STUDY OF α-AMYLASE ENZYME INHIBITION ACTIVITY AND ANTIMICROBIAL ACTIVITY OF LOCAL TEA *Camellia sinensis* (L.) Kuntze, LEAVES OF *Buddleja asiatica Lour*, AND ROOTS OF *Polygala arillata* Buch.-Ham. ex D. Don

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This dissertation work entitled **"STUDY OF α-AMYLASE ENZYME INHIBITION ACTIVITY AND ANTIMICROBIAL ACTIVITY OF LOCAL TEA** *Camellia sinensis* (L.) Kuntze**, LEAVES OF** *Buddleja asiatica lour.***, AND ROOTS OF** *Polygala arillata* Buch.- Ham. ex D. Don**"** submitted by Kabin Chemjong (Exam roll No. 733/073 and T.U Reg. No. 52371812012) under the supervision of Asst. Prof. Bimala Subba [Ph.D.] of Central Department of Chemistry, Tribhuvan University, Nepal is approved for the partial fulfillment of the M. Sc. degree in Chemistry. This dissertation has been accepted for the award of a degree.

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RECOMMENDATION LETTER

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DECLARATION

I, Kabin Chemjong, hereby declare that the work presented herein in this thesis is genuine work carried originally by me and has not been published or submitted elsewhere for the requirement of any degree program. Any literature, data, or work done by others that are cited in this work has been given due acknowledgment and listed in the reference section.

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Kabin Chemjong

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LIST OF ABBREVIATION

WHO	World health organization
DM	Diabetes mellitus
NIDDM	Non- insulin-dependent diabetes mellitus
IDDM	Insulin-dependent diabetes mellitus
GD	Gestational diabetes
ºC	Degree celsius
IC50	Inhibitory concentration 50 %
SC50	Scavenging, 50 %
EC50	Effective concentration, 50%
DPPH	2,2-Diphenyl-1- picrylhydrazyl
DMSO	Dimethyl sulphoxide
FCR	Folin- ciocalteu reagent
g/L	Gram per Liter
mM	Millimolar
mg/mL	Milligram per liter
µg/mL	Microgram per liter
mg/gm	Milligram per gram
TPC	Total phenolic content
GAE/gm	Gallic acid equivalent per gram
TFC	Total flavonoid Content
QE/gm	Quercetin equivalent per gram
DNS	3,5-Dinitrosalicyclic acid
UV	Ultraviolet
FTIR	Fourier Transformed Infrared Spectroscopy
TLC	Thin Layer Chromatography

NMR	Nuclear Magnetic Resonance
GC-MS	Gas chromatography and Mass spectrometry
Nm	Nanometer
N	Normal
MHA	Mueller Hinton agar
T1DM	Type 1 diabetes Milletus
T2DM	Type 2 diabetes Milletus
ZOI	Zone of Inhibition
NPs	Natural Products
v/w	Volume by weight
w/w	Weight by weight
S. aureus	*Staphylococcus aureus*
C. sinensis	*Camellia sinensis*
B. asiatica	*Buddleja asiatica*
P. arillata	*Polygala arillata*
S. typhii	*Salmonella typhii*
A-std	Standard Acarbose

ABSTRACT

A phytochemical and natural product rich medicinal plant is the alternative source for the discovery of new anti-diabetic agent. Diabetes mellitus (DM) is a fatal disease increasing prevalently and their complication then remedial drug with a large number of side effects. Therefore, to search the medicinal plants having the potential to inhibit the α-amylase key enzyme causing the diabetes mellitus is the main goal of the study. In this study, local tea *C. sinensis*, leaves of *B. asiatica*, roots of *P. arillata*, were subjected to phytochemical screening, and quantitative analysis of TPC was found to be 157.17 ± 18.79, 116.47 ± 6.62, and 44.27 ± 2.97 mg GAE/gm in dry weight of the extract. Similarly, TFC was found to be 37.07 ± 2.24, 34.28 ± 2.63 and 5.99 ± 1.00 mg QE/gm dry weight of the extract. Then, antioxidant DPPH assay was performed, IC_{50} values of methanolic extracts of *B. asiatica*, *C. sinensis*, and Quercetin were found to be 26.86±2.00, 19.15±4.32 and 2.36±0.13 µg/mL respectively.

In the case of α-amylase inhibition assay by starch-iodine method, IC_{50} values of methanolic extract of local tea *C. sinensis*, *B. asiatica*, and Acarbose standard were found to be 1737.65 ± 2.21, 1780.00 ± 3.06 and 55.74 ± 2.85 µg/mL respectively. On further analysis of α-amylase inhibition assay by DNS method in the fractions of local tea *C. sinensis*, and *B. asiatica* extracts IC_{50} values were found to be TH-1296.13 ± 94.46, TD-675.97 ± 66.18, TE-338.52 ± 3.78 µg/mL and KH-767.46 ± 18.44, KD-411.50 ± 79.07 and KE- 1051.62 ±12.05 µg/mL respectively in Hexane, dichloromethane, and Ethyl acetate fractions. Antimicrobial activity was performed and their zone of inhibition (ZOI) in local tea *C. sinensis*, leaves of *B. asiatica*, were found to be 17 mm and 12.5 mm against the gram-positive bacteria *S. aureus*. Finally, FT-IR analysis was also performed in ethyl acetate fraction of 20 % methanol in ethyl acetate and 100 % methanol column fractions of *C. sinensis*.

KEYWORD:- Phytochemical screening, 96-well plate microreaders, DPPH assay, α-amylase inhibition assay, Antimicrobial activity, FT-IR spectra.

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CHAPTER I

1. INTRODUCTION

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1. INTRODUCTION

1.1 General background

Nepal is a small country with an area of 147,510 km^2 located in between two giants' nation India in east, west, south, and China in the North. The altitude variation from 70 meter mighty trees in lowlands to world highest mountain range 8848 meters stunted alpines vegetation battling with the harsh environment of the frozen mountains. Climatic difference due to the variation on the geographical structure and topography,118 ecosystems and four types of physiographic zone such as lowlands, mid-hills, high mountains, and high Himalayan range [1], leads to biodiversity in nature abundantly rich with a habitat of flora and fauna in Nepal.

Interdisciplinary, of biodiversity with the culture, and way of life style is directly connected to the each other. The home of the 59 ethnic and indigenous groups and their traditional medicating systems Ayurvedic along with the Unani, Homeopathy and Tibetan medicine, etc plays a pivotal role in delivering health care to the folks. The diverse society with their traditional approach of various medicinal plants might have multiple task. The different way of medical practices handed them orally from generation to generation with the folk medicine.700 hundred species out of 6973 plants species have been used for the medicine in Nepal [1],so far over 1500 plants (1434 flowering plants, 65 ferns, and conifers) have at least one use, including 650 are used as food plants and over 1000 species of wild plants are used for medicine [2]. A large number of the medicinal plant have been used directly to care diseases. Medicinal plants are a good source of income for large numbers of people [3]. Besides that, the increasing side effects of synthetics drugs over the daily life of people leads to an increase in the demand for organic foods products along with the consumption of herbal products. The western world emphasizes more to herbal products. The large varieties of plants and animal resources with the diversified landscape might be the alternative hub and choice for the research on the discovery of the standard drug which has fewer side effects with more efficiency. The challenge in living, like the corona pandemic and the dull life, further enhanced by the quarantine might shift the rate of diabetes patients more vulnerable. Probably the outcome of diabetes after the corona might be vulnerable and cause a fatal mortality rate rather than the coronavirus. The care of problems might be the flora and

fauna. The primary goal of the study was also to investigate the different folk medicinal plants for effective diabetes care.

1.2 Natural product and drugs

Natural products and drugs have been closely linked through the use of traditional medicines and natural poisons for thousands of years ago. Natural products originate from bacterial, fungal, plant, and marine animal sources. The earliest record of uses of the natural product as drugs is illustrated as clay tablets in cuneiform from Mesopotamia in 2600 B.C. which documented oils from *Cupressus sempervirens* (Cypress) and *Commiphora* species are still used to treat coughs, colds, and inflammation, the Ebres papyrus an Egyptian pharmaceutical recorded in 2900 B.C more than 700 plant based drugs along with the garlic, pills, infusions to an ointment. The Chinese natural products are recorded in the Materia Medica in 1100 B.C. Wu Shi Er Bing Fang, contains 52 prescriptions, Shennong Herbal about 100 B.C., 365 drugs and the Tang Herbal in 659 A.D., 850 drugs [4]. Similarly the Greek, English, Arabian, Ireland, France, and Germany also use their natural products along with the Indian and Chinese natural products. However, the isolation of the first plant drug analgesic morphine was from the dried latex of the Opium poppy (*Papaver somniferum*) by Serturner in 1805 [5]. Some of the important drugs obtained from the plants in the nineteenth century were atropine, cocaine, codeine, digitoxin, papaverine, and pilocarpine, and in the twentieth century have been digoxin, ergotamine, ergometrine, reserpine, vincristine, and paclitaxel diterpene alkaloid Taxol found on minor constituent in the bark of Western Yew tree *Taxus brevifolia* [4].

The discovery of filtrate of penicillin in 1928, and commercialize, reisolation, and synthesize by Chain, Florey, and co-workers in the early 1940s from the microorganism revolutionized the discovery of drug research [4]. The output from the early years of this antibiotic research was prolific and included examples such as streptomycin, chloramphenicol, chlortetracycline, cephalosporin C, erythromycin, and vancomycin [5]. In 1990 another revolution in drug research occurs Combinatorial chemistry, later realized that the new structure will not be developed from this technique [6]. The advancement of technology and the development of new techniques lead to the modern era of drug discovery research. Nevertheless, natural products continue to provide unique structural diversity, which presents opportunities for discovering mainly new compounds.

1.3 Phytochemicals

All living creatures directly or indirectly depend on plants as a source of food. Due to plant static and direct exposure to pathogens, herbivores and drastic environmental conditions lead to the development of a biosynthetic pathway to produce toxic and antifeedant secondary metabolites [7]. Plants are open to nature and constantly face numerous biotic and abiotic environmental stress including heat, cold, drought, attacks by pathogens and animals. The interaction of plants with animals and pathogens has been a driving force to synthesize the large diversity of phytochemicals. In this case, plants adapt two major strategies; physical defense and chemical defense. Plant synthesize phytochemical as the secondary metabolites to dissuade insects, other herbivores from eating them. Phytochemical synthesize are toxic if it is consumed in the higher concentration. Synthesize phytochemicals not only provide immediate survival, However, it also increases their reproductive fitness in a challenging environment. Phytochemical plays a vital role in defense against the consumer, diseases causing pathogens and competition from other plants for limited energy resources, the attraction of pollinator species or seed dispersion [8]. Metabolic phytochemical pathway production is natural selection during evolution. Phytochemicals produce by a plant to cope with environmental stress fall into three groups namely, alkaloids, phenolics, and terpenoids.

These groups have various ranges of functions and ecological roles. Alkaloid substance's main role is to deter insects and herbivores. Phenolic substances have multiple adaptive roles to control the overall fitness of plants. The primary role of a phenolic compound is benign interactions with herbivores and symbiotic organisms. As for the example, anthocyanin is a phenolic substance responsible for the vibrant colors, which attract pollinators. Similarly, terpenoids have a wide range of functions which include attractants, repellents, and airborne volatile scents [7]. Besides these three main phytochemical groups plants also synthesize other phytochemicals such as glycosides, glucosinolates.

1.3.1 Polyphenol

Polyphenols are simple and low molecular compounds e.g. gallic acid to be very large and complex polymeric molecules e.g. condensed tannins. Phenolic metabolites contain one or more acidic hydroxyl residues attached to an aromatic arene (phenyl) ring. Hydroxycinnamic acids, flavonoids, anthocyanins, stilbenes, lignans, and tannins are major classes of phenolics, which collectively account for approximately 40% of the

organic carbon in the biosphere [10]. There are over 10000 polyphenolic structures known with half of these being flavonoids [9]. Major of polyphenols present in a wide variety of plant sources are flavonoids consisting common motif of two rings. The amazing arrangement of non-structural phenolics has many functions in plants, including acting as antioxidants. Phenolic compounds are excellent oxygen radical scavengers because the electron reduction potential of the phenolic radical is lower than the electron reduction potential of oxygen radicals and also because phenoxyl radicals are generally less reactive than oxygen radicals [12]. Phenolic compounds can scavenge reactive oxygen intermediates without promoting further oxidative reactions.

1.3.2 Flavonoids

Flavonoids are composed of two aromatic rings with six carbon atoms (ring A&B) interconnected by a heterocyclic ring that consists of 3 carbon atoms (C6- C3-C6) [13]. Flavonoid commonly contains in the diet are flavonols such as quercetin, kaempferol and their glycosides such as rutin. Similarly, flavones are also contained in the diet at lower prominent forms such as luteolin and apigenin. Dietary flavonoids in nature exist almost always as β glycosides. Flavonols are mainly as the C-3 and C-7 glycosides, although the C-4 position glycosylated flavonols are also found in some plants [14]. Other classes of flavonoids are found mainly glycosylated in the C-7 position such as puerarin, and isoflavone-8-C-glucose. Flavonoids are widely distributed in plants and therapeutically, they have shown great promise as an anticancer, antiviral, antioxidant, antibacterial, and anti-inflammatory agent. Flavonoid compounds can be very effective in inhibiting α-glucosidase activity. 103 flavonoids showed a-glucosidase activity these include Xanthones, Flavonones, anthocyanins, and other structural motifs [15]. The presence of a C2=C3 double bond on ring C, a dihydroxyl group (catechol-type) or three adjacent hydroxyl groups (pyrogallol-type) on ring B, and the presence of C-5, and C-7 hydroxyl group on ring A are usually listed as requirements for antioxidant and antiradical activity of flavonoids.

1.3.3 Tannins

Tannin is water soluble phenolic compounds. Tannins are categorized into hydrolyzable and non hydrolyzable or condensed tannins. Hydrolyzables taninns contains centre structure of polyhydric alcohol such as glucose and hydroxyl groups, due to this it undergoes esterified either partially or wholly by gallic acid or Hexahydroxydiphenic acid [7]. Gallotanins on hydrolysis by acid, base, or enzyme yield glucose and gallic acid.

The hexahydroxydiphenic acid of ellagitannins undergoes lactonization to produce ellagic acid [16]. Tannins are polyphenols containing in the berries like Strawberry, raspberry blueberry, blackcurrant, red cabbage, sorghum, millets, barley, dry beans, faba beans, peas, carobs, pigeon peas, winged beans, and other legumes extracts. Other tannin rich extracts are red grape, red wine, and green tea were also effective inhibitors against α-amylase. The inhibitory components were identified as ellagitannins, such as sanguiin H6, sanguiin H10, nobatanin A, lambertianin C, and ellagic acid [17]. Besides, the inhibition there is potential for synergistic effects on starch degradation after ingestion of berries containing appreciable amounts of ellagitannins and anthocyanins. Some examples of gallotannins are Chinese tannin (tannic acid), Turkish tannin, Tara tannin, Acer tannin, and Hamamelis tannin [18].

1.4 Description of plants

1.4.1 Tea [*Camellia sinensis*]

Kingdom:- Plantae

Phylum :- Tracheophyta

Division :- Eudicots

Class:- Magnolioposida

Order:- Ericales

Family:- Theaceae

Genus:- *Camellia*

Species:- *sinensis* (L.) Kuntze

Figure 1:- *Camellia sinensis*

The beverage tea is prepared from the tea plant *C. sinensis*, which is placed in the genus *Camellia*. The genus has over 200 species and is largely habitat to the highlands of Tibet, northern east India, Nepal, and southern China [8]. Since all *Camellia spp*. do not consume as a tea [11]. Taxonomy plays a major role in the identification of the cultivable tea among the *Camellia spp*. The tea plant *Camellia sinensis* (L.kuntze) family Theaceae is only species that can be cultivated and grown in about 30 countries worldwide [8]. Tea is the most common beverage worldwide because of its flavor, taste, and biological activity. Out of the total amount of teas produced and consumed in the world, 78% are black, 20% are green, and 2% are oolong tea [9]. Tea manufacturing is an indispensable

condition for the formation of quality components and functional ingredients. Different tea types have unique quality characteristics and health effects due to different processing techniques. By adjusting and optimizing the tea processing techniques, metabolic flux could be altered, quality ingredients and functional ingredients transformations can be purposefully activated, thereby improving tea quality components formation and health effects [9]. According to the degree of fermentation, teas are classified into three categories, Non-fermented teas, such as green tea, partially fermented teas such as oolong tea, and fully fermented teas such as black and Puerh tea [10].

Nepal is favorable for the cultivation of Tea about 1,804,813 hactor of area are cultivated and these suitable areas exist across 62 districts encompassing 460 municipalities of Nepal [11]. In Nepal, two types of tea are produced: Orthodox tea and CTC (Crush, Tear, Curl) tea. Orthodox tea refers to the process, where the tea is hand-processed or by rolling it in the machines which mimic the hand rolling technique. Most of the specialty teas like green tea, oolong tea, white tea, and hand-rolled tea come under the orthodox category. Orthodox tea is produced and processed in the mountainous regions of Nepal at an altitude ranging from 3,000 – 7,000 feet above sea level. Mostly Orthodox tea is produced in the hilly districts like Ilam, Panchthar, Dhankuta, and Terhathum. Similarly, CTC tea production is concentrated in the plains of the Jhapa district. [11].

1.4.2[*Buddleja asiatica*]

Kingdom :- Plantae

Division :- Eudicots

Class :- Magnolioposida

Order :- Lamiales

Family :- Scrophulariaceae

Genus :- *Buddleja*

Species :- *asiatica. lour*

Figure 2:- *Buddleja asiatica*

Buddleja genus of the family comprises about 100 species [20]. The evergreen shrub, commonly called butterfly bush adapted in the tropical lands of America, Africa, and Asian countries including Nepal and India. It is an erect small tree growing up to the

height of 1 to 5 meters in height [19]. Several *Buddleja* species have been used in traditional medicine in many parts of the world. Roots, barks, and leaves of these plants are used against different kinds of ailments such as dysentery, eye or skin inflammation, as diuretic and antiseptic agents, antispasmodic, wound healing, and anti-inflammatory agents [20]. Traditionally, it is used in the treatment of many health disorders such as inflammation, rheumatism, skin disease, malaria, etc. *B. asiatica Lour* roots, leaves, and stems have been used as a traditional Chinese medicine for the treatment of fever, ache, diarrhea, and articular rheumatism [21]. The leaves and flowers of *B. asiatica* are used as a religious offering to gods and goddesses, squeezed leaves are used as a fish poison and beverage fermentation in Nepal [22]. Pharmacologically, *B. asiatica* was screened for antihepatotoxic, antibacterial, hypotensive, anticancer, antifungal, and antimalarial activities [23]. More than 80 compounds including phenylpropanoids, flavonoids, Phenyl ethanoid glycosides, phenylpropanoid esters, non-phenolic compounds, triterpene saponins, iridoid glucosides, benzoates, triterpenoids, monoterpenes, acetogenins, steroids, shikimates as well as other trace elements were identified in the plant [24].

1.4.3 [*Polygala arillata*]

Kingdom:- Plantae

Phylumb:- Tracheophyta

Class:- Magnolioposida

Order:- Fabales

Family:- Polygalaceae

Genus:- *Polygala*

Species:- *arillata* Buch.-Ham.ex D.Don

Figure 3:- *Polygala arillata*

P. arillata is a shrub plant that widely distributes in the Asian Himalayan range, including southern China, Nepal, northeast India, and northern Vietnam [25]. *Polygala*, a genus of the Polygalaceae family, includes about 500 species growing throughout the World and some of the species are listed in the CITES list as endangered species in Nepal [2]. Some of these species have been traditionally used for the treatment of amnesia, neurasthenia, and inflammation [26]. The root of this plant has been used medically for

wound healing, respiratory troubles, chronic bronchial asthma, chronic bronchitis, whooping cough, and diuretic [27]. Radix Polygala (*P. tenuifolia*) a well-known tonic and for treatment of certain central nervous system dysfunctions including Alzheimer's disease, Parkinson's disease, and depression is also belongs to this genus [28]. The roots of this plant have been used in folk medicine for the treatment of acute arthritis, rheumatism, pain, and also as a tonic ingredient in Vietnam [28].Phytochemical studies on *P. arillata* revealing its roots contain mainly xanthones, oligosaccharide esters, saponins along with several fatty acids and volatile compounds [27]. The oligosaccharide esters, such as sucrose and trisaccharide esters, are hydrophilic and considered as main constituents in its ethnomedicine as a decoction [25].

1.5 Diabetes mellitus

By far diabetes is one of the most common diseases in the world. A metabolic disorder characterized by chronic hyperglycemia or increase blood glucose levels with disturbance in carbohydrate, fat, and protein metabolism consequences of absolute or relative lack of insulin secretion is a diabetes mellitus [29]. Diabetes are of following three types given below. Currently one of the most significant public health challenges worldwide after cancer and heart disease is diabetes. In 2019, 463 million people have diabetes and it is projected to reach 578 million by 2030 and 700 million by 2045. However, it was reported that the prevalence rate of diabetes in Nepalese adults is 4% out of the total adult population which is 17,570,100 and the sufferings are 696,900 [30]. Almost 90% of the cases of diabetes are type 2 diabetes, which is mainly induced by environmental factors such as being overweight and decreased physical activity [31]. Type2 diabetes is a metabolic disease characterized by a high blood glucose level and can cause other health complications, such as cardiovascular disease, neuropathy, high blood pressure, weakness, gangrene, retinopathy, nephropathy, and other dysfunctions [32]. Acarbose or Orlistat is α-glucosidase inhibitor that attenuates type 2 diabetes by limiting the absorption of carbohydrates or lipids. But it is usually accompanied by many side effects such as abdominal distention, flatulence, diarrhea, and meteorism. These serious side effects may be caused by significant inhibition of α-amylase [31]. Previous research suggested that food derived phenolic compounds could delay glucose absorption by inhibition of carbohydrate-hydrolyzing enzymes, which would reduce the postprandial blood glucose level. This could be a suitable approach to treat and prevent type 2 diabetes. The α-glucosidase inhibitors that have fewer inhibitory effects on α-amylase

may be promising agents to prevent postprandial hyperglycemia resulting from type 2 diabetes [31]. Therefore, there is an ongoing search for natural digestive enzyme inhibitors with fewer side effects for prevention or mitigation of the symptom of obesity or diabetes. Effective and non-toxic inhibitors of α-amylase and α-glucosidase from natural products have been searched for the treatment of diabetes

1.5.1 Type 1 Diabetes mellitus (T1DM) (Insulin-dependent mellitus)

Type 1 Diabetes Mellitus (T1DM) form of diabetes, which accounts for only 5–10% of those with diabetes [35]. This type of diabetes results from a cellular mediated autoimmune destruction of the β-cells of the pancreas is so-called insulin-dependent diabetes or juvenile-onset diabetes [30]. Markers of the immune destruction of the β-cell include islet cell autoantibodies, autoantibodies to insulin, autoantibodies to GAD (GAD65), and autoantibodies to the tyrosine phosphatases IA-2 and IA-2b [34]. When fasting hyperglycemia is initially detected, autoantibodies are present in 85–90% of individuals. The rate of β-cell destruction is quite variable, being rapid in some individuals like infants and children whereas slow in adults. Patients rapidly change to severe hyperglycemia and ketoacidosis in the presence of infection or other stress. In this type of diabetes, patients are prone to other autoimmune disorders such as Graves' disease, Hashimoto's thyroiditis, Addison's disease, vitiligo, celiac sprue, autoimmune hepatitis, myasthenia gravis, and pernicious anemia [33].

1.5.2 Type 2 Diabetes mellitus (T2DM) (Non-insulin dependent Diabetes mellitus)

In this type of diabetes, individuals have insulin resistance and usually have relative insulin deficiency rather than absolute failure is called as non-insulin dependent diabetes, type 2 diabetes, or adult-onset diabetes. T2DM diabetes accounts for 90–95% of those with diabetes patients [34]. Patient from this diabetes initially require insulin but does not require insulin throughout their lifetime treatment. These types of diabetes are caused by many reasons and the beta cell destruction also does not occur and have not any symptoms as well. Most patients are obsessed with obesity due to this it acts as insulin resistance. But in the case of not obsess patient, there is an accumulation of fats around the abdominal region. Due to the causes of stress and infection leads to Ketoacidosis [35]. This type of diabetes is undiagnosed for many years and does not show any symptoms, However, the hyperglycemia increases gradually. Patients are at a higher rate of risk as the blood glucose level is periodical.

1.5.3 Gestational diabetes mellitus (GDM)

This type of diabetes is mainly detected during the pregnancy simply define as the degree of glucose intolerance [34]. However, in most cases resolve with delivery, the definition applied whether or not the condition persisted after pregnancy and did not exclude the possibility that unrecognized glucose intolerance may have antedated or begun concomitantly with the pregnancy. The limitations are recognized even after the pregnancy. The epidemic of obesity and diabetes led to more types of diabetes 2 in women of childbearing age, the number of pregnant women with undiagnosed type 2 diabetes has increased. American Diabetes Association (ADA), recommended that high risk women found to have diabetes at their initial prenatal visit, receive a diagnosis of overt, not gestational, diabetes [33]. Approximately 7% of all pregnancies are complicated by GDM, resulting in more than 200,000 cases annually [34].

1.6 Starch

Starch is natural polymeric carbohydrates generated by green plants such as potato, wheat, Barley, Maize, etc. [36]. It has a wide range of applications such as food and feed sectors, pharmaceutical and paper industries, etc. It is also used to control the enzyme activity as a quality control tool. In most applications, starch undergoes a hydrolysis process that leads to conversion of the polymeric chain composed by two high molecular weight units amylose and amylopectin into its primary glucose units mediated by amylolytic enzymes α-amylase and glucosidase [39]. During this process the complementary action of two enzymes: α-amylase and amyloglucosidase cleavages the glycosidic bonds in α-1, 4 for α-amylases and α-1, 4/ α-1,6 for amyloglucosidase respectively [37].

1.7 α-amylase (α-1, 4-glucan-4-glucanohydrolase)

The enzyme is found in microorganisms, plants and particularly high activity in germinating cereals. α-amylase is produced in mammals both in saliva especially in the secretory granules of the cells of the salivary glands and pancreas. Porcine pancreatic α-amylase (PPA)' is an endo-type amylase and is extracted from the pig pancreas. α-amylase has 496 amino acid residues and 170 water molecules. Porcine pancreatic α-amylase (PPA) consists of two isoenzymes (PPA 1 and PPA 2) [38]. They have the same molecular weight but differ slightly in amino acid composition and isoelectric point. The enzyme requires one calcium ion and chloride ions for the integration and activation of the enzyme [38]. The maximum enzyme activity occurs at pH-7. α-amylase catalyzes the

hydrolysis of internal α-(1-4) glucosidic bonds in amylose and α-(1-6), α-(1-4) glycosidic bond in amylopectin through multiple attacks toward the non-reducing end [38].

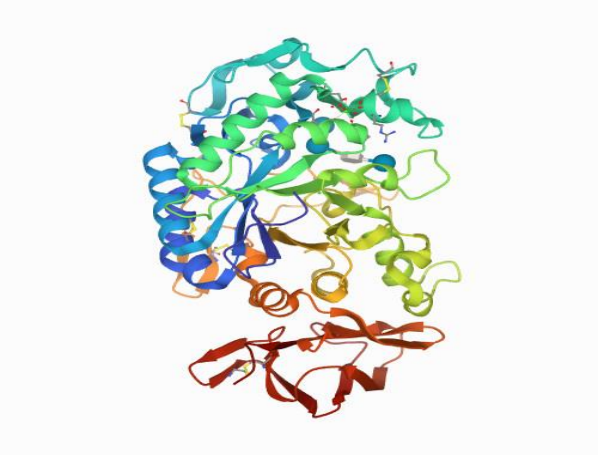


Figure 4:- α- amylase

Based on the kinetic study suggested that pancreatic pig alpha-amylase consists of five subsites for the binding of glucose units, the catalytic attack occurring between subsites 3 and 4 [39]. PPA structure shows a network of water molecules occupying the cleft of the active site and hydrogen-bonding with polar side chains of amino acids, with the chloride ligands, and with the main chain through amino acids.

1.7.1 Mode of action of enzyme inhibition

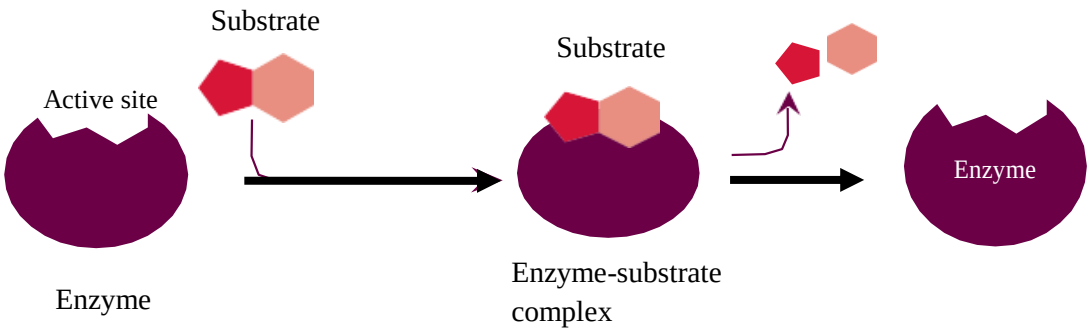


Figure 5:- Mode of action of an enzyme

Enzymes are specific. The mode of action of Alpha-amylase also follows the same pathway as the substrates. The general probable mechanism of the lock and key model is illustrated. In the above figure, enzymes possess the active site. Enzyme catalysis the reaction by binding the substrate to the active site and forms the enzyme-substrate

complex. This binding changes the distribution of electrons in the chemical bonds of a substrate, lowering the activation energy of the reaction and allowing the generation of the final products [42]. The product is released from the active site and regenerates the enzyme for another reaction cycle.

1.7.2Enzyme inhibitor

Enzyme inhibitors are usually low molecular weight compounds that combine with the enzyme to form the enzyme-inhibitors complex either reducing or completely inhibiting the catalytic activity of the enzyme [39]. The most important factors that affect enzyme activity are enzyme concentration, the amount of specific enzyme substrate, P^H of the reaction medium, temperature, and the presence of activators and inhibitors [41]. The binding of the inhibitors to the active sites of the enzyme can block the entry of substrate to the active sites and induce a conformational change that prevents the entry of the substrate to the active site.

1.7.3 Competitive inhibition

During the competitive inhibition, there is a structural similarity between the inhibitors and substrate [46]. There is a competition between the substrate and inhibitors for access to active sites. The inhibitors have an affinity for the active sites, if it binds tightly than the substrates then it is called effective competitive inhibitors. If it binds less strongly then it is called poor competitive inhibitors. In competitive inhibition, inhibitors bind only to the free enzyme not with the enzyme-substrate complex [42].

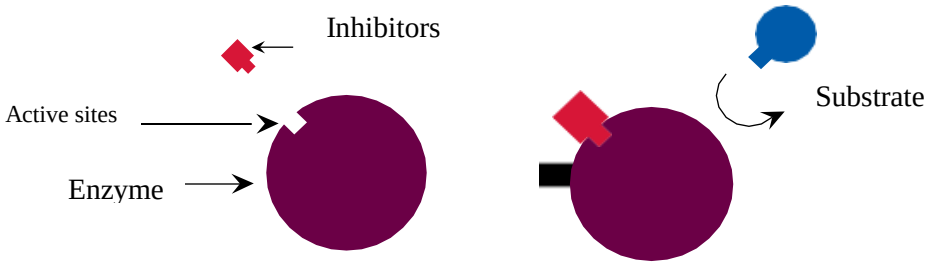


Figure 6:- Competitive Inhibition

1.7.4 Non- competitive inhibition

In non-competitive inhibition, the binding of the inhibitors reduces the enzyme activity. However, it does not affect the binding of the substrate. The affinity of inhibition depends on the concentration of inhibitors only [42]. The inhibitor binds other than the active site of the substrate. The inhibitor's binding substrate does not influence the availability of the

binding site of a substrate. Thus binding of the substrate and the inhibitors are independent of each other and the inhibition cannot overcome by increasing substrate concentration [42]. Non-competitive inhibitors have identical affinity for the enzyme and enzyme-substrate complex.

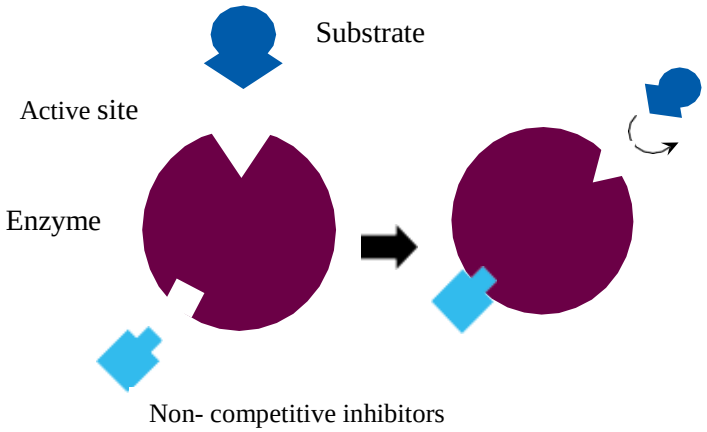


Figure 7:- Non- competitive inhibition

1.7.5 Mixed inhibition

In this type of inhibition, an inhibitor binds to both the free enzyme as well as enzyme-substrate complex, but their affinity is different. Inhibitor binding can be reduced by adding more substrate, but the total inhibition cannot be overcome as in the case of competitive inhibition [42]. This type of inhibition is mostly allosteric, where the inhibitor binds to a site other than active sites to cause a substrate for the active site.

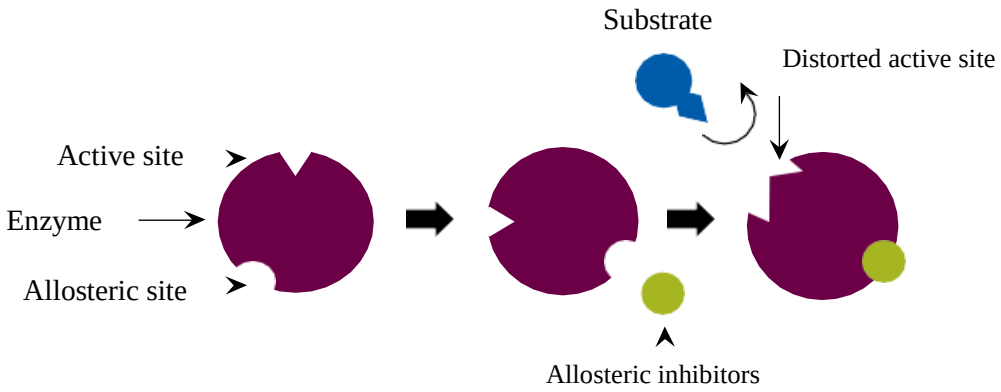


Figure 8:- Mixed inhibition

1.8 Antimicrobial

Gram staining is one of the methods developed by Hans Christian Gram in 1884 A.D [45], to distinguish between the gram-positive and gram-negative bacteria based on their differential staining with crystal violet-iodine complex and safranin as a counterstain. The gram-positive bacteria retain their purple color complex after treatment with 70% alcohol and appear purple. However, gram-negative bacteria decolorized after treatment with alcohol and appear pink in color.

Gram negative bacteria consist of three layers. The first layers consist of the outer membrane (OM), a protective layer that distinguishes gram negative bacteria from gram positive bacteria. The outer membrane has phospholipids bound to the inner membrane and lipopolysaccharide bound to the outer membrane which is responsible to cause endotoxic shock. The outer membrane also contains the porins which assist in the passage of small molecules like amino acid. The second layer is peptidoglycan rigid exoskeleton determines shape to the cell and consists of the repeating unit of disaccharides N-acetyl glucosamine (NAG) and N-acetylmuramic acid (NAM) [43]. The third layer is the inner membrane of the phospholipid bilayer responsible for the multifunctional processes like cell division, transport, and biosynthetic functions.

Gram positive bacteria retain the purple color due to the thick layer of peptidoglycan, under the observation of a microscope. Gram positive bacteria lack the outer membrane and have a thick layer of peptidoglycan that surrounds the plasma membrane to protect the bacteria from harsh environmental conditions [44]. The structural, synthesis, thickness, chemical composition, and extent of cross-linking determine the morphology of bacteria. Bacteria such as staphylococci, streptococci are spherical, and bacillus subtilis is a rod in shape. Furthermore, the cell wall consists of a long anionic polymer of teichioc acids which serves as the sensors and facilitates the movement of different molecules and capsular polysaccharides conveniently attached to the peptidoglycan [44].

1.8.1 *Staphylococcus aureus*

S. aureus is a commensal bacterium in the upper respiratory tract and can act as human health threatening pathogen. About 30 % of populations are colonized with a bacterium. *S. aureus* is among the leading causes of superficial lesions such as skin inflammations and ulcer infections, deep-seated and systemic infections such as osteomyelitis, endocarditis, pneumonia, and bacteremia, and toxemic syndromes such as toxic shock syndrome and staphylococcal scarlet fever both due to toxic shock syndrome toxin and

staphylococcal enterotoxins, staphylococcal scalded skin syndrome and staphylococcal food poisoning [45]. *S. aureus* has numerous mechanisms to evade and subvert the immune system, allowing it to produce infection broadly in immune competent hosts. Cell surface virulence factors containing microbial surface components. These components recognize adhesive matrix molecules, iron-regulated proteins, polysaccharide intercellular adhesion, Protein A, fibronectin-binding proteins, and capsular polysaccharides [44]. *S.aureus* has a large group of exoenzymes, including proteases, glycerol ester hydrolase (lipase), and nucleases. The secreted virulence factors, e.g. are including highly inflammatory cytolysins mainly α, β, γ, and δ toxins and Panton Valentine leukocidin [45], superantigens (SAgs), enterotoxins toxic shock syndrome toxin-1 (TSST-1); and exfoliative toxins A and B.

1.8.2 *Salmonella spp.*

Typhoid fever is one of the fatal diseases causes in humans by *Salmonella typhii* and *paratyhphii*. Both the typhii and paratyphii causes relapsing fever [44]. *Paratyphii* is moderate than *Typhii* but sometimes virulent in outbreaks. Paratyphi causes more resistance to the antibiotics. Typhoid is rare in industrialized countries as improved sanitation but in developing countries high rate and has interrupted transmission through contaminated water. *Typhi* and *Paratyphi* A are members of different O antigen serogroups, D1 and A, based on a minor difference in lipopolysaccharide sugars [43]. Despite this difference, microarray data on shared gene content indicates that *Paratyphi* A and *Typhi* are closely related, with *Paratyphi* A more distant from other members of serogroup A12. Lateral transfer of serogroup genes has been observed, and so *Paratyphi* A arose by transfer of serogroup A genes into a strain very similar to *Typhi* [43]

1.9 Antioxidant DPPH assay

This 2,2-Diphenyl-1-picrylhydrazyl (DPPH) decoloration assay was first reported by Brand Williams and co-workers [47]. The DPPH assay has become quite popular in natural antioxidant studies. One of the reasons is that this method is simple, highly sensitive, and commercially available. This assay is based on the measurement of the reducing ability of antioxidants toward DPPH•. The ability can be evaluated by electron spin resonance (EPR) or by measuring the decrease of its absorbance. The DPPH• radical is one of the few stable organic nitrogen radicals, which bears a deep purple color. The color turns from purple to yellow followed by the formation of DPPH upon absorption of hydrogen from an antioxidant. Antioxidant assays are based on the

measurement of the loss of DPPH color at 517 nm after reaction with test compounds, and the reaction is monitored by a spectrometer. The percentage of remaining DPPH• (DPPH•REM) is proportional to the antioxidant concentration, and the concentration that causes a decrease in the initial DPPH• concentration by 50% is defined as EC_{50}.

The DPPH assay is considered to be mainly based on an electron transfer (ET) reaction, and hydrogen atom abstraction is a marginal reaction pathway [48]. This reaction is stoichiometric concerning the number of hydrogen atoms absorbed. Therefore, the antioxidant effect can be easily evaluated by following the decrease of UV absorption at 517 nm [50]. To standardize the results from various studies, Gallic acid, Quercetin, Trolox equivalent (TE) unit has been used. Trolox is a commercial water-soluble vitamin E. The antioxidant activity of a sample is expressed in terms of micromoles of equivalents of Trolox per 100 g of sample (TE/100 g).

Electron transfer reaction mechanism

$$X^{\bullet} + AH \longrightarrow X^{-} + AH^{\bullet+} \quad (1)$$

$$AH\bullet + \overset{H2O}{\longleftrightarrow} A\bullet + H3O^{+} \quad (2)$$

$$X^{-} + H3O^{+} \longrightarrow XH + H2O \quad (3)$$

$$M(III) + AH \longrightarrow AH^{+} + M(II) \quad (4)$$

Hydrogen transfer reaction mechanism.

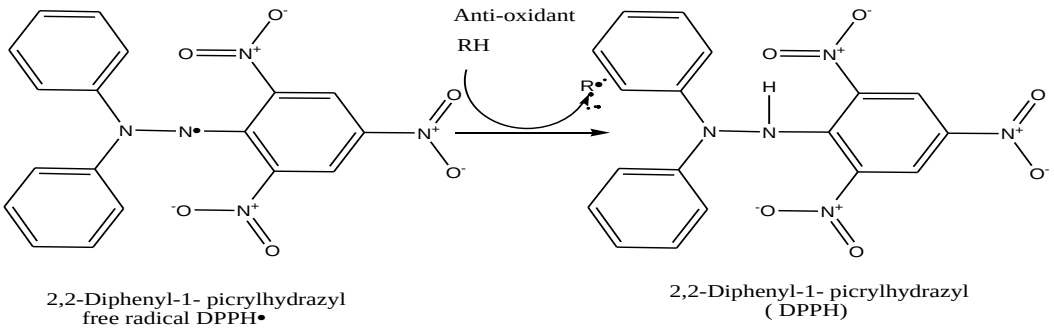


Figure 9:- Reaction involved in DPPH antioxidant assay

Therefore, numerous studies on antioxidants present in plants have been conducted using the DPPH assay, including fruits and vegetables, medicinal plants, cereals, beans, spices, herbs, tea, etc.

1.10 Chemistry of polyphenol content by Folin-Ciocalteu method

The original Folin-Ciocalteu method developed in 1927 A.D originated from chemical reagents used for tyrosine analysis in which oxidation of phenols by a molybdotungstate reagent yields a colored product with λmax at 745-750 nm [49]. The F-C assay has widely used as a measure of total phenolics in natural products, and the basic mechanism is an oxidation/ reduction reaction.

$$Na_2WO_4/Na_2MoO_4 \longrightarrow (\text{phenol-MoW11O40})^{-4}$$

$$\text{Mo (VI) (yellow)} + e^- \longrightarrow \text{Mo (V) (blue)}$$

Although the method is simple, sensitive, and precise the reaction is slow at pH and it lacks specificity. Singleton and Rossim proved the method with Molybdotungsto phosphoric heteropolyanion reagent [47].

$$3H_2O - P_2O_5 - 13WO_3 - 5MoO_3 - 10H_2O$$

And

$$3H_2O - P_2O_5 - 14WO_3 - 4MoO_3 - 10H_2O$$

That reduced phenols more specifically than the λmax for the product is 765 nm. They also made the mandatory steps and conditions to obtain reliable and predictable data for the procedure such as proper volume ratio of alkali and F-C reagent, optimal reaction time and temperature for color development, monitoring of optical density at 765 nm, and use of Gallic acid as the reference standard phenol [50]. However, several papers have replaced the recommended gallic acid reference standard with catechin equivalents, tannic acid equivalents, chlorogenic acid equivalents, caffeic acid equivalents, protocatechuic acid equivalents, vanillic acid equivalents, and ferrulic acid equivalents.

1.11 Chemistry of flavonoid content by Colorimetric method

Total flavonoid content was determined according to two mostly applied spectrophotometric methods based on the formation of aluminum-flavonoid complexes.

This method is modified for the various types of flavonoids. The use of aluminum chloride in this assay is to develop deep yellow-colored complexes of the flavonoids and subsequent measurement of the absorbance at 420 nm using Quercetin as the standard [50]. This method is further modified for the estimation of flavones and flavonols by using $AlCl_3$, while 2, 4-dinitrophenyl hydrazine was used to assess flavanones. In this method, potassium is used after the aluminum chloride and absorbance was taken in 415 nm, which is a very rapid, inexpensive, and widely accepted method. The main use of aluminum chloride in a colorimetric method is to forms acid-stable complexes with the C-4 keto group and either the C-3 or C-5 hydroxyl group of flavones and flavonols and it also forms acid labile complexes with the orthodihydroxyl groups in the A- or B-ring of flavonoids [51]. During the analysis of complex of flavonoid with aluminum-complexes formed by flavonols with C-3 and C-5 hydroxyl groups, such as galangin, morin, and kaempferol and with extra ortho-dihydroxyl groups, such as rutin, quercetin, quercitrin, and myricetin, had maximum absorbance at 415-440 nm. λmax of the complexes formed by chrysin and apigenin which have only the C-5 hydroxyl and C-4 keto groups were at 395 and 385 nm, Flavone compound investigated, luteolin, which has the C-5 hydroxyl group and the ortho dihydroxyl groups in B ring formed a complex that showed a strong absorption at 415 nm respectively [49]. In a compromise, therefore, the wavelength 415 nm was chosen for absorbance measurement.

1.12 In vitro α-amylase inhibition assay

To analysis enzyme activity in the hydrolysis of starch as substrate, two methodological principles can be used, the formation of reducing sugars products of the reaction or the consumption of starch (substrate) [52].

1.12.1 Starch-iodine enzyme assay

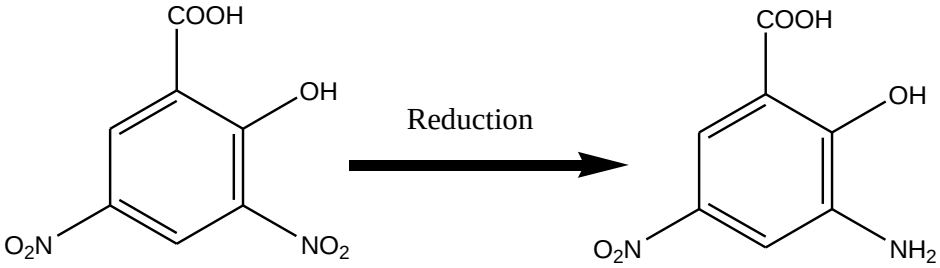
The measurement of the substrate consumption is possible using the starch-iodine staining. This method is based on the binding of iodine to terminals of the starch's polymeric chain that results in a blue colored complex that can be also quantitatively monitored by UV–vis-spectrophotometry [37]. In contrast to the DNS method, the staining is instantaneous with the simple addition of a staining solution containing I_3 that results from the stepwise dissolution of KI and I_2 in water [52]. The analytical protocol can also be conducted in mild conditions (room temperature), bypassing the heating steps and the protocol complexity that is present in the DNS method, as well as in other

alternative methods for the same analytes. The dark blue color indicates the presence of starch only and the yellow color indicates the absence of the starch. Brown color indicates the partially degraded starch in the reaction mixture. The inhibitor from the extracts inhibits the enzyme and cannot hydrolysis the starch so the dark blue color complex remains when the extracts are present [41]. However, in the case of the absence of inhibitor no complex color is developed, which indicates that starch is completely hydrolyzed by α-amylase.

1.12.2 DNS method of enzyme assay

The 3, 5-Dinitrosalicylic acid (DNS) method was first introduced by Sumner in 1921 A.D. for assaying reducing sugars in urine [47]. This method tests for the presence of a free carbonyl group (C=O), the so-called reducing sugars, for example glucose and fructose. Simultaneously, reducing sugar reacts with 3,5-dinitrosalicylic acid (DNS) and reduced to 3-amino,5-nitrosalicylic acid under alkaline conditions.

Reductions



3,5-Dinitrosalicyclic acid (yellow)

3 amino,5-nitro salicyclic acid (Orange-red)

The above reaction scheme shows that one mole of sugar will react with one mole of 3, 5-dinitrosalicylic acid [40]. However, it is suspected that there are many side reactions, and the actual reaction stoichiometry is more complicated than that previously described. The type of side reaction depends on the exact nature of the reducing sugars. The type of side reaction depends on the exact nature of the reducing sugars. Different reducing sugars generally yield different color intensities so, it is necessary to calibrate for each sugar. In addition to the oxidation of the carbonyl groups in the sugar, other side reactions such as the decomposition of sugar also compete for the availability of 3, 5-dinitrosalicylic acid. The DNS method comprises a complex and labor-intensive protocol that includes heating and the use of potentially harmful reagents (e.g. phenol) [52].

1.13 Objectives of the study

1.13.1 General Objectives

The general objective of the study is to carry out in vitro α-amylase inhibition activity and antimicrobial activity of the methanolic extracts of local Tea *C. sinensis, P. arillata,* and *B. asiatica.*

1.13.2 Specific Objectives

The specific objectives of the study are:-

- To collect the ethnomedicnal important plants
- To prepare the methanolic extracts of local Tea *C. sinensis, P. arillata,* and *B. asiatica.*
- To perform the Phytochemical Screening.
- To carry out the Quantitative analysis of total phenolic content and total flavonoid content of the Methanolic extract of local tea *C. sinensis, P. arillata,* and *B. asiatica.*
- To evaluate the Antioxidant activity of Methanolic extracts
- To evaluate the α-amylase inhibition activity of methanolic extract local Tea *C. sinensis, P. arillata,* and *B. asiatica* by iodine-starch enzyme method.
- To perform the fractionization by using three solvents Hexane, Dichloromethane, and Ethyl acetate in separating funnel.
- To evaluate the α-amylase inhibition activity of fractions of Hexane, Ethyl acetate, and Dichloromethane of methanolic extracts of local Tea *C.sinensis,* and *B. asiatica* by DNS enzyme method.
- To perform the column chromatography and Thin layer chromatography.
- FTIR- analysis is done on the 20% methanol in ethyl acetate fractions isolated from the column chromatography of the Methanolic Extract of Tea *C. sinensis.*

CHAPTER-II

2. REVIEW OF LITERATURE

The relationship between medicinal plants and the human use of medicinal plants might be the research for the researchers. The use of medicinal plants plays a vital role in human society from birth to the living and to death too either in the form of drugs or directly. The diversity of the world with the geography and the culture might have provided the different aspect of the importance in the uses of the medicinal plant in human society. The phytochemical constituent might also vary with the challenging climates. Therefore, the natural product and advancement of technology are boon to human society to exist in the globe. However, the evolvement of different diseases such as corona pandemic, an adaptation of microorganisms with drug and challenge of living with the pernicious non-communicable diseases always keeps us in a vulnerable condition. The drug with harmful side effects and less efficiency is a monolithic problem. Based on these challenges a large number of investigations is carried out throughout the world. To sum up the current condition of research on this particular field and their future roadmap, the literature survey on the *B. asiatica, C. sinensis,* and *P. arillata* has been done by collecting different papers published in different journals via google scholar, Pubmed, and depicted as follows.

2.1.1 Medicinal importance of *C. sinensis*

Rasheed A and Haider M (1998) reported that *C.sinensis* extracts were effective against dental caries such as *E. coli, S. salivarius, and S. mutans* [53].

Vinson J. A and Dabbagh Y.A et al (1998) reported that Green tea and Black tea both could improve the risk factors for heart disease [54].

Juhel C et al (2000) reported that obesity is managed by reducing fat digestion through marked inhibition of digestive lipases, such as gastric and pancreatic lipases, especially by saponins, which lead to altered lipid emulsification in gastric or duodenal media [55].

Han Q et. al (2011) reported that Green tea water-soluble polysaccharide fraction has an inhibitory effect against α-amylase, which leads to decreased blood glucose levels [56].

Islam M, et al(2011) reported that 5 % aqueous extract of white tea (*C.sinensis* only buds) is effective to reduce most of the diabetes associated abnormalities in a steptozotocin induced diabetes model of rats [57].

Hartjen P et. al (2012) reported that EGCG can be used to prevent the sexual transmission of HIV [58].

Yang et al. (2014) demonstrated that green tea extract (0.2%–0.5%, w/v) inhibited lipid accumulation during adipogenesis in preadipocytes by reducing expression of transcription factors C/EBPα and PPARγ [59].

San C., et al (2015) reported that pretreatments with black tea extract (0.3–5 μg/mL) and theaflavin-3,3'-digallate (0.03–0.5 μg/mL) for 30 min improved endothelium-dependent relaxations in homocysteine (endoplasmic reticulum stress inductor) treated cultured rat aortic endothelial cells [60].

Bb, Lustosa and Polegato B et. al (2016) reported that reduces cardiac hypertrophy, improves systolic and diastolic dysfunction, restores the antioxidant enzyme activity, and stimulates the glucose pathway and mitochondrial function [61].

Sun T.L and Liu Z et. al (2016) reported that polyphenol in green tea, possesses a potent antioxidant capacity to reduce both arsenic and doxorubicin induced cardiotoxicity [62].

Pang J, et. al (2016) reported that consumption of green tea decreases the risk of myocardial infarction (MI) in a dose-dependent manner up to ≥4 cups/day [63].

Carneiro B.M, et. al (2016) investigated that(-)-epigallocatechin gallate (EGCG) molecule containing in Green Tea capable to inhibits the Brazilian strain of Zika virus entry [64].

Sun L et al (2016) reported that green tea polyphenol containing galloyl moiety in catechin increases the inhibition of pancreatic α-amylase, due to enhanced association with the enzyme active sites [12].

Paudel K.R and Lee U.W et al (2016) reported that Chungtaejeon aqueous extracts Korean fermented tea prevent the risk of atherosclerosis, by decrease hepatic cholesterol, total serum cholesterol, and LDL cholesterol in high fat Atherogenic Wistar rats [65].

Imbe H et al (2016) from trails of one hundred fifty-five participants reported that "Benifuuki" green tea, which is rich in methylated catechins (3 g of green tea extract/three times daily for 12 weeks) contributed significantly to reduce serum total

cholesterol and serum LDL cholesterol compared to “Yabukita” green tea or barley infusion (placebo tea) consumers [66].

Pournourmohammadi S et al (2017) reported that EGCG is useful for resensitizing insulin resistant muscle by activating AMPK through the inhibition of glutamate dehydrogenase in muscle and pancreatic ß-cells [67].

Orem A et al (2017) from clinical trials reported that the effective dose was 2.5 g black tea and phytosterol mixture which contains 1 g plant sterols for 4 weeks consumption of newly developed black tea is an excellent beverage for delivering phytosterols and has several beneficial cardioprotective effects in subjects with mild hypercholesterolemia [68].

Wu M et al (2017) found that lipolysis is increased by Polyphenols such as EGCG, which possess anti-adipogenic effects through being a fatty acid synthase (FAS), which is a possible therapeutic target for appetite and weight control [14].

Ray S et al (2017) reported that Enzyme converts Angiotensin I into angiotensin II (vasoconstrictor properties), black tea (15 μg/mL) can inhibit angiotensin I converting enzyme, Antihypertension properties are mainly attributed by thearubigin and theaflavin [69].

Xu et al. (2018) investigated the effect of large yellow tea manufactured in the Anhui Province of China on metabolic syndrome in high fat diet treated mice. This work revealed that yellow tea improved metabolic abnormalities (changes in lipid profile, hyperglycemia, and body weight) [70].

Deng X et al (2018) reported that tea polypeptides from green tee consumption reduced blood glucose and ameliorated diabetic nephropathy in a streptozocin induced mice model by stimulating the AGEs/RAGE/TGF-β1 signaling pathway and inhibiting the NF-κB pathway [31].

2.1.2 Chemical constituents of local tea *C. sinensis*

Lu, Y., et al (2000) reported three Olean-12-ene type triterpenoid saponins were isolated from the methyl esters from tea roots *C. sinensis* var. assamica after treatment with diazomethane, 3- O-α-L-arabinopyranosyl(1 4 3)-β-D-glucuronopyranosyl-21,22-di-O-angeloyl-Rα-barrigenol-23-oicacid,3-O-α-Larabinopyranosyl(1,4,3)-β-D-glucurono pyranosyl-21-O-angeloyl-22-O-2-methylbutanoyl-R1-barrigenol-23-oic acid and 3-O-α-Larabinopyranosyl (1 4 3)-β-D-glucuronopyranosyl-16α-O-acetyl-21-O-angeloyl-22-O-2-methylbutanoyl-R1-barrigenol-23-oic acid [16].

Lianga Y., et al (2001) isolated eight catechins l-epigallocatechin gallate (EGCG), l-gallocatechin (GC), l-gallocatechin gallate (GCG), l-epicatechin gallate (ECG), l-epicatechin (EC), l-catechin (C), l-catechin gallate (CG), l-epigallocatechin (EGC) [n].

Del Rio, D., et al (2004) reported that high-performance liquid chromatography-mass spectrometry (HPLC-MSn) methods for the rapid and routine analysis of more than 30 phenolics in Green and black tea infusions, identified based on their retention time, absorbance spectrum, and MS fragmentation pattern, included (+)-catechin, (-)-epicatechin, theaflavin, and their various gallate derivatives, quercetin and kaempferol mono-, di-, and triglycosides, quinic acid esters of gallic acid and hydroxycinnamates, and the purine alkaloids, caffeine and theobromine [15].

Yoshikawa, M., et al (2008) reported two types of oleanane oligosaccharides triterpene Chakasaponos 5 and chakasaponin 6 and aromatic glucosidae chakasonoide 1 and acetylated flavonoid oligosaccharide chakaclavonside A from the buds of tea *C. sinensis* [71].

Scoparo, C. T., et al (2012) reported the following compounds, based on retention time, UV-spectra and MS fragmentation patterns: catechins, theaflavins and their gallate derivatives; kaempferol, quercetin, and myricetin mono-, di-, tri-, and tetraglycosides; esters of quinic acid and gallic or hydroxycinnamic acids; purine alkaloids, such as caffeine and theobromine and many lipids. Additionally, many novel compounds were previously undescribed, such as saponin isomers and gallic acid esters of four glycosides of myricetin, quercetin, and kaempferol [72].

J. Tan et al., (2016) reported the quantitation of 61 identified metabolites including catechins, dimeric catechins, flavonol glycosides, amino acids, phenolic acids, alkaloids, and nucleosides revealed distinct changes in the phenol pathway [73].

Dai, W., et al (2016) reported that in green teas, 120 glucosylated/galactosylated, 38 rhamnosylated, 21 rutinosylated, and 23 primeverosylated metabolites were detected simultaneously, Additional 27 novel glycosylated metabolites were tentatively elucidated [74].

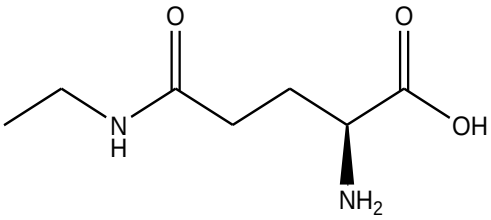
W. Dai., et al (2017) reported the dynamic changes of the metabolites in the tea samples and found theanine, glutamic acid, quercetin, kaempferol, myricetin, apigenin, theaflavin, epigallocatechin gallate, catechin, caffeine [75].

Tan, J., Engelhardt., et al (2017) reported 29 metabolomics White tea. catechins, hydrolysable tannins, phenolic acids, theanine, and caffeine had higher levels in early spring produced than teas in late spring produced. Theaflavins were found highest in mature autumn and lower in spring [13].

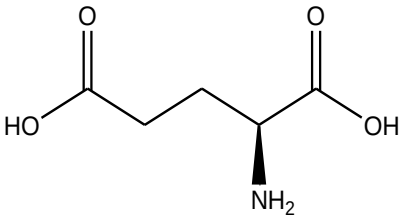
Akhtara N., et al(2020) isolated a total of sixty-three compounds based on their mass and fragmentation pattern by using LC-ESI-QTOF-MS/MS including quantification of caffeine, theophylline, (+)-catechin, (-)-epicatechin, (-)-epicatechin gallate, (-)-gallocatechin, (-)-epigallocatechin gallate and quercetin-3-D-β-glucoside [76].

Yu, X., et al (2020) reported that Untargeted metabolomic analyses detect 129 and 199 annotated metabolites that are differentially accumulated in different tea groups in positive and negative ionization modes, respectively. Each phylogenetic group contains signature metabolites. In particular, diverse classes of flavonoid compounds, such as flavanols, flavonol mono-/di-glycosides, proanthocyanidin dimers, and phenolic acids [77].

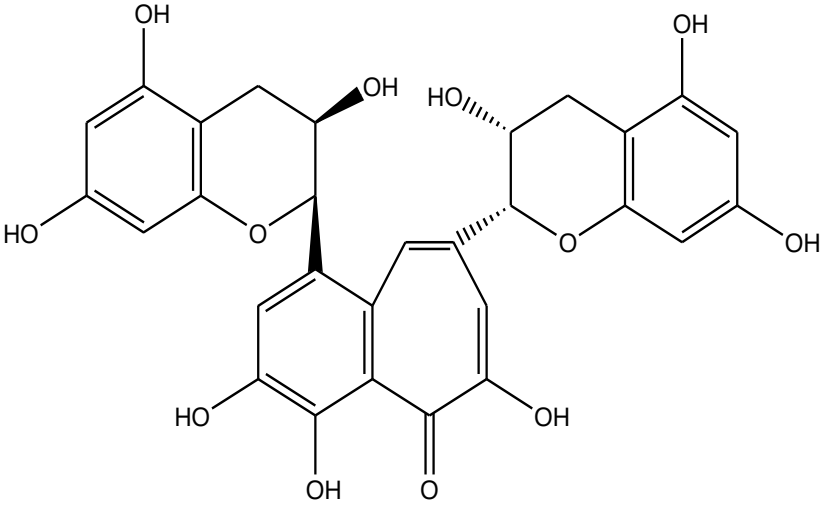
2.1.3 Chemical structures



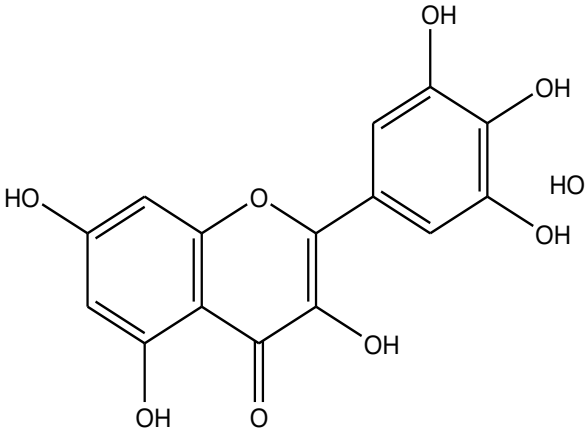
Theanine



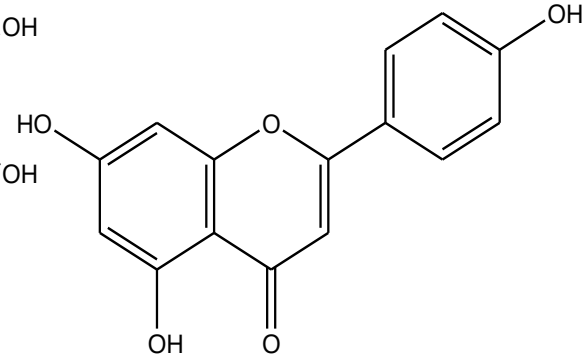
Glutamic acid



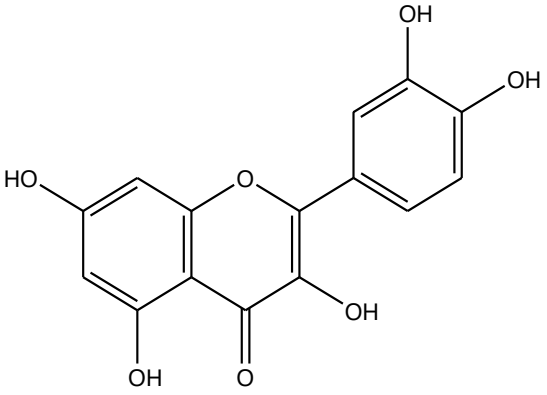
Theaflavin



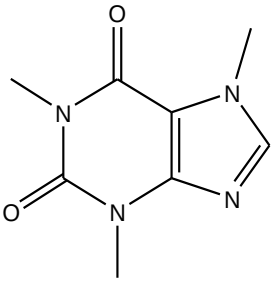
Myricetin



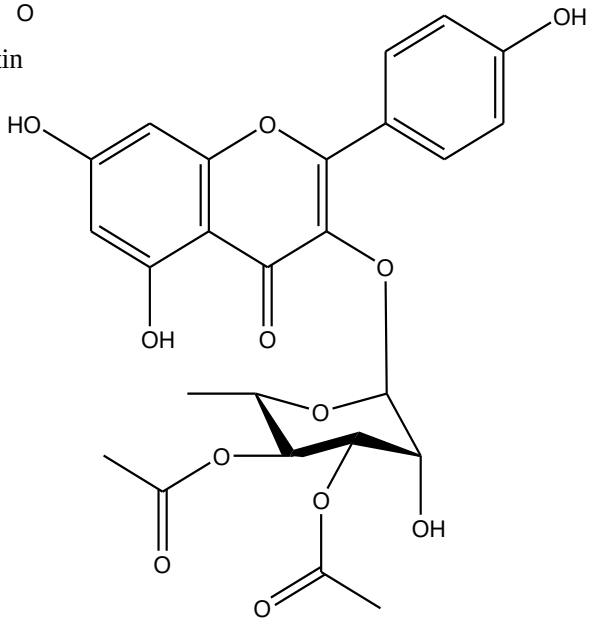
Apigenin



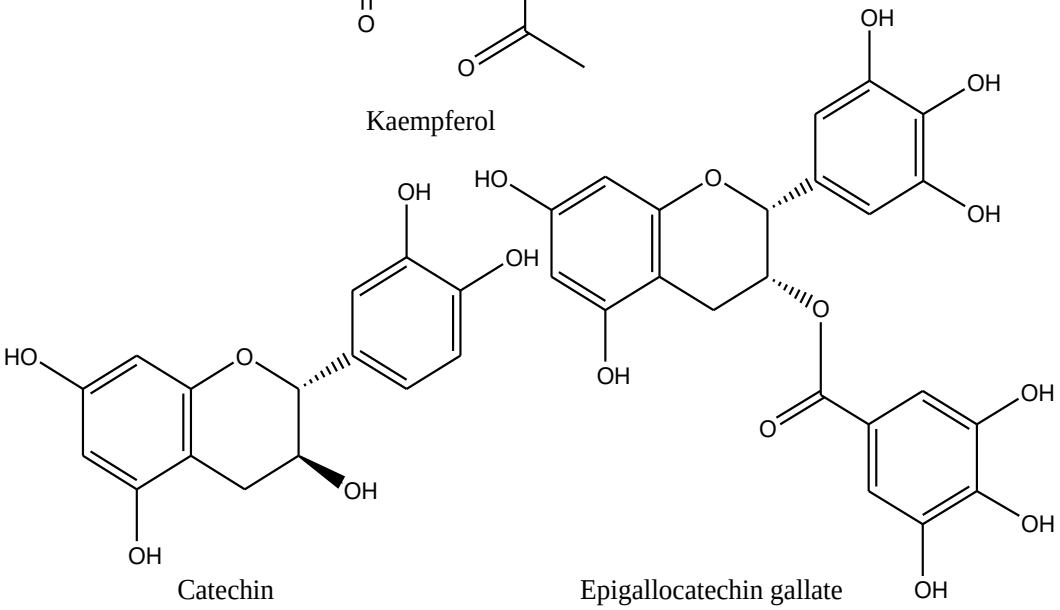
Quercetin



Caffeine



Kaempferol



Catechin

Epigallocatechin gallate

2.2.1. Medicinal importance of *B. asiatica*

Singh S P., et al (1980) illustrated that ethanolic extracts of *B. asiatica* show hypertensive activity [78].

Garg, S. C., et al (1992) reported that the oil has been found to possess good in Vitro antifungal, antibacterial, and anthelmintic activity [19].

Chen et al., (2005) reported that roots, stems, and leaves of *B. asiatica* Lour, have been used in Chinese medicine for the treatment of fever, ache, diarrhea, and articular rheumatism [79].

El-Domiaty M M., et al (2009) reported that antihypotoxicity in the albino rats and illustrate the potential of ethanolic extract of *B.asiatica* [20].

Ali, F., et al (2013) reported a significant inhibitory effect on acetylcholinesterase (AChE) and butylcholinesterase (BChE) in a dose dependent manner. The IC_{50} values of compounds were 5.54 and 8.34 mM against AChE while 30.94 and 35.94 mM against BChE, respectively [23].

Khan, F. A., et al (2015) reported that the potential of ethyl acetate soluble fraction *B. asiatica* is capable to inhibits the Chymotrypsin inhibition and Cytotoxic activity [21].

2.2.2 Chemical constituents of *B. asiatica*

Garg, S. C., et al (1992) reported eighteen various compounds in oil monoterpenoids and sesquiterpenoids and have been found rich in β-caryophyllene oxide (21.7 %), citronellol (16.7 %), and β-caryophyllene (15.8 %). Five minor components (3.1 %) remained uncharacterized [19].

Chen, H., et al (2005) reported that buddlin a new compound was determined from the whole plant of *B. asiatica* [79].

El-Sayed, M M et al (2008) reported the four non phenolic compounds from the methanolic extracts of leaves *B.asiatica* as 1-O-7-d-glucopyranosy l2-methoxy-3-(2-hydroxy-triaconta-3,12-dienoate)-glycerol, 3-O-[α-l-rhamnopyranosyl(154)-7-d-glucopyranosyl-(153)]-[7-d-glucopyranosyl-(152)]-7-d-fucopyranosyl-olean11,13(18)-diene-37,23,28-triol, 3-O-[α-l-rhamnopyranosyl-(154)-7-d-glucopyranosyl(154)-7-d-glucopyranosyl-(153)]-7-d-fucopyranosyl-olean-11,13(18)-diene-37,23,28-triol and 3-O-

[α-l-rhamnopyranosyl-(154)-7-d-glucopyranosyl-(153)]-[7-d-xylopyranosyl(152)]-7-d-glucuronopyranosyl-acid-olean-11,13(18)-diene-37,23,28-triol [80].

Liu, Y.P., et al (2008) reported that four new phenylpropanoid esters of rhamnose A-D, p-methoxycinnamic acid, ferulic acid, and o-methylferulic acid from the arial parts of *B. asiatica*, new compound are 3-O-acetyl-4-O-(p-methoxycinnamoyl)-a-l-rhamnopyranose (1), 3-O-acetyl-4-O-feruloyla-l-rhamnopyranose, 2-O-acetyl-4-O-(O-methylferuloyl)-a-l-rhamnopyranose, 2-O-acetyl-4-O- (p-methoxycinnamoyl)-a-l-rhamnopyranose [67].

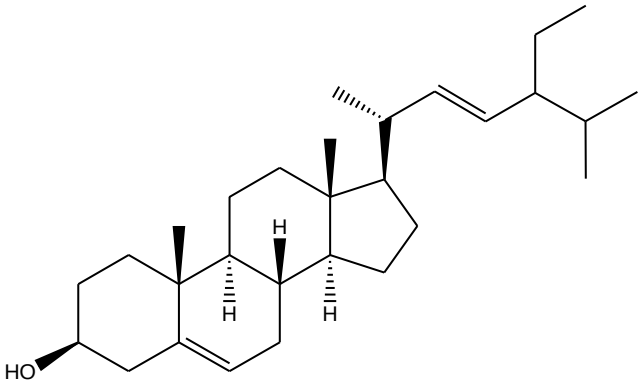
El-Domiaty M M., et al (2009) isolated the new compound as 6-O-(3'',4''-dimethoxycinnamoyl) catalpol from the alcoholic extract of *B.asiatica*, along with the compounds included steroids (β-sitosterol, Stigmasterol, Stigmasterol-O-glucoside, β-sitosterol-O-glucoside), iridoid glucosides (methyl catalpol, catalpol, aucubin), Phenylpropanoids (isoacteoside and acteoside), a triterpene saponin (mimengoside A), flavonoids (diosmin and linarin) in addition to the free sugars mannitol and sucrose [20].

Ali F., et al (2011) reported that seven compounds isolated from the chloroform soluble fraction of *B.asiatica* such as Buddlejone, Dihydrobuddledin-A, Buddledone-B, Ursolic acid,2-phenylethyl-beta-D-glucoside,7-deoxy-8-epiloganic acid, and scutellarin-7-O-beta-D-glucopyranoside [81].

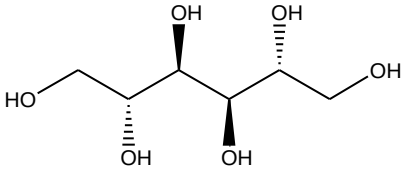
Ali, F., et al (2013) reported that two new benzoates, asiatoate A and asiatoate B, have been isolated from the ethyl acetate soluble fraction of *B. asiatica* whole plant [23].

Khan, F. A., et al (2015) reported that Buddlejol, a new sterol, along with stigmasterol, lignoceric acid, taraxerol, and α-amyrin has been isolated from the ethyl acetate soluble fraction of the antispasmodic plant *B. asiatica* [21].

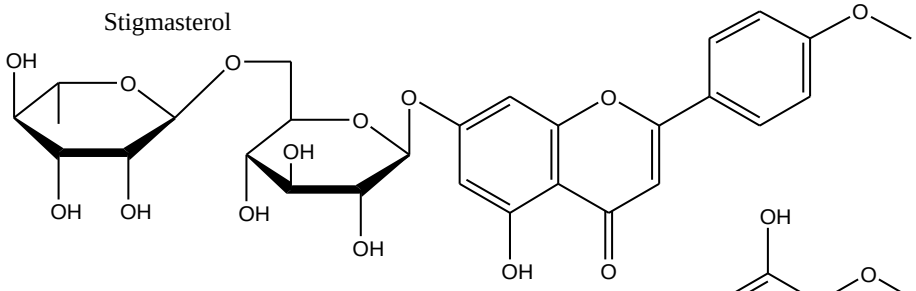
2.2.3 Chemical structures



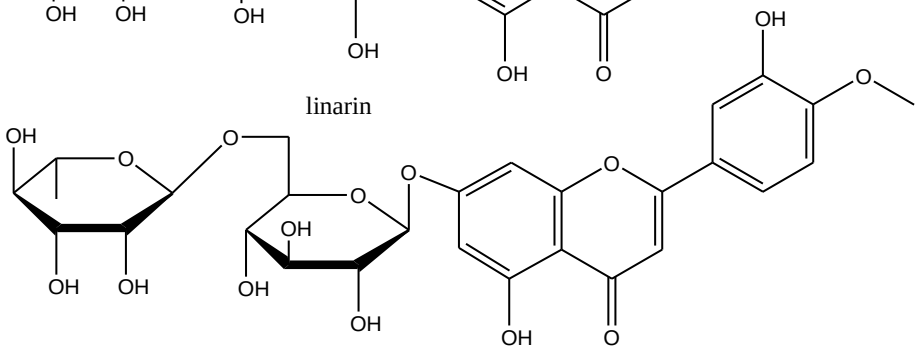
Stigmasterol



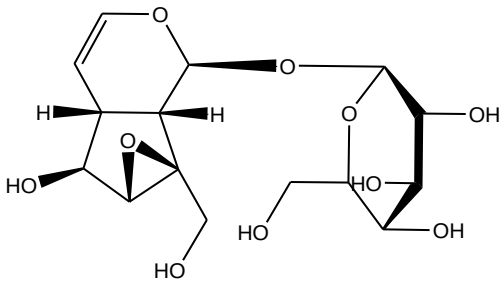
Mannitol



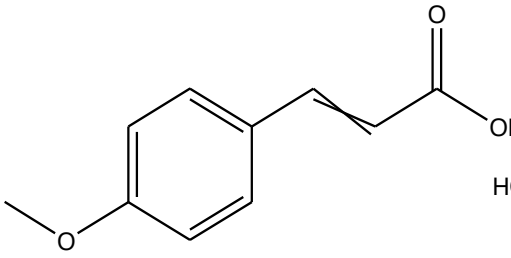
linarin



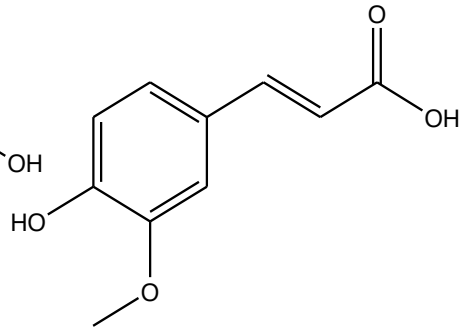
Diosmin



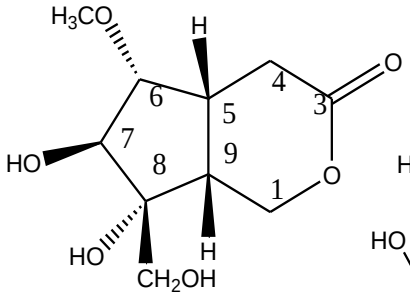
catalpol



p-methoxycinnamic acid



Ferulic acid



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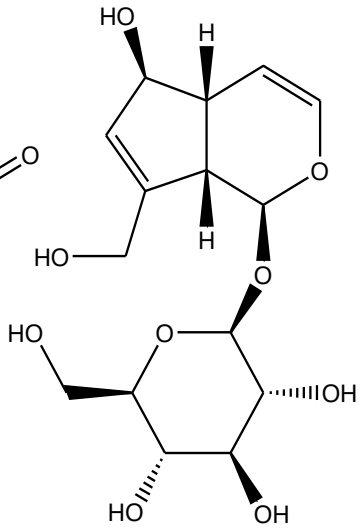
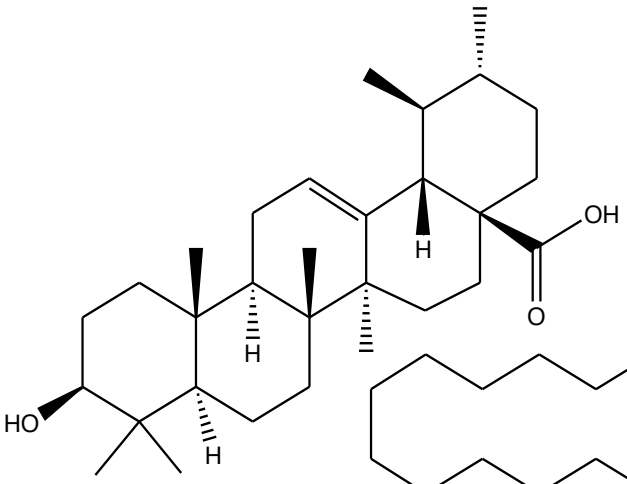
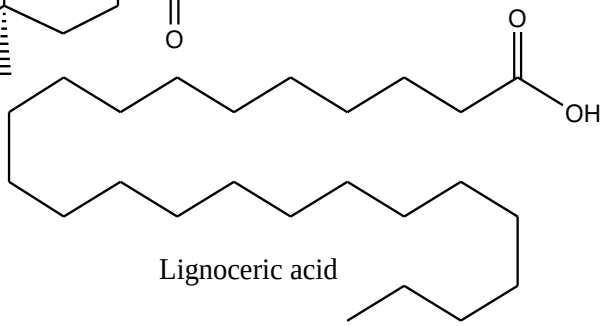
Buddlin



Aucubin



Ursolic acid



Lignoceric acid

2.3.1 Medicinal importance of *P. arillata*

Ghosal et al in (1997) reported that the root of *P. arillata* has been used medically for wound healing, respiratory troubles, chronic bronchial asthma, chronic bronchitis, whooping cough, and diuretic [51].

Fu, J., et al., (2008) reported that *P. japonica* have been used as a well-known tonic and treatment of certain central nervous system dysfunctions including Alzheimer's disease, Parkinson's disease [97].

Chi et al., (2012) reported that the roots of *P. arillata* have been used in folk medicine for the treatment of acute arthritis, rheumatism, pain, and also as a tonic ingredient [98].

2.3.2 Chemical constituent of *P. arillata*

Ghosal, S et al., (1977) reported that stems and roots of *P. arillata* have been shown to contain 1-hydroxy-2,3-dimethoxyxanthone (1), 1,2,3-trimethoxyxanthone, 1-hydroxy-2,3-methylenedioxyxanthone, 1-methoxy-2,3-methylenedioxyxanthone, and a compound that yields 1,3,4-trimethoxyxanthone on methylation. Additionally, senegenic acid and large amounts of esters of glucose and of either protocatechuic acid or gallic acid have been isolated [27].

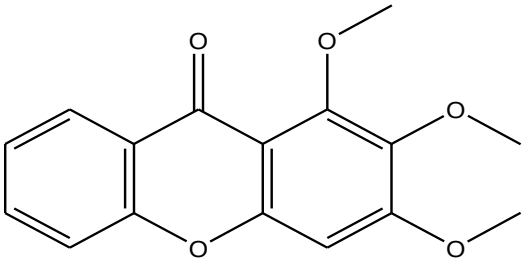
Mingan, O., et al, (1999) reported that six new oleanane- types of saponins, arilloside from the root of *P. arillata* along with the known polygalasaponin [82].

Kobayashi W., et al (2000) isolated that two new sucrose ester arillatoses A and B, four new trisaccharide esters with four known sucrose esters sibiricoses, glomeratose E from the roots of *P. arillata* [25].

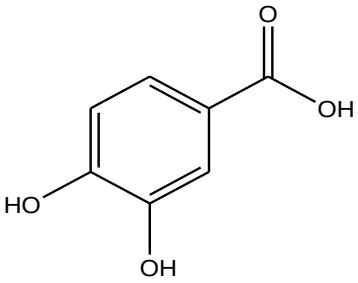
ZhiJun, W., et al, (2000) reported in the stem bark of *P.arillata* fifteen compounds including the derivatives of oligosaccharide esters, xanthones, phenols, and aliphatic acid along with the new seven compounds named arillanin A, B, C, D, and salicyclic acid derivatives [83].

Nguyen D H et al.,(2019) isolated new Oligosaccharide esters polygaloside, a new glucose ester arillatoside with other five known esters from the roots of *P. arillata* [28].

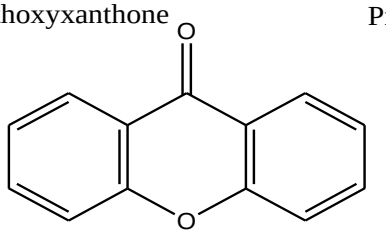
2.3.3 Chemical structures



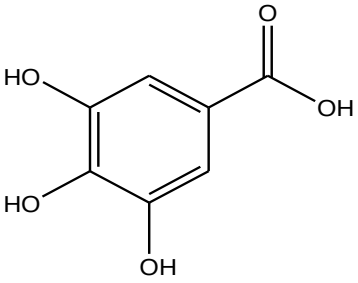
1,2,3-trimethoxyxanthone



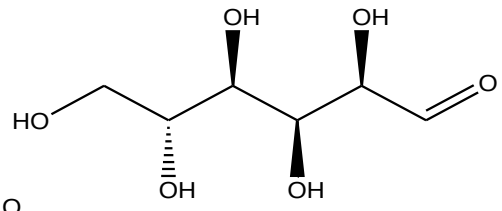
Protocatechuic acid



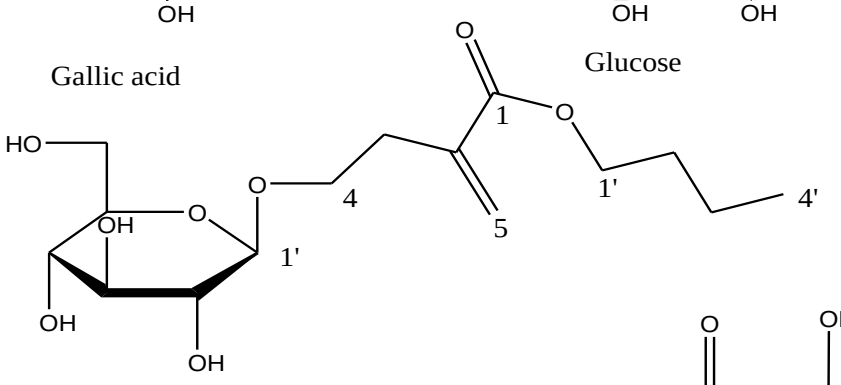
Xanthone



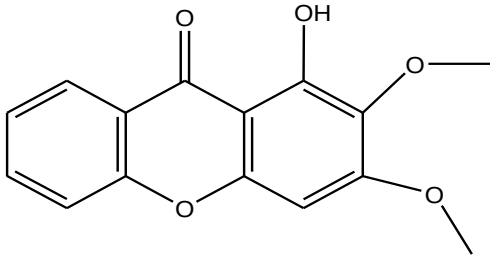
Gallic acid



Glucose



Arillatoside



1-hydroxy-2,3-dimethoxyxanthone

CHAPTER III

3. MATERIALS AND METHODS

3.1 Chemicals and equipment

All the chemicals used in this dissertation were commercially available and analytical grades. Most of the analytical grade chemicals like DMSO, Hydrochloric acid, methanol, ethanol, ascorbic acid, gallic acid, quercetin, aluminum trichloride, sodium chloride, iodine, potassium iodide, sodium carbonate, soluble starch, potassium acetate, folin-ciocalteu reagent, disodium hydrogen phosphate ($Na_2HPO_4.2H_2O$), sodium dihydrogen phosphate ($NaH_2PO_4.2H_2O$), hexane, ethyl acetate, ethanol, etc were from scientific fisher company. Distilled and double distilled were purchased from local vendor. Besides, chemical products like Mueller Hinton agar, Mueller Hinton broth, DNS reagent, DPPH, porcine pancreatic α-amylase (PPA) were analytical grade from sigma Aldrich Company. The equipment and instruments used in this thesis such as 96 well plate, microplate reader, autoclave, FT-IR, micropipette, multiple channel micropipette, etc were the standard grade instruments from Biotek company, Germany. Similarly, rotator evaporator and electric grinder were supplied by the philip harris shenstone, England. Acarbose, neomycin were standard drug from canvax Biotech Company. Lastly, the microorganism *S. typhi, S. aureus* Strain were also a high-grade microorganisms culture at Laboratory in Central Department of Chemistry. Software like graphPad prism 8.0.2, Origin Pro 8.5, Ms. Excel 2007, Chem draw pro 2005, Zotero and Grammarly 2020 updated version were also used during this thesis work.

3.2 Plant Extracts

3.2.1 Collection and identification of the plant

The required different parts of a plant like roots of *P. arillata*, leaves of the *B. asiatica* plant, and local tea *C. sinensis* are collected from the eastern part of Nepal by the ethnomedicinal approach based on used by the local people in different purpose. Similarly, local tea *C. sinensis* is prepared by grinding or rolling the tea leaves by the hands or by using a wooden grinding tool (wookhley). The grinded leaves of tea are further store in the darkroom for (10-12) hours in an airtight condition. Finally, the stored tea grinded is dried in the sunlight. In this way, the traditional tea is prepared which is shown in figure 10. The identification of the plant was done in the National Herbarium and plant laboratories Department of Nepal in the Godavari.

3.2.2 Drying and storage of plant extracts

The collected plant samples were washed with distilled water, dry in shade at room temperature, and finally grounded into fine powder in the electric crushing machine. These dry samples were store in a glass jar for further use.

3.3 Methods of plant extraction

There are various methods of extraction process such as cold percolation, solvent extraction, etc. In this thesis, we applied the cold percolation method by using methanol as a suitable solvent

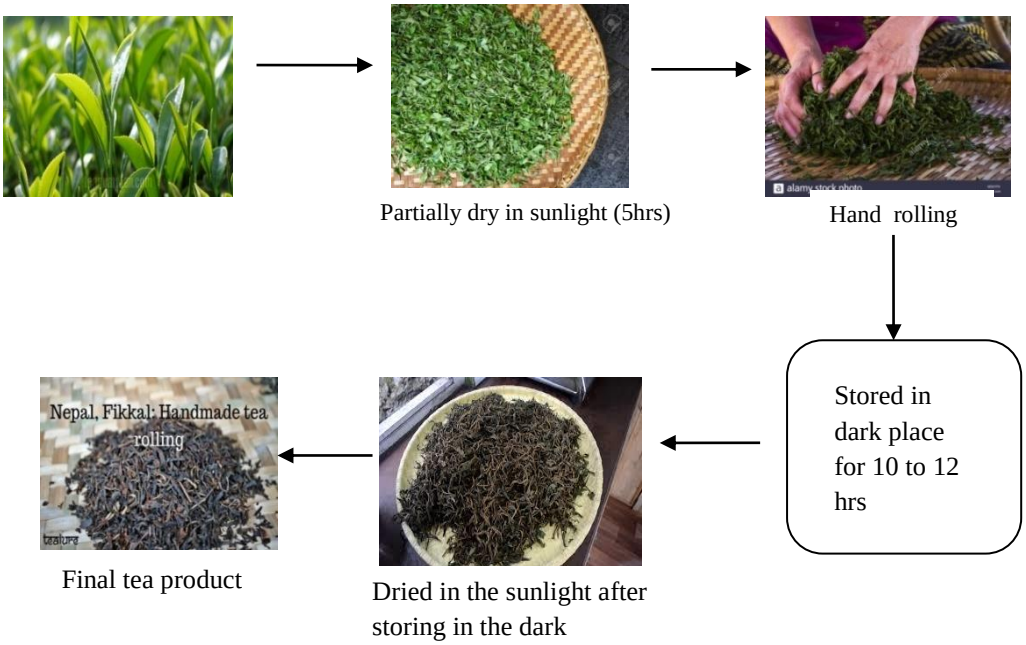


Figure 10:- Hand made local Tea process by local people

3.3.1 Preparation of methanolic extracts

The stored dry powdered of different plants were weighed about 40 grams in physical balance with high accuracy. The weighted extracts were poured into a clean conical flask containing 120 ml methanol solvent in the ratio of 1:3. The solution extracts were shaken in an interval of 24hrs. After 48 hrs, filtration was done by muslin cloth. Then filtrates were collected in another conical flask. Furthermore, methanol was resuspended and the process was repeated three times within a week. Finally, filtrates were filtrated with Whatmann filter paper no.1 and were concentrated in a vacuum rotatory evaporator at 40 ^{o} C, at 150 rpm.

3.3.2 Percentage yield of methanolic extracts

The percentage yield of methanolic extract was calculated by using a physical balance. The final weight obtained was subtracting with the initial weight taken to the initial weight taken, multiple by 100 as shown in the equation.

The percentage yields of every plant extract were estimated by using a simple formula.

$$\text{Percentage yield (\%)} = \frac{\text{weight obtained}}{\text{initial weight is taken}} * 100$$

3.4 Flow chart for Extraction, Screening, and Analysis

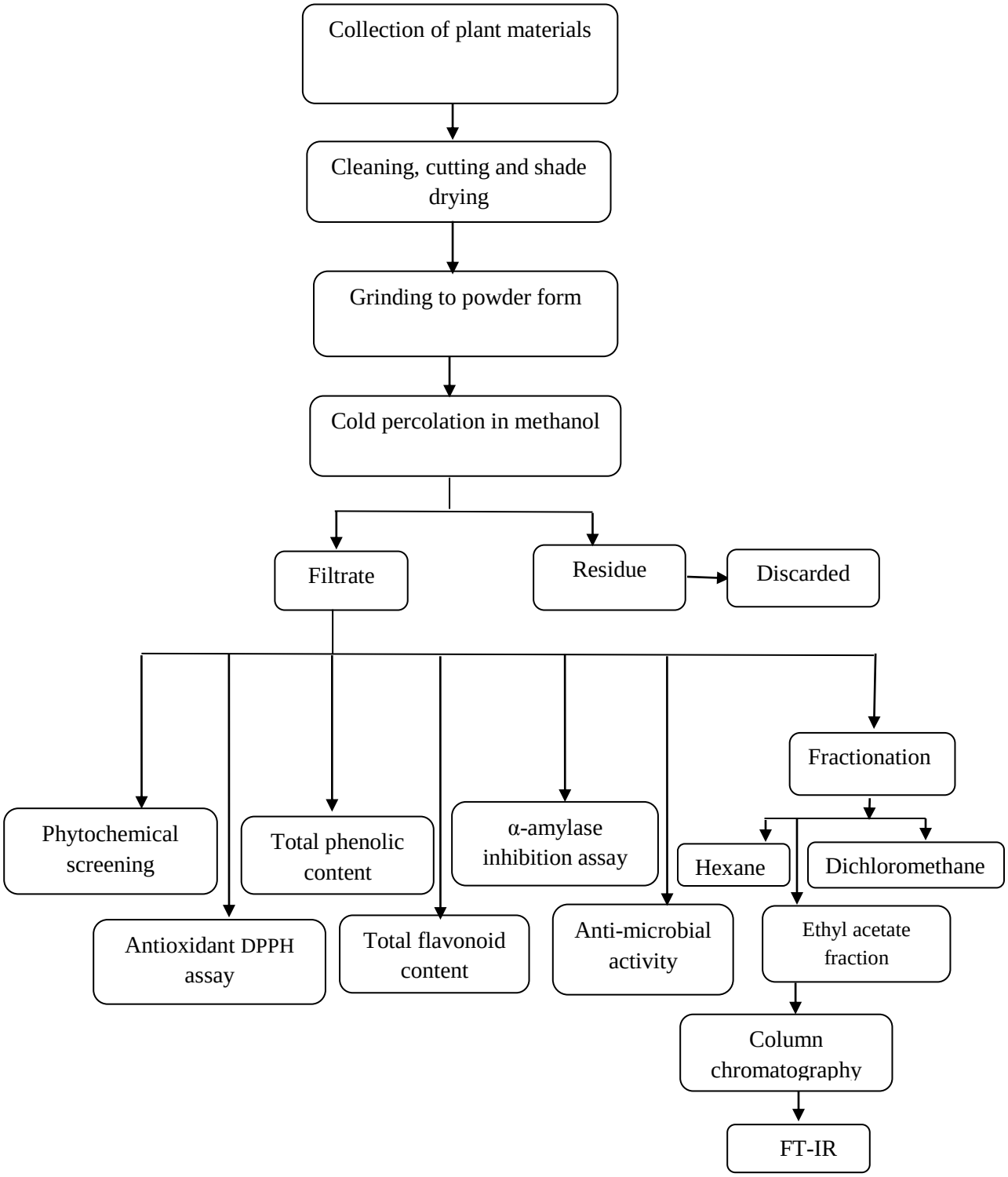


Figure 11:- Flow chart of Analysis (plant extract).

3.5 Phytochemical screening

Phytochemical screening is the qualitative analysis of the identification of bioactive compounds like polyphenol, flavonoids, terpenes, polysaccharides, etc. by using appropriate reagents during the analysis. This type of analysis gives the general idea of different constituents of given plant extracts. The method followed for phytochemical screening was based on the standard protocol [appendix]. The procedure and reagent preparation is given in the index.

3.6 Total phenolic content

The total phenolic content in the plant extract was analyzed by using the Folin calcatueu method of a standard protocol in 96 well plates [49] which was slightly modified.

3.6.1 Preparation of reagents

Sodium carbonate (Na_2CO_3) of 1M was prepared by weighing 5.304 grams in 50 mL of distilled water. Folin-Ciocalteu reagent (1:10) was prepared by diluting 10 mL of commercially available f-c reagent in 100 mL of distilled water.

3.6.2 Preparation of standard Gallic acid

The stock solution of 5000 μg/mL was prepared by dissolving 5 mg in 1 mL ethanol. A prepared stock solution was diluted further to prepare a final solution of 10, 20, 30, 40, 50, 60, 70, and 80 μg/mL. During the analysis, freshly prepared gallic should be used.

3.6.3 Preparation of sample extracts

The plant extracts of the respective plant were prepared in a 1 mL vial of 0.5 mg/ml, from diluting 1 mg/ml stock solution of plant extracts.

3.6.4 Procedure

The total phenolic content of the plant extracts was determined by using Folin-ciocalteau reagent by 96 well plate methods which was a modified form of colorimetric methods. Firstly, 20 μL of different concentrations of standard Gallic acid 10, 20, 30, 40, 50, 60, 70, and 80 μg/mL was loaded on 96 well plates in triplicates which were obtained by diluting a stock solution of 5000 μg/mL with distilled water. Then 20 μL of 50% DMSO and 20 μL plant extract samples of respective plants were loaded on 96 well plates in triplicate and initial reading was taken. After this, each well containing standard gallic acid, 50% DMSO and plant extract sample were loaded with 100 μL of folin ciocalteau reagent followed by 80 μL of sodium carbonate separately. Then, it was left in dark for

15 minutes and finally, after 15 minutes the absorbance was measured at 765 nm by using a microplate reader. Standard gallic acid solutions of different concentrations were used for constructing the standard curve and the total phenolic compound concentration content in the plant extract was expressed as milligram of gallic acid equivalent per gram of dry weight (mg GAE/g) of plant extracts using gallic acid standard curve.

3.6.5 Calculation of total phenolic content

The total phenolic content was calculated using the following relation

$$C = \frac{cV}{m}$$

Where,

C=total content of the phenolic compounds (mg/gm) in gallic acid equivalent (GAE)

c = concentration of gallic acid established from the calibration curve (μg/mL).

V= volume of the extract (mL).

m= mass of extract in gm

3.7 Total flavonoid content

The total flavonoid content in the plant extract was analyzed by using the standard protocol of the colorimetric method [50]. A slight modification was done during the analysis

3.7.1 Preparation of reagent

5% Aluminum trichloride ($AlCl_3$) was prepared by dissolving 5 grams in 100 ml of distilled water. The potassium acetate (CH_3COOK) solution of 0.5 M was prepared by dissolving 2.454 grams in 50 mL distilled water. 50% DMSO solution was prepared by taking 10 mL 99% DMSO in 10 mL distilled water.

3.7.2 Preparation of standard Quercetin solution

Quercetin stock solution (1 mg/mL) was prepared by dissolving 2 mg in 2 mL of methanol solution. Then, initial concentration of 500, 320, 160, 80, 40, 20, and 10 μg/mL was prepared by diluting 1000 μg/mL stock solution, and finally, the final concentration was maintained at 50, 32, 16, 8, 4, 2, 1 μg/ml in a total of 200 μL in each well of 96 well plate.

3.7.3 Preparation of plant extract solution

The stock solution of plant extracts 1000 µg/mL was prepared by dissolving 1 mg plant extract in 1 mL 50%DMSO. The stock solution was further diluted to 500 µg/mL.

3.7.4 Procedure

The total flavonoid content of the extract was measured by the 96 well plate method which was a modified form of the colorimetric method. Firstly, 20 µL of different concentration of standard quercetin 500, 320, 160, 80, 40, 20, and 10 µg/mL were loaded on 96 well plates in a triplicate manner by diluting the standard stock solution of 1000 µg/mL with distilled water and followed by addition of 100 µL distilled water in each well. Then 20 µL of plant extract of 500 µg/mL was loaded on a 96 well plate in triplicate and further added 100 µL of distilled water in each well-containing plant extract to maintain the final volume of 120 µL. Again in each well-containing standard and plant extract sample 60 µL of ethanol, 10 µL 5% aluminum trichloride, 10 µL potassium acetate (0.5 M) was loaded separately. Finally, it was left in dark for 30 minutes and after 30 minutes absorbance was measured at 415 nm by using a microplate reader.

3.7.5 Calculation of total flavonoid content

The following formula was used to calculate the total flavonoid content of the extract:-

$$C = \frac{cV}{m}$$

Where,

C= total flavonoid content mg of quercetin equivalent (QE) per gram (mg of QE/gm)

c = concentration of quercetin established from calibration curve in (µg/mL)

V= volume of extract in mL

m = weight of plant extract in gram (gm).

3.8 Antioxidant activity

The antioxidant activity in the plant extract was analyzed by using a standard protocol as described by the paper [84]. During the analysis slight modifications were done.

3.8.1 Preparation of 0.1 mM DPPH solution

The solution of 0.1 mM DPPH was prepared by dissolving 3.9 mg in 100 mL methanol and stored in dark for further use.

3.8.2 Preparation of Qurecetin solution

The stock solution of 1000 µg/mL was prepared by dissolving 1 mg of Quercetin in 1 mL of 50 % DMSO. Stock solution was further diluted to initial concentration 80, 40, 20, 10, 5, 2.5, 1.25, 0.625 and 0.3125 µg/mL, Then final concentration was maintained at 40, 20,10, 5, 2.5, 1.25, 0.625, 0.3125 µg/mL in total 200 µL volume of each well of 96 well plate.

3.8.3 Preparation of 50 % DMSO solution

50% DMSO solution was prepared by mixing 10 mL of 99% DMSO in 10 mL of distilled water in a 25 mL beaker.

3.8.4 Preparation of plant extracts solution

The stock solution of 500 µg/mL of plant extract was prepared by diluting 50 mg methanolic extract in 1 mL of 50% DMSO. Stock solution was further diluted to different initial concentration of 320,160, 80, 40, 20, 10 µg/mL respectively. Then final concentration was maintained at 160, 80, 40, 20, 10, 5 µg/mL in total volume (200 µL) of each well of 96 well plates.

3.8.5 Measurement of DPPH radical scavenging activity

Firstly, 100 µL of different concentration 40, 20, 10, 5, 2.5, 1.25, 0.625 and 0.3125 µg/mL of positive control Quercetin and extracts of different concentration 160, 80, 40, 20, 10, 5 µg/mL were loaded in a triplicate manner in well of 96 well plates respectively. Then negative control 100 µL of 50 % DMSO was also loaded in triplicate and initial reading was recorded at 517 nm. Immediately, 100 µL of DPPH was loaded in all the triplicate wells of positive control, negative control, and sample extract respectively.

Finally, it was kept in dark for 30 minutes thereafter absorbance was measured at 517 nm by using a microplate reader. A calibration curve was obtained by plotting the graph between % inhibition on the Y-axis and concentration (µg/mL) of qurecetin on the X-

axis. Similarly, the curve of the sample was also plotted and IC_{50} values were calculated. The percentage (%) inhibition was calculated by the following relation:-

$$\% \text{ inhibition} = \frac{(A0-At)}{A0} * 100$$

Where,

A0 = Absorbance of control (i.e negative control)

At = Absorbance of Test sample/ standard

3.9 In vitro α-amylase inhibition assay starch-iodine method.

The Anti-diabetic assay in the plant extracts was analyzed by the iodine-starch method as described by the standard protocol of [41], where slight modification was done during the analysis.

3.9.1 Preparation of iodine solution (5 mM)

At the beginning 5 mM potassium iodide (KI) was prepared by dissolving 0.208 gram in distilled water at 250 mL volumetric flask, then 5 mM iodine solution was prepared by dissolution of 0.0634 gram of iodine in 2 mL of ethanol and finally 100 mL iodine solution was prepared by dissolving in KI solution.

3.9.2 Preparation of phosphate buffer (0.02 M)

Initially, 0.04 M Disodium hydrogen phosphate ($Na_2HPO_4.2H_2O$) and (0.04 M) sodium dihydrogen phosphate ($NaH_2PO_4.2H_2O$) solution were prepared by dissolving 0.712 gram and 0.624 gram in 100 mL of distilled water respectively. Phosphate buffer (0.02 M) was prepared by mixing 50 mL of 0.04 M disodium hydrogen phosphate and 50 mL of 0.04 M sodium dihydrogen phosphate. Then the mixture of phosphate solution was stabilized by further adding 0.072 gram of sodium chloride (NaCl) and P^H6.9 was adjusted. Finally, a mixture of phosphate buffer was diluted with 100 mL of distilled water to obtain 200 mL of 0.02 M phosphate buffer.

3.9.3 Preparation of 0.5 % of a soluble starch solution

The soluble starch solution of 0.5% was prepared by dissolution of 0.05 gram of soluble starch in 10 mL phosphate buffer at P^H 6.9.The solution was slightly warmed at 50ºC.

3.9.4 Preparation of 0.2 Unit α- amylase enzyme

Under the detailed information given, a stock solution of 9 unit α-amylase was prepared by taking 1 mg in 1 mL phosphate buffer in a vial of 2 mL and diluted to 0.4 unit and then to 0.2 unit. The final concentration was maintained at 0.02 units in each well of total volume 200 µL on 96 well plates.

3.9.5 Preparation of stock solution of extract and standard Acarbose solution

Initially, the Stock solution of extract was prepared by weighing 50 mg dry methanolic extract in 1 mL 50% DMSO. The stock solution was further diluted to a different initial concentration of 25000, 12500, 6250, 3125.0, and 1562.5 µg/mL respectively, then final concentration was maintained 2500, 1250, 625, 312.5, and 156.25 µg/mL in each well of total volume 200 µL on 96 well plates.

Similarly, a standard stock solution of Acarbose was prepared by dissolving 25 mg in 2 mL of Phosphate buffer at p^H 6.9. Different initial concentrations 6250, 3125.0, 1562.5, 781.25, 390.6, 195.31, 97.65 and 48.828 µg/mL were diluted from stock acarbose solution of 12500 µg/mL in phosphate buffer. Then, the final concentration was maintained 625, 312.5, 156.25, 78.125, 39.06, 19.531, 9.765, and 4.8828 µg/mL in each well of total volume 200 µL in 96 well plates.

3.9.6 Preparation of 1M Hydrochloric acid 50% DMSO

Initially, the actual strength of Hydrochloric was determined as 11.54 N, from this 1 M hydrochloric acid was prepared by mixing 1 mL Hydrochloric acid (HCL) in 100 mL of distilled water. Similarly, 50% DMSO was also prepared by mixing 10 mL of 99% DMSO in 10 mL distilled water.

3.9.7 Measurement of α-amylase enzyme inhibition by starch-iodine assay

Firstly, negative control, starch only (60 µL phosphate buffer at P^H 6.9), starch + enzyme (40 µL phosphate buffer + 20 µL α-amylase 0.2 unit), Positive control Acarbose (20 µL phosphate buffer + 20 µL α-amylase enzyme + 20 µL Acarbose of different concentration), plant extracts (20 µL phosphate buffer+ 20 µL α-amylase enzyme + 20 µL extracts of different concentration) were loaded in a triplicate manner on 96 well plates and initial reading was taken at 620 nm. Then, the plate was incubated for 10 minutes at 37 º C in an incubator.

After incubation, 20 µL of 0.5%, the soluble starch substrate was loaded in all wells containing negative control, positive control, and plant extracts. Furthermore, incubation of the plate was done for 15 minutes at 37 º C in an incubator. Similarly, after the second incubation 20 µL of Hydrochloric acid 0.1 M solution was loaded in all wells followed by the addition of 100 µL of 5 mM iodine solution. Finally, the optical density (O.D) was measured at 620 nm by using a microplate reader.

The percentage inhibition was calculated by using the following formula as follows:-

$$\% \text{ relative enzyme activity} = \frac{(\text{Enzyme activity of test})}{(\text{Enzyme activity of control})} * 100$$

$$\% \text{ inhibition in the } \alpha\text{-amylase activity} = (100 - \% \text{ relative enzyme activity })$$

The detailed procedure was presented in the flow sheet diagram as follows:-

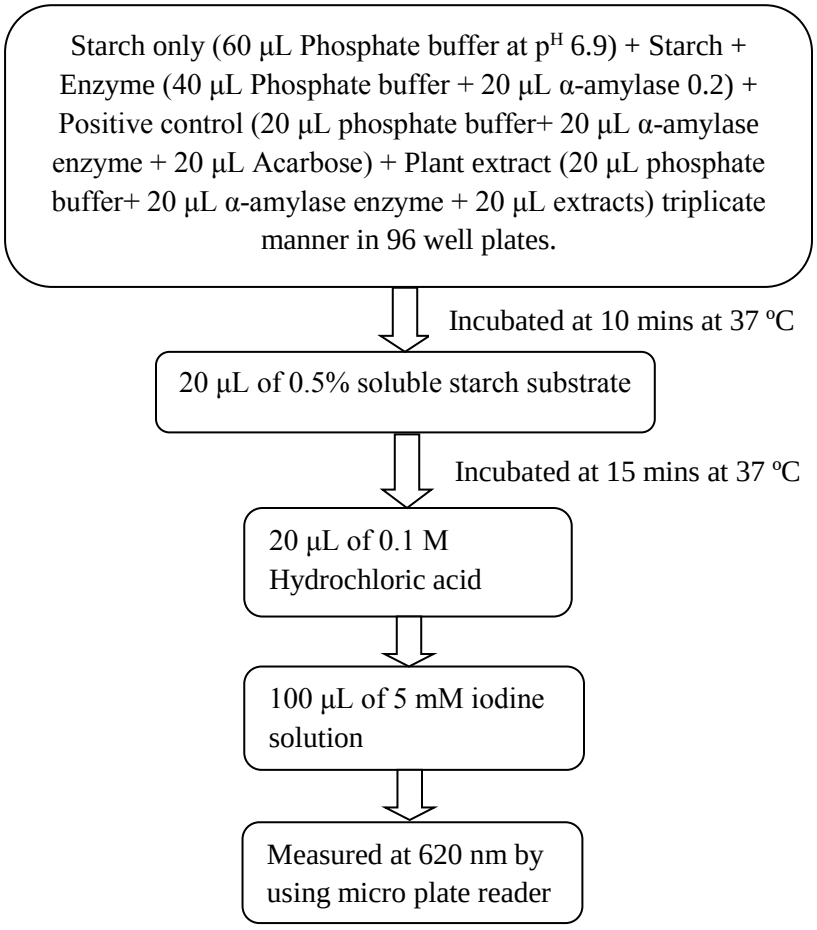


Figure 12.1:- Flow sheet of α- amylase inhibition assay by a Starch iodine method

3.10 In vitro α-amylase inhibition assay by DNS method

This DNS method of alpha-amylase inhibition was carried out by using a standard protocol as mentioned in paper [85], a slight modification was done during analysis.

3.10.1 Preparation of 1 % of a soluble starch solution

The soluble starch solution of 1 % was prepared by dissolution of 1 gram of soluble starch in 10 mL phosphate buffer at p^H 6.9.The solution was slightly warmed at 50 ° C.

3.10.2 Preparation of phosphate buffer (0.02M)

Initially, 0.04 M Disodium hydrogen phosphate ($Na_2HPO_4.2H_2O$) and (0.04 M) Sodium Dihydrogen phosphate ($NaH_2PO_4.2H_2O$) solution were prepared by dissolving 0.712 gram and 0.624 gram in 100 mL of distilled water respectively. Phosphate buffer (0.02 M) was prepared by mixing 50 mL of 0.04 M Disodium hydrogen phosphate and 50 mL of 0.04 M sodium hydrogen phosphate. Then the mixture of phosphate solution was stabilized by further adding 0.072 gram of sodium chloride (NaCl) and maintaining p^H 6.9. Finally, a mixture of phosphate buffer was diluted with 100 mL of distilled water to obtain 200 mL of 0.02 M phosphate buffer.

3.10.3 Preparation of 8 Unit α-amylase enzyme

Under the detailed information given, a stock solution of 9 unit α-amylase was prepared by taking 1 mg in 1 mL phosphate buffer in a vial of 2 mL and diluted to 9 units and then to 8 units. The final concentration was maintained at 4 units in each well of total volume 200 μL on 96 well plates.

3.10.4 Preparation of 2 M sodium hydroxide

The 2 M NaOH solution was prepared in 10 mL of distilled water by dissolving 0.8 grams of NaOH.

3.10.5 Preparation of 5.31 M sodium potassium tartrate in 2M NaOH solution

Sodium potassium tartrate of 5.31 M was prepared by dissolving 12 grams in 8 mL of 2 M sodium hydroxide solution.

3.10.6 Preparation of 96 mM DNS reagent

The DNS color reagent was prepared by dissolving 0.438 gram of DNS reagent in 8 mL sodium potassium tartrate solution and 12 mL of distilled water was added to make the

final volume of 20 mL. Then, the reagent was prepared by heated and stirring in a hot plate magnetic stirrer at 80 º C. Finally, the color reagent 20 mL was prepared.

3.10.7 Preparation of standard Acarbose solution

A standard stock solution of Acarbose was prepared by dissolving 25 mg in 2 mL of Phosphate buffer at p^H 6.9.The stock acarbose solution of 12500 µg/mL in phosphate buffer were further diluted. Then, the final concentration was maintained 781.25, 390.625, 195.3125, 97.65, 48.828, 24.414, 12.207, and 6.103 µg/mL in each well of total volume 200 µL on 96 well plates.

3.10.8 Preparation of plant extracts solution in 2 % DMSO

A stock solution of plant extract was prepared in 2 % DMSO by dissolving 10 mg of extracts in 2 mL vail in vertex and maintaining 5000 µg/mL concentration. The stock solution was further diluted to 2500, 1250, 625, 312.25, 156.125, 78.25, 39.06, and µg/mL respectively.

3.10.9 Measurement of α-amylase inhibition

Initially, 200 µL of α-amylase of 8 unit was taken and added with 200 µL of a fraction of plant extracts of different concentration 2500, 1250, 625, 312.25, 156.125, 78.25, 48.06 µg/mL respectively. As Positive control 200 µL α-amylase was taken with 200 µL of different concentration of acarbose 781.25, 390.62, 195.31, 97.65, 48.82, 24.41, 12.207, 6.10 µg/mL respectively. Similarly, for negative control 200 µL of alpha-amylase was mixed with the 200 µL of 2 % DMSO solvent. Then for the blank 200 µL, phosphate buffer was taken with 200 µL of DNS reagent. Finally, the positive control, negative control, Blank, and extracts in test tubes were incubated at 30 º C for 30 minutes.

After incubation, 200 µL of starch was added to all the test tubes containing the positive control, negative control, blank, and extracts respectively. Then, the solution was incubated at 30 º C for 5 minutes second times. Further, all the solutions containing positive control, negative control, and extracts were treated with the 200 µL of DNS reagent respectively. However, for the blank 200 µL of distilled water was added and heated in a water bath for 15 minutes at 85 º C. Finally, all the solutions of positive control, negative control, Blank, and extracts were diluted with 3 mL of distilled water. The diluted solution was now loaded in the 96 well plates in a triplicate manner and absorbance was read at 540 nm by using a microplate reader.

Percentage inhibition was calculated as the following formula

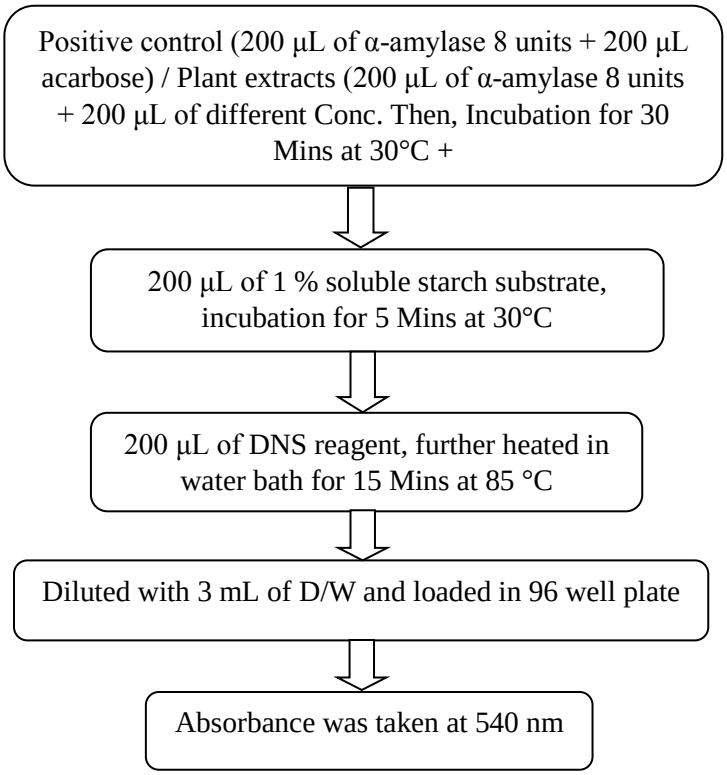
$$\%\ inhibition = \frac{\text{A control} - \text{A Test/Std}}{\text{Acontrol}} * 100$$

Where, Acontrol = $A_{\text{negative control}} - A_{\text{blank}}$

Atest = $A_{\text{test}} - A_{\text{colour sample}} - A_{\text{blank}}$

The detailed procedure was presented in the flow sheet diagram as follows:-

For positive control and plant extracts



For negative control and Blank

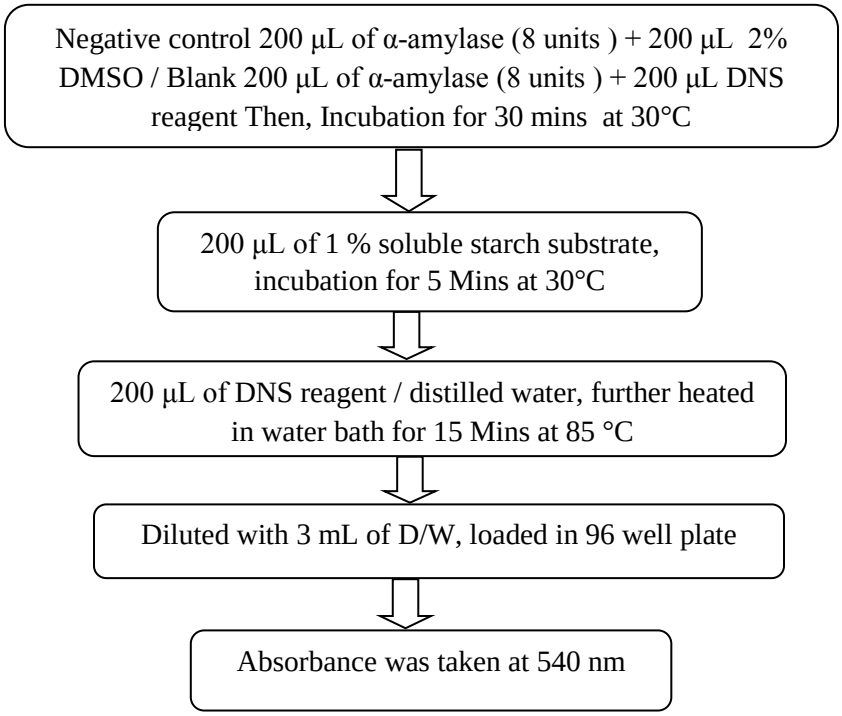


Figure 12.2:- Flow sheet of α- amylase inhibition assay by DNS method

3.11 Antimicrobial activity

The antimicrobial assay was performed by well diffusion technique by using a standard protocol [86]. The equipment like a conical flask, Petri dish, test tubes, micropipette tip, cotton swab, etc. required for this assay was incubated in an autoclave at 120 º C for 15 minutes.

3.11.1 Preparation of Mueller Hinton agar solution

Mueller Hinton agar solution was prepared by dissolving 5.13 gram Mueller Hinton agar in 135 mL of distilled water. The solution was heated for 10 minutes to dissolve completely and further autoclaved at 120 º C for 15 minutes.

3.11.2 Preparation of Mueller Hinton agar plate (MHA-plate)

Mueller Hinton agar plate was prepared in Petri plates by cooling autoclaved Mueller Hinton agar solution at 50 º C then, cooled agar solution was poured at about 25 mL in each Petri plate. Finally, it was cooled for solidification and store in an incubator for further use.

3.11.3 Preparation of Mueller Hinton broth solution

Under the information is given in the Mueller Hinton broth bottle, 0.3 gram of Mueller Hinton broth was dissolved in 15 mL distilled water and autoclaved at 120 º C for 15 Minutes.

3.11.4 Preparation of plant extract sample

50 mg sample extract was dissolved in 1 mL of working solution to prepare 50 mg/mL extract in a vial and finally diluted to 500 µg/ml.

3.11.5 Preparation of positive control and working solution (Negative control)

Neomycin was taken as a positive control. It was prepared by the dissolution of 0.1 gram in 1 mL of distilled water. Similarly, 50 % DMSO was taken as Negative control and prepared by mixing 1 mL of 99% DMSO in 1 mL distilled water.

3.11.6 Preparation of standard culture inoculums

The autoclaved Mueller Hinton broth containing 2.5 mL of each test tube was used as a broth to culture. The colonies of *S. aureus* and *S. typhi* grew in primary culture media on laboratory was transferred by sterilizing inoculating loop to the Mueller Hinton broth (MHB) of a respective test tube, Finally, it was incubated at 37 º C for 24 hours.

3.11.7 Carpet culturing, well drilling, loading of inoculums

Initially, the overnight culture broth was transferred into another test tube containing media and matched the turbidity with standard Mac-farlance solution. Then after matching with standard, Carpet culture was done by using a sterilized cotton swab on Mueller Hinton agar media. The carpeting was done by swabbing all over the Petri dish by rotating at 120º. After carpeting completely, well boreing was done by using cork bores of 30 µL. Finally, sample extract, positive control, negative control were loaded in a respective well of 30 µL by using a micropipette in the sterilized zone and incubated for 24 hrs at 37 º C.

3.11.8 Detection and measurement of a zone of inhibition (ZOI)

After 24 hrs of incubation, the clear zone around the plant extract inhibitor was detected and the Zone of inhibition was measured by using a measuring scale. The diameter of the zone of inhibition was measured from four points of circumference, which gives four diameters.

3.12 Isolation of compound by using thin layer chromatography and silica gel column chromatography (120/60 mesh pore size)

3.12.1 Fractionation

In the beginning, 15 grams methanolic extract of local tea *C. sinensis* was dissolved in 200 ml of distilled water to an obtained aqueous fraction. From the aqueous fraction, the hexane fraction was separated in a separating funnel by adding 300 mL of hexane three successive times. Similarly, the same procedure was followed for the dichloromethane and ethyl acetate fraction for the same aqueous fraction. The three fractions were stored for further analysis.

3.12.2 Selection of solvent and spot

After the fractionization, select the diffuse spot was done by using thin layer chromatography by increasing the polarity of the solvent. The diffuse clear spot was observed at 20 % of methanol in ethyl acetate.

3.12.3 Column chromatography

For the column chromatography ethyl acetate fraction of methanolic extract of local tea *C. sinensis* was chosen, because the extracts show low IC_{50} values in α-amylase inhibition assay and wide diameter of zone of inhibition during the antimicrobial activity test with the microorganisms. Out of the three fractions, ethyl acetate fraction was taken for the column chromatography

Firstly TLC of dry ethyl acetate fraction was performed and distinct spots were observed by the mobile solvent of 20 % methanol in ethyl acetate. Then, 5 gram of dry ethyl acetate fraction was adsorbed in 20 gram of silica (60-120 pore size) by dissolving the extracts in methanol, by gradually churning with a glass rod. The paste of slurry was dried in an oven at 35 º C and powder extract was loaded in a column. Initially, the column was eluted by 100% hexane then gradually polarity of a mobile solvent of ethyl acetate in hexane was increasing up to 100% to obtain the number of fractions. Similarly, for the methanol in ethyl acetate, the mobile solvent was done.

Table 1:- Isolation of compound by column chromatography

S.N	Eluting solvent system	Fraction number	Volume of eluent	Remarks(colour)
1	100 % Hexane	1	100 ml	No
2	5% Ethyl acetate in hexane	2	100 ml	No
3	10% Ethyl acetate in Hexane	3	100 ml	No
4	Up to 95 % Ethyl acetate in hexane	4	100 ml	No
5	100 % Ethyl acetate	5	250 ml	Straw yellow
6	5% Methanol in ethyl acetate	6	300 ml	Straw yellow
7	10 % Methanol in ethylacetate	7	300 ml	Light yellow
8	15 % Methanol in ethylacetate	8	100 ml	No
9	20 % Methanol in ethyl acetate	9	500 ml	Yellow
10	25 % Methanol in ethyl acetate	10	100 ml	No
11	30 % Methanol in ethyl acetate	11	600 ml	Yellow
12	35 % Methanol in ethyl acetate	12	200 ml	Light yellow
13	40 % Methanol in ethyl acetate	13	100 ml	No
14	45 % Methanol in ethyl acetate	14	100 ml	No
15	50 % Methanol in ethyl acetate	15	400 ml	Straw yellow
16	55 % Methanol in ethyl	16	150 ml	Straw yellow

	acetate			
17	60% Methanol in ethyl acetate	17	100 ml	No
18	65 % Methanol in ethyl acetate	18	100 ml	No
19	70 % Methanol in ethyl acetate	19	450 ml	Light green
20	75 % methanol in ethyl acetate	20	100 ml	No
21	80 % Methanol in ethyl acetate	21	100 ml	No
22	85 % Methanol in ethyl acetate	22	300 ml	Green
23	90 % Methanol in ethyl acetate	23	100 ml	No
24	95 % Methanol in ethyl acetate	24	100 ml	No
25	100 % Methanol	25	800 ml	Dark green

3.13 FT-IR analysis

The FT-IR analysis was done for the methanolic extract of local tea *C. sinensis* of ethyl acetate fraction at 20 % methanol in ethyl acetate and 100 % methanol column fraction in Central Department of Chemistry, TU, Kirtipur.

CHAPTER-IV

4.0 RESULT AND DISCUSSIONS

4.1 Percentage yield of sample extracts

Initially, 40 grams of respective powder extract was taken and cold percolation was performed in 1500 mL of methanol three successive times. Then, crude extracts were weighted and the % Yield was calculated. The percentage yield was given in the table as follows:-

Table 2:- Percentage yield of methanolic extracts of local tea *C. sinensis*, leaves of *B. asiatica* leaf, and roots of *P. arillata*.

S.N	Scientific Name	Common Name	Initial weight(gm)	Final weight(gm)	% Yield
1	*C. sinensis*	Local tea	40	20.49	55.14 %
2	*B. asiatica*	Bhimsen pati	40	10.84	27.09 %
3	*P. arillata*	Marcha	40	12.57	31.42 %

The weight of methanolic extract of *C. sinensis*, *B. asiatica*, and *P. arillata* were found to be 20.49 gram, 10.84 gram, and 12.57 gram and yield percentage were found to be 55.14 %, 27.09 %, and 31.42 % respectively.

4.2 Phytochemical screening

Phytochemical screening of the methanolic extract of local tea *C. sinensis*, *leaves of B. asiatica* and *P. arillata* roots are screened with suitable reagent and their result obtained are given in the table below:-

Phytochemical screening indicates contains of phenolic compound, flavonoids, tannins, saponins, terpenoids, quinones, and carbohydrates in tea leaves. *P. arillata* root constituent alkaloids reducing sugar, terpenoids, saponins, glycosides, carbohydrates, sterols. Similarly, *B. asiatica* leaf also contains phenolic compounds, flavonoids, terpenoids, sterols, saponins, reducing sugar, carbohydrates.

Table 3:- phytochemical screening of methanolic extracts of local tea *C. sinensis*, leaves of *B. asiatica*, and *P. arillata* roots.

S.N	Phytochemicals	Local tea *C. sinensis*	*P. arillata* root	*B. asiatica* leaf
1	Alkaloids	-	+	-
2	Flavonoids	+	-	+
3	Reducing sugar	-	+	+
4	Terpenoids	+	+	+
5	Saponins	+	+	+
6	Phenolic compounds	+	-	+
7	Tannins	+	-	-
8	Glycosides	-	+	+
9	Coumarins	-	+	-
10	Sterols	-	+	+
11	Quinones	+	-	-
12	Carbohydrates	+	+	+

Previously, studied papers also prevail that *C. sinensis* leaf constituents polyphenolic compounds like catechin, epigallate-O- catechin, flavonoids like theaflavin, flavin, tannins, saponins. Moreover, *P. arillata* root is not studied extensively, analyze only xanthones, saponins, esters such as sucrose, trisaccharides, and leaves of *B. asiatica* also constituents polyphenolic compounds, flavonoids (diosmin, linarin), triterpene, saponin (Mimengoside A), steroids (Stigmasterol).

The preliminary analysis result of phytochemical screening for the sample may differ for the same plant due to environmental factors like temperature, pressure, minerals contain in soil, and sunlight [23]. The other parameter during analysis like collection time of the plant, size of grinding, percolation, laboratory setup, and chemical grades also causes variation in the analysis. Although it is not reliable, it gives qualitative information about bioactive compounds containing in the plant extract.

4.3 Estimation of total phenolic content (TPC)

During analysis, negative control was 50% DMSO, Positive control was gallic acid and optical density was measured at 765 nm by using 96 well plates microreader in a triplicate manner.

4.3.1Construction of calibration curve

Firstly, the standard curve of gallic acid was plotted between optical density Vs the different concentrations of gallic acid 10, 20, 40, 50, 60, 70, and 80 µg/mL as shown in the figure below.

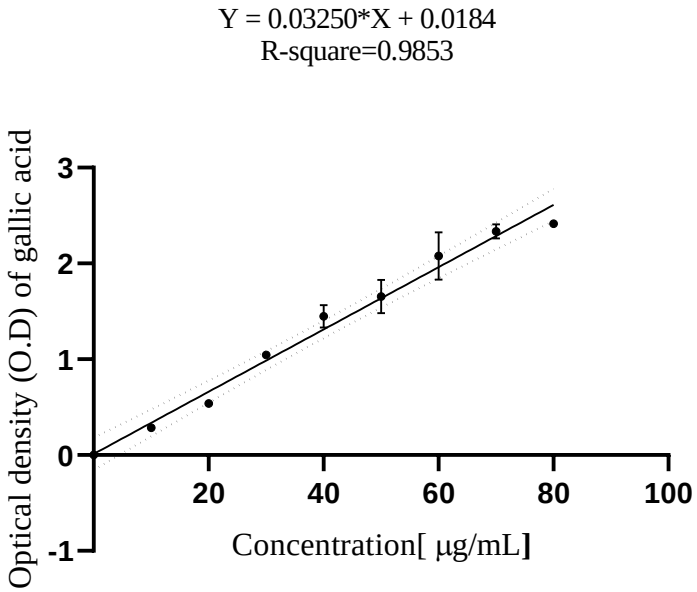


Figure 13:- Standard gallic acid curve for total phenolic Content

4.3.2 Calculation of total phenolic content in plant extracts

The total phenolic content in the plant extract taken under the study was calculated by using regression equation Y = 0.03250*X + 0.01084, R-square = 0.9853 was obtained from the standard curve above. The unknown concentration was calculated by using software GraphPad prism 8.0.2 from the standard curve. Then the total phenolic content of each extract was calculated by using the simple formula C= cV/m. The total Phenolic content was expressed as mg/gm of the tested sample in gallic acid equivalent (GAE) as shown in the table.

Table 4:- Total phenolic content in methanolic extracts of local tea *C. sinensis*, *B. asiatica* leaf, and *P. arillata* roots

S.N	Scientific Name	TPC mg GAE/gm extract
1)	*P. arillata*	44.27 ± 2.97
2)	*B. asiatica*	116.47 ± 6.62
3)	*C. sinensis*	157.17 ± 18.79

Furthermore, it was found that the total phenolic content in *P. arillata* root was 44.27 ± 2.97 mg GAE/gm in dry weight of extract and *B. asiatica* leaf extract contains total phenolic content of 116.47 ± 6.62 mg GAE/gm in dry weight of the extract. Similarly, local tea *C. sinensis* leaf extract contains total phenolic content of 157.17 ± 18.79 mg GAE/gm of the dry weight of the extract. The comparative study among *C. sinensis*, *B. asiatica*, and *P. arillata* plant extract suggested that *C. sinensis* leaf extract contains a higher amount of total phenolic content than *B. asiatica* and total phenolic content in *B. asiatica* leaf extract is higher than *P. arillata* root extracts, which is shown in the Bar-diagram as follows.

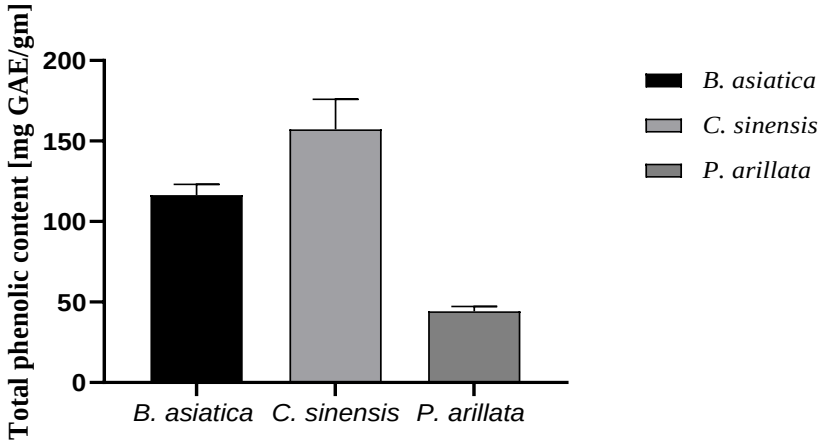


Figure 14:- Bar-graph showing the total phenolic content (mg GAE/gram of dry extract)

On the other hand, previously studied literature suggested that total phenolic content in the black tea extracts was found to be 293.04 ± 4.22 mg/mL GLE as reported by Islam et. al (2011) which is reported higher than our study [57]. Oh., J et al (2013) reported that aqueous and ethanol extracts of black tea contain 82.86 ± 3.18 mg GAE/g and 29.32 ± 0.62 mg GAE/g respectively [87]. Liu., S et al (2017) reported that polyphenol in Quingzhuan dark tea in different fractions of water, ethyl-acetate, n-Butanol fraction contains 18.25 ± 0.21, 62.72 ± 2.63, and 31.09 ± 0.73 mg GAE/g [32]. Tong D.P., et. al (2018) reported that the total polyphenols content of black tea and baked black teas were 362.37 ± 5.00 mg GAE/g and 347.33 ± 1.77 mg GAE/g, respectively [88]. Cheng., Q, et al (2015) found that total Polyphenolic content in water extracts of chloroform, ethyl acetate, and n-butane fractions were 9.8 ± 0.57, 787 ± 26.32, and 310.9 ± 7.25 mg/g [89]. Similarly, Sai K. and Devkota H.P et al (2019) analyzed total polyphenol as 127.48 ± 1.58 mg GAE/g of methanolic extracts of leaves of *B. asiatica* [22]. In the case of roots of *P. arillata* up to this time, no sufficient study was carried out in this particular field.

4.4 Estimation of total flavonoid content (TFC)

During this analysis, Quercetin was taken positive control, 50% DMSO was taken as Negative control respectively. The optical density of each extract taken under the study was measured at 415 nm by using a 96 well plate microreader in a triplicates manner.

4.4.1 Construction of calibration curve

Firstly, to calculate the total flavonoid content in plant extracts. The standard calibration curve was constructed between Optical density Vs Quercetin at different concentrations 50, 32, 16, 8, 4, 2, and 1 µg/ml in a total volume of 200 µL in each well of 96 well plate. The calibration curve of optical density vs concentration is shown below in the figure.

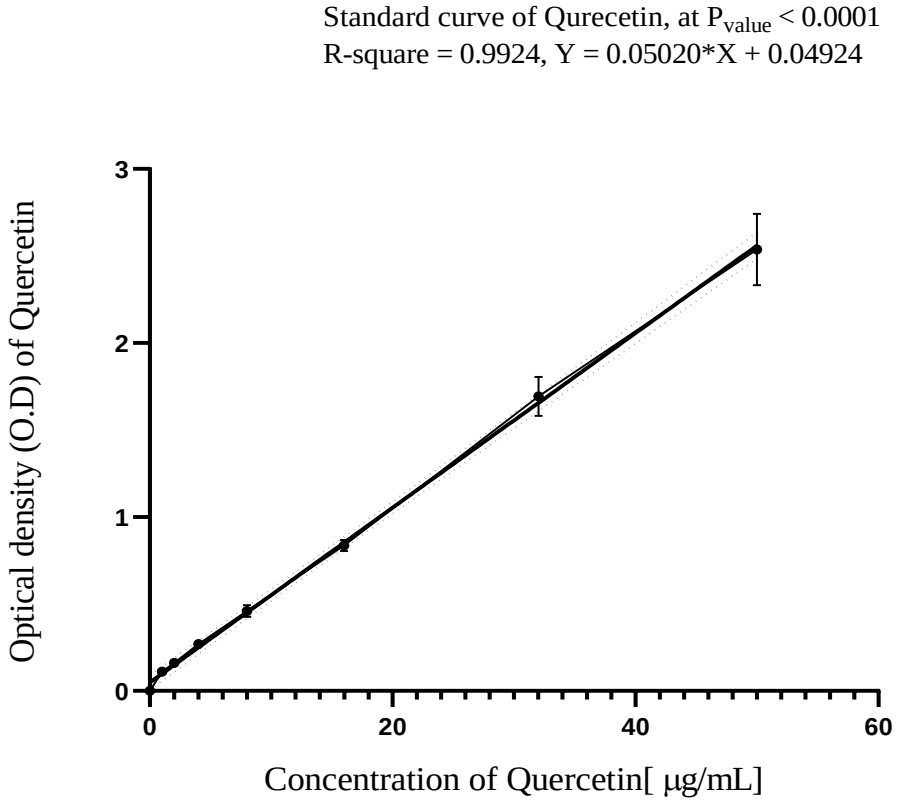


Figure 15:- Standard quercetin curve for total flavonoid content

4.4.2 Calculation of total flavonoid content in plant extract

The total Flavonoid content in plant extracts was calculated by using regression equation Y = 0.05020*X + 0.04924 and R-square = 0.9924, $P_{value} < 0.0001$ were obtained from the standard curve of Quercetin given above. The unknown concentration was obtained by using software graphPad Prism (8.02) from the standard curve. Then total phenolic content in each plant extract was calculated by using the simple formula C= cV/m. The calculated result was expressed in mg QE per gram of extract in dry weight. The total flavonoid content was expressed in mg of quercetin equivalent (QE) per gram dry weight of the test sample is shown in the table.

Table 5:- Total flavonoid content in methanolic extracts of local tea *C. sinensis*, *B. asiatica* leaf, and *P. arillata* roots

S.N	Scientific Name	TFC mg QE/gm extract
1)	*P. arillata*	5.99 ± 1.00
2)	*B. asiatica*	34.28 ± 2.63
3)	*C. sinensis*	37.07 ± 2.24

From the table, it was found that the total flavonoid content in *P. arillata* root extract is 5.99 ± 1.00 mg QE per gram of methanolic extract in dry weight. Then, *B. asiatica* leaf extract contains 34.28 ± 2.63 mg QE per gram of extract in dry weight and the total flavonoid content in local tea *C. sinensis* extracts is 37.07 ± 2.24 mg QE per gram of extract in dry weight. Furthermore, on comparative analysis among the *C. sinensis*, *B. asiatica*, and *P. arillata* plant extracts, it was analyzed that local tea *C. sinensis* extracts contain a higher value of total flavonoid content than *B. asiatica* leaf extracts. Similarly, *B. asiatica* leaf extracts content higher TFC than *P. arillata* root extracts. Therefore, a comparative analysis was elaborated by a simple bar-diagram shown as follows.

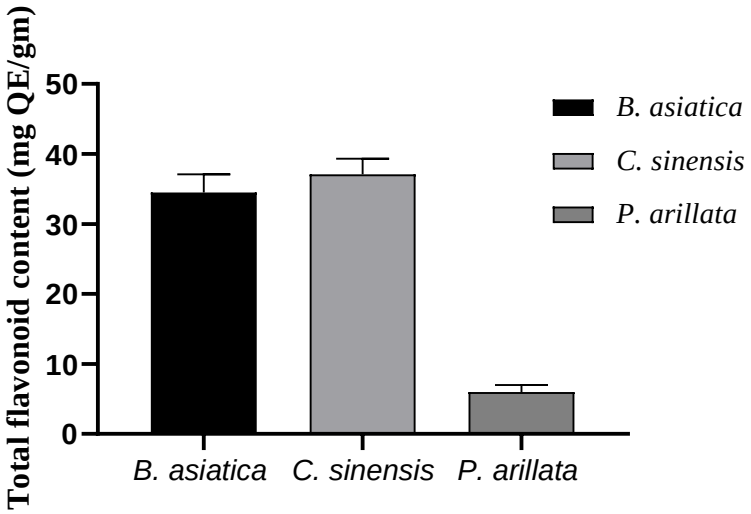


Figure16:- Bar-graph showing the total flavonoid content (mg QE/gram of dry extract)

Furthermore, the survey of literature suggested that total flavonoid content in black tea in water and ethanol extracts is 14.89 ± 0.59 and 5.30 ± 0.03 mg QE/g as reported by Oh., J et. al (2013) [87]. Tong, T., et al (2019) reported that total flavonoid content was 24.80 ± 60 mg of rutin equivalent/gram in Lipton black tea [90]. Similarly, Sai., K and Devkota H, P et. al (2019) found that in the methanolic extracts of *B. asiatica* 648.42 ± 2.88 mg of GAE/g of extract [22].

4.5 Antioxidant activity

4.5.1 DPPH free radical scavenging activity

The DPPH free radical scavenging assay was performed by using quercetin as a standard positive control, 50 % DMSO was taken as negative control and blank in 96 well plates in a triplicate manner. The Optical density was measured at 517 nm by using a microplate reader. The optical density (O.D) decreases with the increase in the concentration of the extracts and the percentage of free radical scavenging is reverse of the optical density.

The Percentage free radical scavenging of plant extracts of local tea *C. sinensis*, *B. asiatica*, *P. arillata*, and standard quercetin was calculated by the given formula. The graph was plotted to compare with different concentration of plant extracts and quercetin Vs concentration which is shown in the graph as follows:-

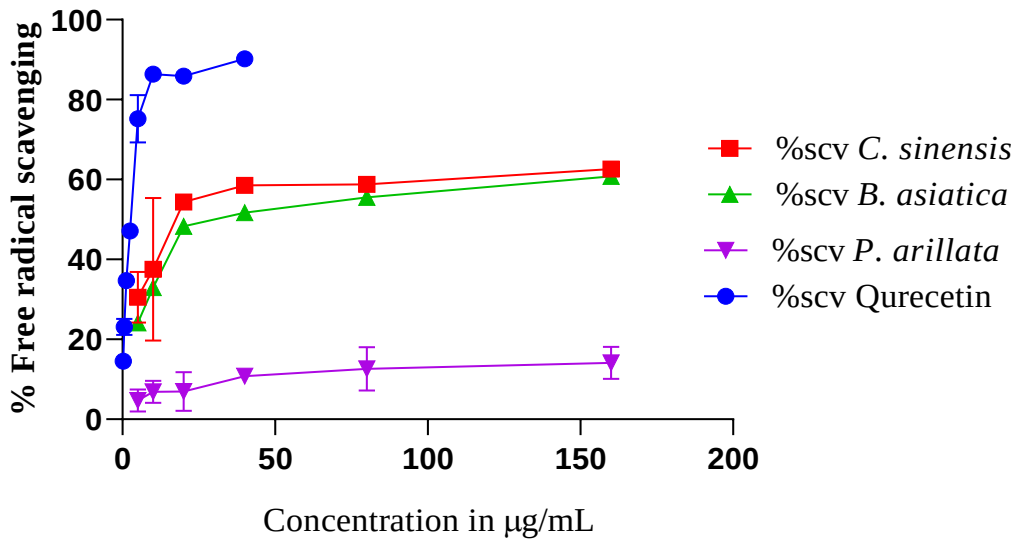


Figure 17:- A plot of % free radical scavenging of methanolic extracts local tea *C. sinensis*, *B. asiatica*, *P. arillata*, and standard Quercetin Vs concentration (µg/mL).

The plot of percentage free radical scavenging versus concentration was used to calculate the concentration of each plant extract for 50 % inhibition of DPPH activity (IC_{50}) by using the software Graph Pad prism 8.0.2. The antioxidant potential activity was compared by calculating the IC_{50} values. The antioxidant potential is inversely proportional to the IC_{50} values. Lower the IC_{50} values higher the antioxidant potential of the plant extracts. The IC_{50} values of methanolic extracts of *C. sinensis*, *B. asiatica* and Quercetin were calculated and shown in the bar- diagram as follows.

IC_{50} values :- Qurecetin = 2.36 ± 0.13 µg/mL
B. asiatica = 26.86 ± 2.00µg/mL
C. sinensis = 19.15 ± 4.32µg/mL

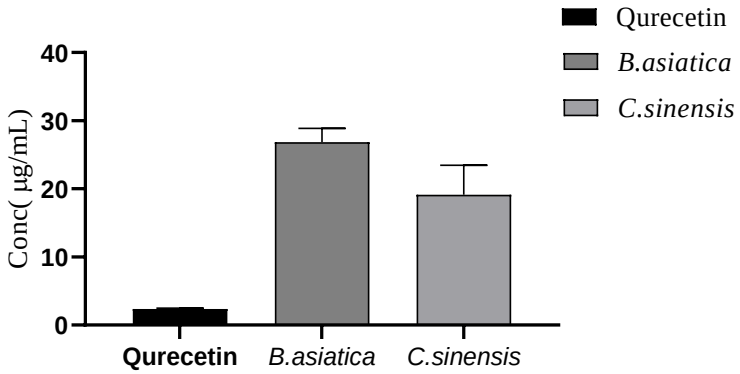


Figure 18:- Bar-graph showing Antioxidant inhibitory activity(IC_{50}) values for Qurecetin and Methanolic plants extract

The IC_{50} values of methanolic extracts of *B. asiatica, C. sinensis*, and Quercetin was found to be 26.86 ± 2.00, 19.15 ± 4.32, and 2.36 ± 0.13 µg/mL respectively. The IC_{50} values of methanolic extracts *B. asiatica* (26.86 ± 2.00 µg/mL), *C. sinensis* (19.15 ± 4.32 µg/mL) was lower than the Quercetin (2.36 ± 0.13 µg/mL). Thus the antioxidant potential activity of both the methanolic extracts of *B. asiatica*, *C. sinensis* was found to be lower than the standard Quercetin. As compare to the Methanolic extracts of *B. asiatica and C. sinensis*, the antioxidant potential of the methanolic extracts of *C. sinensis* was higher than the *B. asiatica*. However, the IC_{50} value of P. arillata was not detected due to percentage free radical scavenging of methanolic extracts was found to lower than the fifty percentage.

Moreover, the fifty percentage inhibitory concentration (IC_{50}) of DPPH was calculated and a comparative study of literature gives the idea about the antioxidant activity of the methanolic extracts. As reported by Isalam., M.S et. al (2011), aqueous Black Tea shows IC_{50} values of 60.26 ± 1.74 mM [57] and Oh J., et. al (2013) analyzed in water fraction and ethanol fraction of black tea as 66.65 ± 1.55 and 28.91 ± 2.15 mg/g [87]. Chang Q., et al (2015) reported water extracts of Qingzhuan tea of chloroform, ethyl acetate, and n-butanol fractions were 244.6 ± 24.62, 6.6 ± 0.90, 17.3 ± 2.89 μg/mL of IC_{50} values [89]. Similarly, Sai K and Devkota H.P., et. al (2019) reported that Methanolic extracts of B. asiatica give the IC_{50} values of 3.04 μg/mL which have a high potential of antioxidant activity as compare to our analysis [22]. According to Sundararajan R., et al (2018) reported that IC_{50} values as 15 μg/mL in methanolic extract of B. asiatica. El-sayed M M., et al (2018) also found that SC_{50} value of 11.99 μg/mL in the methanolic extract of B. asiatica [91]. Tong, T., et al (2019) reported that IC_{50} values of 0.077 ± 0.01 mg/mL in the lipton black tea [90].

4.6.0 In vitro α- amylase inhibition assay

The α-amylase inhibition assay of methanolic extract of local tea *C. sinensis*, *B. asiatica*, and *P. arillata* was analyzed by using acarbose as a positive control, 50% DMSO was taken as Negative control, starch as a substrate, and enzyme as an α-amylase. The fifty percentage inhibitory concentration IC_{50} values were also calculated.

4.6.1 Calculation of α-amylase enzyme inhibition assay by Starch-iodine assay

The α-amylase inhibition assay was calculated by using the formula as given in chapter-3. The calculation was carried out by using software Graphpad prism to determine α-amylase inhibition potential of selected plants from the plot of percentage inhibition Vs concentration curve. Percentage inhibitory concentration of different plant extract and Acarbose were calculated and tabulated. All the reading was carried out in a triplicate manner.

Table 6:- Percentage inhibition of methanolic extracts of local tea *C. sinensis*, *B. asiatica*, roots of *P. arillata* extracts, and Acarbose.

S.N	Concentration (µg/mL)	Acarbose % inhibition	*C. sinensis* % inhibition	*B. asiatica* % inhibition	*P. arillata* % inhibition
1	2500		61.431 ± 7.39	63.511± 5.60	34.670 ± 3.78
2	1250		41.405 ± 2.23	39.185 ± 4.46	23.393 ± 2.86
3	625	94.515 ± 3.29	25.977 ± 1.34	23.738 ± 3.69	21.369 ± 2.34
4	312.5	92.725 ± 1.78	16.640 ± 0.81	12.172 ± 0.27	16.865 ± 0.71
5	156.25	88.163 ± 4.09	13.599 ± 2.25	7.938 ± 0.60	9.514 ± 0.45
6	78.125	64.789 ± 4.84			
7	39.0625	31.536 ± 1.34			
8	19.5313	11.622 ± 1.17			
9	9.765	2.686 ± 1.09			
10	4.8828	1.175 ± 0.40			

Furthermore, the result in the table shows that percentage inhibition of local tea *C. sinensis* plant extracts shows percentage inhibition of 61.431 ± 7.39, 41.405 ± 2.23, 25.977 ± 1.34, 16.640 ± 0.81, and 13.599 ± 2.25 respectively. *B. asiatica* leaf extracts shows percentage inhibition as 63.511 ± 5.60, 39.185 ± 4.46, 23.738 ± 3.69, 12.172 ± 0.27, 7.938 ± 0.60 and *P. arillata* shows the percentage inhibition as 34.670 ± 3.78, 23.393 ± 2.86, 21.369 ± 2.34, 16.865 ± 0.71, 9.514 ± 0.45. Similarly, Standard drug Acarbose shows percentage inhibition of 94.515 ± 3.29, 92.725 ± 1.78, 88.163 ± 4.09, 64.789 ± 4.84, 31.536 ± 1.34, 11.622 ± 1.17, 2.686 ± 1.09, 1.175 ± 0.40 respectively. The comparative graph showing the methanolic extracts of local tea *C. sinensis*, *B. asiatica*, and *P. arillata* have lower percentage inhibition than standard drug acarbose. Among the methanolic extracts of plants, the percentage inhibition of *B. asiatica* was higher than local tea *C. sinensis* and *P. arillata.*

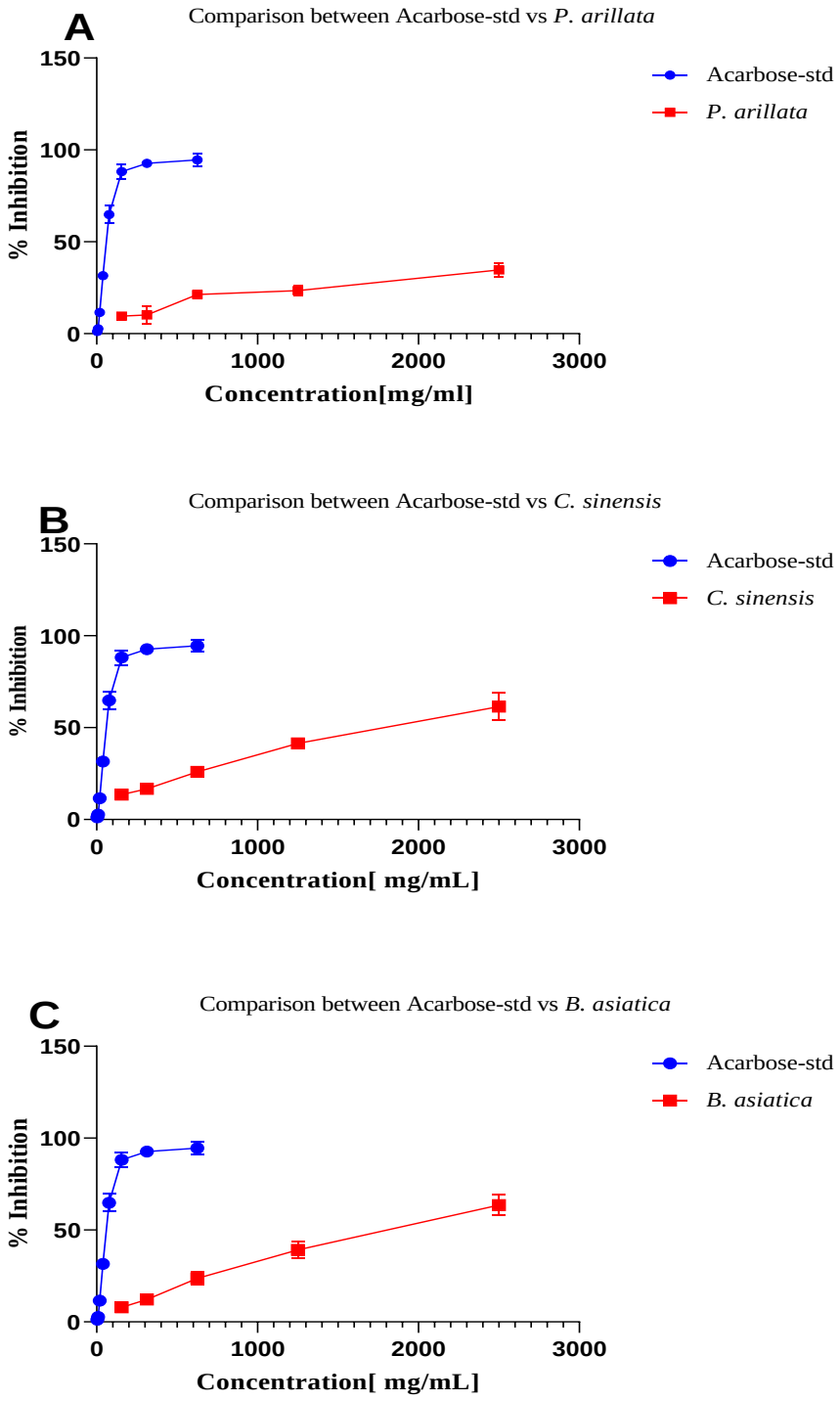


Figure 19:- Graph showing Comparison of a different plant extract with standard Acarbose. A) Comparison of standard acarbose vs *P. arillata*, B) Comparison of standard Acarbose vs *C. sinensis*, C) Comparison of standard Acarbose vs *B. asiatica*.

In addition to, the 50% Percentage inhibitory Concentration (IC_{50}) of different plant extracts of local tea *C. sinensis*, *B. asiatica*, *P. arillata,* and acarbose were calculated and shown in the table. The IC_{50} value of local tea *C. sinensis*, *B. asiatica,* and acarbose were found to be 1737.65 ± 2.21 µg/mL, 1780.00 ± 3.06 µg/mL, and 55.74 ± 2.85 µg/mL respectively. However, *P. arillata* IC_{50} value was not evaluated due to less percentage inhibition. As we know lower the IC_{50} more efficient the extracts. Therefore the local tea *C. sinensis* extracts is more potent compared to the *B. asiatica.* The comparative analysis of *C. sinensis*, *B. asiatica,* and acarbose are represented in the table and bar-diagram as follows.

Table 7:- Showing the IC_{50} value of methanolic extract of local tea *C. sinensis*, *B. asiatica*, and acarbose by starch-iodine assay.

S.N	Sample Extracts	IC_{50}(µg/mL)
1)	*B. asiatica*	1780.00 ± 3.06
2)	*C. sinensis*	1737.65 ± 2.21
3)	Acarbose Tab.	55.74± 2.85

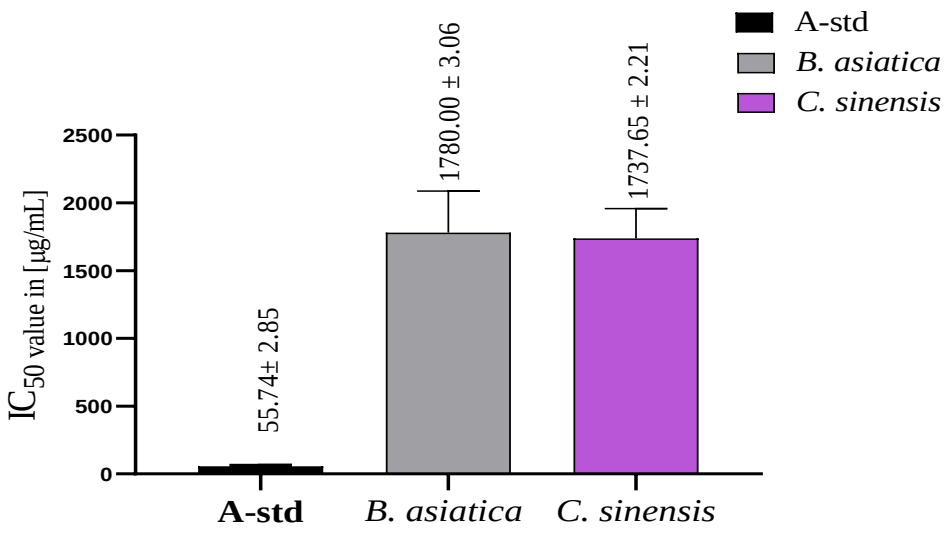


Figure 20 :- Bar-graph showing IC_{50} comparision of plants extracts with Acarbose-std

4.6.2 Calculation of α-amylase inhibition assay by DNS method

The percentage inhibition of local tea methanolic extracts of *C. sinensis* in fraction of Hexane (TH), Dichloromethane (TD), and ethyl acetate (TE) on α-amylase was calculated as given by the formula in the previous chapter. Similarly, the leaves of methanolic extracts of *B. asiatica* fractions hexane (KH), dichloromethane (KD), ethyl acetate (KE) were also evaluated and given in the appendix. And, the standard Acarbose drug percentage inhibition was calculated. However, the methanolic extract of *P. arillata* was not further detected by DNS method because it's TPC, TFC content and even α-amylase inhibition by iodine starch method was found to be lower than *C. sinensis and B. asiatica*. Then, the 50 % inhibitory concentration (IC_{50}) was also calculated from the plot of concentration vs percentage inhibition curve by using the software graph pad prism. The obtained results were given in the table 8 and bar-graph 21 as follows.

Table 8:- IC_{50} values of fractions of local tea *C. sinensis* and *B. asiatica.*

S.N	Plants name	Fractions name	IC_{50}values(μg/mL)
1		Hexane (TH)	1296.13 ± 94.46
2	*C. sinensis*	Dichloromethane (TD)	675.97 ± 66.18
3		Ethyl acetate (TE)	338.52 ± 3.78
4		Hexane fraction (KH)	767.46 ± 18.44
5	*B. asiatica*	Dichloromethane (KD)	411.50 ± 79.07
6		Ethyl acetate (KE)	1051.62 ±12.05
7	Acarbose	A-std	70.89 ± 4.76

IC_{50} values of fractions
A-std = 70.89 ± 4.76
KD = 411.50 ± 79.07
KH = 767.46 ± 18.44
KE = 1051.62 ± 12.05
TE = 338.52 ± 3.78
TD = 675.97 ± 66.18
TH = 1296.13 ± 94.46

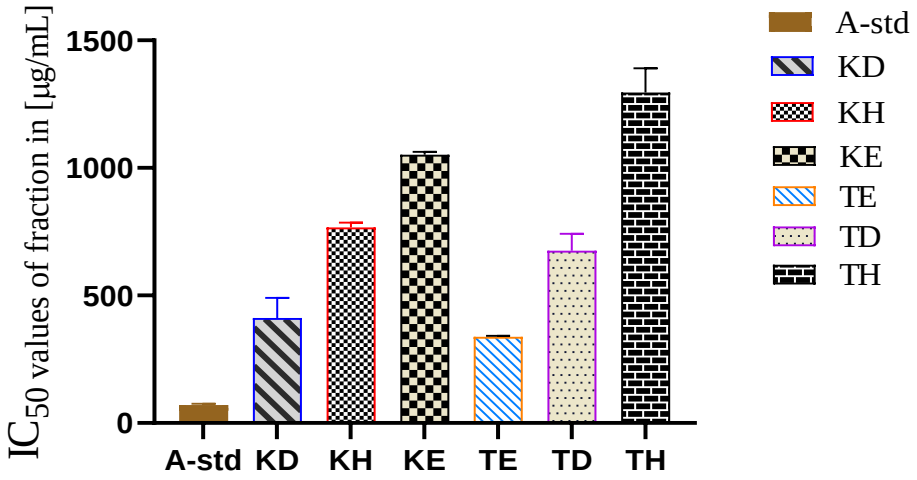


Figure 21:- Bar-graph showing IC_{50} values of fractions of local tea *C. sinensis* (**TE, TD, TH**) and *B. asiatica* **(KD, KH, KE) and Acarbose- std.**

Furthermore, from the table and Bar-graph 50 % inhibitory concentration IC_{50} values of fraction of local tea *C. sinensis* and *B. asiatica* on α-amylase were analyzed. As lower the IC_{50} values higher the inhibition potential content in the extracts. Therefore, the result shows that ethyl acetate fraction of *C. sinensis* (IC_{50} = 338.52 ± 3.78 µg/mL) shows more inhibition potential compare to the dichloromethane (IC_{50} = 675.97 ± 66.18 µg/mL) and Hexane fractions (IC_{50} = 1296.13 ± 94.46 µg/mL). Similarly, the dichloromethane fraction shows more inhibition potential than the hexane fraction. In case of fractions in *B. asiatica* Dichloromethane fraction (IC_{50} = 411.50 ± 79.07 µg/mL) shows greater inhibition potential than the ethyl acetate (IC_{50} = 1051.62 ±12.05 µg/mL) and hexane fraction (IC_{50} = 767.46 ± 18.44 µg/mL). Hexane fraction is more potent as compared to the ethyl acetate fraction. However, all the fractions are less potent as compare to the standard acarbose.

On the literature survey of papers carried out in α-amylase inhibition by methanolic extracts of local tea *C. sinensis*, *B. asiatica*, and *P. arillata* were found as follows. According to Sai., K and Devkota., H.P (2019) reported that methanolic extracts of *B. asiatica show* fifty percentage inhibitory concentration (IC_{50}) values of 1.59 mg/mL [22]. Tong., D.P (2018) reported that IC_{50} of black tea was 1.723 mg/mL and IC_{50} of baked black tea was 1.213 mg/mL [88]. Ylimazer M M., et. al (2012) reported that α-amylase inhibition of IC_{50} values as 34.9 ± 0.90 μg/mL in green tea extract [92]. Gao., J et al (2013) reported that IC_{50} values in green tea extract were 4,020.157 ± 172.363 μg/mL [93]. Xaio., X et. al (2020) reported that tea bags (*C. sinensis*) greater than 1000 μg/mL IC_{50} values [94]. Liu., S, (2017) reported that IC_{50} values vary with the temperature from 20 º C to 100 º C and found IC_{50} values as 12.39 ± 0.15 to 6.31 ± 0.26 mg/mL respectively [32]. Cheng, Q., et. al (2015) reported that IC_{50} of water extract Qingzhuan tea in ethyl acetate and n-butanol fraction were 4.6 ± 0.02, 11.3 ± 0.32 mg/mL [89]. In the case of *P. arillata*, studied data are not found sufficient in this field.

4.7 Antimicrobial activity

Initially, the microbial activity was observed by analyzing plant extracts against the bacteria *S. aureus* and *S. typhi.* Antimicrobial activity of three methanolic plant extracts of local tea *C. sinensis*, *B. asiatica*, and *P. arillata* root was analyzed and their Zone of Inhibition (ZOI) was determined as shown in (figure 22 and table 9). Only two plants extract *C. sinensis* and *B. asiatica* has shown antimicrobial activity towards the *S. aureus* (gram positive bacteria) and their zone of inhibition was found to be 17 mm and 12.5 mm respectively. However, *P. arillata* root does show any antimicrobial activity against *S. aureus* and all the three plant extracts were also unable to show antimicrobial activity toward *S. typhi* (gram negative bacteria).

Furthermore, as compared to the zone of inhibition of drug Neomycin with local tea *C. sinensis* and *B. asiatica* extract. The ZOI of extracts were found to be smaller in diameter than neomycin (25 ± 0.82 mm). Therefore, it was not effective as Neomycin. On comparing the Zone of inhibition of *C. sinensis* and *B. asiatica* extracts, *C. sinensis* extracts show greater than the *B. asiatica.* Thus, *C. sinensis* extract shows more antimicrobial potential than *B. asiatica* extracts. Probably, the purity of extracts might be the possible reason. The comparative study was represented in bar- diagram, and figure as follows.

Table 9:- Antimicrobial screening result of active methanolic plant extracts

	Methanolic plant extracts	Bacteria	Diameter of Zone of Inhibition(ZOI in mm)	Mean ± SD of (ZOI)
1	*C. sinensis*	*S. aureus*	D_1= 17	
2			D_2= 17	17
3			D_3=17	
4			D_4=17	
5	*B. asiatica*	*S. aureus*	D_1=12	
6			D_2=14	12.5 ± 1.00
7			D_3=11	
8			D_4 = 12	
9	*P. arillata*	*S. aureus*	No	No
10	*Neomycin (positive control)*	*S. typhi/ S. aureus*	D_1= 24	
11			D_2= 25	25 ± 0.82
12			D_3=25	
13			D_4=26	
14	*C. sinensis*	*S. typhi*	No	No
15	*B. asiatica*	*S. typhi*	No	No
16	*P. arillata*	*S. typhi*	No	No

Note:- No= No zone of inhibition, D_1, D_2, D_3, and D_4 are the Diameter of the zone of inhibition from the four-point in circumference.

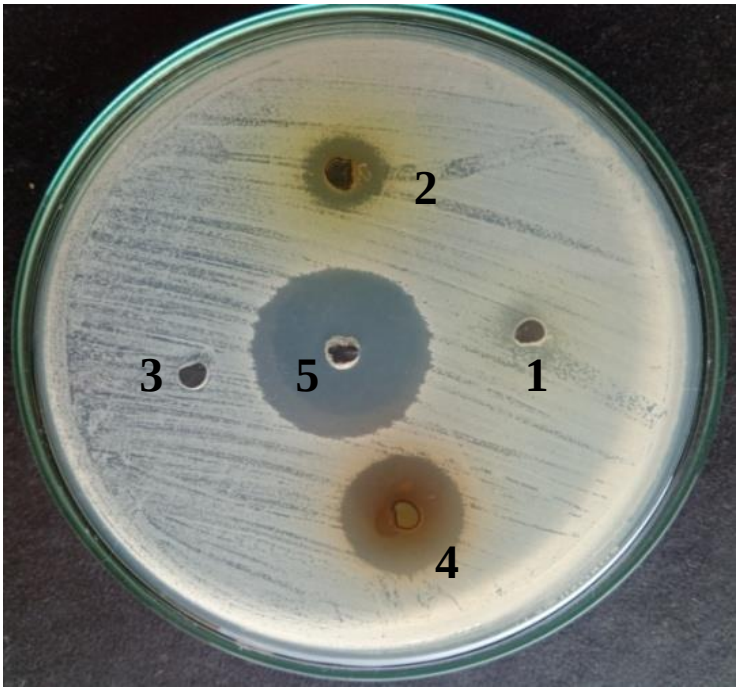


Figure 22:- Antimicrobial screening result against *S. aureus* (gram-positive bacteria) (1) *P. arillata* (2) *B. asiatica* (3) Negative control (50%DMSO) (4) *C. sinensis* (5) Positive control (Neomycin)

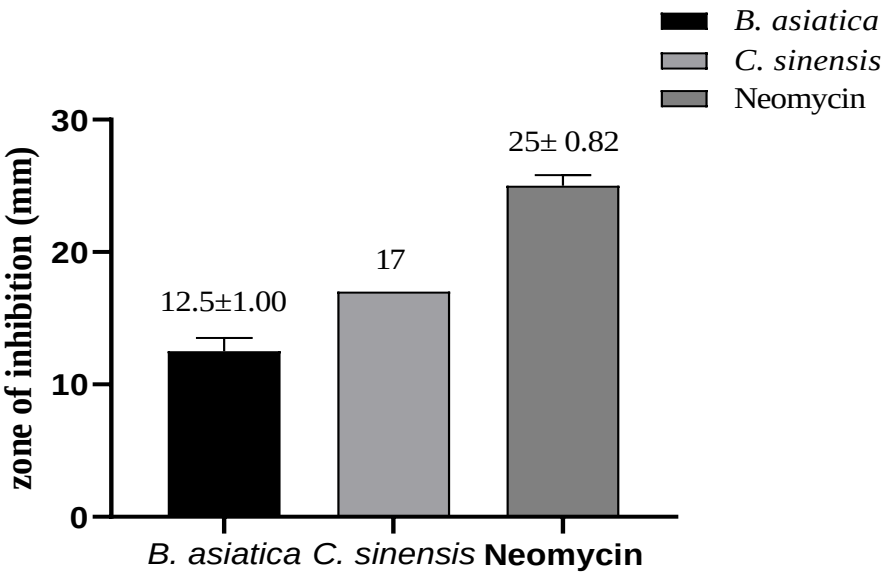


Figure 23:- Bar-graph showing Zone of inhibition (mm) against *S. aureus*

4.8 FT-IR analysis

The FT-IR spectrum of the 20 % methanol in ethyl acetate (M-20) and 100 % methanol (M-100) column fractions of C. sinensis were analyzed as follows

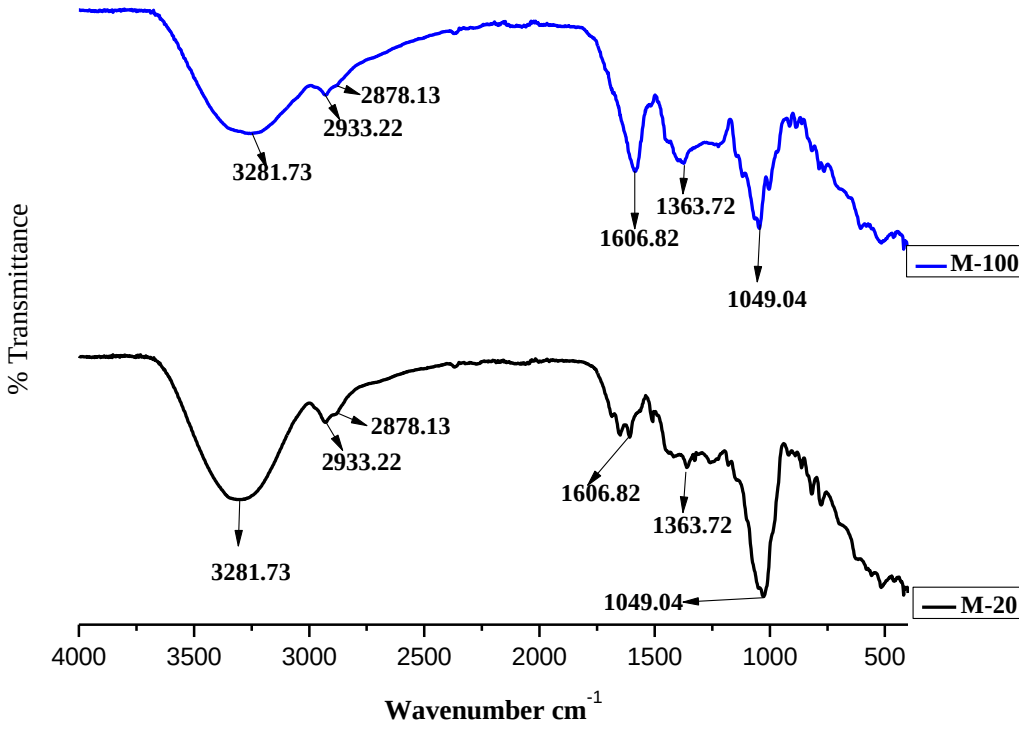


Figure 24:- FT-IR spectra of local tea *C. sinensis*.

The FTIR spectra lead to the identification of different possible functional groups present in it. A wideband was seen at 3281.73 cm^{-1} wave number and it has been reported that it is linked to the O–H and N–H stretching modes of polyphenols. It was reported that the C–H stretch and O–H stretch in alkane and carboxylic acid surfaced at 2933.22 cm^{-1} and 2878.13 cm^{-1} in black tea [95]. It is also possible to allocate a strong band at 1606.82 cm^{-1} to the C=O bond stretching in polyphenols and C=C bond stretching in an aromatic ring. A band has also emerged at 1049.04 cm^{-1} by the C–O stretching in amino acid. A peak at 1363.72 cm^{-1} is also observed in the spectra.

5. CONCLUSIONS AND RECOMMENDATIONS

The phytochemical screening of the methanolic extracts of local tea *C. sinensis*, leaves of *B. asiatica* indicates the presence of bioactive phytochemicals and phytonutrients such as polyphenols, flavonoids, tannins, glucosides, saponins, etc, However, in the case of roots of *P. arillata* the phytochemical constituents are different, therefore it indicates the presence of glucosidase, reducing sugars, carbohydrates, terpenoids, etc. The quantitative analysis of total polyphenol content in the methanolic extract of local tea *C. sinensis*, leaves of *B. asiatica*, and roots of *P. arillata* contains 157.17 ± 18.79 mg GAE/gm, 116.47 ± 6.62 mg GAE/gm, and 44.27 ± 2.97 mg GAE/gm of the dry weight of extract respectively. Similarly, the quantitative content of total flavonoids in the methanolic extract of local tea *C. sinensis*, leaves of *B. asiatica*, and roots of *P. arillata* were found to be 37.07 ± 2.24 mg QE/gm, 34.28 ± 2.63 mg QE/gm, and 5.99 ± 1.00 mg QE per gram of extract in dry weight respectively.

In the case of the antioxidant assay, the fifty percentage inhibitory concentration (IC_{50}) values of methanolic extracts of *B. asiatica, C. sinensis,* and Quercetin were evaluated as 26.86 ± 2.00 µg/mL, 19.15 ± 4.32 µg/mL, and 2.36 ± 0.13 µg/mL respectively. The antioxidant activity of *C. sinensis* was found to be more potent compare to the *B. asiatica*. But, both have less antioxidant activity than the standard Quercetin. whereas in the case of α-amylase inhibition assay, the fifty percentage inhibitory concentration (IC_{50}) values of methanolic extract of *C. sinensis, B. asiatica,* and Acarbose standard were found to be 1737.65 ± 2.21 µg/mL, 1780.00 ± 3.06 µg/mL and 55.74 ± 2.85 µg/mL respectively. The potential of both the extracts is found to be less potent compared to the standard Acarbose. However, Methanolic extracts of *C. sinensis* are quite more efficient than *B. asiatica*. On further analysis in fractions of tea *C. sinensis,* Hexane (TH),dichloromethane (TD) and Ethyl acetate (TE) IC_{50} values were found to be 1296.13 ± 94.46 µg/mL, 675.97 ± 66.18 µg/mL, 338.52 ± 3.78 µg/mL respectively. Similarly, *B. asiatica* extract fractions Hexane (KH), dichloromethane (KD) and Ethyl acetate (KE) IC_{50} values were calculated as 767.46 ± 18.44 µg/mL, 411.50 ± 79.07 µg/mL and 1051.62 ± 12.05 µg/mL respectively.

Furthermore, from the antimicrobial assay among the three methanolic extracts, only the methanolic extracts of local tea *C. sinensis* and *B. asiatica* shows the antimicrobial activity towards the gram-positive bacteria *S. aureus* with the zone of inhibition 17 mm

and 12.5 mm respectively. However, the methanolic extracts of *B. asiatica*, *C. sinensis*, and *P. arillata* were unable to show the antimicrobial activity against the gram-negative bacteria *S. typhi*. The FT-IR spectral analysis of the 20 % methanol in ethyl acetate and 100 % methanol were able to give the functional group peak of the potent compound that might be contained in the methanolic extract of *C. sinensis*.

Finally, the work of our thesis is not limited. There is lots of possible implementation of this work on the industrial level. The isolation of pure compounds, Kinetic study, molecular docking, and their characterization might lead to a noble drug in the future. In vivo analysis in the rat model might be one of the upcoming tasks based on this thesis work. By far in the developing nation, this might be one of the tools to measure the medicinal importance of the medicinal plants and to avoid the illegal trade of highly medicinal value containing plants export and import.

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APPENDICES

A) Phytochemical screening protocol

a) Test of for volatile oils

To about 500 mg extracts, 0.5 mL methanol was added, shaken vigorously, and filtered. Few drops of the filtrate were put on a filter paper utilizing a capillary tube. Yellow spot persistent even after evaporation of the solvent indicates the presence of volatile oils.

b) Test for alkaloids

About 500 mg extracts was dissolved in 3 mL of 2 % (v/v) HCl. The solution was equally divided into two test tubes and the following tests were performed:-

1) Mayer's test

Few drops of Mayer's reagent were added to the first part. The formation of a pale yellow precipitate indicates the presence of alkaloids.

2) Dragendorff's test

Few drops of Dragendroff's reagent were added to the second part. The formation of an orange-red precipitate indicates the presence of alkaloids.

c) Test for terpenoids

To about 200 mg extract, 2 mL of chloroform, and then 3 mL of concentrated sulphuric acid were added carefully. The formation of reddish-brown coloration at the interface indicates the presence of terpenoids.

d) Test for coumarins

To about 1 mL of extract, 1 mL of 10 % NaOH solution was added. The formation of the yellow color indicates the presence of Coumarins.

e) Test for flavonoids

About 200 mg extracts were dissolved in 2 mL methanol. To this solution, a small piece of magnesium and 4-5 drops of concentrated HCl were added. The formation of orange color indicates the presence of flavonoids.

f) Test for quinones

To about 2 mL of extract, 1 mL freshly prepared ferrous sulphate ($FeSO_4$) solution, and a few crystal of ammonium thiocyanate (NH_4SCN) were added and the solution was treated with conc. Sulphuric acid H_2SO_4 drop by drop. The appearance of persistent deep red coloration indicates the presence of Quinines.

g) Test of polyphenols/ $FeCl_3$ test

To about 1 mL extract, 1 mL distilled water was added followed by the addition of a few drops of 10 % (w/v) ferric chloride solution. The appearance of greenish-blue coloration indicates the presence of polyphenols.

h) Test for glycosides

About 500 mg extract was dissolved in 2 mL methanol and divided into two parts and the following tests are performed:-

1) Molish's Test

The first part was treated with 5mL of molishs reagent and conc. H2SO4 was added drop by drop from the side of the test tube without disturbing the solution. The appearance of a violet ring at the junction of two liquids which on shaking turns the solution into violet color indicates the presence of glycosides.

2) To the second part 2 mL of 25 % (v/v) NH_4OH solution was added and vigorously. The appearance of cherry red color indicates the presence of glycosides.

i) Test for reducing sugars

To about 1 mL extract, 1 mL of distilled water was added followed by the addition of 1 mL Fehling s reagents (1:1 mixture of Fehling solution A and B). Then the mixture was warmed over a water bath for 30 minutes. The appearance of brick red precipitates indicates the presence of reducing sugars.

j) Test for saponins

About 500 mg extract was treated with hot water followed by shaking for 30 seconds. The formation of thick forth indicates the presence of saponins.

k) Test for tannins

About 200 mg extract was boiled adding 10 mL distilled water. The mixture was cooled, filtered and few drops of $FeCl_3$ solution were added to the filtrate. The appearance of a blue-black precipitate indicates the presence of tannins.

B) Preparation of reagent

1) Meyer's reagent

Mercuric chloride, $HgCl_2$ (0.679 gm) was weighed in a 50 mL volumetric flask and dissolved in distilled water. To this solution, 2.5 gm potassium iodide (KI) was added. The scarlet red precipitate was dissolved by shaking and volume was made up to the mark by adding distilled water.

2) Dragendorff's reagent

Bismuth nitrate, $Bi(NO_3)$ (4.0 gm) was dissolved in 5N nitric acid (10 mL) to make the solution A. Next potassium iodide, KI (13.5 gm) was dissolved in distilled water (20 mL) to make solution B. These two solutions were mixed in a 50 mL volumetric flask.

Picric acid (0.25 gm) was dissolved in 50 mL distilled water to make an aqueous picric acid solution. The solution was neutralized with sodium bicarbonate ($NaHCO_3$). A strip of whatmann no. 1 filter paper was dipped in the prepared solution. The paper was dried completely and protect from external contamination. Thus prepared sodium picrate paper was used for Cyanogenic Glycoside detection.

3) Molisch's reagent

α-Napthol (5.00 gm) was dissolved in 50 mL methanol to prepare the Molish's reagent.

4) Neutral ferric chloride ($FeCl_3$) solution

1.00 gm of ferric chloride crystals were dissolved in 100 mL distilled water. To this solution, sodium carbonate crystals were added little by little with stirring until the slight turbidity was persistent. Finally, the mixture was filtered and the colorless filtrate was used to neutral Ferric chloride solution.

C:-Tables

Table 10:- Calculation of Antioxidant activity

Concentration in µg/mL	%scv *C. sinensis*			%scv *B. asiatica*		
160	63.492	62.814	61.456	60.171	60.814	61.456
80	58.244	60.493	57.602	56.317	55.353	55.032
40	56.317	59.529	59.850	53.426	51.178	50.535
20	53.747	54.389	55.032	49.251	48.608	47.002
10	20.343	55.996	36.403	33.191	33.833	31.906
5	35.118	23.233	33.191	26.124	23.233	22.912
Concentration in µg/mL	%scv Qurecetin	%scv *P. arillata*				
160				14.239	10.064	18.094
80				8.458	18.737	10.706
40	91.517	88.973	90.198	10.707	9.101	12.634
20	88.124	84.448	85.108	9.101	10.385	1.392
10	84.731	87.559	86.711	3.640	8.779	8.137
5	79.925	68.615	77.097	3.640	7.816	2.677
2.5	47.408	47.408	46.466			
1.25	33.271	35.533	35.344			
0.625	25.353	21.395	22.714			
0.3125	16.305	14.609	12.629			

Table 11:- Total phenolic content calculation

Best-fit values			
Slope	0.0325		
Y-intercept	0.01084		
X-intercept	-0.3337		
1/slope	30.77		
Std. Error			
Slope	0.001501		
Y-intercept	0.07144		
95% Confidence Intervals			
Slope	0.02895 to 0.03605		
Y-intercept	-0.1581 to 0.1798		

X-intercept	-6.102 to 4.462		
Goodness of Fit			
R square	0.9853		
Sy.x	0.1162		
Is slope significantly non-zero?			
F	469.1		
DFn, DFd	1, 7		
P value	<0.0001		
Deviation from zero?	Significant		
Equation	Y = 0.03250*X + 0.01084		
Data			
Number of X values	9		
Maximum number of Y replicates	1		
Total number of values	9		
Number of missing values	0		

	Concentration[µg/mL]	Optical density (O.D) of gallic acid		
	80	2.449	2.394	2.406
	70	2.267	2.415	2.319
	60	2.001	1.878	2.355
	50	1.579	1.855	1.527
	40	1.565	1.454	1.329
	30	1.026	1.039	1.067
	20	0.548	0.535	0.53
	10	0.283	0.274	0.299
	0	0	0	0
Buddleja asiatica B1		0.41		
Buddleja asiatica B2		0.367		
Buddleja asiatica B3		0.391		
*Camellia sinensis*C1		0.591		
*Camellia sinensis*C2		0.498		
*Camellia sinensis*C3		0.476		
Polygala arillata P1		0.144		
Polygala arillata P2		0.163		
Polygala arillata P3		0.157		

	B. asiatica			*C. sinensis*			*P. arillata*		
TPC[mg GAE/gm]	122.81	109.6	117	178.5	149.9	143.1	41	46.8	45

	B. asiatica			*C. sinensis*			*P. arillata*		
TPC[mg GAE/gm]	116.47	6.62	3	157.17	18.79	3	44.27	2.97	3

Table 12:- Total Flavonoid content Calculation

Best-fit values			
Slope	0.0502		
Y-intercept	0.04924		
X-intercept	-0.9808		
1/slope	19.92		
Std. Error			
Slope	0.000935		

Y-intercept	0.02054		
95% Confidence Intervals			
Slope	0.04826 to 0.05214		
Y-intercept	0.006636 to 0.09184		
X-intercept	-1.878 to -0.1290		
Goodness of Fit			
R square	0.9924		
Sy.x	0.0771		
Is slope significantly non-zero?			
F	2885		
DFn, DFd	1, 22		
P value	<0.0001		
Deviation from zero?	Significant		
Equation	Y = 0.05020*X + 0.04924		
Data			
Number of X values	24		
Maximum number of Y replicates	3		
Total number of values	24		
Number of missing values	0		

	concentration[µg/mL]	optical density (O.D) of Quercetin		
	50	2.614	2.305	2.694
	32	1.585	1.682	1.809
	16	0.86	0.799	0.848
	8	0.432	0.446	0.498
	4	0.249	0.273	0.288
	2	0.16	0.167	0.158
	1	0.109	0.12	0.103
	0	0	0	0
Buddleja asiatica k1	0.225			
Buddleja asiatica k2	0.234			
Buddleja asiatica k3	0.208			
Camellia sinensis t1	0.223			
Camellia sinensis t2	0.245			
Camellia sinensis t3	0.238			
Polygala arillata m1	0.074			
Polygala arillata m2	0.08			
Polygala arillata m3	0.084			

	concentration[µg/mL] (Interpolated)	optical density (O.D) of Quercetin (Entered)		
Buddleja asiatica k1	3.501	0.225		
Buddleja asiatica k2	3.681	0.234		
Buddleja asiatica k3	3.163	0.208		

Camellia sinensis t1	3.462	0.223			
Camellia sinensis t2	3.899	0.245			
Camellia sinensis t3	3.760	0.238			
Polygala arillata m1	0.493	0.074			
Polygala arillata m2	0.613	0.08			
Polygala arillata m3	0.692	0.084			

	B. asiatica			*C. sinensis*			*P. arillata*		
TFC mg QE/gram	35.01	36.81	31.63	34.62	38.99	37.60	4.93	6.13	6.93

	B. asiatica			*C. sinensis*			*P. arillata*		
TFC mg QE/gram	34.48	2.63	3	37.07	2.24	3	5.99	1.00	3

Table 13:- Calculation of α-amylase inhibition by an Iodine-starch assay

		Acarbose std (O.D)					
	concentration[µg/mL]				enzyme activity of Acarbose		
	625	3.381	3.594	3.401	0.274	0.061	0.254
	312.5	3.468	3.356	3.36	0.187	0.299	0.295
	156.25	3.354	3.07	3.271	0.301	0.585	0.384
	78.125	2.211	2.425	2.553	1.444	1.229	1.102
	39.0625	1.263	1.177	1.184	2.392	2.478	2.471
	19.53125	0.487	0.46	0.542	3.168	3.195	3.113
	9.765	0.222	0.15	0.159	3.433	3.505	3.497
	4.8828	0.136	0.125	0.108	3.519	3.529	3.547
	Starch Only	Mean	Starch + Enzyme	Mean	enzyme activty of control		
	3.639		0.083				
	3.428	3.655	0.086	0.081	3.574		
	3.897		0.074				
	concentration[µg/mL]	% relative enzyme activity			% inhibition in the α-amylase		
	625	7.658	1.698	7.098	92.342	98.302	92.902
	312.5	5.223	8.357	8.245	94.777	91.643	91.755
	156.25	8.413	16.360	10.736	91.587	83.639	89.264
	78.125	40.397	34.409	30.827	59.603	65.591	69.173
	39.0625	66.924	69.331	69.135	33.075	30.669	30.865
	19.53125	88.639	89.394	87.100	11.361	10.605	12.899
	9.765	96.054	98.069	97.817	3.946	1.93079	2.183
	4.8828	98.461	98.769	99.244	1.539	1.231	0.756

		Final Optical density (O.D)					
		Buddleja asiatica			enzyme activity of test		
	concentration[µg/mL]	B1	B2	B3			
	2500	2.158	2.337	2.557	1.497	1.318	1.097
	1250	1.305	1.524	1.615	2.349	2.131	2.040
	625	1.005	1.006	0.777	2.650	2.649	2.878

	312.5	0.507	0.515	0.526	3.148	3.140	3.129
	156.25	0.388	0.346	0.36	3.267	3.309	3.295
	concentration[µg/mL]	% relative enzyme activity			% inhibition in the α-amylase		
	2500	41.880	36.872	30.715	58.119	63.128	69.285
	1250	65.749	59.621	57.075	34.250	40.379	42.925
	625	74.144	74.116	80.524	25.856	25.884	19.476
	312.5	88.079	87.856	87.548	11.921	12.144	12.452
	156.25	91.409	92.585	92.193	8.591	7.415	7.807

		Final Optical density (O.D)					
		Camellia sinensis					
	concentration[µg/mL]	C1	C2	C3	enzyme activity of test		
	2500	1.802	2.019	2.328	1.626	1.409	1.1
	1250	1.255	1.333	1.414	2.173	2.095	2.014
	625	0.93	0.755	0.838	2.673	2.673	2.59
	312.5	0.429	0.436	0.482	2.999	2.992	2.946
	156.25	0.433	0.3	0.288	2.995	3.128	3.14
	concentration[µg/mL]	% relative enzyme activity			*% inhibition in the α-amylase*		
	2500	45.499	39.427	30.781	54.501	60.573	69.219
	1250	60.806	58.623	56.357	39.194	41.377	43.643
	625	74.797	74.797	72.475	25.203	25.203	27.525
	312.5	83.919	83.724	82.436	16.081	16.276	17.564
	156.25	83.807	87.529	87.865	16.193	12.471	12.135

		Final Optical density (O.D)					
		Polygala arillata					
	concentration[µg/mL]	P1	P2	P3	enzyme activity of test		
	2500	1.167	1.424	1.369	2.488	2.231	2.286
	1250	0.897	0.826	1.028	2.758	2.829	2.627
	625	0.909	0.875	0.75	2.746	2.780	2.905
	312.5	0.297	0.341	0.341	3.357	3.314	3.315
	156.25	0.204	0.203	0.176	3.224	3.225	3.252
	concentration[µg/mL]	% relative enzyme activity			*% inhibition in the α-amylase*		
	2500	69.611	62.420	63.959	30.389	37.580	36.041
	1250	77.166	79.153	73.501	22.834	20.847	26.499
	625	76.831	77.782	81.280	23.169	22.218	18.720
	312.5	93.956	92.725	92.725	16.044	7.275	7.275
	156.25	90.215	90.243	90.999	9.785	9.757	9.001

	Buddleja asiatica			*Camellia sinensis*			Acarbose-std		
Conc[µg/mL]	Mean	SD	N	Mean	SD	N	Mean	SD	N
IC50	1780.00	3.06	3	1737.65	2.21	3	55.73	2.85	3

Table 14:- Calculation of α-amylase inhibition by DNS method

		KE				
conc, µg/mL		final			initial	
2500	0.505	0.498	0.504	0.266	0.283	0.286
1250	0.531	0.522	0.523	0.095	0.098	0.099
625	0.684	0.671	0.681	0.066	0.044	0.054
312.5	0.728	0.707	0.706	0.052	0.046	0.064
156.25	0.728	0.712	0.742	0.041	0.06	0.057
78.125	0.737	0.731	0.732	0.05	0.045	0.04
Blank	0.194	0.2	0.191	0.195		
Negative ctrl	0.784	0.8	0.734	0.772667		
	obt. O.D		Blank	O.D of sample		
0.239	0.215	0.218	0.195	0.044	0.02	0.023
0.436	0.424	0.424	0.195	0.241	0.229	0.229
0.618	0.627	0.627	0.195	0.423	0.432	0.432
0.676	0.661	0.642	0.195	0.481	0.466	0.447
0.687	0.652	0.685	0.195	0.492	0.457	0.49
0.687	0.686	0.692	0.195	0.492	0.491	0.497
	Acontrol	0.772667	0.195	0.5776667		

conc, µg/mL	KD	Final			Initial	
2500	0.664	0.608	0.69	0.392	0.395	0.409
1250	0.684	0.633	0.648	0.282	0.221	0.222
625	0.627	0.624	0.608	0.127	0.117	0.128
312.5	0.522	0.505	0.502	0.068	0.086	0.083
156.25	0.721	0.709	0.713	0.062	0.064	0.051
78.125	0.644	0.738	0.711	0.052	0.057	0.061
39.0625	0.683	0.607	0.709	0.056	0.045	0.046
Blank	0.194	0.2	0.191	0.195		
Negative ctrl	0.784	0.8	0.734	0.772667		
	Obt. O.D			O.D of sample (O1)	O2	O3
0.272	0.213	0.281	0.195	0.077	0.018	0.086
0.402	0.412	0.426	0.195	0.207	0.217	0.231
0.5	0.507	0.48	0.195	0.305	0.312	0.285
0.454	0.419	0.419	0.195	0.259	0.224	0.224
0.659	0.645	0.662	0.195	0.464	0.45	0.467

0.592	0.681	0.65	0.195	0.397	0.486	0.455
0.627	0.562	0.663	0.195	0.432	0.367	0.468
	Acontrol	0.772667	0.195	0.5776667		

	final					
conc, µg/mL	KH			initial		
2500	0.454	0.427	0.432	0.209	0.201	0.198
1250	0.463	0.48	0.484	0.134	0.135	0.132
625	0.632	0.615	0.612	0.067	0.063	0.065
312.5	0.666	0.637	0.636	0.057	0.051	0.051
156.25	0.671	0.642	0.651	0.049	0.048	0.041
78.125	0.681	0.706	0.702	0.046	0.043	0.036
Blank	0.194	0.2	0.191	0.195		
Negative ctrl	0.784	0.8	0.734	0.772667		
	Obt. O.D		Blank		O.D of sample	
0.245	0.226	0.234	0.195	0.05	0.031	0.039
0.329	0.345	0.352	0.195	0.134	0.15	0.157
0.565	0.552	0.547	0.195	0.37	0.357	0.352
0.609	0.586	0.585	0.195	0.414	0.391	0.39
0.622	0.594	0.61	0.195	0.427	0.399	0.415
0.635	0.663	0.666	0.195	0.44	0.468	0.471
	Acontrol	0.772667	0.195	0.5776667		

conc, µg/mL	TD	Final			initial	
2500	0.398	0.373	0.4	0.1	0.096	0.103
1250	0.494	0.459	0.464	0.084	0.079	0.075
625	0.554	0.54	0.546	0.059	0.066	0.054
312.5	0.677	0.64	0.651	0.05	0.066	0.052
156.25	0.784	0.732	0.746	0.133	0.129	0.161
78.125	0.78	0.701	0.719	0.057	0.047	0.053
Blank	0.194	0.2	0.191	0.195		
Negative ctrl	0.784	0.8	0.734	0.772667		
	obt. O.D		Blank	O.D of sample		
0.298	0.277	0.297	0.195	0.103	0.082	0.102
0.41	0.38	0.389	0.195	0.215	0.185	0.194
0.495	0.474	0.492	0.195	0.3	0.279	0.297
0.627	0.574	0.599	0.195	0.432	0.379	0.404
0.651	0.603	0.585	0.195	0.456	0.408	0.39
0.723	0.654	0.666	0.195	0.528	0.459	0.471
	Acontrol	0.772667	0.195	0.5776667		

conc, µg/mL	FINAL	TH			INITIAL	
2500	0.579	0.574	0.61	0.195	0.197	0.2
1250	0.575	0.555	0.567	0.124	0.121	0.129
625	0.561	0.556	0.575	0.051	0.049	0.053
312.5	0.702	0.681	0.675	0.09	0.073	0.078
156.25	0.704	0.696	0.711	0.063	0.133	0.06
78.125	0.723	0.699	0.719	0.049	0.053	0.053
Blank	0.192	0.193	0.183	0.189333		
Neg-Ctrl	0.69	0.702	0.65	0.680667		
	Obt. O.D		Blank		O.D of sample	
0.384	0.377	0.41	0.189	0.195	0.188	0.221
0.451	0.434	0.438	0.189	0.262	0.245	0.249
0.51	0.507	0.522	0.189	0.321	0.318	0.333
0.612	0.608	0.597	0.189	0.423	0.419	0.408
0.641	0.563	0.651	0.189	0.452	0.374	0.462
0.674	0.646	0.666	0.189	0.485	0.457	0.477
	Acontrol	0.680667	0.189	0.491		

conc, µg/mL	TE	Final			Initial	
2500	0.349	0.348	0.351	0.072	0.085	0.082
1250	0.333	0.33	0.338	0.073	0.068	0.071
625	0.392	0.396	0.384	0.069	0.06	0.058
312.5	0.497	0.498	0.491	0.056	0.062	0.054
156.25	0.635	0.628	0.631	0.048	0.049	0.049
78.125	0.654	0.67	0.666	0.045	0.051	0.045
Blank	0.192	0.193	0.183	0.189		
Neg-Ctrl	0.69	0.702	0.65	0.681		
	Obt. O.D		Blank		final O.D	
0.277	0.263	0.269	0.189	0.088	0.074	0.080
0.26	0.262	0.267	0.189	0.071	0.073	0.078
0.323	0.336	0.326	0.189	0.134	0.147	0.137
0.441	0.436	0.437	0.189	0.252	0.247	0.247
0.587	0.579	0.582	0.189	0.398	0.390	0.393
0.609	0.619	0.621	0.189	0.419	0.430	0.432
	Acontrol	0.680667	0.189	0.491		

conc. µg/mL	% inh-KD1	% inh-KD2	% inh-KD3	% inh-TD1	% inh-TD2	% inh-TD3
2500	86.671	96.884	85.113	82.169	85.805	82.343
1250	64.166	62.435	60.012	62.781	67.975	66.417
625	47.201	45.989	50.664	48.067	51.702	48.586
312.25	55.164	61.223	61.223	25.216	34.391	30.063

156.125	19.677	22.100	19.158	21.062	29.371	32.487
78.25	31.275	15.868	21.235	8.598	20.542	18.465
48.06	25.216	36.469	18.984			
conc. µg/mL	%inh-KH1	% inh KH2	%inh-KH3	% inh-TE1	% inh-TE2	% inh-TE3
2500	91.345	94.634	93.249	82.157	85.007	83.786
1250	76.803	74.033	72.822	85.617	85.210	84.193
625	35.949	38.199	39.065	72.795	70.149	72.185
312.25	28.332	32.314	32.487	48.779	49.796	49.593
156.125	26.082	30.929	28.159	19.064	20.692	20.081
78.25	23.832	18.984	18.465	14.586	12.551	12.144

conc. µg/mL	% Inh-TH1	% inh-TH2	%inh-TH3	% inh-KE1	%inh-KE2	% inh-KE3
2500	60.379	61.805	55.088	92.383	96.538	96.018
1250	46.744	50.203	49.389	58.280	60.35776	60.358
625	34.735	35.346	32.293	26.774	25.21639	25.216
312.25	13.975	14.789	17.028	16.734	19.33064	22.619
156.125	8.073	23.948	6.038	14.829	20.88863	15.176
78.25	1.357	7.056	2.985	14.829	15.00289	13.964
Acarbose						

conc. µg/mL	% inh-A1	% inh-A2	% inh-A3		IC50		
				KD	411.50	TH	1296.13
781.25	77.908	77.323	77.713		79.07		94.46
390.625	84.535	83.366	80.052		3		3
195.3125	69.721	72.449	70.695	TD	675.97	KE	1051.62
97.65	68.161	65.432	62.703		66.18		12.05
48.828	43.015	38.142	33.268		3		3
24.414	4.224	17.089	13.385	KH	767.46	A-std	70.89
12.207	9.487	8.122	9.097		18.44		4.76
6.103	8.707	11.241	11.631		3		3
				TE	338.52		
					3.78		
					3		

D) Calculation of IC_{50} values:-

In this dissertation, the IC_{50} value was calculated by using the software GraphPad prism. From the plot of the concentration Vs % inhibition curve, we can calculate IC_{50} values. The percentage inhibition was calculated by using the equation in chapter-3. In the case of TPC and TFC by calculating the unknown concentration of the known optical density values of the extracts from the standard curve (e.g:- Gallic acid and Quercetin) and by using a simple formula as given in chapter-3 we can calculate the TPC and TFC content in the extracts.

Where c = unknown concentration of known optical density (from software)

V = volume in 200 µL

m = mass of extracts taken for analysis mass taken to make a stock solution.

For e.g:- 1 mg in 1 mL

1 µg/µL so, taken volume is 20 µL thus, m= 20 µg.

Then, TPC in mg/g is calculated as

TPC mg/gm = 12.122 µg/mL (c) obtained from software*200 µL/ 20 µg

= 12.122 *0.001 *0.2 mg/20*0.001*0.001 g

= 121.22 mg of GAE/ gram of dry weight