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Screening antimicrobial activity of tropical edible medicinal plant extracts against five standard microorganisms for natural food preservative

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Abstract

Edible medicinal plants are often used in the treatment of various ailments and spice in traditional food preparation. In this study, 45 of tropical edible medicinal plants extracts from Indonesia, Malaysia, and Thailand were screened for their antimicrobial activity against five standard microorganisms for food preservative namely *Aspergillus niger*, *Candida albicans*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus.* The methanol extracts of *Piper nigrum* L. seed, *Piper cubeba* L. seed, and the root of *Ligusticum acutilobum* Siebold and Zucc. showed antimicrobial activity against five species of standard microorganisms. Among them, *P. cubeba* L. extract demonstrated the most susceptible against all tested microorganisms. Minimal inhibitory concentration (MIC) and minimal bactericidal or fungicidal concentration (MBC or MFC) were performed by the broth microdilution techniques as described by the Clinical and Laboratory Standard Institute. MIC values of *P. cubeba* L. extract to *A. niger*, *C. albicans*, *E. coli*, *P. aeruginosa* and *S. aureus* were 12.8, 1.6, 3.2, 6.4, and 1.6 mg/ml, respectively. *P. cubeba* extract killed *A. niger*, *C. albicans*, *E. coli*, *P. aeruginosa* and *S. aureus* with MBC values of 25.6, 3.2, 6.4, 12.8, and 3.2 mg/ml, respectively. The potent antimicrobial activity of *P. cubeba* L. extract may support its use for natural food preservative.

Introduction

The growth of bacteria, yeast, and mould in foods and food products results in waste products and is costly as well as sometimes hazardous. Many different bacterial and fungal species can spoil food products or produce toxins or both. Several food preservation systems such as heating, refrigeration and addition of antifungal compounds can be used to reduce the risk of outbreaks of food poisoning; however, these techniques frequently have associated adverse changes in organoleptic characterizations and loss of nutrient (Valero and Frances, 2006). Although chemical preservatives prevent microbial growth, their safety is questioned by a growing segment of consumers. Moreover, consumer demand of natural, fresh, chemical-additive free and safe food products is increasing at the present (Gould, 1996). Recently, there is interest in the development of natural preservative from edible medicinal plant extracts (EMPE) (Singh *et al*., 2010). Thus, the properties of tropical EMPE for natural food preservative need to be investigated in order to prevent microbial spoilage and therefore to prolong the shelf life of the food or food products, and finally to protect the consumers from potential infection.

Edible medicinal plants are used widely in the food industry as flavors and fragrances, also exhibit useful antimicrobial properties (Rios and Recio, 2005). Many plant-derivate antimicrobial compounds have a wide spectrum of activity against foodborne pathogens and this has led to suggestions that they could be used as natural preservatives in foods (Smith-Palmer *et al*., 1998; Cho *et al.*, 2008). The safest way to look for natural food preservative is to search for activity against classes of standard microorganisms. These include *Escherichia coli* and *Pseudomonas aeruginosa* (Gram positive), *Staphylococcus aureus* and *Bacillus cereus* (Gram negative), *Candida albicans* (yeast), *Aspergillus flavus* and *A. niger* (moulds) (Dweek, 1997).

The objective of this study is to screen the antimicrobial activity of tropical EMPE from Indonesia, Malaysia, and Thailand against standard five species microorganisms mentioned above. The susceptibility of selected tropical EMPE in term of minimum inhibitory concentration (MIC) and minimum bactericidal/fungicidal concentration (MBC/MFC), on the five standard microorganisms will be determined using CLSI methods (Clinical

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Laboratory and Standard Institute) (2002, 2003).

Materials and Methods

Plant materials

The tropical edible medicinal plants were collected from traditional market of Indonesia (IN), Malaysia (MY) and Thailand (TH) and identified by Biopharmaca Research Center (BRC), Bogor Agriculture University (IPB) (Bogor, Indonesia), Institute of Bioscience, Universiti Putra Malaysia (Selangor, Malaysia), and Institute of Science, Walailak University (Nakhon Si Thammarat, Thailand), respectively. The voucher specimens are deposited in the Laboratory of Natural Products, Institute of Bioscience, Universiti Putra Malaysia (Table 1).

Plants extract preparation

The dried plants (100 g) were ground and extracted twice with 400 mL of 100% (v/v) methanol for 48 h at room temperature. Tropical edible medicinal plant extracts (EMPE) were filtered with Whatman filter paper NO.2 (Whatman International Ltd., Middlesex, England) and concentrated with a rotary vacuum evaporator (Heidolph VV2011, Schwabach, Germany) at 50°C, yielding methanol crude extracts. Each methanol tropical EMPE was dissolved in 100% DMSO to obtain 1,024 mg/mL and the solution was dissolved in 1:10 (v/v) sterile double distillated water (ddH_2O) to obtain 102.4 mg/mL stock solutions. Final concentration of DMSO was 10% which was found not to kill the five standard microorganisms tested in this study.

Tested microorganisms and inoculum preparation

Aspergillus niger ATCC 2029, *Candida albicans* ATCC 10231, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 15692 were obtained from the American Type Culture Collection (Rockville, MD, USA). *Staphylococcus aureus* KCCM 11764 was obtained from Korean Culture Center of Microorganisms (Seoul, South Korea).

Aspergillus niger

A. niger was grown on PDA (Difco, Spark, MD, USA) at 35°C for 7 days. A standardized inoculum suspension of *A. niger* was prepared by the method of CLSI M38-A (CLSI, 2002). Briefly, *A. niger* was grown on PDA at 35°C for 7 days (Rukayadi and Hwang, 2007). Seven-day-old colonies were covered with approximately 1 ml of sterile 0.85% saline, and the suspensions were made by gently probing the colonies with the tip of a Pasteur-pipette. The resulting mixture of conidia or sporangiospore and hyphal fragments was withdrawn and transferred to a sterile tube. After heavy particles were allowed to settle for 3 to 5 min, the upper homogenous suspensions were collected and mixed with a vortex mixer for 15 s. The densities of the conidial suspensions were read and adjusted to an optical density (OD) that ranged 80 to 82% transmittance. These suspensions were diluted 1:50 in sterile water distillated water. The 1:50 inoculum dilutions corresponded to 2× density (approximately 0.4×10^4 to 5×10^4 cfu/mL) (Rukayadi and Hwang, 2007). Inoculum quantification was made by plating 0.01 mL of 1:100 dilution of the adjusted inoculum on Sabouraud dextrose agar (SDA) (Difco) to determine the viable number of cfu/mL. The plates were incubated at 28-30°C and observed daily for the presence of fungal colonies. The 2× conidial or sporangiospore inoculum suspension was approximately 5×10^4 cfu/mL.

Candida albicans

The *C. albicans* was cultured in Sobouraud dextrose broth (SDB) or on Sabouraud dextrose agar (SDA) (Difco, Spark, MD, USA) for 48 h at 35°C. Meanwhile, inoculums suspension of *C. albicans* was prepared as follows: the *C. albicans* was propagated in SDB at 35°C for 24 h with 200 rpm agitation. One mL of 24 h old culture in SDB was centrifuged (3900 × g at 4°C for 1 min), and the pellets were washed twice with 1 mL of physiological saline. Sterile physiological saline was added to give a McFarland turbidity 0.5 at 530 nm, corresponding to 5×10^6 cfu/mL (CLSI, 2002).

Escherichia coli, Pseudomonas aeruginosa, and Staphylococcus aureus

E. coli, *P. aeruginosa* and *S. aureus* in Mueller Hinton broth (MHB) or Mueller Hinton agar (MHA) (Difco, Franklin Lakes, NJ, USA). An inoculum cell suspension was prepared as follows: bacterial species was first grown aerobically on MHA plate for 24 h at 37°C. Subsequently, a single colony of each bacterial species was propagated in 10 mL of MHB at 37°C overnight with 200 rpm agitation. A quantity of 1 mL of overnight cultures in MHB was centrifuged (3,000 × g at 4°C for 1 min) and pellets were resuspended in 1 mL of MHB. Standardized inoculums (a McFarland standard) for each strain were 5×10^6 cfu/mL. A standard curve of turbidity against colony forming unit (cfu) was used to obtain the number of cells.

Screening bioassay

Methanol extracts of 45 tropical medicinal plants were screened for antimicrobial activity using the standard paper disk diffusion assay (CLSI, 2002). 100 µL of inoculum of each standard microorganisms

prepared as above was spread on SDA plates with a sterile cotton swab. Sterile filter paper discs, 6 mm diameter (Schleicher and Schuell, Dassel, Germany), were placed on the disks and 50 µL of 102.4 mg/mL (w/v) methanol extract of samples were loaded on the paper discs. 1 mg/mL of amphotericin B (AMB, a positive control for *C. albicans* and *A. niger*) or chlorhexidine (CHX, a positive control for *E. coli*, *P. aeruginosa* and *S. aureus*), and 10% of DMSO (a negative control) were included in the assay. The plates were incubated at 37°C for 12-24 h for bacterial species and 24-48 h for fungi and observed for any clear zones. The experiments were preformed twice to verify the results

MIC and MBC or MFC determination

In vitro susceptibility tests were performed in a 96-well microtiter plate to determine MIC and MBC or MFC of tropical EMPE against *A. niger*, *C. albicans*, *E. coli*, *P. aeruginosa* and *S. aureus* using standard broth microdilution methods with an inoculum of 5 × 10^4 cfu/mL for *A. niger*, and 5 × 10^6 cfu/mL for *C. albicans* and bacterial species, according to the guidelines of CLSI M7-A6 (for bacterial species) (CLSI, 2003), M27-A2 (for *C. albicans*) (CLSI, 2002) and M38-A (for *A. niger*) (CLSI, 2002). Briefly, a 2-fold EMPE stock solution or other antimicrobial agent preparations was mixed with the test organisms MHB, SDB, and PDB for bacterial species, *C. albicans* and *A. niger*, respectively. Column 12 of the microtiter plate contained the highest concentrations of EMPE or other antimicrobial agents, and column three contained the lowest concentrations of EMPE or other antimicrobials agents. Column 2 served as the positive control for all samples (only medium and inoculum or antimicrobial agent-free wells), and column 1 was the negative control (only medium, no inoculum, no antimicrobial agent). Microtiter plates were incubated aerobically at 37°C for 24 h for bacterial species and 48 h for *C. albicans* and *A. niger.* The MIC was defined as the lowest concentration of antimicrobial agent that resulted in the complete inhibition of visible growth.

MBC and MFC values were determined for each of EMPE/microorganim species/medium combination as outlined for MIC by removing the media from each well showing no visible growth and subculturing onto MHA, SDA or PDA plates (Rukayadi *et al*., 2006; 2009; 2010). The plates were incubated at 37°C until growth was seen in the growth control plates. MBC or MFC were defined as the corresponding concentrations required to kill 100% of the microorganisms.

Results and Discussion

The susceptibility of tropical EMPE towards 5 standard species was tabulated on Table 1, based on their inhibition diameter on plates. Previous author have described that an inhibition one of 14 mm or greater which include with diameter of disc was conceived as high antimicrobial activity (Parekh and Chanda, 2007). Based on the results, it showed that the tropical EMPE were more active against *S. aureus*. *S. aureus* is Gram positive bacterium while others, *E. coli* and *P. aeruginosa* are Gram negative bacteria. On the other hand, *A. niger* and *C. albican* are both fungi. The results shows in agreement with previous study which indicated that plant extracts were more active against Gram positive bacteria than those of Gram negative bacteria (Kelmanson *et al.*, 2000; Parekh and Chanda, 2007) (Table 1). Different species of plants influence its activity against microbe tested due to the difference microbe cell wall compound (Grosvenor *et al*., 1995). Three plants extracts namely *L. acutilobum*, *P. cubeba* and *P. nigrum* showed the best potential antibacterial activity against of all microbes tested. Out of 45 tropical EMPE, *C. xanthorrhiza* extract has the strongest potential antimicrobial activity against *A. niger* and *C. albicans.* Moreover, *O. basilicum*, *L. acutilobum* and *P. cubeba* have the strongest antimicrobial activity against *E. coli*, *P. aeruginosa* and *S. aureus*, respectively. In this study, *P. aeruginosa* is the most resistant strain against all tropical EMPE tested. In contracts, *S. aureus* is the most susceptible strain among all microbe tested against all tropical EMPE (Table 1).

Table 2 shows the MIC and MBC or MFC values of *L. acutilobum*, *P. nigrum* and *P. cubeba* on *A. niger*, *C. albicans*, *E. coli*, *P. aeruginosa* and *S. aureus*. MICs and MBCs or MFCs of P. cubeba extract against five standard microbes exhibit relatively stronger than those of *L. acutilobum* and *P. nigrum.* The essential oil of *P. cubeba* contain hydrocarbon terpene and oxygenated terpene, thus, could be used as antioxidant (Hwang *et al*., 2005), antibacterial (Feng *et al*., 2009) and antifungal (Yang *et al*., 2010). *P. nigrum* is used to treat various diseases and has shown to have antimicrobial activity (Rahman *et al*., 2011).

The major phytochemical present in the crude extract of *P. nigrum* was found to be piperine, the active constituent showing inhibitor effect in the crude extract. The fresh berry oil of *P. nigrum* L. recorded MIC values were 2.5 mg/mL against *P. aeruginosa* and 8.5 mg/mL for *A. niger* whereas the dry berry oil needed 4 mg/mL for *C. albicans* (Sasidharan and

Table 1. Species of tropical medicinal plants, plant parts tested and their methanol extract susceptibility to five standard species for food preservative

Voucher specimen number	Plant species	Family	Use part	Antimicrobial activity* (mm)				
				A.n	*C.a*	*E.c*	*P.a*	*S.a*
MY001	*Averrhoa bilimbi* L.	Oxalidaceae	Fruit	-	-	-	-	-
IN001	*Alpinia galanga* (L.) Sw.	Zingiberaceae	Rhizome	-	-	-	-	12
IN002	*Abrus precatorius* L.	Fabaceae	Leaf	-	-	14	-	-
TH001	*Aloe vera* (L.) Burm. f.	Aloeaceae	Resin	-	-	-	-	-
IN003	*Boesenbergia rotunda* (L.) Mansf.	Zingiberaceae	Rhizome	-	-	-	-	12
MY002	*Curcuma longa* L.	Zingiberaceae	Rhizome	-	-	-	-	12
IN004	*Curcuma aeruginosa* Roxb.	Zingiberaceae	Rhizome	-	-	-	-	12
IN005	*Curcuma xanthorrhiza* Roxb.	Zingiberaceae	Rhizome	18	16	14	-	18
IN006	*Caesalpinia sappan* L.	Fabaceae	Wood	-	-		-	-
TH002	*Curcuma mangga*	Zingiberaceae	Rhizome	-	-	-	-	14
IN007	*Carica papaya* L.	Caricaceae	Leaf	-	-	-	-	-
IN008	*Coriandrum sativum* L.	Apiaceae	Seed	-	12	-	-	-
IN009	*Centella asiatica* (L.) Urban	Apiaceae	Leaf	-	-	-	-	14
IN010	*Cinnamomum verum* J. Presl	Lauraceae	Bark	-	-	12	-	12
IN011	*Cryptocarya massoy* Kosterm.	Lauraceae	Stem bark	-	14	14	-	-
IN012	*Colocasia esculenta* (L.) Schott	Araceae	Tuber root	-	12	-	14	16
TH003	*Curcuma hyneana* Val. & Zijp.	Zingiberaceae	Rhizome	-	-	-	-	-
IN013	*Elettaria cardamomum* (L.) Maton	Zingiberaceae	Fruit	-	-	-	-	-
IN014	*Foeniculum vulgare* P. Mill.	Apiaceae	Seed	-	-	-	-	-
MY003	*Glycine soja* Sieb. et Zucc.	Fabaceae	Seed	-	-	-	-	-
IN015	*Hippobroma longiflora* (L.) G. Don	Campanulaceae	Leaf	-	-	12	-	-
TH004	*Kaempferia galanga* L.	Zingiberaceae	Rhizome	-	-	-	-	-
TH005	*Ligusticum acutilobum* S. et Z.	Apiaceae	Root	12	12	12	20	14
MY004	*Leucaena leucocephala* (Lam.) de Wit	Fabaceae	Fruit	-	-	-	-	-
MY005	*Momordica charantia* L.	Cucurbitaceae	Fruit	-	-	-	-	-
MY006	*Moringa oleifera* Lam.	Moringaceae	Leaf	-	-	-	-	-
IN016	*Myristica fragrans* Houtt.	Myristicaceae	Mace	12	-	12	-	-
IN017	*Myristica fragrans* Houtt.	Myristicaceae	Nutmeg	12	-	12	-	18
IN018	*Nigella sativa* L.	Ranunculaceae	Seed	-	14	24	-	14
IN019	*Orthosiphon aristatus Benth.*	Lamiaceae	Leaf	12	-	12	-	12
TH006	*Ocimum basilicum* L.	Lamiaceae	Seed	-	-	26	14	-
IN020	*Plectranthus amboinicus* (Lour.)	Lamiaceae	Seed	-	-	-	-	-
IN021	*Piper nigrum* L.	Piperaceae	Seed	16	24	18	14	16
IN022	*Piper retrofractum* Vahl.	Piperaceae	Fruit	-	-	14	14	20
IN023	*Piper cubeba* L.	Piperaceae	Seed	18	22	24	18	26
IN024	*Piper longum* L.	Piperaceae	Seed	-	12	-	14	-
TH007	*Punica granatum* L.	Punicaceae	Root bark	-	12	-	-	24
TH008	*Piper chantaranothaii*	Piperaceae	Fruit	12	-	-	-	16
MY007	*Psidium guajava* L.	Myrtaceae	Leaf	-	-	16	-	-
MY008	*Physalis angulata* L.	Solanaceae	Whole plant	-	-	-	-	-
IN025	*Pimpinella anisum* L.	Apiaceae	Seed	-	-	-	-	-
IN026	*Urena lobata* L.	Malvaceae	Whole plant	-	-	-	-	-
IN027	*Vanilla planifolia* B. D. Jackson	Orchidaceae	Fruit	-	-	-	-	-
IN028	*Zingiber aromaticum* Vahl.	Zingiberaceae	Rhizome	-	12	-	-	12
IN029	*Zingiber officinale* Roscoe	Zingiberaceae	Rhizome	-	-	-	-	-

*A.n, Aspergillus niger; C.a, Candida albicans; E.c, Escherichia coli; P.a, Pseudomonas aeruginosa; S.a, Staphylococcus aureus

Menon, 2010). The pepper leaf oil which is wasted at present can be utilized against these microorganisms instead of costly synthetic chemicals.

The results show that *P. cubeba* L. extract is more effective in killing all the microorganisms tested. Low concentration of *P. cubeba* extract is needed to kill *C. albicans* and *S. aureus* which was 3.2 mg/mL followed by *E. coli* (6.4 mg/ml), *P. aeruginosa* (12.8 mg/mL) and *A. niger* (25.6 mg/mL). *P. cubeba* is used as antibacterial, expectorant and gastroprotective

Table 2. Minimum inhibitory concentration (MIC) (mg/mL), minimum fungicidal concentration (MFC) (mg/mL), and minimum bactericidal concentration (MBC) (mg/mL) of edible medicinal plant extracts (EMPE) on five standard species for food preservative

	A.n		*C.a*		*E.c*		*P.a*		*S.a*	
Sample and microorganism species*	**MIC**	**MFC**	**MIC**	**MFC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**
Alpinia galanga (L.) Sw.	-	-	-	-	-	-	-	-	12.8	25.6
Abrus precatorius L.	-	-	-	-	25.6	51.2	-	-	-	-
Boesenbergia rotunda (L.) Mansf.	-	-	-	-	-	-	-	-	12.8	25.6
Curcuma longa L.	-	-	-	-	-	-	-	-	12.8	25.6
Curcuma aeruginosa Roxb.	-	-	-	-	-	-	-	-	25.6	51.2
Curcuma xanthorrhiza Roxb.	12.8	25.6	3.2	6.4	3.2	6.4	-	-	3.2	6.4
Curcuma mangga	-	-	-	-	-	-	-	-	3.2	6.4
Coriandrum sativum L.	-	-	6.4	12.8	-	-	-	-	-	-
Centella asiatica (L.) Urban	-	-	-	-	-	-	-	-	12.8	25.6
Cinnamomum verum J. Presl	-	-	-	-	25.6	51.2	-	-	12.8	25.6
Cryptocarya massoy Kosterm.	-	-	6.4	12.8	12.8	51.2	-	-	-	-
Colocasia esculenta (L.) Schott	-	-	1.6	3.2	-	-	6.4	25.6	6.4	25.6
Hippobroma longiflora (L.) G. Don	-	-	-	-	12.8	51.2	-	-	-	-
Ligusticum acutilobum S. et Z.	12.8	51.2	6.4	12.8	12.8	25.6	6.4	25.6	3.2	6.4
Myristica fragrans Houtt. (mace)	25.6	>51.2	-	-	12.8	51.2	-	-	-	-
Myristica fragrans Houtt. (nutmeg)	25.6	51.2	-	-	12.8	25.6	-	-	1.6	6.4
Nigella sativa L.	-	-	6.4	12.8	25.6	51.2	-	-	6.4	25.6
Orthosiphon aristatus Benth.	25.6	51.2	-	-	12.8	51.2	-	-	6.4	12.8
Ocimum basilicum L.	-	-	-	-	256	512	64	256	-	-
Piper nigrum L.	12.8	25.6	3.2	6.4	3.2	6.4	12.8	25.6	1.6	3.2
Piper retrofractum Vahl.	-	-	-	-	25.6	>51.2	12.8	>51.2	25.6	>51.2
Piper cubeba L.	12.8	25.6	1.6	3.2	3.2	6.4	6.4	12.8	1.6	3.2
Punica granatum L.	-	-	12.8	25.6	-	-	-	-	3.2	6.4
Psidium guajava L.	-	-	-	-	3.2	6.4	-	-	-	-
Zingiber aromaticum Vahl.	-	-	3.2	6.4	-	-	-	-	1.6	6.4

* *A.n, Aspergillus niger; C.a, Candida albicans; E.c, Escherichia coli; P.a, Pseudomonas aeruginosa; S.a, Staphylococcus aureus*

(Mohib and Mustafa, 2007). It is widely used in various herbal cough syrups and also as anti-inflammatory, anti-malarial, leukemia treatment. High antioxidant activity was found in *P. cubeba* ethanol extract in comparison to *P. nigrum* extracts (Nahak and Sahu, 2011). *S. aureus* also has the lowest MBC for *P. nigrum* L. extract which was 3.2 mg/mL and followed by 6.4 and 25.6 mg/mL for *C. albicans* and *E. coli*, and *A. niger* and *P. aeruginosa*, respectively. These results indicate that *P. nigrum* L. extract was more susceptible to Gram positive than against Gram negative. These results are consistent with reported by by Karsha and Laskhmi (2010), that *P. nigrum* L. extract is more susceptible to Gram negative compared against Gram negative. It might because *P. nigrum* L. extract altered the membrane permeability results in the leakage of nucleic acid and protein into the extracellular medium. Furthermore, phenols and phenolic compound in the *P. nigrum* L. extract cause injury to membrane function (Davidson and Branen, 1981). The use of piperine alone showed excellent bactericidal activity against Gram positive and Gram negative bacteria. The alkaloids such as piperine, piperidine, volatile oil and resins are responsible for antibacterial activity (Karsha and Laskhmi, 2010). Apart from that, *L. acutilobum* extract also has activity against all the microorganisms tested but higher concentration is needed compared to other two extracts. *S. aureus* has the lowest MBC with 6.4 mg/mL followed by *C. albicans* (12.8 mg/mL), *E. coli* and *P. aeruginosa* (25.6 mg/mL) and A. niger (51.2 mg/mL) for the *L. acutilobum* extract. All the antibacterial activity might be caused by loss control of the bacterial membranes.

Conclusion

The development of resistance in common foodborne pathogens and emergence of new foodborne pathogens intrinsically resistant to the currently available antibiotics demonstrates the urgent importance of identifying novel natural antimicrobial agents. There will be an increasing need for microbial inhibiting substances from plants. The traditional medicinal plants represent a reservoir of antimicrobial agent. Present study shows, *P. cubeba* extract shows the most potent antimicrobial activity against five standard species microorganisms. Therefore, *P. cubeba* extract and its compounds might be potentially valuable as a natural food preservative.

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