INTERNATIONAL JOURNAL OF ADVANCES IN PHARMACY, BIOLOGY AND CHEMISTRY

Research Article

Evaluation of antibacterial, antioxidant and physicochemical properties of formulated polyherbal ointments

A.D.A.H. Kumarasiri, H.I.C. De Silva*, G.H.C.M. Hettiarachchi

Department of Chemistry, University of Colombo, Colombo 3, Sri Lanka.

ABSTRACT

The present study aimed to evaluate the antibacterial, antioxidant and physicochemical properties of newly formulated polyherbal ointments. The 5%, 10% and 20% ointments were prepared mixing crude extracts of *Cyperus brevifolius* (roots), *Asparagus gonoclados* (rhizome) and *Psidium guajava* (leaves) in 1:1:2 ratio, respectively, with simple and emulsifying ointment bases. Antibacterial, antioxidant and physicochemical properties of formulations were evaluated over one month period storing in glass and plastic containers at 4 °C, 25 °C and 38 °C. Both 10% and 20% polyherbal ointments showed antibacterial activity against *Staphylococcus aureus, Bacillus subtilis, Escherichia coli* and *Pseudomonas aeruginosa* with the minimum inhibitory concentrations of 12.5, 12.5, 25 and 50 mg/mL, respectively. Formulations with the two bases showed similar efficacy and stability. Moreover, ointments exhibited satisfactory antioxidant activity, while possessing adequate physicochemical properties. Results conclude that 10% and 20% ointment formulations can be used as a medicament for bacterial skin infections over one month.

Keywords: polyherbal ointment, antibacterial, antioxidant, physicochemical properties

INTRODUCTION

The emergence of antibiotic-resistant is an ever growing threat to global public health while the introduction of novel antibacterial agents is not significant and sufficient for the demand. Therefore, discovery and development of new antibiotics is essential. Traditional knowledge driven drug identification can reduce the cost of drug development through reverse pharmacology. Traditional medicine healers prefer to use crude plant extracts of several herbs, which is to enhance the effectiveness and synergistic action of the treatments known as polyherbal formulations.[1] These herbal preparations can be developed to novel dosage forms which are applicable to modern generations.

In most of the epidermal infections, local application of the drug at the site of the infection would be sufficient and safer as it is lack with systemic side effects.[2] Ointment is a promising external dosage form available for localized treatment which contribute to the therapeutic outcome by increasing absorption of active ingredients.[3] The presence of the antioxidant activity will be an added advantage in antibacterial formulations as antioxidant compounds provide higher skin repairing rate which aid in wound healing and inflammation healing ability.[4]

The plant *Cyperus brevifolius* belongs to the family Cyperaceae. The rhizome of the plant used in the Ayurvedic medicine for the treatment of worm diseases, skin diseases, acne.[5,6] The plant *Asparagus Gonoclados* (Family Asparagaceae) is also used in Ayurvedic remedies for the treatment of wounds, skin diseases, heart diseases, burning sensation.[7,8] *Psidium guajava*, of family Myrtaceae is also important for treatment for skin diseases, wounds, swollen gums, styptic, pulmonary disorders, cough, sprains, diarrhea.[9,10]

The present study was conducted to evaluate antibacterial activity, antioxidant activity and physicochemical properties of polyherbal ointments formulated using above three ethnomedicinal plants, in order to use as medicaments for bacterial skin infections.

MATERIALS AND METHODS

Preparation of plant samples

Herbal plants, *C. brevifolius*, *A. gonoclados* and *P. guajava* were collected from Hikkaduwa, Sri Lanka and authenticated by the National Herbarium at the Department of National Botanical Gardens, Peradeniya, Sri Lanka. The powdered plant parts were extracted with methanol and concentrated until it formed a semisolid residue.

Preliminary Phytochemical Screening

Methanolic extracts of three plant parts were tested for the presence of active phyto-constituents (flavonoids, alkaloids and tannins *etc*.) using standard procedures.[11,12]

Antibacterial bioassays

Agar disc diffusion assay and agar well diffusion assay were used to determine the antibacterial activity of the crude plant extracts and ointment formulations, respectively using *Staphylococcus aureus* (ATCC 25923), *Bacillus subtilis* (MTCC 441), *Escherichia coli* (ATCC 35218) and *Pseudomonas aeruginosa* (ATCC 9027) as test microorganisms. Gentamycin intravenous (IV) solution (1 mg/ml) and sterilized methanol were used as the positive control and the negative control, respectively.[13,14]

Antibacterial bioassays of the crude extracts

Monoherbal plant extracts and polyherbal extracts (600 mg/ml) were prepared using methanol as the solvent. Bioassay was conducted using agar disc diffusion method.

Methanolic extracts of *A. gonoclados*, *C. brevifolius* and *P. guajava* were combined in 10 different ratios according to Table 1 to prepare samples containing 300 mg/ml to find out the most effective combination.

The MIC of the selected polyherbal extract combination was determined by preparing a concentration series of 12.5, 25, 50, 100, 500 and 1000 mg/ml. Antibacterial activities were determined using agar well diffusion assay.

Preparation of ointments and evaluation of antibacterial properties

The selected polyherbal plant extract was incorporated in to both simple and emulsifying ointment bases using "levigation method"[3], to prepare 5%, 10% and 20% ointment formulations. Antibacterial activity studies were carried out using agar well diffusion method. Each well was filled with 100 µl of the polyherbal ointment using 5% DMSO in 1% methanol (4:6) as the solvent.

Determination of physical and chemical properties

The descriptive properties such as colour, odor and consistency, homogeneity, pH and other physical properties such as washability, extrudability and spreadability of the 20% polyherbal ointment formulations were determined were also examined.[15,16]

Determination of the antioxidant activity by reducing power assay

Antioxidant activity of the polyherbal ointment formulation was determined using the reducing power assay using standard methods.[4]

Stability testing

The 10% and 20% ointments were prepared in mass scale, and stored in glass and plastic containers at different temperatures; 4 °C, 25 °C and 38 °C. The stability of the ointments were evaluated over one month by determining the antibacterial and antioxidant activities and physicochemical properties using the standard methods as mentioned above.

Statistical analysis

The inhibition zone diameters were statistically evaluated using two way ANOVA statistics to compare the stability of the ointment formulations.

RESULTS AND DISCUSSION

Phytochemical screening

According to the phytochemical screening studies of methanolic extracts of *A. gonoclados* (roots), *C. brevifolius* (rhizome) and *P. guajava* (leaves) contain flavonoids, alkaloids and tannins. As most antibacterial and antioxidant agents in plants belong to the flavonoid, alkaloid and tannin chemical groups,[12,17] the results confirmed the potent antibacterial and antioxidant activity of methanolic extracts of the selected three plants.[18,19]

Antibacterial bioassays of the crude plant extracts

The monoherbal and polyherbal crude plant extracts were tested by the agar disc diffusion assay to compare its efficacy. The results showed that the polyherbal extracts of 1:1:1 ratio of *C. brevifolius: A.*

gonoclados: P. guajava respectively (600 mg/mL), having a significantly higher antibacterial activity against all four pathogenic bacterial strains tested than the monoherbal extracts (Table 2).[20] This provides an evidence for the increased biological activity due to the synergistic effect of the herbal combinations.[21]

Methanolic extracts of three plants were combined in different ratios (Table 1)[20] to determine the most effective combination. Combination of *C. brevifolius: A. gonoclados: P. guajava* in 1:1:2 ratio (Figure 1), respectively exhibited the best antibacterial activity against Gram-positive bacteria. However, the absence of any antibacterial activity towards Gram-negative bacterial strains may be due to the insufficient concentration (300 mg/mL) of the crude extract.

Minimum inhibitory concentration (MIC) of the polyherbal extract of combination number 4 was examined using a concentration series (12.5, 25, 50, 100, 500 and 1000 mg/mL) in order to identify the minimum active concentration that should be incorporated in to the ointment formulation. The results showed increase in antibacterial activity with increasing concentration against both Gram-positive and Gram-negative bacteria (Figure 2).[1] The polyherbal extract with 12.5 mg/mL displayed an excellence activity against Gram-positive bacterial strains; *B. subtilis* (13.0 ± 1.7 mm)and *S. aureus* (13.0 ± 0.6 mm). Gram-negative bacterial strains exhibited a lower susceptibility to the polyherbal plant extract, where the MIC values were 25 mg/mL against *E. coli* (14.0 ± 0.6 mm) and 50 mg/mL against *P. aeruginosa* (13.0 ± 0.6 mm). Hence, 50 mg/mL concentration was selected in order to incorporate in to the ointment formulation as it was the lowest concentration which exhibited antibacterial activity against all four pathogenic bacterial strains.

Antibacterial bioassays of the ointment formulations

The 5%, 10% and 20% ointment formulations with effective concentrations 25, 50 and 100 mg/mL, respectively were prepared by incorporating the polyherbal extraction to the simple ointment (SO) and emulsifying ointment (EO) bases.[1] All bacterial strains (including Gram-negatives) showed susceptibility towards the sample with 50 mg/mL concentration, where 100 mg/mL concentration showed even higher antibacterial activities (Figure 3). Therefore, MIC of the formulation was about 50 mg/mL which was same as the polyherbal plant extract.

The antibacterial efficacy has not changed after incorporation of the polyherbal extract in to the ointment bases. This implies that the similarities in the diffusion characteristics of the crude polyherbal extract and the ointment formulation.[1] Since, 10% and 20% ointments showed the broad spectrum antibacterial activity those two formulations were used for further studies.

Antioxidant activity of ointment formulations

Antioxidant activities were determined for the simple and emulsifying ointment formulations using the reducing power assay.[4] All the formulations displayed the presence of the antioxidant activity (Figure 4). The antioxidant activity of the 20% ointment samples were higher than the 10% ointment samples. Moreover, reducing power has been increased with the increased concentration of the preparation, where 50 mg/mL sample showed the lowest reducing power and 500 mg/mL sample showed the highest reducing power. Both simple and emulsifying ointment formulations showed very similar antioxidant activity.

Stability test of ointment formulations

When comparing the difference of simple ointment and emulsifying ointment towards the stability, according to the ANOVA test, there is no significant different in antibacterial activity over the time between simple ointment and emulsifying ointment (F value = 0.7 > 0.05). Furthermore there is no significant difference in the effect of container at different temperatures over the one month (F value = 0.9 > 0.05).

When comparing the antibacterial activity of the ointment, the results showed reduction in its activity at all three temperatures (4 °C, 25 °C and 38 °C) over 30 days. Even though activity has been reduced still it showed the activity against both Gram-positive and Gram-negative bacterial strains (Figure 5). According to ANOVA statistics, reduction of the antibacterial activity is statistically significant in samples kept at 4 °C (F value = 0.017 < 0.05) and 38 °C (F value = 0.004 < 0.05). However, there is no significance difference in the reduction of antibacterial activity for samples kept at 25 °C (F value = 0.3 > 0.05). The results concluded that ointment formulations should be kept at 25 °C in order to retain the antibacterial activity as the date of the formulation for 30 days.

The stability of physicochemical activities of both simple and emulsifying ointments were also determined (Table 3). Both simple and emulsifying ointments possessed satisfactory results over one month period. The pH of the ointments were in the

skin pH range of pH 5 to 7. Similar to the antibacterial results, physicochemical properties of 20% ointment formulations kept at 25 °C did not change over 30 days concluding the best storage temperature for ointment is 25 °C.

Antioxidant activity of the 20% simple ointment with 500 µg/µl concentration which stored in glass and plastic containers at 4 °C, 25 °C and 38 °C over one month was tested. When considering the type of containers, there was very little difference in absorbance in all three temperatures (4 °C, 25 °C and 38 °C) (Figure 6). Effect of time over the antioxidant activity was checked and the results showed that though the antioxidant activity was reduced over the time, the reduction was not significant. When comparing the antioxidant activity with the effect of temperature, even though the antioxidant activity reduced with the increasing temperature, the difference of the antioxidant activity was negligible.

CONCLUSION

Combination of methanolic extracts of *C. brevifolius* (root), *A.gonoclados (rhizome)* and *P. guajava (leaves)* in 1:1:2, respectively provided the best antibacterial activity against both Gram-positive and Gram-negative pathogenic bacteria used in the study. Simple ointment and emulsifying polyherbal ointment formulations also exhibited excellence antibacterial activity against both Gram-positive and Gram-negative bacterial strains, where MIC values remained equivalent to the crude polyherbal extract. Both simple ointment and emulsifying ointment formulations showed very similar antibacterial activity, antioxidant activity and physicochemical properties, where the differences were not significant. The type of container (plastic or glass) did not show significant effect on the antibacterial and antioxidant activities and physicochemical stability over the 30 days of storage. The simple and emulsifying ointments kept at 25 °C showed the similar efficacy as the date of formulation, over one month. Hence this formulation can be used as a medicament for skin diseases over one month. However, the formulation need to be further modified to have a proper stability over a considerable time period.

Table 1
Combination of plant extracts in different ratios

Extracts of plants	Extract combination number and ratios									
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
A. gonoclados	1	2	1	1	2	1	1.5	1.5	2	1
C. brevifolius	1	1	2	1	1.5	2	1	2	1	1.5
P. guajava	1	1	1	2	1	1.5	2	1	1.5	2

Table 2
Antibacterial activities of monoherbal versus polyherbal extracts (1:1:1) *(*Average for triplicate trials, (-) mark indicates the absence of an inhibition zone*)*

Bacterial strain	Average inhibition zone (mm) including disc diameter					
	C. brevifolius	***A. gonoclados***	***P. guajava***	**polyherbal extract**	**Positive control**	**Negative control**
B. subtilis	13.0 ± 0.6	8.0 ± 0.6	14.0 ± 0.6	16.0 ± 0.6	22.0 ± 0.6	-
S. aureus	10.0 ± 0.5	8.0 ± 0.5	10.0 ± 0.6	12.0 ± 0.5	21.0 ± 0.5	-
P. aeruginosa	-	7.0 ± 0.5	-	9.0 ± 0.5	18.0 ± 0.5	-
E. coli	-	7.0 ± 0.6	-	10.0 ± 0.6	19.0 ± 0.6	-

Table 3
Physical and chemical properties of the 20% polyherbal ointment formulations kept at 37 °C (as it could be susceptible to physicochemical changes)

Evaluation parameter	Simple ointment formulation		Emulsifying ointment formulation	
	At 0th day	At 30th day	At 0th day	At 30th day
Colour	Yellowish white	Yellowish white	Almost white	Almost white
Odor	Consistency	Consistency	Consistency	Consistency
Consistency	Soft semisolid	Soft semisolid	Soft semisolid	Soft semisolid
Phase separation	Nil	Nil	Nil	Nil
pH	6.45 ± 0.10	6.44 ± 0.03	6.37 ± 0.11	6.37 ± 0.06
Washability	Washable	Washable	Washable	Washable
Extrudability	32.5 ± 1.0	33.0 ± 0.3	30.6 ± 1.1	35.8 ± 0.4
Spreadability	14.2 ± 0.8	14.8 ± 0.9	15.5 ± 0.6	16.1 ± 0.6

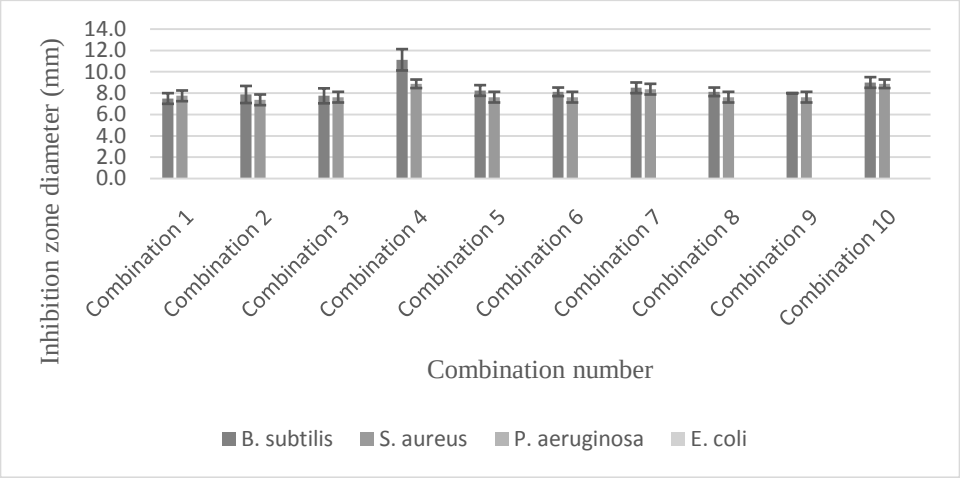

Figure 1
Antibacterial activities of ten different polyherbal combinations

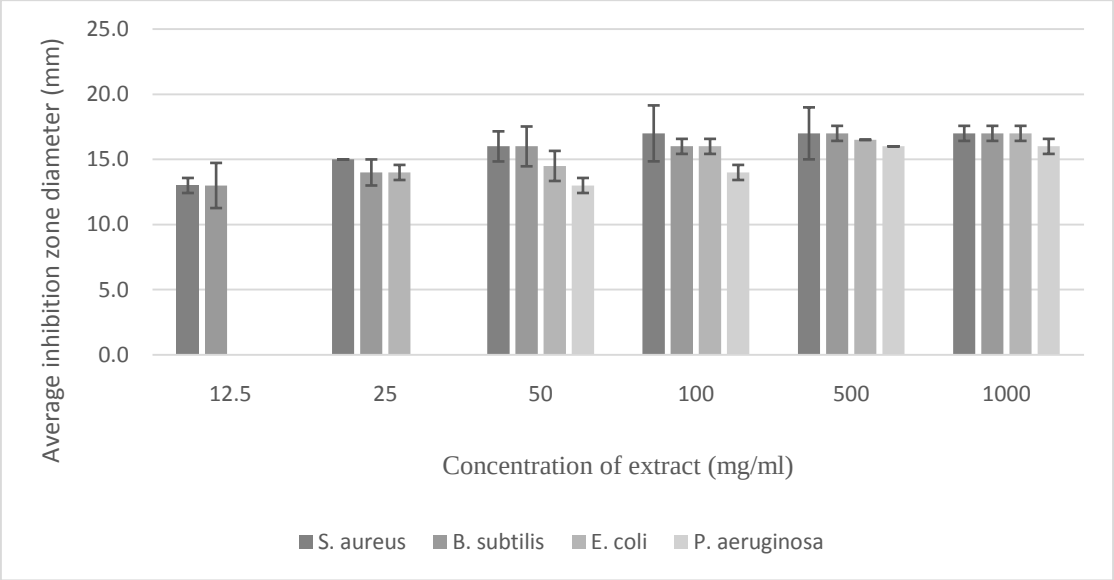

Figure 2
The MIC values of the polyherbal extract *C. brevifolius, A. gonoclados* and *P. guajava* in 1:1:2 (average results for triplicate trials ± standard deviation)

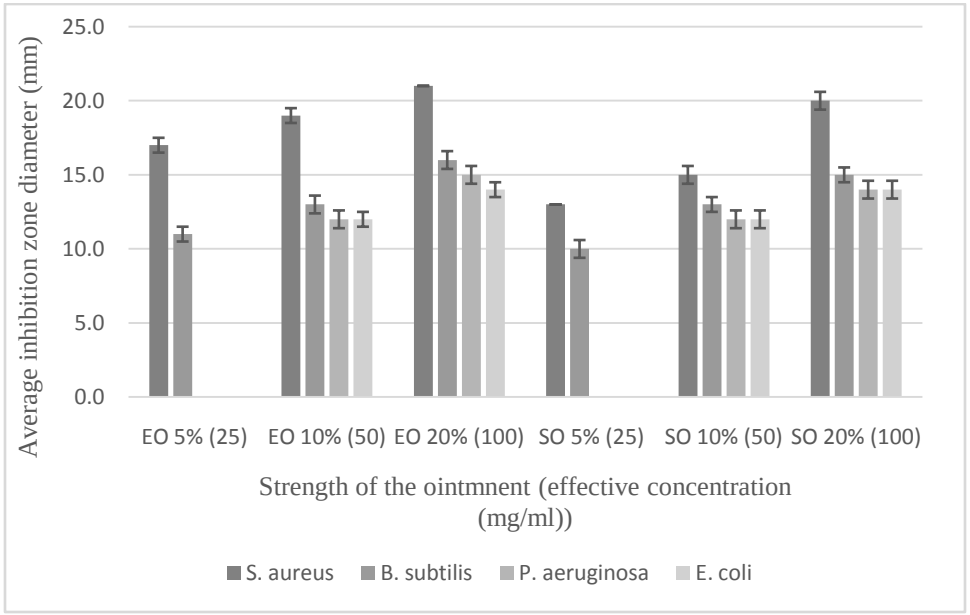

Figure 3
Antibacterial activities of simple ointment (SO) and emulsifying ointment (EO) formulations (average results for triplicate trials ± standard deviation)

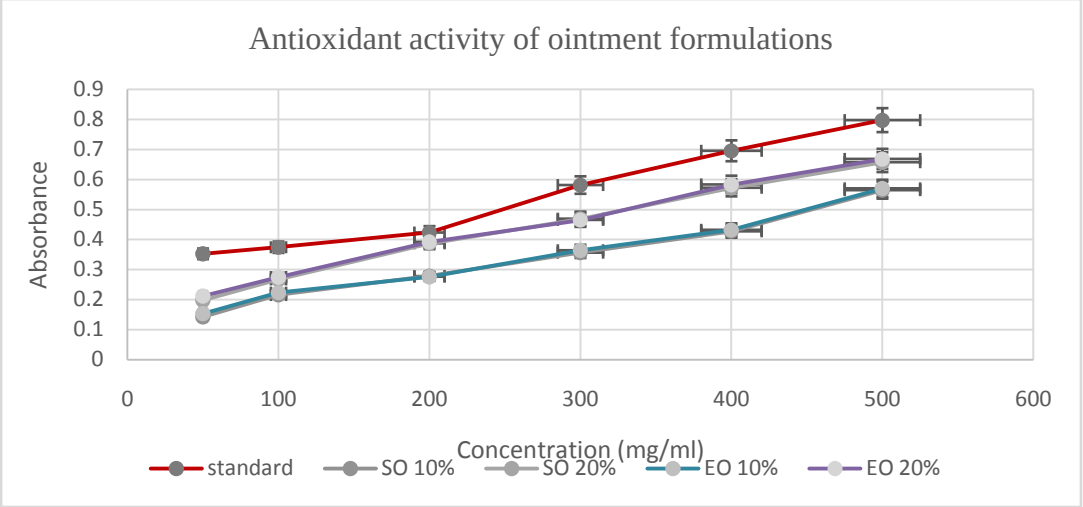

Figure 4
Antioxidant activity of the ointment formulations for (SO- Simple ointment; EO- Emulsifying ointment)

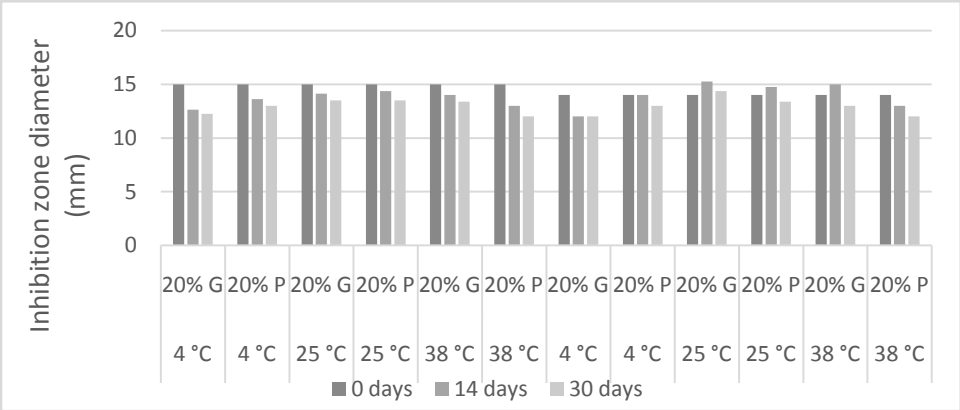

Figure 5
The antibacterial activity of the 20% ointment formulations at different temperatures; 4 °C, 25 °C and 38 °C, over 30 day, against *P. aeruginosa*

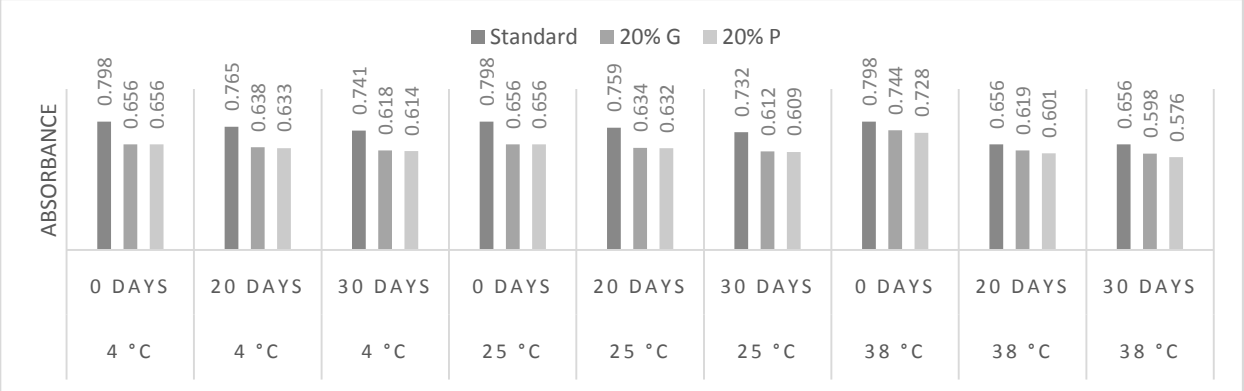

Figure 6
Comparison of antioxidant activity of the simple ointment formulations stored in glass and plastic containers over 30 days. (G- Glass container; P- Plastic container)

REFERENCES

1. Rani GS, Gunda RK, Mansa Y, Vardhan CHK, Kumar VD, Venkateswarlu G. Preparation and Evaluation of Antimicrobial and Antioxidant Activity of Polyherbal Ointment.IntJ Current Trends Pharm Res, 2015; 3(4): 997-1003.
2. Reitamo S, Wollenberg A, Scho E, Perrot JL, Marks R, Ruzicka T, Christophers E, Kapp A, Lahfa M, Rubins A, Jablonska S, Rustin M. Safety and Efficacy of 1 Year of Tacrolimus Ointment Monotherapy in Adults With Atopic Dermatitis. Arch. Dermatol, 2000;136(8): 999-1006.
3. Liu C, Bayer A, Cosgrove SE, Daum RS, Fridkin SK, Gorwitz RJ, Kaplan SL, Karchmer AW, Levine DP, Murray BE, Rybak MJ. Clinical Practice Guidelines by the Infectious Diseases Society of America for the Treatment of Methicillin- Resistant Staphylococcus Aureus Infections in Adults and Children. Clin Infect Dis, 2011;52(3): e18-e55.
4. Jayanthi P, Lalitha P. Reducing power of the solvent extracts of Eichhornia crassipes (Mart) solms. Int J Pharm Pharm Sci, 2011; 3(3): 3-5.
5. Parekh J, Chanda S. In-Vitro Antimicrobial Activities of Extracts of Launaea Procumbens Roxb. (Labiateae), Vitis Vinifera L. (Vitaceae) and Cyperus Rotundus L. (Cyperaceae). Afr J Biomed Res, 2006;9(2): 89-93.
6. Lettre DP, Sharma SK, Singh AP. Antimicrobial Investigations on Rhizomes of Cyperus Rotundus Linn. Der pharmacia Lettre, 2011;3(3): 427-431.
7. Madhavan V, Tijare RD, Mythreyi R, Gurudeva MR, Yoganarasimhan SN. Pharmacognostical Studies on the Root Tubers of Asparagus Gonoclados Baker – Alternate Source for the Ayurvedic Drug Shatavari. Indian J Nat Prod Resour, 2010; 1(1): 57-62.
8. Patel LS, Patel RS. Antimicrobial Activity of Asparagus Racemosus Wild From Leaf Extracts a Medicinal Plant. Int J Sci Res Pub, 2013;3(3): 3-5.
9. Esimone CO, Attama AA, Mundi KS, Ibekwe NN, Chah KF. Antimicrobial Activity of Psidium Guajava Linn Stem Extracts against Methicillin-Resistant Staphylococcus Aureus. Afr J Biotechnol, 2012;11(89): 15556-15559.
10. Ali I, Khan SZ, Nasir H, Ullah I, Ullah A, Ahmad R, Ali M, Khan S. In-Vitro Antibacterial Activity of Leaf Extracts of Psidium Guajava against Selected Pathogenic Bacterial Starins. Int J Innov Sci Res, 2014; 3(1): 10-13.
11 Edeoga HO, Okwu DE, Mbaebie BO. Phytochemical Constituents of Some Nigerian Medicinal Plants. Afr J Biotechnol, 2005; 4(7): 685-688.
12. Khan FA, Hussain I, Farooq S. Phytochemical Screening of Some Pakistanian Medicinal Plants. Middle- East J Sci Res, 2011; 8(3): 575-578.
13. Valgas C, Souza SMD, Smânia EF, Artur SJ. Screening Methods to Determine

Antibacterial Activity of Natural Products. Braz J Microbiol, 2007;38(2): 369-380.

14. Akujobi CO, Njoku HO. Bioassay for the Determination of Microbial Sensitivity to Nigerian Honey. Global J Pharmacol, 2010;4 (1): 36-40.
15. Majumder P, Majumder S. Preparation and characterization of some herbal ointment formulations with evaluation of antimicrobial property. Indian J Res Pharm Biotechnol, 2013; 1(3): 385-390.
16. Rajasree PH, Vishwanad V, Cherian M, Eldhose J, Singh R. Formulation and Evaluation of Antiseptic Polyherbal Ointment. Int J of Pharm & Life Sci, 2012; 3(10): 2021-2031.
17. Nascimento GGF, Locatelli J, Freitas PC, Silva GL. Antibacterial Activity of Plant Extracts and Phytochemicals on Antibiotic-Resistant Bacteria. Braz J. Microbiol, 2000;31(4): 247-256.
18. Arunkumar S, Muthuselvam M. Analysis of Phytochemical Constituents and Antimicrobial Activities of Aloe Vera L. Against Clinical Pathogens. World J. Agric. Sci, 2009; 5(5): 572-576.
19. Ahmad I, Aqil F.In Vitro Efficacy of Bioactive Extracts of 15 Medicinal Plants against ESβL-Producing Multidrug-Resistant Enteric Bacteria. Microbiol Res, 2007; 162 (3): 264-275.
20. Chhetri HP, Yogol NS, Sherchan J, Anupa KC. Formulation and Evaluation of Antimicrobial Herbal Ointment. Kathmandu Uni J Sci Eng Tech, 2010; 6(1): 102-107.
21. Suzuki S, Horinouchi T, Furusawa C. Suppression of Antibiotic Resistance Acquisition by Combined Use of Antibiotics. J. Biosci. Bioeng, 2015; 120(4): 467-469.