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The Effect of Selected Sterilization Methods on Antibacterial Activity of Aqueous Extract of Herbal Plants

[1]S.R. Hashemi, [1]I. Zulkifli, [2]Z. Zunita and [3]M.N. Somchit
[1]Department of Animal Science,
[2]Department of Veterinary Pathology and Microbiology,
[3]Department of Biomedical Sciences, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia

Abstract: The aim of this study was to compare selected sterilization methods to maintain high susceptibility of antibacterial activities of aqueous extracts of herbal plants. Autoclave-sterilized Impregnated disk and Impregnated disk sterilized by Acrodisc syringe filter were embedded on Mueller-Hinton Agar (MHA) plates seeded with the respective test microorganisms. Among five extracts, *Euphorbia hirta* exhibited antibacterial activities. Autoclaving caused less damage to the antibacterial activities of the tested extract in comparison with syringe filtration.

Key words: Antibacterial activity, aqueous extract, *Euphorbia hirta*, sterilization methods

INTRODUCTION

Antimicrobial activities have been exploited intensively in recent years, mainly in respect to the extensive ban on antibiotics in the animal industries and antibiotic overuse in human medicine. Earlier studies have shown that many regional herbs have anti-microbial, anti-pyretic, anti-inflammatory and immunoreactive properties (Habsah *et al*., 2000; Schwikkard and Van Heerden, 2002; Somchit *et al*., 2005). There are several reports on the antimicrobial activity of different herbal extracts (*in vitro*) in different regions of the world (Polasa and Nirmala, 2003; Onyeagba *et al*., 2004; Wiart *et al*., 2004; Kattak *et al*., 2005; Soo Park *et al*., 2005; Sudhakar *et al*., 2006; Yano *et al*., 2006). Several studies have also reported various types of contamination of herbal medicines. Contaminants in herbal medicines that have been reported include other herbs, microorganisms and toxins produced by microorganisms, pesticides and toxic heavy metals (Talaly and Talaly, 2001) which question the rightness of the use of the extracts for different purposes. Food borne pathogens such as diarrheagenic serotypes of *Escherichia coli*, *Salmonella*, *Listeria monocytogenes* and *Aeromonas hydrophila* are widely distributed in nature (Indu *et al*., 2006). They may easily contaminate the obtained herbal extracts.

The main methods for testing the antimicrobial activities of extracts are disk diffusion method, agar dilution method (Luangtongkum *et al*., 2007) and broth dilution method (Kianbakht and Jahaniani, 2003). The most frustrating problem confronting attempts to antibacterial activity of herbal plants by disk diffusion method is contamination by other moulds and fungus which most probably arises when carrying out the aqueous extraction from fresh materials as aseptic techniques are followed when carrying out the other stages of the experiments. The methods used to sterile the extracts may be inappropriate because they seriously change the color and morphology of the herbal extract. The present study has studied a few sterilization methods to compare the antibacterial activity of contamination-free aqueous herbal plants extracts and control (without sterilization).

MATERIALS AND METHODS

Plant materials: Five aqueous extracts obtained from the fruit of *Solanum torvum*, whole plant of *Euphorbia hirta* and rhizomes of *Zingiber officinale*, *Curcuma longa* and *Zingiber zerumbet* were tested for antibacterial activities. Fresh *Euphorbia hirta* was collected in April 2007 from the agriculture gardens in Universiti Putra Malaysia (UPM). *Zingiber officinale*, *Curcuma longa*, *Zingiber zerumbet* rhizomes and *Solanum torvum* fresh fruits were obtained from a local market. All plants were authenticated by the Institute of Bioscience (IBS), Universiti Putra Malaysia.

Preparation of extract: The plants were cleaned and washed with sterile distilled water and oven-dried at 50°C.

Corresponding Author: I. Zulkifli, Department of Animal Science, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia

In order to obtain the plants` extracts, 100 g of each dried plant material powder was placed in a flask and distilled water was added (1:10 w/v). The flasks were incubated in a shaking-water bath at 50°C for 48 h and the obtained extracts were filtered using filter papers (Whatman® No. 1). The flow-through were stored in a deep freezer at -80°C overnight and then subjected to freeze drying (Jouan LP3, France) at -50°C; 0.2 mbar for 48 h to obtain water-free extracts. Extracts were then weighted and stored at 4°C till further use.

Test microorganism: Two Gram-positive (*Staphylococcus aureus* and *Bacillus subtilis*) and two Gram-negative (*Escherichia coli* and *Salmonella entertidis*) were obtained from Bacteriology Laboratory, Faculty of Veterinary Medicine, UPM, Malaysia.

Antibacterial sensitivity test using filter paper method: Isolated colonies of the four bacteria were cultured into tubes containing 10 mL of sterile nutrient broth (OXOID®, CM1, UK) and incubated at room temperature till the cultures` turbidity reached 0.5 McFarland and were aseptically swabbed on the surface of sterile Mueller-Hinton Agar (MHA) plates. Filter paper discs of 6 mm diameter were prepared and sterilized. Using an ethanol dipped and flamed forceps, these discs were aseptically placed over Mueller-Hinton Agar (MHA) plates seeded with the respective test microorganisms. Twenty five microlitres of the various concentration of each spices extract (50, 100 and 200 mg mL^{-1}) were aseptically transferred to these discs to give a final concentration of 1.25, 2.5 and 5 mg $disc^{-1}$, respectively. The plates were incubated in an upright position at 37°C for 24 h. The diameter of inhibition zones were measured in mm and the results were recorded. Inhibition zones with diameter less than 12 mm were considered as having no antibacterial activity. Diameters between 12 and 16 mm were considered moderately active and these with >16 mm were considered highly active (Indu *et al*., 2006).

Antibiotic sensitivity testing: The tested microorganisms were also assayed for their sensitivity against the antibiotics (OXOID®, UK) tetracycline (30 mcg), chloramphenicol (30 mcg), gentamicin (10 mcg), kanamycin (30 mcg), erythromycin (10 mcg) and cephalothin (30 mcg) using the disk diffusion method.

Sterilization methods: After carrying out antibacterial activity test of five different herbal plants` extracts, *Euphorbia hirta* extract was selected to study the effect of the sterilization methods. Two sterilization methods were applied; autoclaving of impregnated discs and filtration of the extracts. For autoclaving, impregnated discs were placed in small glass bottles and then autoclaved at 121°C for 15 min. Filtration was carried out using Acrodisc syringe filter membrane of 0.45 μM (Schleicher and Schuell Bioscience Inc., Keene, NH) and the flow-through was impregnated on blank disks and let stand at room temperature under fume hood to air-dry. The prepared extract-impregnated discs were stored at -5°C before tested (Somchit *et al*., 2003).

Previously isolated classes of constituents: *Zingiber officinale* has two primary extracts: oleoresin and essential oil. The chemistry of these oils is well documented and includes monoterpenes, sesquiterpenes and phenols (Mukherjee *et al*., 1995; Onyeagba *et al*., 2004) Steroidal alkaloids, tannins and saponins from *Solanum torvum* is isolated (Kattak *et al*., 2005). Tannins, flavonoids, phenolic acids, saponin and amino acids are isolated constituents of *Euphorbia hirta* (Sudhakar *et al*., 2006). curcumin. Curcuminoids, a group of phenolic compounds isolated from *Curcuma longa* (Somchit *et al*., 2003). Sesquiterpene, bicyclic monoterpene and monocyclic sesquiterpene obtained from rhizomes of *Zingiber zerumbet* (Polasa and Nimala, 2003).

Statistical analysis: To compare the three methods, a Completely Randomized Design (CRD) with three replicates was used. Means comparison was carried out using Duncan's New Multiple-Range Test ($p<0.05$). All statistical analyses were performed using SAS version 9.1 (SAS Institute, Cary, NC, 2005).

RESULTS AND DISCUSSION

Many studies have been conducted to establish the antimicrobial effect of the medicinal plants (Habsah *et al*., 2000; Sudhakar *et al*., 2006). The results of different studies provide evidence that some medicinal plants might indeed be potential sources of new antibacterial agents even against some antibiotic-resistant strains (Indu *et al*., 2006). Among the five herbal plants studied only the aqueous extract of *Euphorbia hirta* showed considerable growth-inhibiting activity against *Staphylococcus aureus* (Table 1). Significant increase in the inhibitory zone was observed by increasing the concentration of the extract. The range of the inhibition zone`s diameter was measured 19 to 27 mm and the antibacterial activity was a linear function of the concentration (Table 2).

Phytochemicals have been reported to have medicinal uses (Ojo *et al*., 2006). Specifically, saponin has been reported to have antimicrobial effects (Mahato *et al*.,

Table 1: Antibacterial activity of various concentrations of aqueous extracts of *Zinziber officinale*, *Curcuma long*, *Zinziber zerumbet*, Euphorbia hirta and *Solanum torvum*

Plants	Concentration (mg mL^{-1})	Microorganism			
		E. coli	*S. entertidis*	*S. aureus*	*B. subtilis*
Zinziber officinale	50	-	-	-	-
	100	-	-	-	-
	200	-	-	-	-
Curcuma longa	50	-	-	-	-
	100	-	-	-	-
	200	-	-	-	-
Zingiber zerumbet	50	-	-	-	-
	100	-	-	-	-
	200	-	-	-	-
Euphorbia hirta	50	-	-	+	-
	100	-	-	+	-
	200	-	-	+	-
Solanum torvum	50	-	-	-	-
	100	-	-	-	-
	200	-	-	-	-

-: Refers to no antibacterial effect of corresponding medicinal plant to the mentioned bacterial strain at mentioned dose. ±: Refers to antibacterial effect of corresponding medicinal plant to the mentioned bacterial strain at mentioned dose

Table 2: Mean inhibition zones of *Euphorbia hirta* aqueous extracts in comparison with standard antibiotics

Sample	Concentration	Microorganism			
		E. coli	*S. entertidis*	*S. aureus*	*B. subtilis*
Euphorbia hirta	50 (mg mL^{-1})	-	-[1]	19.76±0.26	-
	100 (mg mL^{-1})	-	-	22.26±1.01	-
	200 (mg mL^{-1})	-	-	27.06±0.99	-
Tetracycline	10 (µg disk^{-1})	-	19.96±0.47	-	11.16±0.02
Chloramphenicol	30 (µg disk^{-1})	-	26.96±0.26	29.56±0.64	31.86±0.63
Erythromycin	10 (µg disk^{-1})	-	-	29.40±0.05	30.30±0.24
Gentamicin	10 (µg disk^{-1})	21.66±0.15	19.40±0.45	27.96±0.23	27.16±0.46
Kanamycin	30 (µg disk^{-1})	21.66±0.66	19.13±0.37	26.46±0.35	30.03±0.63
Cephalothin	30 (µg disk^{-1})	13.16±0.63	-	19.53±0.92	37.16±0.60

-: Refers to no antibacterial effect of corresponding medicinal plant to the mentioned bacterial strain at mentioned dose. Values are Mean±SE (mm) of triplicate testes

1992) and could serve as precursors of steroidal substances with a wide range of physiological activities (Madusolomuo *et al*., 1999). The preponderance of saponin in the extracts of *E. hirta* that reported by previous researchers (Jonson *et al*., 1999; Tona *et al*., 2004; Sudhakar *et al*., 2006) could then justify the use of these plants in the treatment of some microbial infections mentioned earlier. However, the observation that Zingiberaceae family contain saponin do not have antibacterial activity but possesses remarkable antibacterial activities suggested that the activities observed may not be due to the presence of saponin only, but that other phytochemical components may be antibacterial active as well. In addition, the biological or therapeutic activities of medicinal plants are closely related to their chemicals compounds. These chemicals are classified into major groups such as essential oils, alkaloids, acids, steroids, tannins, saponins and etc. Each one of these classes of chemicals may have a preferred effective method of extraction which facilitates getting the chemicals out of the plant and into the herbal remedy that is being prepared e.g., some active chemicals found in plants are not soluble or dissolved in water but are more soluble in alcohol. Interestingly, this is also the reason why some plants are prepared in one manner to treat one specific condition, yet are prepared in a different way to treat a completely different condition e.g. preparing an infusion/tea of a plant might extract a delicate group of anti-inflammatory plant steroids to treat arthritis (and leave behind other non-water soluble chemicals), yet when the same plant is prepared in alcohol as a tincture, the delicate steroids are degraded or burned-up in the alcohol but different antibacterial alkaloids (which are only soluble in alcohol) are extracted instead. It has been reported that plant extracts in organic solvent provided more consistent antimicrobial activity compared to those extracted in water (Parekh *et al*., 2005). It can be rationalized in terms of the polarity of the compounds being extracted by each solvent and, in addition to their intrinsic bioactivity, by their ability to dissolve or diffuse in the different media.

The effects of the sterilization methods on the antibacterial effect of the extract are shown in Table 3. It is apparent that sterilization methods have had

Table 3: Comparison of the sterilization methods on the inhibitory zone of *Euphorbia hirta* against *Staphylococcus aureus*

	Concentration of extract		
Method of sterilization	50 (mg mL^{-1}) (1.25 mg disk^{-1})	100 (mg mL^{-1}) (2.5 mg disk^{-1})	200 (mg mL^{-1}) (5 mg disk^{-1})
Extract without sterilization (control)	19.76±0.46^{a}	22.26±1.75^{a}	27.06±1.72^{a}
Impregnated disk, sterilized with autoclave	12.76±0.41^{b}	14.80±0.45^{b}	18.66±0.55^{b}
Impregnated disk, sterilized with Acrodisc syringe filter	9.86±1.01^{c}	12.63±0.61^{b}	16.03±1.00^{c}

Values are Mean±SE (mm) of triplicate testes, Values with different subscripts are significantly different ($p<0.05$)

negative effects on the properties of phytochemical compounds and quality of nutrition. It is believed that high temperature causes serious structural damage and consequently partial or complete loss of important properties (Gliguem and Birlouez-Aragon, 2005). Nelson *et al.* (2007) explained that the antimicrobial substance in the onion extracts, which are mainly phenolic compounds were destroyed or inactivated by heat. In contrast, present findings indicate that autoclaving had less negative effects on the antibacterial properties of herbal extract in comparison with Acrodisc syringe filter. As it was observed in present study, the use of autoclave-sterilized and syringe-filter sterilized both reduced the antibacterial activities of the herbal extracts ($p<0.05$). However, the reduction was far less for autoclaving. Interestingly the reduction in antibacterial activities was significantly related to concentration of the extract. At the concentration of 50, 100 and 200 mg mL^{-1} the inhibitory zone was reduced to 35, 33 and 30% for autoclaved extract and 49, 56 and 59% for syringed-sterilized extract respectively in comparison with the control group (without sterilization). To our knowledge, there has been no report on the use of autoclaving for sterilization of herbal extracts so far as it is believed that it may seriously damage their properties. Indu *et al.* (2006) studied the antibacterial of some syringe-filter sterilized herbal extracts and reported effective inhibitory zone against *E. coli*. Based on present findings, we believe that syringe-filter sterilization could have damaged the herbal extracts` ingredients and if autoclaving had been applied as the means of sterilization, wider inhibitory zone could be observed. Furthermore, Filtration is a mechanical or physical operation. Mechanical filtration is typically achieved by passing water or solvent through materials which act as a sieve, physically trapping the particulate matter. Contaminants or bacteria are removed by nylon syringe filter through a membrane having microscopic holes that allow water or solvent molecules, but not larger compounds, to pass through. It is also possible that some phytochemical compounds cannot pass through the filter.

On the other hand, we experienced difficulty with syringe-filtration of high concentrated extracts. Interestingly, the results showed a decreasing trend of the reduction of the inhibitory zone when compared with the control group by increasing the concentration of the extract, from 49% reduction at the concentration of 50 mg mL^{-1} to 59% reduction at the concentration of 200 mg mL^{-1}.

In conclusion, the results of this study indicate that if it is necessary to apply sterilization, it is recommended to apply autoclaving rather than syringe filtration to maintain the antibacterial properties at the highest possible level.

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