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COMPARATIVE ANALYSIS OF THE ANTIMICROBIAL AND ANTIOXIDANT PROPERTIES OF SELECTED PLANT EXTRACTS AS NATURAL PRESERVATIVES IN OINTMENT FORMULATION

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ABSTRACT

Preservatives are substances added to food and pharmaceutical products to extend their shelf-life. Synthetic compounds have been widely used for such applications, however, reports demonstrated adverse effects of synthetic preservative consumption. Thus, there is a need to develop safe and cheap preservatives to avoid undesirable effects. This study aims to investigate the preservative efficacy of five plants namely *Psidium guajava, Premna odorata, Mimosa pudica, Allium sativum and Zingiber officinale*, as an alternative for synthetic preservatives. Ethanolic extracts of these plants were subjected to phytochemical screening which verified the presence of sapogenins and glycosides in all plant extracts. AAS analysis confirmed that the extracts are safe, as shown by the lead and cadmium contents within the acceptable dietary intake (<0.3 mg/kg and 0.010 mg/kg, respectively). Among the five plants, *P. guajava* and *P. odorata* exhibited the highest antioxidant activity in diphenylpicrylhydrazyl assay at concentrations between 1.00 – 5.00 mg/mL and below 0.50 mg/mL, respectively. Physicochemical stability of the extracts as pre-formulated ointment was observed up to 45 days at different storage conditions, with results comparable to common synthetic preservatives, methyl- and propylparaben. The preservative challenge test performed against fungi and bacteria revealed that *P. guajava* has the highest preservative capability among the pre-formulated ointments. It is concluded that *P. guajava* exhibited the highest antioxidant and antibacterial properties, suggesting its potential use as preservative in ointment formulation.

KEYWORDS: Preservatives; natural; antioxidant; antimicrobial; *P. guajava.*

Abbreviations

DPPH: diphenylpicrylhydrazyl assay

ADI: Acceptable dietary intake

MIC: Minimum inhibitory concentration

USP: United States Pharmacopeia

INTRODUCTION

Preservatives are excipients commonly added to various food and pharmaceutical products in order to extend their shelf life.[1] The addition of preservatives to such products is important to prevent alteration and degradation by microorganisms and avoid physicochemical changes upon exposure to environmental factors such as humidity and temperature upon storage.[2] These preservatives can be classified in terms of their action as antimicrobial, antioxidant or both. Antimicrobial preservatives are introduced during the manufacturing process to kill or inhibit the growth of microorganisms.[3] Guidelines for evaluation of antimicrobial effectiveness of pharmaceutical products against common microbial contaminants and agents of spoilage such as *Candida albicans*, *Aspergillus niger*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* are recommended by USP.[4] Alternatively, antioxidant preservatives are included in the products to avoid degradation from oxidation. The mechanism involves reacting with free radicals, blocking initiation and propagation reactions, thus, slowing the deterioration of products.[5]

According to a survey of Kemi Swedish Chemicals Agency in 2010,[6] the demand for the use of preservatives has been continuously increasing, with high percentage of synthetic (chemical) preservatives utilized in food and pharmaceutical industries. In the Philippines, different synthetic preservatives such as bisulfites, nitrates, butylated hydroxytoluene (BHT), benzoic acid, methylparaben and propylparaben are extensively used for food and pharmaceutical applications. However, reports have demonstrated serious side effects on the use of such chemicals which includes allergy,[7] carcinogenicity,[8] diet-related illness[9] and even behavioral changes.[10] One study on a large non-ADHD group of students revealed that elimination of artificial ingredients on food, including preservatives, increased the academic performance and positive behavior of the students.[3]

With health concerns and risks involved on the use of synthetic preservatives, the search for a natural source is of particular interest. However, very few studies have been conducted on local Philippine plants which evaluate the antimicrobial, antioxidant or both properties for preservative use. Thus, this study aims to screen selected Philippine plants as a source of natural, safe and affordable preservatives for pharmaceutical applications.

MATERIALS AND METHODS

Collection of plant materials

Five locally available plants with existing studies on antimicrobial activity were screened and selected based on their availability and use as food (**Table 1**). The samples were collected from the University of the Philippines-Diliman campus except for *A. sativum* and *Z. officinale* which were bought from the local market in Paco, Manila, Philippines. The samples were submitted to the National Museum Plant Division–Philippines for authentication with control number 0932.

Table 1: Local plants submitted and authenticated by the National Museum Plant Division-Philippines

Family	Scientific name	Common name	Local name
Myrtaceae	*Psidium guajava* L.	Guava	*Bayabas*
Verbenaceae	*Premna odorata* Blanco	Fragnant premna	*Alagao*
Fabaceae	*Mimosa pudica* L.	Sensitive plant, sleepy plant	*Makahiya*
Liliaceae	*Allium sativum* L.	Garlic	*Bawang*
Zingiberaceae	*Zingiber officinale* Roscoe	Ginger	*Luya*

Preparation of crude extract

Plant samples were collected, washed, cut into small pieces and air-dried. The samples were macerated for 48 hours in 95% ethanol and filtered. The ethanolic extracts were subjected to rotary evaporation and the semi-solid extract was dried in a water bath. The resulting extracts were subjected to phytochemical screening, antioxidant activity, cadmium and lead analysis, preservative challenge and stability studies.

Phytochemical Screening: Qualitative phytochemical screening was performed for the presence of alkaloids, glycosides, tannins and other polyphenols, reducing substances, plant acids, saponins and sapogenins, flavones, flavonols and flavonoids adapted from Aguinaldo *et al.*[11]

Determination of *in vitro* antioxidant activity

Evaluation of the free radical scavenging activity of the extracts through their hydrogen donating ability was examined following the method of Subhashini *et al*.[12] A 170 µL DPPH solution was added to the tubes containing 830 µL of different concentrations of the extracts (0.25, 0.50, 1.0 and 5.0 mg/mL) and allowed to react. After 30 minutes, the absorbance values were measured at 517 nm. Ethanol, DPPH solution with ethanol and ascorbic acid were used as blank, negative and positive control, respectively.

Determination of Cd and Pb content

Digestion was done following AOAC[13] with some modifications. The plant extracts were ground and homogenized as needed, weighed in a porcelain crucible and placed in a hotplate at 100°C until dehydrated. The samples were ashed followed by cooling and addition of 5 mL hydrochloric acid. The resulting solution was heated in a hotplate at 120°C until the volume reached 1 mL, then cooled, filtered with Whatman filter paper No. 1 followed by dilution of the filtrate with nitric acid. The same method was performed for blank digestion. Varian AA240FS Fast Sequential AAS with deuterium background corrector was used with Cd and Pb hollow cathode lamp as light source.

Stability Studies

Stability testing was performed following the procedure of Aulton[14] with some modifications. Hydrophilic ointment was prepared using the dried ethanolic extract in place of methylparaben and propylparaben. The formulations were placed in transparent and amber containers and subjected to different temperatures - ambient (30°C) and oven (40°C). Physical changes were observed every 15 days for 45 days based on color, texture and odor in comparison with the combination of methylparaben and propylparaben as control while chemical changes was determined using thin layer chromatography (TLC) with n-butanol: glacial acetic acid:water (4:1:5) as the mobile phase measured at 254 nm and 366 nm.

Preservative Challenge Test

Preservative challenge test was done using USP guidelines.[4] Ointment formulation containing combination of methylparaben and propylparaben were used as positive control and replaced with dried ethanolic extract for test samples. *A. niger* (fungi), *E. coli* (gram negative) and *S. aureus* (gram positive) were diluted with 0.9% sterile saline solution to a density of 0.5 McFarland Standard. Ten mL inoculum was transferred to the formulated product and mixed thoroughly, followed by addition of casein peptone agar, incubation at

room temperature and analysis at 7, 14 and 28 days for minimum inhibitory concentration (MIC).[15]

Statistical treatment

The data gathered were recorded as Mean ± SEM (standard mean error). The statistical significance of differences between groups was determined by one-way analysis of variance (one-way ANOVA), followed by Tukey's HSD test using SPSS 17.0 software. Mean values were considered statistically significant when $p<0.05$.

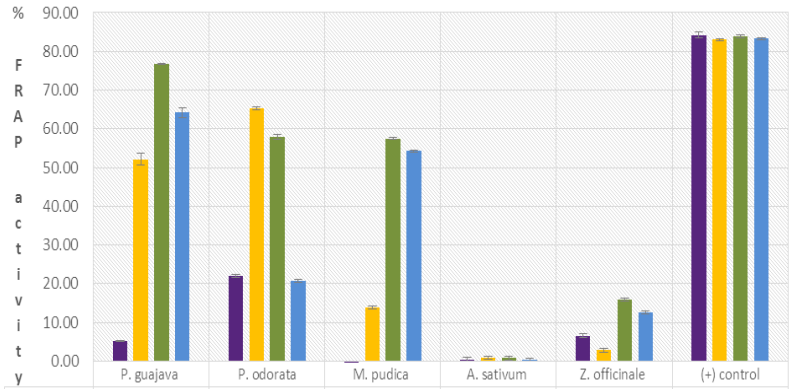
RESULTS

Phytochemical screening of five plant extracts (**Table 2**) demonstrated that *P. odorata* and *M. pudica* have normal pH while the rest of the extracts were at pH 6. Secondary metabolites identified present for most of the extracts were tannins and other polyphenols, glycosides, reducing substances, alkaloids, flavonoids and sapogenins except for flavones and flavonols which were noted only in *P. guajava.* The presence of saponins was not detected in all extracts.

Table 2: Phytochemical screening of five ethanolic plant extracts.

Plant Extract	pH level	Tannins and other Polyphenols	Glycosides	Reducing Substances	Alkaloids				Plant Acid	Saponins	Saponins and Sapogenin	Flavones/ Flavonols	Flavonoids
P.guajava	pH - 6	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(-)	(-)	(+)	(+)	(+)
P. odorata	pH - 7	(+)	(+)	(+)	(-)	(+)	(+)	(-)	(-)	(-)	(+)	(-)	(-)
M. pudica	pH - 7	(+)	(+)	(+)	(-)	(+)	(+)	(+)	(+)	(-)	(+)	(-)	(+)
A. sativum	pH - 6	(-)	(+)	(-)	(-)	(-)	(-)	(-)	(+)	(-)	(+)	(-)	(-)
Z. officinale	pH - 6	(-)	(+)	(+)	(-)	(-)	(+)	(-)	(-)	(-)	(+)	(-)	(-)

Cadmium analysis of five plant extracts showed values ranging from 0.0074 - 0.16 mg/kg which is below the ADI of 0.3 mg/kg set by the WHO.[16] Moreover, lead content of the extracts was below the lowest detection limit (0.010 mg/kg) which is far below the ADI of 0.05 mg/kg established by FAO/WHO[17] in 1987.



	P. guajava	P. odorata	M. pudica	A. sativum	Z. officinale	(+) control
0.25 mg/mL	5.1624	21.9847	-5.1052	0.3956	6.5959	84.2485
0.50 mg/mL	52.1430	65.3134	13.8622	0.8875	2.8057	83.0967
1.00 mg/mL	76.8566	57.8867	57.4253	0.7971	15.9633	83.8398
5.00 mg/mL	64.1565	20.7646	54.2337	0.3077	12.5290	83.2710

Figure 1. DPPH activity of five ethanolic plant extracts with ascorbic acid as positive control.

Figure 1 shows the percentage DPPH activity of the five plant extracts with ascorbic acid as positive control. Among the extracts, *P. guajava* and *P. odorata* showed the highest DPPH activity at 1.00 and 5.00 mg/mL, and 0.25 and 0.50 mg/mL, respectively. Statistical analysis showed significant decrease *($p < 0.00001$)* in DPPH activity of all plant extracts relative to positive control at 0.25 – 5.00 mg/mL concentrations.

Table 3 presents the ethanolic plant extracts which were used as preservatives in ointment formulation against three test organisms, with methylparaben and propylparaben combination as positive control. The results showed that *P. guajava* can prevent the growth of *S. aureus* up to 28 days, followed by *P. odorata* and *Z. officinale* for 7 days. *E. coli* was inhibited by *P. guajava* and *Z. officinale* up to 28 days whereas *A. niger* was inhibited by all plant extracts and positive control except *A. sativum* up to 28 days**.**

Table 3: Preservative challenge test of ointment formulations using five ethanolic plant extracts with methylparaben and propylparaben combination as positive control

Plant	*S. aureus*			*E. coli*			*A. niger*		
	Day 7	Day 14	Day 28	Day 7	Day 14	Day 28	Day 7	Day 14	Day 28
P. guajava	NG	NG	NG	NG	NG	NG	NG	NG	NG
P. odorata	NG	WG	WG	WG	WG	WG	NG	NG	NG
M. pudica	WG	WG	WG	WG	WG	WG	NG	NG	NG
A. sativum	WG	WG	WG	WG	WG	WG	WG	WG	WG
Z. officinale	NG	WG	WG	NG	NG	NG	NG	NG	NG
(+) control	WG	WG	WG	WG	WG	WG	NG	NG	NG

WG – With growth, NG – No growth

Table 4 shows the physical characteristics of ointment packed in transparent and amber container which remained constant at room temperature as there was no difference from baseline (0 day) until the 45th day of the test.

Table 4: Stability of ointment formulations using five ethanolic plant extracts at room temperature.

Sample	Transparent container			Amber container		
	Color	Texture	Odor	Color	Texture	Odor
	Day 1 - 45	Day 1 - 45	Day 1 - 45	Day 1 - 45	Day 1 - 45	Day 1 - 45
Psidium guajava	Off white	Smooth	Odorless	Off white	Smooth	Odorless
Premna odorata	Yellowish	Smooth	Odorless	Yellowish	Smooth	Odorless
Mimosa pudica	Off white	Smooth	Odorless	Off white	Smooth	Odorless
Allium sativum	White	Smooth	Odorless	White	Smooth	Odorless
Zingiber officinale	White	Smooth	Odorless	White	Smooth	Odorless
(+) Control	White	Smooth	Odorless	White	Smooth	Odorless

Stability can also be influenced by temperature which may affect the physical and chemical characteristics of drugs. A change in color was noted on the 30th day of the test which remained until the 45th day in both transparent and amber containers of *P. guajava* and *M. pudica* ointments subjected to 40°C in oven. On the other hand, the rest of the pre-formulated ointment extracts remained constant for 45 days (**Table 5**).

Table 5: Stability of ointment formulations of five ethanolic extracts at 40^0C.

Sample	Transparent container				Amber container			
	Color		Texture	Odor	Color		Texture	Odor
	Day 1 - 15	Day 30 - 45	Day 1 - 45	Day 1 - 45	Day 1 - 15	Day 30 - 45	Day 1 - 45	Day 1 - 45
Psidium guajava	Off-white	Brownish	Smooth	Odorless	Off-white	Brownish	Smooth	Odorless
Premna odorata	Yellowish		Smooth	Odorless	Yellowish		Smooth	Odorless
Mimosa pudica	Off-white	Yellowish	Smooth	Odorless	Off-white	Yellowish	Smooth	Odorless
Allium sativum	White		Smooth	Odorless	White		Smooth	Odorless
Zingiber officinale	White		Smooth	Odorless	White		Smooth	Odorless
(+) Control	White		Smooth	Odorless	White		Smooth	Odorless

Changes in the chemical properties, exhibited by the presence of additional spots in the TLC (degradation products), of *P. guajava* and *M. pudica* pre-formulated ointments were observed at day 30 as a result of increased storage temperature (**Figure 2**).

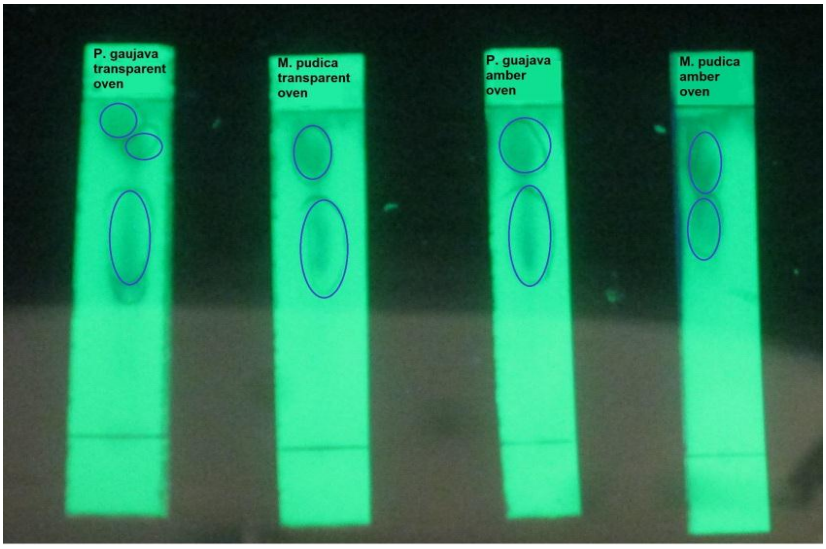


Figure 2: Thin layer chromatography (TLC) plates of ointment formulations of *P. guajava* and *M. pudica* ethanolic extracts exposed to 40°C at day 30. Degradation products are shown in blue circles.

DISCUSSION

Five local plants were tested to determine their capability as substitute for synthetic preservatives. A preservative challenge test is a microbial evaluation of the product's ability to kill or prevent the growth of microorganisms over a specific period of time to ensure product quality and safety.[4] Based on the requirements of the regulatory bodies, only *P. guajava* pre-formulated ointment met the requirements set by USP, European and Japan Pharmacopeia[18] against gram (+) and (-) bacteria while *Z. officinale* met the requirements for gram (-)bacteria. On the other hand, all extracts, except *A. sativum*, conformed to the requirements for fungi. Interestingly, combination of methyl- and propylparaben used as positive control for ointment formulations, both known as antimicrobial preservatives, conformed only to the requirements of preservative challenge test for fungi.

The activity of the extracts against bacteria and fungi may be attributed to the broad range of secondary metabolites present in the plant extracts such as tannins and polyphenols, glycosides, saponins and flavonoids.

Flavonoids are widely known to protect plants from microbial infection by forming complexes with bacterial cell wall, causing disruption of cell envelopes and inactivation of specific bacterial enzymes.[19] Studies revealed that flavonoids from ethanolic extract of *P. guajava* exhibited bactericidal effect against gram-positive and gram-negative bacteria.[20 - 21]

Pinzon *et al.*[22] also isolated two flavonoids, diosmetin and acacetin, from the leaves of *P. odorata* which may be the basis for its antimicrobial activity.

Tannins are also known to exhibit antibacterial action by inactivating bacterial adhesins, enzymes, cell envelope and transport proteins.[23] Olajide *et al.*[24] showed that *P. guajava* leaves contain essential oils rich in tannins which may contribute to its antimicrobial activity. A study [25] demonstrated that *A. sativum* and *Z. officinale* inhibited gram-positive and gram-negative bacteria attributed to secondary metabolites allicin and gingerol present in *A. sativum* and *Z. officinale*, respectively. Various studies have been reported for the antimicrobial activity of saponins against bacteria and fungi,[26 - 28] possibly by its membranolytic properties.[29]

Antioxidant activity of the extracts was also determined using DPPH assay in which *P. odorata* and *P. guajava* exhibited the highest DPPH activity at 0.25 – 0.50 mg/mL and 1 – 5 mg/mL concentrations, respectively. The presence of tannins in *P. guajava* contribute to the antioxidant capacity by increasing the levels of oxidant scavenging proteins [30] thus, reducing the damaging effects of reactive oxygen species (ROS). Alkaloids also promote the antioxidant property by playing a defensive role against pathogens via inhibition of singlet oxygen species, superoxide radical and lipid peroxidation, thus, protecting the cells against ROS.[31] Antioxidant activity can also be attributed to presence of phenolic compounds in the leaves of *P. guajava*.[32]

Safety study conducted on the five plant extracts, in terms of heavy metal testing, demonstrated that the extracts are safe, as shown by cadmium and lead contents below the ADI of 0.3 mg/kg and 0.05 mg/kg, respectively. Moreover, stability studies showed comparable results with the synthetic preservatives on ointment formulations.

Based on the results presented, *P. guajava* exhibited the highest antioxidant capability and showed efficacy as a preservative against *S. aureus*, *E. coli* and *A. niger* which may be attributed to the secondary metabolites present in the extract. It was concluded that *P. guajava* showed good antioxidant and antibacterial activities against common pharmaceutical pathogens which cause spoilage of this product. Moreover, *P. guajava* was noted to be safe and stable in ointment formulation, suggesting its potential use as preservative.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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