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Is AMF inoculation an alternative to maximize the *in vitro* antibacterial activity of *Libidibia ferrea* extracts?

A inoculação com FMA é alternativa para potencializar a atividade antibacteriana *in vitro* de extratos de *Libidibia ferrea*?

¿Es la inoculación con HMA una alternativa para potenciar la actividad antibacteriana *in vitro* de los extractos de *Libidibia ferrea*?

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Abstract
Arbuscular mycorrhizal fungi (AMF) are known to provide plant species with several benefits, such as an increased production of bioactive compounds. However, it is yet to be defined whether extracts of mycorrhizal plants are more efficient *in vitro* antibacterial actions when compared to non-mycorrhizal plants. We tested the hypothesis of whether or not, methanolic extracts of *Libidibia ferrea* fruits, from plants established in the field and inoculated with AMF, have higher antibacterial action when inoculated with *Acaulospora longula*, *Claroideoglomus etunicatum* or *Gigaspora albida*. In addition, native *L. ferrea* fruits collected from the Caatinga area were also tested. The extracts of *L. ferrea* fruits inoculated with *A. longula* had higher *in vitro* antibacterial action in relation to the extracts of fruits from non-inoculated plants (p <0.05) thus characterizing the first record of different antibacterial actions of plant extracts due to inoculation with AMF. The extracts of *L. ferrea* fruits inoculated with *A. longula* were more efficient in inhibiting growth of Gram-negative bacteria. The zone diameters of inhibition ranged from 2.48 % to 7.56 % larger than the zones of the non-inoculated *L. ferrea* fruit extracts. The inoculation of *L. ferrea* with AMF may represent an alternative way of producing fruits with different antibacterial activity.
Keywords: Arbuscular mycorrhizal fungi; Bacterial growth; Biocompounds; Caatinga; Fruits; Mycorrhization.

Resumo
Sabe-se que o uso de fungos micorrízicos arbusculares (FMA) confere benefícios para as espécies vegetais, como aumento na produção de compostos bioativos. Entretanto, não se definiu se extratos de plantas micorrizadas têm atividade antibacteriana in vitro superior àqueles obtidos de vegetais não micorrizados. Foi testada a hipótese de que extratos de frutos de *Libidibia ferrea* inoculada com FMA possuem maior atividade antibacteriana. Para isso, foram utilizados extratos metanólicos de frutos de *L. ferrea*, estabelecida em campo e inoculada ou não com *Acaulospora longula, Claroideoglomus etunicatum, Gigaspora albida*, além daqueles obtidos de frutos de *L. ferrea* coletados em área da caatinga. Os extratos dos frutos de *L. ferrea* inoculada com *A. longula* foram mais eficazes frente a cepas de bactérias Gram-negativas, com halos de inibição que foram de 7.56 % a 2.48 % maiores que halos dos extratos de frutos de *L. ferrea* não inoculada. Para o ensaio de atividade antibacteriana *in vitro* foram utilizadas as cepas *Escherichia coli*,

Escherichia coli, Staphylococcus aureus e Staphylococcus aureus. Os extratos dos frutos de *L. ferrea* inoculada com *A. longula* tiveram maior ação antibacteriana in vitro, frente às cepas testadas, em comparação com os extratos de frutos de plantas não inoculadas ($p < 0.05$), caracterizando o primeiro relato da ação antibacteriana diferenciada de extratos vegetais em função da inoculação com FMA. A inoculação de *L. ferrea* com FMA pode ser alternativa para produção de frutos com ação antibacteriana diferenciada.
Palavras-chave: Biocompostos; Caatinga, Crescimento bacteriano; Frutos; Fungos micorrízicos arbusculares; Micorrização.

Resumen
Se sabe que el uso de hongos micorrízicos arbusculares (HMA) proporciona beneficios para las especies vegetales, como una mayor producción de compuestos bioactivos. Sin embargo, no se ha definido si los extractos de plantas micorrízicas tienen una actividad antibacteriana in vitro superior a los obtenidos a partir de vegetales no micorrízicos. Se probó la hipótesis de que los extractos de los frutos de *Libidibia ferrea* inoculados con HMA tienen una mayor actividad antibacteriana. Para ello se utilizaron extractos metanólicos de frutos de *L. ferrea*, establecidos en campo e inoculados o no con *Acaulospora longula, Claroideoglomus etunicatum, Gigaspora albida*, además de los obtenidos de frutos de *L. ferrea* recolectados en la zona de caatinga. Los extractos de frutos de inoculados con *A. longula* fueron más efectivos contra cepas de bacterias Gram-negativas, con halos de inhibición que fueron 7.56 % a 2.48 % mayores que los halos de extractos de frutos *L. ferrea L. ferrea* no inoculado. Se utilizaron cepas de *Escherichia coli, Escherichia coli, Staphylococcus aureus y Staphylococcus aureus* para el ensayo de actividad antibacteriana in vitro. Los extractos de frutos de *L. ferrea* inoculados con *A. longula* tuvieron mayor acción antibacteriana in vitro, en comparación con las cepas ensayadas, en comparación con los extractos de frutos de plantas no inoculadas ($p < 0.05$), caracterizando el primer reporte de la acción. antibacteriano diferenciado de extractos de plantas dependiendo de la inoculación con HMA. La inoculación de *L. ferrea* con HMA puede ser una alternativa para la producción de frutos con acción antibacteriana diferenciada.
Palabras clave: Biocompuestos; Caatinga; Crecimiento bacteriano; Frutos; Hongos micorrízicos arbusculares; Micorrizas.

1. Introduction

As documented in many studies, arbuscular mycorrhizal fungi (AMF) are microorganisms that provide plant species with several benefits (Wu *et al.*, 2016; Amiri, Nikbakht, Rahimmalek, & Hosseini, 2017; Tavarini *et al.*, 2018). However, only a few papers have recorded whether 'products' with higher mycorrhizal efficiency in plants have a different action, such as mycorrhizal plant extracts.

Some studies reported that the use of AMF inoculation in plants of medicinal interest enhances the production of bioactive compounds (Oliveira, Alves, Silva, & Silva, 2015; Amiri *et al.*, 2017). This may provide the species with medicinal properties, such as antioxidant (Giovannetti *et al.*, 2012), antitumor (Nakamura *et al.*, 2002), and antimicrobial activities (Silva *et al.*, 2013b).

Some of the tested species are natives to Brazil, such as *Anadenanthera colubrina* (Vell.) Brenan, *Inga vera* Willd., *Mimosa tenuiflora* (Wild.) Poir., and *Libidibia ferrea* (Mart. ex Tul.) L.P.Queiroz. These studies revealed extracts of mycorrhizal plants with a higher concentration of phenols, tannins, flavonoids and other biomolecules (Pedone-Bonfim *et al.*, 2013; Silva *et al.*, 2014a, 2014b; Lima, Campos, & Silva, 2015; Santos, Silva, & Silva, 2017; Silva and Silva, 2017).

Even though the plant organs accumulate more compounds whenever the plants are mycorrhizal, only one study has reported whether mycorrhization with *Funneliformis mossae* (T.H. Nilcolson & Gerd.) C. Walker & Schuessler, in addition to increasing the concentration of essential oils extracted from mycorrhizal *Anethum graveolens* L., var. Hanák, maximizes the fungicidal effect of these essential oils against *Colletotrichum nymphaeae* (Pass.) Aa strain CCh32; the fungus which causes anthracnose in strawberries (Karimi *et al.*, 2016).

However, no records have been found in literature about the efficiency of medicinal plant extracts demonstrating a higher concentration of phenolic compounds resulting from mycorrhization. Therefore, the following question arises: Can mycorrhization, in addition to increasing the production of phenolic compounds in medicine plants, also maximize their medicinal actions?

It is crucial to develop investigations that seek to prove the efficiency of mycorrhizal medicinal plant extracts as alternatives to phytochemical studies associated with AMF inoculation. This is because the focus of the field rather the mycorrhizal efficiency at producing phytochemicals (Lima *et al.*, 2015; Almeida, Sawaya, & Andrade, 2018) than the efficiency of mycorrhizal plant extracts. The confirmation of this hypothesis could benefit the phytotherapeutic drugs industry in regards to the preparation of the raw-material used in the production chain of more efficient phytotherapeutic drugs as well as to collaborate with health practices who are seeking alternatives for the use of phytotherapeutic drugs associated with traditional medicine (WHO, 2013).

In this context, considering the increasing worldwide number of reports about the emergence of multidrug resistant bacteria (Rice, 2018), one alternative to mitigate this problem could be the development of medicinal plant extracts with efficient antimicrobial activity. Organs of *L. ferrea* have shown antimicrobial activity (Silva *et al.*, 2013a; Araújo *et al.*, 2014; Biasi-Garbin *et al.*, 2016) and the extracts of mycorrhizal plants have increased the production of compounds such as flavonoids, tannins and gallic acid (Silva *et al.*, 2014a, 2014b; Santos *et al.*, 2017). However, it is yet to be confirmed if these extracts could present a higher antibacterial activity than those of non-mycorrhizal plants. Therefore, our aim was to verify the *in vitro* antibacterial activity of methanolic extracts of *L. ferrea* fruits, from plants inoculated or not with AMF; considering the strains of Gram-positive and Gram-negative bacteria resistant or not to antibiotics. We tested the hypothesis that the extracts of *L. ferrea* fruits from plants inoculated with AMF have higher antibacterial action.

2. Material and Methods

We used *L. ferrea* fruits, mycorrhizal or not, maintained since February 2013, in the Experimental Field of the Laboratory of Mycorrhizal Technology (LTM/UPE), University of Pernambuco, *Campus* Petrolina (9º23'54.1" S; 40º28'49.0" W). After 32 months of field transplant, fruits were collected and used for the testing of antibacterial activity in laboratory.

2.1 Field experiment

For the field experiment, seedlings produced in a greenhouse were inoculated or not with soil inoculum containing 200 spores of each AMF isolate. We used *Acaulospora longula* Spain & N.C. Schenck (UFPE 21), *Claroideoglomus etunicatum* (W. N. Becker & Gerdemann) C. Walker & A.Schussler) (UFPE 06) and *Gigaspora albida* N.C. Schenck & G.S. Sm. (UFPE 01), granted by the Department of Mycology of the Federal University of Pernambuco. Seedlings without AMF were taken as control. After 225 days, the seedlings were transplanted to the experimental field (Silva *et al.*, 2014a).

In the field, the seedlings were distributed using a spacing of 5 m (5 x 5) apart from each other with irrigation through semi-automatic dripping (8.4 L H_2O $plant^{-1}$ h^{-1}). Before the transplant, each pit (40 x 40 x 40 cm) received five liters of vermicompost and 150 g of simple superphosphate. The plants were distributed in six blocks containing 16 plants each, totaling 96 plants in the field area. Surrounding the field, we developed a border which consisted of non mycorrhizal *L. ferrea* plants (Silva *et al.*, 2014b). Two central plants of each portion were used for the analyses.

2.2 Phytochemical and biochemical characterizations and phosphorus content of *L. ferrea* extract

After 32 months of transplantation, the fruits were collected, cleaned using cotton and then kiln-dried (Quimis Ltda, Diadema, Brazil) (45 ºC). Subsequently, 2 g of fruits (with peel and seeds) were cut into four pieces (13.81 mm in width and 12.29 mm in length) with garden shears (Agass Ltd, China) and ground in a multiprocessor (Black+Decker®, USA) for 90 seconds. The resulting material was macerated in 20 mL of methanol (80 %) for 10 days at 20 ºC (Brito, Noronha, França, Brito, & Prado, 2008). The extracts were gauze-filtered, refiltered in qualitative filter paper, and stored in a freezer (-18 ºC).

The methanolic extracts of the fruits were used for the following quantifications: total phenols (Orujei, Shabani, & Sharifi-Tehrani, 2013), total flavonoids (Araújo, Alencar, Amorim, & Albuquerque , 2008), total tannins (Monteiro *et al.*, 2006; Orujei *et al.*, 2013), total proanthocyanidins (Queiroz, Morais, & Nascimento, 2002), total saponins (Vigo, Narita, & Marques, 2003), antioxidant activity (Rufino *et al.*, 2007, modified) and total soluble carbohydrates (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). The phosphorus content was also determined (Santos *et al.*, 2020) (Table 1).

Table 1. Characterization of the methanolic extracts of *Li'bidibia ferrea* fruits (Mart. ex Tul.) L.P.Queiroz inoculated or not with *Acaulospora longula*, *Claroideoglomus etunicatum*, *Gigaspora albida*, and of the methanolic extracts of native *Libidibia ferrea* fruits (Mart. ex Tul.) L.P. Queiroz collected in a Caatinga area, in Petrolina, PE, Brazil.

Characterization	**Contents in extracts methanolic**				
	Control[1]	***A. longula*[2]**	***C. etunicatum*[3]**	***G. albida*[4]**	**Native fruit[5]**
Total phenols (mg g^{-1})	235.50	297.80	265.20	217.60	250.10
Total tannins (mg g^{-1})	156.40	218.60	182.10	148.80	155.50
Total flavonoids (mg g^{-1})	158.60	154.60	146.40	119.60	124.22
Total proanthocyanidins (mg g^{-1})	12.43	10.71	11.32	10.55	4.50
Total saponins (mg g^{-1})	5.50	4.33	3.77	4.40	4.82
AAO (mg g^{-1} of remaining DPPH)	89.20	59.80	104.90	91.60	2.88
Phosphorus content (mg Kg^{-1})	375.70	203.70	452.20	524.40	656.66
Soluble carbohydrates (mg g^{-1})	8.48	7.56	7.00	5.03	1.59

AAO= Antioxidant activity; *A. Longula= Acaulospora longula*; *C. etunicatum= Claroideoglomus etunicatum*; *G. albida= Gigaspora albida; L. ferrea= Libidibia ferrea*; DPPH (2,2 Diphenyl-1-picrylhydrazyl).[1]*L. ferrea* fruits without inoculation; [2]*L. ferrea* fruits inoculated with *A. longula*; [3]*L. ferrea* fruits inoculated with *C. etunicatum*; [4]*L. ferrea* fruits inoculated with *G. albida*; [5]Native *L. ferrea* fruits. Source: Authors.

2.3 *In vitro* antibacterial activity tests

The *in vitro* antibacterial activity analyses were carried out in the Laboratory of Microbial Resistance (LRM), University of Pernambuco - *Campus* Santo Amaro.

2.3.1 Bacterial strains

The strains *Escherichia coli* (Migula) Castellani and Chalmers (ATCC® 25922) and *Staphylococcus aureus* subsp. *aureus* Rosenbach (ATCC® 25923) as well as two strains of resistant bacteria, the ESBL-producing *Escherichia coli* (Migula) Castellani and Chalmers (ATCC® 35218) (ESBL- Extended spectrum beta-lactamase) and the MRSA- *Staphylococcus aureus* subsp. *aureus* Rosenbach (ATCC® 33591) (MRSA- Methicillin resistant *Staphylococcus aureus*) were used. The strains were stored in liquid LB (Luria-Bertani) a medium containing 15 % glycerol, (at -20 ºC), and reactivated in Agar Mueller-Hinton (Kasvi, Brasil) medium at the moment of use.

2.3.2 Screening method for antibacterial activity

Methanolic extracts of *L. ferrea* fruits inoculated with *A. longula*, *C. etunicatum*, *G. albida*, and extracts of *L. ferrea* fruits without AMF inoculation (control) were used. In addition, collected extracts of native *L. ferrea* fruits, from the Caatinga area (9º23'54.1" S; 40º28'49.0" W), were tested. A control, constituted only of methanol (80 %) (Química moderna Ltda, Barueri, Brasil) was also used.

Mueller-Hinton agar (Kasvi, Brasil) plates were inoculated by swabbing a standardized bacterial inoculum, corresponding to 0.5 on the MacFarland (CLSI, 2012) scale. Subsequently, wells with 18 mm in diameter were made, with sterile apparatus, and given150 µL of methanolic extract from *L. ferrea* fruits, or methanol (80 %). The plates were incubated at 37 ºC for 18 h (CLSI 2018). In order to monitor the method's performance, one well containing 1 µg mL^{-1} ciprofloxacin solution was used as a reference for each plate.

The zones of inhibition which were formed surrounding the wells were measured using digital pachymeter (Lee tools, Ltda) (Araújo *et al.*, 2014, modified). Two measures from each well were taken to generate an average for each sample. This test was conducted in technical triplicate for each sample.

2.3.3 Minimum inhibitory concentration (MIC)

The minimum inhibitory concentration was determined by microtritation in Mueller-Hinton broth using microdiluition plates (CLSI, 2012). In the first well of each raw was placed with 200 µL of the extract (100 mg mL^{-1} of initial concentration) from inoculated and non-inoculated plants. From the second well, the extract was mixed with 100 µL of Mueller-Hinton broth, totaling 200 µL per well. Eight dilutions of the extract were made, the concentrations varied between 100 mg mL^{-1} and 0.78 mg mL^{-1}, in technical triplicate for each sample. Each well received 5 µL standardized inoculum of 0.5 McFarland diluted (1:10, in saline solution), the plants were incubated for 20 to 24 h at 37 °C.

2.3.4 Experimental design

The design for each bacterial strain was completely randomized with six treatments: extracts of *L. ferrea* fruits inoculated with *A. longula*; extracts of *L. ferrea* fruits inoculated with *C. etunicatum*; extracts of *L. ferrea* fruits inoculated with *G. albida*; extracts of *L. ferrea* fruits without AMF (control) inoculation; extracts of *L. ferrea* fruits collected in a Caatinga area and a control with methanol (80 %).

2.4 Statistical analysis

The data was subjected to ANOVA and the averages compared by using a Duncan test (5 %) on software Assistat (7.7).

3. Results

The methanolic extracts of *L. ferrea* fruits inoculated with *A. longula* had higher *in vitro* antibacterial activity when compared to those from non-inoculated plants. This was for all the bacterial strains studied (Table 2).

The zones of inhibition produced by methanolic extracts of *L. ferrea* fruits inoculated with *A. longula*, regarding to the strains *E. coli* (ATCC® 25922), *E. coli* (ATCC® 35218), *S. aureus* (ATCC® 25923), and *S. aureus* (ATCC® 33591) were 7.56 %, 5.22 %, 2.74 %, and 2.48 % greater, respectively, than the zones produced by methanolic extract from control (Table 2).

Table 2. Diameters of the inhibition zones (mm) and minimum inhibitory concentration ($mgmL^{-1}$) of bacterial growth in the presence of methanolic extracts of *Libidibia ferrea* (Mart. ex Tul.) L.P. Queiroz fruits inoculated or not with AMF, methanolic extracts of native *L. ferrea* fruits collected in a Caatinga area and 80 % methanol solution

Treatments	**Bacterial strains**							
	***E. coli* (ATCC-25922)**		***E. coli* (ATCC-35218)**		***S. aureus* (ATCC-25923)**		***S. aureus* (ATCC- 33591)**	
	Inhibiton zone	**MIC**	**Inhibition zone**	**MIC**	**Inhibiton zone**	**MIC**	**Inhibition zone**	**MIC**
Control	22.20 d	≥ 100	22.40 c	≥ 100	28.76 b	100	31.82 b	100
A. longula	23.88 a	≥ 100	23.57 a	≥ 100	29.55 a	100	32.63 a	100
C. etunicatum	21.80 e	≥ 100	22.54 b	≥ 100	27.71 c	100	32.26 ab	100
G. albida	23.41 b	≥ 100	21.35 d	≥ 100	28.44 b	100	31.88 b	100
Native fruit	23.22 c	≥ 100	23.18 a	≥ 100	27.13 d	100	31.31 c	100
Methanol (80 %)	18.00 f*	-	18.00 e*	-	18.00 e*	-	18.00 d*	-
CV (%)	0.76	-	2.00	-	1.27	-	1.28	-

Averages followed by the same letter in a single column do not differ by Duncan test ($p<0.05$). *Values equivalents to the absence of inhibition zones. ATTC= American Type Culture Collec ction; *A. longula= Acaulospora longula*; *C. etunicatum= Claroideoglomus etunicatum*; CV= Coefficient of Variance; *E. coli= Escherichia coli*; *G. albida= Gigaspora albida*; *S. aureus= Staphylococcus aureus*; MIC= Minimum Inhibitory Concentration. Source: Authors.

In the MIC test only the highest concentration (100 mg mL^{-1}) showed bacterial growth inhibition for *S. aureus* (ATCC® 25923) and *S. aureus* (ATCC® 33591) strains. For other strains, *E. coli* (ATCC® 25922) and *E. coli* (ATCC® 35218), bacterial growth was observed in all concentrations (Table 2).

4. Discussion

This is the first record of different effects of mycorrhizal *L. ferrea* extracts in regards to the bacteria. This effect was probably due to the increased concentration of phenols (26.4 %) and tannins (39.77 %) observed and compared to the methanolic extract of non-inoculated *L. ferrea* fruits (Table 1). Similar reports have also shown an association of bacterial growth inhibition with the presence of phenols in fruit extracts from plants of Caatinga (Sampaio *et al.*, 2009; Silva *et al.*, 2013a). In addition, the inhibitory effect of tannins on growth of *S. aureus*-MRSA strains has also been reported (Okuda, 2005; Okuda & Ito, 2011).

Similarly, regarding the phenolic compounds, Karimi *et al.* (2016) also reported that essential oils extracted from mycorrhizal *A. graveolens* with *F. mosseae* had higher action against *C. nymphaeae* than essential oils of non mycorrhizal plants. The authors attributed such a benefit to the contents of the essential oils carvone and limonene.

However, the presence of FMA does not always increase the efficiency of plant extracts in inhibiting bacterial growth (Table 2). The zones of inhibition produced by some of the methanolic extracts of mycorrhizal *L. ferrea* fruits tested against *E. coli* (ATCC® 35218) were similar to those formed by extracts of native *L. ferrea* fruits collected in the Caatinga region (Table 2) and the MIC test of these strains showed bacterial growth independent of the concentration tested, what did not occurred with Gram-positive strains (*S. aureus* ATCC® 25923) and *S. aureus* (ATCC® 33591) which showed growth in the highest concentration (Table 2), this fact, demonstrates the importance of complementary tests to verify the antibacterial activity.

It is likely that, different phytochemical and nutritional profiles of the plant extracts may have interfered in the bacterial growth, such as the phosphorus concentration which is an important nutrient for bacterial growth and has different values in the fruits used in this study according to the AMF inoculated (Table 1). The alterations in the phosphorus concentrations activate, or not, the regulation systems which influence the microorganism growth (Behrendes *et al.*, 2014; Shimizu, 2014). In turn, this may have influenced the bacterial growth regarding the extracts of *L. ferrea* fruits inoculated with *A. longula*. In addition, the inhibitory potential of the bacterial growth may vary according to the bacterial species thus giving rise to different efficiencies of the extracts (Conde *et al.*, 2015; Oliveira *et al.*, 2015). As verified in our study (Table 2).

AMF inoculation can be efficient at maximizing the *in vitro* antibacterial activity of extracts of *L. ferrea* fruits and represents an alternative to the production of raw materials for the material manufacture of phytotherapeutic drugs based on *L. ferrea* fruits. The inhibition zones produced by the methanolic extracts of *L. ferrea* fruits inoculated with *A. longula* varied from 21.35 to 32.63 mm (Table 2) and were higher than the zones produced by extracts of *L. ferrea* fruits collected in the Caatinga, which varied between 11 and 18.5 mm (Oliveira *et al.*, 2015; Silva *et al.*, 2013a; Conde *et al.*, 2015).

In general, extracts of *L. ferrea* inoculated with *A. longula* inhibited both Gram-positive and Gram-negative bacteria (Araújo *et al.*, 2014; Nascimento *et al.*, 2015; Ferreira *et al.*, 2016), as recorded in this study (Table 2). However, it is worth highlighting that the extracts of *L. ferrea* inoculated with *A. longula* were also efficient in inhibiting the antibiotic resistant strains of both Gram-negative e Gram-positive bacteria (Table 2).

5. Conclusion

Thus, the inoculation of *L. ferrea* with *A. longula* can be an alternative to producing fruits with different antibacterial activities thus contributing to the World Health Organization guidelines, which seek to integrate alternative and traditional medicine (WHO, 2013).

Our tests are still in their primary stage with regards to the production chain of raw materials which can be used in the phytotherapeutic drugs industry. Therefore, it is necessary to develop studies to verify the efficiency of different concentrations of methanolic extracts of *L. ferrea* fruits, in addition to toxicity tests on the extracts of mycorrhizal plants (Braquehais *et al.*, 2016; Santos *et al.*, 2017). Complementary studies should include assessing the synergic effect of extracts of mycorrhizal *L. ferrea* fruits with antibiotics.

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