THE CHEMICAL CHARACTERISTICS AND ANTIBACTERIAL ACTIVITY OF GREEN TEA (*Camellia sinensis*), STINGING NETTLE (*Urtica dioica*) LEAVES AND THE BLEND

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of Degree of Masters of Science
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ABSTRACT

Infusions such as teas (made from *Camellia sinensis* L.) and tisanes (made from herbs, spices, or other plant parts) have been used for centuries in traditional therapy. In the study, green tea (*Camellia sinensis*) and stinging nettle (*Urtica dioica*) are mixed in different proportions (1.5:1.5, 1.8:1.2, 2.1:0.9, 2.4:0.6, 2.7:0.3) to obtain an optimum formulation of a blended tea using DOE and based on sensory characteristics that were further investigated as potential antibacterial agents. Green tea, *U. dioica* leaves and the selected tea blend was subjected to organic solvent extraction (methanol and ethanol) as well as aqueous extraction. Major phytochemicals (phenols, flavonoid, and tannin) along with the antioxidant activity (DPPH radical scavenging activity, TAC, and FRAP) were determined for the prepared extracts. The antibacterial properties were evaluated by the agar well diffusion method. Minimum inhibitory concentration was evaluated by the micro well dilution method.

The sensory analysis was carried out for brew appearance, aroma, color and overall acceptance of five tea infusions, and from the result, sample C (green tea: *U. dioica* leaves; 2.1:0.9) was found superior to other samples. Among the plant extracts tested, the blend extracts in methanol gave the highest TPC (387.11mgGAE/g), TFC (167.57mgQE/g), Tannin (47.68mgGAE/g), DPPH radical scavenging activity (98.32%), TAC (15.38mgAAE/g), and FRAP (15.12AAE/g) ($p<0.05$). The best antimicrobial actions were observed in the blend extract against the five clinical isolates. The most effective activity was proven against *S. aureus* with a maximum ZOI of 18mm, 18mm, and 10mm in methanol, ethanol, and aqueous solvent respectively, and a minimum inhibitory concentration value of 50µg/ml against *E. faecalis* at 3.2mg/ml. The study concluded that green tea and stinging nettle are in fact more effective as mixtures (2.1:0.9), implying that the combined preparation can synergistically enhance antioxidant as well as antibacterial properties.

Keywords: Tea; green tea (*Camellia sinensis*); stinging nettle (*Urtica dioica*); phytochemicals; antioxidant; antibacterial.

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ABBREVIATIONS

AAE	Amino Acid Equivalent
$AlCl_3$	Aluminium Chloride
ANOVA	Analysis of Variance
ATCC	American Type Culture Collection
DOE	Design of Experiment
DMSO	Dimethyl Sulfoxide
DPPH	2,2-Diphenyl 1-picrylhydrazyl
FRAP	Ferric Reducing Antioxidant Power
$FeCl_3$	Ferric Chloride
GAE	Gallic Acid Equivalent
HCL	Hydrochloric acid
H_2SO_4	Sulphuric acid
MHA	Muller Hinton Agar
MIC	Minimum Inhibitory Concentration
NA	Nutrient Agar
NB	Nutrient Broth
Na_2CO_3	Sodium Bicarbonate
NaOH	Sodium Hydroxide
$NaNO_2$	Sodium Nitrate
TAC	Total Antioxidant Capacity
TCA	Trichoroacetic acid
TFC	Total Flavonoid Content
TPC	Total Phenol Content
WHO	World Health Organization
ZOI	Zone of Inhibition

CHAPTER I

INTRODUCTION AND OBJECTIVES

1.1 Background

Plants kingdom have been an exemplary source of medicines for centuries (Parmar et al 2012). It has provided the foundation for many of today's longstanding medicinal treatments (McCully 2013). According to World Health Organization (WHO), more than 80% of the world's population relies on the use of traditional medicine which is predominantly based on plants (Kirbag et al 2009). Although some of the therapeutic properties attributed to plants have been proven to be erroneous, medicinal plant therapy is based on the empirical findings of hundreds and thousands of years. The most practical and frequently used traditional remedy is preparing an infusion or a decoction from the valuable parts of plants and herbs such as flowers, leaves, and roots (Guimaraes et al 2011).

Infusions such as teas made from the leaves of *Camellia sinensis* L. and tisanes, made from roots, flowers, leaves, seeds, or twigs of plants other than *Camellia sinensis* have been used for centuries in traditional therapy (Shikanga et al 2010). Traditionally, the infusions of cultivated exotic species (e.g., lemon, verbena, spearmint) and wild flora e.g., fennel were popular in the households (Guimaraes et al 2011). Like tea, herbal preparation of stinging nettle, *Urtica dioica* L. has been commonly used since ancient times in traditional medicine to treat several diseases. Infusions of stinging nettle plants can be used for nasal and menstrual hemorrhage, diabetes, anemia, asthma, hair loss and to promote lactation. In particular, leaves are used in the form of herbal infusions, which are rich in vitamins and minerals (Kregiel et al 2018).

Tea is the most popular non-alcoholic beverage in the world, due to the multitude of associated health benefits (Shikanga et al 2010). More recently, the beneficial properties associated with daily consumption of green tea are getting better recognized. The health benefits described in the consumption of teas may

be related to the high content of bioactive ingredients such as polyphenols. The common bioactive compounds found mainly in green teas are flavan-3-ols (catechins) (also called flavanols), proanthocyanidins (tannins), and flavonols while herbal teas contain diverse polyphenols (Malongane et al 2017). Polyphenols have been reported to possess antioxidant, antiviral, and anti-inflammatory activities; modulate detoxification enzymes; stimulate immune function, and decrease platelet aggregation (Lampe 2003). Many of these phytochemicals are reported to be biologically active when combined (Malongane et al 2017).

A combination of teas with other herbs such as ginger, pepper, and tulsi is normally practiced in our households to enhance the benefit and taste of the beverage. Most herbal practitioners and skillful healers have learned from their ancestors that herbal mixtures can be useful to increase the medicinal properties of individual species, reduce some kind of toxicity, and improve the taste of some oral forms. The folk use of mixtures is based on an empirical concept of similarity of the therapeutic effects (e.g., fennel and spearmint are both individually used as a digestive), as well as on the assumption of the sum of the benefits (Carvalho 2010). Moreover, in recent years many species of medicinal plants are widely combined in mixtures to enhance their pharmacological effects as reported in several ethnobotanical surveys (Guimaraes et al 2011).

The concept of synergy is based on the principle that, in combination, the formulation may enhance efficacy, reduce toxicity, decrease adverse side effects, increase bioavailability, lower the dose and reduce the advance of antimicrobial resistance (Vuuren and Viljoen 2011). The therapeutic effects of whole plant matter/extracts tend to be significantly more effective than the sum of the individual effects of each known compound. Synergistic effects can also occur when the extracts of two or more plant species exhibit greater effects than the extracts of one plant species. Enhanced synergy occurs when the final effect is much greater than the sum of the two individual effects. Furthermore, combining different plant extracts reduces the dose of individual plants required, thus preventing the side effects of using large amounts of each plant extract. The combination of different nutrients and phytochemicals gives higher

protection as nutrients act in combination rather than in isolation (Malongane et al 2017). The combination of polyphenolic compounds from different tea types has been used to enhance the antioxidant and antimicrobial effect (Betts et al 2013).

The positive effect of poly-herbal formulation for disease prevention and management assists in disease prevention and minimizing the side effects of pharmaceutical drugs (Malongane et al 2017). The increasing number of disease challenges to access to modern healthcare and the high cost associated with the use of modern healthcare provide a need to investigate the antimicrobial activities of indigenous plants alone and in synergy. Both green tea and *U. dioica* have reportedly shown a synergistic effect when combined with different herbs and antibiotics. However, to our best knowledge, no study has been conducted to observe their interaction. In the study, we aimed to screen the antibacterial activity of *Camellia sinensis* (green tea) and *U. dioica* leaves (stinging nettle) individually and in synergy. Aqueous, methanolic, and ethanolic preparations of plant extracts were tested for synergistic antibacterial activity against clinical isolates.

1.2 Statement of the problem

Microbial infections are the major cause of morbidity and mortality, particularly in the immune-compromised population. Although a wide range of antibiotics is produced for the treatment and management of microbial infections (Singh et al 2012), the increase of infections involving multidrug-resistant (MDR) bacteria and of resistance to last-resort antimicrobial agents have limited therapeutic options for bacterial infections. The frequency of resistance is observed equally among Gram-negative and Gram-positive organisms (Farooqui et al 2015).

Microbial infections are rampant in Nepal. According to National Planning Commission (2020), about 70% of all health problems and deaths in Nepal are attributed to infectious diseases. The burden of microbial infections is only increasing day by day together with the outbreaks of endemic as well as pandemic diseases and infection with drug resistance microbes during recent

years have constituted serious public health as well as a medical problem (Rai 2018).

On the other hand, non-communicable diseases (NCDs), such as cardiovascular diseases, cancer, diabetes, and chronic respiratory diseases, are the leading global cause of death and are responsible for 70% of deaths worldwide (WHO 2017). NCDs account death of 15 million women and men between the ages of 30 and 70 each year. In Nepal, about 65% of death occurs from NCDs (WHO 2017). Majorly, the risk of opportunistic infection has increased in immuno-compromised persons with NCD.

Numerous evidence suggested that several diseases are involved with the production of free radical or reactive oxygen species (ROS) in the cells (Ahmad et al 2013). An excess of free radicals in the body leads to oxidative stress which plays an important role in the pathogenesis of several human diseases that include atherosclerosis, cancer, inflammatory joint disease, asthma, diabetes, senile dementia, and degenerative eye disease. Antioxidants from plants may have greater relevance in the prevention and therapeutics of such diseases for being safer and more effective in the context of their efficiency and non-toxicity than synthetic antioxidants (Ilaiyaraja and Khanum 2011).

1.3 Objectives

1.3.1 General objectives

The general objective of the study was to determine the effect of bioactive compounds on the antibacterial activity of green tea (*Camellia sinensis*) mixed with stinging nettle (*Urtica dioica*) leaves.

1.3.2 Specific objectives

- To prepare stinging nettle (*Urtica dioica*) leaves incorporated green tea (*Camellia sinensis*) in different proportion (1.5:1.5, 1.2:1.8, 0.9:2.1, 0.6:2.4, 0.3:2.7).
- To perform sensory analysis based on a hedonic scale rating and selects the best tea blend.

- To prepare an extract of green tea, *U. dioica*, and the best tea blend using three different solvents viz. methanol, ethanol, and water.
- To quantify major phytochemicals (phenols, flavonoids, and tannins)
- To determine the antioxidant properties by using three different in-vitro methods; DPPH scavenging assay, phosphomolybdenum assay, and reducing power assay.
- To evaluate the antibacterial activity of green tea, *U. dioica* leaves and the blend against six clinical isolates (*S. aureus*, *S. typhi*, *S. flexneri*, *E. faecalis*, and *K. pneumoniae*)
- To determine the minimum inhibitory concentration of green tea, *U.dioica* leaves, and the blend.

1.4 Significance of the study

Herbal beverages such as teas (made from *Camellia sinensis* (L.) Kuntze leaves) and tisanes (made from herbs, spices, or other plant parts) have been used for centuries in traditional medicine for the treatment of several human diseases. Particularly, green tea has been confirmed by several studies for its health-promoting properties comprising antioxidant, anti-inflammatory, anti-diabetic, and antimicrobial activity (Rodrigues et al 2019). In addition, different *Urtica* species are used in many Asian Countries (Nepal, India, and China) in traditional medicines for the treatment of different health problems. Infusions and decoctions of this species, especially from the leaves, are rich in vitamins and minerals and have high in vitro antioxidant and antimicrobial activities (Kregiel et al 2018). However, the fear of the stinging hairs, lack of commercial availability, and the stigma related to stinging nettles being associated with famine/poor man's food are limited to ethno-pharmacological studies and qualitative phytochemical screening with very few studies exploring their biological activities.

Natural products (including herbal beverages) with the capacity to prevent and/or manage microbial infections and chronic diseases, like AD and diabetes, are a promising way to continuously improve human well-being and relieve social systems through health promotion. Moreover, folk medicine frequently combines different plant species in mixtures, called herbal mixtures, with the

purpose to enhance individual efficacy, reduce toxicity, decrease adverse side effects, increase bioavailability, lower the dose, and reduce the advance of antimicrobial resistance (Kuijun et al 2009). For example, green tea is commonly sold in mixtures combining different herbs, fruits, or spices (e.g. lemon, ginger, mint, anise, cinnamon, jasmine, raspberry, pomegranate) in order to improve or add beneficial properties to such beverages (Jain et al 2011).

Over the last decade, clinical studies have revealed several physiological responses to teas that may be relevant to the promotion of health and the prevention or treatment of some chronic diseases. It has been shown in numerous studies that disease resistance is less likely to occur against a combination of compounds than to single active constituents. Green tea in combination with herbs illustrated a strong synergistic effect (Liu et al 2016; Malongane et al 2017; Farooq and Sehgal 2019). A similar synergistic effect was observed when *U. dioica* was combined with antimicrobial and antifungal drugs (Rolta et al 2020). Both the plants have shown effective synergism when combined with herbs or antibiotics, however to the best of our knowledge; no study has been conducted to investigate their combined effect.

Nepal has been known for the production of varieties of teas and other herbs. The introduction of tea along with stinging nettle can be very effective as people of our country mostly rely on herbal medicine rather than modern drugs due to their higher costs and limited access. Besides, this practice of establishing phytochemical or pharmacological interactions of plants could be a positive approach to institutionalizing traditional medicine which can also lead to the development of new drugs, at the same time validating the plant to promote its use and thus supporting its traditional climb.

CHAPTER II

LITERATURE REVIEW

2.1 Green Tea (*Camellia sinensis*)

2.1.1 Introduction

Camellia sinensis is an evergreen shrub belonging to the Theaceae plant family. There are two major tea tree species, *Camellia sinensis* var. *sinensis*, and *Camellia sinensis* var. *assamica*. *Camellia sinensis* var. *sinensis* also known as China tea, is widely planted in China, Japan, and Taiwan, on the other hand, *C. sinensis* var. *assamica* known as Assam tea grows rapidly in South and Southeast Asia, and more recently, Australia (Chan et al 2007). The plant grows best in areas with considerable rainfall, good trenches, and soil that are slightly acidic, such as in tropical and subtropical areas. Based on the processing method with degrees of fermentation as one of the most important parameters, most teas are classified into three major forms, i.e. green teas (unfermented tea), semi-fermented teas (Oolong teas), and completely-fermented tea (black teas), which represent for 24%, 1%, and 75% of the total tea production in the world (Setyopratomo 2014).

Table 2.1.1: The taxonomy of *Camellia sinensis*

Camellia sinensis	
Kingdom:	*Plantae*
Sub kingdom:	*Tracheobionta*
Division:	*Magnoliopsida*
Order:	*Theales*
Family:	*Theaceae*
Genus:	*Camellia* (L.)
Species:	*Camellia sinensis* (L.) Kuntze
Main varieties:	*Camellia sinensis* var. *assamica*
	Camellia sinensis var. *sinensis*

Source: McCully (2013)

Fig1: Figure of a tea shoot

Camellia sinensis var. *assamica*, (Assam tea), is used for the production of green tea (Taylor 2003). It is a faster-growing tea plant having larger leaves with less resistance to cold and the tea is not as delicately flavored as sinensis (Segal 1996). It is processed with fresh leaves; the process starts with roasting or steaming. This steaming process destroys the enzymes responsible for breaking down the color pigments in the leaves, disables polyphenol oxidase activity, particularly catechins, to prevent oxidation. The process also allows the tea to maintain its green color during the subsequent rolling and drying processes (Chacko et al 2010). The leaves are dried and processed immediately after harvesting so that no fermentation occurs (Lee 2009). These processes preserve natural polyphenols concerning health-promoting properties (Chacko et al 2010).

Green tea is originated in China, introducing it to other parts of the world (Lee 2009). The first green tea was reported to have been exported from India to Japan during the 17th century (Chacko et al 2010). Most green teas are produced in Asia with India being the largest tea producer followed by China, Kenya, and Sri Lanka (Lee 2009). Green teas from different countries may differ in appearance, aroma, and flavor characteristics based on different processing methods, harvest times, tea tree varieties, and regions (Jung 2004).

2.1.2 Green tea processing

Processing of tea leaves is one of the important factors for flavor and promotes the development of volatile compounds, reduce bitterness, deactivates enzymes, reduce moisture content, and transforms fresh leaf into a variety of appearance, color, and flavor, that determines different tea types (Ahmed and Stepp 2013).

Green tea is minimally oxidized, non-fermented tea (Kumar et al 2013). The primary step of green tea processing starts with post-harvesting of tea shoots, withering, fixing, rolling, shaping, and drying. The overview of processing steps of green tea is listed below:

2.1.2.1 Plucking/Picking

Post harvesting of tea shoots is known as plucking or picking. Tea leaves are plucked manually or are mechanically harvested by a plucking machine. Many high-quality green teas are hand-plucked. Tea leaves, which include a terminal bud and two young leaves, are plucked from *Camellia sinensis* bushes. Tea leaves are harvested typically twice a year during early spring and early summer or late spring/autumn; winter pickings are much less common, though they occur when climate permits (Ahmed and Stepp 2013).

2.1.2.2 Withering

Once a tea leaf is plucked from the tea plant, a small amount of enzymatic oxidation occurs and the tea leaf will begin to wilt. This is usually done in sunlight (Ahmed and Stepp 2013) or in a cool room with plenty of air movement to remove water from the leaf (McCully 2013). Withering aims to remove moisture and soften the leaves to prepare them for further rolling. The leaves sometimes lose more than a quarter of their weight in water during withering. It leads also to the development of aroma and partial oxidation due to a breakdown of cell walls caused by moisture loss. The process is also important in promoting the breakdown of leaf proteins by peptidases into free amino acid and increases the availability of freed caffeine both of which changes the taste of tea. In general, green tea is withered for at least 8-10 hrs. (Kumar et al 2013).

2.1.2.3 Fixing

After withering, the tea leaves are exposed to heat for 10-15 minutes, in a process called fixing. Fixing deactivates enzymes in tea shoots to prevent oxidation and fermentation and to maintain its green color. The main relevant enzymes in the tea plant include polyphenol oxidase, catalase, peroxidase, and ascorbic acid. These enzymes have high activity after tea leaves are plucked and thus must be deactivated by applying high heat in the fixing process (Ahmed and Stepp 2013).

Fixing methods in green tea processing includes pan-frying and steaming. The pan-frying method developed in China during the Song dynasty (960-1127), is the main way of processing green tea. It involves placing tea leaves directly on a dry pan exposed to a high heat source (Xu and Chen 2002). Steaming, on the other hand, is the prevalent fixing method used in Japan. It involves placing tea leaves on perforated steamers that release steam blast from heated water. Steaming usually preserves more color, polyphenolic content, and antioxidant bioactivity than pan-frying (Xua and Changa 2008).

Fast, even and high-temperature fixing is important for high-quality green tea. Fixing temperature ranges from 100-200^{oC} for artisanal processed green teas and 220-330^{o} for machine-fixed green teas (Xu and Chen 2002).

2.1.2.4 Rolling and shaping

Following fixing, tea leaves are rolled to disrupt cell walls, release leaf moisture and shape the final product (Ahmed and Stepp 2013). The objective of rolling is to break the leaf cells and release the oxidases, including polyphenol oxidase and peroxidase, and initiate the process of catechin oxidation with oxygen in the air. The rolling method provides a unique taste and flavors (Wang and Ho 2009). The rolling of tea leaves was originally done manually, whereas nowadays it is performed by machines. Rolling varies between 10 minutes and 1 hour. Young leaves are rolled under light pressure and for a short duration compared to older leaves, to prevent leaf breakage and yellowing that results from hydrolysis of chlorophyll and auto-oxidation of polyphenols (Xu and Chen 2002).

Rolled leaves are then shaped into various forms including twists, round, flat, needles, flaky, compressed, and ground powder.

2.1.2.5 Drying

Finally, the shaped leaves are dried by pan-drying, basket-drying, sun-drying, or baking (Ahmed and Stepp 2013). The two objectives of drying are to terminate the chemical changes and to remove moisture to impart better storage quality. Depending on the process drying takes from 20 minutes to overnight. Great care must be taken to not overcook the leaves. The drying of the produced tea is responsible for many new flavor compounds particularly important in green teas (Xu and Chen 2002).

2.1.3 Chemical constituents

Tea is reported to contain nearly 4000 bioactive compounds of which one-third is contributed by polyphenols (Parmar et al 2012). The polyphenols found mostly in green tea are flavonoids, in which flavan-3-ols (commonly known as catechins) become the major constituent, which covers up to 30% of their dry weight (Parmar et al 2012; Setyopratomo 2014). Other compounds are alkaloids (caffeine, theophylline, and theobromine), proteins, amino acids, carbohydrates, chlorophyll, volatile organic compounds (aldehydes, alcohols, esters, lactones, hydrocarbons), minerals, and trace elements such as calcium, magnesium, chromium, manganese, iron, copper, zinc, molybdenum, selenium, sodium, phosphorus, cobalt, strontium, nickel, potassium, fluorine, and aluminum; and trace amounts of lipids (linoleic and a-linolenic acids), sterols (stigma-sterol), vitamins (B, C, E), and pigments (chlorophyll, carotenoids) (Horzic et al 2009). There are four kinds of catechins mainly find found in green tea: epicatechin, epigallocatechin, epicatechin-3-gallate, and EGCG (Chacko et al 2010). The most active and abundant catechin in tea is EGCG. Green tea contains much higher concentrations of these catechins than black tea (Parmar et al 2012).

Products derived from green tea are mainly extracts of green tea in liquid or powder form that vary in the proportion of polyphenols (45-90%) and caffeine content (0.4-10%). The preparation methods influence the catechins both quantitatively and qualitatively; the amount of catechins also varies in the

original tea leaves due to differences in variety, origin, and growing conditions (Chacko et al 2010).

2.1.4 Consumption pattern

Green tea has been consumed every day by millions of people around the world since ancient times to maintain and improve health. Tea consumption has its legendary origins in China dating back to more than 4,000 years ago, making it the oldest herbal tea known. According to legend, green tea was first brewed in 2737 BC during the reign of Emperor Shennong (Yadav et al 2016). The normal consumption of tea involves brewing the leaves and then consuming the liquor hot or cold (Wang et al 2008).

People around the world drink tea for cultural reasons, as well as for its desirable sensory properties or its probable health benefits (Lee 2009). Green teas are popular with claimed benefits to slimming, beauty, and relaxation. Green tea is considered to be a functional food (Erba et al 2005) with specific health claims in various countries to promote green tea for respiratory health, reducing cholesterol, and balancing blood pressure (Lee 2009).

2.1.5 Health benefits

Health benefits have been attributed to tea consumption since the beginning of its history, scientific investigation of this beverage and its constituents has been underway for about 30 years (McKay and Blumberg 2002). According to Rusak et al (2008), the health benefit associated with tea consumption is mainly in part to the antioxidant and free radical-scavenging activity. The association has been long appreciated (Chacko et al 2010), as it is reported to contain more content of tea polyphenols, mainly flavanols than oolong and black tea (Zhao et al 2008).

Polyphenols have been reported to possess antioxidant, antiviral, and anti-inflammatory activities; modulate detoxification enzymes; stimulate immune function, and decrease platelet aggregation (Lampe 2003). Green tea also contains methylxanthines: caffeine (1,3,5-trimethylxanthine) and two minors isomeric dimethylxanthines, theobromine and theophylline, which are

responsible for mildly stimulant effects of the tea. The caffeine in green tea is described to enhance mental activity, running performance, treat apnoea and migraine headaches, and implementation of theophylline and theobromine in asthma and bradycardia treatment (Komes et al 2010). Furthermore, these compounds have shown potential for maintaining normal body weight and supporting healthy glucose levels and had a likely advantageous influence on breast cancer (Chacko et al 2010).

Intensive research conducted on green tea concluded that drinking green tea increased plasma total antioxidant activity in humans, showed potential for maintaining normal body weight and supporting healthy glucose levels, presented anticarcinogenic effects, had anticarcinogenic action, showed an inverse relationship with all-cause mortality and cardiovascular disease mortality (Lee 2009).

These positive research results have driven consumption of green tea; thus, has become the fastest-growing non-alcoholic beverage around the world. Besides becoming popular beverages, green teas are also developed regarding their pharmaceutical and industrial application (Wang et al 2008). Various green tea products or products containing green tea are now available in retail food stores, also available to purchase online and several books and journals have been published on green tea, all of which may reflect increased demand for green tea (Chacko et al 2010).

2.2 Stinging nettle (*Urtica dioica*)

2.2.1 Introduction

Stinging nettle, *Urtica dioica* L. (Nepalese tongue –Sisnu,) is a genus of an annual or perennial herb, widely distributed in the temperate and sub-tropical zones (Chopra and Bhargava 1958). The word “nettle” is said to have derived from the Anglo-Saxon word “noedl” meaning “needle” while its genus name is derived from the Latinurere, which means to sting, and more precisely from uro, meaning to burn by friction (Kregiel et al 2018). Four common species of *Urtica*

are used for vegetable purposes found in the Indian region viz. *dioica*, *girardinia*, *plaviflora*, and *trema*. All species are armed with stinging hairs referred to as "trichomes" on the leaves and stems, which when rubbed against the skin cause a burning sensation and temporary rash (Oliver et al 1991). The plant grows in tropical and temperate wasteland areas to a height of 30-50 cm around the world and well tolerates all environments (Kavalali 2003). They prefer to grow in nitrogen-rich soil and are commonly found in soils high in inorganic nitrates and heavy metals (Upton 2013).

Table 2.2.1: The taxonomy of *Urtica dioica*

Urtica dioica	
Kingdom:	*Plantae*
Subkingdom:	*Viridiplantae*
Division:	*Trachaeophyta*
Order:	*Rosales*
Family:	*Urticaceae*
Genus:	*Urtica* L.
Species:	*Urtica dioica* L.

Source: Integrated Taxonomic Information System

Fig 2: Figure of *Urtica dioica* plant

2.2.2 Chemical constituent

Stinging nettle is a powerhouse of nutrients. They are reported to have shown to be richer in individual polyphenols than other wild plants (Augspole et al 2017). The chemical composition varies according to geographic conditions and taxonomical, morphological, and genetic factors (Esposito et al 2019). It

contains on an average 30% protein, 4% fats, 37% non-nitrogen extracts, 9-21% fiber, and 19-29% ash. Nettle is rich in iron, zinc, magnesium, calcium, phosphorus, and potassium. The leaves are rich in protein, fat, carbohydrates, vitamins (C and α-tocopherol), minerals, and trace elements. The content of mineral substances is about 20% of the dry mass. Several chemical constituents such as histamine, serotonin (5- hydroxytryptamine), and acetylcholine are also present. The other chemicals found in stinging nettle are malic acid, aspartic acid, serine, tyrosine, and tryptophan (Kumar et al 2017).

Fresh leaves contain phenolic acid (e.g. hydroxycinnamic acid, hydroxybenzoic acid), tannins, and flavonoids. The main flavonoids are quercetin, kaempferol and rutin. The most active flavonoid is quercetin. These flavonoids have antioxidant and anti-inflammatory properties. It has strong antioxidant and anti-inflammatory actions (Esposito et al 2019). The leaves contain about 4.8 mg chlorophyll per gram of dry leaves, depending on whether the plant was grown in the sun or shade. Surprisingly, more chlorophyll and carotenoids are found in plants that have been grown in the shade (Kukric et al 2012). Leaves content of cobalt, nickel, molybdenum, and selenium have also been determined (Rafajlovska et al 2013). The high content of protein, essential amino acids, vitamins, and iron present in nettle leaves can be an important nutritional supplement. Therefore, they can be a good remedy for the treatment of protein-energy malnutrition in malnourished children, pregnant women, convalescents, and the elderly. Furthermore, the protein content of the leaves widely covers the needs of amino acids, especially the essential amino acids for humans (Kukric et al 2012).

According to Ioana et al (2013), total phenolic content decreased with plant growth, and the decrease in the total phenolic derivatives was due to the decrease of non-tannin phenols (carboxylic acid and flavonoids). They reported total phenol content of 700 mg tannic acid/100g (as is) in young nettle leaves to 145 mg tannic acid/100g (as in) mature leaves. Phytoconstituents in plants show vital pharmacological activity. There are many alkamides and secondary compounds reported from the plants. Extracts and phytoconstituents isolated from nettle have been shown to produce differed pharmacological response,

which includes diuretic, analgesic, anti-inflammatory, and cardioprotective effects (Kumar et al 2017).

2.2.3 Food and feed application

Nettles have a long history of use as a food source as well as for medicinal purposes. The nettle plants have been consumed around the world; both raw and blanched or cooked in the form of soups, curries, and herbal infusions, without showing any report of serious adverse effects (Shonte 2018). Stinging nettles are eaten as famine food in many parts of the world, particularly popular among the lower socioeconomic people (Davidson and Jaine 2006; Khatiwada et al 2011). It is consumed as a vegetable alone or also mixed with cereals esp. millet flour to make porridge (Chopra and Bhargava 1958). Freeze-dried herb is processed for capsules, tablets, and other preparations. Formulations from fresh plant material include homeopathic products, juice, and liquid extracts (Law and Tamime 2010).

In Nepal, the young nettle leaves are cooked and eaten as green vegetables (Manandhar and Maheshwari 2000). It is consumed primarily as a boiled or cooked fresh vegetable whereby it is added to soups, cooked as a potherb, or used as a vegetable complement in dishes (Palikhe 2012). Nettle leaves are also consumed in the form of herbal tea, which is rich in minerals and vitamins. The concentrated form of nettle tea can be used as a soup base or as a component in drinks and cocktails. Nettle tea sometimes can also be used as a nutritional replacement for water (Kregiel et al 2018). *Urtica* plants are used in an alcoholic beverage, brewed similar to that ginger beer (Kovacs 1989). Furthermore, mature nettle leaves have been reportedly used as milk coagulants in the production of Cornish cheese (Fiol et al 2016).

The plant is also used as cattle fodder (Chopra and Bhargava 1958). The nettle fodder was reported with high biological value and was recommended as a valuable poultry feed (Safamehr et al 2012). The use of *Urtica* spp. as a feed component could also positively affect the health of poultry and animal productivity (Kregiel et al 2018). In some parts of Europe, fresh leaves are traditionally fed to pigs and poultry. *Urtica* spp. provides animals with nutrients

and bioactive components, which support antimicrobial activity, immune enhancement, and stress reduction.

2.2.4 Health Benefits

Stinging nettles are among the most commonly used medicinal plants in the world, due to their health-enhancing qualities. Their popularity can be explained by their non-toxic chemical composition, relatively low cost, and wide availability (Kregiel et al 2018). Mithril and Dragsted (2012) reported that nettle plants have been consumed without any report of serious adverse effects. Herbal preparation of the plant is commonly used for diuretic and for treating painful muscles and joints, eczema, gout and anemia, nasal and menstrual hemorrhage, asthma, hair loss and to promote lactation. Studies have shown that all parts of the nettle have antioxidant, antimicrobial, and pro-health capabilities (Khare et al 2012).

The most recognized health benefit of using stinging nettles is activity against Benign Prostatic Hyperplasia (BPH), also known as an enlarged prostate, as well as urinary tract infections. *Urtica* spp. is reported to contain compounds that affect the hormones responsible for BPH (Keland 2000). The herbal extract of *Urtica* plants is useful for bladder disorders, reduces postoperative blood loss, and prevents hemorrhagic and purulent inflammation following adenomectomy. Similarly, root extracts have shown promising activity against breast cancer. The effectiveness of *U. dioica* to treat breast cancer has been proved not only in laboratory conditions but also in in-vivo experimental models. The various extracts of *U. dioica* tested, in fact, prevent cancerogenesis, kill human cancer cells and inhibit their migration. Several studies revealed that nettles possess anti-diabetic properties (Esposito et al 2019).

Studies have also shown that applying nettle leaves directly decreases joint pain and can treat arthritis. Because of their high content of nutritive substances, nettles are also used in folk veterinary medicine. Nettle leaves are rich in proteins, minerals, vitamins, fibers, and phytonutrients, which can help to alleviate nutrition-related health problems. There are many dietary supplements based on *Urtica* spp. now on the market. Most nettle medicines are made from

flowers, stems, and leaves, but roots are also used in pharmacology (Shonte 2017).

2.3 Drying

Drying, in general, refers to the removal of nearly all the free moisture in foodstuff by evaporation or sublimation as a result of the application of heat in controlled conditions (Greensmith 1998). Drying facilitates storing when the plants are not physiologically active; extending the consumption period and utilization at times of shortages (Maanda and Bhat 2010). Drying slows down the action of enzymes but doesn't inactivate them (Harrison and Andress 1914). However, the drying process -changes in aroma and flavor of the plant (Shonte 2018).

The drying technique chosen has a major impact on nutrient degradation and retention (Shonte 2018). Ambient air-drying (such as well-ventilated air-drying and sun drying) was reported as the most common method of the drying process. However, the slow drying process involved in ambient air-drying methods may lead to loss of quality as well as the nutritional property of the leaves (e.g. color changes, losses of ascorbic acid, carotenoids, etc.) (Harbourne et al 2013).

Freeze-drying generally carried out at –45˚C for 5 days; is the most efficient method of drying for medicinal purposes. As this drying process is accomplished by sublimation of ice from the food, very few chemical changes have been reported to occur during this process. Hence this drying process is highly recommended for preserving heat-sensitive antioxidant components such as ascorbic acid, carotenoids, and soluble phenolics. However, the high cost of freeze-drying equipment limits its application to pharmaceutical products and production of highly valued healthy products such as nettle/green leaves, nettle/green leaf powder, nettle/green leaves tea bags, etc. (Shofianet al 2011; Shonte 2018).

Oven drying, on the other hand, is considered to be the most common method of drying used in the food processing industry due to its lower production cost compared to freeze-drying. As it runs between 45 °C to 140 °C, oven drying

results in faster degradation of color and loss of primary metabolite during thermal processing. In contrast to the ascorbic acid and β-carotene, oven drying could increase total phenol content and total antioxidant activity as compared to freeze-drying and fresh nettle leaves (Shonte 2018). The hot drying air removes moisture from the core of the tea leaves by diffusion process (Dutta and Baruah, 2014). The final moisture content of below 7% w.b. is a crucial aspect to get a stable product quality for preservation (Chan et al 2009).

2.4 Sensory Evaluation

The field of sensory evaluation has grown rapidly in the last half of the 20th century, along with the expansion of the processed food and consumer products industries (Lawless and Heymann 1999). Consumers are required to assess a product and report how much they like it which can aid in planning menus such that, foods that are liked by many customers can remain on the menu while those that are disliked by many customers can be removed (Wichchukit and O'Mahony 2015). The sensory evaluation comprises a set of techniques for accurate measurement of human responses to foods and minimizes the potentially biasing effects of brand identity and other information influences on consumer perception. As such, it attempts to isolate the sensory properties of foods themselves and provides important and useful information to product developers, food scientists, and managers about the sensory characteristics of their products (Lawless and Heymann 1999). Stone and Sidel (1993) defined sensory evaluation as a scientific method that is used to evoke, measure, analyze, and interpret those responses to products as perceived by the five senses (sight, smell, touch, taste, and hearing). This definition has been accepted and endorsed by sensory evaluation committees within various professional organizations such as the Institute of Food Technologists and the American Society for Testing and Materials.

Many rating scales have been developed for measuring the extent of liking (Lim 2011; Rosas-Nexticapa et al 2005) among which the 9-point hedonic scale is probably the most used sensory testing scale over the last 60 years in food science. The scale was first introduced as an aid to menu planning for US soldiers in their canteens. The scale comprises a series of nine verbal categories

ranging from from ‘like extremely’ as ‘9’ to ‘dislike extremely’ as ‘1’ for subsequent quantitative and statistical analysis and the responses to the verbal categories are treated as responses to numerical values along a preference continuum, namely a ‘numbers only’ scale (Peryam and Girardot 1952; Peryam and Pilgrim 1957). Typically, a hedonic test today would involve a sample of 75 to 150 consumers who were regular users of the product. The test would involve several alternative versions of the product and be conducted in some central location or sensory test facility. The larger size of an affective test might arise due to the high variability of individual preferences, and thus a need to compensate with increased numbers of people to ensure statistical power and test sensitivity. This also generates an opportunity to look for segments of people who may like different styles of a product, e.g., different colors or flavors. At the same time, it could also provide an opportunity to probe for diagnostic information concerning the reasons for liking or disliking a product (Lawless and Heymann 1999).

2.5 Extraction techniques

Extraction is the separation of medicinally active portions of a plant from the inactive or inert components using selective solvents in standard extraction procedures (Azwanida 2015). It is the crucial first step of any medicinal plant study, which plays a significant role in the final result and outcome. During extraction, solvents diffuse into the solid plant material and solubilize compounds with similar polarity. For successful determination of biologically active compounds from plant material is largely dependent on the type of solvent used in the extraction procedure. The most common solvents used for active component extraction are water, methanol, ethanol, chloroform, ether, and acetone (Pandey and Tripathi 2014). The qualitative and quantitative studies of bioactive compounds from plant materials also rely on the selection of proper extraction methods. Extraction of plant materials can be done by various extraction procedures.

Non-conventional methods are more environmentally friendly due to decreased use of synthetic and organic chemicals, reduces operational time, and better yield and quality of extract that have been developed during the last 50 years.

Supercritical fluid extraction, ultrasound-assisted extraction, enzyme-assisted extraction, and microwave-assisted techniques are a few of those promising non-conventional techniques. At the same time conventional extraction methods, such as Soxhlet are still considered as one of the reference methods to compare the success of newly developed methodology (Azmir et al 2013). Most of these techniques use the extracting power of different solvents and the application of heat and/or mixing. Methanol, acetone, chloroform, petroleum ether, and hexane are the most commonly used solvents for plant extraction. Liquid nitrogen has also been used as a form of extraction in some research work (Karuna et al 2000).

2.6 Phytochemicals in plants

Phytochemicals in plants are natural non-essential chemical compounds produced by plants eliciting pharmacological or toxicological effects in man and animals (Bernhoft et al 2010). These compounds are naturally present to protect the plant from the external environment. However, in most cases, these substances appear to be non-essential to the plant producing them. The active ingredients are found as secondary metabolites in medicinal plants (Hill 1952). These secondary metabolites can be unique to specific species or genera and increase their overall ability to survive and overcome local challenges by allowing them to interact with their environment. Some of the roles of secondary metabolites are relatively straightforward; for instance, they play a host of general, protective roles (e.g. as an antioxidant, free radical-scavenging, UV light-absorbing, and antiproliferative agents) and defend the plant against microorganisms such as bacteria, fungi, and viruses (Harborne 1993). The presence and the quality of these compounds are affected by various factors such as the variety, genotype, climate, soil, vegetative stage, harvest time, storage, processing, and treatment (Doss and Anand 2012). The typical bioactive compounds in plants include tannins, alkaloids, carbohydrates, terpenoids, steroids, and flavonoids.

2.6.1 Phenols/polyphenols

Phenols are widely distributed in plants. They are a highly important class of polyphenols and represent about one-third of consumed phytochemicals. Plant tissue may contain up to several grams per kilogram, and there may be more than 4,000 such compounds. Phenolic compounds in foods originate from one of the main classes of secondary metabolites in plants. They are produced as a response for defending injured plants against pathogens. Their concentrations in plants are also highly related to the level of stress factors such as UV radiation, the intensity of light, low temperature, drought, and deficiency of nutrients (Bernhoft et al 2010).

The main and most important role of phenol is its antioxidant property. They act as free radical scavengers which are formed due to high UV radiation. Similarly, they have also been found to have a wide range of biological effects. Increased bile production, reduced blood cholesterol, and lipid levels, and antibacterial activity against germs like *Staphylococcus aureus* are only a few of the biological roles of phenolic acids (Ghasemzadeh et al 2010).

2.6.2 Flavonoids

Flavonoids are the largest group of phenolic compounds consisting of a central three-ring structure. They occur as plant secondary metabolites that are involved in pigmentation, antioxidants, antimicrobials, anti-stressors, and UV radiation protection. More than 4000 flavonoids have been described so far within the parts of plants normally consumed by humans and approximately 650 flavones and 1030 flavanols are known (Ghasemzadeh et al 2010). Proanthocyanidins are oligomers of flavonoids. All compounds contain phenol groups involved in an effect as a general antioxidant. Other actions are diverse-several structures that reduce inflammation or carcinogenicity (Bernhoft et al 2010). The capacity of flavonoids to act as antioxidants depends upon their molecular structure. The position of hydroxyl groups and other features in the chemical structure of flavonoids are important for their antioxidant and free radical scavenging activities (Heim et al 2002)

Flavonoids are synthesized by plants in response to microbial infection. They are effective antimicrobial substances against a wide array of microorganisms. Their activity is probably due to their ability to complex with extracellular and soluble proteins and to complex with bacterial cell walls (Cowan 1999).

2.6.3 Tannins

Tannins are very widely distributed polyphenols in the plant kingdom (Bernhoft et al 2010). The features distinguishing tannins from plant polyphenols of other types are the properties of the former: binding to proteins, basic compounds, pigments, large molecular compounds, metallic ions, and also antioxidant activities, etc. (Okuda and Ito 2011). They can be found in the root, bark, stem, and outer layers of plant tissue (Saxena et al 2013). There are two distinct types of tannins. Condensed tannins are large polymers of flavonoids and hydrolyzable tannins which are polymers composed of a monosaccharide core (most often glucose) with several catechin derivatives attached. The two types of tannins have the most properties in common, but hydrolyzable tannins are less stable and have greater potential to cause toxicity (Bernhoft et al 2010).

Various studies have identified the toxic character of tannin against filamentous fungi, yeasts, and bacteria. For instance; the growth and protease activity of ruminal bacteria is prevented by the binding of the cell wall of bacteria by tannin (Scalbert 1991).

2.7 Antioxidant activity

An antioxidant is defined as “a substance that, when present in low concentrations compared to that of an oxidizable substrate, significantly delays or inhibits the oxidation of that substrate” (Boots et al 2008). These antioxidants can slow these reactions either by reacting with intermediates and halting the oxidation reaction directly or by reacting with the oxidizing agent and preventing the oxidation reaction from occurring (Pokorny 2007). Antioxidant activity is the total capacity of antioxidants to eliminate free radicals in the cell and food (Hur et al 2014).

Reactive oxygen species (ROS) are generated in the body as by-products of several cellular metabolic reactions. They consist of radical and non-radical oxygen species which is formed by the partial reduction of oxygen. Low levels of these oxygen species are required for cellular processes such as intracellular signaling, cell progression, and cell defense. Conversely, high levels of ROS or the inability of the antioxidant system to regulate ROS levels efficiently results in oxidative stress. The oxidative stress, on the other hand, results in direct or indirect ROS-mediated damage of nucleic acids, proteins, and lipids (Madhanraj et al 2017) leading to chronic diseases, including neurodegenerative diseases: stroke, Alzheimer‘s disease, Parkinson‘s disease, cardiovascular diseases: atherosclerosis and hypertension, diabetes, cancer, and osteoporosis, etc. (Kaczor et al 2016).

The human body produced some of such antioxidants, including glutathione and ubiquinol during normal metabolism. Although there are several enzymes system within the body that scavenges free radicals, the principal micronutrient (vitamins) antioxidants are vitamin E (α-tocopherol), vitamin C (ascorbic acid), and β-carotene. The body cannot manufacture these micronutrients, so they must be supplied in the diet (Lobo et al 2010). Most antioxidants isolated from higher plants are polyphenols, which show biological activity as antibacterial, anti-carcinogenic, anti-inflammatory, antiviral, anti-allergic, estrogenic, and immune-stimulating effects (Guimaraes et al 2011). Polyphenols are reported to exhibit excellent antioxidant and free radical scavenging properties (Hur et al 2014). Thus, consuming dietary antioxidant supplements has become popular among the general public to fight diseases, especially cancer.

2.7.1 DPPH radical scavenging activity

Scavenging of DPPH (2,2-Diphenyl-1-picrylhydrazyl) free radical is the basis of a common antioxidant assay (Rice-Evans et al 1997). It is a rapid, simple, and inexpensive method to test the ability of compounds to act as free radical scavengers and to evaluate their antioxidant capacity. The method is based on the reduction of DPPH, a stable free radical. The free radical reacts with DPPH, it becomes paired off in the presence of a hydrogen donor and then reduced to the DPPH-H, as a consequence of which the absorbance’s decreased from the

DPPH. Radical to the DPPH-H form results in de-colorization (yellow color) to the number of electrons captured. The color absorbance corresponds inversely to the radical scavenging activity of the sample extract i.e. more the de-colorization, more is the reducing ability (Tailor and Goyal 2014).

$$DPPH\bullet + FE \longrightarrow DPPH{-}H + A\bullet \quad (1)$$

$$DPPH + A\bullet \longrightarrow DPPH\text{-}A \quad (2)$$

$$A\bullet + A\bullet \longrightarrow A\text{-}A \quad (3)$$

Where FE is a scavenger and A• is radical

2.7.2 Total antioxidant activity

Phosphomolybdenum assay is a spectrophotometric method developed for the quantification of the total antioxidant capacity of a test sample. The assay is based on the reduction of Mo (VI) to Mo(V) by the sample analyte and the subsequent formation of a green phosphate Mo(V) complex at acidic p^H. The method has been optimized and characterized for linearity interval, repetitive and reproducibility, and molar absorption coefficients for the quantification of several antioxidants, such as ascorbic acid, some phenolics, α-tocopherol, and carotenoids (Prieto et al 1999). A higher absorbance indicates a higher antioxidative activity.

2.7.3 Ferric reducing antioxidant potential

Ferric reducing antioxidant power (FRAP) assay is a robust, sensitive, simple, and automated test used for the quantitative determination of antioxidant capacity in a test sample. This method is based on the principle of increase in the absorbance of the reaction mixtures, the absorbance increases the antioxidant activity increases. The assay uses the reduction of ferric ions (Fe3+) to ferrous ions (Fe2+) at low p^H to form a colored ferrous-tripyridyltriazine complex with potassium ferricyanide, trichloroacetic acid, and ferric chloride. FRAP values are obtained by comparing the absorbance change at 700nm in test mixtures with those containing ferrous ions in known concentrations,

measured by UV-spectrophotometer (Benzie and Strain 1996; Vijayalakshmi and Ruckmani 2016).

2.8 Antimicrobial activity

Antimicrobials are substances that kill or inhibit the growth of microorganisms. The antimicrobials could be in the form of antibiotics, which may be the products of microorganisms or synthesized derivatives or antimicrobial peptides produced by complex organisms as well as some microbes and medicinal plants, which appear to be the focus of mainstream medicine today (Cowan 1999). The first antimicrobial agent in the world was salvarsan, a remedy for syphilis that was synthesized by Ehrlich in 1910. In 1935, sulfonamides were developed by Domagk and other researchers. These drugs were synthetic compounds and had limitations in terms of safety and efficacy. In 1928, Fleming discovered penicillin. Since then, continual improvements have been made for antimicrobial agents in various aspects in addition to the antimicrobial spectrum and activity (Saga and Yamaguchi 2009).

The antimicrobial susceptibility test (AST) is an essential technique for the evaluation of susceptibility of pathogens to the antimicrobial agent while screening new antimicrobials or antibiotics. It is used in pathology to determine the resistance of certain microbial strains to different antimicrobials and in pharmacology research, it is used to determine the efficacy of novel antimicrobials from biological extract against different microorganisms (Das et al 2010). The agar well diffusion methods and broth microdilution are commonly employed methods for in vitro antimicrobial sensitivity screening during the search of novel antimicrobial agents from various sources such as plant extracts, essential oils, synthetic abnormal structures, etc. in test organisms (Das et al 2012).

2.8.1 Agar well diffusion assay

Agar well diffusion assay is widely used to evaluate the antimicrobial activity of plants or microbial extracts while screening new antimicrobials or antibiotics. The antimicrobial activity by the agar well diffusion method is assayed by measuring the zone of inhibition exhibited by the plant extracts. In this assay,

the agar plate surface is inoculated by spreading a volume of the microbial inoculum over the entire agar surface. Then, a hole with a diameter of 6 to 8 mm is punched aseptically with a sterile cork borer or a tip, and a volume (20–100µL) of the antimicrobial agent or extract solution at desired concentration is introduced into the well. Then, agar plates are incubated under suitable conditions depending upon the test microorganism. The antimicrobial agent diffuses in the agar medium and inhibits the growth of the microbial strain tested (Balouiri et al 2016). Organic solvents (DMSO, DMF) used can sometimes exert an inhibitory effect on the test organism, therefore the antimicrobial activity of the test compound is usually determined by subtracting the zone of inhibition produced by solvent from the zone of inhibition given by the mixture of compound and solvent (Sharma and Sharma 2011).

2.8.2 Minimum Inhibitory Concentration (MIC)

On the other hand, the broth microdilution method is used to determine the minimum inhibitory concentration (MIC) of plant extracts. Microplates are used which allows large amounts of data to be generated quickly. Bacterial growth could be assessed either visually by grading turbidity or better spectrophotometrically by measuring optical density. Visual assessment of bacterial growth lacks objectivity and precision. Similarly, spectrophotometric readings may be hampered by additives or antibacterial compounds that affect the spectral characteristics of growth media, the aggregation of bacteria, or bacterial pigments. Thus, the Colorimetric method could be used as an alternative approach. Since tetrazoliums salts are used as colorimetric indicators, bacteria or fungi convert them to colored formazan derivatives that can be quantified (Grare et al 2008).

2.9 Synergistic effects

The basic concept of synergy is based on the idea that using a whole plant containing a group of chemicals working together is more beneficial than using a single compound to achieve a specific effect (Malongane et al 2017). The therapeutic effects of whole plant matter/extracts tend to be significantly more effective than the sum of the individual effects of each known compound

(Wanget al 2011). The term synergy is interchangeably used with ‘potentiation’, i.e., enhancement of one agent by another to exert a greater effect. An enhanced blend occurs when the final effect is much greater than the sum of the two individual effects (Farooqui et al 2015). In simple words, synergy is defined as the interaction of two or more agents to produce a combined effect greater than the sum of their individual effects (Vuuren and Viljoen 2011).

Synergistic effects can also occur when the extracts of two or more plant species exhibit greater effects than the extracts of one plant species (Wang et al 2011). Furthermore, combining different plant extracts reduces the dose of individual plants required, thus preventing the side-effects of using large amounts of each plant extract. The combination of different nutrients and phytochemicals gives higher protection as nutrients act in combination rather than in isolation (Jain et al 2011).

2.10 Antagonist effect

The antagonist effect is also termed sub-additivity (Tallarida 2001), infra-additive (Geary 2013), negative interaction, de-potentiation, and even negative synergy (Berenbaum 1977). It is often considered more of a negative scenario in the bio-medical world, as many researchers are looking to identify synergistic interactions among compounds for some sort of added therapeutic effect. However, in a toxicological sense, it may be beneficial to have an antagonistic effect in a mixture of chemicals (Roell et al 2017).

A study conducted by Langley-Evans (2000) reported that the addition of milk to black tea decreased the antioxidant potential of black tea preparations resulting in the antioxidant antagonist effect. A similar antagonistic effect was observed where both milk and lemon powder was added to matcha tea, which resulted in the reduced antioxidant capacity of the tea (Korir et al 2014). Another study by Belscak et al (2011) showed that the addition of honey to the infusions already containing ascorbic acid antagonistically decreased the total phenolic compounds and antioxidant capacity of that preparations. Therefore, careful consideration must be taken while finding a mixture with an enhanced beneficial effect.

CHAPTER III

MATERIALS AND METHODS

3.1 Materials

3.1.1 Plants used

a. Tea (*Camellia sinensis*): Tea leaves, locally known as "chiyapatta" were collected from Jasbirey, Ilam (26.9857˚N, 87.9298˚E).

b. Stinging nettle (*Urtica dioica*): Stinging nettle leaves were collected locally from inside of Central Campus of Technology premise (26.8213˚N, 87.2887 ˚E), and it is locally known as "Sisnu".

The plants were verified in Botanical Lab at Central Campus of Technology, Hattisar, Dharan.

3.1.2 Bacterial culture

Five bacterial strains; *Staphylococcus aureus* (ATCC 25293), *Salmonella typhi* (ATCC 14028), *Shigella flexneri* (ATCC 12022), *Enterococcus faecalis* (ATCC 29212), and *Klebsiella pneumoniae* (ATCC 20063) were obtained from BPKIHS, Dharan to test antibacterial activities of the extracts. The culture was provided in serum vials and brought to laboratory in an icebox (4^{o}). The culture was then immediately transferred to an appropriate growth medium and incubated under the appropriate conditions.

3.1.3 Chemicals, Reagents and Media

All chemicals, reagents, media and equipment used were available in the laboratory of Central Department of Technology, Dharan. All the chemicals, reagents, media used in our work are listed in **APPENDIX A**.

3.2 Methods

3.2.1 Study site

The study was carried out at the Central Campus of Technology, Dharan, Nepal from January 2021 to April 2021.

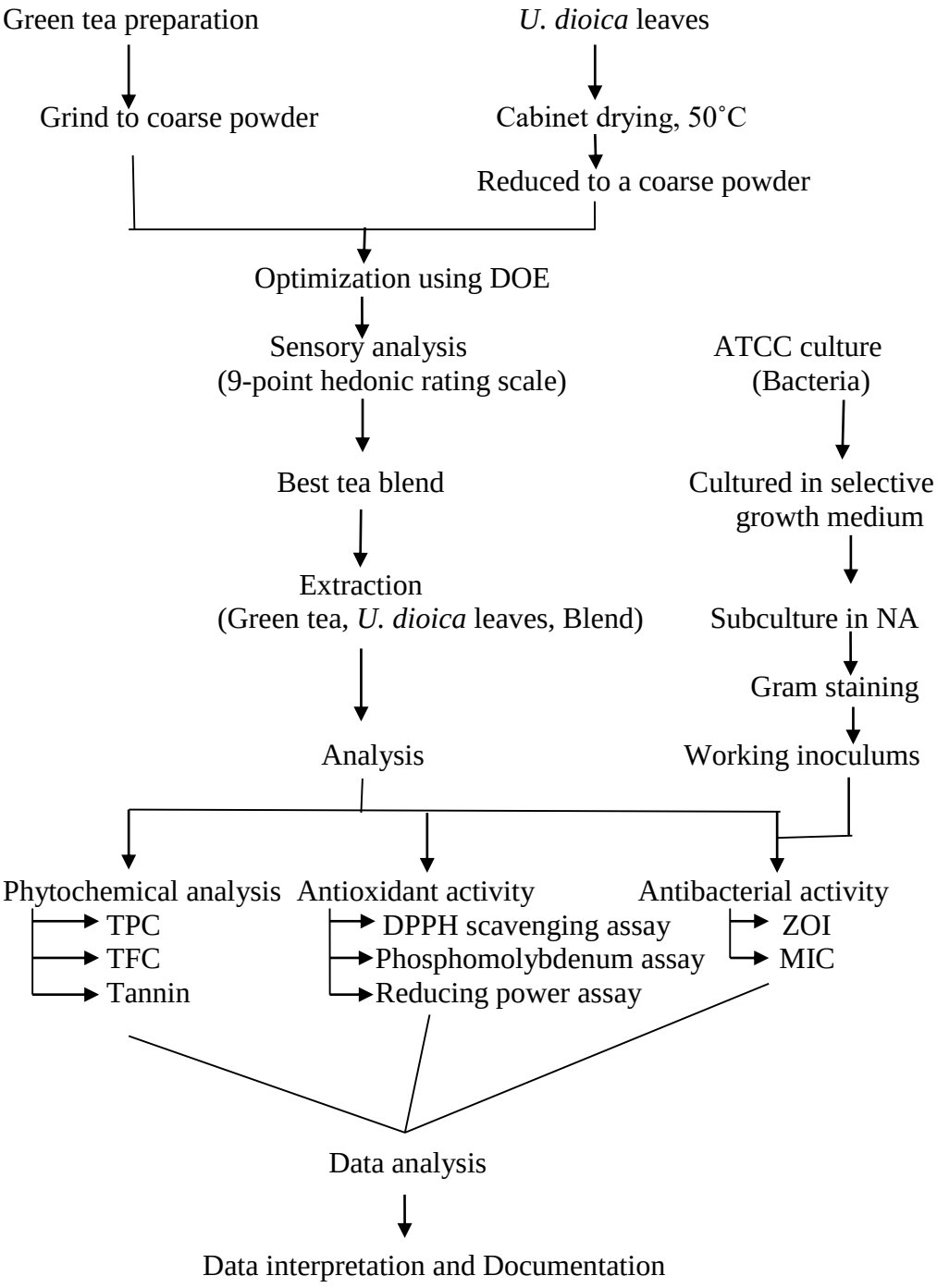


Fig 3: Flow Diagram of the Study

3.2.2 Processing of plant samples

Tea leaves were processed to prepare green tea using a standard procedure (Nagalakshmi 2003) with slight modifications based on local climate and practices as presented in Figure 4.

Plucking (hand plucking, bud + 1st leaf + 2nd leaf)

↓

Steaming (10min, 130°C)

↓

Draining and cooling (10min)

↓

First manual rolling (10min)

↓

Panning (20min, 100°C)

↓

Second manual rolling (30min)

↓

Green tea

Fig 4: Schematic representation of green tea processing

U. dioica leaves on the other hand were washed, sorted and kept in a cabinet dryer at 40-50˚Cto dry. After drying, leaves were reduced to a coarse powder using a grinder, packed and then placed into a well-closed container and stored at 0˚C until further use.

3.2.3 Preparation of mixed tea blend

Different combinations of blend tea were made by mixing different proportions of *U. dioica* leaves with green tea using a response surface methodology. The study's experimental design includes two independent variables: a percentage of green tea powder and a percentage of *U. dioica* leaves. The percentages of *U. dioica* leaves powder are based on the amount of green tea powder used. The experimental design by Design Expert 7.0 software is presented in **APPENDIX A**.

Based on the preliminary research, each level's values were assigned as follows: the percentage of green tea powder ranged from 50-90%, and the percentage of *U. dioica* leaves powder ranged from 10-50%. A total of 13 experimental runs were generated with repetitions, among which five were selected to predict the optimal formula of blended tea for consumer acceptability.

Table 3.2.3 Optimized blend tea samples

Sample code	**Green tea (g)**	***U. dioica* (g)**
A	1.5	1.5
B	1.8	1.2
C	2.1	0.9
D	2.4	0.6
E	2.7	0.3
Control	3.0	0.0

3.2.4 Preparation of blended tea in teabag packaging

The process of making blended tea begins with mixing green tea powder, and *U. dioica* leaves powder according to the percentage specified in the research design. The mixing was performed homogeneously such that the blended tea was perfectly mixed. After that, the tea bags were filled with 3 grams each of the blended tea. The top of the teabag was attached with string and further sealed and labeled. Sealing was done carefully such that there were no leaks in the teabag.

3.2.5 Sensory Evaluation of Tea

Sensory evaluation of tea samples was conducted to establish preference rating of tea for flavor, aroma, appearance, and overall acceptability using a 9point hedonic scale. The hedonic rating test is used to measure the consumer acceptability of food products. This method can be used with untrained panelists as well as with experienced ones (Ranganna 1986). The evaluation was carried out by 11 panelists comprising teachers and non-teaching staff of Central Campus of Technology, Dharan.

Blended tea in teabag packaging was brewed using the same water volume (150ml), water temperature, and brewing time. All samples were served in 150ml cups coded with random alphabets under warm conditions. Each panelist was provided with an evaluation card (**Appendix C**) to record their opinion on sensory observations. They were provided with potable water for rinsing between the samples. Verbal communication among the panelist was prohibited. They were asked to evaluate the samples individually using a scorecard. Sensory parameters based on brew appearance, brew aroma, brew flavor, and brew taste were analyzed. The grading system was based on a 9-point hedonic rating test which was indicated as,

9 = Like extremely; 8 = Like very much; 7 = Like; 6 = Like slightly; 5 = Neither like nor dislike; 4 = Dislike slightly;3 = Dislike moderately; 2 = Dislike; 1 = Dislike extremely

3.2.6 Preparation of extract

Successive solvent extraction was used for the preparation of different extracts. The powdered samples i.e., the blend and the individual plant leaves were subjected to organic solvent (methanol, and ethanol) extraction as well as aqueous extraction.

3.2.6.1 Preparation of aqueous extract

The aqueous extracts were prepared by following the method as described by (Amin et al 2015). 5 gm of the plant powder was taken in a beaker and mixed

with 200 ml of distilled water. The mixture was then heated on a hot plate at 30-40ºC with continuous stirring for 20 minutes. The mixture was filtered using Whattman filter paper No. 1 (pore size 11µm). The filtrate obtained was evaporated in a water bath at 40°C to dry to obtain the solid mass of the extract. Finally, extracts were transferred to brown colored glass bottles, sealed by using caps, and stored at 4±2˚C until analysis.

3.2.6.2 Preparation of methanol and ethanol extract

The organic extraction was performed by using the Soxhlet apparatus as described by Amin et al (2015). Methanol and ethanol were used for the solvent extraction process. This extraction was performed by taking 20 gm of dried plant powder into a glass thimble then extracting with 250 ml of different solvents separately. The extraction process was carried on till the solvent in the siphon tube of the Soxhlet apparatus become colorless. The extracts were concentrated with a Rota-evaporator and then transferred to brown colored bottles and sealed. The dried plant crude extracts were kept in the refrigerator at 4±2ºC for future use.

3.2.7 Calculation of percentage yield of extract

After the complete extraction, the percentage yield of plant extract was calculated. To calculate plant extract yield, the weight of the pre-weighed beaker was subtracted from the weight of the beaker with dry extract. The crude plant extract was then transferred in a sterile bottle using a spatula and was labeled and kept at 4˚C for further use. The percentage yield of the plant extract was calculated as below:

$$\text{Percentage yield (\%)} = \frac{\text{Dry wt. of Extract}}{\text{Dry wt. of plant material}} \times 100$$

3.2.8 Phytochemical screening

The extracts were subjected to qualitative phytochemical screening to detect the major phytochemicals present in them. The plant aqueous was screened out to identify the constituents (phenols, tannins, saponins, alkaloids, flavonoids,

terpenoids) using the following standard procedures as described by Jaradat et al (2015)

3.2.8.1 Test for phenols

Ferric Chloride Test: Extracts were treated with 3-4 drops of ferric chloride solution. The formation of bluish-black color indicated the presence of phenols.

3.2.8.2 Tests for flavonoids

About 2 ml of 2% NaOH solution was mixed with plant crude extract, the intensive yellow color was formed which turns into colorless when added 2 drops of diluted acid to the solution. This indicated the presence of flavonoids.

3.2.8.3 Tests for tannins

About 2 ml of 2% solution of ferric chloride was mixed with the crude extract. A blue-black precipitate indicated the presence of tannins.

3.2.8.4 Test for saponins

5 ml of distilled water was added to the crude plant extract in a test tube and shaken vigorously. The formation of stable foam indicated the presence of saponins.

3.2.9 Quantitative analysis

3.2.9.1 Determination of total phenol

Total phenolic content (TPC) in the plant methanolic, ethanolic, and aqueous extracts were determined using Folin-Ciocalteu method described by Jaradat et al (2015) with some modifications. 1 mg/ml aqueous solutions for all the extracts were prepared in the analysis. The reaction mixture was prepared by mixing 0.5 ml of plant extract solution, 2.5 ml of 10% Folin-Ciocalteu's reagent dissolved in water, and 2.5 ml of 7.5% of Na_2CO_3 aqueous solution. The samples were thereafter incubated in a thermostat at 45 ºC for 45 min. The absorbance was determined using a spectrophotometer at a wavelength of 765 nm against blank. The samples were prepared in triplicate for each analysis and

the mean value of absorbance was obtained. A standard curve was plotted using different concentrations of Gallic acid using the same procedure. Total phenolic content was estimated as mg Gallic acid equivalents (GAE)/g of dried extract.

3.2.9.2 Determination of flavonoids

Total flavonoid content was determined using a modified aluminum chloride assay method as described by Barek et al (2015). 1 ml of each extract solution was mixed with 0.3ml of 5% $NaNO_3$, 0.3ml of 10% $AlCl_3$, followed by the addition of 2ml of 1N NaOH then the volume was made up to 10ml with distilled water. Absorbance was measured after 15 min at 510nm against the reagent blank. The calibration curve was prepared using different concentrations of quercetin following the same above procedures. The total flavonoid content was expressed as mg quercetin equivalents (QE).

3.2.9.3 Determination of tannins

The tannins were determined by Folin - Ciocalteu method (Mythili et al 2014). About 0.1 ml of the sample extract was mixed with 7.5 ml of distilled water, 0.5 ml of Folin-Ciocalteu phenol reagent, and 1 ml of 35 % Na_2CO_3 solution then the volume was made up to 10ml with distilled water. The mixture was shaken well and kept at room temperature for 30 min. Absorbance for test and standard solutions were measured against the blank at 725 nm using a UV/Visible spectrophotometer. The calibration curve was prepared using different concentrations of gallic acid following the same above procedures. The tannin content was expressed in terms of mg of GAE/g of extract.

3.2.10 Antioxidant assay

3.2.10.1 DPPH radical scavenging assay

DPPH free radical scavenging ability of plant extracts was determined by the method described by Hatano et al (1988) with slight modifications. In clean and well-labeled test tubes, 100 µl of extracts were dissolved in a 3.9 ml freshly prepared methanolic solution of DPPH (1mM). The tube was incubated at room temperature for 30 min in the dark and the absorbance of the resulting solutions

was read spectrophotometrically at 517 nm. The percentage inhibition of the radicals due to the antioxidant activity of leaf extracts was calculated using the following formula:

Percentage of inhibition of DPPH activity (%) $= \frac{A_{control} - A_{sample}}{A_{control}} \times 100$

$A_{control}$ is the absorbance of the DPPH solution with nothing added (control).

3.2.10.2 Total antioxidant capacity

The total antioxidant capacity of leaf extracts was analyzed according to the method described by Prieto et al (1999). The tubes containing leaf extract (0.3ml) and 3ml reagent solution (0.6M sulphuric acid, 28mM sodium phosphate, and 4mM ammonium molybdate) were incubated at 95°C for 90 min. After the mixture was cooled to room temperature, the absorbance of each solution was then measured at 695 nm spectrophotometrically against a blank. The antioxidant capacity was expressed as ascorbic acid equivalents (AAE).

3.2.10.3 Reducing power assay

The reducing ability of extracts was measured according to the method described by (Oyaizu 1986). Different concentrations of the plant extracts in corresponding solvents were mixed with 2.5 ml of phosphate buffer (pH 6.6) and 2.5ml of potassium ferricyanide. This mixture was kept at 50ºC in a water bath for 20 minutes. After cooling, 2.5 ml of 10% trichloroacetic acid was added and centrifuged at 3000 rpm for 10 min. The upper layer of solution (2.5 ml) was mixed with 2.5ml of distilled water and 0.5ml of freshly prepared ferric chloride solution. The absorbance was measured at 700 nm. Control was prepared similarly excluding samples. Ascorbic acid at various concentrations was used as standard. Increased absorbance of the reaction mixture indicated higher reducing power.

3.2.11 Antimicrobial assay

3.2.11.1 Preparation of extract solution:

32mg of all crude extracts were dissolved in 10ml DMSO to make a concentration of 3200µg/ml stock solution. After making stock solution the test tubes were capped and stored at 4°C until use.

3.2.11.2 Sterility testing of extracts

All the extracts were tested for growth or contamination. 1 ml each of extracts were spread on nutrient agar and incubated at 37˚C for 24hrs. After incubation, the plates were observed for growth. No growth in the extract after incubation indicated that the extracts were sterile. The extracts were then accessed for microbial assays.

3.2.11.3 Preparation of culture

Appropriate selective media were prepared before the cultures were brought to the lab. Mannitol Salt Agar (MSA) for *S. aureus* and *E. faecalis*, *Salmonella-Shigella* Agar for *S. typhi* and *S. flexneri*, MacConkey Agar for *K. pneumonia* were the selective media prepared for the respective bacterial cultures. The ATCC cultures were first allowed to stand at room temperature in a sterile environment. Using a sterile loop, a loopful of bacterial culture was taken aseptically, one at a time and streaked on the respective agar medium. The plates were then incubated at 37ºC for 24 hours. After incubation, the growth was observed.

3.2.11.4 Streak plate method for determining culture purity

The streak plate method was used to confirm the purity of bacterial stocks. The main aim of this method was to obtain isolated colony forming units (CFUs) of bacteria. For this, a concentrated bacterial colony was taken from selective media and streaked across an NA plate and incubated for 24hrs at 37˚C. After incubation, the colonies were inspected.

3.2.11.5 Gram staining of bacterial cells

An individual CFU was taken from a selected culture using a sterile loop. The loop was spread onto a microscope slide with distilled water to create a thin film. The slide was left to air dry and the bacteria were heat-fixed to the slide. Crystal violet was used to stain the bacteria for 1 min, followed by the application of Gram's iodine for 1 min. The slide was then rinsed with 70% (v/v) ethanol for 15 sec and safranin was applied for 1 min. In between each step, the slide was rinsed with distilled water. Slides were then allowed to air-dry and were viewed under a microscope under oil immersion at a total magnification of 1000x.

3.2.11.6 Preparation of standard culture inoculums

A single colony of the organism on the NA plate was transferred to a tube containing 5ml of NB sterilized by autoclaving. The NB broth containing culture was then incubated at 37°C for 4 hours to maintain the standard turbidity of 0.5 McFarland to obtain the desired cell density of $1.5x10^8$ (cells/ml) as recommended by WHO (1991).

3.2.11.7 Antibacterial screening via Agar Well Diffusion technique

All the sample extracts were investigated for the antibacterial activity by agar well diffusion method against clinical isolates of *Staphylococcus aureus*, *Salmonella typhi*, *Shigella flexneri*, *Enterococcus faecalis*, and *Klebsiella pneumoniae* (Singh et al 2012) obtained from BPKIHS, Dharan, Nepal. The bacterial cultures were grown in nutrient broth medium at 37˚C and kept under refrigeration until use.

The Muller-Hinton agar plates prepared for the assay were seeded with 1.5×10^6 cells ml^{-1} (adjusted to 0.5 McFarland turbidity standards) suspension of tested bacteria using a sterile cotton swab. The inoculated plates were left for 20 minutes at room temperature. Then using cork borer no. 6, wells of 6mm in diameter were made in the inoculated plates and labeled. 100µl of each extract was pipette onto the holes and DMSO itself was tested as a control in a separate well. The plates were then incubated at 37°C for 24 hours. After the incubation,

the plates were observed for the halo zone around the well. All the experiments were performed in triplicates and results were recorded by measuring the zones of inhibition produced by the extracts around the discs.

3.2.11.8 Minimum Inhibitory Concentration

The minimum inhibitory concentration (MICs) assay was determined by the micro-well dilution method (Swanson et al 1992). The 96-well plates were prepared by dispensing 45µL of nutrient broth and 5µL of the inoculum in each well. A 50µL of plant crude extracts initially prepared at the concentration of 3.2mg/ml in DMSO (2.5%) was added into the first wells. Then, 50µL from their serial dilutions was transferred into different consecutive wells to achieve concentrations from 1µg/ml to µg/ml. The last well-containing 95µL of NB without bacterial inoculum was used as a negative control. The final volume in each well was 50µL. The microtiter plates were covered with a sterile lid and incubated at 37°C for 24 hrs. MIC was determined as the lowest concentration of extracts which did not show any growth of the tested organism after macroscopic evaluation, expressed in mg/ml.

3.2.12 Quality control for tests

The quality and accuracy of all tests were maintained by following the standard procedures. All the media, antibiotics, and reagents were prepared, stored, and utilized as recommended by the manufacturing company.

3.2.13 Statistical analysis

All the above determinations were carried out in triplicates. The data were entered in MS Excel, 2019 and all the calculations of standard deviation and preparation of calibration curves were performed in it. ANOVA along with Post hoc test were carried out in JMP Version 14. The difference was considered significant at $p<0.05$. All the graphical outputs were sketched using ggplot2 package in R-programming Version 4.02.

CHAPTER IV

RESULTS

Tea (*Camellia sinensis*) leaves and Stinging nettle (*Urtica dioica*) leaves were collected from Jasbire tea garden, Maipokhari and Hattisar-14, Dharan respectively. Tea leaves were processed further to prepare green tea, which was then mixed with *U. dioica* leaves in different proportions to obtain an optimum formulation of a blended tea using DOE based on sensory characteristics. Green tea, *U. dioica* leaves and the blend extract was prepared using different solvents viz; methanol, ethanol and aqueous. The prepared extracts were then analyzed for their phytochemical contents, antioxidant activity and antibacterial activity.

4.1 Sensory evaluation of blended tea samples

Sensory evaluation was carried out for: tea appearance, aroma, flavor, and overall acceptability of infusion by 11 panelists using a 9-point hedonic scale. The statistical analysis (two-way ANOVA) was done ($p<0.05$). There was a significant difference for three of the sensory attributes evaluated viz., appearance, aroma, and overall acceptability of infusion. The result of the sensory evaluation and statistical analysis is listed in **Table 4.1**.

Table 4.1: Average sensory score of blended tea samples

Sample code	Sensory attributes			
	Appearance	**Aroma**	**Flavor**	**Overall acceptance**
A	6.72 ± 0.90^{a}	6.54 ± 0.82^{b}	7.18 ± 1.07^{ab}	6.54 ± 0.93^{b}
B	6.90 ± 1.13^{a}	6.81 ± 0.98^{ab}	6.72 ± 0.90^{b}	6.63 ± 0.8^{b}
C	7.54 ± 0.93^{a}	7.54 ± 0.68^{a}	7.72 ± 0.78^{a}	7.72 ± 0.78^{a}
D	7.27 ± 1.10^{a}	6.81 ± 0.98^{ab}	6.81 ± 1.40^{ab}	7.00 ± 1.0^{ab}
E	7.09 ± 1.44^{a}	6.54 ± 1.29^{b}	6.27 ± 1.19^{b}	6.45 ± 1.36^{b}

*Figures in the parenthesis are the standard deviations. The same alphabetical values in the superscript within the column are not significantly different at $p<0.05$.

4.1.1 Brew appearance

The mean sensory score for the appearance of samples A, B, C, D, and E was found to be 6.72, 6.90, 7.54, 7.27, and 7.09 respectively. The highest score was obtained for sample C and the least obtained for sample A. There was no significant difference among the samples ($p<0.05$) in brew appearance.

4.1.2 Brew aroma

The mean sensory scores for the aroma of samples A, B, C, D, and E were found to be 6.54, 6.81, 7.54, 6.81, and 6.54 respectively. Sample C was found superior based on aroma from statistical analysis while A, and E were among the least scored samples. The brew aroma was significantly different ($p<0.05$). There was no significant difference in the samples A&E and B, D & C was in brew aroma.

4.1.3 Brew flavor

The mean sensory score for the flavor of five samples A, B, C, D, and E was found to be 7.18, 6.72, 7.72, 6.81, and 6.27 respectively. The flavor of the brew was significantly different ($p<0.05$). The least score for flavor was obtained for sample D while the highest mean score was obtained for sample C. There was no significant difference in the sample A&D and B, C &E in brew flavor.

4.1.4 Overall acceptance

The mean sensory score for the overall acceptance of five samples A, B, C, D, and E was found to be 6.54, 6.63, 7.72, 7.00, and 6.45respectively. The overall acceptability of the samples was significantly different ($p<0.05$). There was no significant difference in the samples A, B, D & E, and C & D.

From the results above, it can be seen that samples C and D were not significant from each other. However, the mean sensory score for sample C was found higher compared to D. Thus, sample C, which has the combinations of green tea (2.1 g) and *U. dioica* leaves (0.9 g) was selected as the best tea blend.

4.2 Physical characteristics of samples

The sample extracts with three different solvents i.e., methanol, ethanol, and water. Different samples showed variations in the percentage yield, texture, and consistency. Obtained mass was sticky to touch with consistency varying from solid to semi-solid in appearance. The percentage yield varied from 10.86% to 28.00%. *U. dioica* leaves had the lowest yield in water whereas green tea had the highest yield in water. The characteristics and percentage yield of the obtained extracts have been listed in **Table 4.2.**

Table 4.2 Physical characteristics of samples

S. No.	Samples	Solvent	Characteristics of extracts		Dry wt. of the sample	Wt. of the extract	% yield
			Color	Consistency			
1.	Green tea	Methanol	Dark brown	Solid	15gm	2.52	16.80
		Ethanol	Dark brown	Solid	15gm	2.45	16.33
		Aqueous	Yellowish	Semi-solid	15gm	4.20	28.00
2.	*U. dioica* leaves	Methanol	Dark green	Solid	15gm	1.67	11.13
		Ethanol	Dark green	Solid	15gm	1.63	10.86
		Aqueous	Green	Semi-solid	15gm	3.66	24.40
3.	Blend	Methanol	Yellowish	Solid	15gm	2.47	16.46
		Ethanol	Yellowish	Solid	15gm	2.68	17.86
		Aqueous	Yellowish	Semi-solid	15gm	3.86	25.73

4.3 Quantitative analysis of phytochemicals in samples

The measured concentrations of total phenols, total flavonoids, and tannins in methanol, ethanol, and water extracts of green tea, *Urtica dioica* leaves and blend is shown in **Table 4.3**.

Table 4.3 Quantitative analysis of Phytochemicals

S. No.	Sample	Solvent	TPC (mgGAE/g)	TFC (mgQE/g)	Tannins (mgGAE/g)
1	Green tea	Methanol	330.95 ± 2.89^{Bc}	140.41 ± 4.40^{Bc}	43.37 ± 0.58^{Bb}
		Ethanol	319.10 ± 0.49^{Bb}	127.68 ± 0.45^{Bb}	41.89 ± 0.50^{Bb}
		Aqueous	262.88 ± 0.93^{Ba}	91.01 ± 0.32^{Ba}	19.41 ± 0.50^{Ba}
2.	*U. dioica* leaves	Methanol	136.84 ± 1.01^{Cc}	51.39 ± 0.78^{Cc}	14.83 ± 0.50^{Cc}
		Ethanol	128.00 ± 0.24^{Cb}	47.78 ± 0.93^{Cb}	12.68 ± 0.33^{Cb}
		Aqueous	30.62 ± 0.74^{Ca}	11.67 ± 0.12^{Ca}	3.79 ± 0.33^{Ca}
3.	Blend	Methanol	387.11 ± 1.28^{Ac}	167.57 ± 1.22^{Ac}	47.68 ± 0.68^{Ac}
		Ethanol	351.86 ± 0.12^{Ab}	163.00 ± 0.90^{Ab}	45.66 ± 0.50^{Ab}
		Aqueous	274.47 ± 0.56^{Aa}	93.82 ± 0.33^{Aa}	20.10 ± 0.51^{Aa}

*Values mean are triplicate results, figures in the parenthesis are the standard deviations. Here, capital alphabets in the superscript represent among the samples and small alphabets represent among the solvents. The different alphabetical values in the superscript within the column indicate they are not significantly different at $p<0.05$.

4.3.1 Total Phenol Content

The total phenol content of all the extracts was determined using the standard procedure. The mean values for total phenol content in different solvent extracts are shown in **Table 4.3**. The blend extracts gave the highest TPC in all three solvents compared to green tea and *U. dioica* leaves extracts. The TPC of the blend in methanol, ethanol, and aqueous was found to be 387.11mgGAE/g, 351.86mgGAE/g, and 274.47mg GAE/g respectively. Among the individual plant extracts, green tea gave significantly higher TPC in all the solvent extracts i.e., 330.95mgGAE/g in methanol, 319.10mgGAE/g in ethanol, and 262.88mgGAE/g in aqueous extracts whereas the TPC of *U. dioica* leaves gave 136.82mgGAE/g, 128mgGAE/g and 30.62mgGAE/g in methanol, ethanol and aqueous solvent respectively.

According to the statistical analysis, there is a significant difference in TPC among the samples in all three solvents, while no significant difference was observed in TPC among the three solvents within each sample ($p<0.05$).

4.3.2 Total Flavonoid Content

The total flavonoid content was determined as per the standard procedure. The results in **Table 4.3** showed that blend extracts gave the highest content of TFC in three solvents compared to green tea and *U. dioica* leaves extracts. The TFC of blend extracts in methanol, ethanol, and aqueous was found to be 167.57mgQE/g, 163.00mgQE/g, and 93.82mgQE/g respectively. Among the individual plant extracts, green tea gave a significantly higher content of total flavonoids in all solvent extracts i.e., 140.41mgqE/g in methanol, 127.68mgQE/gin ethanol, and 91.01mgQE/g in aqueous extracts whereas leaves extracts of *U. dioica* gave a TFC of 51.39mgQE/g, 47.78mgQE/g and 11.67mgQE/g in methanol, ethanol and aqueous solvent respectively.

According to the statistical analysis, there is a significant difference in TFC among the samples in all three solvents, while no significant difference was observed among the three solvents within each sample ($p<0.05$).

4.3.2 Tannin Content

The total tannin content of sample extracts was determined as per the standard provided. The mean values for the tannin content of the sample extracts in different solvents are shown in **Table 4.3**. The blend extracts gave the highest tannin content in all three solvents compared to green tea and *U. dioica* leaves extracts. The tannin content of the blend in methanol, ethanol, and aqueous was found to be 47.68mgGAE/g, 45.66mgGAE/g, and 20.10mgGAE/g respectively. Among the individual plant extracts, green tea gave a significantly higher tannin content in all solvent extracts i.e., 43.37mgGAE/g in methanol, 41.89mgGAE/g in ethanol, and 19.41mgGAE/g in aqueous extracts whereas leaves extract of *U. dioica* gave tannin content of 14.83mgGAE/g, 12.68mgGAE/g and 3.79mgGAE/g in methanol, ethanol and aqueous solvent respectively.

According to the statistical analysis, there is a significant difference in tannin among the samples in all three solvents ($p<0.05$). However, no significant difference was observed between methanol and ethanol extracts of green tea ($p<0.05$).

4.4 Antioxidant assay

Antioxidant activity of methanol, ethanol, and aqueous extracts of samples was investigated by different in-vitro methods namely DPPH scavenging assay, phosphomolybdenum assay, and reducing power assay.

Table 4.4 Antioxidant activities of plant samples

S. No.	Sample	Solvent	DPPH (% inhibition)	TAC (mgAAE/g)	FRAP (mgAAE/g)
1.	Green tea	Methanol	92.06 ± 0.10^{Bc}	15.33 ± 2.42^{Ac}	15.05 ± 1.72^{Ac}
		Ethanol	84.03 ± 0.12^{Bb}	11.29 ± 0.12^{Ab}	11.60 ± 0.39^{Ab}
		Aqueous	78.41 ± 0.43^{Ba}	5.11 ± 1.20^{Aa}	5.43 ± 1.34^{Aa}
2.	*U. dioica* leaves	Methanol	74.63 ± 0.10^{Cc}	2.63 ± 1.20^{Bc}	2.12 ± 0.32^{Bc}
		Ethanol	67.26 ± 0.12^{Cb}	2.11 ± 0.21^{Bb}	2.03 ± 0.10^{Bb}
		Aqueous	60.11 ± 0.20^{Ca}	1.03 ± 2.12^{Ba}	0.90 ± 0.10^{Ba}
3.	Blend	Methanol	98.32 ± 0.44^{Ac}	15.38 ± 1.23^{Ac}	15.12 ± 0.10^{Ac}
		Ethanol	90.49 ± 0.38^{Ab}	13.31 ± 0.10^{Ab}	12.32 ± 1.23^{Ab}
		Aqueous	80.34 ± 0.29^{Aa}	5.42 ± 2.10^{Aa}	5.46 ± 2.73^{Aa}

*Values mean are triplicate results, figures in the parenthesis are the standard deviations. Here, capital alphabets in the superscript represent among the samples and small alphabets represent among the solvents. The different alphabetical values in the superscript indicate they are not significantly different.

4.4.1 DPPH radical scavenging assay

Free radical scavenging activity of the plant extracts has been carried out using DPPH radical. Results in **Table 4.4** demonstrated that all solvent extracts of the blend showed the highest radical scavenging activity among all the plant extracts tested. The highest scavenging activity determined among the sample extracts was from methanol extract. The scavenging activity for the blend, ranged from 80.34 to 98.34% which indicates an increase in the activity as compared to individual plant extracts. Among the plant extracts, green tea was found to have significantly higher radical scavenging activity than *U. dioica* leaves. In green tea, radical scavenging activity ranged from 78.63 to 92.06%

whereas the scavenging activity of leaves extracts of *U. dioica* ranged from 60.11 to 74.63% at 100µg/ml.

Statistical analysis showed that there is a significant difference in antioxidant activity by DPPH radical scavenging activity among the samples in all three solvents, as well as a significant difference was observed among the three samples within each solvent ($p<0.05$).

4.4.2 Total antioxidant capacity

The phosphomolybdenum assay usually detects antioxidants such as ascorbic acid, some phenolics, α-tocopherol, and carotenoids. A higher absorbance indicates a higher antioxidative activity. Results in **Table 4.4** showed all solvent extracts of the blend, showed the highest TAC compared to individual plant extracts. Among the different solvent extracts, methanol extracts were found to show higher activity in all the tested samples. For blend, TAC ranged from 5.42 to 15.38mgAAE/g. Among the plant extracts, the TAC of green tea was significantly higher than in *U. dioica* leaves. For green tea, TAC ranged from 5.11 to 15.33mgAAE/g, while TAC in leaves extracts of *U. dioica* ranged from 1.03 to 2.63mgAAE/g.

Statistical analysis showed that there is no significant difference in TAC between green tea and the blend in three solvents ($p<0.05$). However, a significance difference was observed.

4.4.3 Reducing power assay

Ferric reducing antioxidant power (FRAP) assay was used to evaluate the antioxidant capacity of sample extracts. The result in **Table 4.4** demonstrated that all solvent extracts of the blend showed the higher radical scavenging activity among all the plant extracts tested. The FRAP levels of the blend ranged from 5.46 to 15.12mgAAE/gin three different solvents. Among the individual plant extracts, the FRAP levels were significantly higher in green tea, ranging from 5.43 to 15.05mg AAE/g as compared to *U. dioica* leaves which ranged from 0.90 to 2.12 mg AAE/g.

Statistical analysis showed that there is a significant difference among the solvents of all three samples. Further analysis showed that there is no significant difference in FRAP between green tea and the blend in methanol while *U. dioica* leaves showed a significant difference ($p<0.05$).

4.5 Principal Component Analysis for samples

The principal component analysis for three different sample extracts is shown in figure 4. As we can see the location of TPC, TFC, and DPPH in the same quadrant show that there is a strong correlation between phenol, flavonoid content, and DPPH radical scavenging activity. Similarly, TC, TCA, and FRAP fall in the same quadrant which implies a strong correlation among these parameters. Here, the closest proximity of the green tea extracts (green circles) with tannins, FRAP, and TAC shows that they possess a high value of these parameters. Similarly, the green tea extracts have a good correlation with TPC, TFC, and DPPH but while comparing green tea extracts to tannins, FRAP and TAC there seems to be a poor correlation with TPC, TFC, and DPPH. The farthest proximity leaves extracts of nettle; *U. dioica* (blue circles) with TFC, TC, TPC, DPPH, FRAP, and TAC show that they have the lowest TFC, TC, TPC, DPPH, FRAP, and TAC. Similarly, the blends extract (pink circles) shows closest proximity with TPC, TFC, and DPPH in one quadrant and tannins, TAC, and FRAP in the next quadrant, implying that they possess a high value of these parameters. The figure also shows that the blend extracts have a better correlation with TPC, TFC, DPPH, and tannins content but a low correlation with TAC and FRAP.

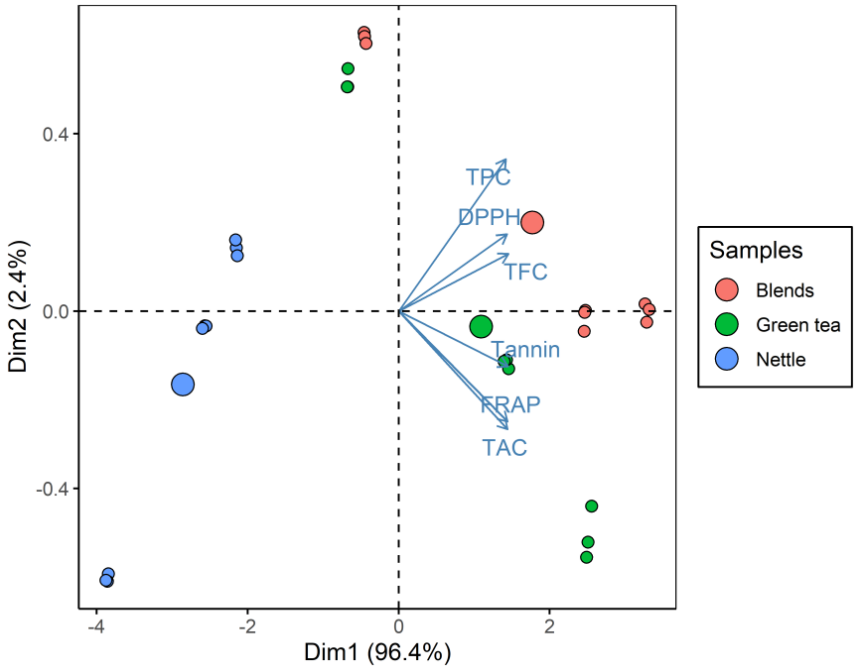


Fig 4: PCA biplot of samples

4.6 Cluster Dendrogram

The cluster dendrogram for different parameters in the blend extracts is shown in figure 5. The blend extracts gave similar values for TFC and DPPH, and TAC and FRAP. However, TAC and FRAP were found to have lower values compared to the rest of the parameters. TPC and Tannin had maximum height implying that the blend extract possesses higher values of these parameters.

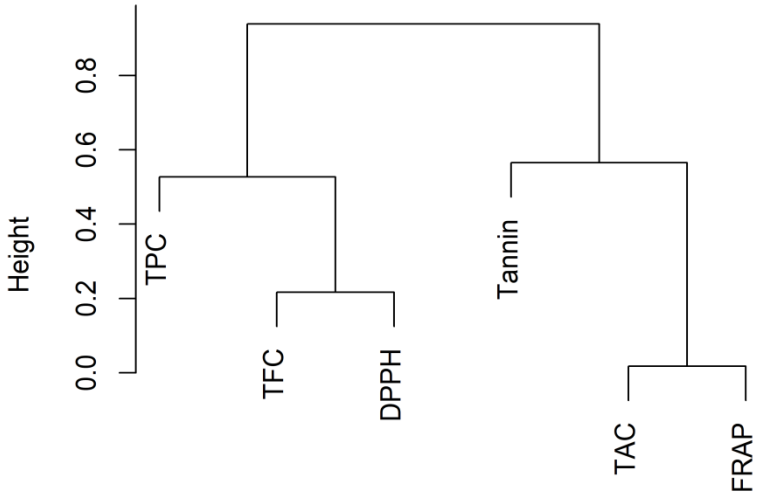


Fig 5: Cluster Dendrogram of blend extracts

4.7 Microbial sensitivity test

4.7.1 Antibiotic sensitivity pattern

The antibiotic sensitivity assay was examined using streptomycin (10µg) to check the sensitivity against clinical isolates. From **Table 4.7.1**, it can be observed that all bacterial isolates were found sensitive to streptomycin.

Among the five isolates, Gram-positive bacteria were found more sensitive than Gram-negative bacteria to streptomycin. The standard streptomycin had the strongest antibiotic activity with 16mm for Gram-positive bacteria (*S. aureus* and *E. faecalis*) whereas; in the case of Gram-negative bacteria streptomycin had the highest activity with 15mm against *S. typhi* and lowest activity with 14mm of the zone of inhibition against *S. flexneri*, and *K. pneumoniae* with 10µg/disc.

Table 4.7.1 Antibiotic sensitivity pattern

	Streptomycin		
Microorganisms	**Disc Potency(µg)**	**ZOI (mm)**	**p-value**
S. aureus	10	16±0.10	
S. typhi	10	15±0.17	
S. flexneri	10	14±0.00	0.0007
E. faecalis	10	16±0.10	
K. pneumoniae	10	14±0.20	

*Values mean are triplicate results, figures in the parenthesis are the standard deviations.

4.7.2 Antibacterial activities of sample extracts

4.7.2.1 Zone of Inhibition of plant extracts in different solvents against bacterial isolates

Plant extracts were investigated for the antibacterial activity by agar well diffusion method against ATCC cultures; *Staphylococcus aureus*, *Salmonella typhi*, *Shigella flexneri*, *Enterococcus faecalis*, and *Klebsiella pneumonia.* The results in **Table 4.7.2.1** illustrated that the methanolic extract of the blend showed the best inhibitory among the bacterial isolates under the study. The

result shows that green tea extracts exhibit a higher inhibitory activity as compared to *U. dioica* leaves. The methanolic extracts showed the highest ZOI of the blend (18mm) against *S. aureus*, green tea (15mm) against *S. aureus* &*S. typhi*, and *U. dioica* leaves (9mm) against *S. aureus*. On the other hand, the ethanolic extracts showed the highest ZOI of the blend (18mm) against *S. aureus*, green tea (14mm) against *S. typhi*, and *U. dioica* leaves (9mm) against *S. aureus*. Similarly, the aqueous extract of the blend showed the highest ZOI (12mm) against *S. typhi* and *S. faecalis*, green tea (11mm) against *S. typhi* while *U. dioica* leaves showed the highest ZOI (6mm) against *S. aureus* and *S. typhi*. However, the aqueous leaves extracts of *U. dioica* showed no antibacterial activity against S. *flexneri, E. faecalis*, and *K. pneumoniae*.

Statistical analysis showed that there is a significant difference in ZOI against the bacterial isolates among the extracts tested. Further analysis states that there is no significant difference among the solvents within each plant extracts tested at $p<0.05$.

Table 4.7.2.1 ZOI (mm) of plant extracts against bacterial isolates

		Zone of inhibition (mm)					
			Bacteria				
Sample	**Solvent**	**DMSO**	***S. aureus***	***S. typhi***	***S. flexneri***	***E. faecalis***	***K. pneumoniae***
Green tea	Methanol	-	15 ± 0.00^{Bc}	15 ± 0.10^{Ac}	11 ± 0.00^{Bb}	13 ± 0.20^{Bc}	11 ± 0.10^{Bb}
	Ethanol	-	13 ± 0.10^{Bb}	14 ± 0.00^{Bb}	11 ± 0.00^{Ab}	13 ± 0.00^{Ab}	11 ± 0.10^{Ab}
	Aqueous	-	8 ± 0.00^{Ba}	11 ± 0.10^{Ba}	6 ± 0.00^{Aa}	10 ± 0.17^{Aa}	9 ± 0.00^{Aa}
U. dioica Leaves	Methanol	-	9 ± 0.20^{Cc}	8 ± 0.17^{Cb}	7 ± 0.00^{Ca}	7 ± 0.00^{Ca}	6 ± 0.00^{C}
	Ethanol	-	9 ± 0.00^{Cb}	8 ± 0.10^{Cb}	7 ± 0.00^{Ba}	7 ± 0.00^{Ba}	-
	Aqueous	-	6 ± 0.00^{Ca}	6 ± 0.00^{Ca}	-	-	-
Blend	Methanol	-	18 ± 0.10^{Ab}	15 ± 0.00^{Ab}	13 ± 0.20^{Ac}	15 ± 0.00^{Ac}	13 ± 0.17^{Ac}
	Ethanol	-	18 ± 1.00^{Ab}	16 ± 0.00^{Ab}	11 ± 0.10^{Ab}	13 ± 0.00^{Ab}	11 ± 0.00^{Ab}
	Aqueous	-	10 ± 0.00^{Aa}	12 ± 0.00^{Aa}	6 ± 0.00^{Aa}	12 ± 0.17^{Aa}	9 ± 0.10^{Aa}

*ZOI diameters are means of triplicates ± standard deviation. The different alphabetical values in the superscript within the column indicate they are not significantly different at $p<0.05$.

4.7.2.2 Minimum inhibitory concentration of plant extracts against bacterial isolates

The minimum inhibitory concentration of plant extracts was evaluated by micro well dilution method. The results in **Table 4.7.2.2** demonstrated that all solvent extracts of blend showed the best inhibitory effect against the bacterial isolates tested. The best inhibitory effect of blend was observed in methanol against *E. faecalis* with the lowest MIC of 50µg/ml, while ethanolic extracts showed the best inhibitory effect against *S. aureus*, and *E. faecalis* with the lowest MIC of 200µg/ml. At the same time, the aqueous extract of the blend showed the best inhibitory effect against all the microbes tested with the lowest MIC 400µg/ml. Similarly, green tea methanolic and ethanolic extract showed the best inhibitory effect against *S. aureus* and *S. typhi* and *E. faecalis* with the lowest MIC of 400 µg/ml, followed by aqueous extracts which showed the best inhibitory effect with the lowest MIC of 800µg/ml against all bacterial isolates tested. On the other hand, the leaves extract of *U. dioica* in methanol showed the lowest MIC of 600 µg/ml *K. pneumoniae* while the ethanolic extracts of *U. dioica* leaves showed the lowest MIC of 800µg/ml against *S. typhi*, *S. flexneri*, *E. faecalis*, and *K. pneumoniae*, at the same time, aqueous leaves extracts showed a lowest MIC of 1600 µg/ml against all bacterial isolates.

Table 4.7.2.2 MIC of plant extracts against bacterial isolates

Sample	Solvent	MIC (µg/ml)				
		Bacteria				
		S. aureus	***S. typhi***	***S. flexneri***	***E. faecalis***	***K. pneumoniae***
Green tea	Methanol	400	600	600	400	600
	Ethanol	400	800	600	400	600
	Aqueous	800	800	800	800	800
U. dioica Leaves	Methanol	800	800	800	800	600
	Ethanol	1600	800	800	800	800
	Aqueous	1600	1600	1600	1600	1600
Blend	Methanol	200	200	200	50	400
	Ethanol	200	400	400	200	400
	Aqueous	400	400	400	400	400

*MIC values were determined in triplicates; values were the same in each replicate.

PHOTOGRAPHS

a) Tea (*Camellia sinensis*) leaves b) Stinging nettle (*Urtica dioica*)

Photograph 1: Plant samples used

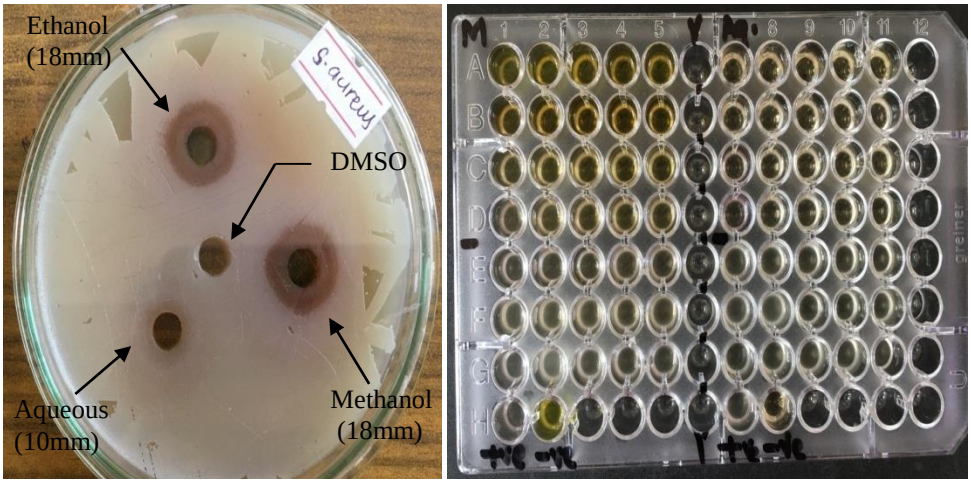
Photograph 2: Green tea

a) Green tea **b)** *U. dioica* leaves

Photograph 3: Powdered samples

Photograph 4: Teabag packaging of blended tea

Photograph 5: Sensory analysis of blend tea samples



a) ZOI (mm) of blend extracts against *S. aureus*

b) MIC of methanolic and aqueous blend extracts against bacterial isolates

Photograph 6: Antibacterial activity of blend extracts

CHAPTER V

DISCUSSION

In recent years, the use of plant natural products in disease treatment has received increasing attention due to the development and spread of resistance of microorganisms to currently available antibiotics. Among various preparations of plant products introduced, infusions and decoction are frequently used to administer an oral dose of these products (Guimaraes et al 2011). Synergistic actions developed from the constituents of the plants are vital for treatment (Rolta et al 2020). Accordingly, this study aimed to investigate the combined effects of two botanicals; *Camellia sinensis* and *Urtica dioica* emphasizing their synergism. Therefore, this research work attempts in the development of tea (infusion) blend by mixing two common plants and studying their antimicrobial properties on an individual basis as well as in synergy.

Following sensory analysis, a sample was selected among five samples as best tea synergy. Individual plant samples along with the selected synergy were then subjected to three different solvents (methanol, ethanol, and water) extraction. The organic extraction was performed by using the Soxhlet apparatus whereas maceration and infusion was used to obtain aqueous sample extracts. The variations in extraction method usually depend upon the solvent used, the length of the extraction period, PH of the solvent, temperature, particle size of the plant tissue, and the solvent to sample ratio. There was a difference in the percentage yield of the solvent extracts from samples. The differences range from 24.40% to 28.00% with water extract followed by 17.86% to 10.86% with ethanol extract and 11.13% to 16.80% with methanol extract. The differences in yield might be due to different plant types, the particle sizes of the plant sample, the extent of dryness, etc.

In our study, the phytochemical screening of both plant samples in all three solvents showed the presence of phytochemicals such as phenols, flavonoids, and tannins. The measured concentrations of total phenols, total flavonoids, and tannins in methanol, ethanol, and water extracts of green tea, *U. dioica* leaves

are shown in Table 4.3. It can be seen that a higher concentration of phenolic compounds was detected in the blend extracts compared to individual plant extracts. This might be due to the incorporation of *U. dioica* leaves in green tea. Between plant extracts, a higher concentration of phenolic compounds was detected in green tea extracts compared to *U. dioica* extracts. The methanol extracts of both plant samples contained the highest quantity of phenolic compounds. This may be due to a better solubility and extractability of phenolic compounds by methanol because of the polarity of the compounds (Garcia et al 2016). Hence, methanol could be a solvent of choice for the extraction of bioactive compounds from these plant materials.

Our result concurs with the previous study conducted by Bhandari et al (2018) who reported that phenolic compounds of *Camellia sinensis* in methanol extract showed higher yield compared to ethanol and water extracts. They reported a TPC of 321.25mgGAE/g and a TFC of 126.88mgQE/g in methanolic extracts. A similar result was observed in our study where green tea methanolic extracts recorded a TPC of 330.95mgGAE/g and TFC of 140.41mgQE/g. On the contrary, Samadi and Fard (2020) reported higher content of total flavonoids and tannin in both ethanolic and aqueous extracts than total phenols. They reported a TFC of 31.55mgQE/g, 16.988mgQE/g, tannin content of 64.30mgGAE/g, 77.75mgGAE/g and TPC of 13.46mgGAE/g, 12.35mgGAE/g in ethanolic and water extracts respectively. Here, a high level of TFC was found in green tea, this may be due to its richness in caffeic and chlorogenic acids and other caffeoyl derivatives (Naczk and Shahidi 2007). Similarly, high level of tannins may be due to its richness in tannic acid (Khasnabis et al 2015).

In our study, *U. dioica* extracts were found rich in total phenols (TPC) compared to flavonoids and tannin contents. A similar result was observed by Rolta et al (2020) who reported a high TPC of 220mgGAE/g and a low TFC of 178.96mgQE/g in methanolic leaves extracts of *U. dioica*. In the work done by Kukric et al (2012), the TPC in ethanol (80%) extract of *U. dioica* leaves was found to be 208.37mgGAE/g, whereas the content of total flavonoid was found to be 20.29mgQE/g. In another study, Guder and Korkmaz (2012) reported a TPC of 132mgGAE/g and a TFC of 65.8mgCatechin/g in hydro-ethanol leaves

extracts of *U. dioica*. On the contrary, Stankovic et al (2019) reported high total flavonoids (TFC) in ethanolic leaves extracts of *U. dioica* which was found to be 55.71mgGAE/g while total phenols content was 17.04mgQE/g and tannin content was 10.54mgGAE/g. The difference in the TPC, TFC, and tannin content maybe due to various factors, including the variety, genotype, climate, soil, vegetative stage of the plant, harvest time, storage, processing, and treatment (Kregiel et al 2018).

Antioxidants play an important role in neutralizing oxidative damage caused by free radicals in blood, cells, and tissue fluids (Stankovic et al 2019). In our study, antioxidant activity measured by DPPH, FRAP, and phosphomolybdenum assay suggests that green tea contains a significantly higher antioxidant activity compared to *U. dioica* leaves. Earlier studies also reported the remarkably higher antioxidant activity to other teas and herbs (Liu et al 2016; Farooq and Sehgal 2019). The higher value in our study may be due to differences in plant species. It has been reported that the primary source of total antioxidant activity in green tea was total polyphenols. The significantly higher antioxidant activity found in green tea could be related to the significantly higher content of polyphenols recorded.

Bhandari et al (2018) reported higher antioxidant activity by DPPH radical scavenging activity of tea leaves in methanol (37.63%) followed by ethanol (23.66%) and lowest in aqueous extracts (9.68%) at 15µg/ml. A similar result was obtained in our study, where the highest activity of 92.06% was obtained in methanol followed by 84.03% in ethanol and the least activity of 78.41%was obtained in aqueous extracts at 1mg/ml. In a study conducted by Kaur et al (2015), the TAC of green tea extracts was found to be 15.866mgAAE/gin methanol. This value was found comparable to our study. In the same study, the FRAP was found to be 5.866mgAAE/g, which was significantly lower compared to our study. The high difference in DPPH and FRAP levels of the green tea extract compared to this study might be due to differences in the TPC in respective extracts, treatment methods or season of harvest.

For stinging nettle, antioxidant activity by DPPH inhibition in our study was found to be higher than that of Thapaliya (2010) who found the inhibition of

66.3% in aqueous extracts. The higher value in our study may be due to the difference in plant species. Guder and Korkmaz (2012) reported the antioxidant activity of *U. dioica* leaves in hydro-ethanol. In the study, antioxidant activity by DPPH scavenging assay gave an inhibition of 54.2% at 250µg/ml and a TAC of 76.4% at 100µg/ml by the ferric thiocyanate (FTC) method. In another study conducted by Gulcin et al (2004), the DPPH scavenging effect of water extract of *U. dioica* was found to be 37% at 60µg/ml. The difference in the value for antioxidant activity compared to our study may be due to differences in the climate, soil, vegetative stage of the plant as well as processing, and treatment methods. Similarly, Kukric et al (2012) reported antioxidant activity by the FRAP of 80% ethanol *U. dioica* leaves extracts which were found to be 7.50AAE/g which is significantly higher than the value obtained in our study. The higher value in the study might be due to corresponding high flavonoids in extracts which are responsible for the antioxidant potential of nettle plants.

The most potent antioxidant activity was found in the blend, suggesting synergistic effects. The combination of tea with other herbs has been shown to synergistically enhance antioxidant activities, giving rise to the development of different tea blends. A similar observation was found by Belscak et al (2011)who reported that the combination of black tea, black pepper (*Piper nigrum*), ginger (*Zingiber officinale*), and tulsi (*Ocimum sanctum*) (1:1:1:1) showed high antioxidant activity compared to that of the individual herb. In the same study, the best synergistic antioxidant activity was obtained for the mixture of tea, ginger, and tulsi (1:1:1). In another study conducted by Buyukbalci and El (2008), it was determined that using lemon (*Citrus limon* L.), bergamot (*Citrus bergamia*), clove (*Syzygiumaromaticum* L.), cinnamon sticks, and ground cinnamon (*Cinnamomum verum*) in preparation of black tea increased the antioxidant activity of the tea. Similar synergistic effects were observed by Jain et al (2011) where the mixture of green tea of *Camellia* species with grape seed (*Vitis vinifera* L.), Amla (*Phyllanthus emblica* L.), Anar (*Punica granatum* L.), Chinese cinnamon (*Cinnamomum cassia* L.) showed a synergistic antioxidant effect.

In the antibiotic assay, streptomycin (10µg) was used as a standard antibiotic against five clinical isolates. At the same dose, streptomycin showed zones of inhibition of 16mm on *S. aureus* and *E. faecalis*, 15mm on *S. typhi*, 14mm on *S. flexneri* and *K. pneumoniae* respectively. This result coincides with the observation of Elangovan et al (2014) in which streptomycin gave the zone of inhibition of 16mm on *S. aureus*, 15mm on *S. typhi*, and 14mm on *E. faecalis*. On the other hand, in the study conducted by Hafidh et al (2011), streptomycin (10µg) tested against clinical isolates, gave the highest ZOI of 19mm on *K. pneumoniae*, 18mm on *S. aureus*, and 15mm on *S. typhi*. However, antibiotic resistivity has been increased among the bacteria. So, there arises a concern for the alternative solution to treat drug-resistant.

ZOI and MIC are two different attributes for the evaluation of the antibacterial effect of obtained plant extract. The MIC value is important to evaluate the dose-response relationship of plant extract with bacteria. The MIC and the ZOI are inversely correlated. The more susceptible the microorganism is to the antimicrobial agent, the lower the MIC and the larger the zone of inhibition. Conversely, the more resistant the microorganism, the higher the MIC and the smaller the zone of inhibition (Pingle et al 2018). Our results indicated that green tea had significantly higher polyphenols content compared to *U. dioica* leaves. The differences in antimicrobial activity between green tea and *U. dioica* leaves may be attributed to different concentrations of polyphenols present. Besides, the composition of active plant compounds has also been reported to play a role in antimicrobial activity. At the same time, bacterial inhibition may also vary according to plant extract; the solvent used for extraction, and the organism tested (Tshivhandekano et al 2014).

Several previous studies have shown green tea extract activity against various Gram-positive and Gram-negative bacteria. These observations may be attributed to green tea catechin compounds and polyphenols. These compounds have been found to possess antibacterial action (Mbata et al 2008). Farooqui et al (2015) reported the activity being displayed by the aqueous and methanol extract of green tea against clinical bacterial pathogens. The most effective activity was proven with a maximum ZOI (17mm and 20mm) and MIC (0.39

and 0.78 mg/ml) against *S. aureus* in aqueous and methanol respectively. This result concurs with our study where all three solvent green tea extracts showed the greatest inhibition against *S. aureus* among six clinical pathogens. In a similar study conducted by Archana and Abraham (2011), methanolic extracts of green tea showed antimicrobial activity (ZOI 14mm and MIC 40µg/ml) against *S. aureus*. Almajano et al (2008) reported ZOI by green tea extracts against *Bacillus cereus* (13.9 mm), *Micrococcus luteus* (11.2 mm), *Pseudomonas aeruginosa* (10.8 mm), *Escherichia coli* (6.2 mm), and *Candida albicans* (8 mm). They also reported that Gram-negative bacteria were found more resistant to polyphenols than Gram-positive bacteria. The disparity in findings could be due to differences in bacterial strains used, and to the differences in concentrations/types of solvent and extracts used by various research laboratories. Similar findings to the antibacterial activity of green tea were also reported in a previous study conducted by Radji et al (2013) against methicillin-resistant *S. aureus* and multidrug-resistant *P. aeruginosa*.

Like tea, stinging nettle (*Urtica* sps.) show noticeable activity against both Gram-positive and Gram-negative bacteria. All parts of the *U. dioica* plant (leaves, stem, and root) have reportedly shown inhibitory effects on various Gram-positive and Gram-negative bacteria including *Staphylococcus aureus, Bacillus cereus, Bacillus subtilis*, *Lactobacillus plantarum*, *Pseudomonas aeruginosa*, and *Escherichia coli* (Kregiel et al 2018). Korpe et al (2013) evaluated the antimicrobial activity of leaves methanol extracts using the disc diffusion method. They found that methanol extract showed the strongest inhibition zone of 10.5mm against *Pseudomonas aeruginosa*, 10 mm against *Streptococcus pyogenes*, 10 mm against *Listeria monocytogenes*,7.8 mm against *Erwin carotovora*, 7.8 mm against *Escherchia coli*, 7.5 mm against *Klebsiella pneumoniae*, and 7.3 mm against *Proteus vulgaris*. Similarly, Rolta et al (2020) reported the antimicrobial activity of methanolic extracts of *U. dioica* (MIC 250µg/ml and 62.5µg/ml) against *S. aureus* and *K. pneumoniae*. In another study, Gulcin et al (2004) reported the antimicrobial activity of water extract showing ZOI(mm) against *Escherichia coli* (8mm), *Proteus mirabilis* (8mm), *Citrobacter koseri* (9mm), *Staphylococcus aureus* (8mm), *Streptococcus pneumoniae* (9mm), *Enterobacter aerogenes* (9mm),

Micrococcus luteus (13mm), *Staphylococcus epidermidis* (11mm) and *Canidida albicans* (8mm). The antimicrobial activity of *U. dioica* leaves was also reported in previous study conducted by Kukric et al (2012). The study revealed an inhibitory effect of *U. dioica* extracts on various Gram-positive and Gram-negative bacteria including *Bacillus subtilis*, *Lactobacillus plantarum*, *Pseudomonas aeruginosa* and *Escherichia coli*.

Green tea in combination with *U. dioica* leaves showed higher antibacterial activity against five clinical isolates compared to the individual plant extracts. Among the microbial strains tested, *K. pneumoniae* was the most resistant microbes while *S. aureus* and *E. faecalis* were the most susceptible strains. Methanolic and ethanolic extracts of the blend exhibit a higher inhibitory effect with maximum ZOI against *S. aureus* and *S. typhi*. Similarly, all solvent extracts of the blend showed possibly higher synergistic potential with a decrease in MIC against all the bacterial isolates. Micro-organisms differ in their resistance to given plant extracts. Combinations of the different plant extracts in several cases demonstrated synergistic or additive effects on microorganisms.

A synergistic effect occurs when the effect of two drugs together is greater than the effect of either alone while indifference occurs when the effect of two drugs together is less than the effect of either alone (Vuuren and Viljoen 2011). Thus, comparing the obtained results of the antibacterial activity of the tested extracts it was noted that the combination of green tea and *U. dioica* leaves possess antimicrobial activity with a possible synergistic effect on the microbes tested. Our results concur with Das et al (2012) who reported that the combination of different spices (cumin and fenugreek, cumin, and mustard), showed synergistic activity against micro-organisms. A similar result was observed by Bag and Chattopadhyay (2015) who reported that the antimicrobial activity of the coriander and cumin seed oil combination showed a selective degree of activity. Combinations of different plant species were found to inhibit bacterial growth with variation in their effectiveness. Another study by Rolta et al (2020) reported that three medicinal plants (*Juniperus communis*, *Urtica dioica*, and *Coleus forskohlii*) in combination with antibacterial and antifungal antibiotics yield synergistic effects against bacterial and fungal pathogens. Farooq and

Sehgal (2019) studied the synergistic interaction between green tea with *Ocimum gratissimum*. Similarly Jyoti et al (2016) studied the synergistic effect of silver nanoparticles of *U. dioica* in combination with streptomycin, amikacin, kanamycin, tetracycline, ampiciline, cefepime, amoxicillin, and cefetaxime against *B. cereus*, *S. epidermidis*, *S. aureus*, *B. subtilis*, *E. coli*, *S. typhimurium*, *K. pneumoniae* and *S. marcescens.*

In conclusion, the results of this preliminary study highlight the potential beneficial synergies between green tea and *U. dioica* leaves. The combination exhibited both possible synergistic antibacterial and antioxidant activity, thus may be used as a potential source of safe and potent natural antibacterial and antioxidant agents in pharmaceutical for developing new drugs for the treatment of infectious diseases and in food industries. Their interactions may increase their antibacterial and antioxidant efficacy at a sufficiently low concentration which may reduce their adverse side effects and facilitate their use in developing plant-based formulations.

CHAPTER VI

CONCLUSION AND RECOMMENDATION

6.1 Conclusion

Present work was carried out on sensorial properties, phytochemical, antioxidant, and antimicrobial of green tea (*Camellia sinensis*), stinging nettle (*Urtica dioica*) leaves, and the blend. The following were the conclusion drawn from the work:

1. Green tea in combination with *U. dioica* leaves at a proportion of 2.1:0.9 was found highly acceptable to consume.
2. Among the plant extracts tested, the blend extracts in all three solvents were found to have a significant increase in their phytochemical contents compared to green tea and *U. dioica* leaves extracts.
3. The blend extracts were found to exhibit a stronger antioxidant activity by DPPH radical scavenging assay compared to green tea and *U. dioica* leaves extracts in methanol, ethanol, and aqueous solvent.
4. The blend extracts were found to exhibit higher antimicrobial activity with a significant increase in the zone of inhibition and a significant decrease in the minimum inhibitory concentration against the bacterial isolates.
5. From this work, it can be concluded that green tea and *U. dioica* are infact more effective as mixtures (2.1:0.9), implying that the combined preparation can synergistically enhance antioxidant as well as antibacterial properties.

6.2 Recommendations

From the present study following recommendations were made for further work.

1. Other varieties of stinging nettle (*Urtica* sps.) can be used.
2. Although antibiotics disc had revealed good action against the isolates, the plant extract, especially, the blend had also shown good antimicrobial properties. Therefore, plants extract in combination could be discovered for further evaluation of antimicrobial properties.
3. The combination of these plants will also help in avoiding undesirable side effects due to higher doses of a single herb. Therefore, further investigation is needed to determine the bioavailability of the active compounds and to determine the dose and toxicity before they can be used as therapeutic agents.

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APPENDICES

APPENDIX A

Materials and Equipment

1. **Equipment**

   Micropipette, Pipette, Microscope, Grinder, Incubator, Refrigerator, Digital Balance, Hot Plate, Cabinet Dryer, Water Bath Shaker, Soxhlet Apparatus, Rotatory evaporator, Autoclave, Spectrophotometer, Hot air oven.

2. **Materials**

   Beakers, Conical flasks, Volumetric Flasks, Measuring Cylinders, Glass Slides, Coverslips, Petri plates, Inoculating Loop, Spatula, Wire Gauge, Test Tubes, Screwcap Tubes, Glass rods, Filter papers, Funnel.

3. **Chemicals**

   Methanol, Ethanol, Gallic acid, DPPH, Quercetin, Aluminum chloride, Ascorbic acid, Ferric chloride, Sodium nitrate, Sodium hydroxide, Gram's iodine, Crystal violet, Safranin, DMSO, Conc. H_2SO_4, Conc HCL etc.

4. **Media**

   Nutrient Broth (NB), Nutrient Agar (NA), Mueller Hinton Agar (MHA), Mueller Hinton Broth (MHB), Mannitol Salt Agar (MSA), Salmonella-Shigella Agar (SSA), MacConkey Agar (MAC).

5. **Antibiotic disc**

   Antibiotic disc of Streptomycin (10µg) was used.

APPENDIX B

Combinations of the experimental runs as per DOE

Standard run	**Green tea (g)**	***U. dioica*(g)**
1	2.700	0.300
2	1.953	1.047
3	2.700	0.300
4	2.100	0.900
5	2.100	0.900
6	1.500	1.500
7	1.802	1.198
8	2.549	0.451
9	1.651	1.349
10	1.500	1.500
11	2.398	0.602
12	1.500	1.500
13	2.700	0.300

APPENDIX C

SPECIMEN CARD FOR SENSORY EVALUATION

Hedonic rating test

Name of the panelist:……

Date:……

Product: Tea (Green tea + *U. dioica* leaves)

Control: Green tea

Dear panelist, you are five coded samples of the combination tea, please conduct the sensory analysis based on the following parameter using the table given;

	Sensory parameters			
Sample code	Appearance	Flavor	Aroma	Overall acceptance
A				
B				
C				
D				
E				

Perceptions	**Points**
Like extremely	9
Like very much	8
Like moderately	7
Like slightly	6
Neither like/ dislike	5
Dislike slightly	4
Dislike moderately	3
Dislike very much	2
Dislike extremely	1

Comments (if any)

……

……

……

APPENDIX D

Calibration curves

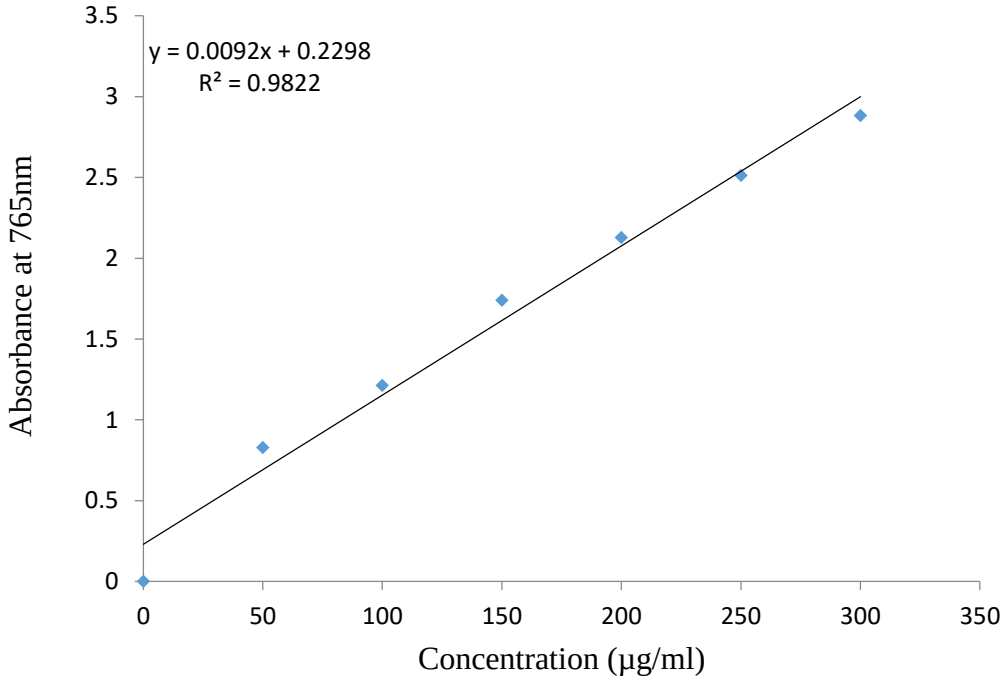

Fig D.1 Gallic acid calibration curve in methanol for TPC

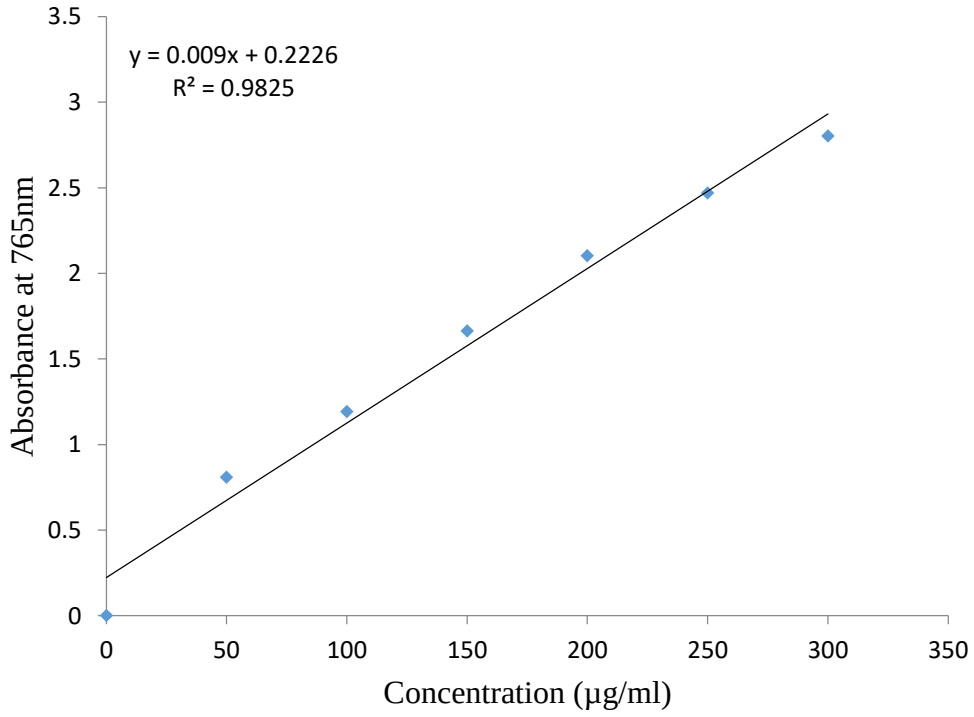

Fig D.2 Gallic acid calibration curve in ethanol for TPC

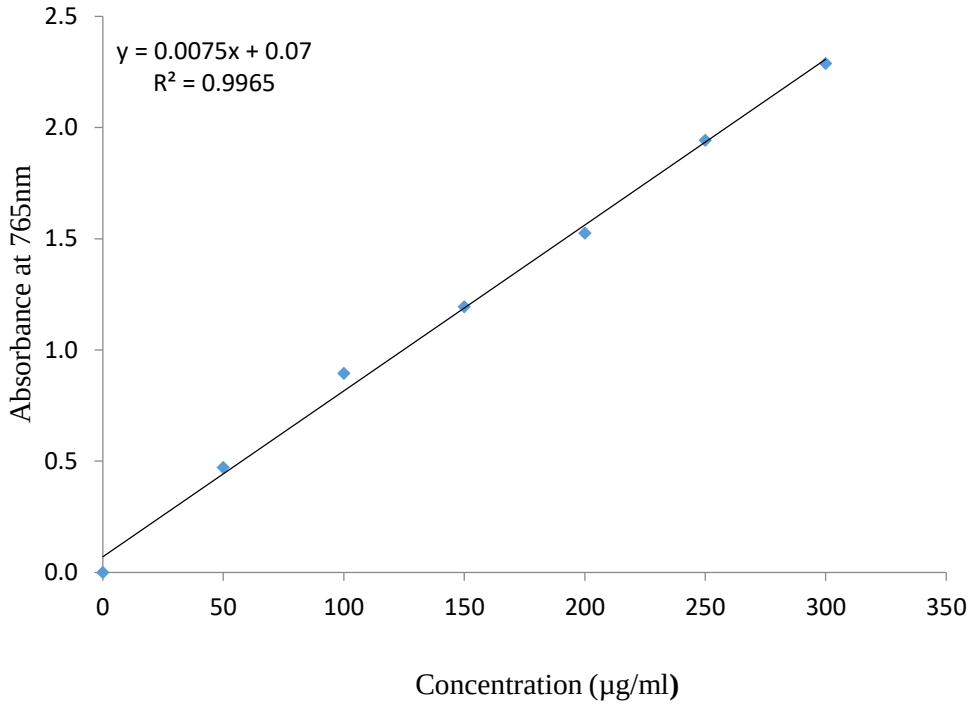

Fig D.3 Gallic acid calibration curve in water for TPC

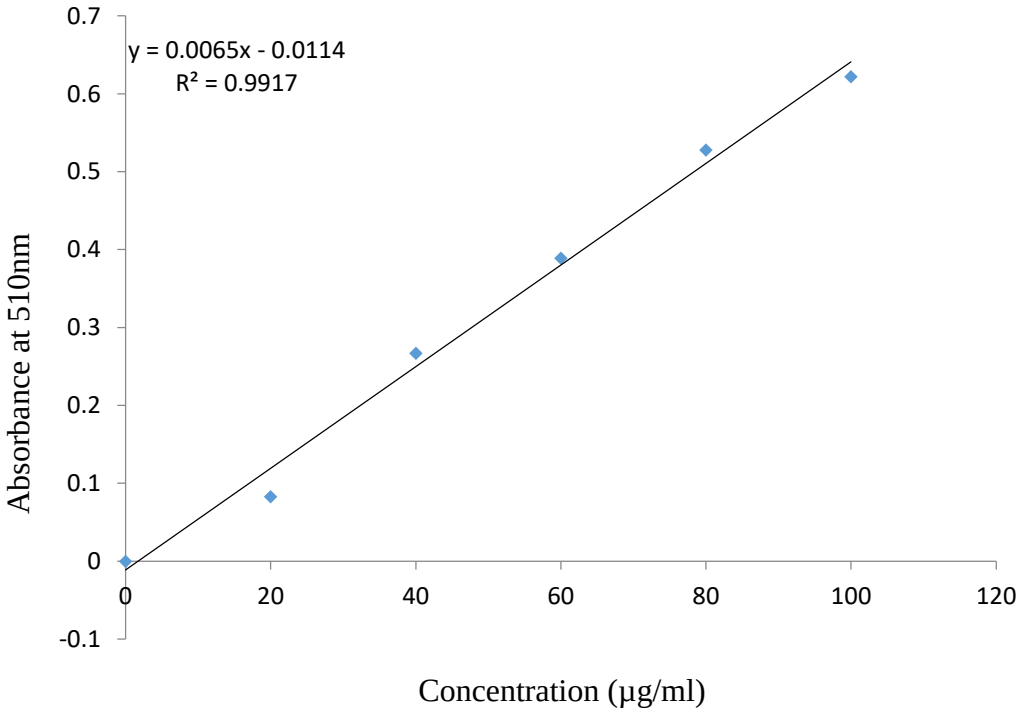

Fig D.4 Quercetin calibration curve in methanol for TFC

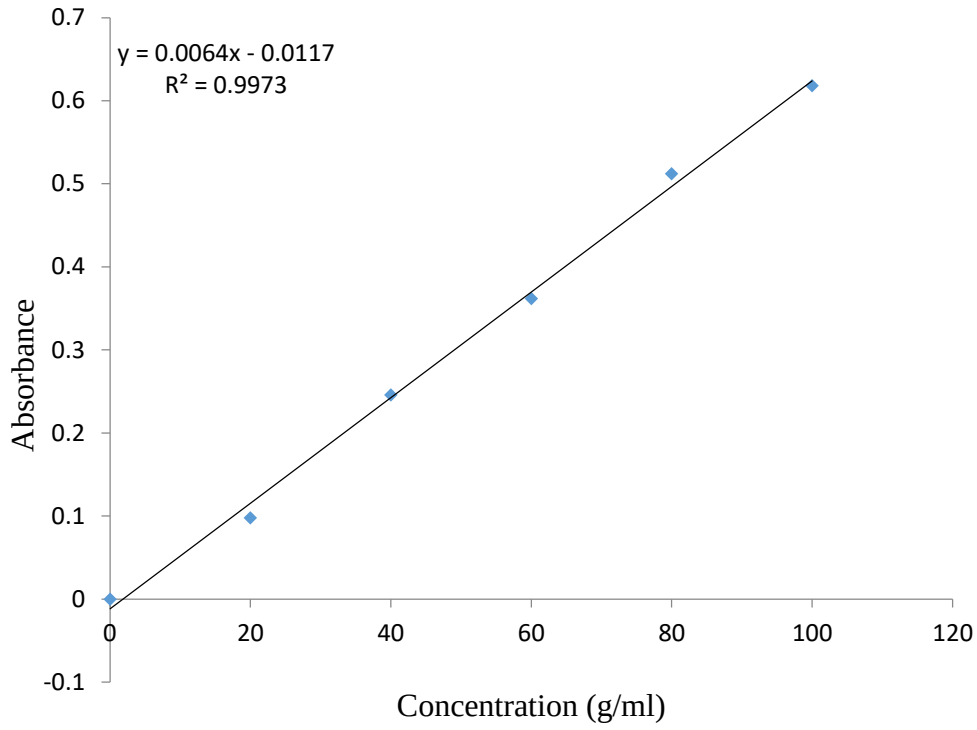

Fig D.5 Quercetin calibration curve in ethanol for TFC

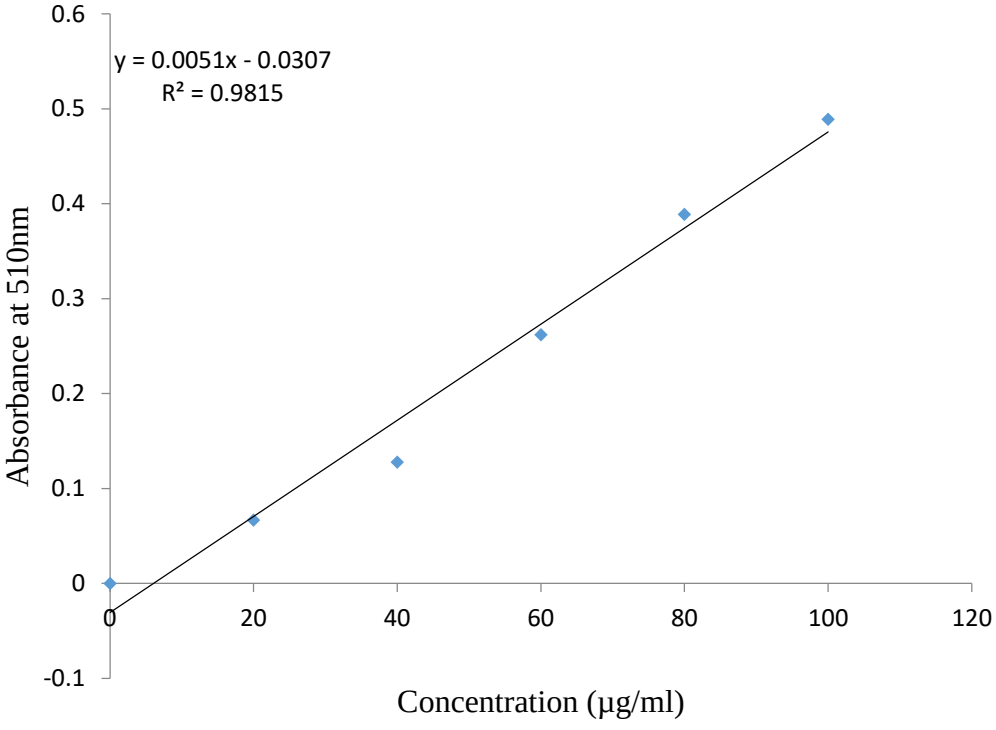

Fig D.6 Quercetin calibration curve in ethanol (80%) for TFC

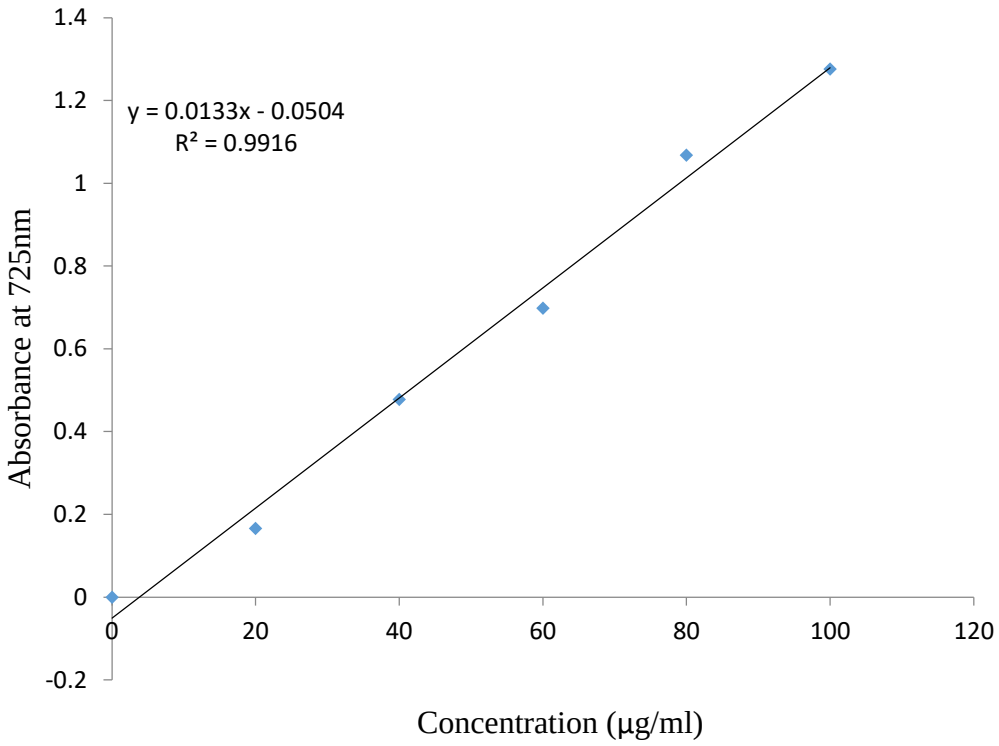

Fig D.7 Gallic acid calibration curve in methanol for Tannin

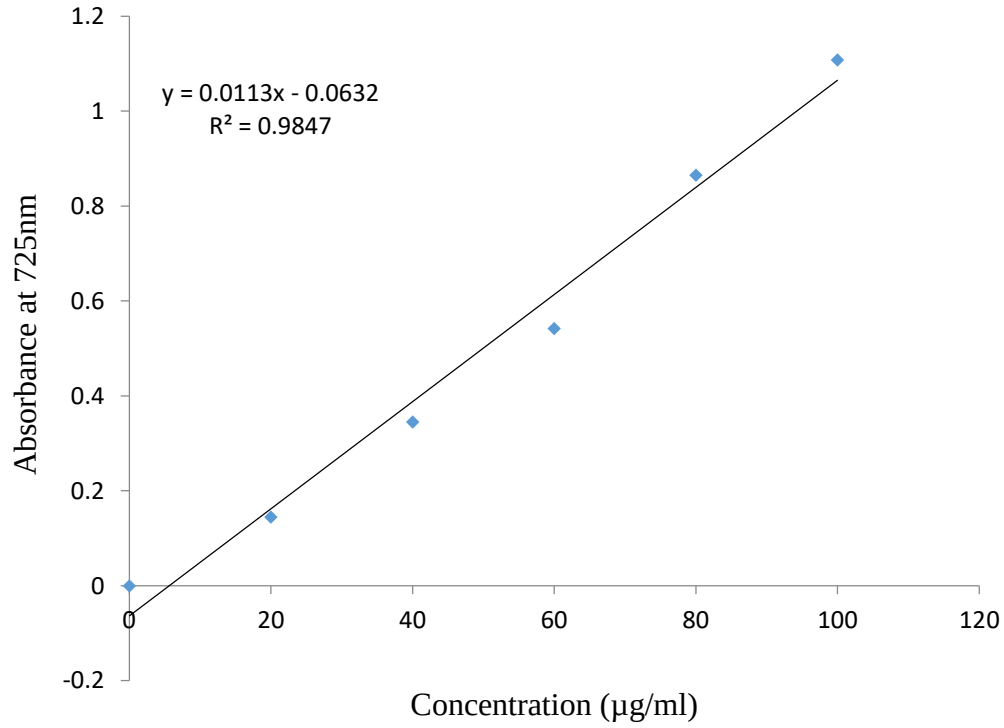

Fig D.8 Gallic acid calibration curve in ethanol for Tannin

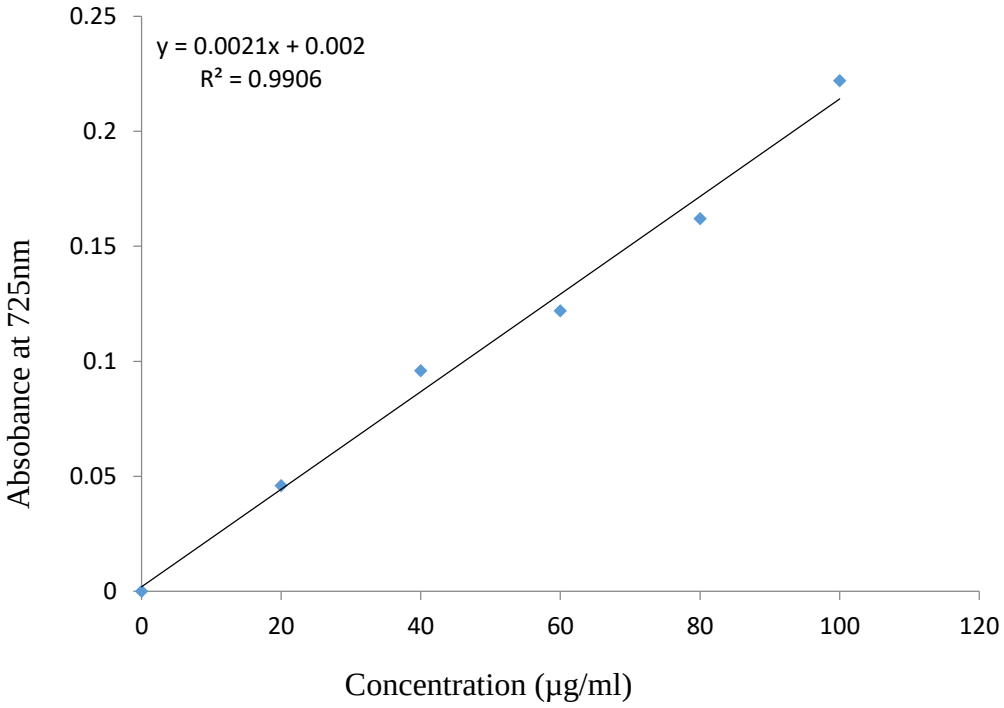

Fig D.9 Gallic acid calibration curve in water for tannin

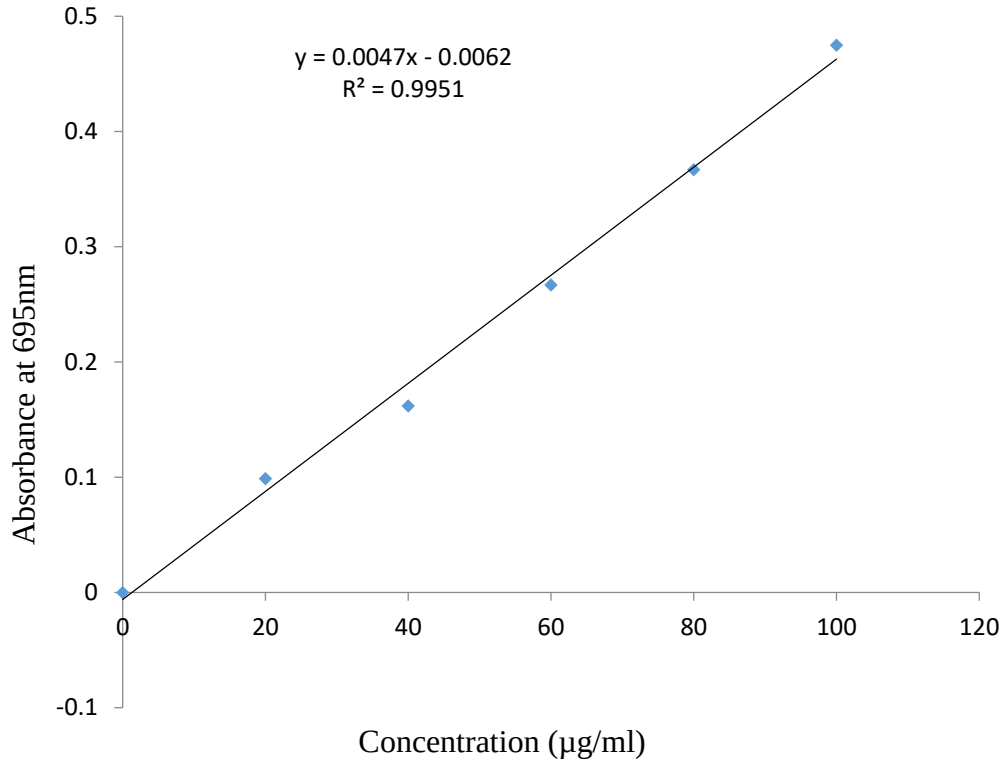

Fig D.10 Ascorbic acid calibration curve in methanol for TAC

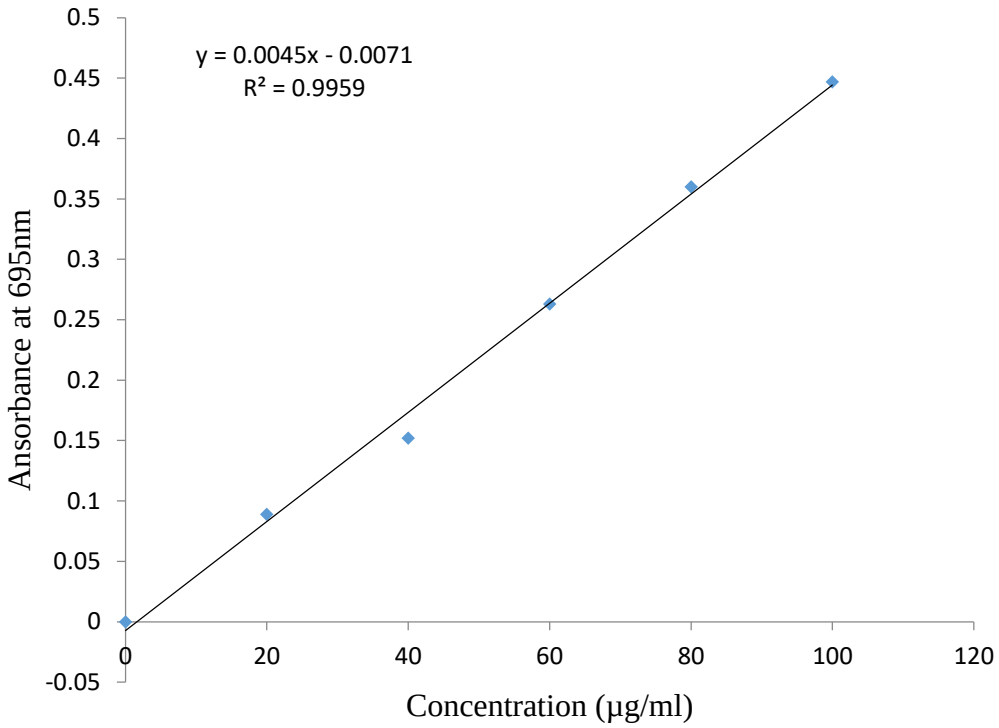

Fig D.11 Ascorbic acid calibration curve in ethanol for TAC

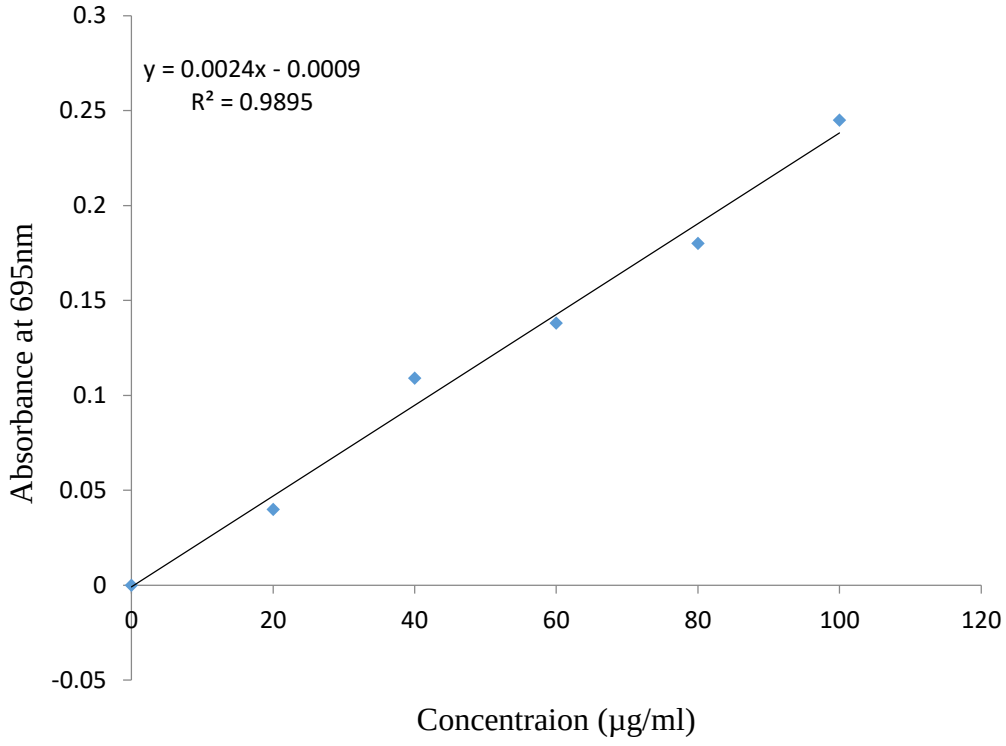

Fig D.12 Ascorbic acid calibration curve in water for TAC

APPENDIX E

Table E.1 ANOVA for Brew appearance

Source	df	Sum of squares	Mean Square	F Ratio	Prob > F
Sample Code	4	4.436364	1.10909	0.8815	0.4818
Error	50	62.909091	1.25818		
	54	67.345455			

Table E.2 ANOVA for Brew aroma

Source	df	Sum of Squares	Mean Square	F Ratio	Prob > F
Sample Code	4	7.381818	1.84545	1.9444	0.1176
Error	50	47.454545	0.94909		
C. Total	54	54.836364			

Table E.3 ANOVA for Brew flavor

Source	df	Sum of Squares	Mean Square	F Ratio	Prob > F
Sample Code	4	13.018182	3.25455	2.7204	0.0398
Error	50	59.818182	1.19636		
C. Total	54	72.836364			

Table E.4 ANOVA for Overall acceptability

Source	df	Sum of Squares	Mean Square	F Ratio	Prob > F
Sample Code	4	11.927273	2.98182	2.9710	0.0281
Error	50	50.181818	1.00364		
C. Total	54	62.109091			

Table E.5 ANOVA for TPC (Samples)

1. Methanol

Source	df	Sum of Squares	Mean Square	F Ratio	Prob > F
Samples	2	87704.016	43852.0	285536.2	<.0001
Error	6	0.921	0.153578		
C. Total	8	87704.938			

2. Ethanol

Source	df	Sum of Squares	Mean Square	F Ratio	Prob > F
Samples	2	87704.016	43852.0	285536.2	<.0001
Error	6	0.921	0.153578		
C. Total	8	87704.938			

3. Aqueous

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Samples	2	113535.51	56767.8	65963.00	<.0001
Error	6	5.16	0.8606		
C. Total	8	113540.67			

Table E.6 ANOVA for TPC (Solvents)

1. Green tea

Source	df	Sum of Squares	Mean Square	F Ratio	Prob > F
Solvent	2	7935.9973	3968.00	839.8019	<.0001
Error	6	28.3495	4.72		
C. Total	8	7964.3468			

2. *U. dioica* leaves

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Solvent	2	20843.108	10421.6	12769.60	<.0001
Error	6	4.897	0.816122		
C. Total	8	20848.005			

3. Blend

Source	df	Sum of Squares	Mean Square	F Ratio	Prob > F
Solvent	2	19921.516	9960.76	10072.22	<.0001
Error	6	5.934	0.99		
C. Total	8	19927.450			

Table E.7 ANOVA for TFC (Samples)

1. **Methanol**

Source	df	Sum of Squares	Mean Square	F Ratio	Prob > F
Samples	2	20907.161	10453.6	11031.64	<.0001
Error	6	5.686	0.9476		
C. Total	8	20912.846			

2. **Ethanol**

Source	df	Sum of Squares	Mean Square	F Ratio	Prob > F
Samples	2	20907.161	10453.6	11031.64	<.0001
Error	6	5.686	0.9476		
C. Total	8	20912.846			

3. **Aqueous**

Source	df	Sum of Squares	Mean Square	F Ratio	Prob > F
Samples	2	13307.524	6653.76	805973.9	<.0001
Error	6	0.050	0.008256		
C. Total	8	13307.574			

Table E.8 ANOVA for TFC (Solvents)

1. **Green tea**

Source	df	Sum of Squares	Mean Square	F Ratio	Prob > F
Solvent	2	3938.3406	1969.17	200.7182	<.0001
Error	6	58.8637	9.81		
C. Total	8	3997.2043			

2. ***U. dioica* leaves**

Source	df	Sum of Squares	Mean Square	F Ratio	Prob > F
Solvent	2	3004.3595	1502.18	2010.203	<.0001
Error	6	4.4837	0.75		
C. Total	8	3008.8432			

3. **Blend**

Source	df	Sum of Squares	Mean Square	F Ratio	Prob > F
Solvent	2	10220.109	5110.05	4398.268	<.0001
Error	6	6.971	1.16		
C. Total	8	10227.080			

Table E.9 ANOVA for Tannin (Samples)

1. **Methanol**

Source	df	Sum of Squares	Mean Square	F Ratio	Prob > F
Samples	2	1911.8210	955.910	1814.714	<.0001
Error	6	3.1605	0.527		
C. Total	8	1914.9815			

2. **Ethanol**

Source	df	Sum of Squares	Mean Square	F Ratio	Prob > F
Samples	2	1954.9478	977.474	3191.809	<.0001
Error	6	1.8375	0.306		
C. Total	8	1956.7853			

3. **Aqueous**

Source	df	Sum of Squares	Mean Square	F Ratio	Prob > F
Samples	2	586.25042	293.125	946.0399	<.0001
Error	6	1.85907	0.310		
C. Total	8	588.10949			

Table E.10 ANOVA for Tannin (Solvents)

1. **Green tea**

Source	df	Sum of Squares	Mean Square	F Ratio	Prob > F
Solvent	2	1081.3128	540.656	1292.543	<.0001
Error	6	2.5097	0.418		
C. Total	8	1083.8226			

1. ***U. dioica* leaves**

Source	df	Sum of Squares	Mean Square	F Ratio	Prob > F
Solvent	2	205.40336	102.702	438.4381	<.0001
Error	6	1.40547	0.234		
C. Total	8	206.80882			

2. **Blend**

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Solvent	2	1213.3064	606.653	1237.282	<.0001
Error	6	2.9419	0.490		
C. Total	8	1216.2483			

Table E.11 ANOVA for DPPH scavenging assay (Samples)

1. **Methanol**

Source	df	Sum of Squares	Mean Square	F Ratio	Prob > F
Samples	2	903.66042	451.830	6463.952	<.0001*
Error	6	0.41940	0.070		
C. Total	8	904.07982			

1. **Ethanol**

Source	df	Sum of Squares	Mean Square	F Ratio	Prob > F
Samples	2	862.79536	431.398	5181.608	<.0001
Error	6	0.49953	0.083		
C. Total	8	863.29489			

2. Aqueous

Source	df	Sum of Squares	Mean Square	F Ratio	Prob > F
Samples	2	774.34687	387.173	4741.544	<.0001
Error	6	0.48993	0.082		
C. Total	8	774.83680			

Table E.12 ANOVA for DPPH scavenging assay (Solvents)

1. Green tea

Source	df	Sum of Squares	Mean Square	F Ratio	Prob > F
Solvent	2	282.38780	141.194	1983.060	<.0001
Error	6	0.42720	0.071		
C. Total	8	282.81500			

2. *U. dioica* leaves

Source	df	Sum of Squares	Mean Square	F Ratio	Prob > F
Solvent	2	316.27282	158.136	7412.644	<.0001
Error	6	0.12800	0.021		
C. Total	8	316.40082			

3. Blend

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Solvent	2	454.94649	227.473	1598.797	<.0001
Error	6	0.85367	0.142		
C. Total	8	455.80016			

Table E.13 ANOVA for TAC (Samples)

1. Methanol

Source	df	Sum of Squares	Mean Square	F Ratio	Prob > F
Samples	2	326.39776	163.199	10784.07	<.0001
Error	6	0.09080	0.015		
C. Total	8	326.48856			

2. Ethanol

Source	df	Sum of Squares	Mean Square	F Ratio	Prob > F
Samples	2	212.92207	106.461	22077.17	<.0001
Error	6	0.02893	0.004822		
C. Total	8	212.95100			

3. Aqueous

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Samples	2	38.984600	19.4923	11245.56	<.0001
Error	6	0.010400	0.0017		
C. Total	8	38.995000			

Table E.14 ANOVA for TAC (Solvents)

1. Green tea

Source	df	Sum of Squares	Mean Square	F Ratio	Prob > F
Solvent	2	147.26976	73.6349	17439.84	<.0001
Error	6	0.02533	0.0042		
C. Total	8	147.29509			

2. *U. dioica* leaves

Source	df	Sum of Squares	Mean Square	F Ratio	Prob > F
Solvent	2	4.0648222	2.03241	952.6927	<.0001
Error	6	0.0128000	0.00213		
C. Total	8	4.0776222			

3. Blend

Source	df	Sum of Squares	Mean Square	F Ratio	Prob > F
Solvent	2	168.45576	84.2279	5493.122	<.0001
Error	6	0.09200	0.0153		
C. Total	8	168.54776			

Table E.15 ANOVA for FRAP (Samples)

1. Methanol

Source	df	Sum of Squares	Mean Square	F Ratio	Prob > F
Samples	2	335.50176	167.751	24995.99	<.0001
Error	6	0.04027	0.006711		
C. Total	8	335.54202			

2. Ethanol

Source	df	Sum of Squares	Mean Square	F Ratio	Prob > F
Samples	2	197.68916	98.8446	10718.09	<.0001
Error	6	0.05533	0.0092		
C. Total	8	197.74449			

3. Aqueous

Source	df	Sum of Squares	Mean Square	F Ratio	Prob > F
Samples	2	42.119622	21.0598	30570.69	<.0001
Error	6	0.004133	0.000689		
C. Total	8	42.123756			

Table E.16 ANOVA for FRAP (Solvents)

1. Green tea

Source	df	Sum of Squares	Mean Square	F Ratio	Prob > F
Solvent	2	138.52829	69.2641	7132.463	<.0001
Error	6	0.05827	0.0097		
C. Total	8	138.58656			

2. *U. dioica* leaves

Source	df	Sum of Squares	Mean Square	F Ratio	Prob > F
Solvent	2	2.7772667	1.38863	2231.732	<.0001
Error	6	0.0037333	0.00062		
C. Total	8	2.7810000			

3. Blend

Source	df	Sum of Squares	Mean Square	F Ratio	Prob > F
Solvent	2	150.81647	75.4082	11990.71	<.0001
Error	6	0.03773	0.0063		
C. Total	8	150.85420			

Table E.17 ANOVA for AST vs ZOI

	Sum of Squares	df	Mean of Squares	F-ratio	P-value
Between Groups	12	4	3	12	0.0007
Within Groups	2.5	10	0.25		
Total	14.5	14			

Table E.18 ANOVA for ZOI (Samples)

1. Methanol

i. *S. aureus*

Source	df	Sum of Squares	Mean Square	F Ratio	Prob > F
Samples	2	158.00000	79.0000	4740.000	<.0001
Error	6	0.10000	0.0167		
C. Total	8	158.10000			

ii. *S. typhi*

Source	df	Sum of Squares	Mean Square	F Ratio	Prob > F
Samples	2	98.000000	49.0000	3675.000	<.0001
Error	6	0.080000	0.0133		
C. Total	8	98.080000			

iii. *S. flexneri*

Source	df	Sum of Squares	Mean Square	F Ratio	Prob > F
Samples	2	56.000000	28.0000	2100.000	<.0001
Error	6	0.080000	0.0133		
C. Total	8	56.080000			

iv. *E. faecalis*

Source	df	Sum of Squares	Mean Square	F Ratio	Prob > F
Samples	2	96.000000	48.0000	3600.000	<.0001
Error	6	0.080000	0.0133		
C. Total	8	96.080000			

v. *K. pneumoniae*

Source	df	Sum of Squares	Mean Square	F Ratio	Prob > F
Samples	2	78.000000	39.0000	2925.000	<.0001
Error	6	0.080000	0.0133		
C. Total	8	78.080000			

1. Ethanol

i. *S. aureus*

Source	df	Sum of Squares	Mean Square	F Ratio	Prob > F
Samples	2	122.00000	61.0000	181.1881	<.0001
Error	6	2.02000	0.3367		
C. Total	8	124.02000			

ii. *S. typhi*

Source	df	Sum of Squares	Mean Square	F Ratio	Prob > F
Samples	2	104.00000	52.0000	15600.00	<.0001
Error	6	0.02000	0.0033		
C. Total	8	104.02000			

iii. *S. flexneri*

Source	df	Sum of Squares	Mean Square	F Ratio	Prob > F
Samples	2	32.000000	16.0000	4800.000	<.0001
Error	6	0.020000	0.0033		
C. Total	8	32.020000			

iii. *E. faecalis*

Source	df	Sum of Squares	Mean Square	F Ratio	Prob > F
Samples	2	72.000000	36.0000	0	0
Error	6	0.000000	0.0000		
C. Total	8	72.000000			

iv. *K. pneumoniae*

Source	df	Sum of Squares	Mean Square	F Ratio	Prob > F
Samples	1	0.00000000	0.000000	0.0000	1.0000
Error	4	0.02000000	0.005000		
C. Total	5	0.02000000			

2. Aqueous

i. *S. aureus*

Source	df	Sum of Squares	Mean Square	F Ratio	Prob > F
Samples	2	24.000000	12.0000	.	.
Error	6	0.000000	0.0000		
C. Total	8	24.000000			

ii. *S. typhi*

Source	df	Sum of Squares	Mean Square	F Ratio	Prob > F
Samples	2	62.000000	31.0000	9300.000	<.0001
Error	6	0.020000	0.0033		
C. Total	8	62.020000			

iii. *S. flexneri*

Source	df	Sum of Squares	Mean Square	F Ratio	Prob > F
Samples	1	0	0	0	0
Error	4	0	0		
C. Total	5	0			

iv. *E. faecalis*

Source	df	Sum of Squares	Mean Square	F Ratio	Prob > F
Samples	1	6.0000000	6.00000	200.0000	0.0001
Error	4	0.1200000	0.03000		
C. Total	5	6.1200000			

v. *K. pneumoniae*

Source	df	Sum of Squares	Mean Square	F Ratio	Prob > F
Samples	1	0.00000000	0.000000	0.0000	1.0000
Error	4	0.02000000	0.005000		
C. Total	5	0.02000000			

Table E.19 ANOVA for ZOI (Solvents)

1. Green tea

i. S. aureus

Source	df	Sum of Squares	Mean Square	F Ratio	Prob > F
Solvents	2	78.000000	39.0000	11700.00	<.0001
Error	6	0.020000	0.0033		
C. Total	8	78.020000			

ii. S. typhi

Source	df	Sum of Squares	Mean Square	F Ratio	Prob > F
Solvents	2	26.000000	13.0000	1950.000	<.0001
Error	6	0.040000	0.0067		
C. Total	8	26.040000			

iii. S. flexneri

Source	df	Sum of Squares	Mean Square	F Ratio	Prob > F
Solvents	2	50.000000	25.0000	0	0
Error	6	0.000000	0.0000		
C. Total	8	50.000000			

iv. E. faecalis

Source	df	Sum of Squares	Mean Square	F Ratio	Prob > F
Solvents	2	14.000000	7.00000	300.0000	<.0001
Error	6	0.140000	0.02333		
C. Total	8	14.140000			

v. K. pneumoniae

Source	df	Sum of Squares	Mean Square	F Ratio	Prob > F
Solvents	2	8.0000000	4.00000	600.0000	<.0001
Error	6	0.0400000	0.00667		
C. Total	8	8.0400000			

2. *U. dioica* leaves

i. S. aureus

Source	df	Sum of Squares	Mean Square	F Ratio	Prob > F
Solvents	2	14.000000	7.00000	525.0000	<.0001
Error	6	0.080000	0.01333		
C. Total	8	14.080000			

ii. S. typhi

Source	df	Sum of Squares	Mean Square	F Ratio	Prob > F
Solvents	2	8.0000000	4.00000	300.0000	<.0001
Error	6	0.0800000	0.01333		
C. Total	8	8.0800000			

iii. S. flexneri

Source	df	Sum of Squares	Mean Square	F Ratio	Prob > F
Solvents	1	0	0	0	0
Error	4	0	0		
C. Total	5	0			

iv. E. faecalis

Source	df	Sum of Squares	Mean Square	F Ratio	Prob > F
Solvents	1	0	0	0	0
Error	4	0	0		
C. Total	5	0			

v. K. pneumoniae

No ZOI was observed

3. Blend

i. S. aureus

Source	df	Sum of Squares	Mean Square	F Ratio	Prob > F
Solvents	2	128.00000	64.0000	190.0990	<.0001
Error	6	2.02000	0.3367		
C. Total	8	130.02000			

ii. S. typhi

Source	df	Sum of Squares	Mean Square	F Ratio	Prob > F
Solvents	2	26.000000	13.0000	0	0
Error	6	0.000000	0.0000		
C. Total	8	26.000000			

iii. S. flexneri

Source	df	Sum of Squares	Mean Square	F Ratio	Prob > F
Solvents	2	78.000000	39.0000	2340.000	<.0001
Error	6	0.100000	0.0167		
C. Total	8	78.100000			

iv. E. faecalis

Source	df	Sum of Squares	Mean Square	F Ratio	Prob > F
Solvents	2	14.000000	7.00000	700.0000	<.0001
Error	6	0.060000	0.01000		
C. Total	8	14.060000			

v. K. pneumoniae

Source	df	Sum of Squares	Mean Square	F Ratio	Prob > F
Solvents	2	24.000000	12.0000	900.0000	<.0001
Error	6	0.080000	0.0133		
C. Total	8	24.080000			

Table E.20 ANOVA for MIC

1. Methanol

	Sum of Squares	df	Mean of Squares	F	P-value
Between Groups	590416.6667	2	295208.3	21.8	0.0003
Within Groups	121875	9	13541.67		
Total	712291.6667	11			

2. Ethanol

	Sum of Squares	df	Mean of Squares	F	P-value
Between Groups	406666.7	2	203333.3333	16.6363	0.0009
Within Groups	110000	9	12222.22222		
Total	516666.7	11			

3. Aqueous

	Sum of Squares	df	Mean of Squares	F	P-value
Between Groups	2986666.667	2	1493333	65535	.000
Within Groups	0	9	0		
Total	2986666.667	11			