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Antibacterial Activities of Extracts from four Wild Food Fruits

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Aims: This study aimed to assess the *in vitro* antibacterial effects of extracts from four wild food fruits: *Balanites aegyptiaca, Saba senegalensis, Ziziphus mauritiana,* and *Raphia sudanica.* **Place and Duration of Study:** The samples of plant material were collected at Banamba and Sikasso, Mali between January and May 2018. The bacterial strains were collected at Research Centre for Biological Food and Nutritional Sciences (CRSBAN), University Professor Joseph Ki-Zerbo; Ouagadougou, Burkina Faso. The experimental parts were also carried out at CRSBAN from

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Methodology: The fruit extracts were screened for antibacterial activity against *Escherichia coli*, *Salmonella typhi*, *S. aureus*, *B. cereus*, and *L. monocytogenes* strains. The diameters of the inhibition zones (ID), the minimum inhibitory concentrations (MIC) as well as the minimum bactericidal concentrations (MBC) were evaluated using agar diffusion method.

Results: The findings revealed that these parameters have varied as a function of fruit species and/or their zones of provenances. All fruit extracts showed significant growth reducing effect against all the tested bacteria. The extracts from *R. sudanica* have exhibited the strongest growth-inhibiting activity specifically against *E. coli* (ID = 15.33 ± 0.58 mm) and *Salmonella typhi* (ID = 18.00 ± 1.00 mm) with lower MIC (from 2.08 ± 1.44 to 5.83 ± 1.44 mg/mL). Moreover, the MBC/MIC ratios revealed that the extracts from the studied fruits possess mainly bacteriostatic effects towards the tested strains.

Conclusion: These findings support local therapeutics properties attributed to these fruits. They also demonstrate that, in addition to their nutritional values, these edible fruits could be used for developing antibiotics to treat infectious diseases and food poisoning.

Keywords: Wild food fruits; antibacterial sensitivity; zones; Banamba and Sikasso.

1. INTRODUCTION

In Africa, the use of folk medicine and pharmacopeia is a very common practice. This practice could be explained by the poverty of populations, the lack of infrastructures and sociomedical personnel in modern or conventional medicine, etc. The cosmogonic perceptions of evil, religious or superstitious beliefs reinforce the trend [1,2]. So, the practitioners of traditional medicine perceive it as an alternative to the modern one. After an unsuccessful treatment in the high hospitals, the patients can sometimes recover its health miraculously and cheaply by having recourse to the traditional therapy [2]. In other side, to face up the resurgence of antimicrobial-resistant pathogens. the researchers worldwide are hardworking to find other alternative sources of drugs and bioactive chemical compounds of plant origin [3] that would serve as antimicrobial agents less expensive, less toxic, and more effective [4]. This is why, many plants have been explored as alternative sources of drugs and in particular antimicrobial and anti-inflammatory agents [5-8].

In this struggle, the plants that are considered to be primarily food have not been sidelined [9,10]. Regarding to the high frequent use of traditional medicine for the primary care by a large part of the population in both developed and developing countries [11], it is then important to extend the investigations on this category of food plants. Nowadays, the therapeutic foods are very coveted to fight strongly against the malnutrition especially in children [12].

In a recent study, we showed that a wide range of wild edible fruits are used by the local populations in Mali not only for their nutritional needs but also for the management of certain recurrent pathologies [13,14]. Among these species, the fruits of *Balanites aegyptiaca* L. (Del.), *Saba senegalensis* A.DC. (Pichon), *Ziziphus mauritiana* Lam. and *Raphia sudanica* A. Chev. hold an important place. Although their nutritional virtues have often been investigated throughout the continent [15-18], very little scientific data exist on their antibacterial properties. Therefore, further investigations are needed in order to have a better understanding of their therapeutic virtues for the management of certain diseases related to the consumption of unhealthy foods such as salmonellosis, cholera and hepatitis A.

This work is part of this framework through a comparative study of the *in vitro* antibacterial activities of extracts from four wild edible fruits: *B. aegyptiaca, S. senegalensis, Z. mauritiana* and *R. sudanica.* The extracts are tested on few of the most common infectious bacterial agents found in Mali.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Plant material

The plant material was constituted of the fruit pulps of *B. aegyptiaca, S. senegalensis, Z. mauritiana* and *R. sudanica.* These plants were chosen following an ethnobotanical survey conducted in Banamba and Sikasso cities in Mali. They are among the most cited and least investigated species.

2.1.2 Biological material

The used biological material is presented in the Table 1. These bacterial strains were chosen due to their high frequency of foodborne contaminations and for their pathogenicity.

2.2 Methods

2.2.1 Extract preparation

After drying in the laboratory at the ambient temperature, the fruits pulps are recovered and pulverized. Then 100 g of powder were dissolved in 1000 mL of solvent (methanol – water: 80; 20; v/v). The mixture was stirred for 48 hours at room temperature (about 30 °C), then filtered under vacuum. The filtrate obtained was freeze-dried and the extract was dissolved in of 5% DMSO to obtain a stock solution of 40 mg/mL for the antibacterial tests.

2.2.2 Preparation of the bacterial inoculum

A suspension of each bacterial strain (young colonies aged of 24 h hours) was prepared in sterile physiological water. The bacterial charge was then adjusted to McFarland 0.5 standard which corresponds approximately to 1x10⁸ forming units in millimeters (CFU/mL) [19].

2.2.3 Disk preparation

The blank discs (from Liofilchem SRL) with 5 mm of diameter were impregnated with 100 μ L of the stock solution (4 mg of extract). The commercial antibiotic discs of Amikacine and Doxycycline were used as positive controls and a 5% DMSO solution as negative control.

2.2.4 Disk diffusion

The agar disk diffusion method according to the Clinical and Laboratory Standards Institute [20] with some modifications was used to perform the *in vitro* bacterial sensitivity towards the extracts. Fifteen millimeters (15 mL) of Mueller-Hinton agar medium (Himedia Ref® M173-500G),

previously sterilized, are poured in a petri dish. After medium solidification, the petri dish was seeded with 100 μ L of bacterial suspension. Then the blank discs are deposited on the surface of the seeded medium. After 1 h at room temperature, the petri was incubated at 37°C for 24 h.

The inhibitory activities of extracts were estimated through the diameters (mm) of the inhibition zones (ID) measured after incubation. These diameters were classified according to the scale used by Mihin et al. [10]: if ID < 8 mm, the bacterial strain is considered not sensitive to the extract tested, if $8 \le ID < 14$ mm, sensitive strain, if $14 \le ID < 20$ mm, very sensitive strain and if ID ≥ 20 mm, extremely sensitive strain.

2.2.5 Minimum inhibitory concentrations (MIC)

The Clinical and Laboratory Standards Institute [20] adjusted by Mihin et al. [10] were used. The microplates of 96 wells were used to assess the MIC. For each extract, sequential dilutions from 40 to 0,625 mg/mL were prepared with distillated water. Thus, 100 µL of Mueller Hinton broth (from Liofilchem SRL Ref 610031) were introduced into all the wells of microplate. A volume of 100 µL of diluted extract at different concentrations was added to the content of the wells of columns 1 to 10. The bacterial inoculum (50 µL), previously prepared in Mueller Hinton broth and adjusted to 0.5 McFarland, was added to the contents of these wells. The wells of column 11 (without inoculum) have served negative control. Those of column 12 containing 100 µL of Muller-Hinton broth and 50 µL of bacterial suspension represented positive control. The microplates were closed and incubated for 48 h at 37°C.

After incubation, the bacterial growth was examined in each well, which is indicated by turbidity. The MIC of extract for a strain correspond to the lowest concentration showing no visible germ growth to the naked eye [21].

Table 1. Bacterial strains used for the tests

N°	Bacterial strains	References	Gram
1	Escherichia coli	ATCC 25922	Newsters
2	Salmonella typhi	SKN 1152	Negative
3	Bacillus cereus	LMG 13569	
4	Enterococcus faecalis	ATCC 19433	Positive
5	Listeria monocytogenes	NCTC 9863	FOSILIVE
6	Staphylococcus aureus	ATCC 25923	

2.2.6 Minimum bactericidal concentrations (MBC)

After 48 h incubation at 37° C, 50 µL were taken from the wells which presented no detectable bacterial growth and seeded on Mueller-Hinton agar. The control wells (negative and positive) were also seeded in the same conditions as the extracts. The minimum bactericidal concentration (MBC) was considered as the lowest extract concentration at which 99.99% of bacteria strains were inhibited after 24 h of incubation [21].

2.2.7 Bactericidal and bacteriostatic properties

The determination of the bactericidal and bacteriostatic properties of our extracts were performed using the ratio MBC/MIC used by Mihin et al. [10]. In fact, the extract was considered bacteriostatic when the ratio MBC/MIC > 4; on the opposite that is to say MBC/MIC \leq 4 the extract has a bactericidal property.

2.2.8 Data statistical analysis

The Minitab 18.1 software has been used for the statistical analysis. The collected data were summarized as mean \pm standard deviation. The statistical differences were determined using the Fischer test and the differences were considered significant if p < 0.05.

3. RESULTS

3.1 Antibacterial Sensitivity in vitro

The inhibition zones diameters (ID) of the extracts towards the tested strains and their interpretations are showed in the Table 2 below. These data revealed that all the tested bacterial strains were sensitive towards all the fruit extracts apart of *Enterococcus faecalis* with the fruits of *B. aegyptiaca* from Sahelian and those of *Z. mauritiana*. However, this sensitivity has varied sometimes according to the species fruits and the provenance zones. Moreover the fruits extract of *R. sudanica* have shown a very sensitive effect on the *Escherichia coli* (ID =

 15.33 ± 0.58 mm) and Salmonella typhi (ID = 18.00 ± 1.00 mm) strains (Table 2).

3.2 Minimum Inhibitory Concentrations (MIC)

The Table 3 summarized the minimum inhibitory concentrations.

The extracts MIC have varied from one species fruit to another (p < 0.05) except for S. typhi and L. monocytogenes (p > 0.05). The extracts of Raphia sudanica have been the most effective with the lowest minimum inhibitory concentrations (MIC) from 2.08±1.44 mg/mL on S. aureus to 5.83±1.44 mg/mL on L. monocytogenes and E. faecalis. For all extracts, the highest MIC has recovered on E. faecalis, from 5.83 à 20.00 mg/mL (Table 3).

3.3 Minimum Bactericidal Concentrations (MBC)

The minimum bactericidal concentrations are recorded in the Table 4.

This Table 4 indicates that the minimum bactericidal concentrations (MBC) have fluctuated from 21.67 ± 2.89 mg/mL with the extracts of *Raphia sudanica* to 40.00 mg/mL with the extracts of *Saba senegalensis* (Table 4).

3.4 Bactericidal and Bacteriostatic Properties

The ratio MBC/MIC has allowed determining the Bactericidal and bacteriostatic effects. The interpreted results are presented in the Table 5.

This Table 5 showed that the fruits extracts have bacteriostatic effects towards the tested strains, except those of *Z. mauritiana* which showed a bactericidal effect on the *S. aureus*, *B. cereus* and *L. monocytogenes* strains. The fruits extracts of *R. sudanica* have also presented a bactericidal effect on *L. monocytogenes*.

4. DISCUSSION

The objective of this work was to assess the *in vitro* antibacterial activities of four wild food fruits.

				Bacterial strains			
Samples		E. coli	S. typhi	S. aureus	B. cereus	L. monocytogenes	E. faecalis
Balanites S	udanese	9.67 ± 0.58 ^{cd} S	13.00 ± 1.00 ^b S	11.67 ± 0.58 ^{abc} S	11.67 ± 1.53 ^ª S	8.33 ± 0.58 [°] S	8.33 ± 0.58 ^b S
Balanites S	ahelian	8.67 ± 0.58 ^d S	11.00 ± 1.00 ^{cd} S	12.83 ± 1.26 ^a S	9.67 ± 0.58 ^{cd} S	9.50 ± 0.87 ^{cd} S	7.67 ± 0.58 ^{bc} R
Saba Sudai	nese	10.33 ± 0.58 ^c S	12.00 ± 1.00 ^{bc} S	10.33 ± 0.58 ^{cd} S	10.00 ± 1.00 ^{bcd} S	11.17 ± 0.76 ^{ab} S	8.33 ± 0.58 ^b S
Saba Sahel	lian	12.67 ± 0.58 [♭] S	10.67 ± 0.58 ^{cd} S	12.33 ± 0.58 ^{ab} S	9.33 ± 0.58 ^d S	10.17 ± 0.76 ^{bc} S	9.50 ± 0.87 ^a S
Ziziphus Sudanese		8.67 ± 0.58 ^d S	8.33 ± 0.58 ^e S	9.67 ± 0.58 ^d S	11.33 ± 0.58 ^{ab} S	10.17 ± 0.76 ^{bc} S	6.67 ± 0.58 [°] R
Ziziphus Sahelian		10.33 ± 1.53 ^c S	9.67 ± 0.58 ^{de} S	11.00 ± 1.00 ^{bcd} S	12.00 ± 1.00 ^a S	9.67 ± 0.58 ^c S	5.33 ± 0.58 ^d R
Raphia Sud	lanese	15.33 ± 0.58 ^a VS	18.00 ± 1.00 ^a VS	10.33 ± 0.58 ^{cd} S	11.00 ± 1.00 ^{abc} S	12.33 ± 0.58 ^ª S	8.33 ± 0.58 ^b S
Positive	Amikacine	25.33 ± 0.58 ^e ES	26.33 ± 1.53 ^f ES	26.00 ± 1.00 ^e ES	27.00 ± 1.00 ^e ES	24.33 ± 1.53 ^e ES	24.67 ± 0.58 ^d ES
controls	Doxycycline	$29.67 \pm 0.58^{\dagger} ES$	22.67 ± 0.58 ⁹ ES	29.67 ± 1.15 [†] ES	33.00 ± 1.00 [†] ES	$32.33 \pm 0.58^{t} ES$	21.33 ± 0.58 ^e ES
р		.48E-6 < .05	.04E-6 < .05	.002 < .05	.022 < .05	.0002 < 0.05	.32E-4 < .05

Table 2. Inhibition diameters of extracts (ID) in mm and their interpretations

*For each bacterial strain, the means which do not share any letters are considered as significantly different (p < .05). *R: Resistant; S: Sensitive; VS: Very Sensitive and ES: Extremely Sensitive.

Bacterial strains								
Samples	E. coli	S. typhi	S. aureus	B. cereus	L. monocytogenes	E. faecalis		
Balanites Sudanese	6.67 ± 1.44 ^a	6.67 ± 1.44^{a}	6.67 ± 1.44 ^b	6.67 ± 1.44 ^b	7.50 ± 0.00^{ab}	19.17 ± 1.44 ^a		
Balanites Sahelian	6.67 ± 1.44 ^a	5.83 ± 1.44^{ab}	5.83 ± 1.44 ^{bc}	5.83 ± 1.44 ^b	7.50 ± 0.00^{ab}	20.00 ± 0.00^{a}		
Saba Sudanese	3.33 ± 1.44 ^b	5.00 ± 0.00^{ab}	5.83 ± 1.44 ^{bc}	5.83 ± 1.44 ^b	6.67 ± 1.44^{ab}	9.17 ± 1.44 ^b		
Saba Sahelian	3.33 ± 1.44^{b}	5.00 ± 0.00^{ab}	4.17 ± 1.44 ^{cd}	5.83 ± 1.44 ^b	5.83 ± 1.44^{b}	9.17 ± 1.44 ^b		
<i>Ziziphus</i> Sudanese	6.67 ± 1.44 ^a	6.67 ± 1.44^{a}	9.17 ± 1.44 ^a	9.17 ± 1.44 ^a	7.50 ± 0.00^{ab}	19.17 ± 1.447		
Ziziphus Sahelian	6.67 ± 1.44 ^a	6.67 ± 1.44^{a}	9.17 ± 1.44 ^a	9.17 ± 1.44 ^a	8.33 ± 1.44^{a}	19.17 ± 1.44 ^a		
Raphia Sudanese	4.17 ± 1.44 ^{ab}	4.17 ± 1.44 ^b	2.08 ± 1.44^{d}	$2.50 \pm 0.00^{\circ}$	5.83 ± 1.44 ^b	5.83 ± 1.44 ^c		
p	.016 < .05	.123 > .05	.0002 < .05	.0004 < .05	.099 > .05	.23E-8 < .05		

*For each bacterial strain, the means which do not share any letters are considered as significantly different (p < .05).

			Bacterial strains			
Samples	E. coli	S. typhi	S. aureus	B. cereus	L. monocytogenes	E. faecalis
Balanites Sudanese	31.67 ± 2.89 ^a	31.67 ± 2.89^{a}	33.33 ± 2.89^{a}	33.33 ± 2.89 ^a	33.33 ± 2.89 ^{ab}	nd
Balanites Sahelian	31.67 ± 2.89 ^a	33.33 ± 2.89^{a}	31.67 ± 2.89 ^a	36.67 ± 2.89 ^a	36.67 ± 2.89^{a}	nd
Saba Sudanese	33.33 ± 2.89^{a}	33.33 ± 2.89 ^a	33.33 ± 2.89^{a}	33.33 ± 2.89 ^a	33.33 ± 2.89^{ab}	40.00 ± 0.00^{a}
Saba Sahelian	31.67 ± 2.89^{a}	33.33 ± 2.89^{a}	33.33 ± 2.89^{a}	33.33 ± 2.89^{a}	35.00 ± 0.00^{ab}	40.00 ± 0.00^{a}
Ziziphus Sudanese	31.67 ± 2.89 ^a	31.67 ± 2.89 ^b	nd			
<i>Ziziphus</i> Sahelian	31.67 ± 2.89^{a}	31.67 ± 2.89 ^a	28.33 ± 2.89^{a}	31.67 ± 2.89 ^a	31.67 ± 2.89 ^b	nd
Raphia Sudanese	23.33 ± 2.89 ^b	23.33 ± 2.89 ^b	21.67 ± 2.89 ^b	21.67 ± 2.89 ^b	21.67 ± 2.89 [°]	38.33 ± 2.89^{a}
p	.016 < .05	.009 < .05	.002 < .05	.001 < .05	.0002 < .05	.422 > .05

Table 4. Minimum bactericidal concentrations (MBC) in mg/mL of extracts

* For each bacterial strain, the means which do not share any letters are considered as significantly different (p < .05). nd: Not determined.

	Table 5.	Bactericidal	and	bacteriostatic	effects of	f the f	fruits extracts
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			Bacterial strains			
Samples	E. coli	S. typhi	S. aureus	B. cereus	L. monocytogenes	E. faecalis
Balanites Sudanese	Bacteriostatic	Bacteriostatic	Bacteriostatic	Bacteriostatic	Bacteriostatic	nd
Balanites Sahelian	Bacteriostatic	Bacteriostatic	Bacteriostatic	Bacteriostatic	Bacteriostatic	nd
Saba Sudanese	Bacteriostatic	Bacteriostatic	Bacteriostatic	Bacteriostatic	Bacteriostatic	Bacteriostatic
Saba Sahelian	Bacteriostatic	Bacteriostatic	Bacteriostatic	Bacteriostatic	Bacteriostatic	Bacteriostatic
Ziziphus Sudanese	Bacteriostatic	Bacteriostatic	Bactericidal	Bactericidal	Bacteriostatic	nd
Ziziphus Sahelian	Bacteriostatic	Bacteriostatic	Bactericidal	Bactericidal	Bactericidal	nd
Raphia Sudanese	Bacteriostatic	Bacteriostatic	Bacteriostatic	Bacteriostatic	Bactericidal	Bacteriostatic

*nd: Not determined.

4.1 Antibacterial Sensitivity in vitro

The assessment of inhibition zones diameters (ID) revealed that all the tested bacterial strains were sensitive towards all the fruit extracts apart of Enterococcus faecalis with the fruits of B. aegyptiaca from Sahelian and those of Z. mauritiana. However, this sensitivity has varied sometimes according to the species fruits and the provenance zones. The fruit extracts of Raphia sudanica have shown the largest inhibitory effects on the growth of Escherichia coli (ID = 15.33 ± 0.58 mm) and Salmonella typhi $(ID = 18.00 \pm 1.00 \text{ mm})$ strains (Table 2). These data are consisted with those from our recently survey (data not published) which had already mentioned the use of R. sudanica fruits in the traditional management of typhoid fever, mainly caused by the Salmonella bacteria genus. These results agree with those of Yahia et al. (2020) [22] who registered the ID from 10.4 ± 0.45 to 14.2 ± 0.27 mm on the same strains with leaf hydromethanolic extracts of Z. mauritiana. But, our obtained ID are higher compared to those of Keita et al. [23] with the leaf tannic extracts of Z. mauritiana (ID = 6-7 mm) on clinical strains of E. coli, S. aureus and S. typhi. This difference would be related to the synergy of action between the chemical components of our extracts [24]. The antibacterial activity of ethanolic and methanolic fruit extracts of Balanites have been reported by Murthy et al. [25].

Different studies had also indicated the use of R. sudanica in the traditional management of numerous pathologies: Gastro-enteritis, gonococci, dracunculiasis, bilharzia, diabetes, hypertension, etc. [2,11]. Likewise, it is known that the organs of B. aegyptiaca, S. senegalensis and Z. mauritiana are also involved in the treatment of several pathologies such as dysentery, cough, conjunctivitis, paralysis of hernia, children, diabetes, hypertension [2,26,13,14].

4.2 Minimum Inhibitory (MIC) and Bactericidal Concentrations (MBC)

The extracts of *Raphia sudanica* have been the most effective (p < 0.05) with the lowest minimum inhibitory concentrations (MIC) from 2.08 \pm 1.44 mg/mL on *S. aureus* to 5.83 \pm 1.44 mg/mL on *L. monocytogenes* and *E. faecalis*. For all extracts, the highest MICs have recovered on *E. faecalis*, from 5.83 à 20.00 mg/mL (Table 3). As for the minimum bactericidal concentrations

(MBC), they have varied from 21.67 ± 2.89 mg/mL with the extracts of Raphia sudanica to 40.00 mg/mL with the extracts of Saba senegalensis (Table 4). These values are more than those obtained with Desmodium ramosissimum (another food species) extracts (MIC = 1.25 mg/mL) on the reference strains of S. aureus and E. coli [27]. These fairly high MIC and MBC values obtained in our study could be due to the non-miscibility of the extracts in agar medium or to their low impermeability through the bacterial walls. In other side, they could be related to the involvement of a resistance mechanism towards our extracts similar to that involved in the resistance to conventional antibiotics. Since it is known that the bacteria have an outer phospholipid membrane that makes their cell wall hardly penetrable by antimicrobial agents [28]. In a recent work, we have demonstrated that these four wild fruits are rich sources of protein, minerals, and vitamins [15]. So, these molecules could reduce the effect of extracts against bacteria [29].

4.3 Bactericidal and Bacteriostatic Properties

The MBC/MIC report revealed that our fruits extracts have mostly presented bacteriostatic effects towards the tested strains (Table 5).

For each plant species and within the same species, the chemical components nature would be responsible of the observed effects and their differences as it is known that the inhibitory activities are dependent to the contents of active biological substances [30]. The inhibitory effects (bacteriostatic or bactericidal) of our four species fruit extracts recovered on the strains would be linked to their richness in secondary metabolites (terpenoids, polyphenols, flavonoids,, saponines, etc.) as reported by Konaré et al. [31]. Indeed. these components are endowed with antibacterial and anti-inflammatory activities [32,8]. Some of them counteract the bacteria by disrupting its membrane (tannins, terpenoids, simples phenols), others by forming complexes with its wall or else by inactivating its enzymes (flavonoids, phenolic acids) [33,28]. Another study has also shown that these antibacterial effects are generally the result of a coordinated action between the different constituents of extracts [24]. Based on these therapeutic virtues in addition to their nutritional ones, these food plants could be useful to fight the malnutrition especially in children who are the first victims [12], since the malnutrition is most often due to the microbial poisoning.

Regarding to these findings, the fruits of these species deserve to be valorized in order to better contribute in the fight against certain infectious diseases especially those of foodborne (cholera, salmonellosis, enterohemorrhagic *E. coli* infections, etc.).

5. CONCLUSION

This work has shown that the fruit extracts of *B.* aegyptiaca, *S.* senegalensis, *Z.* mauritiana and *R.* sudanica possess the inhibitory activities towards the tested bacterial strains. The extracts of *R.* sudanica have presented the highest inhibitory activities on *Escherichia coli* and *Salmonella typhi* strains. Considering these properties, these wild edible fruits could be recommended to contribute in the fight against the foodborne poisoning from these strains. Lastly, for a better valorization of these wild fruits, *in vivo* tests would be undertaken to confirm these activities presented *in vitro*.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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