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Selected Philippine Plant Extracts as Alternative Preservatives for a Pharmaceutical Liquid Preparation

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Preservatives play an essential role in enhancing quality and prolonging shelf-life of pharmaceutical products by improving their antimicrobial stability or reducing the amounts of oxidative degradation products. Persistent use of synthetic compounds as preservatives resulted in several reports of undesirable effects. Hence, development of alternatives is necessary to maintain their vital function while minimizing adverse effects. In this study, ethanolic extracts of five plants with known antimicrobial activities, *Psidium guajava, Premna odorata, Mimosa pudica, Allium sativum* and *Zingiber officinale*, were formulated into suspensions and evaluated for preservative activity using the United States Pharmacopeia (USP) (2015) guidelines. Phytochemical test, antioxidant activity and compatibility test were also conducted on the extracts. *Premna odorata (p=0.999)* and *Mimosa pudica (p=0.054)* at 5.00 mg/mL concentration exhibited comparable antioxidant activity against the standard antioxidant preservative, butylated hydroxytoluene, using ferric reduction antioxidant power assay. Based on the criteria for product category 4 of the USP, suspensions of *Premna odorata* and *Psidium guajava* demonstrated acceptable preservative activity against selected microorganisms, *Escherichia coli* and *Staphylococcus aureus*. These bioactivities can be attributed to the phytochemicals present in the extracts such as glycosides, reducing substances, flavonoids and alkaloids. In conclusion, for the USP category 4 products such as antacid suspensions, *Psidium guajava* can be utilized as an alternative source of antimicrobial preservative, *Mimosa pudica* as an alternative source of antioxidant preservative, and *Premna odorata* as an alternative source of preservative with both antimicrobial and antioxidant efficacy.

Key words: Compatibility test, plant extracts, *Premna odorata*, preservatives, preservative challenge test, *Psidium guajava*

INTRODUCTION

The pharmaceutical industry utilizes preservatives on their products to improve quality by retaining color, texture and flavor, and prolong shelf-life. Pharmaceutical preparations are prone to the growth of microorganisms due to contamination while frequent exposure to light and air may yield products of oxidative degradation, both of which are detrimental to the health of consumers. Synthetic or chemical preservatives, such as bisulfites, nitrates, butylated hydroxytoluene (BHT), benzoic acid, methylparaben and propylparaben, have been used for decades in pharmaceutical preparations (Abdel-Azi et al. 2016) and thus, are readily available. Their mechanisms of action are based on their antioxidant, antimicrobial or anti-enzymatic properties. However, various studies have been conducted which demonstrated their ability to cause undesirable effects such as allergic reactions (Wilson and Bahna 2005), carcinogenicity (Gharavi et

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al. 2007), behavioral changes (McCann et al. 2010) and even mental performance (Shee et al. 2010). This growing awareness on these effects resulted in the search for alternative sources.

Numerous reports demonstrated the antibacterial and antioxidant activities of the extracts of common Philippine plants such as *Psidium guajava* (Chen and Yen 2007; Rattanachaikunsopon and Phumkhachorn 2010), *Premna odorata* (Pinzon et al. 2011), *Mimosa pudica* (Abirami et al. 2014), *Allium sativum* and *Zingiber officinale* (Karrupiah and Rajaram 2012). With such bioactivities, these plants may be potential sources of natural preservatives. In this study, selected Philippine plant extracts were assessed for their potential preservative capability in an antacid suspension.

MATERIALS AND METHODS

Collection and extraction of plant materials

Five local plants with known antimicrobial property were selected based on their availability and use as food. *Psidium guajava* (PG), *Premna odorata* (PO) and *Mimosa pudica* (MP) were collected from the grounds of Fine Arts Building, University of the Philippines Diliman while *Allium sativum* (AS) and *Zingiber officinale* (ZO) were bought from the Paco, Manila market. The plant samples were authenticated by the National Museum Plant Division Philippines with control number 0932.

The leaves of PG, PO and MP, cloves of AS, and rhizomes of ZO were washed thoroughly with water to remove dirt. The samples were cut into small pieces and air-dried. The dried samples were macerated with 95% ethanol for 48 hours followed by filtration, evaporation and drying. The dried extracts were subjected to phytochemical screening and testing for antioxidant activity for the characterization of the plant extracts.

Phytochemical Screening

Qualitative phytochemical screening was done following the standard method of Trease and Evans (1989) with some modifications. The plant extracts were tested for the presence of secondary metabolites (glycosides, reducing substances, alkaloids, plant acids, saponins, saponins and sapogenins, flavones/flavonols and flavonoids).

in vitro antioxidant activity

Assessment of antioxidant activity was done using the ferric reduction antioxidant power (FRAP) assay following the method of Subhashini et al. (2011) with some modifications. This assay depends on the reduction of ferric tripyridyltriazine complex to ferrous tripyridyltriazine by a reductant at low pH. Different tubes containing 750 µL of phosphate buffer and 750 µL of potassium ferricyanide were added with 300 µL of ethanolic plant extracts at different concentrations (0.25, 0.50, 1.00 and 5.00 mg/mL). The mixtures were incubated at 50°C for 30 mins followed by the addition of 750 µL trichloroacetic acid and centrifugation for 10 min at a speed of 3000 rpm. A 500-µL upper layer of the mixtures were transferred to a tube, diluted with 500 µL distilled water and added with 100 µL fresh ferric chloride. The mixtures were measured at 700 nm using a UV-Vis spectrophotometer (UH5300, Hitachi, Tokyo, Japan). The reducing power of each extract was calculated using Equation 1. Distilled water was used as blank while the standard antioxidant preservative, butylated hydroxytoluene, BHT (Brewer 2011), was used as positive control.

$$\% \text{ Reducing power} = 100 \times \frac{\text{Absorbance}_{\text{sample}} - \text{Absorbance}_{\text{blank}}}{\text{Absorbance}_{\text{sample}}} \quad (1)$$

Preparation of antacid suspension

Ethanolic extracts of the plant samples were used to replace the synthetic preservative, propylparaben, in the formulation. Special and meticulous processing and handling of the formulations were observed to limit microbial load. The composition of the suspension is presented in Table 1.

Compatibility studies

The compatibility of each excipient/active pharmaceutical ingredient (API) in the suspension, with plant extracts as preservatives, was determined by TLC analysis. Each

Table 1. Formulation of the oral antacid suspensions (qs – *quantum sufficiat*).

Component	Composition (%w/v)	Use
Aluminum hydroxide	6	Active pharmaceutical ingredient
Sorbitol solution	40	Humectant
Syrup	10	Filler, flavour enhancer
Glycerin	20	Co-solvent, viscosity enhancer
Xanthan gum	0.5	Suspending/gelling agent
Propylparaben (replace with plant extract)	0.2	Antimicrobial preservative
Strawberry flavor	4	Flavouring agent
Purified water	qs	Diluent

excipient/API was spotted into separate TLC plates, and the TLC profiles were determined by visualization under long (366 nm) and short (254 nm) wavelength UV to serve as baseline profiles. The same procedure was done on the plant extracts.

The compatibility of each plant extract with individual excipients/API was then assessed. A 1:1 weight ratio of plant extract and excipient/API was mixed and dissolved in 5 mL of 99% ethanol. The resulting solution was spotted onto TLC plates and the R_f value was determined using Equation 2. Significant changes in the R_f values of the spots in the mixture profile when compared to the corresponding baseline profile of the extracts, and the appearance of additional spots served as indications of possible incompatibility between the specific excipient/API and extract.

$$\text{Rf value} = \frac{\text{Distance travelved by solute front}}{\text{Distance travelved by solvent front}} \quad (2)$$

Preservative challenge test

The preservative capacity of the formulated suspensions was evaluated following the guidelines of antimicrobial effectiveness testing of the USP (USP 2015). *Escherichia coli* (ATCC 25922) and *Staphylococcus aureus* (ATCC 25923) were used as test microorganisms, and each was diluted with 0.9% sterile saline solution to a density of 0.5 McFarland standard (0.05 mL 1.175% barium chloride in 9.95 mL 1% sulfuric acid), equivalent to $1x10^8$ colony forming units (cfu)/mL. A 1-mL inoculum was transferred to the suspensions and mixed thoroughly. The resulting solutions were incubated at 22.5 ± 2.5°C. A 1-mL aliquot of each solution was sampled at 0, 14 and 28 days, diluted with sterile saline solution, transferred to soybean casein digest agar and incubated at 32.5 ± 2.5°C for 24 hours to determine the number of cfu/mL using dilution pour-plate method. The formulated suspension containing propylparaben as antimicrobial preservative was used as positive control. Bacteria should have no increase from the initial calculated count at 14 and 28 days to conform with the USP criteria for product category 4 (antacids made with an aqueous base).

Statistical treatment

The data gathered in the antioxidant, compatibility and antimicrobial effectiveness tests were recorded as mean ± SEM (standard error of mean). The number of cfu/mL from the preservative challenge test was converted to log values. Log reductions were computed based on the change in log values for each sampling day and at day 0, and were used to compare the preservative activity of the positive control and the plant extract formulations. Significant differences between the positive control and plant extracts were determined by one-way analysis of variance (ANOVA) followed by Tukey's *post hoc* test using SPSS 17.0 software. Mean values were considered statistically significant when $p<0.05$.

RESULTS

Phytochemical screening

PO and MP extracts were at neutral pH while PG, AS and ZO extracts were at pH 6.0. All extracts revealed the presence of glycosides and sapogenins, and absence of saponins. The results of the phytochemical screening were summarized in Table 2**.**

Table 2. Phytochemical screening of ethanolic extracts of five selected plants. PG – *Psidium guajava*, PO – *Premna odorata*, MP – *Mimosa pudica*, AS – *Allium sativum*, ZO – *Zingiber officinale*.

Phytochemicals	**Plant extracts**				
	PG	PO	MP	AS	ZO
Glycosides	(+)	(+)	(+)	(+)	(+)
Reducing substances	(+)	(+)	(+)	(-)	(+)
Alkaloids	(+)	(-)	(-)	(-)	(-)
Plant acids	(-)	(-)	(+)	(+)	(-)
Saponins	(-)	(-)	(-)	(-)	(-)
Saponins and sapogenins	(+)	(+)	(+)	(+)	(+)
Flavones and flavonols	(+)	(-)	(-)	(-)	(-)
Flavonoids	(+)	(-)	(+)	(-)	(-)

***in vitro* antioxidant activity**

An increase in antioxidant activity of the extracts is noted with increasing concentration (Figure 1). However, most of the extracts exhibited antioxidant activity below the positive control BHT, except for PO, at 5.00 mg/mL. The highest FRAP activity among the extracts was demonstrated by PO, with significant reduction in the antioxidant activity compared to BHT at 0.25 *(p=0.001),* 0.50 *(p=0.001)* and 1.00 *(p=0.001)* mg/mL concentrations. Only the 5.00 mg/mL concentration of PO *(p=0.999)* and MP *(p=0.054)* produced comparable antioxidant activity relative to BHT.

Compatibility studies

Baseline TLC profiles of the plant extracts showed that PO and PG produced two spots while MP, AS and ZO displayed only one spot (Table 3). On the other hand, all the components of the suspension produced a weak, non-distinguishable spots on the TLC plates, except for strawberry flavor which exhibited one faint spot.

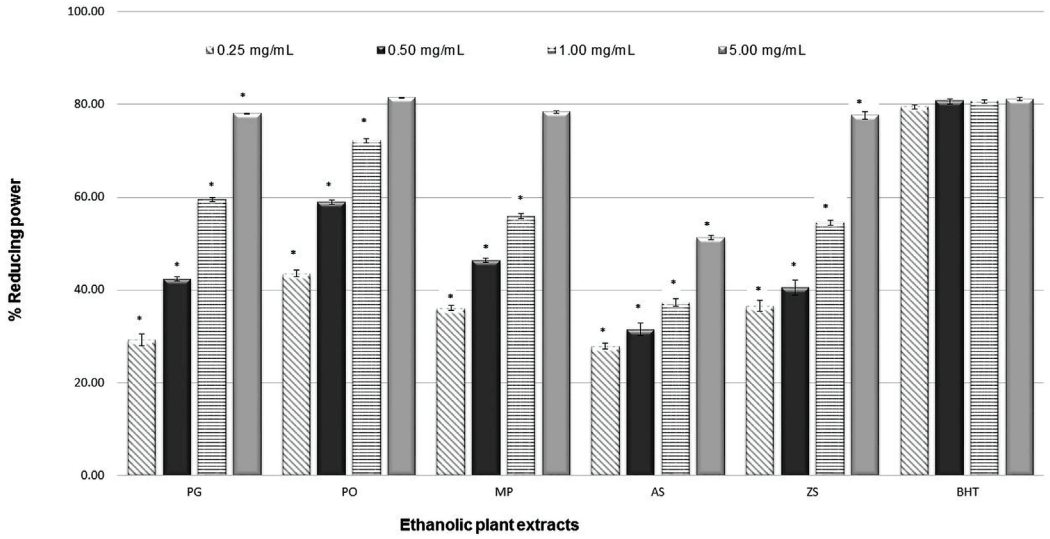

Figure 1. Ferric reduction antioxidant power (FRAP) assay of five ethanolic plant extracts with butylated hydroxytoluene as positive control. PG - *Psidium guajava*, PO - *Premna odorata*, MP - *Mimosa pudica*, AS - *Allium sativum*, ZO - *Zingiber officinale*. * - Significant difference with positive control ($p<0.05$).

Table 3. Baseline TLC profiles of the individual plant extracts and components of the antacid suspension.

Components	R_f value ± SEM
Plant extract	
Psidium guajava (PG)	0.69 ± 0.082, 0.54 ± 0.13
Premna odorata (PO)	0.84 ± 0.052, 0.67 ± 0.039
Mimosa pudica (MP)	0.94 ± 0.17
Allium sativum (AS)	0.95 ± 0.041
Zingiber officinale (ZO)	0.97 ± 0.028
API: Aluminum hydroxide	0
Excipient	
Sorbitol	0.93 ± 0.023
Syrup	0.95 ± 0.033
Glycerin	0.83 ± 0.022
Xanthan gum	0.92 ± 0.031
Strawberry flavor	0.41 ± 0.012

Table 4. TLC profiles of ethanolic plant extracts and excipient/API combination.

Mixture	R_f value ± SEM	p values
Psidium guajava (PG) extract		
PG-aluminum hydroxide	0.84 ± 0.047, 0.66 ± 0.054	0.98, 0.79
PG-sorbitol	0.94 ± 0.17, 0.69 ± 0.033	0.84, 0.56
PG-syrup	0.94 ± 0.17, 0.74 ± 0.023	0.84, 0.27
PG-glycerin	0.91 ± 0.11, 0.67 ± 0.056	0.91, 0.75
PG-xanthan gum	0.87 ± 0.15, 0.69 ± 0.082	0.96, 0.61
PG-strawberry flavor	0.93 ± 0.19, 0.66 ± 0.054	0.86, 0.79
Premna odorata (PO) extract		
PO-aluminum hydroxide	0.84 ± 0.047, 0.73 ± 0.034	1.00, 0.76
PO-sorbitol	0.83 ± 0.0078, 0.77 ± 0.012	1.00, 0.25
PO-syrup	0.85 ± 0.016, 0.72 ± 0.0039	1.00, 0.91
PO-glycerin	0.82 ± 0.076, 0.72 ± 0.027	1.00, 0.91
PO-xanthan gum	0.87 ± 0.14, 0.75 ± 0.036	1.00, 0.48
PO-strawberry flavor	0.83 ± 0.0067, 0.71 ± 0.034	1.00, 0.95
Mimosa pudica (MP) extract		
MP-aluminum hydroxide	0.84 ± 0.042	0.94
MP-sorbitol	0.91 ± 0.031	1.00
MP-syrup	0.91 ± 0.054	1.00

Table 4 continuation . . .

MP-glycerin	0.90 ± 0.016	1.00
MP-xanthan gum MP-strawberry flavor	0.90 ± 0.023 0.90 ± 0.0078	1.00 1.00
Allium sativum (AS) extract		
AS-aluminum hydroxide	0.95 ± 0.039	1.00
AS-sorbitol	0.93 ± 0.19	1.00
AS-syrup	0.98 ± 0.040	1.00
AS-glycerin	0.86 ± 0.086	0.98
AS-xanthan gum AS-strawberry flavor	0.92 ± 0.034 0.74 ± 0.023	1.00 0.56
Zingiber officinale (ZO) extract		
ZO-aluminum hydroxide	0.93 ± 0.039	0.99
ZO-sorbitol	0.97 ± 0.030	1.00
ZO-syrup	0.95 ± 0.039	1.00
ZO-glycerin	0.99 ± 0.031	1.00
ZO-xanthan gum	0.99 ± 0.043	1.00
ZO-strawberry flavor	0.97 ± 0.030	1.00

*Significant difference ($p<0.05$) with the Rf value of the corresponding plant extract.

The R_f values of the combination of extracts and excipients/API (Table 4) showed similar values with the baseline TLC profiles of the plant extract, with no additional spots appearing on the TLC plates.

Preservative challenge test

All the formulated suspensions showed a decrease in the number of *E. coli* at days 14 and 28 from the initial count (day 0) which indicates their potent preservative activities (Table 5). Significant decrease in the log reduction values of all the formulated suspensions compared to the positive control formulation was observed at day 14. However, at day 28, statistically significant increase was demonstrated by all the formulated suspensions *(p=0.001)* with respect to the positive control formulation, except for AS *(p=0.542)* which displayed comparable results.

For *S. aureus*, only PG and PO displayed reduction of count on days 14 and 28 while the rest demonstrated decrease only at day 14. It is noteworthy that the standard preservative, propylparaben, also showed an increase in *S. aureus* growth at days 14 and 28. Statistical analysis revealed significant increase in the log reduction values of all the formulated suspension *(p=0.001)* when compared with the positive control formulation at day 14. However,

Table 5. Antimicrobial effectiveness testing of different ethanolic plant extracts against *S. aureus* and *E. coli* analyzed at days 0, 14 and 28, with propylparaben as positive control.

Plant extracts	**Microbial count ± SEM, cfu/mL x 10^5**			**Log reduction ± SEM**	
	Day 0	Day 14	Day 28	Day 14	Day 28
E. coli					
PG	117 ± 3.48	28.3 ± 5.08	0.033 ± 0.0030	0.63 ± 0.076*	3.55 ± 0.029*
PO	7.58 ± 0.83	33.3 ± 3.87	0.033 ± 0.0050	0.36 ± 0.054*	3.37 ± 0.070*
MP	128 ± 3.18	3.40 ± 0.46	0.10 ± 0.020	1.58 ± 0.054*	3.12 ± 0.092*
AS	58.5 ± 1.12	3.00 ± 0.48	0.10 ± 0.011	1.30 ± 0.065*	2.77 ± 0.056
ZO	132 ± 11.6	13.9 ± 1.81	0.10 ± 0.015	0.98 ± 0.018*	3.13 ± 0.10*
PP	39.0 ± 3.83	0.15 ± 0.029	0.10 ± 0.020	2.43 ± 0.050	2.60 ± 0.056
S. aureus					
PG	102 ± 4.67	81.0 ± 3.24	9.00 ± 0.12	0.10 ± 0.018*	1.05 ± 0.024*
PO	213 ± 44.2	135 ± 21.6	7.50 ± 0.40	0.19 ± 0.078*	1.43 ± 0.081*
MP	270 ± 37.7	150 ± 37.7	810 ± 33.5	0.27 ± 0.081*	<0.10
AS	330 ± 31.6	159 ± 25.7	990 ± 26.3	0.33 ± 0.080*	<0.10
ZO	180 ± 18.0	129 ± 10.8	540 ± 26.4	0.14 ± 0.068*	<0.10
PP	9.00 ± 0.23	108 ± 14.9	15.4 ± 7.36	<0.10	<0.10

PG – *Psidium guajava*, PO – *Premna odorata*, MP – *Mimosa pudica*, AS – *Allium sativum*, ZO – *Zingiber officinale*, PP – propylparaben (positive control formulation), cfu – colony forming units. *Significant difference with positive control formulation at p<0.05.

only PG *(p=0.001)* and PO *(p=0.001)* demonstrated significant increase in the log reduction values at day 28 in comparison to the positive control formulation.

DISCUSSION

For years, it has been demonstrated that the antimicrobial and antioxidant activities of the plants have been attributed to the bioactive components present in the plant extracts. In the present study, five locally available plants in the Philippines were analyzed to determine their antimicrobial and antioxidant properties for preservative use.

Premna odorata and *Mimosa pudica* ethanolic extracts showed potent antioxidant activities at 5.00 mg/mL concentration, with comparable activity to the standard antioxidant preservative, butylated hydroxytoluene. Glycosides, present in both *Premna odorata* and *M. pudica* extracts, contain hydroxyl groups which chelate cations, thus, inhibiting generation of reactive species and decomposition of lipid peroxides (Brewer 2011). The study by Otsuka et al. (1989) showed two iridoid glycosides, 2”- and 3”-caffeoyl-6-α-L-rhamnopyranosylcatalpol, from *Premna odorata* while *M. pudica* contains phenolic and purine glycosides, turgorins (Johnson et al. 2014). Reducing substances, also found in the ethanolic extracts of two plants, are potent antioxidant agents that can scavenge species that initiate peroxidation and reduce localized O_2 concentrations, leading to termination of oxidative chain reactions (Nawar 1996), which are damaging to cells.

Chemical interactions between extracts and excipients that yield new chemical entities may manifest through significantly different R_f values from baseline profiles, or the appearance of new spots in the TLC plates. The results showed no significant difference between the R_f values of baseline profiles with that of combination profiles. Appearance of additional spots on any of the TLC profiles was also not observed. The results were conclusive that the excipients used in the formulation had no compatibility issues with any of the plant extracts used.

Two microorganisms were used for the antimicrobial effectiveness test, including *S. aureus* for gram (+) bacteria and *E. coli* for gram (-) bacteria. Based on the criteria set by the USP, only *Premna odorata* and *Psidium guajava* conformed to the preservative standards of antacid suspensions against *E. coli* and *S. aureus* while the rest of the extracts complied with the requirements for *E. coli* only. Glycosides and reducing substances present in both extracts also work as antimicrobial agents by disrupting the bacterial cell wall, which results in leakage of proteins and other compounds necessary for the bacteria’s growth and survival. This leakage then results in bacterial cell death (Anandhi et al. 2014). Flavones and flavonoids found in the phytochemicals of *Psidium guajava* extracts contribute to the antimicrobial activity by forming complexes with bacterial cell wall and extracellular soluble proteins, thus, disrupting the bacterial cell membrane (Enwa et al. 2014). Alkaloids, also present in the ethanolic extracts of *Psidium guajava,* are potent antimicrobials that work by inhibiting nucleic acid synthesis and division of bacterial cells. Alkaloids also act by disrupting bacterial outer membrane and cytoplasmic membrane integrity, leading to leakage of cytoplasmic content and eventually, cell death (Cushnie et al. 2014).

CONCLUSION

For antacid suspensions, *Psidium guajava* can be utilized as an antimicrobial preservative while *Mimosa pudica* as an antioxidant preservative. On the other hand, *Premna odorata* can be used as an alternative source for both antioxidant and antimicrobial preservatives. A better detail on the preservative capability of these extracts can be achieved by testing with other microorganisms indicated in the USP, such as yeasts and molds. Isolation of the metabolite/s responsible for the bioactivities and their mechanisms of action can also be further investigated.

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CONFLICT OF INTEREST

The authors declare no conflict of interests in this study.

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