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Andrew GK Mtewa
Chemistry Section,
Department of Applied Studies,
MIT, Malawi University of
Science and Technology, Limbe,
Malawi

Antibacterial potency stability, pH and phytochemistry of some Malawian ready-to-serve aqueous herbal formulations used against enteric diseases

Andrew GK Mtewa

Abstract

Aqueous herbal formulations against enteric diseases in Malawi are stored in different containers and for different durations, a practice whose implications to the stability of the antimicrobial potency, pH and phytochemical presence has not been explored in Malawi. Formulations from *P. guajava* leaves and roots, *M. azadirachta* leaves and *C. frutescens* roots were stored in clay pots and plastic bottles and checked for their stability in antibacterial potency against *Escherichia coli* using the disc method, its pH changes and phytochemical presence. Phytochemicals were present mostly from day 2, pH was storage duration-dependent. Antibacterial potency depended on storage duration and was not affected by container material. Maximum antibacterial activity was observed in *P. guajava* leaves formulation in plastic bottle at 62% that of Erythromycin. The study showed that aqueous formulations can be stored in either plastic containers or clay pots without adversely affecting their potency.

Keywords: Aqueous herbal formulation stability, phytochemical screening, enteric diseases, ready-to-use formulations, antibacterial activity, extractions

1. Introduction

Enteric bacteria are responsible for high mortality rates in numerous developing countries with as many as 50,000 people dying daily as a consequence of infection [1]. Among many others, enteric diseases include diarrheal and dysentery. They are commonly caused by ingesting contaminated food in poor hygienic conditions. Most herbal plants used to prepare anti-enteric disease formulations in Malawi are already proven to have anti-bacterial activities. Extracts of *P. guajava* have anti-diarrheal and anti-dysentery activities [2, 3] and *C. frutescens* extracts are also reported to have antibacterial activity [4, 5]. However, studies on the changes that occur in the antibacterial potency during storage of the actual freshly made formulations which are ready to be served to patients have not been widely done on Malawian formulations.

It is reported that medicinal plants are tradition of yesterday and drugs of tomorrow [6]. Actually, they are medications for Malawian societies even today. Ready-To-Serve (RTS) aqueous herbal formulations are very common in Malawian societies mainly as alternative remedy for various ailments including those caused by enteric bacteria. Their use as antibiotics spurns from tradition and cost benefits compared to conventional medicines. An anti-microbial is a substance that kills or inhibits the growth of microorganisms such as bacteria, fungi or protozoans [7]. *Escherichia coli* are an example of bacteria responsible for enteric diseases [8].

Herbal formulations are stored in various forms, including powder [9], aqueous [10], paste and tablets. Aqueous formulations can be prepared from either single medicinal plants or a combination [11, 12]. Some aqueous formulations are conveniently taken as beverages [13, 14].

Traditional herbal formulations are kept in different containers over various durations for different reasons. It is reported that certain societies believe that the potency of a herbal formulation depends on the containers used [15]. In Malawi, containers usually used are calabash, glass bottles, plastic bottles, clay pots, plastic bags and metallic pots. Aqueous formulations are stored for 5 days by most practitioners but some extend up to 14 days and get administered to patients until they get well.

Although formulations are used even several days after preparation, storage of herbal formulations may lead to variations in chemical profiles of the herbs [16]. It is reported that such variations includes deterioration which leads to loss of active components [15].

This paper evaluates how the antibacterial potency in some Malawian aqueous herbal formulations against *E. coli*, pH and phytochemical presence change during storage in two container materials and check if the containers used for storage affect these parameters.

Correspondence

Andrew GK Mtewa
Chemistry Section,
Department of Applied Studies,
MIT, Malawi University of
Science and Technology, Limbe,
Malawi

1.1 Principles of herbal antimicrobial activity determination

The determination of antimicrobial activity is commonly done through the agar well diffusion and the paper disc method. Both involve the preparation of live strains of bacteria and appropriate dilutions in normal saline solution.

Aliquots of the diluted cultures are inoculated on growth media favorable for specific bacteria being cultured. For the paper disc method, paper discs are then moisturized using the herbal formulation extracts and then placed on top of the inoculations^[1, 4]. For the well diffusion method, wells (4 to 5 mm deep) are created in the inoculums using sterile borers and then aliquots of the herbal formulations are poured into the wells^[5, 17, 18]. After incubation, the diameter of the clear inhibition zone is measured.

2. Materials and methods

2.1 Fresh herbal plant collection

Fresh plant samples of *P. guajava*, *C. frutescens* and *M. azadirachta* were collected from Chizalo village, *Capsicum frutescens* roots were obtained from pepper farms in Domasi and *Melia azadirachta* leaves were collected from Thom Allan village all outside Zomba city. The plants' identities were confirmed by technicians at the Biology department of the University of Malawi's Chancellor College.

2.2 Preparation of traditional medicinal formulations

All raw plant material sampling and formulation preparation procedures were as directed by two practising herbalists through participant observations and discussions. The herbalists involved were selected due to their affiliation to the state-registered Malawi Traditional Healers Umbrella Organization (MTHUO) in their areas.

2.2.1 *P. guajava* leaves' formulation

Tender leaves of *P. guajava* (75g) were crushed using a mortar and pestle then put in tap water (1l). The samples were shaken/ stirred and allowed to stand for 5 minutes and they were ready for administration.

2.2.2 *P. guajava* roots' formulation

Roots of *P. guajava* (2g) were crushed using a mortar and pestle then put in tap water (1l). Edible table salt (Cerebos iodated, 1g) was added and stirred. The mixture was heated until boiling. Once the formulation showed slight signs of boiling, it was removed from the hot plate. After 5 minutes from the heater, the formulation was ready for analysis.

2.2.3 *C. frutescens* roots' formulation

Maize grains (500g) were pound at a maize mill. The husks were removed by winnowing and the gains were transferred into a plastic bucket containing water (5l), covered and stored for 3 days. Stale corn water from this storage bucket was used to prepare medicinal samples. Roots of *C. frutescens* (5g) were crushed using a mortar and pestle and put in the prepared stale corn water (1l) and then heated to simmering. After 5 minutes from the heater, the samples were ready for administration.

2.2.4 *M. azadirachta* leaves' formulation

Tender leaves of *M. azadirachta* (100g) were crushed using a mortar and pestle and put in tap water (1l). The samples in plastic bottles were shaken and those in clay pots were stirred and allowed to stand for 5 minutes and then they were ready for administration.

2.3 Determination of pH

pH was determined according to method from literature^[20] with a few modifications. Samples (10ml) were drawn into a beaker and a handheld bench pH meter (Martini instruments) was dipped directly into the samples.

2.4 Phytochemical Screening in the formulations

The methods used were according to literature^[21, 22, 23].

2.4.1 Flavonoids

Dilute ammonia solution (5ml) was added to aqueous extracts of medicinal formulations (5ml) of the extract followed by the addition of concentrated sulphuric acid (2ml). Yellow coloration indicated the presence of flavonoids.

2.4.2 Tannins

Aqueous extracts of medicinal formulation (1 ml) was mixed with 2ml of FeCl₃. A dark green color indicated the presence of tannins.

2.4.3 Anthocyanins (Anthocyanosides)

Aqueous extracts of formulations (1ml) were mixed with 5ml of dilute HCl. Pale pink colour indicated presence of anthocyanosides.

2.4.4 Terpenoids

Aqueous extracts of formulation (5ml) were mixed with 4ml of chloroform and concentrated sulphuric acid (4ml). A reddish brown coloration on the interface indicated the presence of terpenoids.

2.4.5 Alkaloids

Aqueous extracts of formulations (2ml) of filtrate was mixed with Mayer's reagent (4ml), formation of a yellow coloration with some precipitates was positive indication of alkaloids.

2.5 Changes in antibacterial activity in herbal formulations during storage

The aqueous formulations were prepared as above and stored in 5 bottles and in clay pots. 400ml of each were collected and then filtered using number 4 filter paper (Whatman, 20-25 μm poresize) and centrifuged at 3000 rpm for 10 minutes. The supernatant was discarded and the semi solid matrix was filtered using number 6 filter paper (Whatman, 3 μm poresize). The residues were freeze dried for 18 hours and used in the antimicrobial potential determination of the formulations.

2.5.1 Microbial nutrient media preparation

MacConkey agar (Oxoid, England) was prepared according to manufacturer's instructions by dissolving 52 g in 1 litre of distilled water, boiled and then sterilized using an autoclave (Dixons surgical instruments, Wickford) at 121°C for 15 minutes. The media was let dry before inoculation.

2.5.2 Bacterial strain plate inoculation

E. coli strains were isolated from infected fecal samples with the help of technicians at the National Microbiology Reference Laboratory (NMRL) through the NMRL-EQA programme number 2015-1 involving Valid Nutrition microbiology laboratory. The strains were confirmed by indicating dry, pink (lactose positive) colonies with surrounding pink reason MacConkey and further it was positive on indole and methyl red biochemical tests. Sterile petri dishes were carefully inoculated with the *E. coli* strains using MacConkey agar and standardized to 1.0 x 10⁴ cfu/ml.

2.5.3 Observation of changes in *E. coli* growth zone of inhibition during storage

The well diffusion method was used according to literature [18, 24, 25]. A standard cork borer of 4mm India meter was used to create well (4mm deep) at the center of the inoculated plate and the agar was removed from the well. 0.1ml of the extracts was introduced into the wells. The plates were then incubated at 37°C for 24 hours after which the zone of inhibition of *E. coli* growth was observed, measured with a transparent ruler and recorded in millimeters. The screening was done in triplicates. Sterilized distilled water was used as negative control and erythromycin (250mg) was used as standard antibiotic.

3. Results and discussions

3.1 Phytochemical screening in the formulations

All the phytochemicals of interest in this study were present except anthocyanins contrary to related research work [26] which reported the presence of anthocyanins in *P. guajava* aqueous extracts. Boiling involved in the preparation of roots' samples formulations could have degraded anthocyanins into chalcones [27] due to their thermal labile nature [28]. The absence in the formulations that did not require boiling on preparation according to literature [28] could be due to the liability of anthocyanins to undergo numerous reactions during storage like the transformation of monomeric forms to oligomeric or polymeric compounds, a reaction which increases with storage time. The alkaloids were detected at least by day 7 in all formulations probably due to slow evolution from chelates.

3.2 Herbal formulation pH during storage

pH in all the formulations under study are shown in figure 1.

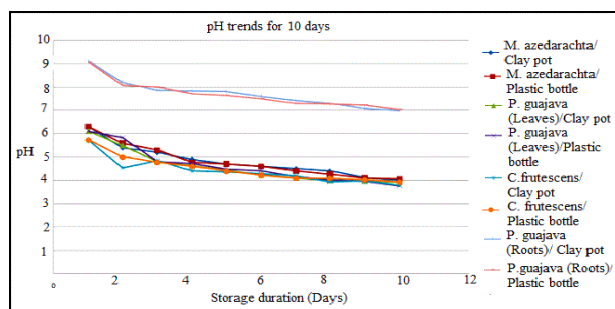


Fig 1: pH trends in aqueous herbal formulations during storage in different containers presented by their means, with n=3. The coloured lines indicate different formulations in the two containers.

Observations in clay pots from day 15 were not made as the clay pots had a tendency to lose the solutions during storage. Formulation pH in clay pots and plastic bottles containing the same formulations over 10 days were not significantly different ($p > 0.05$) which indicates that storage containers did not affect pH. The lowering of pH from day 1 to 10th day was observed in all formulations which shows that storage duration affects pH value for the first 10 days.

Comparing to other aqueous herbal formulations, the current trends are slightly similar to the ones reported [29] on *P. guajava* fruit jelly and for other aqueous herbal formulations on banana and sapota beverage [30], on cucumber-melon functional drink [31], on *Aloe vera* RTS herbal beverage [32] and on whey based pine apple and bottle gourd mixed herbal beverage [33].

According to literature [30, 31, 32, 33] the decrease in pH for aqueous herbal formulations can be attributed to the

production of organic acids and amino acids leading to an increased acidity in the solutions. It was also reported that the degradation of polyphenolic compounds in the herbal formulations and the action of some acids on sugars [33] and protein components in the formulations [32] influence the decrease in pH.

CO₂ produced from the decay process of organic matter by microorganisms with water to give organic acids of which H⁺ is also responsible for the lowering of pH [34] in the formulations. The evolution of flavonoids and terpenoids from the plant tissues also reduced pH values of the formulations [32]. In the current study, flavonoids and terpenoids were evolved in the formulations during storage. The decrease in pH is advantageous as low pH inhibits growth of pathogenic microorganisms and the acidic environment created acts as a preservative [32] to the formulations.

In the current study, pH in herbal formulation samples at storage duration from day 4 in *M. azadirachta* clay pot and plastic bottle, from day 3 in *P. guajava* leaves in clay pot and plastic bottle, from day 3 in *C. frutescens* plastic bottle and from day 2 in *C. frutescens* clay pot to day 30 had a potentially low risk of bacterial proliferation as bacterial growth is optimal at pH of around 5.00 to 8.50 [35].

3.3 Antibacterial activity during storage of herbal formulations

Figure 2 presents results for antibacterial activity by growth inhibition zone using Erythromycin (250mg) as a standard antibiotic. The lower antibacterial activity in all the studied formulations than that of erythromycin reinforces the position reported in literature¹ that commercially perfected and tested antibiotics should be used in treatments whenever available. The maximum activity was observed in *P. guajava* leaves formulation in plastic bottle on day 5 at 62% that of erythromycin.

There was no significant difference ($p > 0.05$) between formulations from the same plants in different containers which show that storage containers did not affect antibacterial activity of the formulations but duration for the first 10 days did since more bioactive components were now being evolved and used up at the same time.

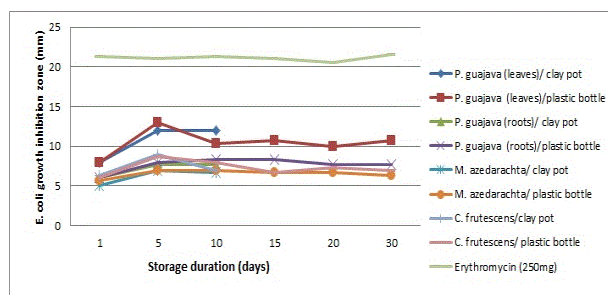


Fig 2: Changes in antibacterial activity of aqueous herbal extracts by growth inhibition zone presented by their means, with n=3. Formulations in clay pots were studied for 10 days.

The results in this study are in agreement with those reported in other studies where antibacterial activity increased during 3 days storage for *P. guajava* extracts [19]. The antibacterial activity observed also confirms what was for *C. frutescens* and *P. guajava* extracts [4]. Other research reported some antibacterial activity of *C. frutescens* on *E. coli* strains [24] which were almost constant during storage [36].

The higher activities observed in the formulations than those on day 1 are due to pH differences^[17] where lower pH favours

antibacterial activity than higher pH on day 1. This confirms that *P. guajava* leaves can serve as good anti-diarrhea and related ailments' medicines.

4. Conclusions and Recommendations

Container material does not affect the pH, evolution of phytochemicals and antibacterial activities of aqueous herbal formulations during storage. However, storage duration does. Practitioners and patients can get the best formulae in terms of the three parameters above in both clay pot and plastic bottle at around day 5 with high antibacterial activity and at optimal pH for preservation.

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