PHYTOCHEMICALS AND ANTIOXIDANT ACTIVITIES OF NEEM (*Azadirachta indica)* LEAVES

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Approval Letter

This *dissertation* entitled Phytochemicals and Antioxidant Activities of *Neem leaves* (*Azadirachta indica)* presented by Adit Shree Adhikari has been accepted as the partial fulfillment of the requirements for the degree of Bachelor of Science in Nutrition and Dietetics.

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Abstract

Fresh *Neem* leaves were collected from Dharan (Pindeswori Campus), Sunsari district, Nepal and were well washed with distilled water and subjected to cabinet drying at 50ºC until constant weight of sample was obtained. Thus, obtained dry powder was extracted using soxhlet apparatus in two different solvents i.e. methanol and ethanol and further concentrated using rotatory vacuum evaporator that was used for total phenol content (TPC), total flavonoid content (TFC) and tannin content determination. The mean values of methanolic and ethanolic extract were then statistically analyzed at 5% level of significance by paired t-test. Similarly, 99% methanol was used to determine total antioxidant capacity (TOAC), DPPH radical scavenging activity and reducing power assay of fresh *Neem* leaves.

The preliminary phytochemical analysis of aqueous extract of *Neem* showed presence of protein, carbohydrates, phenols, tannin, steroids, terpenoids and glycosides. Total phenol content (mg GAE/g), total flavonoid content (mg QE/g) and tannin content (mg GAE/g) of methanolic and ethanolic extract of *Neem* were respectively 28.09± 0.31 and 22.88±0.24, 98.76±6.4 and 67.1 ±2.86, 1.55±0.79and 1.4±0.14 respectively. The methanol and ethanol extract showed significant difference in the phytochemical contents (p-value<0.05). Similarly, total antioxidant capacity (TOAC), DPPH radical scavenging assay and reducing power of fresh *Neem* leaves was found to be 91.49±0.24%, 71.91±0.04%, 51.08±0.85% of dry mass respectively. Overall, the study shows that *Neem* contains significant amount of phytochemicals and possess good antioxidant properties too.

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List of Abbreviations

Abbreviation	Full Form
AAE	Ascorbic Acid Equivalent
CE	Catechin Equivalent
DPPH	2,2 -Diphenyl-1- picrylhydrazyl
dw	Dry Weight
GAE	Gallic Acid Equivalent
NCDs	Non-Communicable Diseases
QE	Quercetin Equivalent
RE	Rutin Equivalent
RONS	Reactive Oxygen Nitrogen Species
ROS	Reactive Oxygen Species
TFC	Total Flavonoid Content
TPC	Total Phenol Content
TOAC	Total Antioxidant Capacity

Part I

Introduction

1.1 Background

Medicinal plants are the richest bio-resource of drugs for traditional systems of medicine. Since evolution, man has been using plant extracts to improve his health and life-style. Prime sources of naturally occurring antioxidants for humans are fruits, vegetables and spices. Search for the novel natural antioxidants from tea, fruits, vegetables, herbs, and spices are continued as efforts have been made by researchers all over the globe. Herbs have given worldwide focus as a source of novel antioxidant compounds due to their safety as compared to synthetic antioxidants. Many plants have been screened for their antioxidant potential and there is growing interest in replacing synthetic antioxidants because of the concern over the possible carcinogenic effects of these in foods with natural ingredients (Shahidi and Zhong, 2010). Medicinal plants contain a wide variety of free radical scavenging molecules such as phenolic compounds (phenolic acids, flavonoids, catechins, proanthocyanidins, quinons, coumarins, tannins etc.), nitrogen compounds (alkaloids, amines, betalains etc.), vitamins, terpenoids, carotenoids and other secondary metabolites (Khalil, Arshad, Saboon, Amjad, & Akhtar, September, 2019)

Among the reported medicinal plants, *Neem leaves* is used as a popular folk medicine throughout the world (Arunkumar and Muthuselvam, 2009). It is a succulent plant with triangular fleshy green leaves bearing white teeth at the margins. Neem leaf grows 60–100 cm in length. The plant can survive at 40ºC temperature and also below freezing temperature depending on the root health (Baruaha *et al.*, 2016). *Neem leaves (L.)* Burn.f (Synonym *A. barbadensis Miller*) is the legitimate name for *Neem leaves* according to International Rules of Botanical Nomenclature (Sahu *et al.*, 2013).

Neem leaves juice has been used traditionally for its purgative effects and fresh leaf gel used in different formulations and cosmetic preparations. *Neem leaves* contains over hundreds of nutrients and bio-active compounds, including vitamins, enzymes, minerals, sugars, lignin, anthraquinones, saponins, salicylic acid and amino acids, which are responsible for their medicinal properties. Its secondary metabolites have multiple properties such as anti-inflammatory, antibacterial, antioxidant, immune boosting,

anticancer, anti-diabetic, anti-ageing and sunburn relief. Several uses of *Neem leaves* also have been reported such as for burn injury, eczema, cosmetics, inflammation and fever in traditional medicine systems (Kumar *et al.*, 2017a).

Interestingly, the genus Neem comprises 2 individual species, however, the primary focus of most nutritional, pharmacological, and natural product studies is concentrated on *Neem leaves* only. This might be because the rationale of pharmacognostic studies is rooted down in the traditional alternative and complementary medicines, where mostly *Neem leaves* is used in the treatment of diverse ailments (Dey *et al.*, 2017). Among various species of *Neem* , *Neem leaves* is considered to be the most potent, commercially important and the most popular plant in the research field (Misir *et al.*, 2014). *Neem leaves* is blessed with all the phytochemicals and marked to be efficient in sustaining in medical field (Sonam and Tiwari, 2016). *Neem leaves* gained many reputations, such as "champions among health care medicines," "the best health food in 21st century" and "star in plant" due to its unique effective ingredients and special functions (Kumar and Yadav, 2014).

1.2 Statement of the problem

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). Premature death from non-communicable diseases (NCDs) continues to be one of the major development challenges in the 21st century. NCDs account death of 15 million women and men between the ages of 30 and 70 each year, and leave no country untouched. In Nepal, 65% of death occurs from NCDs where as 22% of population has risk of premature death from NCDs (WHO, 2017). The lives of too many people in the world has been shortened by chronic diseases (WHO, 2005).

Reactive species, or free radicals, are a group of highly reactive chemical molecules with one or more unpaired electrons. These molecules arise from chain reactions that include three steps: initiation, propagation, and termination. In this manner, formation of free radicals is able to trigger amplification. Collectively, reactive species, present in living organisms, are called reactive oxygen nitrogen species (RONS). Some of RONS are superoxide ($O^{2\bullet-}$), hydroxyl ($OH^{\bullet}$), Peroxyl ($RO^{2\bullet}$), hydrogen peroxide (H_2O_2), ozone(O_3),

$NO^{\bullet}$, $NO^{2\bullet}$ etc. (Ferreira *et al.*, 2018). Some internally generated sources of free radicals are mitochondria, xanthine oxidase, peroxisomes, inflammation, phagocytosis, arachidonate pathways, exercise, ischemia/reperfusion, injury etc whereas externally generated sources of free radicals are cigarette smoking, environmental pollutants, radiation, certain drugs, pesticides, industrial solvent, ozone etc (Lobo *et al.*, 2010).

During daily activities, with advanced age, consumption of additives, alcohol, coffee, foods of animal origin, foods that have been barbecued, broiled, fried, grilled, or otherwise cooked at high, temperatures, foods that have been browned or burned, herbicides, hydro vegetable oils, pesticides, sugar etc. is on increasing demand that in turn results in production of ROS sources (Mohammed et al., 2015). Thus, unbarred generation of these free radicals leads to attack on various bio-molecules, cellular machinery, cell membrane, lipids, proteins, enzymes and DNA causing oxidative stress and ultimately cell death. Overproduction of free radicals can lead to many chronic diseases such as rheumatoid arthritis, atherosclerosis, cancer, diabetes, post-ischemic perfusion injury, cardiovascular diseases, myocardial infarction, chronic inflammation, stroke and septic shock, aging and other degenerative diseases in humans (Kumar *et al.*, 2017a). Thus, the present study takes into consideration the phytochemical analysis and antioxidant activities of *Neem leaves* since it is gaining interest among people as a functional food.

1.3 Objectives

1.3.1 General objective

The general objective of this work was to analyze the phytochemicals and antioxidant activities of Neem leaves.

1.3.2 Specific objectives

- To perform the preliminary qualitative phytochemical analysis of Neem leaves aqueous extract.
- To prepare methanolic and ethanolic extract of Neem leaves powder by soxhlet extraction for determination of total phenolic content (TPC), total flavonoid content (TFC) and total tannin content.
- To compare the differences in methanolic and ethanolic extract of phenolic, flavonoids and tannin content of Neem leaves.

- To carry out the antioxidant assays i.e., total antioxidant capacity (TOAC), DPPH radical scavenging assay and reducing power of Neem leaves.

1.4 Significance of the study

World Health Organization (WHO) estimates that up to 80% of people still rely on traditional remedies such as herbs for their medicine. One of such herbs is *Neem leaves* which has caught the global commercial interest in the world of Science, particularly medical science (Adesuyi *et al.*, 2012). It is rich in phenolic compounds, such as flavonoids, phenolic acids, stilbenes and coumarins, vitamins, minerals, amino-acids etc. These structures are ubiquitous bioactive compounds and a diverse group of secondary metabolites. Accordingly, bioactive polyphenols have attracted special attention because they can protect the human body from the oxidative stress. The antioxidant properties of phenolic acids and flavonoids are due to their redox properties, ability to chelate metals and quenching of singlet oxygen (Aljesri, 2015). Plants containing beneficial phytochemicals may supplement the needs of the human body by acting as natural antioxidants (Sharma, Shah, & Dave, 2020) (Altemimi *et al.*, 2017). Phytochemical analysis of plants is commercially important being great interest of pharmaceutical industries for the production of new drugs to cure various diseases (Wadood, 2013) (Kumar *et al.*, 2017a).

Many new antioxidants molecules have been secluded and determined from herbs and spices. The daily intake of these foods might be one of the major and promising sources against major disease leading to a healthier life. Biochemical results on edible plants wealthy in antioxidants are expected to lead to chemoprevention of lipid per-oxidation, cancer, inflammation and aging of human organs (Nakatani, 2000). To consider a natural composite as an antioxidant substance, it is important to investigate its antioxidant activities in vitro (Benzidia *et al.*, 2018).

This study adopts this approach and it aims at showing the value of extracts from whole leaf of *Neem leaves* since it is one of the highly investigated medicinal plant that has also been a commercial success as pharmacological, nutraceutical, and skin care products (Dey *et al.*, 2017). Important pharmaceutical properties that have recently been discovered for whole leaf extract include the ability to improve the bioavailability of co-administered vitamins in human subjects (Hamman, 2008) (Nejatzadeh-Barandozi, 2013). The present

study will analyze the phytochemical and antioxidant activities of *Neem leaves*. Phytochemical data may offer a tool to study the complexity of maculate species with the aim of identifying unique constituents which may be used as markers with possible chemotaxonomic significance (Sayeda *et al.*, 2016). Also in view of high interest shown by the people of different countries for its medicinal value and commercial uses, it is quite worthy to review the active constituents and clinical effectiveness of Neem leaves (Baruaha *et al.*, 2016). Since *Neem leaves* is produced in local levels, consumption of *Neem leaves* should be promoted. This is the new attempt on analysis of phytochemical content of absolute methanol and absolute ethanol extracts, TOAC and reducing power determination of methanol extracts, total carotene and chlorophyll-a and chlorophyll-b determination of whole leaf extract of *Neem leaves*.

1.5 Limitation of the study

Due to time limit, this study had the following limitations.

a. Aqueous extract was not taken for quantitative analysis.

1.6 Delimitation of the study

a. Only one species of *Neem* was used for research work.
b. Aqueous solution was not done due to time limit.

Part II

Literature review

2.1 *Neem leaves*

2.1.1 Introduction

Azadirachta indica (Neem) is a fast-growing evergreen popular tree found commonly in India, Africa and America. It has been used in Ayurvedic medicine for more than 4000 years due to its medicinal properties (Pandey *et al.*, 2014). The Sanskrit name of the neem tree is 'Arishtha' meaning 'reliever of sickness' and hence is considered as 'Sarbaroganibarini'. The tree is still regarded as 'village dispensary' in India (Susmitha *et al.*, 2013). The neem tree has been described as A. indica as early as 1830 by De Jussieu *et al.*, 1830 (Barman, Ahmed, & Chakraborty, 2018) and its taxonomic position is as follows:

Order	Rutales
Suborder	Rutinae
Family	Meliaceae
Subfamily	Melioideae
Tribe	Melieae
Genus	Azadirachta
Species	Indica

Neem (*Azadirachta indica*), the versatile medicinal plant is the source of several compounds having diverse chemical structure and biological effects (Biswas et al., 2002). A significant amount of research has already been carried out during the past to understand the chemistry and medicinal uses of different parts of neem for use therapeutically and industrially (Maragathavalli et al., 2012). *Azadirachta Indica* is now used in traditional medicine as a source of many therapeutic agents. *A. indica* (seed) is known to contain antibacterial, antifungal activities against different pathogenic microorganisms and antiviral activity against vaccinia, chikungunya, measles and coxsackie B Viruses (Biswas et al., 2002). Neem seed have been shown to exhibit wide pharmacological activities including; antioxidant, antimalarial, anti-carcinogenic, anti- inflammatory (Maragathavalli et al., 2012). The biological activities are attributed to the presence of many bioactive compounds in different parts of the plant.

2.1.2 History of *Neem leaves*

Along with Ayuerveda most of world's other reputed medicinal system like Unani, Chinese, and European "Materia Medica" have announced and acknowledged neem tree as "Panacea of all Disease". However, in India it is famous with many other names like 'Divine Tree', "Heal All", "Nature's Drugstore", and "Village Dispensary". Traditional Ayurvedic uses of neem include the treatment of fever, leprosy, malaria, ophthalmia and tuberculosis. Various folk remedies for neem include use as an anthelmintic, antifeedant, antiseptic, diuretic, emmenagogue, contraceptive, febrifuge, parasiticide, pediculocide and insecticide. Traditional routes of administration of neem extracts included oral, vaginal and topical use. It is honored colloquially in these circles as 'The Village Pharmacy', millions with exposure to the tree brush their teeth with its twigs, use its juice on their skin disorders and place its leaves throughout their homes to keep away insect. Few most important traditional uses of the different parts of plants are below stated; ·Neem twigs are used for brushing teeth in India and Pakistan. This practice is perhaps one of the earliest and most effective forms of dental care. ·All parts of the tree (seeds, leaves, flowers and bark) are used for preparing many different medical preparations. ·Neem oil is useful for skin care such as acne, and keeping skin elasticity. ·Traditionally, patients suffering from Chicken Pox sleep on the leaves in India owing to its medicinal value. ·In Ayurvedic, Unani and folklore traditional medicine, different parts of neem were preferred in the treatment of a wide range of afflictions (Subapriya & Nagini, 2005).

The *Neem leaves* plant has been known and used for centuries. It is native of southern and eastern Africa along Nile in the Susan and then it was introduced in North Africa and Mediterranean countries (Sahu *et al.,* 2013). It is a true gift from nature. Ancient records show that the benefits of *Neem leaves* have been known for centuries with its therapeutic advantages and healing properties. Many ancient works including the Bible refer to the use of *Neem*. The first detailed discussion of *Neem* 's medicinal value is probably that which is found in the Papyrus Ebers, an Egyptian document written around 1550 B.C. 2000 years ago, the Greek scientist regarded *Neem leaves* as the Universal panacea. The medicinal use of *Neem* was already mentioned more than 4000 years ago in a collection of Sumerian clay tablets dated 2100 B.C. 6000 years old carvings of the *Neem leaves* plant were discovered in Egypt. It was considered the "Plant of immortality" and was offered as a burial gift to the deceased pharaohs. Egyptian queen Nefertiti and Cleopatra used it as part of their

regular beauty regimes. Alexander the Great and Christopher Columbus used it to treat soldier's wounds.

Neem leaves is the most efficacious natural plant used both externally and internally and there are numerous benefits that are derived from this wonderful plant. The health benefits of *Neem leaves* have been propagated throughout the world. Among the existing species, *Neem leaves* is best known for its medicinal properties. Today *Neem leaves* is grown commercially in different parts of the world to meet the great demand for *Neem leaves* gel, juice and latex that have varied applications and uses in the cosmetic, food and alternative medicine industries (Mehta, 2017).

2.1.3 Distribution and cultivation

Neem is native of dry areas of the Indian subcontinent, Myanmar and China (Rojas-Sandoval et al., 2014). It was naturally distributed in Thailand, Malaysia and Indonesia and has become one of the most widespread trees in tropical and subtropical areas. It has become invasive in the Caribbean (Puerto Rico, Dominican Republic), sub-Saharan Africa (Kenya, Gambia, Senegal, Guinea Bissau, Ghana, Tanzania), and the Pacific (Australia, Fiji, Marshall Islands) (Rojas-Sandoval et al., 2014). Neem naturally occurs in dry deciduous and thorn forests, or acacia forests. In its exotic range, it has become invasive in a number of habitats including fallow agricultural land, savannah, and dry arid forests (coastal forest in Ghana, lowland monsoon forest in Indonesia, evergreen and dry deciduous forest in Africa) (Orwa et al., 2009). Neem can be found from sea level up to an altitude of 1500 m in places where average annual rainfall ranges from 400 to 1200 mm and where average annual maximum temperatures may be as high as 40°C. Adult trees tolerate some frost but seedlings are sensitive to it.

Neem can grow on a wide range of soils, from acidic to alkaline pH, but it does better on shallow, stony, sandy, poor soils, in marginal sloping places or on rocky crevices (Puri, 1999). Neem is a full sunlight species but it can withstand some shade in its first years (Orwa et al., 2009). Neem is able to extract nutrients from highly leached sandy soils and can survive extreme pH conditions, from 3 to 9 (Rojas-Sandoval et al., 2014). In well-drained soils, neem withstands up to 2500 mm rainfall. Neem has some tolerance of salinity and has been used in sugarcane plantations with a significant soil salinity (Orwa et al., 2009; Ahmed et al., 1997).

However, the exact origin is uncertain: some say neem is native to the whole Indian subcontinent; others attribute it to dry forest areas throughout all of South and Southeast Asia, including Pakistan, Sri Lanka, Thailand, Malaysia, and Indonesia. It is in India that the tree is most widely used.

2.1.4 Botanical Description

A. indica commonly known as neem, is a large, evergreen tree, 12-18m in height and 1.8-2.4m in girth, with a straight and long, spreading branches forming a board crown, commonly found throughout the greater part of India. Stem has a girth 1.8-2.4m and the bark is rough, hard, grey or dark grey, reddish brown inside with numerous oblique furrows and scattered tubercles. The leaves as alternate, impair pinnate and 20-38 cm long. The leaflets are 8-19 cm alternate or opposite. Leaves are ovate-lanceolate, oblique or sub foliate, glossy and bluntly serrate. The flowers are hermaphrodite. White or pale yellow, small, scented and numerous. Flowers are very lax and in axillary panicles. The calyx is five lobed. The sepals are small in size. The petals are five in number, polypetalous. Stamina tube is a little shorter than the petals. There are 9-10 lobes at the apex; the lobes are truncate, again slightly toothed. The anthers are within the tube opposite to and shorter than the lobes. The ovary is called, style elongate, slender and stigma shortly cylindrical. There are two collateral ovules in each cell. The fruit is one seeded, drupe and endocarp is woody. The seeds are ellipsoid, cotyledons thick, fleshy cordate at base and radical superior.

2.1.5 Chemical constituents

Table 2.1 Proximate composition of *Neem leaves* on dry matter basis

Parameters	**Composition (%)**
Crude protein	7.1 ± 0.16
Crude fat	1.01 ± 0.07
Carbohydrates	22.35 ± 0.30
Total ash	13.08 ± 0.04

Source: (Ahmed and Hussain, 2013)

Table 2.2 Chemical composition and properties of *Neem leaves*

S. N.	Constituents	Types of constituents	Properties and activity
1	Amino acids	Provides 20 of the 22 required amino acids and 7 of the 8 essential ones	Basic building blocks of proteins in the body and muscle tissues
2	Anthraquinones	Provides Neem emodin, Neem tic acid, alovin, anthracine	Analgesic, antibacterial
3	Enzymes	Peroxidase, aliiase, catalase, lipase, carboxypeptidase, amylase, alkaline phosphatase	Facilitate biochemical reaction
4	Hormones	Auxins and gibberellins	Wound healing and anti-inflammatory
5	Minerals	Calcium, chromium, copper, iron, manganese, potassium, sodium and zinc	Essential for good health
6	Salicyclic acid	Aspirin like compounds	Analgesic
7	Saponins	Glycosides	Cleansing and antiseptic
8	Steroids	Cholesterol, campesterol, lupeol, sistosterol	Anti-inflammatory agents, lupeol has antiseptic and analgesic properties
9	Sugars	Monosaccharides: Glucose and Fructose Polysaccharides: Glucomannans/polymannose	Anti-viral, immune modulating activity of acemannan
10	Vitamins	A, B, C, E, choline, B12, folic acid	Antioxidant (A, C, E), neutralizes free radicals

Source: (Mahor and Ali, 2016; Sahu *et al.*, 2013)

2.1.6 Uses of *Neem leaves*

Pharmacological Activity of *Azadirachta indica*

Analgesic effect

In a Study done by Kumar et al., (2012) by using albino rats, it was found that Neem seed oil (NSO) of 2ml/kg body weight is comparable to morphine with a dose of 1mg/kg body weight, NSO produces a better analgesic effect than morphine with 45 minute of interval and in another similar study done by Srinivasa et al., (2014) it was stated that neem resembles indomethacine.

Antipyretic effects

Methanol extract of Neem leaves shows antipyretic effects when administrated orally in rabbits and rats

Antifungal effects

In a study done by Mondali et al., (2009) shows that the ethanolic extract of A.indica leaves is more effective against Rhizopus and Aspergillus compared to aqueous leaf extract. Aqueous and ethanolic extract of neem leaves were found effective against Candida albicans by which these organism shows sensitivity at the concentration of 15% and 7.5% on aqueous extract and the Minimum Inhibitory Concentration (MIC) was 7.5%. In the ethanolic extraction Candida albicans were found to be susceptible at the concentration of 15%, 7.5% and 3.75%, besides that; the MIC were 3.75% (Aarati et al., 2011).

Antibacterial

The methanol extract of A. indica leaves shows antibacterial activity against Bacillus subtilis, Staphylococcus aureus, Proteus vulgaris, Salmonella typhi, and showed low activity on Pseudomonas aeruginosa but it is infective against Escherichia coli. The petroleum ether and methanol extract of A. Indica leaves were highly effective against Candida albicans (Grover et al., 2011). Furthermore, the hexane extract from A.indica bark shows antimicrobial activity against Escherichia coli (Abalaka et al., 2012). In another study done by Vashist and Jindal, (2012) the Azadirachta indica seeds poses an antibacterial activity against the bacteria that causes eye infection (Ophthalmic infection) such as Staphylococcus aureus, Staphylococcus pyogenes, Escherichia coli and Pseudomonas aeruginosa.

Antiviral

Neem leaves is found to be effective against Dengue virus type -2 in which it halts the replication of the virus itself in an invitro environment and in the laboratory animals (Rao et al., 1969). The aqueous extract of Neem bark were found to be effective against Herpes simplex virus type 1 by blocking its entry into natural target cell (Tiwari, Darmani, Yue, & Shukla, 2010), even though Neem does not cure but it shows the ability to prevent smallpox, chickenpox and fowl pox (Bhowmik, Bhattacharjee, & Kumar, 2010).

Contraceptive

According to Bansal et al., (2010) the addition of sodium nimbidinate salt in aqueous form to semen of rat and human results in death of sperm in different percentage. Neem oil claimed spermicidal activity against rhesus monkey human spermatozoa in invitro condition, and when the oil is used in intra vaginally it prevents pregnancy in rats with concentration of 20 micro-litre and in rhesus monkey and women were about 10 milli-litre (ml) and the oral dose as low as 25 micro litre prevents implantation in rats and does not show any side effects upon repeated application. Similarly, Neem extract (Nim-76) is found to be effective than raw neem oil which act as spermicidal with no alteration in hormonal values. According to Khillare and Shrivastav (2003), aqueous extract of old and tender leaves shows 100% of mortality of the sperms without altering its morphology (head, midpiece and tail).

Hepatoprotective

Young stem bark extract of Azadirachta indica were used to analyze the hepatoprotective activity by inducing carbon tetrachloride as acute hepatotoxic agent in rats and uses Silymarin as a standard hepatoprotective agent. A dose of 200mg/kg and 500mg/kg were choosen for the studies. Upon administration of Azadirachta indica, it stabilizes the levels of Serum glutamate oxaloacetate transaminase (SGOT), Serum Glutamate Pyruvate Transaminase (SGPT), Alkaline Phosphatase (ALP), Serum bilirubin and elevates total protein amount. Thus, this plant clearly notifies the improvement of the functional status of liver cells (Gomase, Rangari , & Verma, 2011).

Antihyperglycemic agent

In a dose of 800 mg/kg Neem root bark extract shows anti hyperglycemic effects upon tested with overnight fasted wistar albino rats of either sex and in alloxan induced diabetic rats but it is not significant as glibenclamide (Patil, Banagar, Banagar, & B, 2013). A dose of 250 mg/kg of aqueous extract of fresh leaves of Neem was administrated orally onto

streptozotocin induced and its associated retinopathy in rats for 16 weeks and resulted in significant fall in blood glucose level and serum lipids and there was slight increase in HDL level. The slight increase indicates the extract as positive effect in lipid metabolism of diabetic rats. Furthermore, the plant completely reversed the unusual changes in the retina of the rats (Hussain, 2002). Aqueous neem fruit extract was found to be effective as blood glucose lowering agent at the dose of 500mg/kg in normoglycemic albino rabbits upon oral administration (Rao & Agrawal, 2000).

2.2 Phytochemicals

2.2.1 Introduction

Phytochemicals are non-nutritive bioactive chemical compounds found naturally in plants that protect against diseases. The active ingredients are the main effective compounds of medicinal plants, the presence and quality vary from one plant to the other. A number of phytochemicals are known, some of which include: alkaloids, saponins, flavonoids, tannins, glycosides, anthraquinones, steroids and terpenoids. They do not only protect the plants but have enormous physiological activities in humans and animals. These include cancer prevention, antibacterial, antifungal, anti-oxidative , hormonal action, enzyme stimulation and many more (Doss and Anand, 2012). Many phytochemicals possess antioxidant activity and reduces the risk of different diseases by serving structural and functional role as well as an electrolyte (Abidemi, 2013).

Phytochemical constituents are the biochemical compounds used as the precursors for the development of drugs (Sonam and Tiwari, 2016).

2.2.2 Classes of phytochemicals

The different classes of phytochemicals are discussed below.

2.2.2.1 Alkaloids

These are the largest group of secondary chemical constituents made largely of ammonia compounds comprising basically of nitrogen bases synthesized from amino acid building blocks with various radicals replacing one or more of the hydrogen atoms in the peptide ring, most containing oxygen. The compounds have basic properties and are alkaline in reaction, turning red litmus paper blue. In fact, one or more nitrogen atoms that are present in an alkaloid, typically as 1°, 2° or 3° amines, contribute to the basicity of the alkaloid.

The degree of basicity varies considerably, depending on the structure of the molecule, and presence and location of the functional groups (Sarker and Nahar, 2007).

2.2.2.2 Glycosides

Glycosides in general, are defined as the condensation products of sugars (including polysaccharides) with a host of different varieties of organic hydroxy (occasionally thiol) compounds (invariably monohydrate in character), in such a manner that the hemiacetal entity of the carbohydrate must essentially take part in the condensation. Glycosides are colorless, crystalline carbon, hydrogen and oxygen-containing (some contain nitrogen and sulfur) water-soluble phyto-constituents, found in the cell sap. Chemically, glycosides contain a carbohydrate (glucose) and a non-carbohydrate part (aglycone or genin) (Firn, 2010; Kar, 2007).

2.2.2.3 Flavonoids

Flavonoids are a group of polyphenolic compounds ubiquitously found in fruits and vegetables. Flavonoids occur in both in the free state and a glycoside. These compounds occur as yellow and white plant pigments (Latin flavus = yellow). Till date more than 4000 flavonoids have been discovered, out of which 500 are found in free-state.

Flavonoid is known for its anti-inflammatory and anti-allergic effects, antioxidant, antithrombotic and vaso protective properties. Many flavonoid containing plants are diuretic or antispasmodic (e.g. liquorice & parsley). Some flavonoids have antibacterial and antifungal properties. They also play a role in protecting the plants from microbe and insect attacks. More importantly, the consumption of foods containing flavonoids has been linked to numerous health benefits. They are secondary metabolites, meaning they are organic compounds that have no direct involvement with the growth or development of plants. Flavonoids are widely disbursed throughout plants and gives vibrant colors to the flowers and fruits of many plants (Kesarkar *et al.*, 2009).

2.2.2.4 Phenolics

Phenolic compounds constitute a large group of phenyl-propanoids produced by plants as secondary metabolites. They are involved in different functions in the ecology, physiology, and biochemistry of plants such as chemical defense against predators, reproduction, and in plant–plant interference. So far, 8000 PCs have been identified in 16 different classes

with very diverse chemical structures and molecular masses (Velderrain-Rodriguez *et al.*, 2014).

They contain benzene rings, with one or more hydroxyl substituents, and range from simple phenolic molecules to highly polymerized compounds (Lin *et al.*, 2016). Their consumption has been linked with reduced risk of multiple non-communicable chronic diseases (NCCD), e.g., cardiovascular (CVD) and neurodegenerative diseases, certain cancers, type II diabetes and osteoporosis, putatively attributed to their multifaceted bio actions, e.g., anti-oxidation, anti-inflammation, modulation of signal transduction, anti-microbial activity, and anti-proliferation (Velderrain-Rodriguez *et al.*, 2014). Table 2.5 shows the major classes of phenolic compound.

Table 2.3 The major classes of phenolic compound

S.N.	Number of carbon atom	Basic skeleton	Class
1	6	C_6	Simple phenols, Benzoquinones
2	7	C_6-C_1	Phenolic acids
3	8	C_6-C_2	Acetophenones, Tyrosine derivatives
4	9	C_6-C_3	Hydroxycinnamic acid, Coumarins
5	10	C_6-C_4	Naphthoquinones
6	13	C_6-C_1-C_6	Xanrhones
7	14	C_6-C_2-C_6	Stilbenes
8	15	C_6-C_3-C_6	Flavonoids
9	18	$(C_6$-$C_3)_2$	Lignans
10	30	$(C_6$-C_3-$C_6)_2$	Biofalvonoids
11	N	$(C_6$-C_3-$C_6)_n$	Condensed tannins

Source: (Saxena *et al.*, 2013)

2.2.2.5 Saponins

The term saponin is derived from *Saponaria vaccaria* (*Quillaja saponaria*), a plant, which abounds in saponins and was once used as soap. Saponins therefore possess ‘soap like’ behaviour in water, i.e., they produce foam. On hydrolysis, an aglycone is produced, which is called sapogenin. There are two types of sapogenin: steroidal and triterpenoidal.

Usually, the sugar is attached at C-3 in saponins, because in most sapogenins there is a hydroxyl group at C-3. Saponins are regarded as high molecular weight compounds in which, a sugar molecule is combined with triterpene or steroid aglycone. There are two major groups of saponins and these include: steroid saponins and triterpene saponins. Saponins are soluble in water and insoluble in ether, and like glycosides on hydrolysis, they give aglycones. Saponins are extremely poisonous, as they cause heamolysis of blood and are known to cause cattle poisoning (Kar, 2007).

2.2.2.6 Tannins

These are widely distributed in plant flora. They are phenolic compounds of high molecular weight. Tannins are soluble in water and alcohol and are found in the root, bark, stem and outer layers of plant tissue. Tannins have a characteristic feature to tan, i.e. to convert things into leather. They are acidic in reaction and the acidic reaction is attributed to the presence of phenolics or carboxylic group (Kar, 2007). They form complexes with proteins, carbohydrates, gelatin and alkaloids. Tannins are divided into hydrolysable tannins and condensed tannins (Bule, Khan, Nisar, & Niaz, 2020) (Doughari, 2012).

Hydrolysable tannins, upon hydrolysis, produce gallic acid and ellagic acid and depending on the type of acid produced, the hydrolysable tannins are called gallotannins or egallitannins. On heating, they form pyrogallic acid. Tannins are used as antiseptic and this activity is due to presence of the phenolic group. Tannin rich medicinal plants are used as healing agents in a number of diseases. In Ayurveda, formulations based on tannin-rich plants have been used for the treatment of diseases like leucorrhoea, rhinnorhoea and diarrhea (Rao V. , 2012). Tannins have the ability to bind and precipitate macromolecules such as protein, carbohydrates, and digestive enzymes, thus causing harmful nutritional effects. However, their potency as antioxidants and free radical scavengers can have positive effects on cardiovascular diseases and certain types of cancer (Shahidi *et al.*, 2018).

2.2.2.7 Terpenes

Terpenes are among the most widespread and chemically diverse groups of natural products. They are flammable unsaturated hydrocarbons, existing in liquid form commonly found in essential oils, resins or oleoresins (Akhtar & Swamy, 2018). Terpenoids includes hydrocarbons of plant origin of general formula $(C5H8)n$ and are

classified as mono-, di-, tri- and sesquiterpenoids depending on the number of carbon atoms (Rao V. , 2012).

2.2.2.8 Anthraquinones

These are derivatives of phenolic and glycosidic compounds. They are solely derived from anthracene giving variable oxidized derivatives such as anthrones and anthranols (Akhtar & Swamy, 2018).

2.2.2.9 Essential oils

Essential oils are the odorous and volatile products of various plant and animal species. Essential oils have a tendency evaporate on exposure to air even at ambient conditions and are therefore also referred to as volatile oils or ethereal oils. They mostly contribute to the odoriferous constituents or 'essences' of the aromatic plants that are used abundantly in enhancing the aroma of some spices (Rao V. , 2012) (Martinez *et al.*, 2008). Essential oils are either secreted either directly by the plant protoplasm or by the hydrolysis of some glycosides and structures such as directly Plant structures associated with the secretion of essential oils include: Glandular hairs, Oil tubes (or vittae), modified parenchymal cells, Schizogenous or lysigenum passagage. Essential oils have been associated with different plant parts including leaves, stems, flowers, roots or rhizomes. Chemically, a single volatile oil comprises of more than 200 different chemical components, and mostly the trace constituents are solely responsible for attributing its characteristic flavour and odour (Rao V. , 2012) (Firn, 2010).

2.2.2.10 Steroids

Plant steroids (or steroid glycosides) also referred to as 'cardiac glycosides' are one of the most naturally occurring plant phyto-constituents that have found therapeutic applications as arrow poisons or cardiac drugs (Mousavi, 2018). The cardiac glycosides are basically steroids with an inherent ability to afford a very specific and powerful action mainly on the cardiac muscle when administered through injection into man or animal. Steroids (anabolic steroids) have been observed to promote nitrogen retention in osteoporosis and in animals with wasting illness (Rao V. , 2012).

2.3 Factors affecting phytochemical content

a. Cultivar effect

Genetic composition is the main determining factor which can directly influence the phytochemicals of vegetables, since it has been shown that the differences in phytochemical compounds between cultivars are greater than those between plants of the same cultivar grown under different conditions (Hu, 2012). The strong effect of genetic materials on the phenolic profile has been demonstrated in several horticultural crops such as potato, faba bean, tomato, garlic, globe artichoke and cardoon (Rouphael, et al., 2016).

b. Extraction method and solvent used

Solvent type and extract preparation methods affect phytochemical concentration and different activities of plants. Polar solvent enable extraction of significant amounts of phenolics and flavonoids (Anwar and Przybylski, 2012). The study done by (Vidic *et al.*, 2014) shows that there is significant difference in the phytochemical contents due to the difference in the method of extraction used where soxhlet extraction was better than ultrasound extraction of the sample.

Methanolic extract induced the best extraction yield and more complex composition of phenolics (Truong & Nguyen, 2019) (Anwar *et al.*, 2013; Kumar *et al.*, 2017b; Sonam and Tiwari, 2016). Moreover, scientists have discovered that highly polar solvents, such as methanol, have a high effectiveness as antioxidants (Altemimi *et al.*, 2017). Another study reveals that solvent used in extraction affects the yield, total phenolic, total flavonoid, flavonols, condensed tannins, carotenoids contents and their antioxidant and antimicrobial properties in various degrees. The highest contents of phytochemical contents were obtained with methanol extracts (Felhi *et al.*, 2017). Similar result was given by the study of (Widyawati *et al.*, 2014) where methanolic extract showed better phytochemical content than ethanol, aquadest, ethyl acetate and hexanes.

c. Environmental conditions

Environmental factors including sunshine radiation, temperature variation and climatic conditions within a geographical location may influence the level of phytochemicals (Kumar, Yadav, Yadav, & Yadav, 2017). Mineral composition, soil type, temperature, light and water content are among the frequently reported factors that affect the total phytochemical contents in plants (Li, Tsao, & Deng, 2012). Optimum fertilization is important to ensure favorable levels of phytochemicals. High levels of fertilizer (nitrogen,

phosphorus and potassium) application may result in increased vegetative growth and yield with a decrease in the level of phytochemicals (Adhikari, 2009). Similarly, the application of nitrogen increased the chlorophyll content in leaves of the Neem leaves plants and the highest levels of chlorophyll 'a' and 'b' were obtained in the highest levels of nitrogen. Soil fertility management may be one of the strategies to increase yield of Neem leaves (Hasanuzzaman *et al.*, 2008).

Highland and Semi-arid zone samples possessed higher antioxidant activity whereas Tropical zone samples possessed minimum. Different agro-climatic conditions have effects on phytochemical diversity and antioxidant potential of Neem leaves plant (Kumar *et al.*, 2017b). The study by (Kumar *et al.*, 2017b) demonstrated that antioxidant activity was higher in Neem leaves plants grown in Northern India in comparison to Southern India. More phytochemicals are produced in plants under cold stress conditions. Different agro-climatic conditions have effects on the phytochemicals, total phenolic content (TPC) and antioxidant potential of the *A. vera* plant (Kumar *et al.*, 2017a).

d. Growth conditions

Growth stages of plants contribute to the level of phytochemical content. Based on the finding of (Zhang *et al.*, 2010), during the early growth stage, leaves have low phenolic and flavonoids contents while the total alkaloids content gradually increases during the growth and development stages. Neem leaves of different developmental stages contain different active components and possess antioxidant capacity to different degrees (Y. Hu *et al.*, 2003).

e. Post- harvest factors

Phytochemicals are very susceptible to deterioration during postharvest, due to fungal decay, physiological disorders, pests, mechanical injury, over-ripeness, and inadequate temperature and relative humidity during storage or transport, which may result in great losses in quality components including phytochemical (Yahia, 2018).

Food-processing operations also have a major influence on the stability of phytochemicals in fruit and vegetables and their products. Conventional (thermal), modern or non-thermal (e.g. high pressure processing pulsed electric field, ultrasound/sonication, ozone, ultraviolet), domestic (e.g. washing, peeling, cutting) and industrial (e.g. canning, drying)

processing are widely reported to degrade the level of phytochemicals in processed food products (Rawson, Koidis, Rai, Tuohy, & Brunton, 2010).

f. Post- harvest storage conditions

Storage temperature, atmosphere gas composition and use of chemicals are major factors that influence the quantity and quality of phytochemicals (Li *et al.*, 2012).

Lower temperature can slow the deterioration of phytochemicals. Opposed to lower temperature, high temperature also brings a significant change in total phenolic, flavonoid, tannin content and antioxidant activity compared with its fresh form. However their concentration may vary according to the drying methods used and the duration of exposure to hot air (Michalczyk *et al.*, 2009). Exposure to high temperature leads to discharge of phenolic compounds through disintegration of cellular constituents which then results in the migration of components, leading to losses by leakage or breakdown by various chemical reactions involving enzymes, light and oxygen as well as (Davey *et al.*, 2002)

g. Others

An appropriate extraction of phenolic compounds depends on multiple factors, such as their chemical nature, raw material, storage time and conditions. Not least it depends on the extraction and quantification methods, choice of standards, and presence of interference (Dimcheva and Karsheva, 2018).

2.4 Importance of phytochemicals

Different mechanisms of action of phytochemicals have been suggested. They may inhibit microorganisms, interfere with some metabolic processes or may modulate gene expression and signal transduction pathways (Kris-Etherton *et al.*, 2002; Manson, 2003).

Phytochemicals may either be used as chemotherapeutic or chemo preventive agents with chemoprevention referring to the use of agents to inhibit, reverse, or retard tumorigenesis. In this sense chemo-preventive phytochemicals are applicable to cancer therapy, since molecular mechanisms may be common to both chemoprevention and cancer therapy (George, Chandran , & Abrahamse, 2021). Plant extracts and essential oils may exhibit different modes of action against bacterial strains, such as interference with the phospholipid’s bilayer of the cell membrane which has as a consequence a permeability increase and loss of cellular constituents, damage of the enzymes involved in the production of cellular energy and synthesis of structural components, and destruction

or inactivation of genetic material. In general, the mechanism of action is considered to be the disturbance of the cytoplasmic membrane, disrupting the proton motive force, electron flow, active transport, and coagulation of cell contents (Rao V. , 2012). Some specific modes of actions are antimicrobial activity, antioxidants, anti-carcinogenesis, anti-ulcer, anti-diabetic, anti-inflammatory (Doughari, 2012).

2.5 General method of extraction of phytochemicals

Extraction is the separation of medicinally active portions of plant using selective solvents through standard procedures (Handa *et al.*, 2008). There are different methods of extraction, the purpose of which is to separate the soluble plant metabolites, leaving behind the insoluble cellular residues. The crude extract obtained through these methods contains complex mixture of many plant metabolites, such as alkaloids, glycosides, phenolics, terpenoids and flavonoids. Some of the crude extracts obtained may be ready for use as medicinal agents in the form of tinctures and as fluid extracts where as some need further processing. Several of the commonly used extraction methods are maceration, infusion, decoction, soxhlet extraction, supercritical fluid extraction etc. (Azwanida, 2015).

2.5.1 Soxhlet extraction or hot continuous extraction

In this method, finely ground sample is placed in a porous bag or "thimble" made from a strong filter paper or cellulose, which is place, is in thimble chamber of the Soxhlet apparatus. Extraction solvents is heated in the bottom flask, vaporizes into the sample thimble, condenses in the condenser and drip back. When the liquid content reaches the siphon arm, the liquid contents emptied into the bottom flask again and the process is continued.

The advantage of this method is it requires a smaller quantity of solvent compared to maceration. Similarly, large amounts of drug can be extracted with a much smaller quantity of solvent. This effect tremendous economy in terms of time, energy and consequently financial inputs. At small scale, it is employed as a batch process only, but it becomes much more economical and viable when converted into a continuous extraction procedure on medium or large scale (Al-Naqqash, 2018).

Hot solvent systems under reflux state are more efficient for the recovery of antioxidant components, thus offering higher extract yields (Sultana *et al.*, 2009). This is in agreement with the findings that methanol and hot water are more efficient to extract antioxidant compounds from *Phellinus baumii* (Shon et al., 2004).

2.5.2 Maceration

Maceration has become a popular and inexpensive way to extract essential oils and bioactive compounds, and it generally consists of several steps. First, the plant materials are ground into small particles to increase the surface area for proper mixing with the solvent. Next, an appropriate solvent, called menstruum is added in a closed vessel. The liquid is then strained off, and the solid residue is pressed to recover as much occluded solution as possible. The strained and pressed liquids are mixed and separated from impurities by filtration. In order to facilitate extraction, occasional shaking can be used, increasing the diffusion and the removal of concentrated solution from the sample surface, while carrying fresh menstruum inward for higher extraction yields (Carmo *et al.*, 2017).

2.5.3 Supercritical fluid extraction (SFE)

Supercritical fluid (SF) or also called as dense-gas is a substance that shares the physical properties of both gas and liquid at its critical point. Factors such as temperature and pressure are the determinants that push a substance into its critical region. SF behaves more like a gas but have the solvating characteristic of a liquid. An example of SF is CO2 that become SF at above 31.1°C and 7380 kPa. A major drawback of this method is the initial cost of the equipment is very high (Azwanida, 2015).

2.5.4 Ultrasound assisted extraction (UAE)

UAE is a modern extraction technique used sometimes for cyclitols extraction, which possesses strategical advantages (reduction of solvent amount, lower energy consumption and time saving) compared to classical methods. The operating principle is the involvement of ultrasounds (at frequencies between 20-100 MHz) to develop bubbles by cavitation phenomenon inside the liquid solvent. These bubbles will induce plant wall cell disruption and will speed-up the solvent penetration into the plant material (Ratiu *et al.*, 2018).

2.6 Different solvents used in extraction

Successful determination of biologically active compounds from plant material is largely dependent on the type of solvent used in the extraction procedure. Properties of a good solvent in plant extractions includes, low toxicity, ease of evaporation at low heat, promotion of rapid physiologic absorption of the extract, preservative action, inability to cause the extract to complex or dissociate. The factors affecting the choice of solvent are

quantity of phytochemicals to be extracted, rate of extraction, diversity of different compounds extracted, diversity of inhibitory compounds extracted, ease of subsequent handling of the extracts, toxicity of the solvent in the bioassay process, potential health hazard of the extractants. The choice of solvent is influenced by what is intended with the extract. Since the end product will contain traces of residual solvent, the solvent should be nontoxic and should not interfere with the bioassay. The choice will also depend on the targeted compounds to be extracted (Tiwari et al., 2011). The Table 2.6 shows different solvents uses for active component extraction (Tiwari *et al*., 2011).

Table 2.4 Solvents used for active component extraction

Water	**Ethanol**	**Methanol**	**Chloroform**	**Ether**	**Acetone**
Anthocyanins	Tannins	Anthocyanins	Terpenoids	Alkaloids	Phenol
Starches	Polyphenols	Terpenoids	Flavonoids	Terpenoid	Flavonol
Tannins	Polyacetylenes	Saponins		Coumarins	
Saponins	Flavonol	Tannins		Fatty acids	
Terpenoids	Terpenoids	Xanthoxyllines			
Polypeptide	Sterols	Totarol			
Lectins	Alkaloids	Quassinoids			
		Lactones			
		Flavones			
		Phenones			
		Polyphenols			

2.7 Drying

2.7.1 Introduction

Drying is one of the oldest methods of food preservation, resulting in shelf-stable products that are of utmost relevance in this era of convenience. The preservation effect is achieved by reducing the water content to a level that reduces the incidence of microbial growth and retards deteriorative chemical reactions such as enzymatic and non-enzymatic browning and rancidity due to lipid oxidation. Nevertheless, owing to the complex nature of foods, the effect of drying is not just limited to reduction in water content. The application of heat during drying causes structural modifications of macromolecular components in foods (carbohydrates, proteins and lipids), by which the final product acquires significant functional characteristics. Also, moisture diffusion from within the product is accompanied

by various physical changes, including shrinkage, puffing, crystallization and glass transitions (Anandharamakrishnan, 2017). Scientists usually employed a dried powder of plants to extract bioactive compounds and eliminate the interference of water at the same time (Stankovic, 2020).

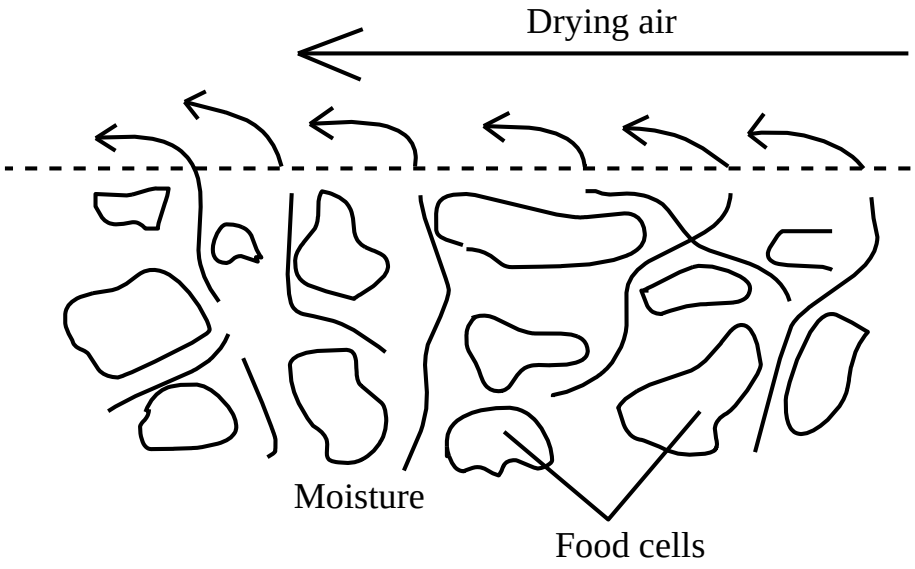
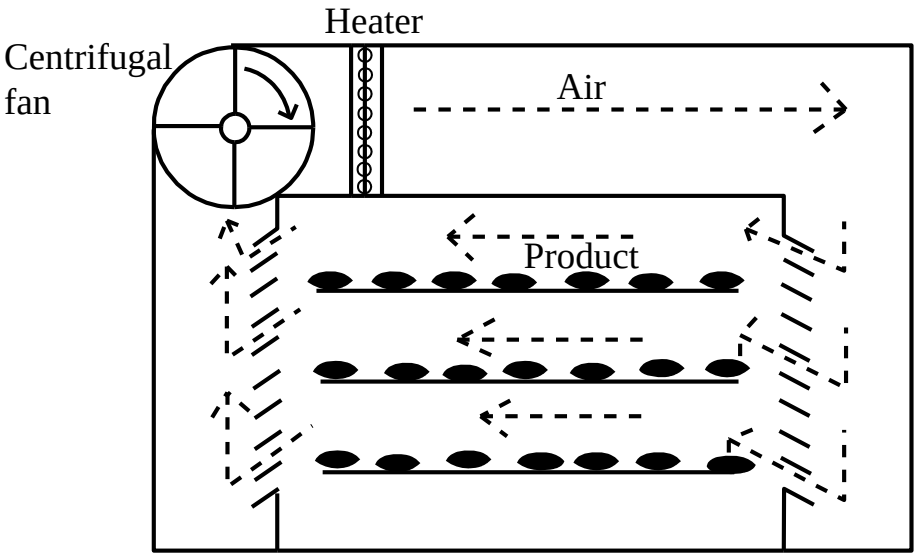


Fig. 2.1 Torturous pathway for removal of moisture during drying (Geankoplis, 2003).

2.7.2 Cabinet drying

Cabinet drier consist of an insulated cabinet fitted with shallow mesh or perforated trays, each of which carries a thin layer of food. Hot air is circulated through the cabinet tray. A system of duct and baffles is used to direct air, over and/ or through each tray, to promote uniform air distribution either horizontally between the trays of food materials or vertically through the trays and food (Fellows, 2000). Air heaters may be direct gas burners, steam coil exchangers or electrical resistance heaters. The air is blown past the heaters and thus heated air is used for dying. It is relatively cheap to build and maintain, flexible in design, and produces variable product quality due to relatively poor control. It is used singly or in groups, mainly for small- scale production (1-20 ton/day) of dried fruits and vegetables (Fellows, 2000).



Source: (Geankoplis, 2003)

Fig. 2.2 Cabinet dryer

2.8 Evaporation

2.8.1 Introduction

Evaporation is the separation technology to separate a solvent – in most of the cases water – from a solution, emulsion or suspension by means of boiling, while the dissolved or suspended substance has no or only a slight steam pressure. It is the objective of this separation technology to recover the solvent as pure as possible, for example, in the production of drinking water from sea water. In other cases, the concentration of the dissolved, emulsified or suspended substance is given priority. For example, for the production of condensed milk, it would be desirable to achieve a higher dry substance content in the product. Another objective of evaporation is the oversaturation of the solution until precipitation of crystals. For the production of kitchen salt, for example, sodium chloride solution is evaporated in order to product salt crystals. Frequently both objectives, that is, concentration of the solution and production of a possibly pure solvent are expected from an evaporation plant (Klinke, 2014).

2.8.2 Rotary evaporation

The rotary evaporator is an essential piece of equipment in most organic laboratories. Commonly vacuum is provided by a water aspirator (or by other mild vacuum sources), and the flask is rotated by an electric motor. This provides a thin film of solution for

evaporation, hopefully preventing the solution from bumping. The flask rotates in a temperature-controlled water bath, which provides the heat of vaporization. Little heat may be needed to evaporate highly volatile solvents like ether, whereas heat is definitely required for solvents like toluene (Pirrung, 2007).

It is essential that a glassware bulb be inserted between the flask and the evaporator to make provision for bumping of the solution being evaporated. If a bump does occur, the solution is trapped in the bulb, enabling it to be transferred back into the flask. Without the bulb, the solution would be drawn up into the evaporator itself, where it could mix with condensed solvents, be contaminated by other compounds that have been earlier evaporated in the equipment, or be lost altogether. This bulb naturally must be rinsed with solvent after evaporation of each sample. The flasks used on a rotary evaporator may be of many sizes and types, but typically round-bottom or pear-shaped flasks are used (Pirrung, 2007).

Flat-bottom flasks are not recommended because they are less robust to vacuum. The evaporator generally has a condenser or trap (circulating cold water or dry ice) that can condense evaporated solvents to prevent their being drawn into the waste water or vacuum source and/or to potentially permit solvent recovery and recycling (Pirrung, 2007).

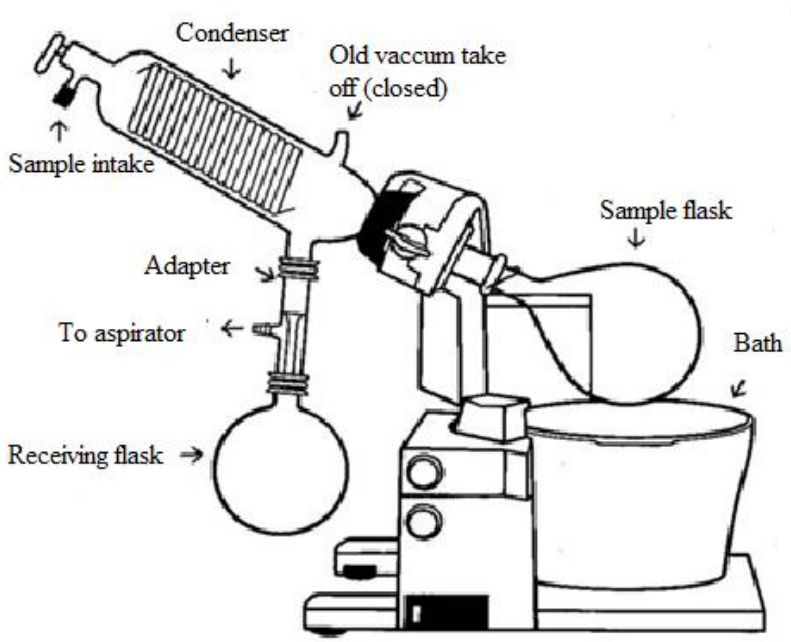


Fig. 2.3 Rotatory evaporator

2.8.2.1 Uses of rotary vacuum evaporator

- Solvent recycling
- Solvent distillation
- Powder drying
- Chemical synthesis
- Extraction
- Research
- Active agent concentration
- Vacuum distillation of sensitive substances
- Liquid mixture separation (LabTech, 2018).

2.9 Antioxidants

An antioxidant can be defined as: “any substance that, when present in low concentrations compared to that of an oxidizable substrate, significantly delays or inhibits the oxidation of that substrate” (Halliwell and Gutteridge, 1995) but later the word “oxidation” was altered to “oxidative damage” that suggests an in vivo biological process: “any substance that delays, prevents or removes oxidative damage to a target molecule” (Halliwell, 2007). Most recently, Apak et al. (2016a) gave a more specific definition: “natural or synthetic substances that may prevent or delay oxidative cell damage caused by physiological oxidants having distinctly positive reduction potentials, covering reactive oxygen species (ROS)/reactive nitrogen species (RNS) and free radicals (i.e. unstable molecules or ions having unpaired electrons).”These definitions demonstrate the roles of antioxidants at cellular levels in humans as they are related to oxidative stress and free radicals and further to potential health effects in human.

Free radical production occurs continuously in all cells as part of normal cellular function. However, excess free radical production originating from endogenous or exogenous sources might play a role in many diseases. Antioxidants prevent free radical induced tissue damage by preventing the formation of radicals, scavenging them, or by promoting their decomposition (Lobo, Patil, Phatak, & Chandra, 2010). Occurrence of chronic diseases were recognized to be associated with the oxidative stress, where the reactive oxygen species (ROS) and reactive nitrogen species (RNS) including free radicals were continuously produced in human cells and led to oxidative damage to cell components (Bagchi and Puri, 1998; Valavanidis *et al.*, 2013). In concern by that, there is an increasing

interest in antioxidants; chemical compounds that possess the ability to neutralize these free radicals in the body by reducing or scavenging its activities (Lobo, Patil, Phatak, & Chandra, 2010). The antioxidant properties are the basis of preventive function towards many chronic diseases, including neurodegenerative diseases: stroke, Alzheimer's disease and Parkinson's disease, cardiovascular diseases: atherosclerosis and hypertension, diabetes, cancer and osteoporosis etc.(Kaczora *et al.*, 2016).

Two principal mechanisms of action have been proposed for antioxidants. The first is a chain- breaking mechanism by which the primary antioxidant donates an electron to the free radical present in the systems. The second mechanism involves removal of ROS/reactive nitrogen species initiators (secondary antioxidants) by quenching chain-initiating catalyst. Antioxidants may exert their effect on biological systems by different mechanisms including electron donation, metal ion chelation, co-antioxidants, or by gene expression regulation (Lobo *et al.*, 2010).

Some of the most widely used in vitro antioxidant methods as described by Mermlestein (2008) are Oxygen Radical Absorbance Capacity method (ORAC), Hydroxyl Radical Antioxidant Capacity (HORAC) assay, Trolox Equivalent Antioxidant Capacity (TEAC) method, Ferric Reducing/Antioxidant Power (FRAP), metho Peroxyl Radical Scavenging Capacity (PSC) method, Total Oxyradical Scavenging Capacity (TOSC) method, The DPPH method, Total Radical-Trapping Antioxidant Parameter (TRAP) method.

2.9.1 Total antioxidant capacity

The measurement of different antioxidant molecules separately is not practical and their antioxidant effects are additive, the total antioxidant capacity of a sample is measured, and this is called total antioxidant capacity (TAC), total antioxidant activity (TAA), total antioxidant power (TAOP), total antioxidant status (TAS), total antioxidant response, or other synonyms (Erel, 2004).

One major advantage of TAC assays is that, by definition, estimate the antioxidant components of a sample in a global way. Measuring each antioxidant component individually is labor-intensive and time-consuming, requiring complex and costly techniques (Erel, 2004). Other advantages of using TAC assays include simplicity of the techniques, low cost per sample, speed of reactions and possibility to be performed using automated, semi-automated, or manual methods (Bhattarai , 2019).

2.9.2 DPPH radical scavenging assay

DPPH assay measures the ability of a substance to scavenge the DPPH radical, reducing it to hydrazine. When a substance that acts as a donor of hydrogen atoms is added to a solution of DPPH, hydrazine is obtained, with a change in color from violet to pale yellow (Flieger & Flieger, 2020). The DPPH• test is based on the ability of the stable 2, 2-diphenyl-1-picrylhydrazyl free radical to react with hydrogen donors. The $DPPH^{\bullet}$ radical displays an intense UV-VIS absorption spectrum. In this test, a solution of radical is decolorized after reduction with an antioxidant (AH) or a radical ($R^{\bullet}$) in accordance with the following scheme:

$$DPPH^{\bullet} + AH \rightarrow DPPH^{\bullet} - H + A^{\bullet},$$

$$DPPH^{\bullet} + R^{\bullet} \rightarrow DPPH^{\bullet} - R$$

The $DPPH^{\bullet}$ radical is one of the few stable organic nitrogen radicals, which bears a deep purple color. It is commercially available and does not have to be generated before assay. This assay is based on the measurement of the reducing ability of antioxidants toward $DPPH^{\bullet}$. The DPPH assay is considered to be mainly based on an electron transfer (ET) reaction, and hydrogen-atom abstraction is a marginal reaction pathway. The test is simple and rapid and needs only a UV-vis spectrophotometer to perform, which probably explains its widespread use in antioxidant screening (Prior *et al.*, 2005; Sochor *et al.*, 2010).

2.9.3 Reducing power

The reducing power assay is a relatively simple, quick, and inexpensive direct method of measuring the combined (“total”) antioxidant activity of reductive (electron donating) antioxidants in a test sample (Benzie & Strain 1996a, 1999). The assay uses the reduction of ferric ions (Fe^{3+}) to ferrous ions (Fe^{2+}) as the signal, or indicator, reaction, and this is tied to a color change (Benzie and Devaki, 2018).

In this assay, a key oxidant (in the form of a ferric salt in aqueous solution) is reduced by the electron donating (reductive) antioxidants in the reaction mixture that have a redox potential, under the reaction conditions employed, lower than that of the half reaction:

The flavonoids and phenolic acids are present in the medicinal plant exhibit strong antioxidant activity which is depending on their potential to form the complex with metal atoms, particularly iron and copper. This method is based on the principle of increase in the absorbance of the reaction mixtures, the absorbance increases the antioxidant activity increases. The antioxidant compound present in the samples forms a colored complex with

potassium ferricyanide, trichloroacetic acid and ferric chloride, which is measured at 700 nm by UV-Spectrophotometer (Vijayalakshmi and Ruckmani, 2016).

Part III

Materials and methods

3.1 Raw materials

The plant under the study was *Neem leaves* (*Neem barbadensis).*

3.2 Identification of the plant

The plant specimen was taxonomically identified as *Neem leaves* from the Department of Biology of Central Campus of Technology.

3.3 Equipment and chemicals

The equipment and chemicals used during the study were made available in the campus. The list of chemical and equipment used for the analysis is shown in Appendix A.1 and Appendix A.2 respectively.

3.4. Collection and preparation of sample

The plant specimen under study i.e., *Neem leaves*, leaves were collected in October 2021 from Dharan (Pindeswori Campus), Sunsari District of Nepal. The basic flow diagram of methodology is made by modifications from the methodologies described by Jaradat *et al.* (2015) and is shown in Fig 3.1.

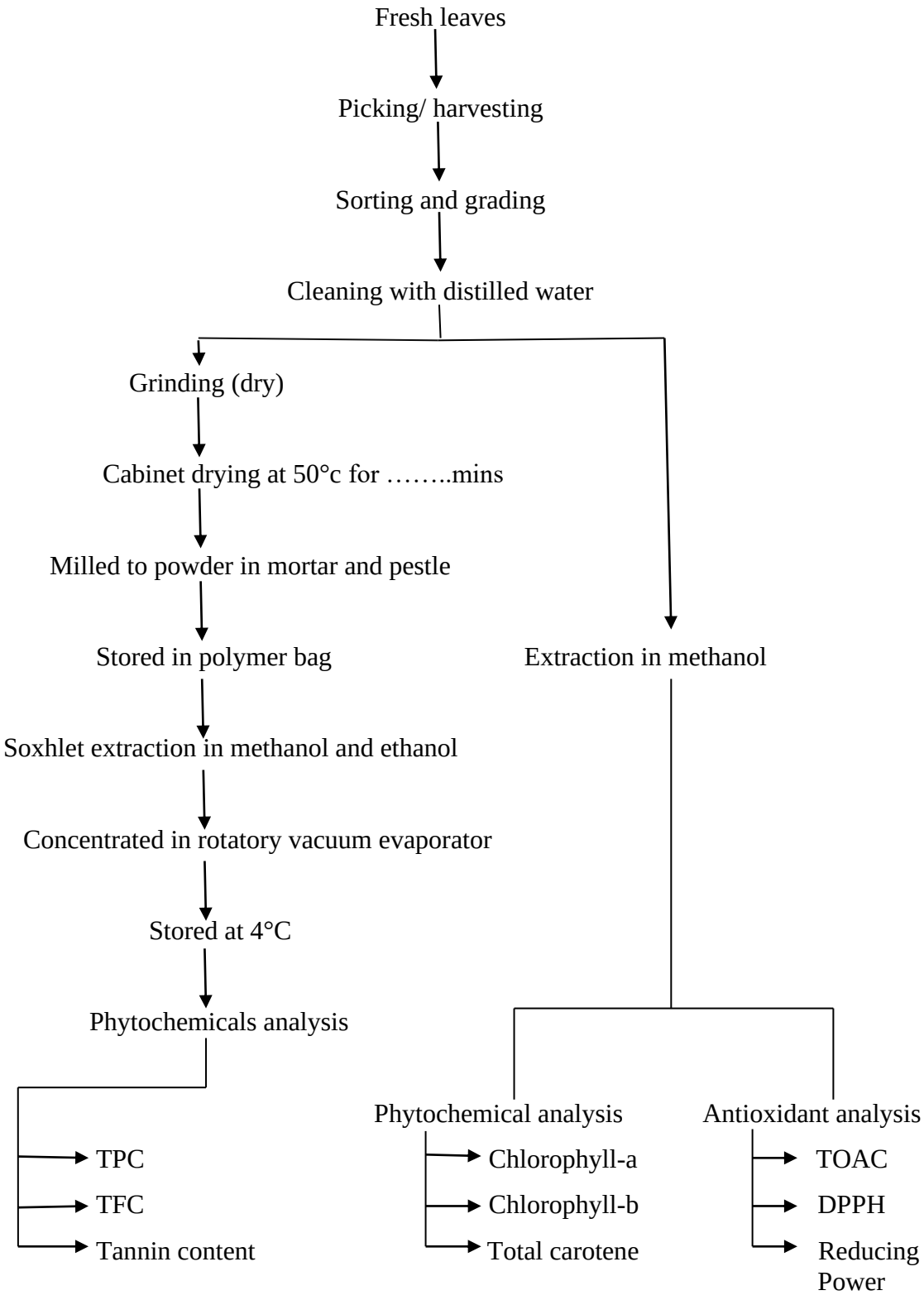


Fig. 3.1 Flow diagram of methodology

3.5 Drying

The fresh leaves of *Neem leaves* were well washed with distilled water and then dried at 40ºC-50ºC in cabinet dryer until all plant parts become well dried. After drying, the plant materials were then powdered by using grinder and placed into a well closed container.

3.5.1 Water and dry matter contents

The water content is determined following the method of Benzidia *et al.* (2018).

Calculation,

$$\text{Water content (\%)} = [(W_1 - W_2)/ W_1] \times 100$$

W_1 stands for = Weight of the sample before drying; whereas W_2 = stands for Weight of the sample after drying. Thus, dry matter content was extracted from water content as shown in the formula below:

$$\text{Dry matter content (\%)} = 100 - \text{Water content (\%)}$$

3.6 Preliminary qualitative phytochemical screening of plant specimen (Jaradat *et al.*, 2015)

3.6.1 Preparation of aqueous extract

The aqueous extraction is done by taking 5 grams of the plant powder and mixed with 200 ml of distilled water in a beaker. The mixture is heated on a hot plate at 30ºC-40ºC and mixed with continuous stirring for 20 minutes. The mixture is filtered using Whatmann filter paper and the filtrate is used for further preliminary phytochemical analysis.

3.6.2 Qualitative analysis for phytochemicals

The plant methanolic extracts were screened for the presence of the phytochemical classes by using the standard following methods (Jaradat *et al.*, 2015).

a. **Tests for proteins**

- Ninhydrin test: Boil 2 ml of 0.2% Ninhydrin solution with the entire plant crude extract, appeared violet color indicate the presence of proteins and amino acids.

b. **Tests for carbohydrates**

- Fehling's solutions test: Boil a mixture of Fehling solutions A and B with equal volumes were added to crude plant extract. A red color precipitate indicated the presence of reducing sugars.
- Benedict's reagent test: Boil 2 ml of Benedict's reagent with a crude extract, a reddish-brown color indicated the presence of the carbohydrates.

- Molisch's solution test: Shake 2 ml of Molisch's solution with crude plant extract then add 2 ml of H_2SO_4 concentrated and poured carefully along the side of the test tube a violet ring appeared at the inter phase of the test tube indicated the presence of carbohydrate.
- Iodine test: 2 ml of iodine solution mixed with crude plant extract. Purple or dark blue colors prove the presence of the carbohydrate.

c. **Test for phenols and tannins**:

- Two milli-liter of 2% solution of $FeCl_3$ mixed with crude extract. Black or blue-green color indicated the presence of tannins and phenols.

d. **Tests for flavonoids**

- Alkaline reagent test: 2 ml of 2% NaOH solution was mixed with plant crude extract, intensive yellow color was formed, which turned into colorless when added 2 drops of diluted acid to solution, this result indicated the presence of flavonoids.

e. **Test for saponins**

- Five milliliter of distilled water was added to crude plant extract in a test tube and it was shaken vigorously. The foam formation indicated the presence of saponins.

f. **Tests for glycosides**

- Liebermann's test: 2 ml of acetic acid and 2 ml of chloroform mixed with entire plant crude extract. The mixture was then cooled and added H_2SO_4 concentrated, green color indicated the entity of aglycone steroidal part of glycosides.
- Salkowski's test: H_2SO_4 concentrated (about 2 ml) was added to the entire plant crude extract. A reddish-brown color produced indicated the entity of steroidal aglycone part of the glycoside.
- Keller-kilani test A mixture of Acetic acid glacial (2 ml) with 2 drops of 2% $FeCl_3$ solution was added to the plant extract and H_2SO_4 concentrated. A brown ring produced between the layers which indicated the entity of cardiac steroidal glycosides.

g. **Test for steroid**

- Two milliliter of chloroform and concentrated H_2SO_4 were mixed with the entire plant crude extract. In the lower chloroform layer produced red color that indicated the presence of steroids. Another test was performed by mixing 2 ml of each of acetic acid with H_2SO_4 concentrated and crude extract with 2 ml of chloroform. Green color indicated the entity of steroids.

h. **Test for terpenoids**

- Two milliliter of chloroform was mixed with the plant extract and evaporated on the water path then boiled with 2 ml of H_2SO_4 concentrated. A grey color produced indicated the entity of terpenoids.

3.7 Phytochemicals quantitative analysis

The plant aqueous is screened for the presence of the phytochemical classes by using the standard following methods as per (Sharma, et al., 2020)

3.7.1 Preparation of plant extract

The phytochemical extraction was performed using organic solvent extraction. The organic extraction was performed by Soxhlet extraction method. This extraction was done by taking 20 gm of dried plant powder and was placed into a glass thimble then extracted with 250 ml of different solvents separately (methanol and ethanol). The extraction processes carry on till the solvent in siphon tube of Soxhlet apparatus become colorless. After that the extract was heated on rotatory vacuum evaporator at 35ºC until all the solvent evaporated. The dried plant crude extract was kept in refrigerator at 2-8 ºC for their future use (Jaradat *et al.*, 2015).

3.7.2 Total phenolic content of plant

Total phenolic content (TPC) in the plant methanolic and ethanolic extracts was determined using spectrophotometric method with some modifications. 1 mg/ml aqueous solutions for both methanolic and ethanolic extracts were prepared in the analysis (Jaradat *et al.*, 2015).

TPC was determined using the Folin–Ciocalteu method (Singleton *et al.*, 1999) with slight modifications. 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 spectrophotometer at wave length = 765 nm. The samples were prepared in triplicate for each analysis and the mean value of absorbance was obtained. The same procedure was repeated for the standard solution of gallic acid and the calibration line was construed. Based on the measured absorbance, the concentration of gallic acid equivalent expressed in terms of (mg of GAE/g of extract).

3.7.3 Total flavonoid content of plant

Flavonoid content in the plant methanolic and ethanolic extracts was determined using spectrophotometric method with some modifications. 1 mg/ml aqueous solutions for both methanolic and ethanolic extracts were prepared in the analysis (Jaradat *et al.*, 2015).

Total flavonoid content was determined using a modified aluminum chloride assay method as described by (Barek and Hasmadi, 2015). 2 ml of solution was pipette out in a test tube in which 0.2 ml of 5% Sodium Nitrate ($NaNO_3$) was mixed and stand for 5 minutes. 0.2 ml of 5% Aluminium Chloride ($AlCl_3$) was pipetted out, mixed in the tube and allowed to stand for 5 minutes. This followed addition of 2 ml of 1N Sodium Hydroxide (NaOH) in the tube and finally volume was made up to 5ml. The absorbance was measured after 15 minutes at 510nm against a reagent blank. The test result was correlated with standard curve of Quercetin (20, 40, 60, 80, 100µg/ml) and the total flavonoid content is expressed as mg QE/g of dry weight.

3.7.4 Tannin content of plant materials

Tannin content in the plant methanolic and ethanolic extracts was determined using spectrophotometric method with some modifications. (Hayat, et al., 2020) 1 mg/ml aqueous solutions for both methanolic and ethanolic extracts were prepared in the analysis (Jaradat *et al.*, 2015).

The tannins were determined by Folin-Ciocalteu method. About 0.1 ml of the sample extract was added to a volumetric flask (10 ml) containing 7.5 ml of distilled water and 0.5 ml of Folin-Ciocalteu phenol reagent, 1 ml of 35 % Na2CO3 solution and dilute to 10 ml with distilled water. The mixture was shaken well and kept at room temperature for 30 min. A set of reference standard solutions of gallic acid (20, 40, 60, 80 and 100 µg/ml) were prepared in the same manner as described earlier. Absorbance for test and standard solutions were measured against the blank at 725 nm with an UV/Visible spectrophotometer. The tannin content was expressed in terms of mg of GAE /g of extract (Marinova *et al.*, 2005; Miean and Mohamed, 2001; Singh *et al.*, 2012).

3.8 Antioxidant assays

3.8.1 Preparation of plant extracts for antioxidant evaluation

About 10 g of the grounded plant were soaked in 1 Liter of methanol (99%) and put in a shaker device at 100 rounds per minute for 72 hours at room temperature and stored in

refrigerator for 4 days. The extracts were then filtered using filter papers and concentrated under vacuum on a rotator evaporator. The crude extract was stored at 4 ºC and the antioxidant test was done directly within five minutes (Jaradat *et al.*, 2015).

3.8.2 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 (0.3 mL) and 3 mL reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate) were incubated at 95ºC for 90 min. After the mixture had cooled to room temperature, the absorbance of each solution was measured at 695 nm spectrophotometrically against a blank. The antioxidant capacity was expressed as ascorbic acid equivalents (AAE).

3.8.3 DPPH radical scavenging assay

The capacity of prepared extracts to scavenge the 'stable' free radical DPPH was monitored according to the method of (Hatano *et al.*, 1988) with slight modifications. Extracts (100µL) were dissolved in 3.9 mL freshly prepared methanolic solution of DPPH (1 mM, 0.5 mL). The mixture was vortexed for 15 seconds and then left to stand at room temperature for 30 min in the dark. The absorbance of the resulting solution was read spectrophotometrically (UV/VIS spectrometer) at 517 nm. The percentage inhibition of the radicals due to the antioxidant activity of leaf extracts was calculated using the following formula.

% inhibition = $(A_{control} - A_{sample})/A_{control} \times 100$

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

Lower absorbance of the reaction mixture indicates higher free radical scavenging activity (Biswas, Haldar, & Ghosh, 2010).

3.8.4 Reducing power assay

The reducing power of the prepared extracts was determined according to the method of (Oyaizu, 1986). Briefly, each extract (1mL) was mixed with 2.5 mL of a 0.2M phosphate buffer (pH 6.6) and 2.5 mL of a 1% (w/v) solution of potassium ferricyanide. The mixture was incubated in a water bath at 50ºC for 20 min and then 2.5 mL of 10% (w/v) trichloroacetic acid solution was added and the mixture was then centrifuged for 10 min at 3000rpm. A 2.5 mL aliquot of the upper layer was combined with 2.5 mL of distilled water

and 0.5 mL of 0.1% (w/v) ferric chloride solution. Absorbance of the reaction mixture was read using UV/VIS spectrometer (SP-3000) at 700 nm. Mean values from three independent samples were calculated for each extract. Here, ascorbic acid was used as a reference standard, the reducing power of the samples were comparable with the reference standard.

3.9 Statistical analysis

Analysis was carried out in triplicate. Statistical calculations were performed in Microsoft office Excel 2010. All the data obtained in this experiment were analyzed for significance by paired t- test by using Microsoft office Excel 2010. Significant difference of means was analyzed with paired t-test at 5% level of significance.

Part IV

Results and discussion

A common variety of *Neem* (*Neem leaves*) was collected from Sunsari district which was used to test the presence of phytochemicals. The *Neem leaves* were collected fresh in November 2021. Fresh *Neem leaves* were subjected to drying at 50ºC.

4.1 Preliminary phytochemical qualitative analysis of *Neem leaves*

The aqueous extract of *Neem leaves* was prepared to conduct preliminary phytochemical analysis. Table 4.1 shows the phytochemicals present in *Neem leaves*.

Table 4.1 Phytochemical screening of *Neem leaves* aqueous extract

S.N.	Test	Aqueous extract
i	Protein	+
ii	Carbohydrate	+
iii	Reducing sugar	+
iv	Starch	+
v	Phenols and Tannins	+
vi	Flavanoids	+
vii	Saponins	-
viii	Glycosides	+
ix	Steroids	+
x	Terpenoids	+

Where, Plus (+) = positive test;

Minus (-) = negative test

The phytochemicals screening of aqueous extract of *Neem leaves* showed that bioactive compounds such as flavonoids, steroids, terpenoids, proteins, phenols, carbohydrates, reducing sugar, starch, tannins, glycosides were detected to be present in the leaves of *Neem leaves* whereas saponin was negative as shown in Table 4.1. These findings are in total agreement with those existing in the literature (Dharajiya *et al.*, 2017; Kumar *et al.*, 2016).Other studies have shown that the presence of saponins depends on extraction solvents. They are positive with ethanol, methanol, ethyl acetate, petroleum ether, acetone and hexane extracts and negative with the aqueous extract (Truong & Nguyen, 2019).

Since this plant had been used in the treatment of different ailment such as malaria, dysentery, diarrhea, skin burn etc., the medicinal roles of these plants could be related to such identified bioactive compounds. The presence of these biologically active compounds in the extracts has made the plant to be known for its medicinal use especially for antimicrobial activity against pathogenic organisms (Wintola & Afolayan, 2015).

4.2 Quantitative analysis of phytochemicals in *Neem leaves*

The table 4.2 shows the phytochemical content of *Neem leaves*

Table 4.2 Phytochemical content of *Neem leaves* showing p-value

Assays	**Methanolic extract**	**Ethanolic extract**	**P-value**
Total phenol content (TPC)	28.09±0.30[a]	22.88±0.24 [b]	0.00*
Total flavonoid content (TFC)	98.70 ±6.4 [b]	67.1±2.86[c]	0.0005*
Tannin content	1.55±0.79[c]	1.4±0..14[c]	0.01*

Data expressed as mean ± SD; n =3; [a] mg GAE/g dw; [b] mg RE/g dw; [c] mg GAE/g dw;

* Indicates significant difference

4.2.1 Total phenolic content (TPC) of *Neem leaves*

TPC of methanolic extract of whole *Neem leaves* was found to be 28.09± 0.31 mg GAE/g of dry weight which is shown in Table 4.2. This finding is in agreement with the study done by Kumar *et al.* (2017a) where the values ranged from 32.9 to 65.7 mg GAE per g of dry weight. And this finding is concomitant with sample from Kerela whose TPC of methanolic extract of whole *Neem leaves* plant was 32.9 ± 0.19 mg GAE/ g of dry weight. Maximum values of TPC were obtained for Punjab, Jammu and Himachal accessions. Kerala, Telangana and West Bengal showed low TPC values as compared with other accessions in the study by Kumar *et al.* (2017a). Different agro-climatic conditions have effects on phytochemical diversity and antioxidant potential of *Neem leaves* plant (Sarkar , Singh, & Bhattacharya, 2021).

In this study, ethanolic extract of *Neem leaves* showed TPC of 22.88±0.24 mg GAE/g of dry weight. Whereas study done by Nejatzadeh-Barandozi (2013) shows that 95% ethanol leaf gel extracts (ELGE) gives TPC of 413 ± 9.88 mg GAE/100g dw of extract which is less, it may be due to the difference in the degree of solvent used for extraction of

polyphenols (Bista, Ghimire, & Subedi, 2020) (Felhi *et al.*, 2017; Kumar *et al.*, 2017a; Prabu *et al.*, 2018) and difference in the part of the plant used (Vidic *et al.*, 2014) where ethanolic extract of gel and peel were 2.06±0.25 mg GAE/g and 7.99±0.26 mg GAE/g respectively. The findings of *Neem leaves* is also concomitant to the study of *Ficus racemosa* where TPC of ethanolic extract was found to be 12.36 mg GAE/g (Sulaiman and Balachandran, 2012).

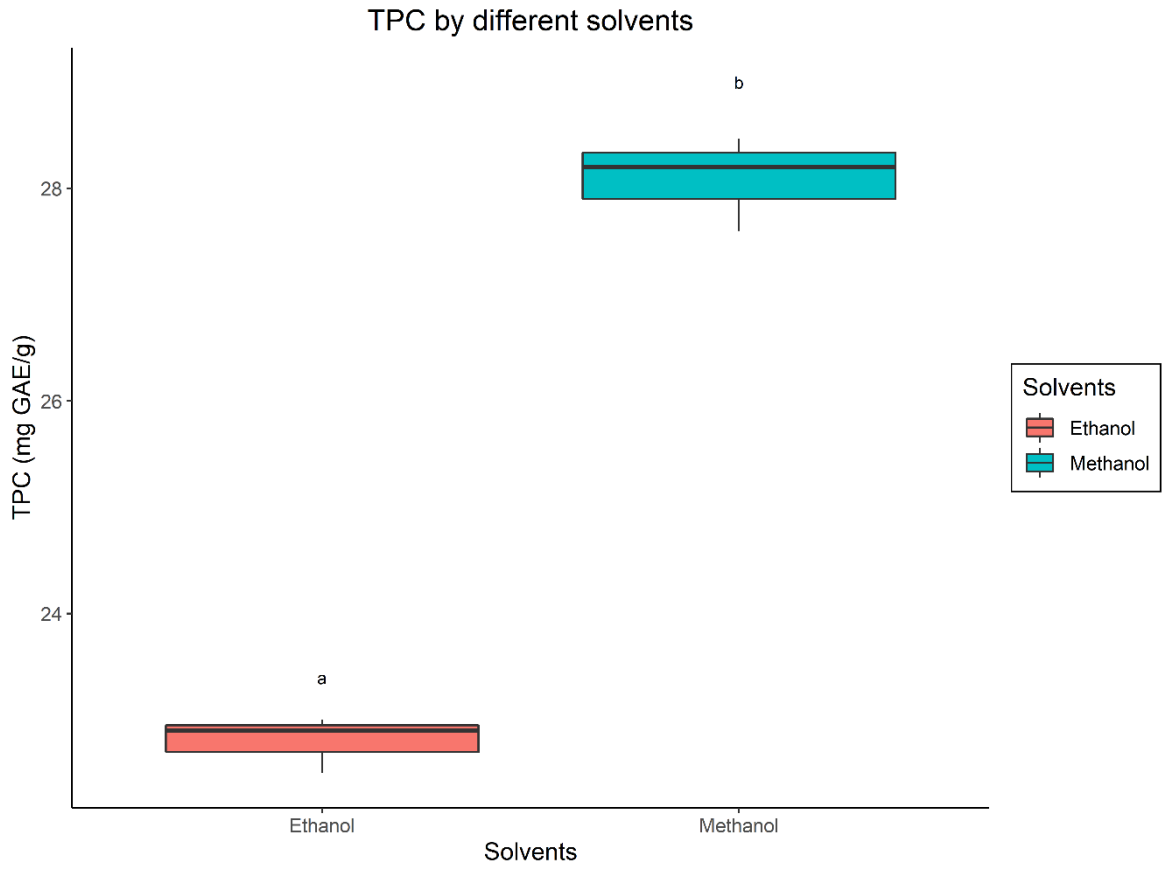


Fig 4.1: TPC by different processing methods

TPC of methanolic and ethanolic extract of the plant was significantly different from each other (p-value <0.05) as shown by Table 4.2. Methanolic extract yielded high TPC content than ethanolic extract. Similar result was found in the study by (Anwar *et al.*, 2013; Kumar *et al.*, 2017b; Sonam and Tiwari, 2016).

Plant polyphenols have been implicated in diverse functional roles, including plant resistance against microbial pathogens and animal herbivores such as insects (antibiotic and anti-feeding actions), protection against solar radiation, besides reproduction,

nutrition, and growth. Phenolic compounds have also been reported to prevent diseases resulting from oxidative stress (Rahman, et al., 2022) (Dimcheva and Karsheva, 2018; Lopez *et al.*, 2013).

4.2.2 Total flavonoid content (TFC) of *Neem leaves*

This study showed total flavonoid content (TFC) of methanolic extract of *Neem leaves* to be 98.70 ±6.4 mg QE/g of dry weight as shown in Fig. 4.3. The findings of this study is more than the study done by Taukoorah and Mahomoodally (2016) where total flavonoid content of methanolic extract of crude gel was found to be 60.95 ± 0.97µg RE/mg of crude extract. The difference is probably due to the difference in the part of the plant used (Asuk *et al.*, 2015) and also due to difference in the chemical used to prepare standard curve (Dimcheva and Karsheva, 2018). The flavonoid concentration of methanolic extract of *P. capillacea* was 91.58 ± 3.74QE/mg which is near to the flavonoid content of *Neem leaves* (Formagio *et a*l., 2014). The findings of *Neem leaves* is also concomitant to the study of leaves of *Zapoteca portoricensis* where TFC of methanolic extract was found to 63.67 ± 0.20 mg QE/g (Agbo *et al.*, 2015).

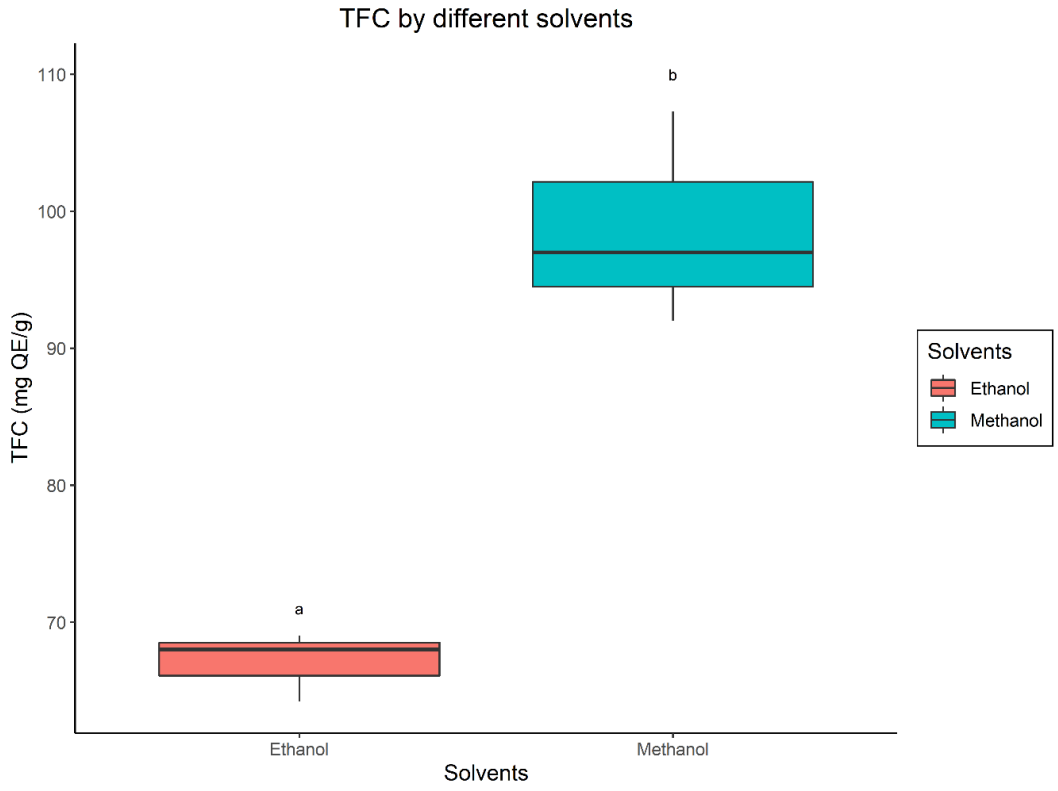


Fig 4.2: Total flavonoids by different processing methods

This study showed total flavonoids of ethanolic extract of *Neem leaves* to be 67.1 ±2.86 mg QE/g dry weight of extract as shown in Fig. 4.3. This result is higher than the study done by Botes *et al.* (2008) which showed that total flavonoids (mg of CE/100g ± SD) of 95% Aqueous Ethanol Leaf Gel Extracts (ELGE) was 20.2 ± 0.50. Other study showed flavonoid content of ethanol extract of *Neem barbadensis* flower was 13.20 ± 0.09 mg CE/g of dry mass (Bista, Ghimire, & Subedi, 2020) (Debnath *et al.*, 2017).The difference is probably due to difference in the part of plant used for analysis and also standard curves were prepared from two different chemicals and maximum value was obtained at quercetin equivalent than catechin and rutin equivalents. This decrease is due to the unstable nature of rutin and catechins. The flavonoid content of *Cistus incanus* expressed as quercetin, rutin and catechins equivalent were respectively 40.80 ± 4.52mg/g, 6.00 ± 0.75mg/g and 20.40 ± 2.26mg/g (Dimcheva and Karsheva, 2018).

TFC of methanolic and ethanolic extract of the plant was significantly different from each (p-value <0.05) other as shown by Table 4.2 above. Methanolic extract yielded high TFC content than ethanolic extract. Similar result was found in the study by (Anwar *et al.*, 2013; Asuk *et al.*, 2015; Kumar *et al.*, 2017b; Sonam and Tiwari, 2016).

Some flavonoids are antioxidants and has been proved to exhibit a wide range of biological activities like anti-microbial, anti-inflammatory, anti-angionic, analgesic, anti-allergic, cytostatic and antioxidant properties (Ullah, et al., 2020).

4.2.3 Tannin content of Neem leaves

Methanolic extract of *Neem leaves* showed total tannin content of 1.55± 0.79 mg GAE/g of dry weight of extract. This finding is concomitant with the study of Bael (*Aeglemarmelos)* powder from cold treated pulp where tannin content was 2.90±1.13 mg GAE/ g dw Aryal *et al.* (2018).

Ethanolic extract of *Neem leaves* showed total tannin content of 0.844±0.04 mg GAE/g of dry weight. A study of Wintola and Afolayan (2011) showed tannin content of ethanolic extract of *Neem ferox* is 0.026 ± 0.01 mg CE/g of dry plant materials which is less than the value of *Neem leaves*. The tannin content of *Neem leaves* is concomitant to the study by (Mohammed and Manan, 2015) where tannin content of *Moringa olifera* seed extract was 0.890 ± 0.020 mg GAE/g of dry weight.

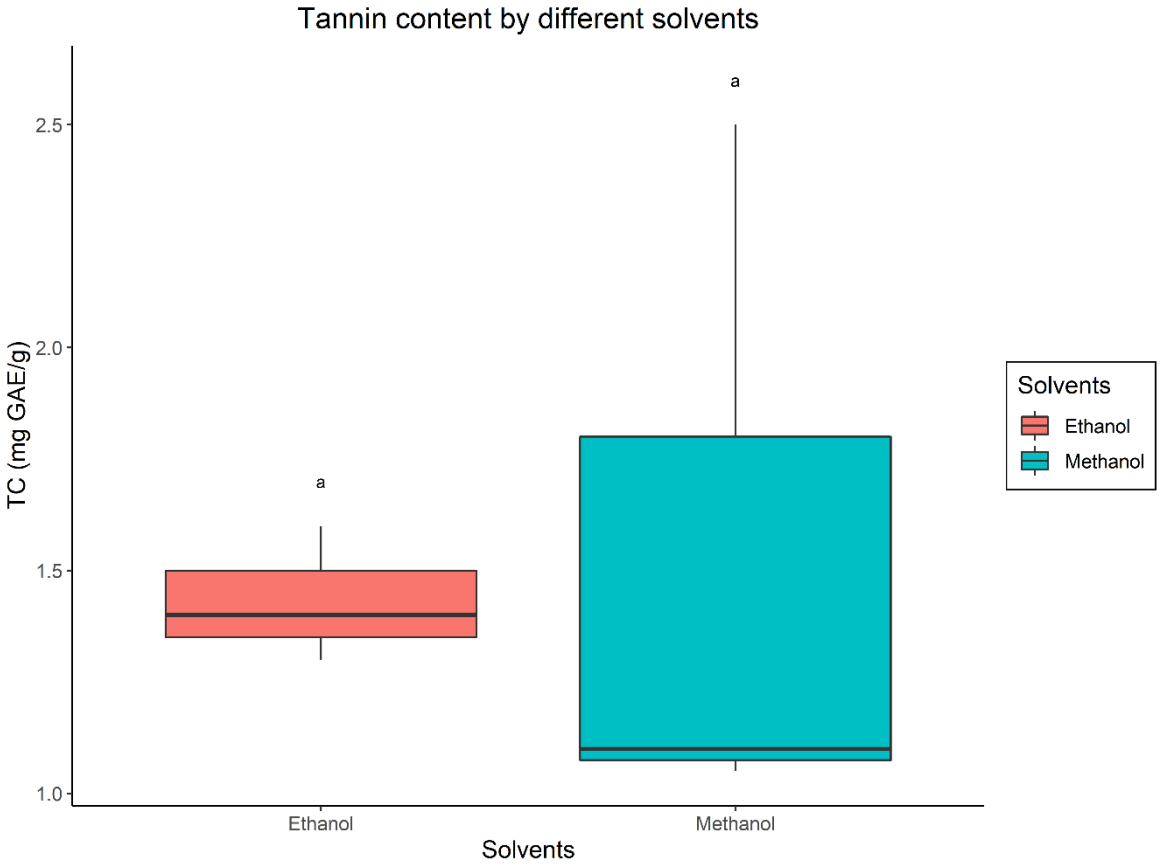


Fig 4.3: Tannin by different processing methods

Tannin content of methanolic and ethanolic extract of the plant was significantly different from each other (p-value<0.05) as shown by Table 4.2 above. Methanolic extract yielded higher tannin content than ethanolic extract. Similar result was found in the study by (Anwar *et al.*, 2013; Kumar *et al.*, 2017b; Sonam and Tiwari, 2016).

Tannin has been reported to interfere with bacterial cell protein synthesis and is important in the treatment of ulcerated or inflamed tissues and also in the treatment of intestinal disorders (Sieniawska, 2015). Thus, these observations therefore support the use of *Neem leaves* in herbal cure remedies.

4.2.4 Total antioxidant capacity (TOAC), DPPH radical scavenging activity and Reducing power of *Neem leaves*

In the study, **TOAC** of 99% methanolic extract of *Neem leaves* was found to be 91.49±0.24%. This finding is concomitant to the study done in Ethiopia in green tea where

TOAC was 80.0 ± 0.63% (Tadesse, Hymete, Bekhit, & Mohammed, 2015). The antioxidative potential of plant extracts can be measured using various in vitro assays and each assay is based on at least one feature of antioxidant activity. However, total antioxidant properties of plants cannot be evaluated by any single method because of their complex nature of phytochemicals. Therefore, two or more methods should always be employed in order to evaluate the total antioxidative effects of plant extracts (Gunathilake, 2016). Antioxidants prevent free radical induced tissue damage by preventing the formation of radicals, scavenging them, or by promoting their decomposition (Lobo, Patil, Phatak, & Chandra, 2010).

The Table 4.3 shows antioxidant potential of methanolic *Neem leaves* extract.

Table 4.3 Antioxidant potential of methanolic *Neem leaves* extract

Parameter	**Antioxidant potential**
Total antioxidant capacity (TOAC) (%)	91.49±0.24
DPPH radical scavenging assay (%)	71.91±0.04
Reducing power (%)	51.08 ±0.85

Data expressed as mean ± SD

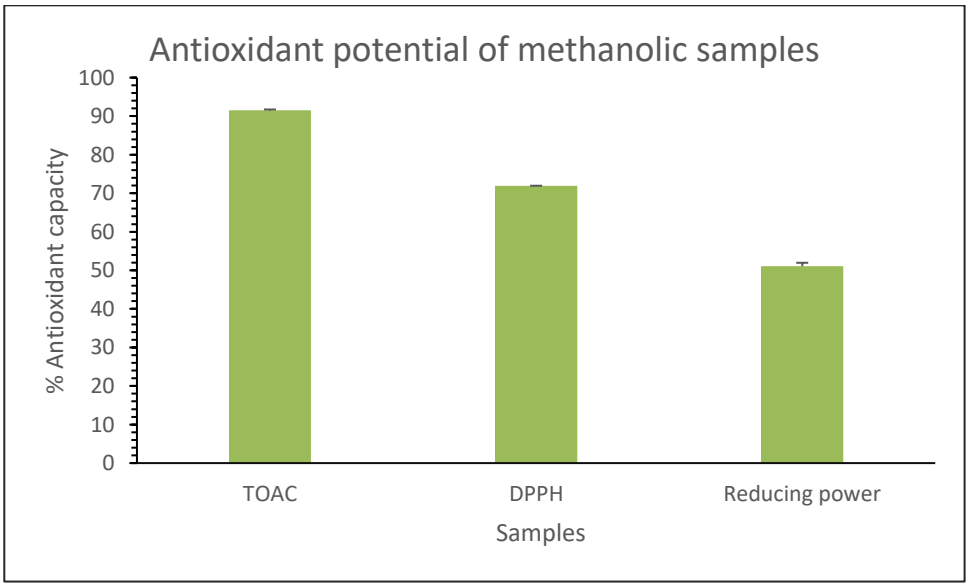


Fig 4.4: Antioxidant potential of methanolic samples

The **DPPH radical scavenging activity** of 99% methanolic extract was found to be 71.91±0.04% which is similar to the study done by Sultana *et al.* (2009) where 80% methanolic and ethanolic extract shows DPPH scavenging activity was 80.1±2.3% and

70.7±1.2% respectively. It has been documented that growth periods of *A. vera* is critical in the regulation of the DPPH radical scavenging activity of gel (Ray, Gupta, & Ghosh, 2013) .

Environmental temperature plays a significant role on antioxidant activity evaluation and it is more pronounced in cold weather (Kumar, Yadav, Yadav, & Yadav, 2017). Thus, the higher value of DPPH indicates that majority of free radicals produced can be scavenged by *Neem leaves* methanolic extract which protects the cells from being oxidized and thus help prevent many degenerative diseases (Baliyan, et al., 2022).

The **reducing power** of 99% methanolic extract of *Neem leaves* was found to be 51.08 ±0.85%. One study by Akhtar *et al.* (2015) showed that reducing power of *Berberis lycum Royale* was 80±2% and is nearly equal to the values from our study. The various biological and environmental factors in which the plant grew also contribute to the plant antioxidant power (Xie , et al., 2019).

The reduction of Fe^{3+} has been described as an indicator of electron donating activity which can demonstrate the antioxidant potential of different phenolic compound of Phyto-origin (Bajpai, Agrawal, Bang, & Park, 2015). The reducing power is generally associated with the presence of reductones (Kolli, Amperayani, & Parimi, 2015), which has been shown to exhibit antioxidant potential by splitting the free radical chains by donating hydrogen atoms. Reductones can prevent the peroxide formation by reacting with the precursors of peroxides.

Part V

Conclusions and Recommendations

5.1 Conclusion

The present study can conclude following things.

- Drying of fresh *Neem leaves* showed water and dry matter content to be 97.53% and 2.47% respectively.
- Total phenols, flavonoids, tannins, steroids, terpenoids, protein, carbohydrates, glycosides were detected in the leaves of *Neem leaves* whereas saponin was not detected in aqueous extract.
- Total phenol content (TPC), total flavonoid content (TFC) and tannin content of methanolic and ethanolic extract of *Neem leaves* were respectively 28.09± 0.31 and 22.88±0.24, 98.76±6.4 and 67.1 ±2.86, 1.55±0.79and 1.4±0.14 as demonstrated in fig 4.1, 4.2 and 4.3 respectively.
- The methanol extract yielded better phytochemical content significantly (p-value<0.05) than ethanol extract.
- Total antioxidant capacity (TOAC), DPPH radical scavenging and reducing power of fresh *Neem leaves* in 99% methanolic solution at 1mg/1ml concentration were found to 91.49±0.24%, 71.91±0.04%, 51.08±0.85%respectively.

5.2 Recommendations

The following recommendation can be drawn from conclusion.

- Since *Neem leaves* is a good source of phytochemicals and antioxidants, its cultivation and utilization should be promoted in local levels.
- Storage stability of phytochemicals can be studied.
- Downstream processing of phytochemicals can be studied.
- Extraction of phytochemicals using different extraction technique can be studied.

Part VI

Summary

Azadirachta indica commonly referred to as *Neem leaves* is a cactus like perennial plant belonging to family Xanthorrhoeaceae, widely in distribution in tropical and subtropical regions of the world. There are many plants with interesting pharmaceutical activities but *Neem leaves* is probably the most applied medicinal plant worldwide. Since biblical times, *Neem* has been used for its purgative effect, skin disorders healing and beauty treatments. Diverse biologically active compounds in *Neem leaves* are associated with curing different ailments and therefore, many times is called the wonder plant.

Fresh *Neem leaves* were collected from Sunsari, district and after cleaning, subjected to cabinet drying at 50°C so as to avoid the interference of moisture in the phytochemical analysis. Phytochemical preliminary analysis shows presence of protein, carbohydrates, steroids, terpenoids, phenols, tannin, glycosides, flavonoids whereas saponin was negative with aqueous extract. Water and dry matter content of the plant under study was found to be 97.53% and 2.47% respectively. The phytochemicals were extracted in methanol and ethanol in soxhlet apparatus and used for further determination of total phenol content (TFC), total phenol content (TPC) and tannin content. TPC, TFC and tannin content of methanolic and ethanolic extract were found to be 28.09±0.31 mg GAE/g and 22.88±0.24 mg GAE/g, 98.7±6.4 mg QE/g and 67.1±2.86 mg QE/g, 1.55±0.79 mg GAE/g and 1.4±0104 mg GAE/g respectively. Phytochemical content was better in methanolic extract that significantly differed from ethanolic extract i.e. (p-value <0.05) showed by paired t-test.

Determination of chlorophyll-a, chlorophyll-b and total carotene content was carried by extraction of fresh *Neem leaves* in 96% methanol and values were found to be 0.088±0.007 mg/100g, 0.017±0.006 mg/100g and 35±0.23 mg/100g respectively. Fresh leaves of plant were extracted in 99% methanol to carry out the antioxidant assays. Total antioxidant capacity (TOAC), DPPH radical scavenging assay and reducing power at 1mg/1ml concentration were found to be 91.49±0.24%, 71.91±0.04%, 51.08±0.85% respectively. The results obtained in this work are noteworthy, not only with respect to the antioxidant activities of the methanol extracts, but also with respect to its content of various phytochemical compounds as well as could be a source for the exploitation of these

phytochemicals beneficial in the pharmaceutical and alternative medicine industries. So, the promotion of cultivation and utilization of this plant should be carried out from the local levels. Furthermore, studies in isolation and quantification of the compounds to elucidate their different antioxidant mechanisms and the existence of possible synergism, if any, and their effects through in vivo studies are needed to evaluate their natural biological function.

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Appendices

Appendix A.1

Appendix A.1 List of chemicals

1) Aluminium chloride (S.D fine-chem Ltd.)
2) Ammonium molybdate (Thermo Fisher Scientific India Pvt. Ltd.)
3) Ascorbic acid (Thermo Fisher Scientific India Pvt. Ltd.)
4) Boric acid (Merck (India) Ltd.)
5) Disodium hydrogen orthophosphate (Thermo Fisher Scientific India Pvt. Ltd.)
6) DPPH (Thermo Fisher Scientific India Pvt. Ltd.)
7) Ethanol (Merck (India) Ltd)
8) Ferric chloride (Thermo Fisher Scientific India Pvt. Ltd.)
9) Folin-ciocalteu's reagent (Thermo Fisher Scientific India Pvt. Ltd.)
10) Galic acid (Thermo Fisher Scientific India Pvt. Ltd.)
11) Hydrochloric acid (Thermo Fisher Scientific India Pvt. Ltd.)
12) Methanol (Merck (India) Ltd.)
13) Ninhydrin solution (Central drug house Pvt.Ltd.
14) Petroleum ether (Merck (India) Ltd.)
15) Sodium carbonate (Qualigence fine chemicals)
16) Sodium dihydrogen orthophosphate (Thermo Fisher Scientific India Pvt. Ltd.
17) Sodium hydroxide (Thermo Fisher Scientific India Pvt. Ltd.)
18) Sodium nitrate (Thermo Fisher Scientific India Pvt. Ltd.)
19) Sulphuric acid (Thermo Fisher Scientific India Pvt. Ltd)
20) Trichloroacetic acid (Thermo Fisher Scientific India Pvt. Ltd.)

Appendix A.2

Appendix A.2 List of equipments used

1) Cabinet dryer (Y.P. Scientific industries)
2) Centrifuge (Victolab, India)
3) Electric balance (Phoenix instrument, 620g)
4) Spectrophotometer (Labtronics, India)
5) Soxhlet apparatus (Y.P. Scientific industries)
6) Hot air oven (Victolab, India)
7) Incubator (Victolab, India)
8) Muffle furnace (Accumax, India)
9) Rotatory Vacuum Evaporator (IKA RV 10)
10) Refrigerator (Victolab, India)

Appendix B (Standard curves)

Appendix B.1

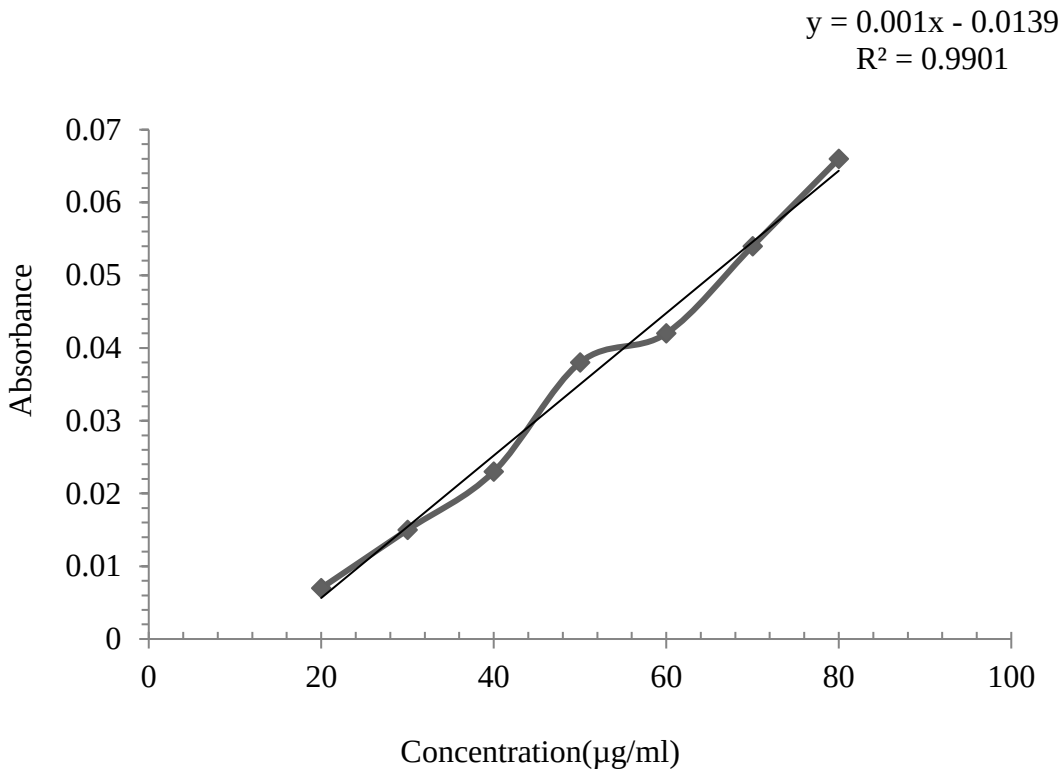

Fig. B.1 Gallic acid standard curve in methanol for TPC determination

Appendix B.2

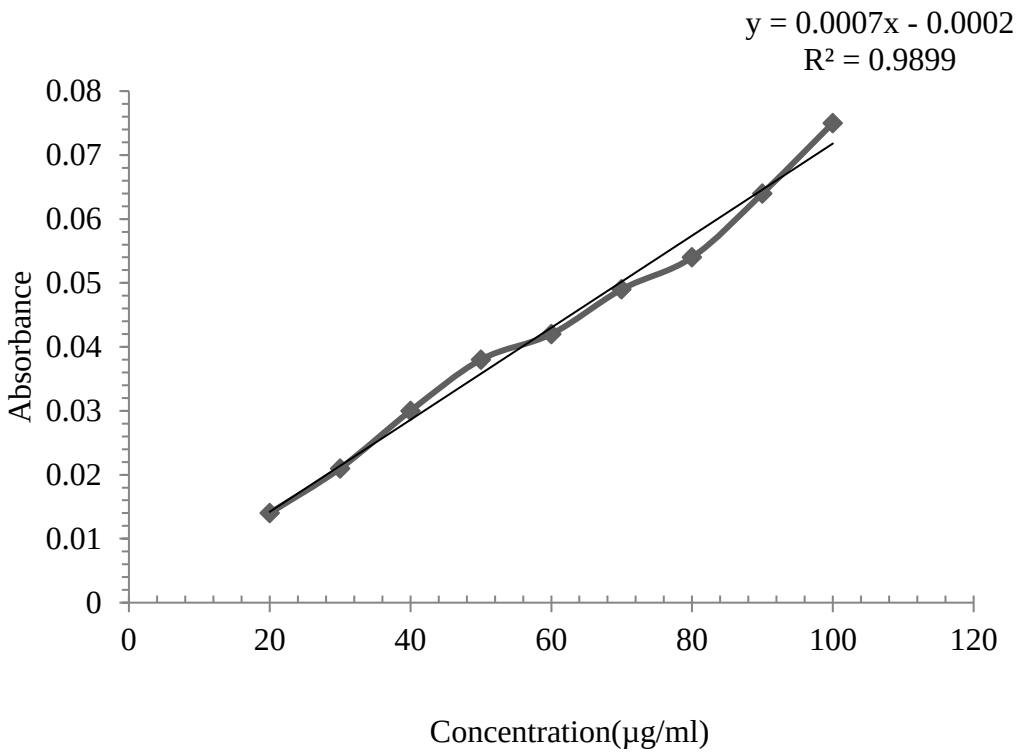

Fig. B.2 Gallic acid standard curve in ethanol for TPC determination

Appendix B.3

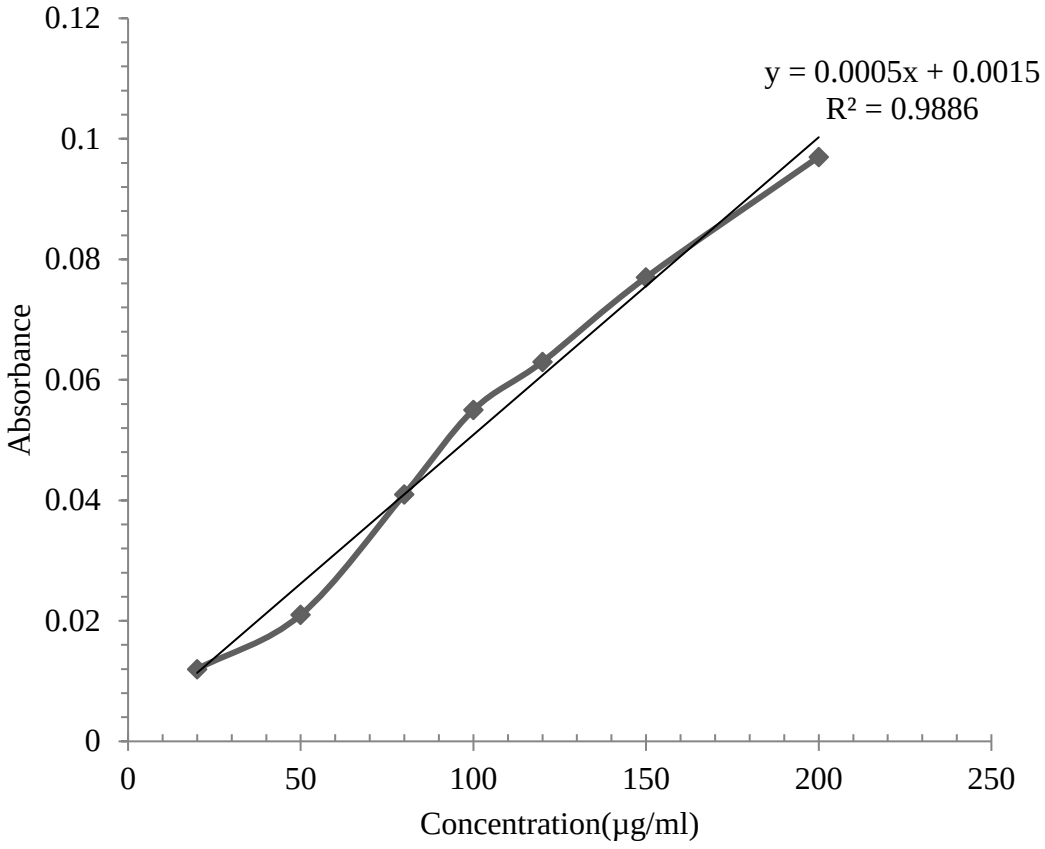

Fig. B.3 Quercetin standard curve in methanol for TFC determination

Appendix B.4

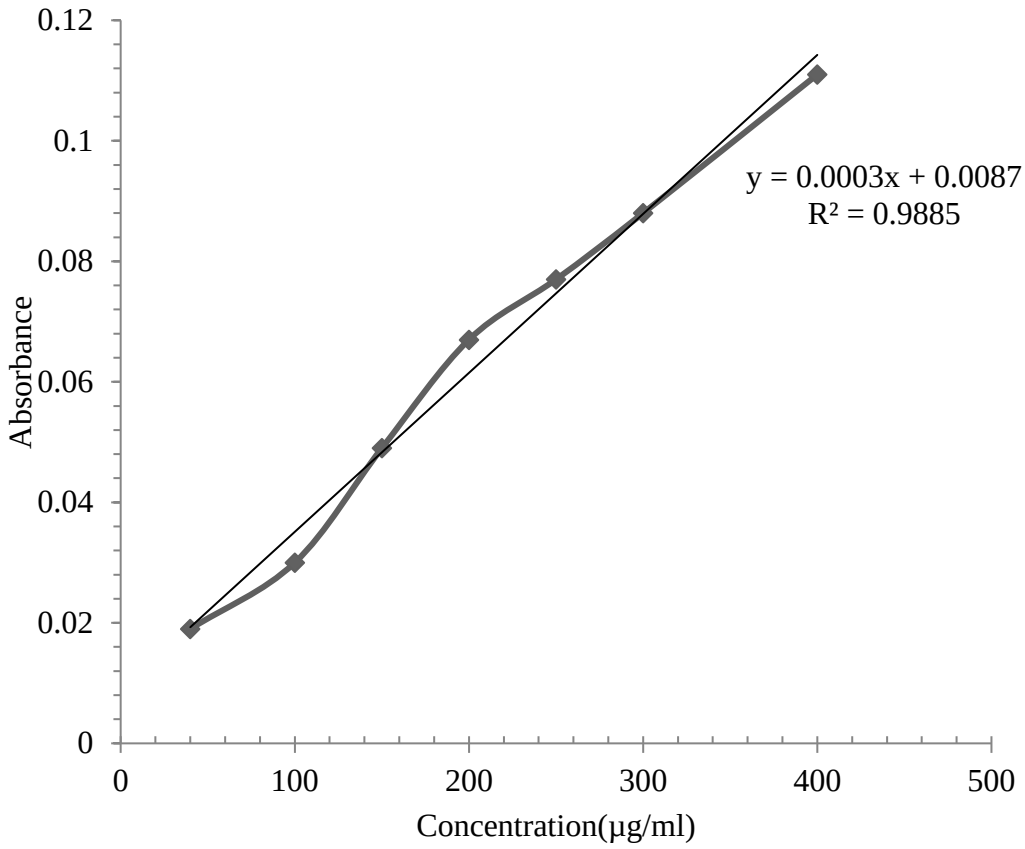

Fig. B.4 Quercetin standard curve in ethanol for TFC determination

Appendix B.5

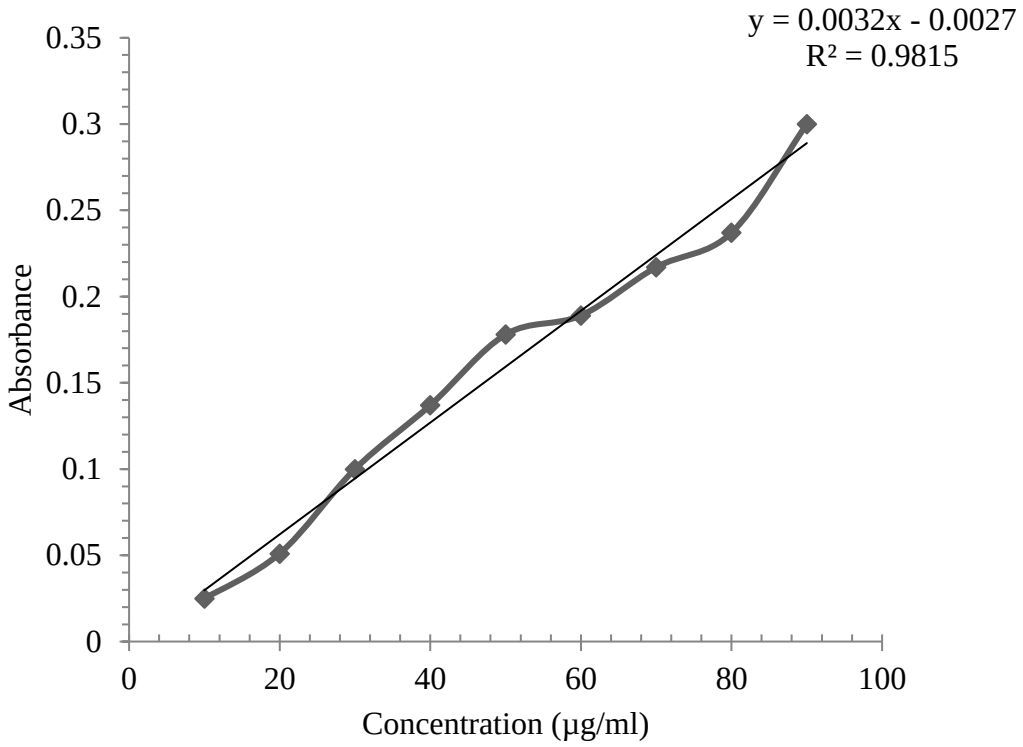

Fig. B.5 Gallic acid standard curve in methanol for tannin content determination

Appendix B.6

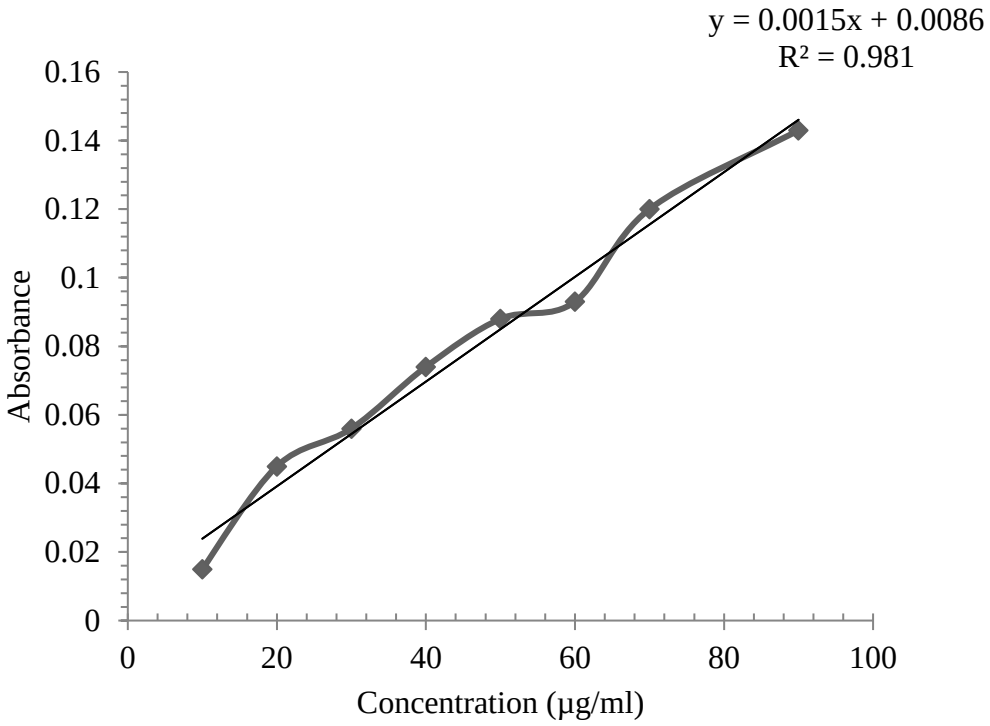

Fig. B.6 Gallic acid standard curve in ethanol for tannin content determination

Appendix C.1

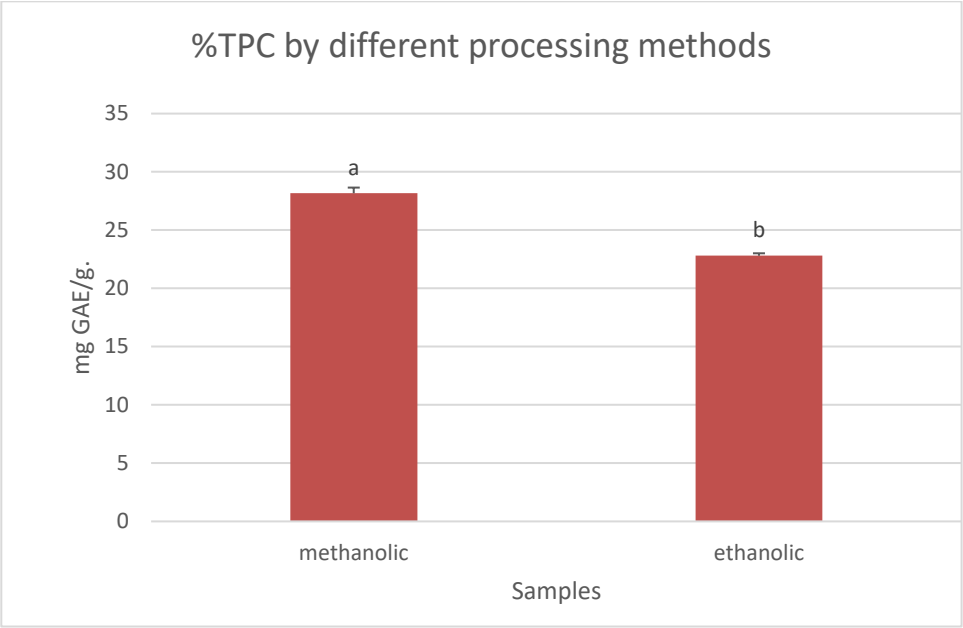

Fig. C.1 % TPC by different processing methods

Appendix C.2

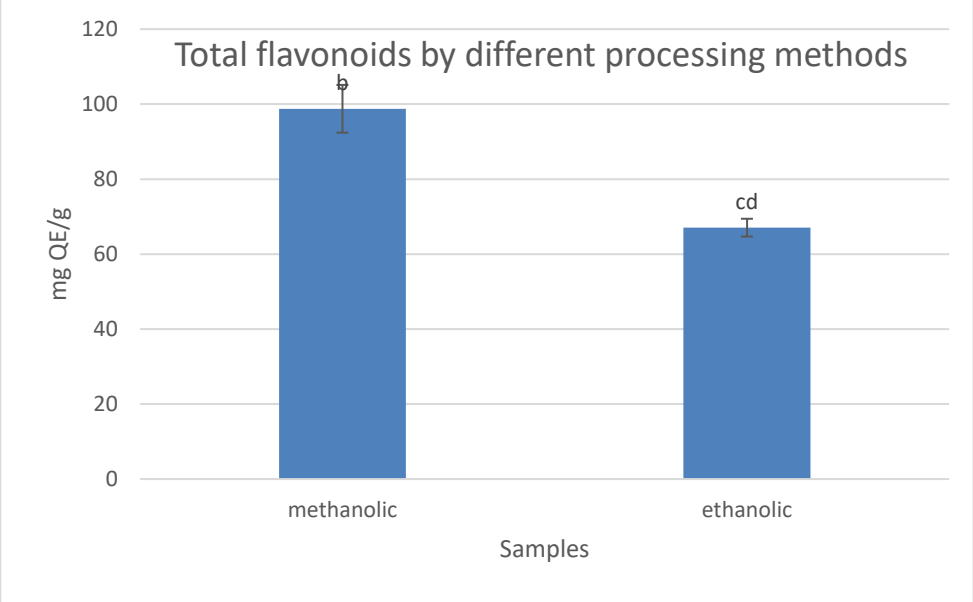

Fig. C.2 Total flavonoids by different processing methods

Appendix C.3

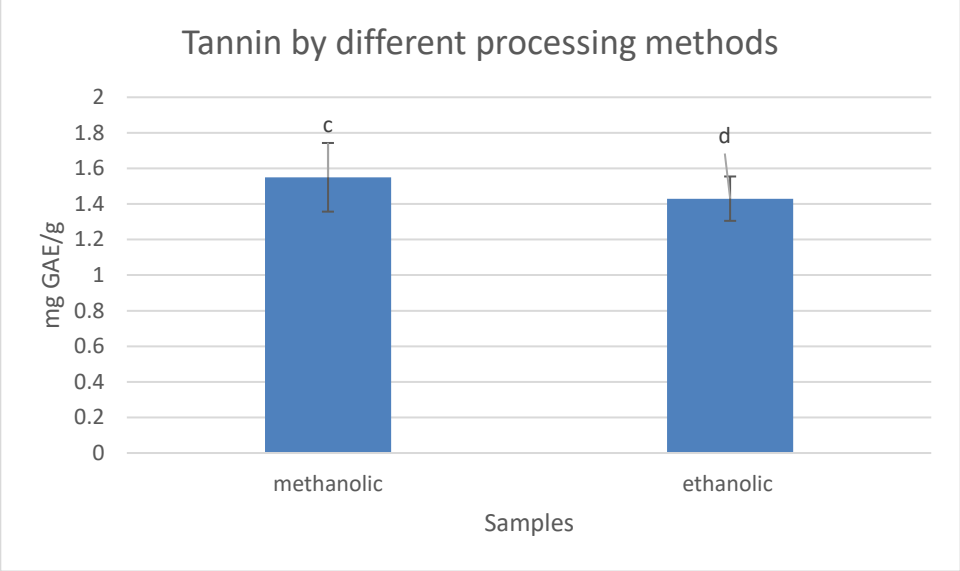

Fig. C.3 Tannin by different processing methods

Appendix C.4

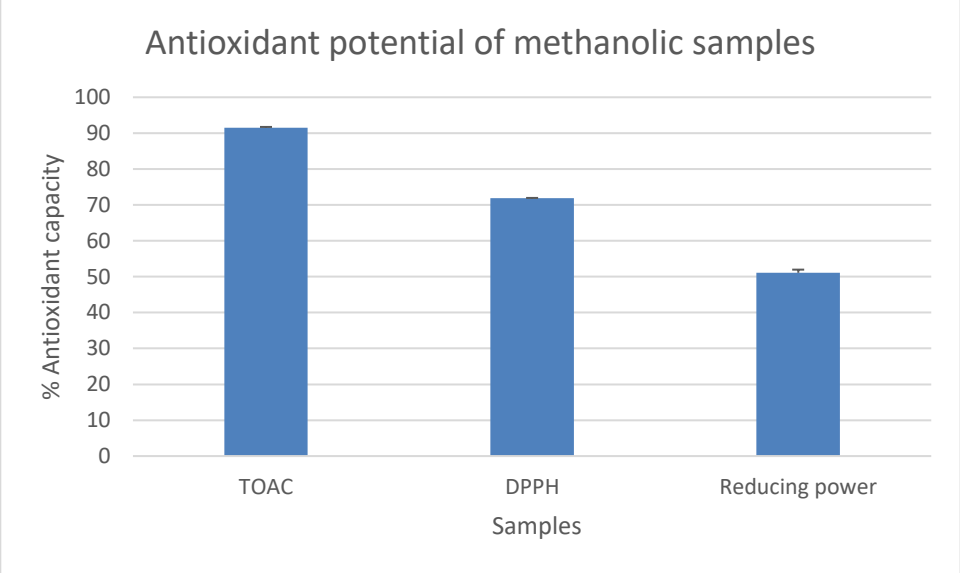

Fig. C.4 Antioxidant potential of methanolic samples

Appendix D.1

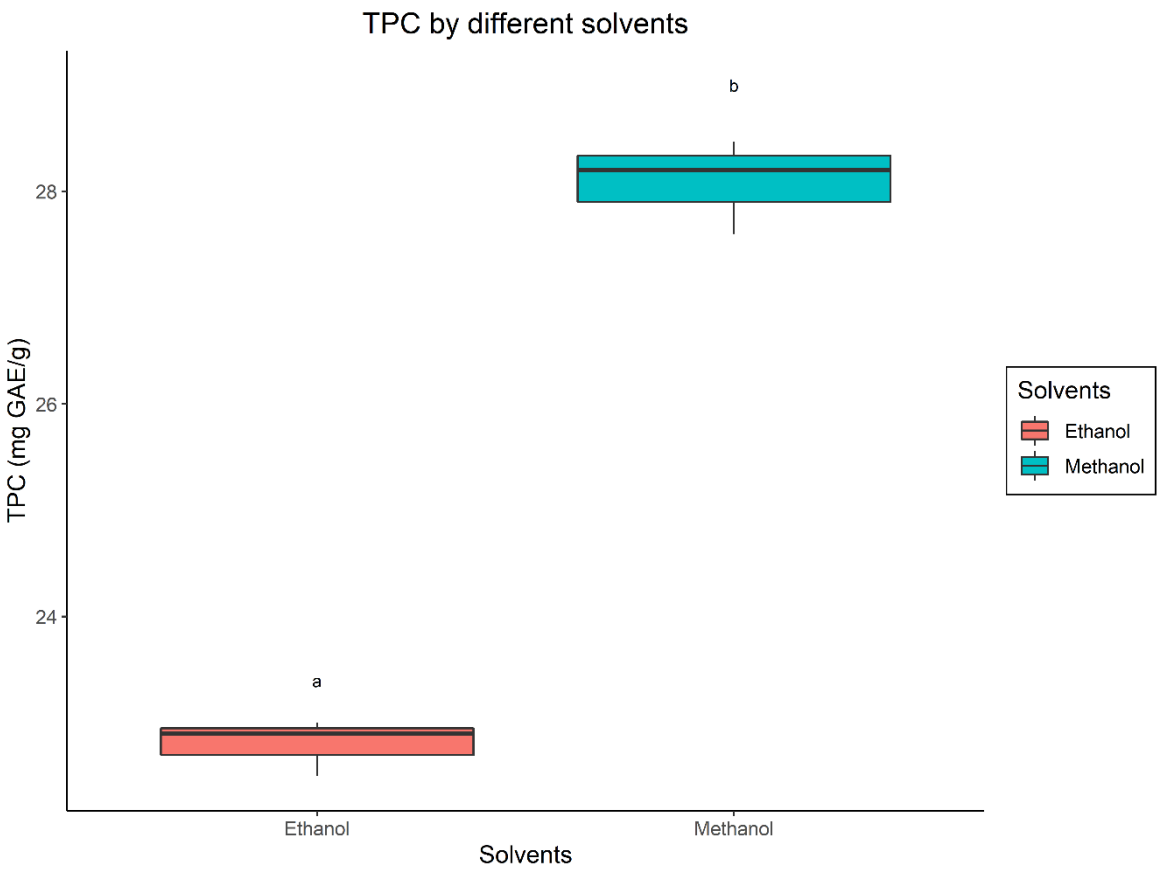

Fig: TPC by different solvents

Appendix D.2

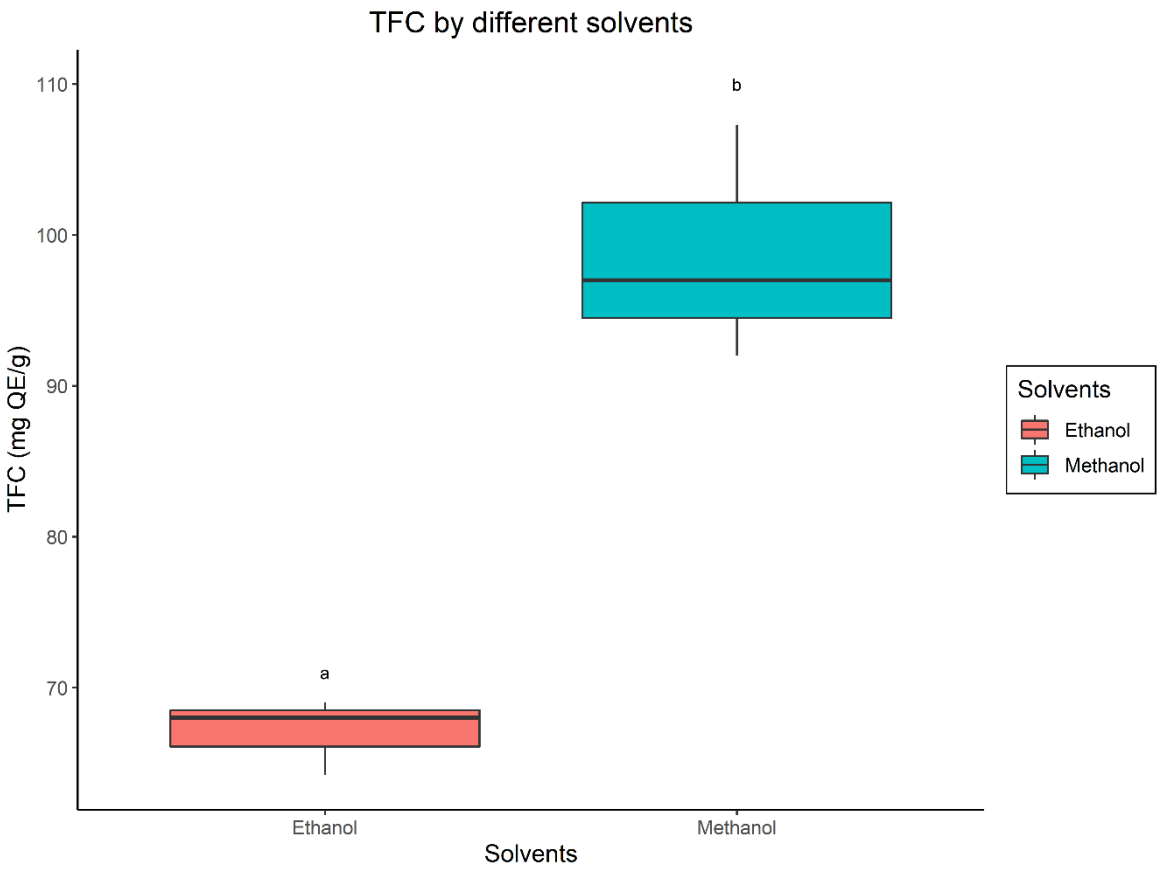

Fig: TFC by different solvents

Appendix D.3

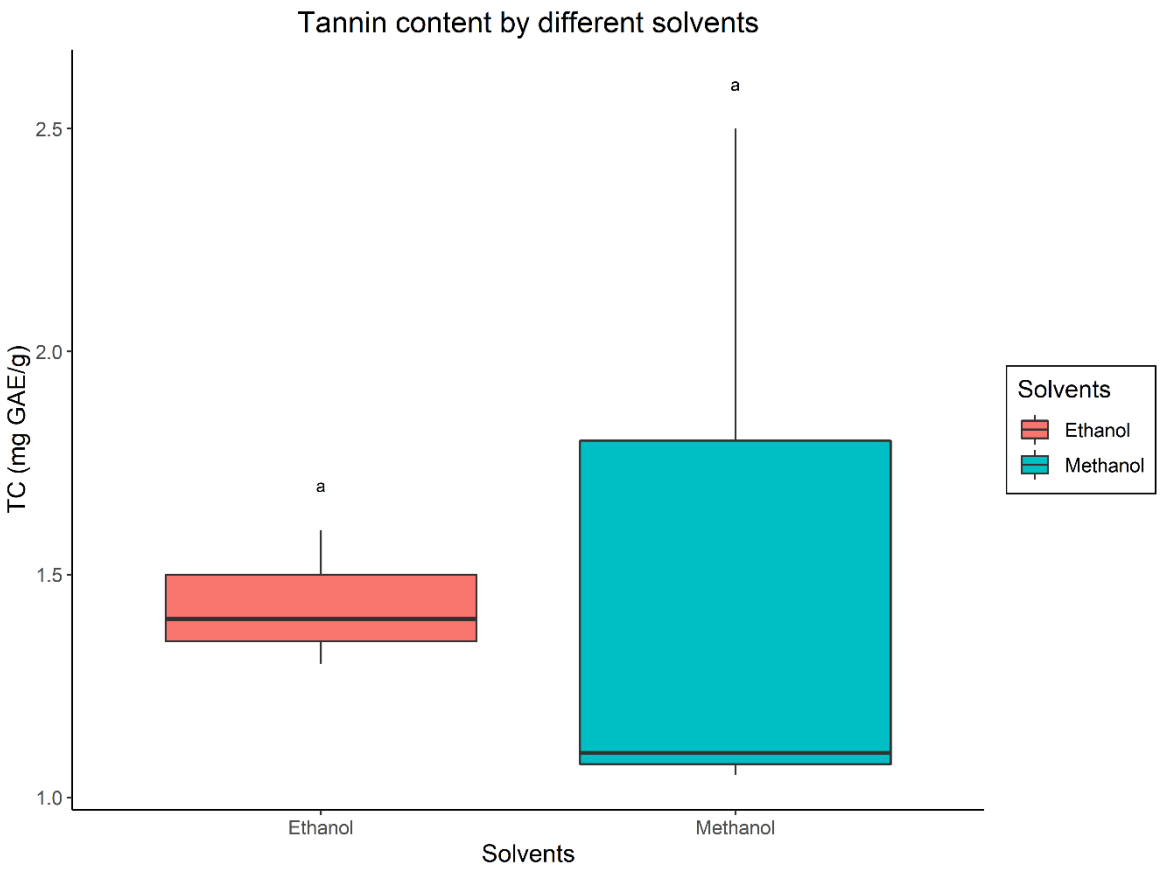

Fig: Tannin content by different solvents

Appendix D.4

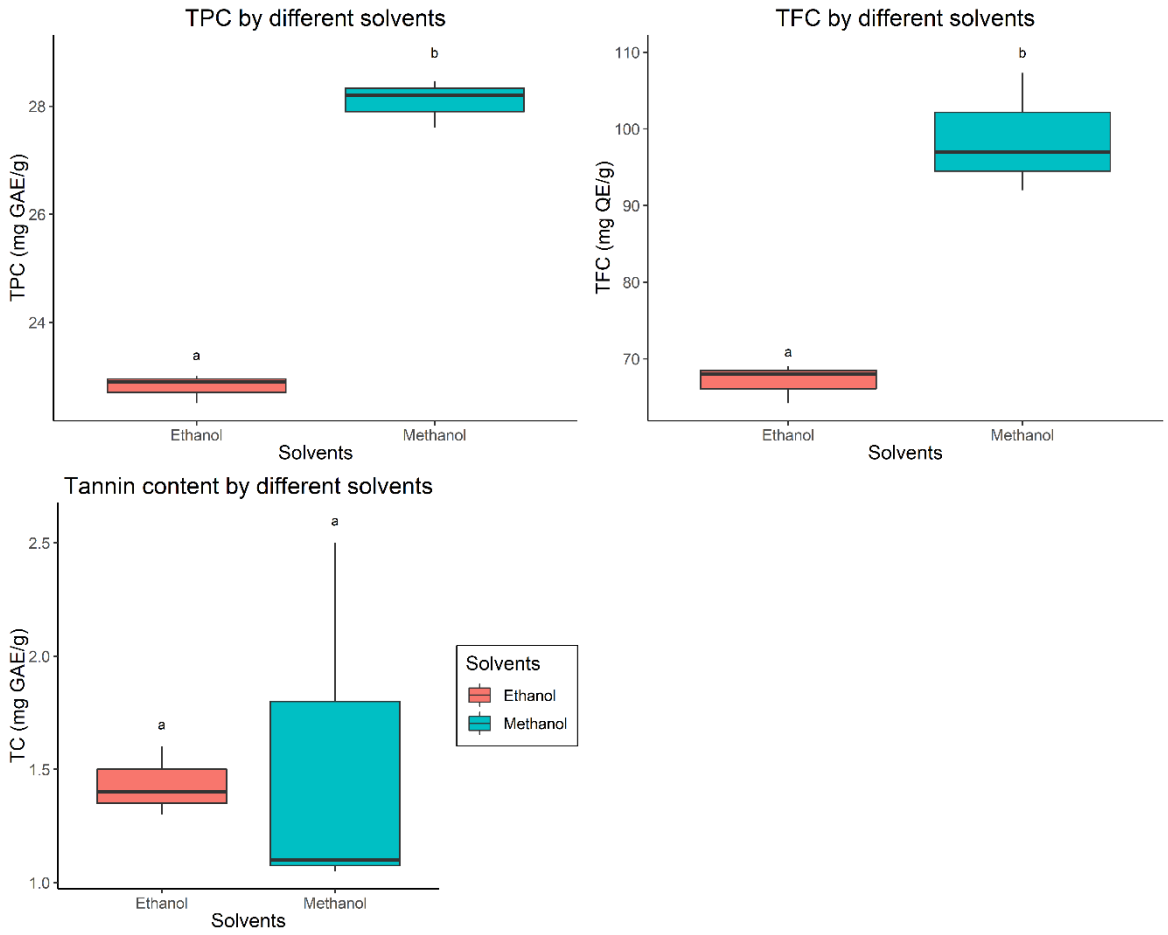

Fig: Box Plot

PHOTO GALLERY