



Research Journal of  
**Medicinal  
Plant**

ISSN 1819-3455



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## **Time-kill Curve Studies of Ampucare Against *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella pneumoniae* and *Proteus vulgaris***

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**Abstract:** Present study attempts to determine antimicrobial efficacy of Ampucare stored at different conditions by time kill curve studies against *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella pneumoniae* and *Proteus vulgaris*. In all storage conditions, a rapid killing time was achieved by Ampucare. Bacterial count was less than  $3 \text{ Log}_{10} \text{ cfu mL}^{-1}$  after 6 h of study in all organisms under study. No deviation in pattern of bacterial inhibition was found in all conditions of storage of Ampucare. There was no re growth reported even after exposure for longer time under influence of Ampucare. In conclusion, Ampucare has good antimicrobial activity under all storage conditions of study against *E. coli*, *S. aureus*, *P. vulgaris* and *K. pneumoniae*.

**Key words:** Ampucare, wound healing, time kill curve

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### **INTRODUCTION**

The infection of wounds in hospitals and in the community cause problems for both the treatment of patients and infection control. The unique properties of Ampucare, a polyherbal preparation for wound healing, are mainly due to presence of *Azadirachta indica* (Neem) and *Curcuma longa* (Turmeric) as major ingredients. *Azadirachta indica* is regarded as The Wonder Tree and Nature's Drug Store and is reported to possess antimicrobial, wound healing, antiinflammatory, fungistatic, fungicidal, antioxidant, bactericidal and free radical scavenging activities. It is also useful in control of ulcer infections and skin disease infections (Fabry *et al.*, 1996; Bandyopadhyay *et al.*, 2004; Subapriya and Nagini, 2005; Jain and Bansal, 2003; Gomes *et al.*, 2007). *Azadirachta indica* originated from Sri Lanka, India and Burma (Gomes *et al.*, 2007). It is now grown in Bangladesh, Cambodia, Nepal and in many other countries around the world (Schmutterer and Asche, 1986). In Kenya it grows along the coast and is traditionally used to treat several diseases. The *Kiswahili* name for the plant is Mwarubaini which means a cure for forty diseases. *Curcuma longa* is reported to have antibacterial (Ruskin, 1992; Kundu *et al.*, 2005), antimicrobial and antifungal (Costa-Lotufo *et al.*, 2002) properties useful in wound healing (Wuthi-udomlert *et al.*, 2000) and diabetic impaired healing (Sidhu *et al.*, 1998) have been reported.

Therapeutic efficacy of many indigenous plants for several disorders has been described by practitioners of traditional medicine in India. *Azadirachta indica* is a tree which has been used for a long time in agriculture and medicine. *Azadirachta indica* is an indigenous plant widely distributed in India. Antimicrobial properties of *A. indica* were studied by several authors (Sidhu *et al.*, 1999) who reported the antimicrobial activity of the seed oil against a variety of pathogens.

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Ampucare derives its name from the word amputation i.e., intentional surgical removal of a limb or body part, to remove diseased tissue or relieve pain. Ampucare is indicated for treatment of diabetic leg ulcer, burns, bedsores, venous ulcer, arterial ulcer, skin infections, traumatic wounds and post-operative wounds. Efficacy and wound healing trend of Ampucare in patients suffering from diabetic leg ulcer, venous ulcer, arterial ulcer, burns, cellulitis is, traumatic wounds, post operative wounds and bedsores has also been studied and successful results have been demonstrated. Several reports on antifungal activities of Ampucare are available (Lund *et al.*, 1997; Wolfe *et al.*, 1996).

The present study is conducted to evaluate antimicrobial activity of Ampucare under normal storage, freeze thawed and sedimented condition against *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella pneumoniae* and *Proteus vulgaris*.

## MATERIALS AND METHODS

### Microorganisms

The following strains obtained from Microbial Type Collection Center of Institute of Microbial Technology, Chandigarh, India were used for the study-*Escherichia coli* (MTCC No-739), *Staphylococcus aureus* (MTCC No-737), *Proteus vulgaris* (MTCC No- 426) and *Klebsiella pneumoniae* (MTCC No-109).

### Ampucare

Ampucare which contains extracts of *Azadirachta indica* and *Curcuma longa* as major ingredients was used for the study. Ampucare was provided by manufacturer, Venus Remedies Limited, India for the study. Ampucare was kept under following conditions for this study:

- **Ampucare-A:** Stored under normal room temperature
- **Ampucare-B:** Frozen for 5 h at 0°C and thawed at room temperature
- **Ampucare-C:** Forced to sediment under centrifugation at 5000 rpm for 1 h

### Media and Reagents

Following media and reagents were used in study-Sterile water, 0.65% saline, Phosphate buffer, Chlorine, Tryptic Soy broth, Lactose broth, DE neutralizing broth with tween 80, Tryptic Soy agar, Sabouraud Dextrose Agar, MacConkey agar, Mannitol salt agar and Sterile deionized water. All media were manufactured by Hi Media, India and prepared as per manufacturer's instructions.

### Preparation of Inocula

The inoculum suspension were enumerated in duplicate by standard microbiological procedures at the initiation and completion of testing appropriate dilution are prepared and enumerated by standard microbiological procedure. To prepare the inoculum suspension from an agar plate, the microbial growth was washed from the agar surface with buffered phosphate dilution to make 10 fold dilutions from  $10^{-1}$  to  $10^{-10}$  of the organism. An inoculum of 1.0 mL of each dilution was plated on the appropriate media. Plates were incubated at 37°C for 48 h. Count of surviving organisms were recorded. Average triplicate plate (3 plates from each dilution) counts were multiplied by the dilution factor to arrive at cfu mL<sup>-1</sup>.

### Time-Kill Curve Studies

One-way Analysis of Variance (ANOVA) was used to determine statistical difference between Ampucare A, Ampucare B and Ampucare C in all organisms under study.

$p < 0.05$  were considered statistically significant. A kill curve ( $\log_{10}$  cfu mL<sup>-1</sup> vs. time) was drawn for each product and linear regression analysis for each set of data was made. For each strain, time-kill bacterial kill studies were performed in Ampucare with an inoculum of  $5 \times 10^6$  to  $1 \times 10^6$  cfu mL<sup>-1</sup>. A flask of inoculated MH broth with no Ampucare served as a control. The surviving bacteria were counted after 0, 1, 2, 3, 4, 5 and 6 h incubation at 37°C by sub culturing 1 mL serial dilutions in to DE neutralizing broth.

#### Determining Time-Kill Endpoints

A bactericidal effect is defined as a 3 log decrease in the cfu mL<sup>-1</sup> or a 99.9% kill over a specified time (Wolfe *et al.*, 1997). The definition of kill for this study has been used as per National Committee for Clinical Laboratory Standards (1992) together with modifications based on a suggestion by Handwerger and Tomasz that a kill can be determined at 6 h. (May *et al.*, 1998). A constant logarithmic rate of kill has been assumed during a time-kill. A 90% kill at 6 h is equivalent to a 99.9% kill at 24 h. In this study the kill measurement was determined by the actual reduction in viable counts at 6 h for each isolate.

This study was conducted at Venus Medicine Research Centre, India between June to August 2008.

## RESULTS

#### Time-Kill Curve Analysis

For *E. coli* time-kill curve analysis demonstrated bacterial killing in 0 to 6 h. In all conditions under study, there was non significant change in colony count in each time point. For Ampucare-A, 6.16 to 2.30 log<sub>10</sub> cfu mL<sup>-1</sup>; for Ampucare-B 6.22 to 2.30 log<sub>10</sub> cfu mL<sup>-1</sup> and for Ampucare-C 6.50 to 2.30 log<sub>10</sub> cfu mL<sup>-1</sup> of colony count was recorded (Fig. 1).

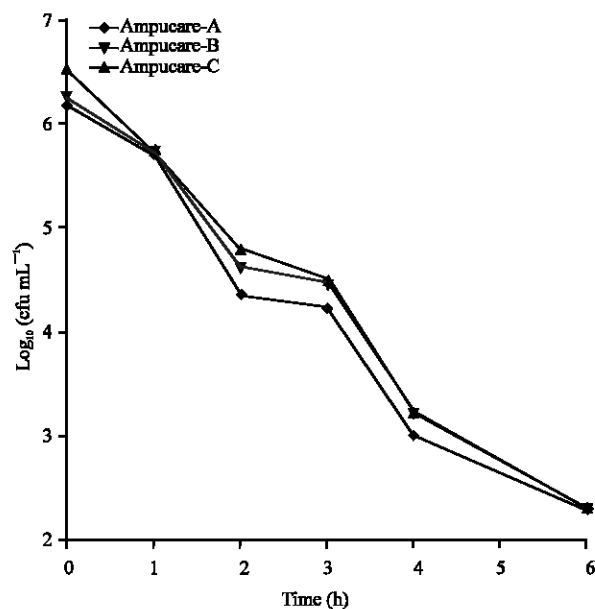


Fig. 1: Time kill curve of *E. coli*

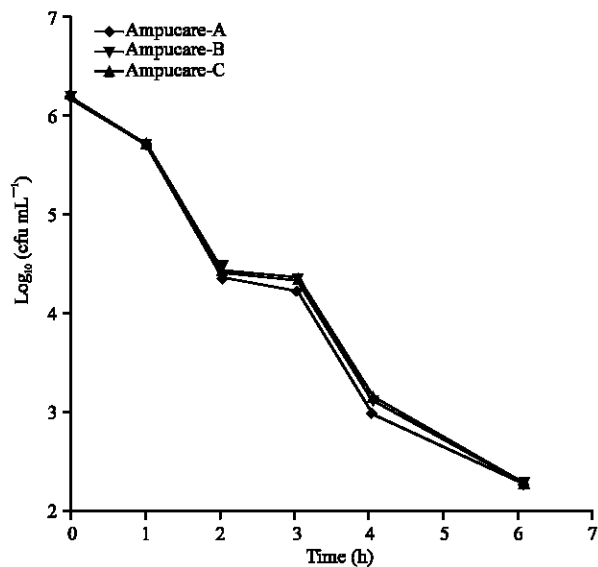


Fig. 2: Time kill curve of *S. aureus*

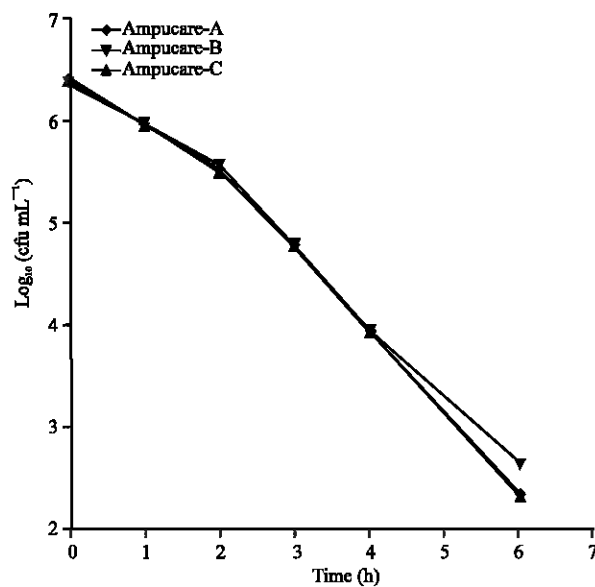


Fig. 3: Time kill curve of *P. vulgaris*

With *S. aureus* time-kill curve analysis demonstrated that under all conditions of study, there was non significant change in colony count in each time point. For Ampucare-A, 6.16 to 2.30 log<sub>10</sub> cfu mL<sup>-1</sup>; for Ampucare-B 6.18 to 2.30 log<sub>10</sub> cfu mL<sup>-1</sup> and for Ampucare-C 6.17 to 2.30 log<sub>10</sub> cfu mL<sup>-1</sup> of colony count was recorded (Fig. 2).

In *P. vulgaris* also there was non significant change in colony count in every time point. For Ampucare-A, 6.36 to 2.70 log<sub>10</sub> cfu mL<sup>-1</sup>; for Ampucare-B 6.33 to 2.60 log<sub>10</sub> cfu mL<sup>-1</sup> and for Ampucare-C 6.30 to 2.78 log<sub>10</sub> cfu mL of colony count was recorded (Fig. 3).

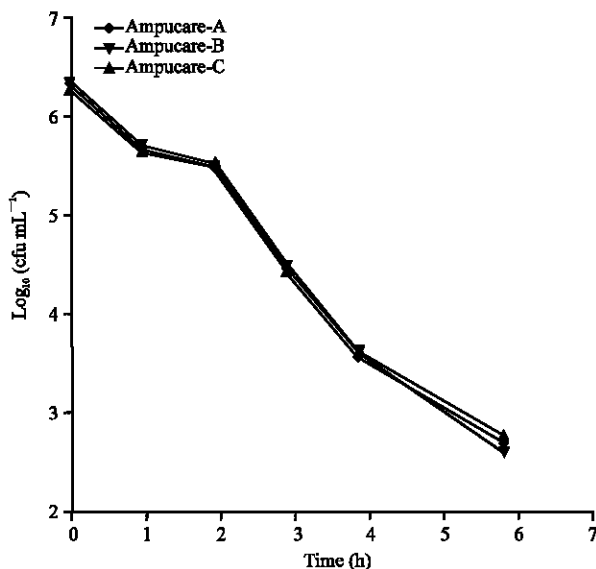


Fig. 4: Time kill curve of *K. pneumoniae*

Similarly, for *K. pneumoniae* time-kill curve analysis demonstrated good bacterial killing in 6 h and all conditions under study, there was non significant change in colony count in each time point. In Ampucare-A, 6.41 to 2.30 log<sub>10</sub> cfu mL<sup>-1</sup>; in Ampucare-B 6.36 to 2.60 log<sub>10</sub> cfu mL<sup>-1</sup> and in Ampucare-C 6.39 to 2.30 log<sub>10</sub> cfu mL<sup>-1</sup> of colony count was seen (Fig. 4).

There is no re growth of these organisms in the culture media under study even after exposure for longer time under influence of Ampucare.

## DISCUSSION

A study of neem oil obtained from *A. indica* seed has been performed (Handwerger and Tomasz, 1985). The use of plant extracts and phytochemicals with known antimicrobial properties may have great significance in therapeutic treatments. Use of plant extracts in the treatment regimen of various diseases are gaining importance as antimicrobial, antibacterial, antiviral and anti fungal activities of many plants are reported particularly against skin diseases such as eczema, burns, ulcers and herpes (Gandhi *et al.*, 1988). Traumatic wounds are the most common type of wounds and may include abrasions, contusion, incision tunneled wound lacerations, bites, cuts and thermal wounds (Leaper, 2006). Most of patients that visit emergency department are victims of traumatic lacerations. Burns refer to any type of extremity of damage experienced by the skin. The causal factor may be heat, cold, chemicals, radiation, steam, gases or electric current. First degree burns are confined to erythema only, second degree burns involve papillary dermis and may also involve reticular dermis layer whereas third degree burns include charring of the skin and produce hard eschars (Singer *et al.*, 1997; Sevitt, 1976). These wounds delay in the healing process by multiple microbial infections.

There are no reports of microbial efficacy studies of Ampucare, although the activity of this therapeutic preparation has been established as clinical useful in wound healing. There are only some reports available, which indicate that ingredients used in Ampucare are

antibacterial and antifungal in nature (Gandhi *et al.*, 1988). Combination of these ingredients into finished formulation has not been studied so far. In the present study, Ampucare addition to a culture lead to a clear reduction for the bactericidal population over a period of time. *Escherichia coli* in the concentration of  $1.7 \text{ Log}_{10} \text{ cfu mL}^{-1}$  is reduced to  $0 \text{ Log}_{10} \text{ cfu mL}^{-1}$  with in of exposure to Ampucare. Similarity, *S. aureus* also show reduction of bactericidal population by 5 hof exposure. *P. vulgaris* and *K. pneumoniae* in this studies show reduction in much lesser time than other organisms of the study. All conditions of storage under this study have shown similar pattern of results. Ampucare has shown excellent microbial killing properties in *in vitro* conditions. There is not much variation in antimicrobial activity of Ampucare under normal storage, freeze thawed and sedimented condition against *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella pneumoniae* and *Proteus vulgaris*. Moreover, present study has also demonstrated that Ampucare has excellent bactericidal properties which is evident by reduction in number of colonies only in 6 h.

In conclusion, Ampucare has bactericidal properties which is expressed over a period of time in time-kill experiments. There is no impact of freezing/thawing or sedimentation of Ampucare on the pattern of time-kill curve in organisms under study.

#### ACKNOWLEDGMENT

Authors are thankful to CGM, Domestic operations of Venus Remedies Limited for providing the samples of Ampucare for this study.

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