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Phytochemical Studies, Antimicrobial and Anti-Inflammatory Properties of the Hydroethanolic Extract of *Kalanchoe pinnata* (Lam.) Pers. from Togolese Flora

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Abstract

Kalanchoe pinnata (Lam.) Pers. is known as a plant that has many special benefits such as anti-inflammatory and antibacterial. The present study was carried out to perform a phytochemicals study and evaluate the antimicrobial and anti-inflammatory activity of the hydroethanolic extract of *Kalanchoe pinnata* (Lam.) Pers. leaves. After phytochemicals screening, the content of phenolic compounds, proanthocyanidol and flavonoids in the extract of this plant was determined spectrophotometrically. Antimicrobial activity was assessed using the micro-dilution technique on 96-well plates in liquid medium, combined with agar spreading. Anti-inflammatory activity was assessed using the 1% carrageenan induced rat paw oedema model. Phytochemical screening revealed the presence of alkaloids, saponins, triterpenes and sterols, phenols and flavonoids in the plant extract in varying proportions. The extract contained (0.049 ± 0.03 µg EAG/mg extract) total polyphenols, (0.215 ± 0.025 µg CE/mg extract) proanthocyanidins and (385.435 ± 0.0328 µg ER/mg ES) flavonoids. The hydroethanolic extract of the leaves of this plant inhibited the in vitro growth of the microbial strains studied to varying degrees. The MIC of the extract varied from 12.5 to 25 mg/mL and the BMC from 12.5 to 50 mg/mL. The plant did not show any activity on 1% carrageenan-induced rat

paw edema.

Keywords

Kalanchoe pinnata, Hydroethanolic Extract, Phytochemistry, Pharmacological Activities, Togo

1. Introduction

Today, despite advances in modern medicine, a large proportion of the world's population uses and relies on traditional medicine for their health needs. *Kalanchoe pinnata* is a plant used as a medicinal herb by almost all traditional medicines in the tropical regions of Africa, Asia, India, China, Australia, America, Madagascar and Hawaii [1]. In Togo, *Kalanchoe pinnata* leaves are used as an anti-inflammatory and antibacterial remedy for bronchitis, genito-urinary diseases and various forms of fungal infections. The leaves are also used to treat vomiting and diarrhoea [2]. In recent years, antibiotic resistance in pathogenic micro-organisms has become an increasingly important public health problem worldwide [3]. It is therefore necessary to look for new substances with a lower risk of toxicity. The aim of the present work is therefore to evaluate some of the pharmacological properties of *Kalanchoe pinnata* (Lam.) Pers. specifically a phytochemical study and then the antimicrobial and anti-inflammatory properties of the hydroethanolic extract of the leaves of *Kalanchoe pinnata* (Lam.) Pers.

2. Materials and Methods

2.1. Plant Material

The plant material consisted of young leaves of *Kalanchoe pinnata* (Lam.) Pers. (Family: Crassulaceae). The fresh leaves of the plant were harvested in the early morning of October to November 2021 in Togblecope-Akoin, Lomé. The plant was then identified at the Botany and Plant Ecology Laboratory of the Faculty of Science at the University of Lomé. The leaves were then cut into very small pieces and dried at room temperature in the shade, before being ground into a coarse powder using an appropriate mill. The powder was then stored in an airtight container and kept in a cool, dark, dry place until the analysis was carried out.

2.2. Extraction Procedure

The leaves were then cut into very small pieces and dried at room temperature in the shade, before being ground into a coarse powder using a suitable grinder. The powder was then stored in an airtight container in a cool, dark, dry place until analysis. The powders obtained from grinding the plants were delipidated with petroleum ether. To 400 g of ground plant material, we added 2 L of petroleum ether for 24 hours. After delipidation, we proceeded with extraction by maceration in 2000 mL of a hydroethanolic solution composed of 70% alcohol 95˚ and

30% distilled water in a clean, flat-bottomed plastic container. The whole was stirred continuously for 72 hours at laboratory temperature, then filtered through Whatman No.1 paper, then evaporated under vacuum at 50˚C at 125 rpm using a Heidolph-type electric rotavator. The resulting concentrated hydroethanol extract was lyophilized at low temperature and stored at 4˚C in a dry bottle until use [4].

2.3. Micro-Organisms Tested

The micro-organisms tested included Gram-positive bacteria (*S. aureus*), Gram-negative bacteria (*E. coli*, *P. aeruginosa*, *Salmonella* spp., *K. pneumoniae*) and yeasts (*Candida albicans*, *Candida* spp.). These included six (6) reference strains from the American Type Culture Collection (ATCC) collection, obtained at the National Institute of Hygiene of Lomé, and thirty-four (34) clinical strains obtained from cultures of various samples taken in the bacteriology laboratories of the Sylvanus Olympio and Campus University Hospital Center of Lomé. The bacterial stock cultures were maintained on nutrient agar and all cultures were subcultured weekly and then stored at 4˚C. Gentamicin injection (40 mg/mL) and nystatin (10 mg/mL) were used as standard antibiotics and antifungals respectively in this research work. They were purchased from pharmacies.

2.4. Animals

The animal material consisted of albino rats of the Wistar strain. These animals were bred in the animal house of the Microbiology and Food Quality Control Laboratory of the Higher School of Biological and Food Techniques of the University of Lomé. The animals were fed a standard diet made up of proteins, carbohydrates and lipids, and water ad libitum. Aeration and lighting conditions were good.

2.5. Phytochemical Screening of the Extract

Phytochemical screening was carried out using standard procedures and the following qualitative tests were performed [5]:

- Detection of alkaloids (Dragendroff test): 2 mL of the extract and 0.2 mL of dilute hydrochloric acid were taken in a test tube. Next, 1 mL of Dragendroff's reagent was added. Observation of an orange-brown precipitate indicated the presence of alkaloids.
- Detection of flavonoids: A few drops of methanol and magnesium turnings were added in addition to a few drops of concentrated hydrochloric acid (Hcl) to 2 mL of extract. The appearance of red colour indicates the presence of flavonoids in the extract.
- Detection of triterpenes and sterols: A few drops of chloroform and H_2SO_4 sulphuric acid were added to 2 mL of extract. The appearance of a red-brown ring between two phases, one clear at the bottom and the other green (not so much) at the top, indicates the presence of triterpenes and sterols in the extract.
- Identification of phenolic compounds: A few drops of iron perchlorate Fe_2CL_3

were added to 2 mL of extract at 1 mg/mL. The appearance of a blackish-brown colour indicates the presence of gall tannins in the extract.

- Detection of saponins: Whether or not a persistent foam appeared after shaking a test tube containing a few millilitres of the extract indicated the presence of saponins.

2.6. Quantitative Phytochemical Analysis of the Extract

- Evaluation of total phenol content

Total polyphenols were determined using the method described by Karou (2006) [6]. The total phenol content was determined by extrapolation on a standard curve, obtained from a series of dilutions with distilled water of gallic acid (200 mg/L) ranging from 0.05 to 0.25 mg/mL. A mixture consisting of 0.2 mL of extract at 1 mg/mL and 0.5 mL of FCR diluted 1/2 in distilled water was added to the test tubes. After 5 minutes of incubation at room temperature protected from light, 0.5 mL sodium carbonate (20 g/L) was added to the mixture. The volume in each tube was made up to 4 mL. After shaking, the different solutions were left to stand in the dark for 30 min. The OD was read at 760 nm using a UNICO model 12 spectrophotometer against a negative blank consisting of a mixture of 0.5 mL FCR, 0.5 mL sodium carbonate and distilled water and a positive blank consisting of the extract to be determined and distilled water. Two readings were taken per sample.

- Evaluation of proanthocyanidol content

The proanthocyanidol content was evaluated using the Butanol-HCL method developed by Porter *et al.* (1986) [7] and taken over by [8]. The test consisted of adding 0.2 mL of ammoniacal iron sulphate (20 g/L) and 7 mL of a butanol/HCL solution (95/5 mL) to 0.2 mL of each extract in tubes. After 40 min incubation in a water bath at 95˚C, the tubes were cooled and the absorbances were read at 550 nm. The absorbances were duplicated for each tube. The concentration of procyanidin in the extract was obtained using the following relationship: X = (OD × 1 CE/g)/0.280 (OD = optical density measured at 550 nm; X = concentration of pro anthocyanidins in the extract. It is expressed in mg Catechin Equivalent (CE)/g extract or in catechin equivalent per gram (% CE/g); OD = 280 corresponds to 1% catechin used as a standard).

- Determination of flavonoid content

Flavonoid content was estimated using the method described by Andzi-Barhé *et al.* (2015) [9]. This involved preparing plant extracts at 1 mg/mL in distilled water, aluminium chloride ($AlCl_3$) 2% in distilled water, and rutin at different concentrations of 0; 5; 25; 50; 75; 100; 150; 200 µg/mL in methanol. The operation involved vortexing 1 mL of the 1 mg/mL extract solution or 1 mL of each rutin concentration with 1 mL of 2% aluminium chloride ($AlCl_3$). After 10 minutes incubation, absorbance was measured directly with a UV-visible spectrophotometer (METASH UV-5200PC UV/VIS Spectrophotometer) at 415 nm against a blank. Rutin was used as a standard. The total flavonoid content of the extracts was deduced from the calibration curve established with Rutin (0 - 200 µg/mL) and the

results are expressed in microgram equivalent of Rutin per milligram of dry extract (µg ER/mg ES).

2.7. Assessment of Antimicrobial Activity

The evaluation of the antimicrobial properties of the hydroethanol extract of *Kalanchoe pinnata* was carried out using the microplate dilution method described by Anani *et al.* (2015) [10]. Stock solutions of the extracts sterilised by filtration through a 0.45 µm diameter millipore membrane were prepared at 100 mg/mL in distilled water. A sterility test was carried out by spreading 100 µl of the extract on MH agar and incubating at 37˚C for 18 h to 24 h. The extract is considered pure in the absence of culture. Successive 2-fold dilutions ranging from 100 to 12.5 mg/mL of extract were prepared using Muller Hinton Broth (MHB) for bacteria and Sabouraud Broth (SB) for yeast. Microbial suspensions corresponding to a turbidity of 0.5 Mac Farland (107 CFU/mL) were made with microorganisms from a 24-hour pure culture at 37˚C after a Gram control. Next, 100 µL of the microbial suspension was brought into contact with the extracts at different concentrations, involving a further dilution to half the concentration. Positive control wells (microbial suspension without extract) and negative control wells (BMH or BS + Extract) were also constructed. Gentamicin (40 mg/mL) and nystatin (10 mg/mL) were used as reference antibiotics and antifungals. The plates were then shaken and incubated at 37˚C for 24 hours. The lowest concentration that completely inhibited visible growth was recorded as the MIC. Subcultures were made from clear wells which did not show any growth on nutrient agar to appreciate MBC. The lowest concentrations without growth after the subculturing were MBCs) [10]. The tests were carried out in an aseptic environment.

2.8. Assessment of Anti-Inflammatory Activity: Carrageenan Oedema Model

The animals were fasted 12 h before the start of the experiment. The rats were weighed and divided into batches according to their weight. The tibiotarsal joint of each rat was marked to delimit the part to be immersed. The volumes of the rats' paws were measured before and after oedema induction using a water plethsmometer (the volume of liquid displaced corresponds to the volume of the paw) [11]. The rats in batch 1 did not undergo oedema induction, thus serving as a control for the test. In the other batches, the solutions were administered by gavage prior to oedema induction. Batch 2 received physiological water, while batch 3 was given ketoprofen at a dose of 20 mg/kg body weight as a reference. Batches 4 and 5 received the extract at a dose of 400 and 800 mg/kg body weight respectively. 30 min after administration of these different solutions, inflammation was induced by inducing oedema by injecting 100 µL of 1% carrageenan solution into the plantar fascia of the animal's left hind leg. After induction of oedema, paw volume was measured every hour for 5 hr. Treatment of the animals continued every day for three days after induction of oedema and on the third day

with daily gavage. The anti-inflammatory activity of the extract was assessed by calculating the volume of oedema using the formula: Vo = Vt – Vi (Vo: Volume of oedema; Vt = Volume of paw at time t after injection of carrageenan; Vi = Volume of paw before induction of oedema).

2.9. Data Processing

The results were analyzed using Excel 2019 and Graph Pad Prism 8.0.2 (Boston, USA) software. Comparison of means and variances were compared using a one-factor ANOVA followed by Tukey's test at the significant level of $P < 0.05$. The data were presented in the form of mean values ± standard deviation (SD) and a figure.

3. Results

3.1. Qualitative Phytochemical Analysis

The results of various qualitative phytochemical tests on the hydroethanolic extract of *Kalanchoe pinnata* (Lam.) Pers. showed the presence of alkaloids, total phenols, triterpenes and sterols, flavonoids and saponins. Table 1 shows the results of the qualitative analysis of the hydroethanol extract of the plant.

Table 1. Results of the qualitative phytochemical analysis of the hydroethanolic extracts of *Kalanchoe pinnata* (Lam.) Pers.

Extract	Total phenols	Alkaloids	Triterpenes and sterols	Saponins	Flavonoids
EHKP	+	++	+	+	+++

(+): Presence; HEKP: Hydroethanolic extract of *Kalanchoe pinnata* (Lam.) Pers.

3.2. Quantitative Phytochemical Analysis

The total phenol, pro anthocyanidin and flavonoid contents of the plant extract were determined. Table 2 shows the results of the qualitative analysis of the hydroethanol extract of the plant.

Table 2. Results of the qualitative phytochemical analysis of the hydroethanolic extracts of *Kalanchoe pinnata* (Lam.) Pers.

Compounds	Total polyphenol (µg EAG/mg)	Proanthocyanidols (µg CE/mg)	Flavonoids (µg ER/mg ES)
EHKP	0.049 ± 0.03	0.215 ± 0.025	385.435 ± 0.0328

µg EAG/mg: equivalent microgram of gallic acid per milligram of extract; µg EC/mg: equivalent microgram of catechin per milligram of extract; µg EAA/mg: equivalent microgram of ascorbic acid per milligram of extract; µg RE/mg ES: equivalent microgram of rutin per milligram of dry extract; HEKP: Hydroethanolic extract of *Kalanchoe pinnata* (Lam.) Pers.

3.3. Antimicrobial Activities

Tables 3-5 and Table 6 show the minimum inhibitory concentrations (MIC), the minimum bactericidal concentrations (MBC) and the antibiotic potency (MBC/MIC)

of the extract. The extract is considered bactericidal if (MBC/CMI) ≤ 1; it is bacteriostatic if (MBC/MCI) > 1.

Table 3. MIC and MBC of the hydroethanolic extract of *Kalanchoe pinnata* obtained for the reference strains.

REFERENCE STRAINS	MIC	MBC	MBC/MIC
S. aureus ATCC 29213	25	25	1
E. coli ATCC 25922	25	25	1
P. aeroginosa ATCC 29263	25	25	1
S. typhi murium ATCC 14028	12.5	25	2
S pneumoniae ATCC 49619	25	50	2
C. albicans ATCC 10231	25	25	1

Table 4. MIC and MBC of the hydroethanolic extract of *Kalanchoe pinnata* obtained for the clinical strains (Gram positive bacteria).

Clinical strains (Gram positive bacteria)	MIC	MBC	MBC/MIC
S. aureus 1	25	25	1
S. aureus 2	25	25	1
S. aureus 3	25	25	1
S. aureus 4	25	50	2
S. aureus 5	25	25	1
S. aureus 6	25	50	2
S. aureus 7	25	25	1
S. aureus 8	12.5	25	2
S. aureus 9	25	25	1
S. aureus 10	12.5	25	1

Table 5. MIC and MBC of the hydroethanolic extract of *Kalanchoe pinnata* obtained for the clinical strains (Gram negative bacteria).

Clinical strains (Gram negative bacteria)	CMIC	MBC	MBC/MIC
E. coli 1	12.5	25	2
E. coli 2	12.5	25	2
E. coli 3	12.5	25	2
E. coli 4	12.5	25	2
E. coli 5	12.5	50	4
E. coli 6	12.5	50	4
E. coli 7	25	25	1
P. aeroginosa 1	25	25	1
P. aeroginosa 2	25	50	2

Continued

P. aeroginosa 3	12.5	25	1
K. pneumoniae 1	25	25	1
K. pneumoniae 2	25	25	1
K. pneumoniae 3	25	25	1
K. pneumoniae 4	25	50	1
K. pneumoniae 5	50	50	1
Salmonella spp. 1	12.5	25	2
Salmonella spp. 2	25	25	1
Salmonella spp. 3	12.5	25	2
Salmonella spp. 4	25	50	2

Table 6. MIC and MBC of the hydroethanolic extract of *Kalanchoe pinnata* obtained for the clinical strains (Yeasts).

Clinical strains (Yeasts)	MIC	MBC	MBC/MIC
C. albicans 1	25	50	2
C. albicans 2	25	50	2
C. albicans 3	25	25	1
Candida spp. 1	25	50	2
Candida spp. 2	12.5	25	2

3.4. Anti-Inflammatory Activities

The average volume of edema at each hour for each batch of rats was calculated and the results are reported in Table 7.

Table 7. Edema volume over time.

Volumes (mL)		Physiological water	Ketoprofen	EHEKP 400 mg/kg bw	EHEKP 800 mg/kg bw
Vo	1H	0.21 ± 0.05**	0.06 ± 0.01	0.27 ± 0.03***	0.25 ± 0.01***
Vo	2H	0.24 ± 0.05***	0.04 ± 0.01	0.39 ± 0.03***	0.30 ± 0.01***
Vo	3H	0.34 ± 0.04***	0.04 ± 0.01	0.44 ± 0.01***	0.43 ± 0.02***
Vo	4H	0.33 ± 0.05***	0.07 ± 0.04	0.36 ± 0.01***	0.46 ± 0.02***
Vo	5H	0.33 ± 0.04***	0.06 ± 0.00	0.40 ± 0.03***	0.50 ± 0.02***
Vo	72H	0.08 ± 0.03	0.09 ± 0.03	0.05 ± 0.04	0.18 ± 0.00*

Vo: Edema volume; H: Time; EHEKP: Hydroethanolic extract of *Kalanchoe pinnata*, Values are expressed as mean ± SD. These values were analysed by Fisher's test (Chi-square). Differences are statistically significant *P < 0.05; **P < 0.01; ***P < 0.001 when we compare the groups having taken the extract and physiological water to the reference groups. The volumes of the edemas thus obtained allowed us to draw the graph of the evolution of the edema induced after administration of the various treatments over time.

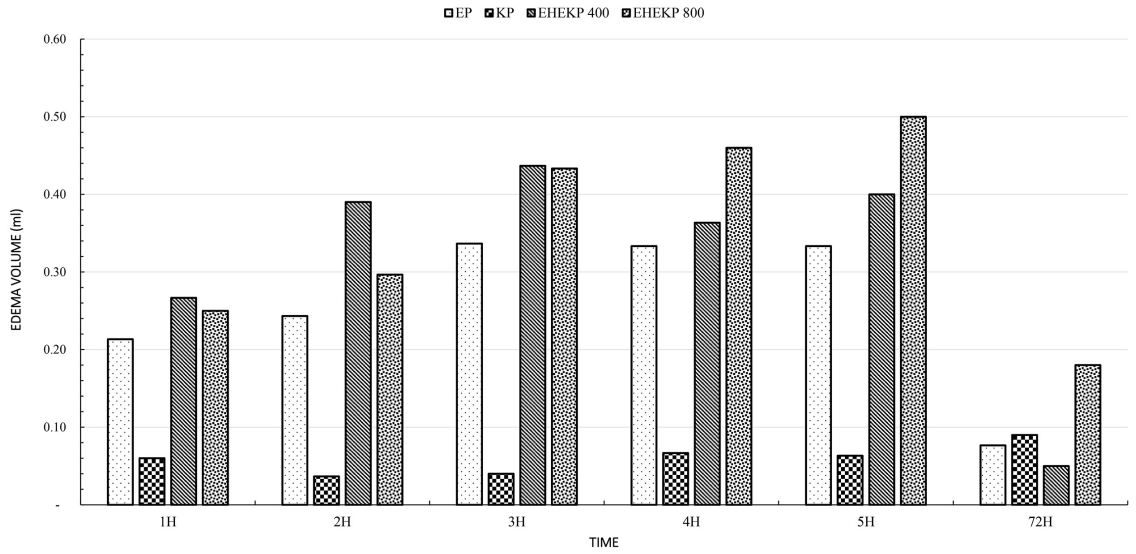

Figure 1. Evolution of paw edema in rats over time. EP: Physiological Water; KP: Ketoprofen; HEKP: Hydroethanolic extract of *Kalanchoe pinnata*.

By analyzing the histogram (**Figure 1**), we notice that after administration of the treatments, the inflammation increases rapidly during the first five hours in the batches treated with the extract much more than those treated with physiological water and decreases considerably on the third day whereas in those treated with Ketoprofen the volume of the edema hardly varies during the 72 hours.

4. Discussion

Plants are used as an important source of medication in many traditional medicines [12]. Phytochemical screening of the hydroethanolic extract of *Kalanchoe pinnata* (Lam.) Pers. showed the presence of alkaloids, total phenols, triterpenes, sterols, flavonoids and saponins. Quantitative analysis showed relatively very low amounts of total phenols and proanthocyanidins and a low number of flavonoids. According to the work of Pattewar *et al.* (2013) [13], the leaves of *Kalanchoe pinnata* contain Alkaloids, Flavonoids, Saponins, steroids, tannins, terpenoids and phenols. The revelation of these secondary metabolites in the plant could depend on certain factors, namely the type of solvent and the method used for the extraction. [14] showed the presence of flavonoids and total polyphenols in 95% ethanolic, methanolic and 65% methanolic extracts and their absence in the aqueous extracts of *K. pinnata* leaves. The results of the work of Bourgou *et al.* (2016) [14], also showed that the extraction method significantly influences the yield and the levels of secondary metabolites. According to Mamadou *et al.* (2014) [15], the solubility of secondary metabolites depends on their chemical nature in the plant, which varies from simple to highly polymerized compounds. Plant materials may contain varying amounts of phenolic acids, phenylpropanoids, anthocyanins, and tannins. This structural diversity is responsible for the great variability of physicochemical properties influencing the extraction of polyphenols. Among other things,

the solubility of secondary metabolites is affected by the polarity of the solvent used.

The hydroethanolic extract of the leaf of *K. pinnata* was active on all strains with an MIC ranging from 12.5 mg/mL to 25 mg/mL. *S. aureus* strains were inhibited with a MIC of 25 mg/mL while E. coli was inhibited with a MIC of 12.5 mg/mL. This could be related to the difference in membrane structures of these microorganisms. The difference in sensitivity of the microbial strains to the hydroethanolic extract of the plant subjected to the study could be related to the capacity of diffusion of the extract through the bacterial membrane. In fact, the wall of Gram-negative bacteria is thin and rich in lipopolysaccharides, whereas that of Gram-positive bacteria is thick and rich in peptidoglycans. The fact that in our study the Gram-negative bacteria are more sensitive to the extract than the Gram-positive ones would suggest that our extract is more fat-soluble than water-soluble [10]. Saleem *et al.* (2015) [16] showed that 90% and 95% methanolic extracts of *Kalanchoe pinnata* leaves inhibit the growth of microorganisms in vitro with the minimum inhibitory concentration (MIC) which varied between 1.45 and 22.7 mg/mL. Their results could be explained by the difference in extraction solvent and the difference in the method used.

The evaluation of the anti-inflammatory activity shows us that after administration of the treatments, an increase in the volume of edema during the first five hours in the batches treated with the extract much more than those treated with physiological water and decreases considerably on the third day whereas in those treated with Ketoprofen, the volume of the edema hardly varies during the 72 hours. Matthew *et al.* (2013) [17] showed that *Kalanchoe pinnata* has anti-inflammatory activity by reducing paw edema in rats. This difference could be related to the type of extraction and the plant organ used for the studies. Indeed, in their study, the aqueous extracts and the pure ethanolic extracts of the stems of the plant were used while the hydroethanolic extract of the leaves of the plant was used to carry out our study. The differences could arise from the phytochemical composition of the plant, climatic and soil factors, time of harvest, method of brief drying, and specificity of secondary metabolites.

5. Conclusion

Finally, we can suggest that the hydroethanolic extract of the leaves of *Kalanchoe pinnata* (Lam.) Pers. have antimicrobial and immunomodulatory activities. The studied plant can be considered as a potential source of useful antimicrobial drug. The experiment was conducted with only one extract. Therefore, further research is essential to evaluate other plant extracts against other species of bacteria, fungi, viruses and other microorganisms. Further studies are however recommended on the plant to determine the pharmaceutical potential of the plant as a medicine and to isolate and elucidate the structure of the bioactive compounds.

Conflicts of Interest

The authors have declared no conflicts of interest.

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