Positive effects of Moringa oleifera and Moringa stenopetala seed and leaf extracts against selected bacteria

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Abstract

Moringa oleifera is hailed as the "miracle tree" for its impressive catalog of nutritional, medicinal, and water purification benefits. A (sub)tropical plant with a rapid growth rate (3–5 m in a single season), Moringa has proven beneficial in multiple ways in developing regions around the world. In addition to its high nutrient content and water clarifying properties, Moringa seed and leaf extracts have shown potential as natural antibacterial agents. Based on this, we anticipated that extracts from multiple species of *Moringa* would exhibit potentially useful antibacterial properties against a range of bacterial species. To explore this, both disk diffusion and minimum inhibitory concentration (MIC) culture techniques were employed to assess the inhibitory effects of seed and leaf extracts from M. oleifera and *M. stenopetala* against species of bacteria commonly used in research and teaching laboratories. Aqueous seed extracts from both Moringa species showed broad-spectrum activity but were especially effective at inhibiting the growth of Gram-positive bacteria, including species of Staphylococcus, Streptococcus, and Bacillus. Moringa leaf extracts also exhibited antibacterial activity, with ethanolic leaf extracts showing greater efficacy than aqueous leaf extracts in disk-diffusion assays. Temporary acidification (1 h at pH 2) of Moringa seed and leaf extracts had a detrimental effect on their antibacterial activity. MIC assays using Moringa leaf extracts also showed more pronounced inhibition of Gram-positive bacteria (MIC = 12.5% v/v) versus Gram-negative species (MIC = 25% v/v). These results are of particular relevance in tropical areas where pharmaceutical drugs are scarce but Moringa is widely available and often used as a nutritional supplement. Moreover, the rising threat of multi-drug resistant pathogens lends greater importance to the study of antibacterial plant products that ultimately may find application in the clinical setting.

Introduction

Recent explorations in herbal medicine among the scientific community have shown potential for the development of new antimicrobial products from various plant species for use in both developing regions having little access to pharmaceutical drugs and in developed areas that have experienced a rise in bacterial infections caused by antibiotic-resistant strains (2, 3, 4, 7, 17, 28, 30). One such medicinal plant that has experienced a degree of commercialization in recent years is Moringa oleifera (also called the "horseradish tree," "drumstick tree," and, more recently, the "miracle tree"), a drought-resistant and rapidly growing tree species native to India but which is now widespread in tropical and subtropical regions around the world. Growing to a maximum height of about 12 m but usually pruned yearly to ease harvesting, the use of *M. oleifera* leaves as a nutritional supplement and herbal medicine has been well documented (12, 15, 23, 24, 25, 27). M. oleifera leaf powder contains unusually high levels of protein, iron, calcium, magnesium, potassium, several vitamins (e.g., β -carotene and vitamins B_2 , B_6 , and C), and dietary fiber and other complex carbohydrates (15). Because of this, *M. oleifera* products have been incorporated into feeding programs where malnutrition is prevalent. M. stenopetala also has an unusually high nutrient content, but as this species occurs only in northeastern tropical Africa, the use of *M. stenopetala* as a nutritional supplement is limited mainly to regions in which the plant is indigenous (19).

In addition to their nutritional benefits, the potential of *Moringa spp*. as new sources of

antimicrobial products is becoming more widely recognized (5, 8, 11, 20, 22, 27, 29). Extracts of seeds and leaves of Moringa spp. exhibit antibacterial properties through direct inhibition of growth in culture-based experiments. As has been shown using leaf extracts in experiments with Xanthomonas campestris (13) and Erwinia amylovora (14), this inhibition is primarily due to the production of compounds that compromise the structural integrity of the cytoplasmic membrane. Several decades ago, Eilert et al. (9) described both antibacterial and antifungal activity of Moringa extracts and identified 4-(a-L-rhamnosyloxy) benzyl isothiocyanate as an active compound in aqueous preparations. However, other compounds, including phenolic acids, flavonoids, and alkaloids, likely also contribute to the antibacterial activity of these extracts (13). Manilal et al. (20) recently showed antibacterial activity of Moringa stenopetala extracts on par with clindamycin and vancomycin controls against clinical isolates of methicillin-resistant Staphylococcus aureus (MRSA), and several other studies have shown inhibitory activity of Moringa extracts against a variety of human pathogens, including Proteus mirabilis, Helicobacter pylori, and Bacillus cereus (5, 11, 20, 27). Although documentation of the antibacterial activity of Moringa seed and leaf extracts is expanding, the clinical potential of these natural products is unknown, and further testing of these materials is both prudent and needed.

Based upon studies from other research groups and our own observations (21), we predicted that crude seed and leaf extracts from multiple species of *Moringa* would exhibit broad-spectrum activity against a variety of bacterial species. To test this prediction, we compared the growth-inhibitory activity of aqueous seed extracts and both aqueous and ethanolic leaf extracts from two Moringa species, M. oleifera and M. stenopetala, against selected species of Gram-positive and Gram-negative bacteria, some of which are known to cause opportunistic infections. In addition, the minimum inhibitory concentration (MIC) of aqueous Moringa leaf extracts was determined for several species of Gram-positive and Gram-negative bacteria. Finally, as Moringa leaves and seeds are commonly consumed as dietary supplements and could potentially have an inhibitory effect on susceptible members of the intestinal microbiota, we have simulated passage of these plant products through the stomach by testing the effect of a temporary shift to acid pH on the antibacterial activity of both aqueous and ethanolic Moringa extracts. Abolishment of the antibacterial efficacy of Moringa extracts following exposure to low pH would suggest that these plant products can be consumed for nutritional purposes without concern that the composition of the gut microbiota would be detrimentally or otherwise affected.

Materials and Methods

Bacterial Cultures

Species of bacteria used in this study consisted of strains commonly employed in general microbiology teaching laboratories. Gram-positive species included *Bacillus cereus* (ATCC 14579), *Staphylococcus aureus* (ATCC 29213), *Enterococcus durans* (ATCC 6056), *Corynebacterium xerosis* (ATCC 373), *Listeria monocytogenes* (ATCC 15313), *Streptococcus* agalactiae (ATCC 13813), and Micrococcus luteus (ATCC 4698). Gram-negative test bacteria included Proteus vulgaris (ATCC 6380), Yersinia kristensenii (ATCC 33639), Serratia liquefaciens (ATCC 27592), and Escherichia coli (ATCC 25922). Source cultures of all bacteria used to inoculate experimental media were routinely grown in BD Bacto[™] tryptic soy broth (Becton, Dickinson and Company) at pH 7 and 37 °C.

Moringa Seed and Leaf Extract Preparation

Seeds of *Moringa oleifera* and *Moringa stenopetala* used in this study were obtained from Educational Concerns for Hunger Organization (ECHO; www.echonet.org, Fort Myers, Florida, USA). Leaves from each *Moringa* species were obtained from plants propagated from seeds in our laboratory. To obtain the seed extracts, the dried *Moringa* seed pods were husked by hand, revealing the small, white pit in each pod. The seeds were then ground into a fine powder using a mortar and pestle.

For preparation of *Moringa* seed extracts, 3 g of the powdered seed were combined with 15 ml of warm (60 °C) ddH2O and mixed by hand for 5 minutes using a glass rod. The slurry was then transferred to tubes and placed in a centrifuge at 3000 rpm for 10 minutes at room temperature. After centrifugation, the supernatant was extracted using a micropipette and vacuum filtered to remove any remaining suspended particles. The vacuum-filtered extract was then sterilized by pushing through a 0.45 μ m cellulose acetate (CA) membrane filter into a sterile petri dish. *Moringa* leaves used for extract preparation were washed and dried thoroughly prior to processing. Leaf extracts from *Moringa spp*. were obtained by macerating 12 g of leaf tissue in 20 ml ddH₂O. This slurry was then filtered through five layers of cheesecloth into two 15 ml conical tubes, which were placed in a centrifuge at 3000 rpm for 10 min. For each *Moringa* species, the leaf extract supernatant was collected, and 10 ml were filtered through a 0.45 μ m CA membrane filter into a sterile 17 ml test tube. Preparation of the ethanolic leaf extracts was the same as water extraction except that the ddH₂O was replaced with 20 ml of 70% ethanol.

Disk Diffusion Assays

Using ethanol-flamed forceps, pre-sterilized blank disks (6 mm in diameter; Becton, Dickinson and Company) were aseptically placed in Petri dishes that contained a single, sterile plant extract (either *M. oleifera* or *M. stenopetala* seed or leaf extract) in order to test for antibacterial activity using a modification of the disk-diffusion method described by Bauer et al. (6). Other Petri dishes used for soaking blank disks contained either filter-sterilized ddH₂O or 70% ethanol to be used as negative controls. All disks were soaked for 15 min before experimental use.

To perform the disk diffusion plate assays, each species of bacteria was inoculated onto the entire surface of triplicate plates of Difco[™] Mueller-Hinton agar. Plates were inoculated from fresh tryptic soy broth cultures (OD₆₀₀ of 0.05) of each bacterial species using pre-sterilized cotton swabs that had been inserted into the cultures and spread in multiple directions on the agar surface to produce a "lawn" of

bacterial growth after incubation. Disks containing either crude Moringa extract or sterile solvent (either ddH₂O or 70% ethanol, depending on the experiment) were placed on the inoculated plates in labeled quadrants using ethanol-flamed forceps. Four disks were placed on each agar plate: 1) a disk impregnated with M. oleifera seed or leaf extract; 2) a disk impregnated with M. stenopetala seed or leaf extract; 3) a disk infused with 30 μ g of the broad-spectrum antibiotic tetracycline (Becton, Dickinson and Company) as a positive control; and 4) a disk containing only sterile solvent (i.e., no Moringa extract) as a negative control. Once in place, the disks were pressed gently into the agar surface with the forceps to ensure they would not move or become detached when inverted for incubation. All test plates were incubated at 37 °C for 24 h.

Following incubation, antibacterial activity was assayed by measuring the diameter (to the nearest mm) of zones of inhibition around the disks on each plate culture. Measurements of zones of inhibition shown in each graph are averages of triplicate cultures for each bacterial species tested and are expressed as the mean \pm standard error. Statistical significance (P ≤ 0.05) determinations of growth inhibition among tested groups and controls were performed using one-way analysis of variance (ANOVA) followed by Tukey's honest significant difference (HSD) test.

To simulate and test the effect of oral ingestion of *Moringa* leaf and seed products on antibacterial activity, we subjected both aqueous and ethanolic *Moringa* extracts to temporary acidification. For this procedure, the pH of each extract was dropped to pH 2 using a sterile solution of 1 M HCl. After 1 h at room temperature (~23 °C), the pH of each extract was raised to neutral (pH 7) using sterile 1 M NaOH. Sterile blank disks were then soaked in the extracts, and disk diffusion experiments were conducted as described above.

Minimum Inhibitory Concentration (MIC) Assay

MIC assays were performed in triplicate using Moringa oleifera aqueous leaf extract. Ten test tubes were used for each MIC experiment, with tube 1 initially containing 10 ml of 100% crude Moringa leaf extract (obtained as described above) and tubes 2-10 containing 5 ml sterile tryptic soy broth (TSB). A serial dilution was then performed in which 5 ml of leaf extract from tube 1 was aseptically transferred to tube 2, producing a 1:2 dilution. After mixing well, 5 ml from tube 2 was transferred to tube 3 and mixed. Dilutions continued in this way through tube 9. After mixing, 5 ml was removed from tube 9 and discarded. This resulted in all 10 tubes having a final volume of 5 ml, with tube 1 containing 100% Moringa extract, tubes 2-9 containing two-fold decreasing concentrations of extract, and tube 10 serving as a control containing 100% TSB (i.e., no extract). All ten tubes were then inoculated with a test species of bacteria using a loop (a different 10-tube set was required for each species of bacteria tested for all three trials).

After a 24-h incubation (unshaken) at 37 °C, growth in each of the tubes was assessed by visual inspection for turbidity, and the MIC for each bacterial species was recorded as the tube containing the highest dilution of extract that showed no growth. The recorded MIC was confirmed by transferring a portion of the contents of each tube onto separate plates of tryptic soy agar (TSA) by streaking for isolation and checking for growth after incubation (37 °C for 24 h).

Results and Discussion

Antibacterial Activity of Aqueous *Moringa* Seed Extracts

In recent years, several studies have demonstrated the antibacterial activity of extracts from various species of Moringa, especially Moringa oleifera (5, 8, 11, 13, 14, 16, 18, 20, 21, 24, 26, 29). In agreement with these reports, most of which employed extracts from a single Moringa species against one or a few species of bacteria, we observed antibacterial activity of seed extracts made from two different Moringa species against a diversity of both Gram-positive and Gram-negative bacteria, some species of which, to our knowledge, have not been included in previous studies (e.g., Enterococcus durans, Yersinia kristensenii, Serratia liquefaciens, Streptococcus agalactiae, and Corynebacterium xerosis).

Disk diffusion assays showed that aqueous seed extracts of both *M. oleifera* and *M. stenopetala* inhibited the growth of a wide variety of bacteria, with Gram-positive bacteria being especially susceptible. Zones of inhibition from *Moringa* seed extracts against *Staphylococcus aureus* were equal in size (~22 mm) to zones of inhibition produced by a tetracycline control disk (a zone of inhibition of >22 mm for *Staphylococcus* spp. indicates susceptibility to tetracycline based on data from the European Committee on Antimicrobial Susceptibility Testing [10]). Growth inhibition by Moringa extracts against other Gram-positive cocci, including Streptococcus agalactiae and Micrococcus luteus, produced zones of inhibition that were of similar size (20-25 mm) to those observed with S. aureus (Figure 1). For Bacillus cereus, an endospore-forming Gram-positive rod, the sizes of the observed zones of inhibition by Moringa seed extracts indicated significant growth inhibition ($P = 1.6 \times 10-6$) and actually exceeded that from tetracycline at about 20 mm versus 13 mm, respectively (Figure 1). Another opportunistic Gram-positive rod, Listeria monocytogenes, was also inhibited by seed extracts from both Moringa species. However, with zones of inhibition having an average diameter of 16 mm

versus 30 mm, the antibacterial effect of the *Moringa* extracts was less than that observed with the tetracycline control (Figure 1).

Although an inhibitory effect was still evident against them, Gram-negative bacteria as a whole showed less sensitivity to aqueous *Moringa* seed extracts than Gram-positive bacteria. The four Gram-negative bacteria tested were all of the family *Enterobacteriaceae*. Of these species, *Proteus vulgaris*, an opportunistic pathogen often responsible for urinary tract infections, had the largest zones of inhibition (~15 mm) and was significantly affected ($P = 4.6 \times 10-6$) by the *Moringa* seed extracts (Figure 1). By contrast, *Escherichia coli*,

Figure 1

Antibacterial activity of aqueous seed extracts of Moringa oleifera and Moringa stenopetala against selected mostly Gram-positive bacteria, as compared to tetracycline and sterile water controls.



Note. The average sizes (from triplicate samples) of zones of inhibition on disk diffusion plate assays are shown. A measurement of 6 mm is a baseline representing the diameter of the disks themselves and equates to no visible zone of inhibition.

Serratia liquefaciens, and Yersinia kristensenii exhibited no significant susceptibility (P > 0.05) to the Moringa extracts over the sterile water control (data not shown). As was observed with the Gram-positive bacteria, there was no significant difference (P > 0.05) between the antibacterial activity of Moringa oleifera seed extract versus that of Moringa stenopetala.

Antibacterial Activity of Moringa Leaf Extracts

In addition to testing the antibacterial activity of *Moringa* seed extracts, we also collected and tested the potential for bacterial growth inhibition using both aqueous and ethanolic leaf extracts. Although a significant ($P \le 0.05$) effect was observed over sterile water controls (Figure 2A), inhibition of bacterial growth by aqueous leaf extracts was less pronounced than that observed from aqueous seed extracts. This may be due to the antibacterial constituents being more concentrated in the seed extracts than in the leaf extracts, although other explanations, such as differences in the effectiveness of the extraction methods, are also possible.

For the Gram-positive bacteria tested, zones of inhibition around tetracycline control disks were approximately twice as large in diameter as zones of inhibition using *Moringa* leaf extracts, which ranged from 12–16 mm in diameter (mean = 13 mm) for both *Moringa* species (Figure 2A). As was the case using seed extracts, the inhibitory effect of the leaf extracts was generally less pronounced against the Gram-negative bacteria tested, which had zones of inhibition ranging from 7–11 mm in diameter (data not shown).

To potentially augment the antibacterial effect

of the Moringa leaves, we repeated the test using ethanolic rather than water-based leaf extracts. Against the same bacteria, ethanolic Moringa leaf extracts produced larger zones of inhibition and, thus, a greater antibacterial effect than observed with water extraction. Other studies have shown a similar increase in antibacterial activity of ethanolic over aqueous M. oleifera extracts, and Hagos et al. observed greater antibacterial activity from methanol extraction over water extraction of M. stenopetala leaves (16, 26, 31). In the present study, sizes of the zones of inhibition using ethanolic leaf extracts from both Moringa species were similar and had average diameters ranging from 12–21 mm (mean = 17 mm) (Figure 2B), resulting in an approximately 30% increase in efficacy over aqueous leaf extracts.

Effect of Acidification on the Antibacterial Activity of *Moringa* Seed and Leaf Extracts

Moringa seed pods and leaves can be an important nutritional supplement for peoples living in tropical and subtropical regions around the world (15, 23). Although considerable work has been done to characterize the nutritive properties of *Moringa* products when prepared in various ways for human consumption (15), to our knowledge, the potential effect of ingestion—and in particular the temporary acidification that occurs during passage through the stomach—on the antibacterial properties of *Moringa* has not been investigated.

In testing the effect of low pH on the antibacterial properties of *Moringa* extracts, a significant decrease ($P \le 0.05$) in antibacterial activity occurred against most species and under most conditions as a result of temporary acidification.

Figure 2

Antibacterial activity of A) aqueous Moringa leaf extracts, and B) ethanolic Moringa leaf extracts against selected Gram-positive bacteria, as compared to tetracycline and sterile solvent (ddH_2O for part A; 70% ethanol for part B) controls.



Note. The average sizes (from triplicate samples) of zones of inhibition on disk diffusion plate assays are shown. A measurement of 6 mm is a baseline representing the diameter of the disks themselves and equates to no visible zone of inhibition.

As aqueous seed extracts and ethanolic leaf extracts both showed strong antibacterial activity in previous experiments, we limited testing to these preparations from both *M. oleifera* and *M. stenopetala* against a selection of Gram-positive and Gram-negative bacteria.

After 1 hour at pH 2 (23 °C), activity against the tested Gram-positive bacteria was significantly $(P \le 0.05)$ diminished, and in several cases entirely abolished, for aqueous seed extracts of both M. oleifera and M. stenopetala versus the untreated (nonacidified) control extract (Figure 3A, B). The same trend was observed for the seed extracts against the tested Gram-negative bacteria; in every case, antibacterial activity was severely diminished or entirely absent after the acidification treatment (Figure 3C, D). Similarly, with the exception of *Staphylococcus aureus* among the Gram-positive bacteria (Figure 3E) and Escherichia coli among the Gram-negative bacteria (Figure 3G), acidification of Moringa ethanolic leaf extracts consistently produced smaller zones of inhibition around the test disks compared to the untreated controls (Figure 3E-H). Against the Gram-negative bacteria in particular, zones of inhibition were entirely absent around most of the disks containing acid-treated extracts (Figure 3G, H).

These results suggest that antibacterial constituents occurring in seeds and leaves of *Moringa spp.* are acid-labile and are likely broken down by digestive chemicals of the stomach when ingested (1, 9, 12). This brings into question the curative potential of ingested natural *Moringa* seed and leaf products for the treatment of gastrointestinal bacterial infections, such as gastric ulcers caused by *Helicobacter pylori* [even if antibacterial activity has been demonstrated in vitro (11)], and is a topic warranting further study.

MIC Assays Using Moringa Leaf Extracts

The results of minimum inhibitory concentration (MIC) assays confirmed the antibacterial effect of Moringa leaf extracts. Crude aqueous leaf extracts from M. oleifera inhibited the growth of all bacteria tested to varying degrees. As with the disk diffusion test, the MIC assays showed that Gram-positive bacteria were generally more susceptible to the inhibitory effect of M. oleifera leaf extracts, with most species showing susceptibility at >50% dilution of crude extract (Table 1). A representative MIC preparation is shown in Figure 4. Only two of the four Gram-negative bacteria tested (P. vulgaris and Y. kristensenii) were inhibited beyond a 50% dilution of crude leaf extract, and no Gram-negative species were inhibited beyond a 25% dilution (Table 1).

Conclusions

The results of this study and others cited in this report show that seed and leaf extracts of multiple species of the fast-growing, tropical to subtropical tree *Moringa* exhibit clear antibacterial properties. Both disk diffusion and MIC assays indicated that among the species tested, Gram-positive bacteria were more susceptible to *Moringa*-derived antibacterial compounds than were Gram-negative bacteria, possibly due to the presence of an outer membrane in the cell wall of the latter. Moreover, this work presents evidence that the antibacterial components of crude *Moringa* extracts are acid-labile and

Figure 3

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Effect of acidification on the antibacterial activity of Moringa seed and leaf extracts.



Note. The average sizes (from triplicate samples) of zones of inhibition on disk diffusion plate assays are shown. A measurement of 6 mm is a baseline representing the diameter of the disks themselves and equates to no visible zone of inhibition. A) *Moringa oleifera* aqueous seed extract against selected

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Gram-positive bacteria; B) *Moringa stenopetala* aqueous seed extract against Gram-positive bacteria; C) *M. oleifera* aqueous seed extract against selected Gram-negative bacteria; D) *M. stenopetala* aqueous seed extract against Gram-negative bacteria; E) *M. oleifera* ethanolic leaf extract against Gram-positive bacteria; F) *M. stenopetala* ethanolic leaf extract against Gram-positive bacteria; G) *M. oleifera* ethanolic leaf extract against Gram-negative bacteria; H) *M. stenopetala* ethanolic leaf extract against Gram-negative bacteria; H) *M. stenopetala* ethanolic leaf extract against Gram-negative bacteria. Controls are sterile ddH_2O (A–D) or sterile 70% ethanol (E–H).

Table 1

Antibacterial minimum inhibitory concentration (MIC) assay results for Moringa oleifera aqueous crude leaf extract.

Bacterial Species	Gram	Two-Fold Tube Dilution Series*				
	Reaction	1	2	3	4	5
Bacillus cereus	Positive	-	-	-	-	+
Corynebacterium xerosis	Positive	-	-	-	+	+
Enterococcus durans	Positive	-	-	+	+	+
Listeria monocytogenes	Positive	-	-	-	+	+
Micrococcus luteus	Positive	-	-	+	+	+
Staphylococcus aureus	Positive	-	-	-	+	+
$Streptococcus\ agalactiae$	Positive	-	-	+	+	+
Escherichia coli	Negative	-	-	+	+	+
Proteus vulgaris	Negative	-	-	-	+	+
Serratia liquefaciens	Negative	-	-	+	+	+
Yersinia kristensenii	Negative	-	-	-	+	+

Note. *Although the dilution series for all species tested contained 9 tubes, the results for only five tubes are shown because growth occurred for all species in tubes 6-9; growth also occurred in every case in tube 10, a positive control containing no *Moringa* extract. The concentration (v/v) of total extract in each tube was as follows: tube 1, 100%; tube 2, 50%; tube 3, 25%; tube 4, 12.5%; tube 5, 6.25%. (+) growth present; (-) growth absent.

Figure 4

Representative minimum inhibitory concentration (MIC) assay using crude aqueous leaf extract from M. oleifera.



Note. A two-fold MIC serial dilution protocol was employed using 9 tubes, as well as a positive control containing no *Moringa* extract; the numbers indicate the % dilution of the extract.

subject to inactivation by conditions of low pH. Thus, although still highly nutritive, it is likely that the antibacterial properties of *Moringa* plant products are diminished upon ingestion.

Antibacterial compounds isolated from *Moringa* seeds and/or leaves may prove beneficial and find application in the clinical setting, either in native form or by contributing to the development of highly effective semi-synthetic pharmaceuticals. The many nutritional benefits of *Moringa* products have been well documented for some time and have gained considerable commercial promotion in recent years. With the rise in antibiotic resistance reaching alarming levels in the contemporary healthcare sector, the search for new antimicrobial drug options is of utmost importance. There could hardly be a more pertinent time than now to invest in the study of promising alternatives to traditional antibiotics, including those offered by species of *Moringa* and other medicinal plants.

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