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Effects of aqueous and ethanolic extracts of costus (*Saussurea lappa*) against Pneumococcal TIGR4 growth

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A long historical reputation for folk plant therapy has gained the trust of people. The World Health Organisation **(**WHO) states that 80% of the world's population relies on traditional medicinal plants. The roots of *Saussurea lappa* (*S. lappa*) were extracted by both aqueous and ethanol solutions before being investigated against *Streptococcus pneumoniae* (*S. pneumoniae*) TIGR4. Inhibition zones were also measured. Statistical analysis demonstrated that the aqueous extract of *S. lappa* had an obvious bacteriostatic effect, whereas the ethanolic extract suppressed the bacterial cell densities at all experimental times. The bacterial viable counts showed that all pneumococci died at 5 hr post-inoculation when using 1% of 4 mg/ml *S. lappa*. On the other hand, live bacterial cells were detected until 11 hr in the absence of the plant extract. Moreover, disc diffusion methods illustrated a clear biological effect against *S. pneumoniae*. This highlights strong promise for *S. lappa* against this aggressive bacterium and might offer an alternative natural preventative candidate that possibly facilitates complications following respiratory viral infection (RVIs), such as coronaviruses.

Keywords: Medicinal plants. *S. lappa*. *S. pneumoniae*. Antibiotic. Respiratory viral infection.

INTRODUCTION

Due to major concerns about the rapid increase in resistance to antibiotics (McCarthy et el. 2015; Ansari et al. 2019; Saeedi et al. 2017) and about capsular switching leading to ineffectiveness of vaccines (Jin et al. 2009; Park et al. 2007; Song et al. 2012), there is an urgent need for alternative antimicrobial agents. Consequently, plant extracts have been of interest to alternative medicine physicians as potential drugs, particularly extracts of plants that have been used traditionally in some folk practices (Alsharif and Waznah, 2020). The WHO has stated that 80% of the world's population relies primarily on plant extracts used traditionally. Moreover, there are practical advantages of using medicinal plants instead of antibiotics. For example, plant extracts usually have fewer side effects, are relatively inexpensive, tolerated well by patients, widely accepted because of the long history of their use and because they are natural and renewable products.

The dried root of the herb *S. lappa* (Decne.) Sch.Bip. belongs to the family Asteraceae. It has been used by Muslims round the world as it was prescribed medically by the Prophet Muhammad peace be upon him 1444 years ago, particularly for respiratory infections (Sahih al-Bukhari 5692, 5693, book 76, hadith 15). Historically, it is a well-known plant used in folk remedies including Ayurvedic, Chinese and Tibetan medicinal systems. They have been used *S. lappa* to treat several ailments such as cough, asthma, chronic wounds, rheumatism, ulcer, dysentery and stomachache (Yashvanth et al. 2010, Pandey et al. 2007).

Additionally, *S. lappa* has been reported as an antibacterial agent that decreased the severity of clinical complications in the gut that were caused by bacterial strains (Irshad, 2012). Furthermore, Indian alternative medicine physicians describe this root as a drug for pulmonary tuberculosis, fever, chronic rheumatism and skin diseases (Kapoor, 2001). Kapoor also reported that the herb is used as a prophylactic, anthelmintic, sedative, and antiseptic drug and in the treatment of malaria and leprosy diseases.

WHO states that 14.5 million children under the age of five become infected with pneumococcal disease every year, leading to approximately 826,000 deaths (WHO, 2011). *Streptococcus pneumoniae*, or pneumococcus, is considered one of the most serious pathogens worldwide, and it is implicated in most of the respiratory infections in the United Kingdom (Kadioglu et al. 2008). Although there have been improvements produced by modern intensive care and the introduction of antibiotics, there are numerous morbidities and mortalities associated with pneumococcal diseases, and the organism *S. pneumoniae* is responsible for most hospital cases that occur after infection with the influenza virus (Palacios et al. 2009).

The WHO also revealed that respiratory viral

infections (RVIs) cause death in approximately 4 million people every year. Moreover, it has been reported that coronaviruses increase bacterial adhesion to lung cells, resulting in increased bacterial cultures in the human respiratory tract (Golda et al*.* 2011).

Coronaviruses can cause several symptoms as reported recently in Wuhan (Guan et al. 2020) and are known to be followed by bacterial pneumonia, mostly from *S. pneumoniae* (Jackson et al. 2003; Morris et al. 2017; Christenson et al. 2004). This bacterium has been the primary source of the secondary infection that has been reported post-RVI, such as in influenza outbreaks (Palacios et al. 2009).

Bacterial infection following respiratory viral incidences have also been demonstrated during both corona and influenza outbreaks (Golda et al. 2011; Smith et al. 2018; Hedlund et al. 2003; Gill et al., 2010; Berendt et al. 1975; Weinberger et al. 2009). Clinical samples showed that the presence of S. pneumoniae in severe cases of people diagnosed with H1N1 influenza infection was 56.4%, and 25% in mild cases, whereas other bacteria were not remarkably high (Palacios et al. 2009). Additionally, antibiotic therapy has been successful in treating RVIs (Velasco et al. 1995). Scientific experiments revealed that S. pneumoniae was responsible for most of the pneumonia incidences following RVI and caused up to 75% of deaths just 40 hr post-viral infection (Velasco et al. 1995). In addition, a significant increase in pneumococcal pneumonia hospitalizations was noted to correspond to the influenza pandemic (Christenson et al. 2004; Berendt et al. 1975). Accordingly, it is believed that pneumococcal vaccines might protect against pneumonia caused by S. pneumoniae that followed RVIs (Alsharif 2020).

Pneumococcus possesses number of virulence factors responsible for pneumococcal pathogenicity (Kadioglu et al. 2008), with the pore-forming toxic protein pneumolysin (Ply) being one of the most important factors. It is a 53 kDa protein that is believed to be a potent pneumococcal virulence factor that plays a major role in pneumococcal pathogenicity and is conserved in virtually all pneumococci (Berry et al. 1992; Mitchell et al. 1997; Price et al. 2012; Yoo et al. 2010). *In vivo* studies have illustrated that Ply exerts its effects as a key determinant in pathogenesis (Feldman et al*.* 2002), possibly through some mechanisms resulting in the destruction of respiratory tract tissue, complement activation and stimulation of the production of cytokines (Martner et al*.* 2007).

The effects of *S. lappa* on the important pathogen *S. pneumoniae* TIGR4 have not been studied. In order to clarify the medicinal value of this herb and to study its biological activity against microorganisms, it was investigated in this study, as a first report, the potential antibacterial agents of the *S. lappa* root against *S. pneumoniae* and demonstrated the effects of different extracts on growth rates. Discs diffusion methods were also investigated in this study.

MATERIALS AND METHODS

Plant extraction

The root of *S. lappa* was purchased from a local traditional herb market in Madinah, Kingdom of Saudi Arabia. In order to prepare an aqueous extract, 100 g of root powder of the medicinal herb *S. lappa* was soaked in sterile distilled water at room temperature as it is used traditionally. Ethanolic extraction was performed with slight modifications according to several other laboratories (Prakash and Kumar 2006; Karuppusamy et al*.* 2011; Prakash 2012; *Sarraj* et al*.* 1985). The powder (100 g) was soaked for 72 hr in 1 L of 70% ethanol for extraction at room temperature. A rotary evaporator was used under reduced pressure after filtering the ethanolic extract, giving 4.9 g of the crude extract. After completely evaporating the ethanol, 100 ml of the organic solvent dimethyl sulfoxide (DMSO) was used to dissolve the extracted herb, giving a final concentration of 49 mg/ml as a stock solution. A ten percent solution was prepared from this stock solution and serially diluted up to 10×10^8.

Pneumococci and media

The pneumococcal strain used in this study was *S. pneumoniae* TIGR4. It is a medically referenced and sequenced strain (Salvia et al*.* 2006). It was streaked on blood agar (B.A.), (Oxoid, UK) with 5% horse blood (E&O Laboratories, Scotland), and colonies were inoculated in the liquid culture Brain Heart Infusion (BHI., Oxoid, UK) at 37°C until the growth reached an Optical Density of ~0.6 (OD_{600nm}). Pneumococci were then stored with 15% glycerol (Riedel-de Häen, Germany) at –80°C. After 16 hr of freezing, each strain was tested by streaking on B.A. provided with 5% horse blood with an optochin disc for confirmation (Mast Diagnostics, UK). All bacterial growth measured at time points as ODs and Viable Counts (VCs). Live cells represented by Colony-Forming Unit (CFU).

Antibacterial activity (sensitivity test)

The ethanolic plant extract was assessed against the bacterium *S. pneumoniae* at two different concentrations, 49 mg/ml, and 10 mg/ml, using the agar disc (filter paper) diffusion method according to the BSAC (Alhadhrami et al. 2016) with a slight modification. Optochin discs were applied as positive controls. All discs were 6 mm in size and prepared and soaked in 20 µl of 10% plant extract after each concentration was serially diluted up to 1/10.0000 before the addition of the discs to the agar plate. The plates were incubated for 24 hr at 37°C before the inhibition zones were recorded by measuring the diameter in mm to determine bacterial susceptibility.

Statistical analysis

Differences in the median survival time between tested groups of pneumococci were analysed by the one-tailed Mann-Whitney U test. Differences in the geometric mean

number of pneumococci in the broth groups were calculated using the unpaired two-tailed *t* test. All analyses were performed using GraphPad Prism version 5.01. A *p-value* of <0.05 was considered significant.

RESULTS AND DISCUSSION

Phytochemical analysis

S. lappa roots showed the presence of monoterpenes, sesquiterpenoids, flavonoids, lignans, triterpenes, steroids, and glycosides, etc. *S. lappa* roots are a rich source of sesquiterpenoids, especially sesquiterpene lactones. The essential oil of dried and crushed *S. lappa* roots obtained by hydro-distillation showed a higher content of sesquiterpenoids (79.80%) than monoterpenoids (13.25%) (Liu et al. 2012).

Aqueous extract of *S. lappa* and TIGR4 growth

Pneumococci grown in 4 mg/ml aqueous *S. lappa* were able to establish their log phase of growth at 7 hr post-inoculation (Figure 1), whereas control started the same growth phase after 3 hr. Consequently, the difference of 4 hr reflected a bacterial growth delay that was not observed when *S. lappa* was extracted in ethanol.

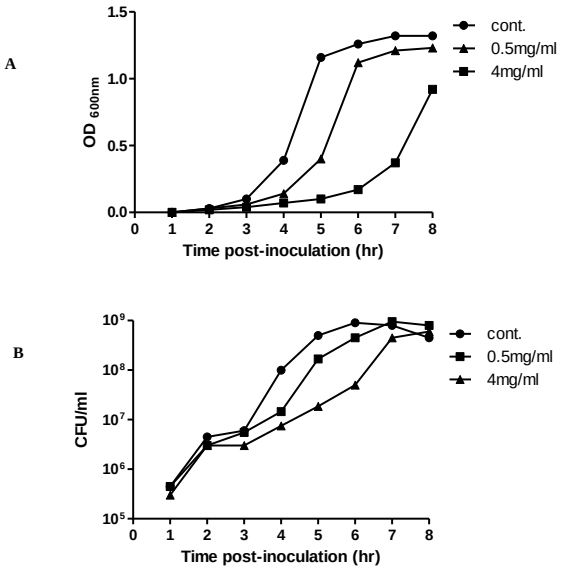


Figure 1: Effects of the aqueous extract of *S. lappa* growth curves over 8hr. (A) Optical Density (OD) of TIGR4 cells. (B) Viable Counts (VC) of TIGR4 cells. A and B representing bacterial growth in different concentrations of the aqueous extract of *S. lappa* (0.5 mg/ml, 4 mg/ml, cont. means no plant extract). CFU is Colony-Forming Unit. Each time point recording was performed in triplicate.

On the other hand, the bacterial viable counts (B) in (Figure 1) illustrate a very large change in the bacterial population; for instance, the TIGR4 viable counts at 5 hr were almost 10^7, but when these bacteria grew for the same amount of time without *S. lappa*, they were approximately 10^9, although both generally had similar growth curve patterns.

Effects of the *S. lappa* aqueous extract on pneumococcal TIGR4 growth

Bacterial growth of TIGR4 at 5 hr demonstrated an obvious effective role of the aqueous extract of *S. lappa* when TIGR4 bacteria were grown at a concentration of 4 mg/ml as can be seen in (Figure 2).

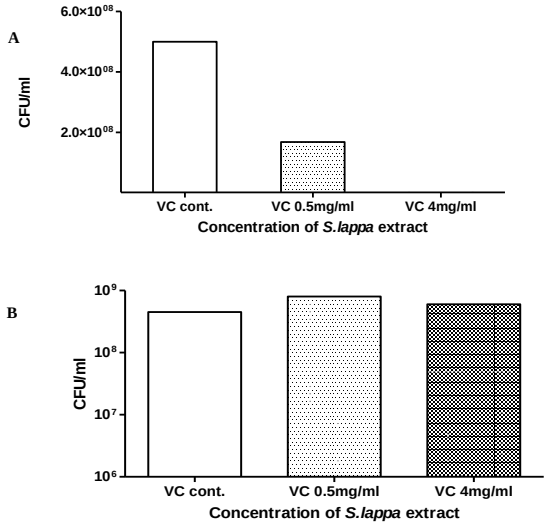


Figure 2: Effects of the aqueous extract of *S. lappa* growth curves at 5 and 8hr Post-Inoculation (PI). (A) Viable Counts (VC) of TIGR4 cells at 5hr. (B) Viable Counts (VC) of TIGR4 cells at 8hr. A and B representing bacterial growth in different concentrations of the aqueous extract of *S. lappa* (0.5 mg/ml, 4 mg/ml, cont. means no plant extract). CFU is Colony-Forming Unit. Each time point recording was performed in triplicate.

The lower *S. lappa* concentration (0.5 mg/ml) showed better growth, and an approximately 3-fold greater bacterial population was detected in the control compared to the moderate bacterial growth of the middle bar (0.5 mg/ml).

Ethanolic root extract of *S. lappa* on the *S. pneumoniae* growth curves

The OD growth curve after 12 hr (Figure 3: A) shows that the first pneumococcal log phase was the control, which started at 3 hr and sharply increased until approximately 5.30 hr before the growth curve entered its stationary phase, taking only 3 hr to enzymatically prepare itself to grow. In contrast, the bacteria growing under the effects of 1% *S. lappa* at a concentration of 4 mg/ml did not increase at any of the experimental times. However, *S. lappa* at a concentration of 0.1% delayed the pneumococcal growth rate for almost 7 hr post-inoculation.

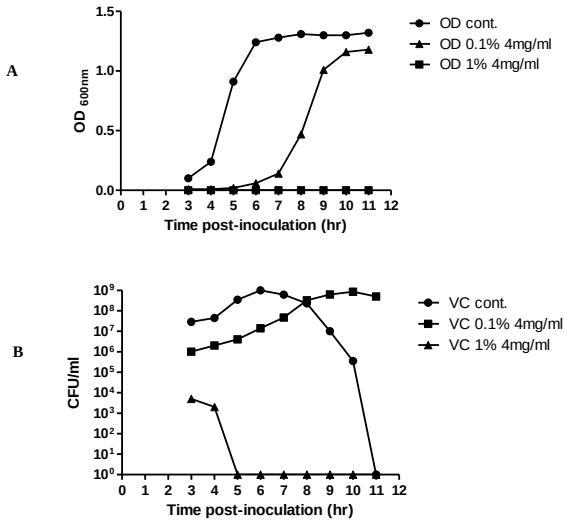


Figure 3: Effects of the ethanolic extract of *S. lappa* growth curves over 12hr. (A) Optical Density (OD) of TIGR4 cells. (B) Viable Counts (VC) of TIGR4 cells. A and B representing bacterial growth in different concentrations of the ethanolic extract of *S. lappa* (0.1% 4mg/ml, 1% 4mg/ml, cont. means no plant extract). CFU is Colony-Forming Unit. Each time point recording was performed in triplicate.

The VC growth curves (Figure 3: B) represent live cells. All TIGR4 pneumococci died at 5 hr post-inoculation with the 1% ethanolic extract (4 mg/ml *S. lappa*). On the other hand, pneumococci were alive until 11 hr in the absence of the *S. lappa* plant extract. Pneumococci started their log growth phase after 7 hr under 0.1% *S. lappa* following a very weak log phase before reaching the stationary phase, reporting very long and weakened growth. OD stands for optical density. VC is viable count.

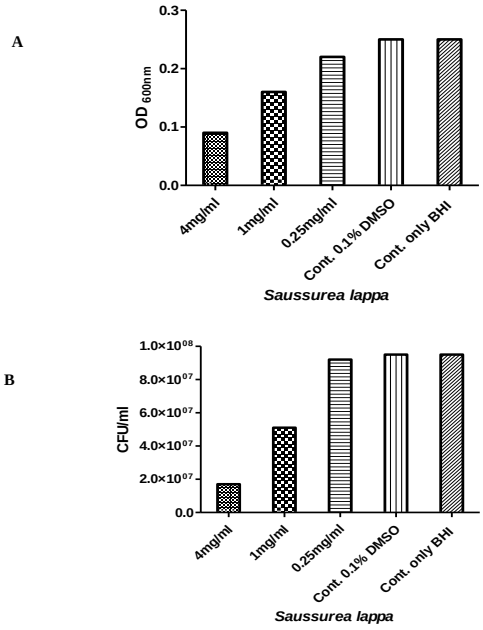
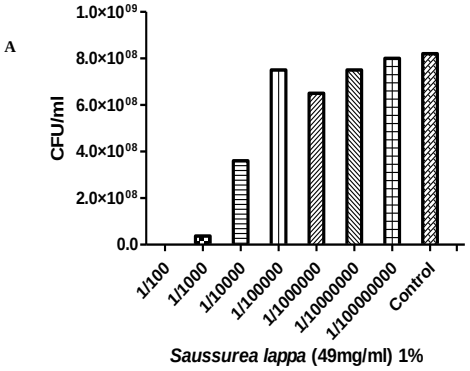


Figure 4:Effects of the ethanolic extract of *S. lappa* on VC at 3hr Post-Inoculation (PI). (A) Optical Density (OD) at 3hr. (B) Viable Counts (VC) of TIGR4 cells at 3hr. A and B representing bacterial growth in different concentrations of the ethanolic extract of *S. lappa* (4 mg/ml, 1 mg/ml, 0.25 mg/ml, cont. 0.1% DMSO and cont. only media Brain Heart Infusion (BHI). CFU is Colony-Forming Unit. Each time point recording was performed in triplicate.

The diagram A in (Figure 4) demonstrates the pneumococcal count as the OD at 3 hr post-inoculation when grown under several concentrations of *S. lappa* (0.2% of 4 mg/ml, 0.1% of 4 mg/ml, 0.1% of 1 mg/ml, and 0.1% of 0.25 mg/ml from a 10% original concentration). Moreover, the bacteria were grown in 0.1% DMSO as well as in BHI, the bacterial media used for all cultures in this study. The highest concentration tested was 0.2% of 4 mg/ml, which had the greatest effect of TIGR4 bacteria, and at this concentration, the pneumococci were not able to grow to an OD greater than 0.05, as seen at the level of 0.1 OD in 0.1% 4 mg/ml; however, in 0.1% of 1 mg/ml of *S. lappa*, the growth reached and OD of 0.2. This growth increased at 0.1% of 0.25 mg/ml, where the OD increased gradually, exceeding 0.2 OD. All these weak pneumococcal growths were absent when *S. lappa* was not added to the bacterial media. This result is clear from both the DMSO and BHI bars, where the ODs reached a peak approaching 0.3 OD.

Similarly, the B diagram shows the pneumococcal live cells at 3 hr post-inoculation under several concentrations of the plant extract of *S. lappa*. The live TIGR4 bacteria reached almost 0.5×10^7 when grown with 0.2% 4 mg/ml *S. lappa*. This number doubled after pneumococcal growth in 0.1% 4 mg/ml of the same plant extract. The growth increased to almost 6×10^7 at a lower concentration of *S. lappa*: 0.1% of 1 mg/ml. The other bars, DMSO and BHI, both had approximately 1×10^8 growth, which showed greater bacterial growth than under the concentration of 0.1% 0.25 mg/ml. All diagrams demonstrated that the *S. lappa* plant ethanolic extract has a clear dose-dependent relationship that affects the bacterial clinical isolate TIGR4.



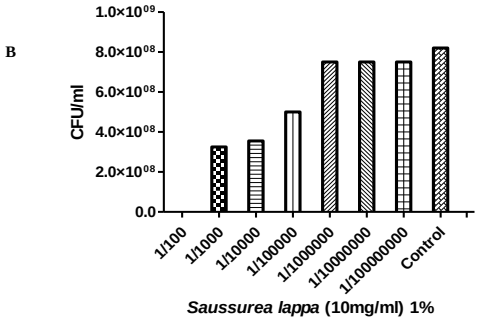


Figure 5: Effects of different ethanolic extract concentrations of *S. lappa* on TIGR4 VCs at 5hr Post-Inoculation (PI). (A) Viable Count (VC) of TIGR4 growth in serially diluted 49 mg/ml. (B) VC of TIGR4 in serially diluted10 mg/ml. CFU is Colony-Forming Unit. Each time point recording was performed in triplicate.

The ethanolic extract, giving 4.9 g of the crude extract. After completely evaporating the ethanol, 100 ml of the organic solvent dimethyl sulfoxide (DMSO) was used to dissolve the extracted herb, giving a final concentration of 49 mg/ml as a stock solution. A ten percent solution was prepared from this stock solution and serially diluted up to 10×10^8.

The above bars (A) in (Figure 5) show that the pneumococcal growth representing live cells at 5 hr post inoculation under the 1% ethanolic extract of 49 mg/ml *S. lappa* has a clear gradual increase associated with a decrease in the plant extract concentration starting from 1/100 up to 1/1.000.000.00 (10×10^8) in (Figure 5). The B graph demonstrates that 1% of the 10 mg/ml ethanolic extract, which was serially diluted for the same plant extract, reflects a slight similarity in the affected live bacterial cells under the different tested *S. lappa* concentrations with obviously less affected cells, which might be due to the low concentration of *S. lappa*.

Sensitivity test

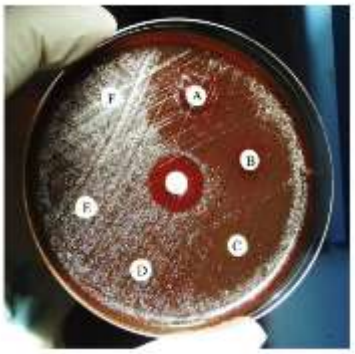


Figure 6: Disc diffusion test of streptococci TIGR4 susceptibility. Thin filter paper discs were loaded with different concentrations of the *S. lappa* ethanolic extract. The concentration of the *S. lappa* plant extract tested was 10 mg/ml. (A) Stock solution of *S. lappa*. (B) The concentration 1/10. (C) the concentration 1/100. (D) the concentration 1/1000. (E) the concentration 1/0000. (F) the concentration 1/100000. The disc in the centre is the positive control disc optochin.

The data illustrate that TIGR4 bacteria were not able to grow around high concentrations of the *S. lappa* ethanolic extract, and there was a gradual decrease in the pneumococcal population represented by the inhibition zones around each disc (Figure 6: A, B, C, D, E and F). Disc A had the highest concentration of the ethanolic *S. lappa* extract, whereas disc F had the lowest.

Recent studies have revealed that the alcoholic extract of the herb *S. lappa* possesses high concentrations of phytochemical compounds, including phenols and flavonoids, and antioxidant activities that might be due to the high phenolic and flavonoid contents (Singh and Chahal 2018; Randhir et al. 2004).

In the current study, a control group of the same bacteria growing under the same conditions but without the addition of *S. lappa* was shown to start the log phase of growth at 3 hr post inoculation. The delay in pneumococcal growth under *S. lappa* indicates that an aqueous pharmaceutical of an *S. lappa* extract might be prescribed as continuous nasal doses every day and could have an effective and potent result against pneumococcal growth.

The roots of *S. lappa* in this study were dried, ground and extracted in both ethanol and water to investigate the antibacterial activity against the life-threatening pneumococci *S. pneumoniae* (TIGR4). The aqueous extract of *S. lappa* is widely and traditionally used. Interestingly, the aqueous extract showed an obvious bacteriostatic effect against pneumococcal growth at different time points. Bacterial cells were not able to grow until 5 hr post-inoculation, after which they recovered and grew.

In the ethanolic extract group, only one percent of the plant extract at a concentration of 4 mg/ml was sufficient to suppress *S. pneumoniae* bacterial cell density at all experimental times tested. However, *S. lappa* at a concentration of 0.1% delayed the pneumococcal growth rates for almost 7 hr post-inoculation. This gives insight into a possible use for this plant as a clinical drug dose that can be given pharmaceutically to individuals. The viable bacterial count showed that all TIGR4 pneumococci died at 5 hr post inoculation under 1% 4 mg/ml *S. lappa*. On the other hand, live bacterial cells remained until 11 hr in the absence of the plant extract of *S. lappa*. TIGR4 pneumococci started their log phase of growth after 7 hr under 0.1% *S. lappa* following a very weak log phase before reaching the stationary phase, reporting very long and weakened growth rates.

This outcome is in agreement with other similar studies published in 2019 by Omer and colleagues (Omer et al. 2019). They compared the effects of aqueous and ethanolic extracts as antibacterial agents. Their data

analysis resulted in a significant effect of the ethanolic extract on the bacterial cells of *Staphylococcus aureus*. Similarly, a stronger effect of the ethanolic extract of *S. lappa* was found compared with the aqueous extract. Other researchers have demonstrated that ethanolic extraction leads to more chemical compounds than aqueous extraction (Barrero et al. 2011; Sonawane et al. 2010), which may explain why the ethanolic extract in the current study is more active against pneumococci than the aqueous extract.

Disc diffusion methods with the plant extract were serially diluted in order to measure the pneumococcal inhibition zones on blood agar. The findings clearly showed that the root ethanolic extract had a biological effect against the clinically isolated S. pneumoniae TIGR4 based on the concentration used. This highlights strong possible promise for utilizing S. lappa to treat such an aggressive bacterium (Trappetti et al. 2011a; Williams et al. 2012).

Extraction of *S. lappa* has been studied against bacteria causing serious complications, for example, *S. aureus* (Wang et al. 2010), but pneumococcal growth of TIGR4 representing live cells at 5 hr post inoculation under 1% of 49 mg/ml *S. lappa* revealed a general gradual increase associated with decreasing plant extract concentration (from 1/100 and serially diluted up to 1/1.000.000.00 (10×10^8)). Additionally, 1% of 10 mg/ml extract reflected a slight similarity in the affected bacterial live cells under the different tested concentrations of *S. lappa* with notably fewer affected cells, which might be due to the low *S. lappa* concentration of 10 mg/ml compared to the *S. lappa* concentration of 49 mg/ml.

CONCLUSION

It is believed that due to the effects of S*. lappa* on *S. pneumoniae*, which is the most causative complication associated with RVIs, *S. lappa* might be a promising candidate to prevent potential complications for those infected with RVIs, including coronaviruses (Alsharif 2020).

To conclude, the data showed a clear anti-pneumococcal effect based on different extract concentrations. Hence, the potential health benefits of *S. lappa* as an alternative natural antibacterial product rather than traditional antibiotics against *S. pneumoniae* are high. Further investigation is required to examine the root to overcome pneumococci, particularly different pneumococcal serotypes, and to determine the novel and active chemical components.

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