbance recorded for the reaction mixtures, i.e., urease–DTNB–plant extract.

Dosage of thiol groups

The experiment was carried out to determine the RA, and the active fractions were tested at the concentration of 100 µg/mL. After different pre-incubation times, instead of the substrate was added 1 mL of DTNB. The tubes were then incubated at room temperature for 15 minutes, and the absorbance read at 412 nm against the blank. The calculation of the modified thiol (SH) groups was carried out on the basis that the 100% active urease has 36 thiol groups in its active site (23).

Statistical analysis

All experiments were performed in triplicate and the results are presented as mean ± SE (standard error) values. GraphPad Prism version 8.0.2 was used to represent Lineweaver–Burk graph. Statistical analysis was carried out by one-way ANOVA followed by Dunnett's tests using GraphPad Prism 8.0.2. Results were considered significantly different when $P < 0.05$.

Results

Total phenolic content and flavonoids of plant extracts and fractions

Table 1 represents the content of polyphenols and flavonoids of the crude extracts of KG and EA. For crude extract, the total polyphenols content 129.72 and 130.08 mg QE/g extract respectively; and flavonoids of KG and EA content 1.20 and 2.27 mg GAE/g extract.

Table 1: Total phenolic and flavonoids content in the crude extracts

Crude extract	Total phenols content (TPC) (mg QE/g)	Flavonoids content (FC) (mg GAE/g)
KG	129.72 ± 0.39	1.20 ± 0.01
EA	130.08 ± 0.22	2.27 ± 0.12

TPC: Total phenolic content, FC: Flavonoids content, KG: *Khaya grandifoliola*, EA: *Entada africana.*

Regarding the different fractions, table 2 shows that the fraction FMeOH of KG and EA were found to contain the higher amount of the target metabolites, 131.02 ± 0.25 and 132.83 ± 0.32 mg GAE/g extract respectively of total phenolic and 0.28 ± 0.01 and 1.54 ± 0.01 mg QE/g extract of flavonoids.

Table 2: Total phenolic and flavonoids content in the fractions

	Fractions				
KG	**FC**	**F5**	**F10**	**F25**	**FMeOH**
TPC (mg QE/mg)	26.13± 0.78	36.16±0.19	50.81±0.25	109.41±0.03	131.02±0.225
FC (mg GAE/mg)	0.2± 0.1	0.1± 0.2	0.08± 0.12	0.06±0.01	0.28±0.01
EA	**FC**	**F5**	**F10**	**F25**	**FMeOH**
TPC (mg QE/mg)	67.01±0.88	119.5±0.55	127.57±0.40	130±0.40	132.83±0.32
FC (mg GAE/mg)	0.3±0.01	0.54±0.05	0.9±0.03	1.2±0.01	1.54±0.01

TPC: Total phenolic content, FC: Flavonoids content, KG: *Khaya grandifoliola*, EA: *Entada africana*, FC: Fraction of methylene chloride, F5: Fraction of methylene chloride/methanol (95%), F10: Fraction of methylene chloride/ methanol (90%), F25: Fraction of methylene chloride/methanol (75%), FMeOH: Fraction of methanol.

Effect of plant extracts and fractions on urease activity

Crude extracts of KG and EA were tested as potential inhibitors of urease. Figure 1 shows the inhibition potential of extracts of KG and EA. It was found that the inhibition was concentration dependent. The IC50 value calculated were 31.61 ± 0.23 for KG and 35.70 ± 0.14 mg/mL for EA. There is no difference ($P > 0.05$) compared to the reference Thiourea (IC50 30.37 ± 0.30 µg/mL) (Table 3).

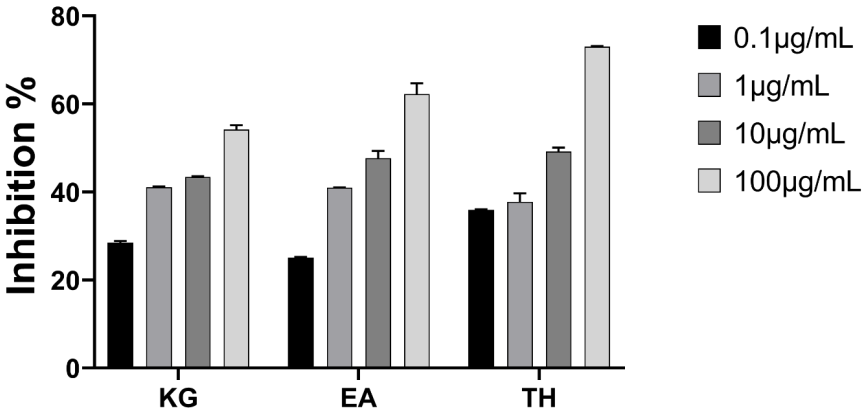


Figure 1: Inhibition of Jack-Bean urease by extracts of *Khaya grandifoliola* and *Entada africana.*

KG: *Khaya grandifoliola*, EA: *Entada africana*, TH: Thiourea.

Table 3: IC50 values of the crude extracts of KG and EA

Crude extract	Thiourea	KG	EA
IC_{50} (µg/mL)	30.37 ± 0.30	31.61 ± 0.23	35.70 ± 0.14

KG: *Khaya grandifoliola*, EA: *Entada africana*, IC50: Half-maximal inhibitory concentration

After fractionation of crude extracts of KG and EA, the five fractions obtained were tested on urease inhibitory activity. Figures 2a and 2b show that the inhibition is concentration dependent manner for all the fractions of KG and EA respectively. The methanolic fractions of KG and EA display a higher inhibition on urease activity. However, the fraction of EA showed a more potent activity (IC50: 29.00 ± 1.05 µg/mL), which is comparatively higher than the standard Thiourea (IC50: 30.37 ± 0.30 µg/mL) (Table 4).

Figures 3 show the type of inhibition of the most active fraction (FMeOH) of EA and KG. Lineweaver-Burk plots indicated that the FMeOH of EA and KG exert a mixed inhibition on Jack-Bean urease, as a change in both Vmax and affinity (Km values) of urease toward the substrate (urea) was observed. The constants of inhibition Kic and Kia were 9.96 and

91.15 µg/mL for KG and 13.24 and 226.59 µg/mL for EA respectively.

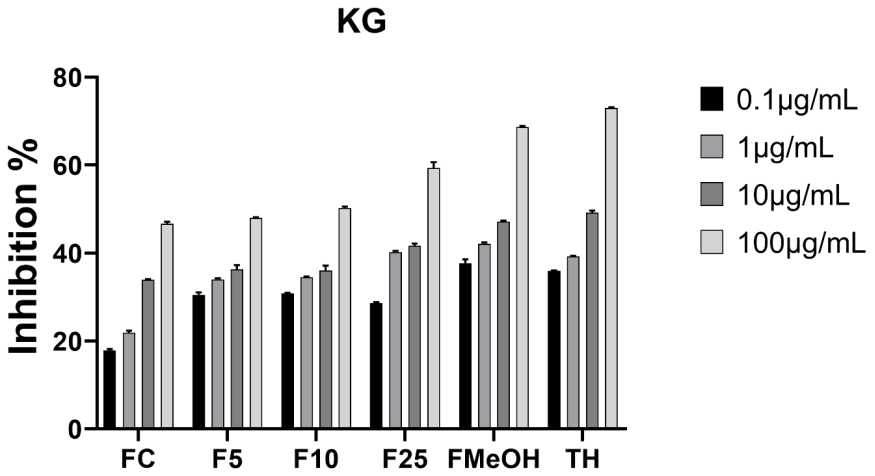


Figure 2a: Inhibition of Jack-Bean urease by fractions of *Khaya grandifoliola.*
FC: Fraction of methylene chloride, F5: Fraction of methylene chloride/methanol (95%), F10: Fraction of methylene chloride/ methanol (90%), F25: Fraction of methylene chloride/methanol (75%), FMeOH: Fraction of methanol, TH: Thiourea

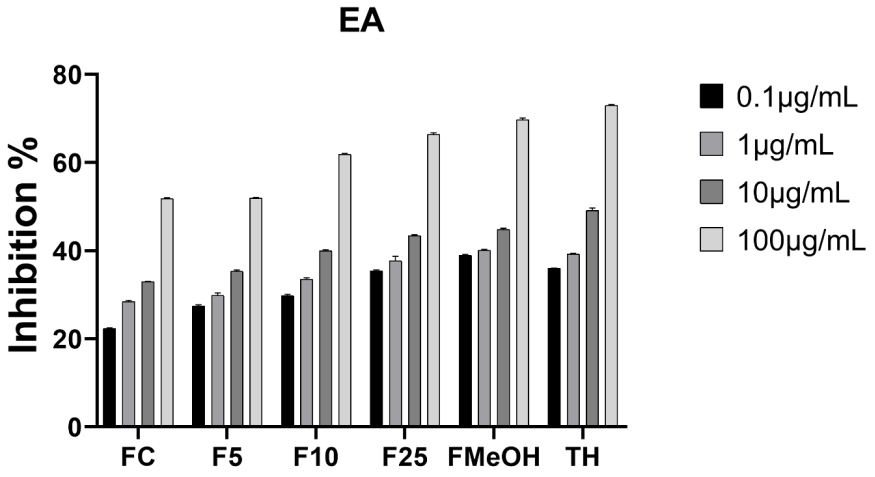


Figure 2b: Inhibition of Jack-Bean urease by fractions of *Entada africana.*
FC: Fraction of methylene chloride, F5: Fraction of methylene chloride/methanol (95%), F10: Fraction of methylene chloride/ methanol (90%), F25: Fraction of methylene chloride/methanol (75%), FMeOH: Fraction of methanol, TH: Thiourea

Table 4: The IC50 values of the fractions of KG and EA

	KG	EA
Fractions	**IC_{50} (µg/mL)**	
FC	ND	91.45 ± 1.34
F5	ND	89.15 ± 2.05
F10	98 ± 1.00	52.0 ± 1.53
F25	90.45 ± 1.25	33.60 ± 0.70
FMeOH	47.05 ± 2.25	29.00 ± 1.05

KG: *Khaya grandifoliola*, EA: *Entada africana*, FC: Fraction of methylene chloride, F5: Fraction of methylene chloride/methanol (95%), F10: Fraction of methylene chloride/ methanol (90%), F25: Fraction of methylene chloride/methanol (75%), FMeOH: Fraction of methanol. ND: Not Determined.

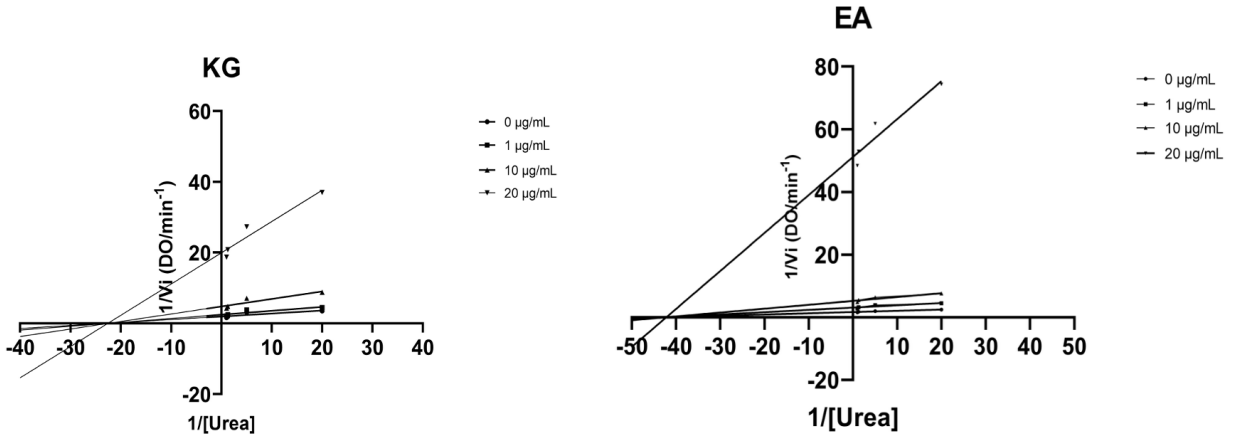


Figure 3. Inhibition of Jack-Bean urease by FMeOH of EA and KG Lineweaver–Burk plots of the reciprocal of initial velocities vs. reciprocal of four fixed substrate concentration in absence (●) and presence of 1 µg/mL (o); 10 µg/mL (▲); 20 µg/mL (△)

Mechanism of inhibition exerted by FMeOH moieties on urease

Residual urease activity in the presence of plant fractions

Figure 4 below show residual urease activity at different incubation times in the presence of FMeOH fractions EA and KG respectively at concentrations of 10 and 100 µg/mL. After 5 min of contact between the plant fraction at the concentrations tested and the enzyme, the enzymatic activity suddenly drops by nearly 50%. A gradual decrease in residual urease activity is noted as a function of incubation time. In addition, a total absence of residual urease activity is observed after 100 minutes of incubation with the FMeOH fraction of EA at the highest concentration (100 µg/mL).

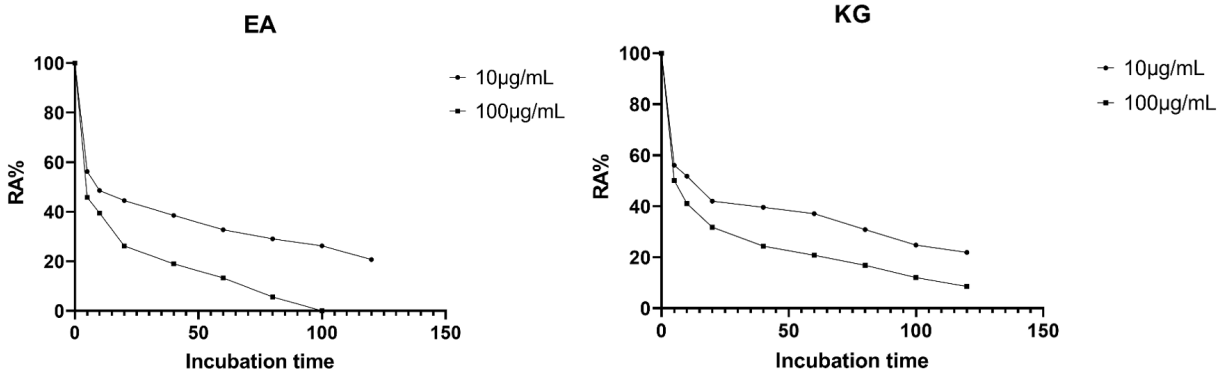


Figure 4: Residual urease activity after inhibition by the FMeOH fraction of EA and KG. Values are the means ± standard deviation of 3 different assays in duplicate.

Number of thiol groups modified by the FMeOH fractions of *K. grandifoliola* and *E. africana*

The Figure 5 below show the residual urease activity as a function of the number of thiols modified in the enzyme by the FMeOH fraction of KG and EA respectively. The test was carried out with the highest concentration (100µg/mL) of the plant fraction. An increase in the number of modified urease thiols is noted as the residual activity of the enzyme decreases for *K. grandifoliola* and no modification in the number of urease thiols as the residual activity decreases for EA.

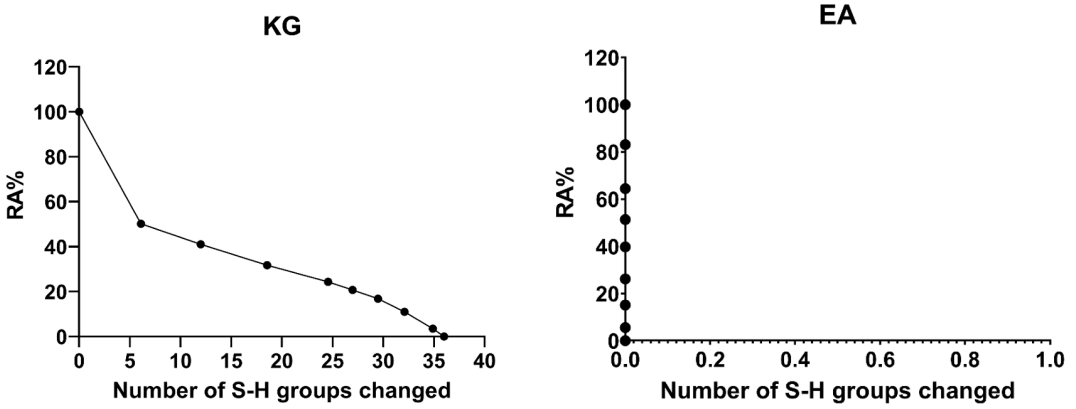


Figure 5: Residual activity of urease decreasing not as a function of the number of thiols modified in the enzyme by the FMeOH fraction of KG and EA.

Discussion

Urease, a nickel-containing enzyme, is an important target for agent development in both agricultural and medicinal practice. In medicine, targeting urease has been reported as a potential target for discovery of potential antiulcer, as well as anti-gastric cancer drug candidates. In this work, we examined the effect of extracts and fractions of *Khaya grandifoliola* and *Entada africana* on the inhibition of activity of urease (Jack-Bean).

Recently, natural therapy based on medicinal plants has drawn a lot of attention. Herbs have been used to treat a wide range of illnesses due the fact that the synthetic medications display a while range of side effects. For that reason, many studies have been done by scientists in closely monitoring and identifying the pharmacological properties of herbal active compounds from the suitable sources of active chemicals for developing more effective treatments with fewer side effects (24). *K. grandifoliola* and *E.africana* are used in Cameroonian traditional medicine for the treatment of skin and hepatic disorders. The extracts and fractions of these two plants revealed promising inhibitory potential against urease with IC50 value 31.61 ± 0.23 and 35.70 ± 0.14 µg/mL, respectively, equivalent to the standard Thiourea (IC50=30.37 ± 0.30 µg/mL). Therefore, these plants are considered as potential urease inhibitors worthy of development. Many studies previously reported the antiureasic effect of medicinal plants. This activity could be explained by the presence of secondary metabolites in these extracts such as polyphenols which are a group of compounds where molecules with antiurease activity are found (25). Several other natural compounds have been identified as urease

inhibitors, including certain flavonoids, phenolic compounds, and alkaloids derived from various plants (26). Our results are similar to those found by (8,11). To the best of our knowledge, there are no studies describing the antiurease activities of the two medicinal plants and their active and identified ingredients. Our study is an opportunity for the discovery of new anti-Helicobacter pylori and anti-ulcer agents.

An important approach to determine the inhibition mechanism and binding sites is the analysis of enzyme kinetics. The Lineweaver-Burk plots has been widely used to illustrate the type of inhibition in enzyme kinetics (27). According to the changes of Km and Vmax in the Lineweaver-Burk plots, it is proposed that the kinetic behavior of KG and EA concerning to Jack-Bean urease was mixed inhibition. These changes in Vmax and Km highlight that plant extracts not only reduce the rate of the enzymatic reaction, but also modify the interaction of the enzyme with its substrate, thus demonstrating the complexity of the inhibition mechanism. Several phenolic compounds present in the extracts of *K. grandifoliola* and *E. africana* could be responsible for this mixed inhibition. Also, the mechanism of inactivation of urease was studied by dosage of the modified thiol groups of urease with the FMeOH fraction of *K. grandifoliola* and *E. africana*. The number of thiols modified in urease by the extracts was calculated assuming that the absorbance recorded for urease under the non-denaturing conditions would correspond to 36 thiols per enzyme molecule. There is an existing relationship between residual urease activity and the number of modified thiols in the enzyme. Figure 5 shows a decrease in urease activity without involvement of the number of modified thiols of the enzyme, so the inhibition of urease by FMeOH of *E. africana* would occur by other mechanisms. On the other hand, the inactivation of urease by FMeOH of *K. grandifoliola* would occur by a modification of the number of thiols until a total loss of activity. This indicates that cysteine 592, an amino acid residue critical for the catalytic function of urease, remains unmodified at the beginning of the incubation period and is modified at later incubation time. This supports the observation that Cys592 located at the active site of urease is less reactive and more resistant to chemical modification than other urease thiols and plays an essential role in the hydrolysis mechanism of urease. (28). When this residue is chemically modified, the molecule loses its activity and the reaction is disrupted. Our investigations show that the FMeOH fraction of *K. grandifoliola* is capable of reacting with urease thiols, completely blocking the action of urease. These results highlight the potential of these extracts as a source of bioactive molecules to inhibit the enzymatic activity of urease.

Conclusion

In conclusion, this study offers valuable insights into the potential therapeutic mechanisms of extracts and fractions of *Khaya grandifoliola* and *Entada africana* on inhibition of the activity of Jack-Beans urease. The methanolic fractions of KG and EA are strong potent urease inhibitors, exerting a comparable inhibitory effect to the recognized urease inhibitor Thiourea. Further, kinetic and mechanical experiments revealed that FMeOH fraction of KG and EA exert the mixed inhibition by binding to the active site thiol groups of the enzyme in a concentration−time-dependent. This research contributes to the expansion of knowledge for the development of natural drugs and offers prospects for precision medical interventions. Our findings provide a scientific support of these two plants to be used as herbal remedies to manage gastric ulcer. The

potential of investigated urease inhibitors could be the novel therapeutics and need to be explored further. However, to fully validate these results, further research, using in vivo models, is needed to support the results obtained in this study.

Funding

The authors declare that there is no funding to report.

Author contributions

Conceptualization: CRN, DPL, FNN, and PFM; Writing – original draft: CRN, FAE; Writing –review and editing: CRN, DPL and FAE; Read and corrections: CRN, DPL, FAE, MAA, AFK, JNM, BFN, FNN and PFM. All authors have read and agreed to the published version.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgments

The authors are grateful to the Laboratory of Pharmacology and Toxicology, Department of Biochemistry, Faculty of Science, the University of Yaoundé 1 for providing the all-laboratory facilities.

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