Safe food Preparation with Natural Antimicrobial Polyphenol: a Preventing Strategy for Microbial Pathogens Infection during and after a Flood

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Foods can be contaminated with pathogenic microbes during and after the flood following poor hygiene practices by food handlers. Consequently, the number of pathogenic microbe infections will increase via the ingestion of contaminated food and water as the primary source of the organism during and after the flood following poor hygiene practices. Therefore, the main objective of this chapter is to discuss the efficacy of natural antimicrobial polyphenol against pathogenic microbes (such as Leptospirosis) food contamination during and after a flood as preventing strategies for safe food preparation. Use of natural antimicrobial polyphenol to control the growth of pathogens is one strategy that has been adopted for the safe food preparation. Phenolic compounds are secondary metabolites and one of the most widely occurring phytochemicals in plants. They contribute to the sensory properties when added to food and have antioxidant and antimicrobial properties characteristics that are valuable in extending the shelf-life of food. This chapter provides a detailed description of the various methods such as antimicrobial activity evaluation, investigation of the mode of action of polyphenol against pathogens by electron microscopy observation and evaluation of survivability of pathogen in cooked rice and tea to explore the role of polyphenol to prevent pathogenic microbial infection. The methods presented in this chapter are illustrated on the model of Leptospirosis. As such, the intervention and proper practices can be determined to reduce the risk of pathogens outbreak by food handlers, especially street foods during and after a flood.

Keywords: pathogens outbreak; Leptospirosis; natural polyphenol; flood; preventing strategies; safe food preparation

1. Introduction

Contaminated foods and beverage may be a big problem following flooding. Flood waters may carry silt, raw sewage, or wastes contaminated with various microbes. Filth and bacteria such as *Leptospira interrogans* in flood water will contaminate food and drinks, making it unsafe to consume. Moreover, ready-to-eat (RTE) foods can be contaminated with bacteria during and after flood following poor hygiene practices by food handlers, and the organism can multiply rapidly at ambient temperatures. The number of leptospirosis cases has tripled in Kelantan following recent massive floods in Malaysia. Twenty to twenty five leptospirosis cases were recorded in Kelantan, Malaysia before the floods, but the number of cases went up to 94 after floods hit the state. Leptospirosis, which is characterized by hemorrhage, diarrhea, jaundice, severe renal impairment, and aseptic meningitis, etc., has emerged as a global zoonotic infectious disease in the past decade [1-3]. *Leptospira interrogans* is a Gram negative, obligate aerobic spirochete, with periplasmic flagella. The pathogenic *Leptospira interrogans* has several serovars [5], and reported to cause Leptospirosis. Faine [4] reported that Leptospiral lesion disrupt the integrity of the cell membrane of endothelial cells lining small blood vessels in all parts of the body, and leading to capillary leakage and hemorrhage. Damage of blood vessels in the renal cortex leads to renal failure [6] and in the liver, cause haemorrhagic jaundices.

Human infection is accidental, usually occurring after direct or indirect contact with urine from leptospiuric animals [7]. Other mechanisms like animal bites, handling of infected tissues, spreading via the ingestion of contaminated food and water are unusual. However, the recent association of Leptospirosis in Kelantan, Malaysia may be via the ingestion of contaminated food and water as the primary source of the organism. Recently, the Malaysian Medical Association (MMA) Sibu Sub-Branch advisor Dr Hu Chang Hock cautioned the publics that flood waters mixed with septic tank water can contain both animal and human faeces, and breed lots of bacteria (Such as *Leptospira* sp) and viruses. The main reservoir hosts of *L. interrogans* are wild rodents and domestic animals, which can persistently excrete *L. interrogans* through urine (Fig. 1). The shed leptospiral cells can survive in moist soil and water for a long time before infecting a new host [8]. Therefore, *L. interrogans* adapts to diverse natural environments and evades host immune defense during infection to maintain transmission. This makes *L. interrogans* an important pathogen in understanding leptospirosis. Therefore, introducing of natural antimicrobial polyphenol to food product might be another attraction candidate process for safe food preparation and also as preventing strategies for Leptospirosis infection during and after a flood.
Fig. 1  Cycle of leptospirosis

1. Spiral shaped Leptospira bacteria prevalent in fresh water, soil and mud
2. Leptospirosis spreads via urine of infected animals usually rodents like rats
3. Urine of infected animals contaminates the water or soil nearby and eventually infects the animals
4. Urine of infected animals excrete the bacteria into environment and spreads rapidly to human through fresh water or flooding when open wounds like cuts get exposed to the contaminated water or soil
5. Consumption of contaminated water may infect the humans
6. Non-hygienic cooking practices with the contaminated food and ingestion of contaminated food may infect the human population
2. Leptospire

Leptospire are spirochetes (Fig. 2) which are about 0.1 mm in diameter and 6 to 20 mm in length. It includes both pathogenic and saprophytic species compromising the genus Leptospira, where by it belongs to the family Leptospiraceae and order Spirochaetales [9]. Leptospires have distinct hooked ends, with two periplasmic flagella with polar insertions are located in periplasmic space, and are accountable for motility. The flagellar sheath and core are constituted by FlaA and FlaB proteins respectively [10]. Leptospires have a double membrane structure where the cytoplasmic membrane and peptidoglycan cell wall are closely associated and overlaid by outer membrane [11].

During the meeting of Subcommittee on the Taxonomy of Leptospiraceae held in the year 2007 at Quito, Ecuador, a decision was made to give the status species to previously described genomospecies 1, 3, 4 and 5, which results in a family encompassing 13 pathogenic Leptospira species namely *L. alstonii* and *L. alexanderi*, *L. borgpetersenii*, *L. fainei*, *L. inadai*, *L. interrogans*, *L. kirschneri*, *L. santarosai*, *L. terpstrae*, *L. weillii* and *L. wolffii* with more than 260 serovars. The saprophytic species of Leptospira are *L. biflexa*, *L. kmetyi*, *L. meyeri*, *L. vanthieltii*, *L. wolbachii* and *L. yanagawae* and it contains more than 60 serovars. The serovar classification of Leptospira is based on the expression of surface-exposed epitopes in a mosaic of the lipopolysaccharide (LPS) antigens whereby the specificity of the epitopes is dependent on their sugar composition and orientation [12].

Leptospirosis in human is always attained from animal source and this disease is regarded as zoonosis globally. Pathogenic leptospires live in the proximal renal tubules of the kidneys of carriers. However, other organs or tissues might also serve as source of infection. From the kidney, leptospires will be excreted in urine which then may contaminate soil, surface water, streams and rivers. Direct contact with the urine or indirectly from the contaminated water will cause infection in humans or animals. The carriers might be wild or domestic animals, mainly rodents, cattle, small marsupials, dogs and pigs. Humans suffer acute infections and at times long term sequelae but almost never become chronic carriers [12].

![Fig. 2 Leptospira organisms.](image-url)
3. Phytochemicals in plants as antimicrobial agents

Medicinal plants and herbs are good candidate source for natural antimicrobial polyphenol. Use of natural antimicrobial to control the growth of food borne pathogens is one of the strategies that have been adopted. Phytochemicals in plants are broadly grouped into polyphenolic compounds, terpenoids and essential oils, alkaloids, lectins and polypeptides. The polyphenolic compounds also include simple phenols and phenolic acids, quinones, flavonoids and tannins [13, 14]. Polyphenolic compounds are secondary metabolites and one of the most widely occurring phytochemicals in plants. They contribute to the sensory properties when added to food and have antioxidant and antimicrobial properties [14], characteristics that are useful in extending the shelf-life of food. The antioxidant and other biological properties in polyphenolic compounds have been attributed to beneficial health effects when consuming foods rich in polyphenols [15].

3.1 Flavanoids

Polyphenolics are phytochemicals found to be composing a large portion of our diet. These metabolites are active constituents reportedly capable of impeding maladies through oxidative stress induction and enzyme and cell receptor regulation [16]. These polyphenols include over 8000 diversiform compounds that can be additionally grouped into 10 general classes [17]. Flavonoids are substantial part of polyphenol family with over 4000 derivative compounds categorised based on their respective molecular structure. The molecule structures appear to subsist on two benzene rings linked together through three-carbon chain, often forming an oxygenated heterocycle (C6-C3-C6).

Flavonoid compounds normatively materialize as glycosides given the possible hexose and pentose sugars association such as glucose, galactose, rhamnose, arabinose and xylose. The sugars are linked individually or found as blend within the sugars. The glycosylation process allows flavonoids to develop into water-soluble hence aids during storage within cell vacuoles. Flavonoids accretion takes places in epidermal tissues, with a rough 70% estimation of flavonoid accumulation in the upper and 30% in the lower epidermal region. Even though, flavanoids are synthesized in the cytoplasm, their accumulation eventuates in vacuoles [18].

Over the years, scientists and food manufacturing industrialists have been exploiting flavonoids to serve an expedient purpose in curbing various pathological diseases. The molecular action of flavonoids generally ascribes to their antioxidant traits through the reduction strength or feasible impact on intracellular redox level [19]. The numerous types of flavonoids are abundantly found in fruits, roots, nuts, seeds, bark, stems and flowers. The diversification of flavonoids is attributed to the combined existence of functional groups such as hydroxyl, methoxyl, and O-glycoside group substituent on the basic benzo-pyrene (C6-C3-C6) moiety [20] as described in Fig. 3. There are four prominent flavonoid groups known as flavones, flavanone, catechin and anthocyanin.

The flavones are visible as colourless to yellow crystalline matter, able to dissolve in water and ethanol. Mixing flavones in alkali solution, observes a solution in yellow colour. Flavones posses moderate to strong oxygen bases enabling them to readily solubilise in acids. The flavones are classified on the basis of its 2-phenylchroomen-4-one (2-phenyl-1-benzopyran-4-one) backbone structure, resulting with a molecular formula of C15H10O2. These molecules co-exist with the presence of three functional groups; hydroxyl, carboxyl and conjugated double bond [21]. Flavanones differ from flavones by a C2-C3 bond [22]. The flavanone class incorporates a large selection of compounds with O- and/or C-substituent at the two aromatic ring structures. These substituent is in a wide array of hydroxy, methoxy, methylenedioxy, O- and C-glycosyl, C-methyl, C-benzyl, C-hydroxymethyl, C-formyl, C-isoprenyl substituents (including furano or dihydrofurano rings), conjugations to stilbene, anastatin, phenolic acid, and diarylleptanoid moieties [23, 24].

The catechins exist as the isomer of epicatechin. The catechin occurs in trans configuration while the epicatechin exists as cis configurations, all due to the absent of 2,3-double bond [25]. The anthocyanins are the most colourful compounds built from a typical structure called anthocynidins. Their colour covers from deep red to purple and across deep blue. The presence of carotenes is the contributing factor for the colourful characteristics. The production of anthocyanins arises when plants are exposed to ultraviolet radiation, insect wounds, nutrient depletion, and during abnormal metal concentration [18]. When anthocynidins are bound to sugar moieties, they form glycosides known as anthocynins [26]. The varieties of anthocyanins are extinguished by the presence of hydroxylated groups, the feature and the amount of bonded sugars on their structure, the alipathic or aromatic carboxylates linking to the sugar in the molecule and the location of these bonds.
Fig. 3 Main groups of flavonoids and their respective derivative components
4. Safe food Preparation with Natural Antimicrobial Polyphenol

In this chapter, we describe and discuss plant sample extraction technique, antimicrobial screening with determination of minimum inhibition concentration, minimum bactericidal concentration and Evaluation of Survivability of *L. interrogans* in Cooked Rice and Tea. Beside that the *in situ* antibacterial study method by using various microscopy techniques such as, scanning and transmission electron microscopy (SEM and TEM) also will be discussed. Fig. 4 shows the various steps involved in the evaluation of the natural antimicrobial polyphenol for safe food preparation.

![Evaluation of Antibacterial Activity](image)

Disk Diffusion Technique

Minimum Inhibitory Concentration (MIC)

Scanning (SEM) Electron Microscope Observations

Transmission (TEM) Electron Microscope Observations

Time-Kill Study

Polyphenol Extraction

Flow Chart

Safe food Preparation

A & d = Rice or Tea alone
b & e = Rice or Tea + LI
c & f = Rice or Tea + P + LI

The survival will be determined by spread plate method on TCBS agar

**Fig. 4** Various steps involved in the evaluation of the natural antimicrobial polyphenols for safe food preparation
The ability to produce a safe food product with extended storage life which is acceptable to the consumer according to the relevant food standard guidelines is the objective of food preservation. The demands of consumers for processed foods with a limited refrigerated shelf-life have promoted research to improve processing technologies that may lower the risk of microbial poisoning such as Leptospira poisoning. Natural antimicrobial substances, such as *Eclipta alba* L., can be investigated for food preservation. The efficacy of *E. alba* against *L. interrogans* serogroups was previously investigated by evaluating the minimum inhibitory concentration (MIC) of the extracts like acetone, water and saponified lipid by using standard tube dilution in comparison with micro dilution technique [27]. Hence, in this chapter we reported the methods to study the antileptospiral capacity of medicinal plants polyphenols. Antimicrobial properties of plant extracts against food spoilage and pathogenic bacteria have been reviewed extensively in the literature [28]. Most of these reported studies have looked at the efficacy of plant extracts against food related bacteria. Screening for antimicrobial activity against the native microbiota of the food or microbiota at different times of the storage period, changes during processing and packaging would be a more accurate representation of the bacteria that could be in the food as suggested in this chapter. Therefore this chapter have been proposed to look at the efficacy of polyphenol against *L. interrogans* poisoning during and after a flood as a preventing strategies for safe food preparation with natural antimicrobial polyphenol.

### 4.1 Sample Collection

Medicinal plants parts such as flower, leaf, fruit or root (Fig. 5) can be collected or purchase from the local market. The plants parts firstly should be washed with tap water and then with distilled water. The collected plant parts will be then dried in open air, 25°C for 7 days, after which the dried samples will be ground into fine powder using a grinder.

![Medicinal plants](Image)

**Fig. 5** Different parts of medicinal plant which represent a rich source of novel polyphenols
4.2 Polyphenol Extraction Procedure

The extractions of polyphenol can be carried out as described Crozier et al. [29] with some modification. Briefly, air-dried powder of plants parts (0.5 g) will be weighed and placed into a 100 mL conical flask. Forty mL of methanol will be added, followed by 10 mL and 6 M HCL solution. The mixture will be stirred with a magnetic stirrer. The mixture will then be placed in a sample flask (250 mL), attached to the reflux and heated for 2 hours at 90°C, then filtered with a Whatman No.1 filter paper (Whatman, England). Filtrates will be dried by using a vacuumed rotary evaporator at 40°C. The extracts will be preserved in refrigerator for further studies. Fig. 5 show the various steps involved in the extraction technique of natural products such as medicinal plants polyphenol.

Fig. 6 Polyphenol extraction methods
4.3 Total Flavanoid Content

The total flavonoids content of plants parts can be estimated by method described by Zhishen et al. [30]. Based on this method, each sample (1.0 mL) will be mixed with 4 mL of distilled water and subsequently with 0.30 mL of a NaNO₂ solution (10%). After 5 min, 0.30 mL AlCl₃ solution (10%) will be added followed by 2.0 mL of NaOH solution (1%) to the mixture. Immediately, the mixture will be thoroughly mixed and absorbance will then determined at 510 nm versus the blank. Standard curve of quercetin will be prepared (0-12 mg/mL) and the results will be expressed as quercetin equivalents (mg quercetin/gm dried extract of medicinal plants).

4.4 Bacterial Strains

ATCC reference strain of L. interrogans (23581) can be obtained from the American Type Culture Collection (ATCC) and will be used as model bacteria in this chapter. L. interrogans can be maintained by continuous culture (stock culture) in semisolid Ellinghausen-McCullough-Johnson-Harris (EMJH) medium (2.3 g/liter; Difco, USA) containing 3% rabbit serum (RS) and 0.1% bacteriological agar.

4.5 Antimicrobial activity evaluation

4.5.1 Preparation of antibiotic

Stock solution of penicillin G (1 mg/mL) will be prepared by dissolving 1 mg reagent grade of penicillin G powder (Amresco, USA) in 1 mL sterile-distilled water. The working solution (100 μg/mL) will be prepared by diluting the stock solution with sterile-distilled water.

4.5.2 Antimicrobial Disc Diffusion Assay

Antimicrobial assay of natural polyphenol will be performed by Disc diffusion method. Culture of L. interrogans will be developed on EMJH medium using sterile cotton swabs. The sterile disc (5 mm) will be saturated with polyphenol (1000 μg/mL). Disc with solvents (10% DMSO) will be used as control and the respective antibiotic disc penicillin G used as positive control. The sterile impregnated disc with E. alba leaves polyphenol will be placed on the Noble agar base supplemented with 10% rabbit serum (named LVW agar) or EMJH medium containing 3% rabbit serum (RS) and 0.1% bacteriological agar surface with flamed forceps and gently pressed down to ensure complete contact of the disc with the agar surface. After the incubation at 30°C for 7 days the size of the inhibition zone will be measured. Antibacterial activity will be determined by measuring the diameter of the zone of inhibition. Controls will be included that comprised pure solvents instead of the polyphenol. The experiments will be repeated three times and the mean values will be presented as results.

4.5.3 Determination of MIC of polyphenol

Bacterial susceptibility testing antileptospiral assay can be carried out using broth microdilution test as recomended by Eloff [31] and Murray and Hospenthal [32]. Active leptospiral cultures will be prepared in EMJH medium and will be grown at 30°C for 7 days. The culture then will be diluted in EMJH medium to reach a bacterial density of 2 × 10⁶ cells/ml [31].

Two fold serial dilution of the test polyphenol concentration ranging from 50 to 800 μg/mL will be prepared in EMJH medium containing 10% DMSO in a sterile 96-well round bottomed plate, final volume of 100 μL per well. A 100 μL volume of L. interrogans suspension (2 × 10⁶ cells/mL) will be added to each well. Each plate will be included positive controls (EMJH containing 10% DMSO and L. interrogans without polyphenol) and negative control (EMJH containing 10% DMSO) [32]. The plate will be mixed and incubated at 30°C for 7 days. Then, each well will be added with 20 μL of 0× alamar blue which is an oxidant reduction indicator that changes colour from dark blue to bright pink in response to chemical reduction of the growth medium in the presence of bacterial viability. The plate will be further incubated at 30°C for 1 day. The bacterial growth will be observed by colour changing of the indicator and confirmed by measuring absorbance at 570 nm and 600 nm using ELISA reader. The MIC will be defined as the lowest concentration of the E. alba leaves polyphenol that exhibited complete inhibition of microbial growth. The MIC of penicillin G can be also performed as mentioned above, but test concentrations ranged from 0.025 to 50 μg/mL. All tests can be carried out in triplicate.

4.5.4 Growth profile of L. interrogans in the presence of E. alba leaves polyphenol

To assess the antimicrobial effect with MIC concentration (Section 3.5.3) over time, growth profile curve can be plotted. A 7 days culture was harvested by centrifugation, washed twice with phosphate saline and resuspended in phosphate saline. The culture then will be diluted in EMJH medium to reach a bacterial density of 2 × 10⁶ cells/mL. Polyphenol will be added to aliquots of 25 mL EMJH medium in 50 mL Erlenmeyer flask in a water bath at 37°C in
amount which would achieve concentration of 0 (control) and Minimal Inhibitory Concentration (MIC), 1/2 MIC, and 2 MIC after addition of the inocula. After that a 1-mL inoculum will be added to all Erlenmeyer flasks. After addition of the inoculums 1 mL portion will be removed from Erlenmeyer flask and the growth of L. interrogans will be monitored using this portion by measuring the Optical Density at 540 nm (UV-9100, Ruili Co., China). The growth of L. interrogans can be measured every 12 hours until 7 days by the above method.

4.5.5 Ultrastructural observations for identification of preliminary antimicrobial mechanism of polyphenol against L. interrogans

4.5.5.1 Scanning Electron Microscopy (SEM) observation of the L. interrogans treated with E. alba leaves polyphenol

Culture of L. interrogans will be cultured on EMJH medium containing 3% rabbit serum (RS) and 0.1% bacteriological agar using sterile cotton swabs. The medicinal plants polyphenol will be loaded (50 μL) on the agar surface with pipette and will ensure complete contact of the polyphenol with L. interrogans. The treated L. interrogans will be observed at various incubation times such as 24 h, 2th day, 4th day and 6 day after adding the polyphenol. The microbe will be fixed for SEM with Macdowell-Trump fixavative prepared in 0.1 M phosphate buffer (pH 7.2). SEM analyses will be performed by locating the agar with microbes on double-stick adhesive tabs on a planchette and the planchette will be placed in a Petri plate. In a fume hood, a vial cap containing 2% osmium tetroxide in water was placed in an unoccupied quadrant of the plate. After being covered, the plate will be sealed with parafilm, and vapor fixation of the sample proceeded for 1 h. Once the sample will be vapor fixed, the planchette was plunged into slushy nitrogen (~210°C) and will be transferred on to the “peltier-cooled” stage of the Freeze Dryer (Emitech K750), and freeze drying of the specimen will be proceeded for 10 h. Finally, the freeze dried specimen will be sputter coated with 5–10 nm gold before viewing in the SEM (LEO SUPRA 50 VP Field Emission SEM, Carl Zeiss, Oberkochen, Germany) operating at 15 kV at various levels of magnification.

4.5.5.2 Transmission Electron Microscopy (TEM) Observation of the L. interrogans treated with E. alba leaves polyphenol

Culture of L. interrogans will be developed on EMJH medium using sterile cotton swabs. The E. alba leaves polyphenol will be loaded (50 μL) on the agar surface with pipette and will ensure complete contact of the polyphenol with L. interrogans. The treated L. interrogans will be observed at various incubation times such as 24 h, 2th day, 4th day and 6 day after adding the drugs. L. interrogans will be harvested at 24 h, 2th day, 4th day and 6 day after adding the drugs and will be re-suspended in 1 mL ice-cold PBS, will be transferred to 1.5 mL Eppendorf tubes, and will be centrifuged (5,000×g, 5 min, 4°C). Pellets will be re-suspended in 100 mM cacodylate (pH 7.3) containing 2.5% glutaraldehyde and fixed overnight at 4°C. Pellets will be then washed three times in 100 mM cacodylate buffer and post fixed in 100 mM cacodylate containing 1% OsO4 for 2 h. Pellets will be then washed three times in distilled water and directly dehydrated in an ethanol series (50%, 70%, 90%, 3 × 100%). Subsequently, the samples will be embedded in Epon 820 resin [33]. The resin will be polymerized at 65°C over a period of 48 h. Ultrathin sections will be cut on a ultramicrotome and loaded onto 300-mesh copper grids (Plano GmbH, Marburg, Germany). Staining with uranyl acetate and lead citrate will be performed. Finally, grids can be viewed on a transmission electron microscope (LIBRA 120-ZEISSL, Oberkochen, Germany) operating at 5.00 kV.

4.6 Evaluation of Survivability of L. interrogans in Cooked Rice and Tea

4.6.1 Preparation of Rice

Nonglutinous white rice (Oryza sativa L.) will be used throughout this study. Plain rice will be prepared using 5 g of white rice washed twice with 10 mL of sterile distilled water. The washed rice will be transferred to a large test tube, then 8 mL of sterile distilled water will be added, and the mixture will be cooked in a beaker with boiling water [34]. The time will be taken to cook the rice in boiling water was about 15 min for the white rice. Three rice preparations can be used in this study such as rice alone, L. interrogans inoculated rice and L. interrogans inoculated rice with polyphenol. The cooked rice with E. alba leaves polyphenol will be prepared by using 8 mL of distilled water together with 0.5 g of polyphenol. All the tea preparations will be left in the incubator to cool down to 27°C before inoculation with L. interrogans. A new batch should be freshly prepared on the day of each experiment.

4.6.2 Preparation of Tea

Loose black tea (Camelia sinensis) can be used throughout this study. Plain tea will be prepared by adding 1 g of loose black tea to 100 mL of boiled distilled water [34]. All the tea preparations will be left in the incubator to cool down to 27°C. Three tea preparations can be used in this study such as tea alone, L. interrogans inoculated tea and L. interrogans inoculated tea with E. alba leaves polyphenol. The tea with E. alba leaves polyphenol will be prepared by
using 1 g of loose black tea together with 0.1 g of *E. alba* leaves polyphenol in 100 mL of boiled distilled water. All the tea preparations will be left in the incubator to cool down to 27°C before inoculation with *L. interrogans*. A new batch should be freshly prepared on the day of each experiment.

### 4.6.3 Survival of *L. interrogans* Inoculated onto Cooked Rice

Three grams of the cooked rice will be transferred to each of the five universal bottles. An estimated $1 \times 10^8$ CFU of *L. interrogans* in 20 μL of PBS will be dispensed randomly as small droplets on each of the rice clumps for incubation time of 0, 1, 3, 6, and 24 h at 27°C in the incubator. The bottles will be loosely capped. Immediately after the last sample was inoculated (0 h exposure), 5 mL of PBS will be added to the bottle and the rice grains in it will be mixed by vortex for 1 min. Determination of viable counts of *L. interrogans* will be performed with 100 μL of serial diluted rice suspension on Noble agar base supplemented with 10% rabbit serum (named LVW agar) or EMJH medium containing 3% rabbit serum (RS) and 0.1% bacteriological agar (any suitable agar). The same procedure will be performed with the remaining inoculated samples after 1, 3, 6, and 24 h of incubation time. *L. interrogans* inoculated into empty bottles will be kept under similar conditions to serve as control. Three replicated experiments can be performed on each rice preparation.

### 4.6.4 Survival of *L. interrogans* Inoculated onto Tea

Ten millilitres of the prepared tea will be transferred to each of the five universal bottles. An estimated $1 \times 10^8$ CFU of *L. interrogans* in 20 μL of PBS will be inoculated into the tea for incubation time of 0, 1, 3, and 6 h at 27°C in incubator. The bottles will be loosely capped. Immediately after the last sample was inoculated (0 h exposure), the sample will be mixed by vortex for 1 min. Determination of viable counts of *L. interrogans* will be performed with 100 μL of serial diluted tea suspension on on Noble agar base supplemented with 10% rabbit serum (named LVW agar) or EMJH medium containing 3% rabbit serum (RS) and 0.1% bacteriological agar (any suitable agar). The same procedure will be performed with the remaining inoculated samples after 1, 3, and 6 h of incubation time. *L. interrogans* inoculated into 10 mL of sterile distilled water was kept under similar conditions and served as a control. Three replicated experiments can be performed on each tea preparation.

### 4.7 Statistical Analysis

Data collected during the experiment can be analyzed using SPSS 17.0 software. The data will be analyzed using one way ANOVA, Kruskal-Wallis one-way analysis of variance, t-test and Mann-Whitney *U* test. The significance level can be set at $P < 0.05$.

### 5. Conclusion

Rice is a staple food in the Southeast Asian countries and is thus maybe a main source for *Leptospira* contamination during and after flood. There is currently very little information regarding the survivability of *Leptospira* in various types of cooked food and beverages that are commonly found in third world countries such as cooked rice and tea. Therefore, the current chapter provide the useful information with regards to methods to evaluate *Leptospira* behaviour in food and beverages after treated with medicinal plants polyphenols that are common in third world countries. As such, the intervention and proper practices can be determined to reduce the risk of *Leptospira* outbreak by food handlers, especially street foods during and after a flood.

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