Acta Scientific MICROBIOLOGY (ISSN: 2581-3226)

Volume 6 Issue 9 September 2023

Research Article

An Assessment of Phytochemical, Anti-Microbial and Anti-Oxidant Properties of *Berberis Aristata* and *Tinospora Sinensis* of Nepal

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DOI: 10.31080/ASMI.2023.06.1299

Received: July 24, 2023

Published: August 30, 2023

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Abstract

The majority of Nepalese people are dependent on indigenous use of medicinal plants. Medicinal plants play vital role in Nepalese livelihood, health and socio-economic prospects. Very few systematic studies have been done on the medicinal plants of Nepal for their anti-oxidant and anti-microbial activities. Thus, in the present study, we collected and screened the Nepalese medicinal plants from different ecological region of Nepal for the anti-oxidant activity leading to the isolation of active compounds.

Our research is focused on collecting medicinally important plant "*Tinospora sinensis*" and "*Berberis aristata*" and conducting their phytochemical analysis, anti-microbial assay and antioxidant assay. The plant samples of *T. sinensis* and *B. aristata* were collected from an altitude of 1161m and 1666m respectively from the hills of Dhunibesi-7, Dhading. The crude methanolic extracts of these plant species were prepared by Soxhlet apparatus method and concentrated by rotary vacuum evaporation method. Then these extracts were further subjected to get more concentrated in water bath. Then, the plant extracts were subjected to preliminary phytochemical analysis for the detection of alkaloids, carbohydrates, tannin, flavonoid, saponins, sterol, fatty acid and fixed oil, resins, protein and amino acid.

Likewise, the anti-microbial assay of the plant extracts were tested by Agar-disc diffusion method with Gram-positive bacteria (*Bacillus subtilis* and *Staphylococcus aureus*) and Gramnegative bacteria (*Klebsiella pneumoniae* and *Enterobacter*). Furthermore, the anti-oxidant assay of the plant extracts were conducted by DPPH assay using ascorbic acid as reference. The IC_{50} were also evaluated and compared. The proximate value analysis of the plant extracts were also carried out.

The preliminary phytochemical analysis of the crude extracts of the medicinal plants (*Tinospora sinensis* and *Berberis aristata*) indicated the presence of major phytochemical compounds, including alkaloids, carbohydrates, tannin, flavonoid, saponins, resins, protein and amino acid which may be responsible for the observed anti-microbial activities and anti-oxidant activities. The observed results further support the view that these Nepalese medicinal plants are promising sources of potential anti-oxidants and medicinal compounds.

All the plant extracts have shown anti-microbial activity against Gram-positive bacteria (*Bacillus subtilis* and *Staphylococcus aureus*) and Gram-negative bacteria (*Klebsiella pneumoniae* and *Enterobacter*). The highest zone of inhibition was shown by *Berberis aristata* leaves collected against *Bacillus subtilis* with the diameter being 1.6cm. The leaf extract of *Berberis aristata* showed better anti-microbial activities than other plant extracts.

The strongest anti-oxidant activity was shown by methanolic extract of stem of *Berberis aristata* with IC_{50} value of 82.101µg/ml whereas the lowest anti-oxidant activity was shown by methanolic extract of leaves of *Tinospora sinensis* with IC_{50} value of 128.53µg/ml. Among all the plant extracts, the *Berberis aristata* stem extract and the *Tinospora sinensis* leaves extract showed higher anti-oxidant activity.

Keywords: Anti-Microbial; Antioxidant; Preliminary Phytochemical Analysis; Ic_{50}; Zone Of Inhibition

Abbreviations

IC_{50}: Half Maximal Inhibitory Concentration; m: Metre(s); DPPH: 2,2-Diphenyl-1-Picrylhydrazyl; cm: Centimeter(s); µg: Microgram(s); mm: Millimeter(s); mM: Millimolar(s); %: Percentage; ft: Feet; in: Inch(es); ml: Milliliter(s); mg: Milligram(s). oz: Ounce(s); CCl_4: Carbon Tetrachloride; STZ: Streptozotocin; min: Minute(s); i.e.: That is eg. For Example; AMR: Anti-Microbial Resistance; µM: Micromolar; pTLC: Preparative Thin Layer Chromatography; NMR: Nuclear Magnetic Resonance; fMLP/CB: Formyl-L-Methionyl-L-Leucyl-L-phenylalanine/Cytchalasin B; nm: Nanometre; Rf: Retention Factor; HPTLC: High Performance Thin Layer Chromatography; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide; °C: Degree Centigrade; w/v: Weight/Volume; DMSO: Dimethyl Sulfoxide; No.: Number; µl: Microlitre; I%: Percentage of Inhibition; A_c: Absorbance of the Control (1 ml Methanol + 0.5 ml DPPH Solution); A_o: Absorbance of the Sample Solution; MeOH: Methanol

Introduction

Background

Nepal has significantly diverse ecosystem producing a wide range of unique and valuable medicinal plant resources. Nepal is ranked as 9th among the Asian countries for its floral wealth with an estimated 9,000 species of flowering plants [1]. So far, 6,653 species of flowering plants have been reported [2]. Among these, about 50% fall under the rubrics "useful" and "ethnobotanical", and about 25%–50% are ethnomedicinals [2,3]. Catalogues have recorded 1,792 to 2,331 useful medicinal and aromatic plants in Nepal, reporting their importance in alleviating human suffering because they have long been used for subsistence, home remedies, and traditional therapies [4]. These plants are also important for local livelihoods [5] and income generation, and they do fetch higher market prices [6].

National and regional demands for herbal medicine are accelerating and globalization of herbal medicine, along with uncontrolled exploitative practices and lack of concerted conservation efforts, now threaten the country's medicinal plants [7,8]. Numerous drugs have been introduced to international markets through validation of traditional medicines, indigenous therapies and ethnopharmacological practices. Sustainable utilization and management of medicinal plants based on traditional knowledge is therefore necessary. It is imperative that the medicinal plants, their traditional uses, and management practices be catalogued as part of a larger conservation effort toward "rescuing a global heritage". This effort should acknowledge empirical data and ethnoecological knowledge. Far-west Nepal is the least studied area in Nepal [9]. The present study was thus an attempt to catalogue the important medicinal plants of Far-west Nepal along with their traditional uses and management interventions.

The ayurvedic health care system depends solely on the use of these highly valued native medicinal plants. Plants that possesses therapeutic properties or exert beneficial pharmacological effects on the human body are generally designated as medicinal plants. Two of the medicinal plant of economic importance are described below in terms of their taxonomical classification, habitat and botanical description.

Tinospora sinensis

Among the vast library of important medicinal plants, *Tinospora sinensis* is a deciduous climbing shrub which belongs to the family Menispermeaceae. The plant family Menispermeaceae consists of about 70 genus & 450 species that are found in tropical regions. The plant is designated as Rasayana in Ayurveda and is very well known for building up the immune system and body's defense against definite infecting micro-organisms. It is regularly observed growing up mango or neem trees. It is engendered either by stem or seeds. It's leaves, barks and root contains different bio-active compounds like alkaloids, lactone, steroids, polysaccharides, glycosides and aliphatic compounds having different restorative significances. It has the bio medical properties like anti-diabetic, anthelminthic, anti-pyretic, mitigating, cerebrum tonic, sexual enhance, hepato-protection, blood purifier, immunomodulatory or immuno-stimulatory, anti- tuberculosis, cell-reinforcement, anti-tumor, anti-stress, anti-malarial, anti-allergic, anti-arthritic, and further more helps in a version of reactions of the tumor chemotherapy [10].

Scientific classification

Kingdom: Plantae

Clade: Angiosperm

Clade: Eudicots

Super order: Rananculanae

Order: Rananculales

Family: Menispermeaceae

Genus: *Tinospora*

Species: *sinensis*

Local name: Gurjo

Habitat

Tinospora sinensis is known by the common name heart-leaved moonseed

(http://famemyanmar/heartleavedmoonseed/), gudchi, gurjo (Nepali name) which is an herbaceous vine of the family Menispermeaceae indigenous to the areas of Nepal, India, Sri Lanka and Myanmar.

Botanical description

Tinospora sinensis is a large deciduous, extensively spreading climbing shrub with number of coiling branches. Stem of this plant is rather succulent with long, filiform, fleshy and climbing in nature. Aerial root arise from the branches. The bark creamy white to grey in colour and deeply left. Arial roots are present; these aerial roots are characterized by tetra to penta-arch primary structure. However, cortex of root is divided into outer thick walled and inner parenchymatous. Leaves of this plant are simple, alternate, exsitipulate, long petiole approximately 15 cm round, pulvinate, heart shaped, twisted partially and halfway round. Lamina is ovate, 10-20 cm long, 7 nerved and deeply cordate at flowers are unisexual, racemes, greenish yellow in colour, appears when plant is leaf less. Male flowers are clusters and female flowers exist in solitary inflorescence. Sepals are 6 in 2 series of 3 each. Outer ones are smaller than the inner sepals. Petals are also 6, smaller the sepals, free and membranous. Flowering occurs during March to June they are orange-red colour, fleshy, aggregate of 1-3 and ovoid, smooth, drupelets on thick stalk [11].

Chemical constituents

The preliminary studies on the phytochemical analysis determined that the plant contains steroids, glycosides, carbohydrates, steroids, mucilage and oxalic acids in the stems and carbohydrates, glycosides, saponins, tannins, phenols, flavonoids and alkaloids in the leaves. The plant also constituted four chemical constituents like trans-syringing, 3'-dimethyl-phillyrin, sesquiterpene glycoside, vanillin, daucosterol and I-sitisterol. However, the number of bioactive compounds is found to be low in *Tinospora sinensis* (Lour.) Merr. when compared to the other two species like *T. cordifolia* (Wild.) Miers and *T. crispa* (L.) [12].

The plant mainly contains alkaloids, glycosides, steroids, sesquiterpenoids, aliphatic compounds, essential oils, mixture of fatty acids and polysaccharides. The alkaloids include berberine, bitter gilonin, and non-glycoside gilonin gilosterol. The major phyto-constituent in Tinospora sinensis include tinosporine, tinosporide, tinosporaside, heptacosanol, clerodane furano diterpene, diterpenoid furano lactone, tinosporidine, columbin, b-sitosterol. Berberine, palmatine, tembertarine, magniflorine, choline and tinosporin are reported from the stem of the plant. The new clerodane furano diterpene 2 with the molecular formula $C_{20}H_{20}O_8$, has been isolated from the stems of the plant. Phytochemical investigation of the methanol extract of Tinospora sinensis aerial parts led to the isolation of four new and seven known compounds [26]. The structure of the new aporphine alkaloids, Nformylasimilobine 2-O-β-D-glucopyranosyl -(1-2)-β-D-glucopyranoside (tinoscorside A) and Nacetylasimilobine 2-O-β-D-glucopyranosyl –(1-2)-β-D-glucopyranoside (tinoscorside B), a new clerodane diterpene, tinoscorside C and a new phenylpropanoid , sinapyl 14-O- β-Dapiofuranosyl-(1-6)-O- β-D-glucopyranoside (tinoscorside D) [13].

Uses

Tinospora sinensis is widely used medicinal plant in Ayurvedic system for its general tonic, antiperiodic, anti-spasmodic, anti-inflammatory, antipyretic, anti-arthritic, anti-lepritic, antiallergic and anti-diabetic properties [13].

The plant is used to improve the immune system and the body resistance against infections. The root of this plant is known for its anti-stress and anti-malarial activities. The stem is bitter, stomachic,

diuretic, stimulates bile secretions, allays thirst, enriches the blood and cures jaundice. The extract of the stem is useful in skin problems. The root and stem of *T. sinensis* is prescribed in combination with other drugs as an antidote to snakebite and scorpion [14]. The plant is also used in the treatment of wounds, pneumonia, asthma and cough. *T. sinensis* has anti-cancer, immune stimulating, nerve cell protecting, anti-diabetic, cholesterollowering and liver-protective actions. *Tinospora sinensis* is also responsible for decreasing the tissue damage caused by radiation, the side effects of some forms of chemotherapy and speeding healing of diabetic foot ulcers [12].

The tribal Baiga, living in the interior areas of Naugarh and Chakia Block of Varanasi district, Uttar Pradesh make the paste of stem of the Guduchi (*T. sinensis*) and the roots of Bhatkatiaya (*Solanum surattense*). The pills are prepared and used in the treatment of fever for three days. The tribals of Mumbai and its neighboring areas and the fishermen along the sea coast use *T. sinensis* as drug in the treatment of fever, jaundice, chronic diarrhoea and dysentery. The tribals of Khedbrahma region of North Gujarat use the plant in their day-to-day life as food or medicine. They use powdered root and stem bark of *T. sinensis* with milk for the treatment of cancer; decoction of root is used for the cure of dysentery and diarrhoea and decoction of old stems is preferred in the treatment of periodic fever. Decoction of stem is administered orally by the people of Jammu (J&K) and Bigwada (Rajasthan) for the treatment of fever. The inhabitants of Bhuvneshwar (Orissa) use the warm juice of root of *T. sinensis* orally for the treatment of fever. Juice or decoction of leaves is administered orally with honey in fever by the local people of Punjab. The Muslim tribals of Rajouri, Jammu (Tawi) comprising Gujjar and Backwals used the plant in bone fracture [10,15].

Pharmacological activities reported from *tinospora sinensis*

S. no	Activity	Part/extract	Animal model/cell line
1.	Anti-oxidant activity	Whole plant or Ethanol	N-nitrosodiethylamine induced liver cancer in male wistar albino rats
2.	Anti-inflammatory	Stem or aqueous extract	Carrageenan induced paw edema model in rats
3.	Anti-tumor activity	Aqueous alcoholic extract	C6 glioma cells were used, extract reduced the cell proliferation in dose
4.	Anti-Bacterial	Stem/Aqueous and ethanolic Extract	Microorganisms used: *E. coli, P. vulgaris, E. faecalis, S. typhi, S. aureus, S. marcesenses*
5.	Anticancer activity	Aqueous and ethanolic extract	IMR 32 human neuroblastoma cell lines as a model system
6.	Immunomodulatory activity	Whole plant or aqueous extract	Swiss male albino mice

Table a

Berberis aristata

Berberis aristata, also known as Indian barberry, "Chutro" or tree turmeric, is a shrub belonging to the family Berberidaceae and the genus Berberis. The plant of Berberis genus contains barberine, oxyberberine, berbamine, aromoline, karachine, palmatine, oxyacanthine and taxilamine. The genus comprises approximately 450-500 species of deciduous evergreen shrubs. *Berberis aristata* is one of the plants used in Ayurveda for several remedies. *B. aristata* commonly known as -Daru haldhi and Chitral is spinous herb native to northern Himalaya region. There are 12-13 varieties like *Berberis asiatica, Berberis lycium, Berberis vulgaris, Berberis nepalensis* etc [16]. *Berberis aristata* is used in ayurvedic medicines from very long time. It is used as a tonic, alternative, demulcent, diaphoretic, and diuretic, in the treatment of diarrhoea, jaundice

and skin diseases, syphilis, chronic rheumatism and urinary disorders. The root and wood are rich in a yellow alkaloid berberine, a bitter substance, which dissolves in acids and forms salts of the alkaloid. The root contains two more alkaloids. A protoberberine alkaloid karachine is isolated and characterized, and taxilamine is also isolated. A protoberberine alkaloid – karachine – isolated and characterized and also taxilamine isolated [17].

Scientific classification

Kingdom: Plantae

Sub-kingdom: Tracheobionta-Vascular plants

Super-division: Spermatophyta-Seed plants

Division: Magnoliophyta-Flowering plants

Class: Magnoliopsida-Dicotyledons

Subclass: Magnoliidae

Order: Ranunculales

Family: Berberidaceae

Genus: *Berberis*

Species: *B. aristata, B. vulgaris, B. lyceum, B. nepalensis*

Habitat

Berberis aristata is known as Indian berry, chutro or tree turmeric is a shrub belonging to the family Berberdaceae and the genus *Berberis.* The genus comprises approximately 450-500 species of deciduous evergreen shrubs and is found in the temperate and sub- tropical regions of Asia, Europe and America. B. aristata is native to Himalayas in India and in Nepal. It is also naturally found in the wet zone of Sri Lanka [16].

Botanical description

Berberis aristata is characterized by an erect spiny shrub, ranging between 2 to 3 m (6.6 to 9.8 ft) in height. It is a woody plant, with bark that appears yellow to brown from the outside and deep yellow from the inside. The bark is covered with three-branched thorns, which are modified leaves, and can be removed by hand in longitudinal strips. The leaves are arranged in tufts of five to eight and are approximately 4.9 cm (1.9 in) long and 1.8 cm (0.71 in) broad. The leaves are deep green on the dorsal surface and light green on the ventral surface. The leaves are simple with pinnate venation. The leaves are leathery in texture and are toothed, with several to many small indentations along the margin of the leaf [16,17].

The flowering season begins in mid-March and lasts throughout the month of April. The yellow flowers that develop are complete and hermaphroditic. The average diameter of a fully opened flower is 12.5 mm (0.49 in). The flowers form a racemose inflorescence, with 11 to 16 flowers per raceme, arranged along a central stem. The flower is polysepalous, with three large and three small sepals, and polypetalous, with six petals in total. The male reproductive structure, the androecium, is polyandrous and contains six stamens, 5-6 mm (0.20-0.24 in) long. There is one female reproductive structure, the gynoecium, which is 4–5 mm (0.16-0.20 in) long and is composed of a short style and a broad stigma. The plant produces bunches of succulent, acidic, edible berries that are bright red in color and have medicinal properties. The fruits start ripening from the second week of May and continue to do so throughout June. The berries are approximately 7 mm (0.28 in) long, 4 mm (0.16 in) in diameter, and weigh about 227 mg (0.0080 oz) [18].

Chemical composition

Alkaloids are the major type of phytoconstituents present in *B. aristata*. *Berberis aristata* contains protoberberine and bis isoquinoline type of alkaloid. The root bark of the plant contains a protoberberine alkaloid - karachine along with aromoline, oxyberberine, oxyacanthine, berbamine, and berberine chloride [19]. *B. aristata* flower contains various polyphenolic flavonoids like quercetin, rutin, meratin and acids like E-caffeic acid and chlorogenic acid (Saied S. et. al., 2007). Alkaloids like pakistanine, 1-O-methylpakistanine, pseudopalmatine chloride and pseudoberberine chloride were isolated from the bark of the plant [20]. A secobisbenzlisoquinoline or simple isoquinoline alkaloid was isolated from *Berberis* aristata. The major alkaloid found in *B. aristata* is Berberine having yield of 2.23% followed by palamatine. Another alkaloid taxilamine was also obtained from this plant [21]. Alcoholic extraction of the powdered bark of *B. aristata* after concentration and filtration gave berberine, tetrahydropalmatine, tetrahydroberberine palmatine and palmatine chloride or its mixtures. The ethanolic extract of heartwood of *B. aristata* reveals the presence of n-docosane which is an aliphatic hydrocarbon [22].

Uses

In India, *B. aristata* is used in traditional herbal medicine. Its stem, roots, and fruits are used in Ayurveda. A preparation called rasaunt is prepared by boiling the bark of the root and of the lower part of the stem in water. The solution is then strained and evaporated until a semi-solid mass, rasaunt, is obtained. It is mixed with either butter or alum, or with opium and lime-juice. The root bark contains the bitter alkaloid berberine, which has been studied for its potential pharmacological properties [21]. The fruits of the species are eaten by people living in areas where the plant is found, often as a dessert. The fruits are juicy and contain plenty of sugars and other useful nutrients that supplement their diet. The roots can also be used for making an alcoholic drink. The plant as a whole is a good source of dye and tannin which is used for dyeing clothes and for tanning leather [23].

Ethno-pharmacological activities of the different of *B. aristata*

S.N	Part of the Plant	Ethno-pharmacological/clinical application
1.	Fruits	Preventive and curative effects on paracetamol and CCl_4 induced hepatotoxicity
2.	Root	Anti-platelet activating factor activity 85
3.	Stem, root bark and wood	Protection against ethanol-induced mitochondrial damage
4.	Fruit extract	Inotropic effect
5.	Root	Antihyperglycemic and antioxidant effect
6.	Root bark	Scientific evidence for the folklore use of *B.aristata* DC in urinary troubles.
7.	Stem bark	Blood glucose lowering potential
8.	Root	Anti-diabetic activity
9.	Bark	Anti-diarrhoeal activity
10.	Stem	Hypoglycemic and hypolipidemic activity
11.	Root	Anti-osteoporotic activity in ovariectomized rats
12.	Leaves and root	Broad spectrum antimicrobial activity for the treatment of ear infection
13.	Root	Potentiation of thiopentone sodium induced hypnosis in rodents
14.	Stem bark	Hypoglycemic activity of aqueous extract in STZ-induced rats
15.	Leaves	Used in hepatobiliary disorders

Table b

General methods of plant extraction

- **Plant tissue homogenization:** Dried or wet, fresh plant parts are grinded in a blender to fine particles, put in a certain quantity of solvent and shaken vigorously for 5- 10 min or left for 24hrs after which the extract is filtered. The filtrate then may be dried under reduced pressure and are dissolved in the solvent to determine the concentration [25].
- **Serial exhaustive extraction:** It is another common method of extraction which involves successive extraction with solvents of increasing polarity form a non-polar (hexane) to a more polar solvent (methanol) to ensure that a wide polarity range of compound could be extracted [25].
- **Soxhlet extraction:** It is only required where the desired compound has a limited solubility in a solvent, and the impurity is insoluble in that solvent. If the desired compound has a high solubility in a solvent then a simple filtration can be used to separate the compound form the insoluble substance. The advantage of this system is that instead of many portions of warm solvent being passed through the sample, just one batch of solvent is recycled. This method cannot be used for thermolaible compounds as prolonged heating may lead to degradation of compounds [26].

- **Maceration:** In maceration (for fluid extract), whole or coarsely powdered plant drug is kept in contact with the solvent in a stoppered container for a defined period with frequent agitation until soluble matter is dissolved. This method is best for suitable for use in case of the thermoliable drugs [27].
- **Decoction:** This method is used for the extraction of the water soluble and heat stable constituents from crude drug by boiling it in water for 15 min, cooling, straining, and passing sufficient cold water through the drug to produce the required volume [28].
- **Percolation:** A percolator (a narrow, cone-shaped vessel open at both ends) is generally used. The solid ingredients are moistened with an appropriate amount of the specified menstrum and allowed to stand for approximately 4 h in a well closed container, after which the mass is packed and the top of the percolator is closed. Additional menstrum is added to form a shallow layer above the mass, and the mixture is allowed to macerate in the closed precolater for 24 hrs. The outlet of the percolator then is opened and the liquid contained therein is allowed to drip slowly. Additional menstrum is added as required, until the percolate measures about three quarters of the required volume of the finished product. The marc is then pressed and the expressed liquid is added to the percolate. Sufficient menstrum is added to produce the required volume and the mixes liquid is clarified by filtration or by standing followed by decanting [29].

Preliminary phytochemical analysis

Preliminary phytochemical analysis refers to the extraction, screening and identification of the medicinally active substances found in the plants. Some of the bioactive substances that can be derived from the plants are alkaloids, flavonoids, tannins, carotenoids, anti-oxidants and phenolic compounds. Medicinal plants have bioactive compounds which are used for curing various human diseases and also play an important role in healing. Phytochemicals have two categories i.e., primary and secondary constituents. Primary constituents involve the metabolic products which are required for growth, multiplication and development and also provide direct energy. These include carbohydrates, proteins, fats, chlorophyll, sugars and amino acids. Secondary constituents involve the metabolic products which are required for the defense mechanism. These include alkaloids, glycosides, terpenoids, flavonoids and so on. Medicinal plants have anti-microbial, anti-fungal, anti-diuretic, anti-analgesic, anti-cancer, anti-viral, anti-malarial and antiinflammation activities due to the presence of the phytochemicals. The preliminary phytochemical analysis determines the presence or absence of the phytochemicals in the plants. The preliminary phytochemical analysis of the medicinal plants are also important for the commercial interest in both research institutes and pharmaceutical companies for the manufacturing of the new drugs for the treatment of various diseases [30]. Preliminary phytochemical analysis is a valuable step in the detection of the bioactive principles present in the medicinal plants and subsequently may lead to drug discovery and development [31].

Anti-microbial assay

Bacterial and fungal pathogens are the etiological agents of human infection which have raised concern in the healthcare field over the years. With the advancement in science and technology, remarkable progress has been made in the field of medicine with the discoveries of many natural and synthetic drugs [32]. Antibiotics are undeniably one of the most important therapeutics discoveries of the 20th century that had effectiveness against serious bacterial infections. However, only third of the infectious diseases known have been treated from these synthetic products. This is because of the emergence of resistant pathogens that is beyond doubt the consequences of years of widespread indiscriminate use incessant and misuse of antibiotics [33]. Antibiotics resistance has increased substantially in the recent years and is posing an ever increasing therapeutic problems. One of the methods to reduce the resistance to antibiotics is by using antibiotic resistance inhibitors from plant [34]. Plants are known to produce a variety of compounds to protect themselves against a variety of pathogens. It is expected that plant extracts showing targets sites other than those used by antibiotics will be active against drug resistant pathogens [35]. Medicinal plants have been used as traditional treatments for numerous human diseases for thousands of years and in many parts of the world. Antimicrobial screening is done to specify the presence of antimicrobial molecule in the plant against pathogens and microbes. An antimicrobial susceptibility test is done to determine the least amount of an antimicrobial chemotherapeutic agent that will inhibit the growth of microbes and pathogen in-vitro using agar well diffusion and disc diffusion method.

Antioxidant assay

The anti-oxidant assay of the plants is done by the DPPH radical scavenging method. The DPPH assay is based on the capability of an antioxidant to donate hydrogen radical or an electron to DDPH radical, which is stable free radical with deep violet colour [36]. When an odd electron become paired in the presence of free radical scavenger of antioxidant agent, DPPH radicals get reduced to corresponding hydrazine, DPPH-H form and the solution gets decolorized from its initial deep violet to light yellow colour [37]. The degree of fall in the absorbance is measured spectrophotometrically and is proportional to the concentration of the antioxidant [38]. The measurement of the scavenging of DPPH radical allows one to determine exclusively the intrinsic ability of the substance to donate hydrogen atoms or electrons to this reactive species in a homogenous system [39]. The primary reaction which takes place is the formation of free radical.

R′ and the reduced form of DPPH as shown in the figure below. The free radical produced can undergo further reactions which control the number of the molecules of DPPH reduced by one of the reductant. The DPPH radical-scavenging capacity is studied was reported after 30 min reaction time for all samples evaluated. The parameter used to measure the radical scavenging activity of extracts and fractions evaluated is IC_{50} value, defined as the concentration of antioxidant required for 50% scavenging of DPPH radicals in this specified time period. The smaller IC_{50} value, the higher antioxidant activity of the plant extracts [40].

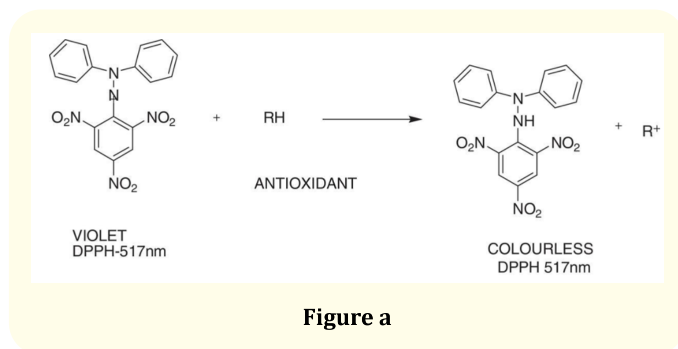


Figure a

Proximate value analysis

The proximate value analysis is defined as the determination of the compounds contained in the mixture as distinguished from ultimate analysis, which is the determination of the elements contained in a compound. It is also known as the determination, by prescribed methods, of moisture, volatile matter, fixed carbon (by difference), and ash. The term 'proximate analysis' does not include determinations of chemical elements or determinations other than those names.

A typical proximate value analysis includes the moisture, ash, volatile matter, and fixed carbon contents. It is important for economic reasons to know the moisture and ash contents. The proximate analysis of a fuel provides the percentage of the material that burns in a gaseous state (volatile matter), in the solid state (fixed carbon) and the percentage of inorganic waste material (ash), and is therefore of fundamental importance for biomass energy use [41].

Aims and objectives.

General

- To conduct phytochemical analysis of the medicinal plants "*Berberis aristata*" and "*Tinospora sinensis*" and their anti-microbial, anti-oxidant and phytochemical analysis.

Specific

Following general objectives are framed to accomplish the aim of the present study:

- For sample collection, identification and extract preparation.
- For phytochemical screening.
- For anti-microbial assay.
- For anti-oxidant assay

Statement of problem

Anti-microbial resistance (AMR) has emerged as one of the most serious global health threats in this century. The problem of AMR is especially urgent regarding antibiotic resistance in bacteria. The quest for the development of new anti-microbial agents to avert a developing global crisis in health care is imperative. This has led to the search of new raw materials that can be used in developing new anti-microbial compounds that can combat the increasing resistance by the pathogens.

Plants have served as a valuable source of ingredients in the traditional treatment of infectious diseases for millennial as being

highlighted by historical records and modern ethno-botanical studies. Plants are capable of producing a vast array of structurally diverse compounds, each of which serves as a specific role for the plant itself (e.g., defense against phytopathogens). Sometimes, these compounds are also active against human pathogens. There are four major groups of antimicrobial compounds made by plants: phenolics and polyphenolics, terpenoids and essential oils, lectins and polypeptides, and alkaloids. Thus, crude extracts of medicinal plants stands out as veritable sources of potential resistance modifying agents. Hence, the evaluation of the combined effect of herbal extracts with commercially produced anti-microbial agents and formulation of herbal extracts concoctions can be used as means of developing new and improved anti-microbial agents.

Rationale of study

The Himalayas of Nepal are famous for the medicinal plants and have even been mentioned in the Ayurveda. Many of the herbs and plants found in the Himalayas are used in the traditional healing systems like Ayurvedic, Homeopathic, Amchi, and Allopathy and so on. A study has reported 690 species of plants from all over Nepal are considered having medicinal properties (Malla and Shakya, 1984); out of which 510 species are found in wild in Nepal; 120 species are in cultivation and 60 species are exotic.

Though, there has been an extensive use of *Tinospora sinensis* and *Berberis aristata* by traditional healers based on the claim that these plants treat ailments including gastrointestinal tract disorders, inflammatory infections, respiratory tract infections, skin disorders and rheumatism, systematic investigations are still needed.

Therefore, it seems imperative to prepare the extracts by pressurized liquid extraction, and to analyze the phyto-constituents and important medicinal properties of *T. sinensis* and *B. aristata*.

The aim of the present study is to investigate the phytochemical and bio-medicinal properties of *T. sinensis* and *B. aristata* by determining the anti-microbial activity of the stem and leaves extracts on clinical isolates of *Staphylococcus aureus, Bacillus subtilis, Escherichia coli* and *Klebsiella pneumoniae* and anti-oxidant activity by DPPH free radical scavenging assay.

Literature review

Jiang H et. al. Filoterapia; "New terpenoid and phenylpropanoid glycosides from *Tinospora sinensis*" (2018); conducted the examination of the extract of *T. sinensis* for finding out more bioactive compounds and isolated two new cadinane sesquiterpene glucosides (1,2), two new phenyl propanoid glycosides (3,4) and two new diterpenoid glucosides (5,6), as well as six known compounds from the stems of *T. sinensis*. The structure of these compounds were elucidated by extensive spectroscopic analysis and chemical methods; which named these new compounds as 'Tinosinenoside G' (1), 'Tinosinenoside H' (2), 'Tinosinenoside I' (3), 'Tinosinenoside J' (4), '4-epimer of 2-deacetoxytinosinenoside D' (5) and 'Tinosinenoside K' (6). The six known compounds were identified as 'Tinocordiside' (7), 'Si-napyl alcohol-9O-(E)-p-coumaroyl-4-O-β-D-glucopyanoside' (8), 'Betulalbuside A' (9), 'Vervenone-10-Oβ-D-glucopyranoside' (10), 'Syringin' (11) and 'Tinosiene' (12) by comparison with their NMR data with those reported. The absolute configuration of Compound (1) was determined by time-dependent density functional theory (TDDFT) electronic circular dichroism (ECD) calculations and in-situ dimolybdenum CD method. They also conducted the test for in-vitro cytotoxicity against BV-2 microglial cells and inhibition on NO release using LPS induced BV-2 microglial cells with positive control of minocycline (MINO) for all the isolated compounds except for the Compound (7), (11) and (12). From this, they concluded that all the tested compounds showed no cytotoxicity in vitro against BV-2 microglial cells at the concentration of 10.0, 30.0 and 100.0 μM. However, it also showed that Compound (4) showed weak inhibitory activity on NO release with IC_{50} values of 42.90 μM in vitro assay and the IC_{50} values of positive control was 5.41 μM. Hence, they suggested that *T. sinensis* plant may be a good leading compounds resource for treating neuro-inflammation and related ailments.

Lam SH., *et al*. Molecules; "Chemical Constituents from the Stems of *Tinospora sinensis* and Their Bioactivity" (2018); conducted the experiment to determine the chemical constituents from the stems of *T. sinensis* and evaluate their bioactivity. They refluxed the dried stems of *T. sinensis* with methanol and the obtained extract was divided into chloroform ($CHCl_3$) and water (H_2O) soluble fractions by liquid-liquid partition. Upon further purification

over silica gel column and preparative thin layer chromatography (pTLC), fifty-seven compounds were isolated from the stems of *T. sinensis*, including three new compounds, namely 'Tinosporide A', 'Tinosporin A' and 'Tinosporin B'. The other fifty-four known compounds were identified, including eight lignans, five pyrrole alkaloids, seventeen benzenoids, ten terpenoids, eight sterols, four amides, one coumarin and last two, lichexanthone and 2,6-dimethoxy-pquinone. The chemical structures of the three new compounds were determined on the basis of 1D and 2D NMR and mass spectrometric analysis. Fifteen purified compounds were examined for their inhibition bioactivity of superoxide anion generation and elastase release by human neutrophils in response to fMLP/CB. However, most displayed weak inhibition percentages at the test concentration (10μM). Among them, three purified compounds displayed higher inhibitions of superoxide anion generation at 10μM and one compound also exhibited inhibitory effect on elastase release. They also concluded that Columbin exhibited significant antiinflammatory activities in a dose-dependent manner and malabarolide was not the pre-dominant component, might be due to the different parts of plant materials. Hence, they suggested that the extracts and purified compounds of the stems of *T. sinensis* have the potential to be developed as novel anti-inflammatory lead drugs as health foods, but also require further investigation of the anti-inflammatory mechanism.

Javed Ahamed., *et al*. IRJP 2012, 3 (2); "Development of Quality Standards of *B. aristata* stem bark", Phytochemical Research Laboratory, Department of Pharmacognosy and Phytochemistry, Faculty of Pharmacy, Hamdard, New Delhi-110062, India, investigated the physio-chemical parameters like moisture content, total ash content, acid insoluble ash content, water soluble ash content, water extractable matter and phytochemical screening of *Berberis aristata* stem bark. The preliminary phytochemical screening of *B. aristata* revealed the presence of alkaloids, glycosides, phenolic compounds, carbohydrates, flavonoids and sterols. From the physio-chemical parameter, it was concluded that the total ash value, acid insoluble ash and water soluble ash was found to be 12.27%, 5.87% and 1.95% respectively. This percentage clearly indicates that the stem bark of *B. aristata* is best for drug action and effects. The successive extractive value revealed that the percentage yield of methanolic extract was highest with 5.47% and lowest percentage yield was that of dichloromethane with 1.09%. Hot extractive value revealed that the percentage yield of methanolic extract was highest with 6.4% and lowest percentage yield was that of petroleum ether with 2.52%. Cold extractive value revealed that the percentage yield of methanolic extract was highest with 4.25% and lowest percentage yield was that of petroleum ether with 1.56%. With this study, it is concluded that methanol, being the most polar solvent, extracted the highest amount of constituents from the plant material. The moisture content was found to be 6.5%, swelling was not found and the foaming index was less than 100. The methanolic extract in the solvent system of n-butanol-ethyl acetate: formic acid: water (3:5:0.5:1) revealed 4 spots at UV 366nm with Rf values being 0.47, 0.52, 0.74 and 0.86. Hence, all these parameters, which are being reported, could be of great significance in identification of distinctiveness features of the drug and also valuable in manufacturing as raw material or in prescription medicine.

Khan M.I., *et al.* (2018); "Quality Control Standardization and Evaluation of Antimicrobial Potential of Daruhaldi (*Berberis aristata* DC) Stem Bark", investigated the quality standards and antimicrobial potential for the stem bark of *Berberis aristata*. The determination of botanical, physico-chemical, pharmaco-toxicological, fluorescence, microbial load, and phytochemical parameters of the stem bark were carried out. Using the CAMAG-HPTLC system, High-performance thin-layer chromatography (HPTLC) was carried out. Anti-microbial screening revealed that the extract were sensitive against the bacteria *Bacillus* subtilis and *Escherichia coli* and anti-fungal screening revealed it's sensitivity against the fungi *Penicillium citrinum* and *Aspergillus terreus*. The foreign matter, foaming index, swelling index, bitterness value, resin content, loss on drying, total ash, acid-insoluble ash, water-soluble ash, heavy metals, microbial load, berberine content, total phenolic content, and total flavonoid content were found to be 0, 0, 5, 1.34, 0.86%, 2.07%, 4.33%, 0.28%, 2.66%, within limits, 6 colonies in 1/100 dilution, 0.032 mg/g, 144.04 mg/ml, and 85.61 mg/ml, respectively. Hot extractive value revealed that the percentage yield of aqueous extract was highest with 9.36 ± 0.31% and lowest percentage yield was that of petroleum ether extract with 2.98 ± 0.52%. Cold extractive value revealed that the percentage yield of aqueous extract was highest with 1.60 ± 0.07% and lowest percentage yield was that of hydro methanolic extract with 0.48 ± 0.04%. Likewise, successive extractive value

revealed that the percentage yield of petroleum ether extract was highest with 3.16 ± 0.10% and lowest percentage yield was that of methanolic extract with 1.56 ± 0.21%. The preliminary phytochemical screening of the methanolic extract revealed the presence of phytochemicals such as alkaloids, glycosides, flavonoids, phenolics, sterols, carbohydrates, and proteins. In the HPTLC study, the methanolic extract of *B. aristata* stem bark in the solvent system of toluene: ethyl acetate: formic acid (15:10:2) revealed 5 and 6 spots at UV 254 and 366 nm, respectively, with max Rf values in the range of 0.32 to 0.91 indicating the occurrence of 6 different constituents in it. Chloroform extract of *B. aristata* stem bark in toluene: ethyl acetate: glacial acetic acid (10:5:1) revealed 12 and 8 spots at UV 254 and 366 nm, respectively, with max Rf values in the range of 0.16 to 0.84 indicating the occurrence of 12 different constituents. Hence, from the results obtained, it is concluded that the methanolic extract of *B. aristata* stem bark possesses potent antimicrobial activity and antifungal activity and could be of great significance in quality control standardization and preventive and therapeutic approaches to infectious diseases.

Lamichhane Basant., *et al.* (2014); "Study of phytochemical, anti-oxidant, anti-microbial and anti-cancer activity of *Berberis aristata*", investigated the phytochemical presence, antioxidant potential, anti-microbial activity, and anti-cancer activity of methanolic extract of Berberis aristata stem bark. The preliminary phytochemical screening of methanolic extract of B. aristata stem showed high amount of alkaloid to be 2.45%. One of the active constituents berberine; being a major alkaloid; and tannin was calculated to be 3.55% and 0.935% respectively. Other phytochemicals like saponin, terpenoids, coumarin, flavonoids and steroids were also present in trace amount. Anti-oxidant assay like DPPH free radical scavenging activity, hydrogen peroxide activity and reducing power activity was carried out by expressing in % inhibition with L-Ascorbic acid. DPPH free radical scavenging activity and hydrogen peroxide radical scavenging activity was compared with standard; IC_{50} for L-Ascorbic acid was 9.6 µg/ml and that of extract was 33.31 µg/ml and IC_{50} for L Ascorbic acid was 54.23 µg/ml and that of extract was 60.6 µg/ml respectively. Similarly, reducing power of plant extract at different concentration was compared with L-Ascorbic acid. Anti-microbial screening revealed that the extract were sensitive against *C. albicans*, *S. typhii*, *P. aeruginosa* and *E. coli*, while it was neutral to *K. pneumonia* and *S. aureus*. Further, MTT assay showed a significant cytotoxicity to breast cancer cell line (MDA-MB-231) and brain tumor cell line (U-87 MG) in compared to standard embryonic, fibroblast cell lines of mouse (NIH/3T3). Hence, with this study, it was concluded that the stem bark of *B. aristata* has anti-microbial activity against major pathogens, anti-oxidant activity comparable to that of L Ascorbic acid and also has potential anti-cancer properties and a detailed investigation was suggested.

Methodology

Collection of plant samples

The stem and leaves samples of *Tinospora sinensis* and *Berberis aristata* were collected from an altitude of 1161m and 1666m from the hills of Dhunibesi-7, Dhading by the method of 'Quadrant sampling' using sterile tools and techniques. The collected stem and leaves samples of the two medicinal plants were then kept separately in 4 separate airtight plastic container and the top of the plastic container was further sealed with parafilm to ensure that the container was airtight.

Drying of plant samples

The collected plant samples were dried separately under shade at room temperature in dark, clean and hygienic conditions for about 2-3 weeks. The dried samples were grinded into powdered form with the help of an electrical grinder and the respective weights of the powdered samples were taken (Gawai, Das, and Rout, 2013).

Crude extract preparation

The crude extract preparation of the dry powdered plant samples were done using the Soxhlet apparatus. The Soxhlet apparatus was placed in the place where there was no direct sunlight. The dry powdered plant samples were taken in a cylindrical pouch of Whatman filter paper and both the ends of the pouch were sealed with the help of stapler and the pouch was placed in the thimble. The round bottomed flask of the Soxhlet apparatus was filled with 750 ml of methanol and the thimble was attached to the top of the round bottomed flask and finally, the cooler was attached at the top of the thimble. The cooler was attached with inlet and outlet pipes for the circulation of cold water. After the water was circulated in the cooler, the heating device of the Soxhlet apparatus was turned

on and set to the temperature of (55-60) °C and the process was allowed to operate for about 72 hours. After the completion of 72 hours, the heating device was turned off along with the water circulation. Then, the Soxhlet apparatus was disassembled and the extract solution contained in the round bottomed flask was poured into sterile culture bottles having screw caps. This process was carried out separately for each of the four plant samples. Then they were subjected to evaporation by the use of rotary vacuum evaporator at 37°C until the fully dried extract was obtained. Thus obtained extracts were again transferred into beakers and further placed in water bath to obtain more concentrated extracts. Finally, the dried crude extracts were weighed, and the opening of the beakers were covered with aluminium foil and stored in refrigerator at 4°C for further use (Jha., *et al*., 2017).

Preliminary phytochemical analysis

Few milligrams of the obtained crude extracts were taken and dissolved in a conical flask containing about 100 ml of methanol separately. These were considered as the 'test solution' for the preliminary phytochemical analysis. The following procedure was carried out for each of the plant extract samples.

Test for alkaloids

Mayer's Test (Potassium Mercuric Iodide Reagent)

Few ml of test solution was taken in a clean test tube and a few drops of Mayer's reagent was added to it. If the cream coloured precipitate was formed, it showed the presence of alkaloids.

Wagner's Test (Iodine-Potassium Iodide)

1.27 grams of iodine and 2 grams of potassium iodide were dissolved in 5 ml of distilled water and the solution was diluted to 100 ml with distilled water. This was known as Wagner's reagent.

Few ml of test solution was taken in a clean test tube and few drops of Wagner's reagent was added to it. If the brown flocculent precipitate was formed, it indicated the presence of alkaloids.

Hager's Reagent Test

A saturated solution of picric acid was prepared which was known as Hager's reagent. Few ml of test solution was taken in a clean test tube and few drops of Hager's reagent was added to it. If the orange-yellow precipitate was formed, it indicated the presence of alkaloids.

Test for carbohydrate

Molisch's Test

Few ml of test solution was taken in a clean test tube containing 0.5 ml distilled water, and it was mixed with 2 drops of Molisch's reagent. To that solution, 1 ml of conc. sulphuric acid was added from the side of the inclined test tube, such that the acid formed a layer beneath the aqueous solution without mixing with it. If the red brown ring was appeared at the common surface of the liquids, sugars were present.

Fehling's solution test

Fehling's solution A and Fehling's solution B were mixed in equal volumes immediately before use and was termed as Fehling's solution. Few ml of test solution was taken in a clean test tube and few ml of distilled water was added to it, followed by the addition of few ml of the Fehling's solution. The mixture was then warmed. If a red precipitate of cuprous oxide was obtained, reducing sugars were present.

Benedict's test

Few ml of test solution was taken in a clean test tube and few drops of Benedict's reagent was added to it followed by boiling on water bath. If reddish brown precipitate was formed, the reducing sugars were present.

Test for tannin

Ferric chloride reagent test

A 5% w/v solution of ferric chloride was prepared in 90% ethanol. Then few ml of test solution was taken in a clean test tube and few drops of ferric chloride reagent was added to it. If dark green or deep blue color was obtained, tannins were present.

Gelatin solution test

1% w/v solution of gelatin was prepared in water, containing 10% sodium chloride. Few ml of test solution was taken in a clean test tube and few ml of gelatin solution was added to it. If white precipitate was obtained, tannins were present.

Test for flavonoid

Alkaline reagent test

Few ml of test solution was taken in a clean test tube and few drops of sodium hydroxide solution was added to it. If an intense

yellow color was formed which when treated with few drops of dil. acetic acid turned to colorless, flavonoids were present.

Zinc-hydrochloride reduction test

A mixture of zinc dust and conc. hydrochloric acid was prepared. Few ml of test solution was taken in a clean test tube and few drops of the mixture was added to it. If red color was observed after few minutes, flavonoids were present.

Ammonia test

A mixture of 5 ml of dil. ammonia and conc. sulphuric acid was prepared. Few ml of test solution was taken in a clean test tube and few ml of the mixture was added to it. If yellow color was formed which disappeared within 10-15 seconds, flavonoids were present.

Lead acetate test

Few ml of test solution was taken in a clean test tube and few ml of lead acetate was added to it.

If yellow or white precipitate was formed, flavonoids were present.

Shinoda test

Few ml of test solution was taken in a clean test tube and few ml of ethanol was added which was followed by filtration. Few pieces of magnesium chips were added to the filtrate followed by the addition of few drops of conc. hydrochloric acid. If pink, orange, red or purple was observed, flavonoids were present.

Sulphuric acid test

Few ml of test solution was taken in a clean test tube and few drops of conc. sulphuric acid was added to it. Of orange color was formed, flavonoids were present.

Test for saponins

Foam test

Few ml of test solution was taken in a clean test tube and was shaken vigorously with a small amount of sodium bicarbonate and distilled water. If a stable, characteristic honeycomb like froth was obtained, saponins were present.

Test for Sterol

Salkowaski reaction test

Few ml of test solution was taken in a clean test tube, and 2 ml of chloroform and 2 ml of conc. sulphuric acid were added from the side of the test tube, followed by shaking for few minutes. If red colour was developed in the layer of chloroform, sterols were present.

Test for fatty acid and fixed oil

Spot test

A small quantity of plant extract was pressed between two filter papers and allowed to dry at room temperature. If the first filter paper had stain, fixed oils were present.

Detection of resins

Acetone-water test

Few ml of test solution was taken in a clean test tube and few ml of acetone was added to it. Small amount of water was added to it and the test tube was shaken. If turbidity was appeared, resins were present.

Detection of protein and amino acid

Ninhydrin test

Few ml of test solution was taken in a clean test tube and few ml of 0.25% ninhydrin reagent was added to it, followed by boiling for a few minute. If blue color was formed, amino acid was present.

Biuret test

Few ml of test solution was taken in a clean test tube and was treated with 1 ml of 10% sodium hydroxide solution, followed by heating. To that, a drop of 0.7% copper sulphate solution was added. If purplish violet color was formed, proteins were present.

Anti-microbial assay

The entire process of anti-microbial assay was carried out in biosafety cabinet. The following processes were carried out for each of the plant extracts.

Micro-organisms used

- *Bacillus subtilis*
- *Staphylococcus aureus*

- *Klebsiella pneumoniae*
- *Enterobacter*

Preparation of plant extract

- For each of the plant extracts, the stock solution of 400 mg/ml was prepared by weighing 400 mg of plant extract in 5 ml culture tube and 1 ml of DMSO was added to it by micropipette.
- The extract was completely dissolved by vortexing for 5-10 minutes.
- The test solution of 400 mg/ml, 200 mg/ml, 100 mg/ml and 50 mg/ml concentration were prepared.

Preparation of inoculums

- The ATCC culture of the test organisms (*Bacillus subtilis*, *Staphylococcus aureus*, *Klebsiella pneumoniae* and *Enterobacter*) were taken out of the refrigerator and kept at room temperature to allow the temperature to equilibrate.
- Then few ml of each test organisms were inoculated in four separate conical flasks containing 150 ml of nutrient broth each and the conical flasks were allowed to be incubated at 37°C in incubator for 24 hours.
- Each culture to be tested was streaked onto nutrient agar to obtain isolated colonies.
- Overnight incubation was done at 37°C.
- Then, the isolated colonies were transferred by the help of sterile loop onto nutrient broth.
- Overnight incubation was done in rotary shaker incubator at 37°C.
- Thus, the pure culture of the four test organisms were obtained.

Inoculation procedure

- For this, 68 petri plates were prepared containing Muller-Hinton Agar.
- For inoculation, a sterile cotton swab was dipped into the suspension of pure culture of test organism and was pressed firmly against the inside wall of the conical flask just above the fluid level and the swab was rotated to remove excess liquid.
- The cotton swab was streaked over the entire surface of the medium four times, rotating the petri plate approximately 90 degrees after each application to ensure an even distribution of the inoculum.
- Finally, swabbing was done all around the edge of the agar surface.
- This process was done for all of the four pure cultures of test organisms.

Anti-microbial discs and plant extract discs

- Upon removal of the anti-microbial discs from the refrigerator (4°C), the package containing the disc was left unopened at room temperature for approximately one hour to allow the temperature to equilibrate which reduced the amount of condensation on the discs.
- For the plant extract, discs were made from No. 1 Whatman filter paper by the help of punching machine and the discs were sterilized by autoclaving.
- After sterilization, the discs were dipped into each of the concentration of the plant extracts prepared initially in a sterile condition.
- The discs were applied to the petri plates in which the test organisms were swabbed beforehand as soon as possible, but no longer than 15 minutes after inoculation.
- The discs were placed individually with the help of sterile forceps, and then gently pressed down onto the agar.
- Four discs, one of each concentration were placed in each of the petri plate swabbed with the four test organisms.
- Then the petri plates were labeled and parafilm was used to seal the edges of the petri plates.

Recording and interpreting results

- Now the petri plates were inverted and incubated at 37°C for 6-12 hours.
- After incubation, the diameter of the zones of complete inhibition was measured (including the diameter of the disc) in between 6-12 hours and was recorded it in millimeters.
- The measurements were done with the help of a ruler on the undersurface of the plate without opening the lid and recorded in a notebook.

Antioxidant assay

This is a quick and easy method to analyze the scavenging potential of antioxidants. Free radical scavenging activity of selected extract was measured by using 2, 2 diphenyl-1-picrylhydrazyl (DPPH) radical (Bhandari Laxman, 2013).

Preparation of DPPH solution (0.1mM)

- 0.39 mg of the DPPH was weighed carefully and was dissolved in methanol and finally the volume was maintained to 100 ml.

Preparation of extract solution

- A stock solution of plant extract of 1 mg/ml was prepared by dissolving 10 mg of each extract in 10 ml of methanol.
- From the sample stock solution, 10, 25, 50, 75, 100 µg/ml solutions were prepared.
- For 10 µg/ml, 10 µg/ml of extract solution from 1 mg/ml was mixed with 990 µl methanol.
- For 25 µg/ml, 10 µg/ml of extract solution from 1 mg/ml was mixed with 975 µl methanol.
- For 50 µg/ml, 25 µg/ml of extract solution from 1 mg/ml was mixed with 950 µl methanol.
- And, similarly other different concentrations were also prepared.

Evaluation of anti-oxidant potential

- To the sample solutions of different concentration, 1.5 ml DPPH solution was added.
- Samples were incubated at room temperature (in dark) for 30 minutes.
- The absorbance was then measured at 517 nm spectrophotometrically.
- Ascorbic acid was used as the standard for anti-oxidant.
- The percentage of inhibition was calculated by using formula,

$$I\% = (A_C - A_O/A_C) * 100\%$$

Proximate value analysis

The process was carried out for each of the four plant extracts.

Determination of moisture content

- About 1 gram of powdered plant extract was taken in a petri plate and kept in a hot air oven at 105°C for about 30 minutes and then the weight was taken.
- This process was continued till the constant weight was obtained.
- The final weight of the powder was noted.
- Loss on drying was calculated.
- Three samples were taken to calculate total loss on drying.
- The total moisture content was calculated by the formula:

Total Moisture Content = (Loss on Drying/Fresh Weight) * 100%

Determination of Ash Value

- A clean silica crucible was taken and weighed and dried in the hot air oven for a few minutes.
- About 1 gram of the powdered plant extract was weighed and place in the silica crucible.
- Fine layer of powdered plant extract was spread uniformly on the silica crucible.
- Crucible was kept inside the muffle furnace for 6 hours at 450°C to make the crucible dull red hot until free from carbon.
- Crucible was allowed to cool down at room temperature and weighed.
- The same procedure was repeated till constant weight.
- The percentage of total ash was calculated and noted down.
- The total ash percentage was calculated by the formula

Total ash percentage = (Ash Weight/Fresh Weight) * 100%

Result and Findings

Preliminary phytochemical analysis

The results of the preliminary phytochemical analysis of the four plant extracts were determined as follows:

Tinospora sinensis (Stem)

S. No.	Tests for	Result
1.	Alkaloids	+
2.	Carbohydrate	+
3.	Tannin	+
4.	Flavonoid	+
5.	Saponins	+ + +
6.	Sterol	-
7.	Fatty Acid and Fixed Oil	-
8.	Resins	-
9.	Protein and Amino Acid	+

Table 1: Phytochemical Screening of *Tinospora sinensis* (Stem).

Tinospora sinensis (Leaf)

S. No.	Tests for	Result
1.	Alkaloids	+
2.	Carbohydrate	+
3.	Tannin	+
4.	Flavonoid	+
5.	Saponins	+
6.	Sterol	-
7.	Fatty Acid and Fixed Oil	-
8.	Resins	+
9.	Protein and Amino Acid	-

Table 2: Phytochemical Screening of *Tinospora sinensis* (Leaf).

Berberis aristata (Stem)

S. No.	Tests for	Result
1.	Alkaloids	+ + +
2.	Carbohydrate	+
3.	Tannin	+ +
4.	Flavonoid	+
5.	Saponins	+ +
6.	Sterol	-
7.	Fatty Acid and Fixed Oil	-
8.	Resins	-
9.	Protein and Amino Acid	+

Table 3: Phytochemical Screening of *Berberis aristata* (Stem).

Berberis aristata (Leaf)

S. No.	Tests for	Result
1.	Alkaloids	+
2.	Carbohydrate	+
3.	Tannin	+ + +
4.	Flavonoid	+
5.	Saponins	+
6.	Sterol	-
7.	Fatty Acid and Fixed Oil	-
8.	Resins	+
9.	Protein and Amino Acid	-

Table 4: Phytochemical Screening of *Berberis aristata* (Leaf).

Anti-microbial assay

The results of the anti-microbial activity of the different concentrations of the four plant extracts were determined as follows

Zone of Inhibition of Tinospora sinensis (Stem)

	Bacillus subtilis **(mm)**	***Staphylococcus aureus*** **(mm)**	***Klebsiella pneumoniae*** **(mm)**	***Enterobacter*** **(mm)**
400 mg/ml	9	9	5	9
200 mg/ml	8.5	8.75	7.5	8.25
100 mg/ml	8.25	8	7	7.5
50 mg/ml	7.5	7.5	6.5	7

Table 5: Zone of Inhibition of *Tinospora sinensis* (Stem).

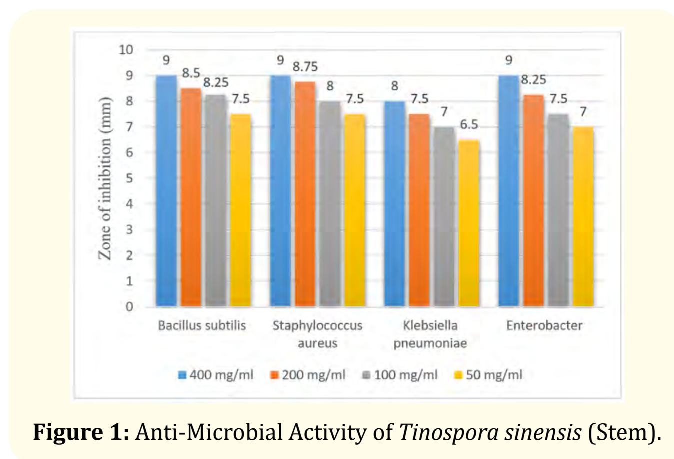


Figure 1: Anti-Microbial Activity of *Tinospora sinensis* (Stem).

Tinospora sinensis (Leaves)

	***Bacillus subtilis* (mm)**	***Staphylococcus aureus* (mm)**	***Klebsiella pneumoniae* (mm)**	***Enterobacter* (mm)**
400 mg/ml	8.5	8.75	8.5	8.5
200 mg/ml	8	8.5	7.5	7.75
100 mg/ml	7.75	8.25	6.5	7
50 mg/ml	7.5	8	6.25	6.75

Table 6: Zone of Inhibition of *Tinospora sinensis* (Leaves).

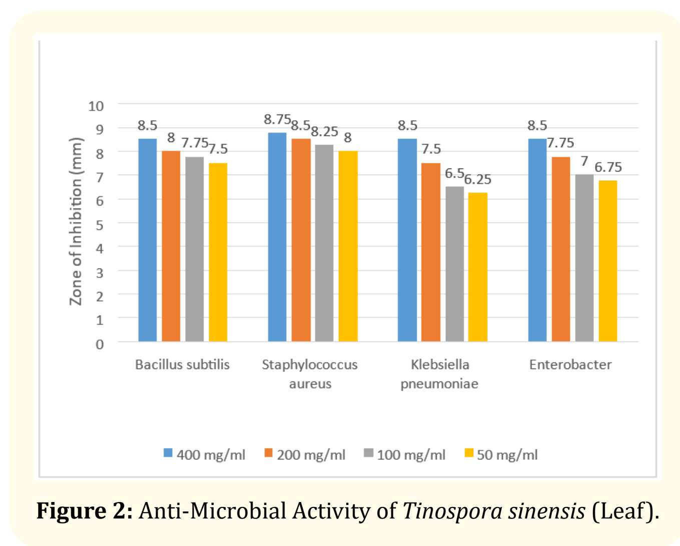


Figure 2: Anti-Microbial Activity of *Tinospora sinensis* (Leaf).

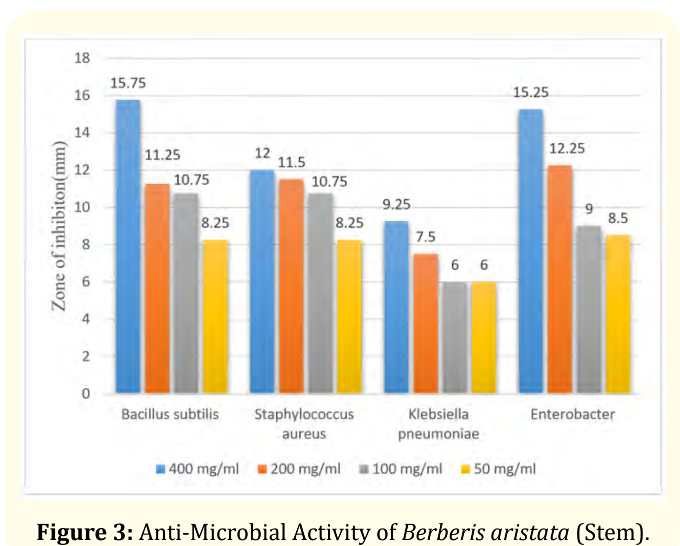


Figure 3: Anti-Microbial Activity of *Berberis aristata* (Stem).

Berberis aristata (Stem)

	***Bacillus subtilis* (mm)**	***Staphylococcus aureus* (mm)**	***Klebsiella pneumoniae* (mm)**	***Enterobacter* (mm)**
400 mg/ml	15.75	12	9.25	15.25
200 mg/ml	11.25	11.5	7.5	12.25
100 mg/ml	10.75	10.75	6	9
50 mg/ml	8.25	8.25	6	8.5

Table 7: Zone of Inhibition of *Berberis aristata* (Stem).

Berberis aristata (Leaves)

	***Bacillus subtilis* (mm)**	***Staphylococcus aureus* (mm)**	***Klebsiella pneumoniae* (mm)**	***Enterobacter* (mm)**
400 mg/ml	9.75	8.75	8.75	8.5
200 mg/ml	8.25	8.5	8.25	8.5
100 mg/ml	7.75	8	8	7.5
50 mg/ml	7.5	7.25	7.75	6.75

Table 8: Zone of Inhibition of *Berberis aristata* (Leaves).

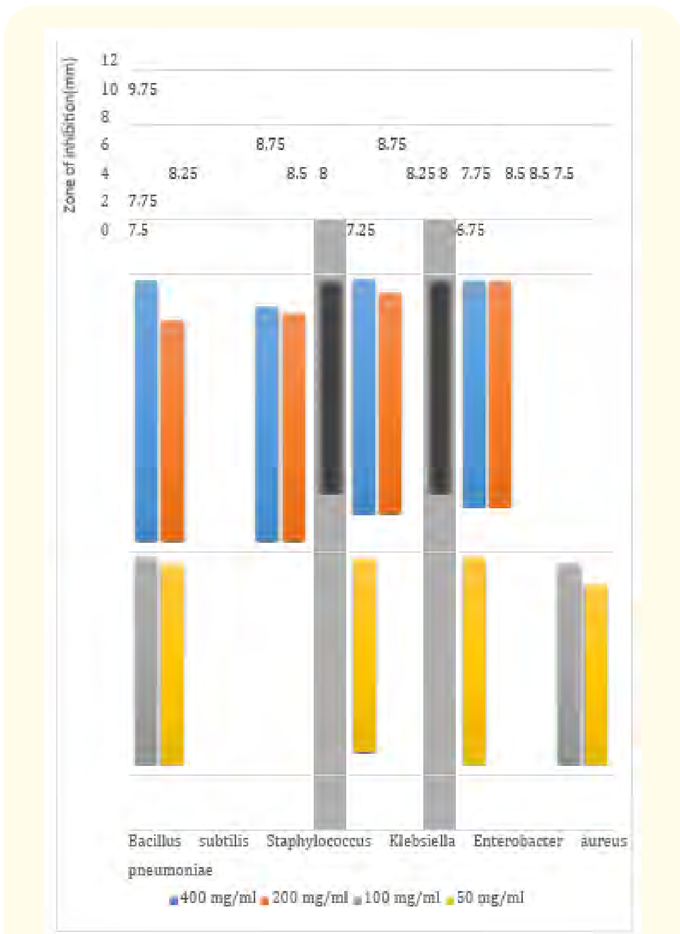


Figure 4: Anti-Microbial Activity of *Berberis aristata* (Leaf).

Anti-oxidant assay

The results of the anti-oxidant activity of the four plant extracts were determined as follows

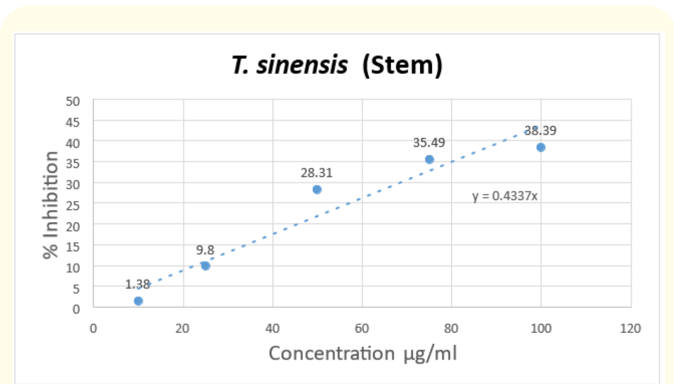


Figure 5: Anti-oxidant activity of *Tinospora sinensis* (Stem)

Plants/MeOH	Conc. (µg/ml)	Absorbance	Mean Absorbance	% Inhibition	IC_{50} (µg/ml)
Tinospora sinensis Leaves (*Ac = 0.824)	100	0.552	0.548	33.49	128.53
		0.544			
	75	0.565	0.567	31.18	
		0.569			
	50	0.59	0.593	28.03	
		0.595			
	25	0.74	0.745	9.58	
		0.749			
	10	0.812	0.808	1.94	
		0.804			

Tinospora sinensis Stem (*Ac = 0.724)	100	0.443	0.446	38.39	115.47
		0.449			
	75	0.465	0.467	35.49	
		0.469			
	50	0.515	0.519	28.31	
		0.523			
	25	0.65	0.653	9.8	
		0.656			
	10	0.716	0.714	1.38	
		0.712			
Berberis aristata Leaves (*Ac = 0.724)	100	0.403	0.4	44.75	96.15
		0.397			
	75	0.42	0.422	41.71	
		0.424			
	50	0.456	0.46	36.46	
		0.464			
	25	0.625	0.621	14.22	
		0.617			
	10	0.7	0.703	2.9	
		0.706			
Berberis aristata Stem (*Ac = 0.824)	100	0.358	0.361	50.13	82.101
		0.363			
	75	0.379	0.376	48.06	
		0.373			
	50	0.387	0.39	46.13	
		0.393			
	25	0.58	0.585	19.19	
		0.489			
	10	0.661	0.666	8.01	
		0.671			

Table 9: Anti-Oxidant Activity.

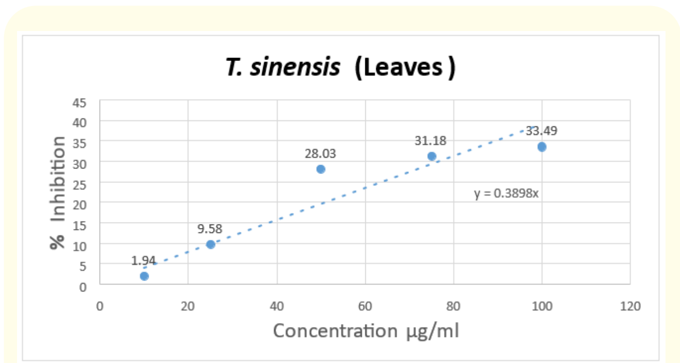


Figure 6: Anti-oxidant activity of *Tinospora sinensis* (Leaves).

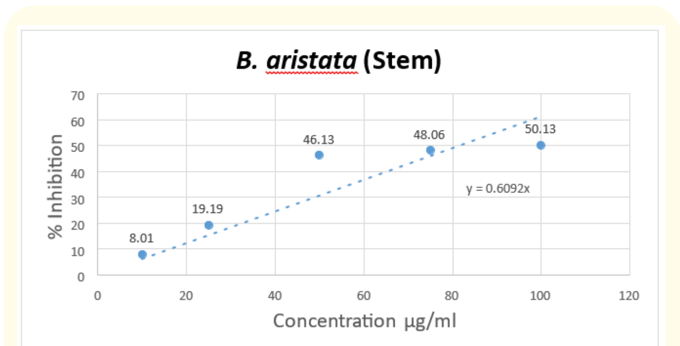


Figure 7: Anti-oxidant activity of *Berberis aristata* (Stem).

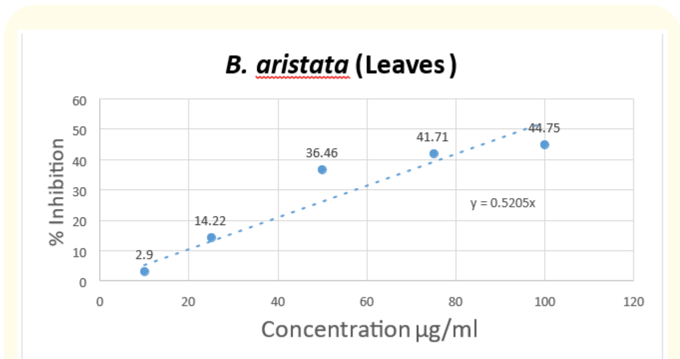


Figure 8: Anti-oxidant activity of *Berberis aristata* (Leaves).

Proximate value analysis

Determination of moisture content

Plant Sample	Fresh Weight (gm)	Dry Weight (gm)	Loss on Drying (gm)	Loss on Drying (%)
Berberis aristata (Stem)	2.0	1.83	0.17	8.5
Berberis aristata (Leaf)	2.0	1.77	0.23	11.5
Tinospora sinensis (Stem)	2.0	1.81	0.19	9.25
Tinospora sinensis (Leaf)	2.0	1.75	0.25	12.5

Table 10: Determination of Total Moisture Content.

Determination of ash value

Plant Sample	Fresh Weight (gm)	Ash Weight (gm)	Total Ash (%)
Berberis aristata (Stem)	3.0	0.27	9.01
Berberis aristata (Leaf)	3.0	0.15	4.99
Tinospora sinensis (Stem)	3.0	0.25	8.32
Tinospora sinensis (Leaf)	3.0	0.16	5.31

Table 11: Determination of Ash Value.

Discussion

In this project, we have studied about *Berberis aristata* and *Tinospora sinensis* (medicinal plants) for their proximate, phytochemical, anti-microbial and anti-oxidant properties collected from an altitude of 1666 m and 1161 m respectively from the hills of Dhunibesi07, Dhading. The extraction process was done by maceration method by use of methanol.

We performed nine phytochemical tests from the extracts of the collected plants. When methanol was used as solvent, all plant extracts showed positive phytochemical results except for the test of sterol and fatty acid/fixed oil. The positive results of the preliminary phytochemical analysis depicted that those four plant extracts have anti-microbial and antioxidant properties.

Anti-microbial assay was also performed with all the four plant extracts by agar disc diffusion method. Four antibiotics were used in this process. In this study, we performed anti-microbial assay with four pathological bacteria. Among the four plant extracts, the stem extract of *Berberis aristata* showed highest anti-microbial activity whereas the leaf extract of *Tinospora sinensis* showed lowest anti-microbial activity. Zone of inhibition was larger in 400 mg/ml and least in 50 mg/ml concentration of extracts. The largest zone of inhibition (15.75 mm) was obtained by the stem extract of *Berberis aristata* with *Bacillus subtilis* in 400mg/ml concentration. Anti-microbial activity was comparatively higher in 400 mg/ml concentration of plant extracts. When 200 mg/ml concentration of plant extracts were used, the largest zone of inhibition was 12.25 mm, obtained by the stem extract of *Berberis aristata* against *Enterobacter*. When 100 mg/ml concentration of plant extracts were used, the largest zone of inhibition (10.75 mm) was obtained by the stem extract of *Berberis aristata* with *Bacillus subtilis*. When 50 mg/ml concentration of plant extracts were used, the largest zone of inhibition was 8.25 mm, obtained by the stem extract of *Berberis aristata* against *Bacillus subtilis* and *staphylococcus aureus*. Among the four pathogenic bacteria used, all were found susceptible (Zone of inhibition<10) to plant extracts at different concentrations. In the stem extracts of *Berberis aristata* and *Tinospora sinensis*, and the leaf extract of *Tinospora sinensis*, *Klebsiella pneumoniae* was found less susceptible whereas in the leaf extract of *Berberis aristata*, *Staphylococcus aureus* was found less susceptible.

We also performed anti-oxidant assay of the four plant extracts by calculating the IC_{50} value. The IC_{50} value of reference standard ascorbic acid was found to be 25.38 µg/ml. In our study, the stem extract of *Berberis aristata* showed effective anti-oxidant activity with IC_{50} value of 82.101 µg/ml.

We also performed the proximate value analysis for all of the four plant extracts by determining the total moisture content and the total ash percentage. From the study of total moisture content, it was observed that the stem extract of *Berberis* aristata had the lowest moisture content being 8.5% while the leaf extract of *Tinospora sinensis* had the highest moisture content being 12.5%. From the study of total ash percentage, it was observed that the leaf extract of *Berberis aristata* had the lowest ash percentage (4.99%) while the stem extract of *Berberis aristata* had the highest ash percentage (9.01%). Hence, from the proximate value analysis it was concluded that the stem and leaf extracts of *Berberis aristata* have higher physical and chemical stability.

This study revealed that the selected plant extracts possessed significant anti-oxidant activity and this could be due to the presence of phenolic compounds in the preliminary phytochemical analysis.

Conclusion

Plant and plant based medicaments are the basis of many modern pharmaceuticals which we use today for our various ailments. The extraction from medicinal plants have been tested to identify the sources of the therapeutic effects. As a result, great interest in traditional medicine and increase in demand for more and more drugs from plant sources have been observed. This interest in plant derived drugs was mainly due to the current wide spread belief that "green machine" is safe and more dependable than the costly synthetic drugs which have adverse side effects (Parekh and Chanda, 2005).

Successive extraction of medicinal plant (crude materials) is largely dependent on the type of solvent used in the extraction process. The traditional healers use primarily water as the solvents but a number of researchers found that plant extracted with alcoholic solvents (ethanol/methanol) provide more consistency, complete extraction including less polar compounds which possesses the ability to isolate tannins, polyphenols, terpenoids, saponins etc. and water solvent extract contains only starch, saponins, polypeptides and lactins (Zhang, X., 2000).

The preliminary phytochemical screening of the plant extracts were found to exhibit positive tests for alkaloids, carbohydrates, tannins, flavonoid and saponins. However, the phytochemical tests for sterol and fatty acid/fixed oil were found to be negative for all the plant extracts. The stem extracts of *B. aristata* and *T. sinensis* were found to exhibit negative test for resin while they exhibited positive test for protein and amino acid. The leaf extracts *B. aristata* and *T. sinensis* were found to exhibit positive test for resin while they exhibited negative test for protein and amino acid.

The result of phytochemical analysis revealed that the active principle responsible for the antibacterial activity is phenolic

and alkaloid compound. The flavonoids are responsible for the antioxidant activity. The present study will also be successful in identifying medicinal plants with different antimicrobial activity and this can further be exploited for isolation and characterization of the novel phytochemical in the treatment of infectious disease when drug resistant microorganisms are being developed in today's world. In Nepal, there are various medicinal plants with effective antimicrobial activities that can serve in developing drugs to various diseases so effective study and analysis of these plants need to be considered.

Acknowledgements

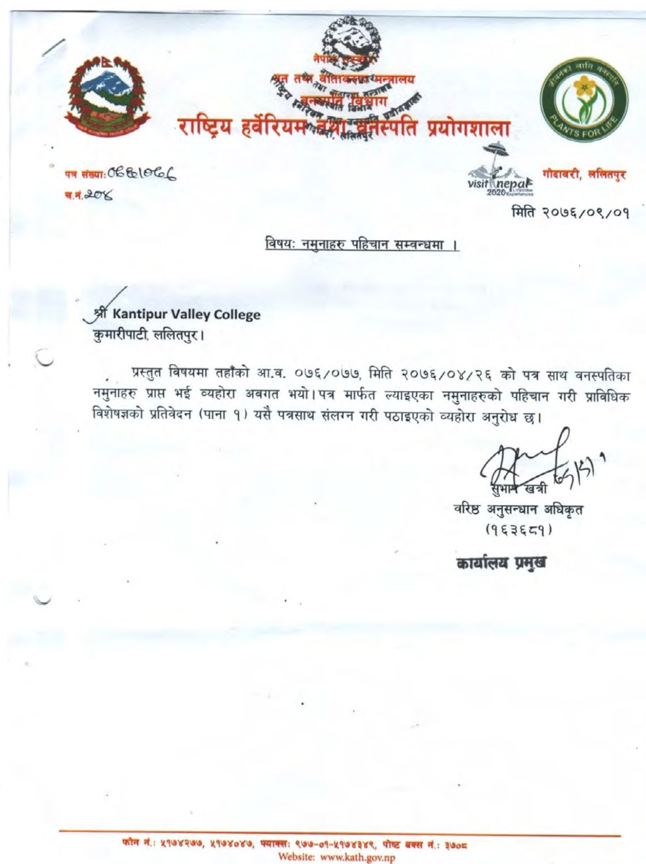
Foremost, we would like to express our sincere gratitude to Kantipur Valley College for funding on this project. We would like to acknowledge our Principal Mr. Pushpa Man Amatya, Academic Director Prof. Dr. Mukunda Ranjit, and Department Co-ordinator Mr. Sagar Thapa for facilitating in making our project work successful.

We would also like to thank our Supervisor, Mr. Sagar Atri for his continuous support of our Thesis study and research, for his patience, motivation, enthusiasm and immense knowledge. His guidance helped us in all the times of research and writing of this thesis. We could not have imagined having a better supervisor and mentor for our thesis.

Besides our Supervisor, we would like to thank Mr. Ramesh Raj Padhaya for his guidance to conduct the Phytochemical Analysis.

Our sincere thanks also goes to Mr. Laxman Bhandari, Department of Plant Resources, Natural Products Research Laboratory, Thapathali, Kathmandu for their immense help in conducting the anti-oxidant assay by DPPH method.

Last but not the least, we would like to thank our parents, families and friends for being the source of inspiration, support and guidance in every step of our life.



राष्ट्रिय हर्वेरियम तथा वनस्पति प्रयोगशाला

PLANTS FOR LIFE

पत्र संख्या: ०७६/०७७
च.नं. २०४

गोदावरी, ललितपुर

मिति २०७६/०९/०१

विषयः नमुनाहरु पहिचान सम्वन्धमा ।

श्री Kantipur Valley College
कुमारीपाटी, ललितपुर।

प्रस्तुत विषयमा तहाँको आ.व. ०७६/०७७, मिति २०७६/०४/२६ को पत्र साथ वनस्पतिका नमुनाहरु प्राप्त भई व्यहोरा अवगत भयो। पत्र मार्फत ल्याइएका नमुनाहरुको पहिचान गरी प्राविधिक विशेषज्ञको प्रतिवेदन (पाना १) यसै पत्रसाथ संलग्न गरी पठाइएको व्यहोरा अनुरोध छ।

सुभाष खत्री
वरिष्ठ अनुसन्धान अधिकृत
(१६३६८१)

कार्यालय प्रमुख

फोन नं.: ५१७४२७७, ५१७४०४७, फ्याक्स: ९७७-०१-५१७४३४९, पोष्ट बक्स नं.: ३७०८
Website: www.kath.gov.np

Appendix 1: Certification.

Bacillus subtilis

Bacillus subtilis known also as the hay bacillus or grass bacillus, is a Gram-positive, catalasepositive bacterium, found in soil and the gastrointestinal tract of ruminants and humans. A member of the genus *Bacillus* (Euzeby JP, 2008)). *B. subtilis* is rod-shaped, and can form a tough, protective endospore, allowing it to tolerate extreme environmental conditions. *B. subtilis* has historically been classi-

fied as an obligate aerobe, though evidence exists that it is a facultative anaerobe (Ambrosiano N, 1999). *B. subtilis* is considered the best studied Grampositive bacterium and a model organism to study bacterial chromosome replication and cell differentiation (Cohn F, 1872). It is one of the bacterial champions in secreted enzyme production and used on an industrial scale by biotechnology companies. *B. subtilis* cells are typically rod-shaped and are about 4-10 micrometers (μm) long and 0.25–1.0 μm in diameter (Hong HA et. al., 2009).

This species is commonly found in the upper layers of the soil and *B. subtilis* is thought to be a normal gut commensal in humans. The number of spores found in the human gut was too high to be attributed solely to consumption through food contamination. *B. subtilis* has been linked to grow in higher elevations and act as an identifier for both eco-adaptability and honey bee health (Sudhagar S. et. al., 2017). **B.**

Enterobacter sp.

Enterobacter is a genus of common Gram-negative, facultatively anaerobic, rod-shaped, nonspore-forming bacteria of the family ‘Enterobacteriaceae’. It is the type genus of the order ‘Enterobacterales’ (Adeolu M et. al., 2016). Several strains of these bacteria are pathogenic and cause opportunistic infections in immuno-compromised (usually hospitalized) hosts and in those who are on mechanical ventilation. The urinary and respiratory tracts are the most common sites of infection (Tan Wen-Si et. al., 2014). The genus *Enterobacter* is a member of the coliform group of bacteria. It does not belong to the fecal coliforms (or thermotolerant coliforms) group of bacteria, unlike *Escherichia coli*, because it is incapable of growth at 44.5 °C in the presence of bile salts. Some of them showed quorum sensing properties as reported before (Cabral, JPS, 2010).

Klebsiella pneumoniae

Klebsiella pneumoniae is a gram-negative, non-motile, encapsulated, lactose-fermenting, facultative anaerobic, rod-shaped bacterium. It appears as a mucoid lactose fermenter on MacConkey agar (Ryan KJ et. al., 2004). It naturally occurs in the soil, and about 30% of strains can fix nitrogen in anaerobic conditions. *Klebsiella pneumoniae* (*K. pneumoniae*) are bacteria that normally live in our intestines and feces. These bacteria are harmless when they’re in your intestines. But if they spread to another part of your body, they can cause severe infections. The risk is higher if we are sick. *K. pneumoniae* can infect our: eyes blood wounds lungs bladder brain liver (Postgate J, 1998).

Staphylococcus aureus

Staphylococcus aureus is a Gram-positive, round-shaped bacterium. It is often positive for catalase and nitrate reduction and is a facultative anaerobe that can grow without the need for oxygen (Masalha M. et. al., 2001). An estimated 20% to 30% of the human population are longterm carriers *S. aureus* which can be found as part of the normal skin flora, in the nostrils, and as a normal inhabitant of the lower reproductive tract of women (Kluytmans J. et. al., 1997).

Appendix 2: Micro-organisms used.

The types of media used were Mueller Hinton Agar (MHA) for antimicrobial assay, Nutrient Broth and Nutrient Agar for preparation of test micro-organisms. Methanol was used for Soxhlet extraction process and DPPH for anti-oxidant assay.

Chemical composition of Mueller Hinton Agar

Ingredient	gm/l
HM infusion B from	300
Acicase	17.5
Starch	1.5
Agar	17
Final pH (at 25^{0}C)	7.3 ± 0.1

Chemical composition of Nutrient Agar

Ingredient	gm/l
Peptone	5
HM Peptone B	1.5
Yeast Extract	1.5
Sodium chloride	5
Agar	15
Final pH (at 25^{0}C)	7.4 ± 0.2

Chemical composition of Nutrient Broth

Ingredient	gm/l
Peptone	5
Sodium chloride	5
HM peptone B	1.5
Yeast extract	1.5
Final pH (at 25^{0}C)	7.4 ± 0.2

Appendix 3: Composition of media used.

Figure: Collection of Plant Samples.

Figure: Drying of Stem and Leaves of

Tinospora sinensis

Figure: Drying of Stem and Leaves of

Berberis aristata

Figure: Grinding Process using Electrical Grinder

Figure: Rotary Vacuum Evaporator

Figure: Crude Plant Extracts

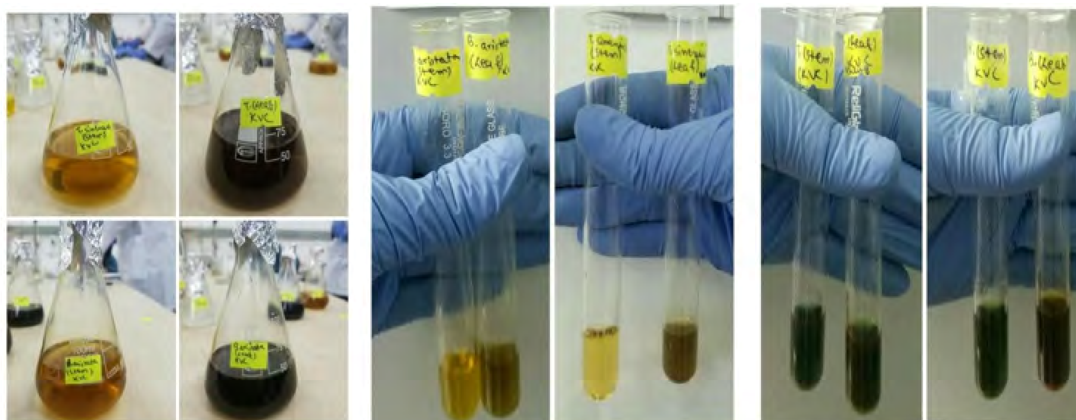
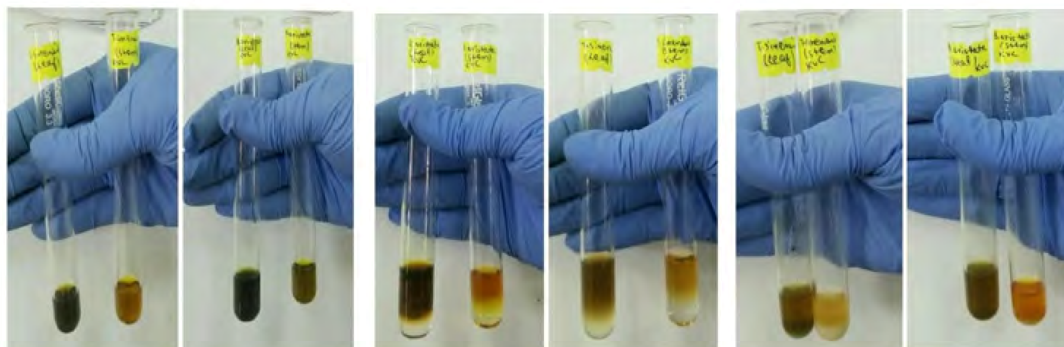


Figure: Test solutions for Preliminary Phytochemical Analysis

Test for Alkaloids

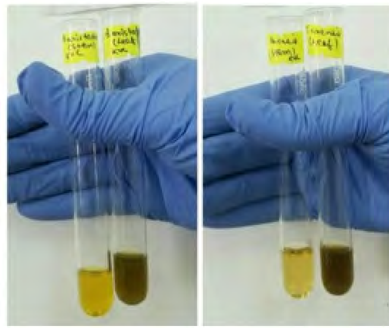
Test for Carbohydrate



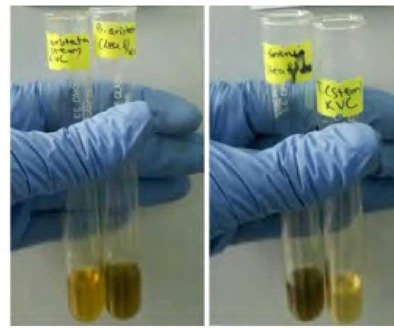
Test for Tannin

Test for Flavonoid

Test for Saponins



Test for Resins



Test for Protein and Amino Acid

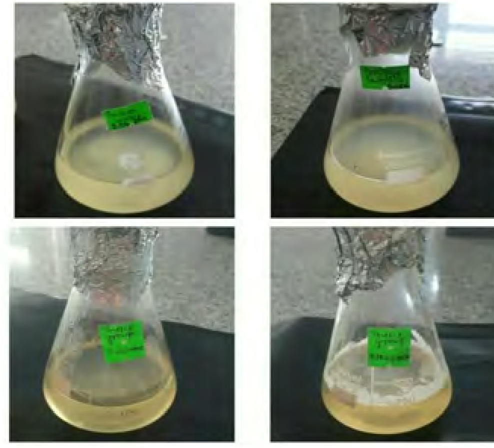


Figure: Preparation of Plant Extracts at Figure: Preparation of Inoculums Different Concentrations

Figure: Working in Biosafety Cabinet

Figure: *B. subtilis* (*B.* Figure: *Enterobacter* (*B.* Figure: *B. subtilis* (*B. aristata*-Stem) *aristata*-Stem) *aristata*-Leaves)

Figure: *B. subtilis* (*B.* Figure: *Enterobacter* (*T.* Figure: *Enterobacter* (*T.* *aristata*-Leaves) *sinensis-Leaves*) *sinensis*-Stem)

Figure: UV-Vis Spectrophotometer Figure: Determination of Total Moisture Content Figure: Determination of Ash Value using Muffle Furnace

Figure: Nutrient Agar

Figure: Muller Hinton Agar

Figure: DPPH Chemical

Appendix 4: Photos.

Bibliography

1. Bhattarai KR., *et al*. "Medicinal Plant Knowledge of the Panchase Region in the Middle Hills of the Nepalese Himalayas". *Banko Janakari* 21.2 (2013): 31-39.

2. Kunwar Ripu M., *et al*. "Medicinal plants, traditional medicine, markets and management in far-west Nepal". *Journal of Ethnobiology and Ethnomedicine* 9.24 (2013).

3. Uprety Yadav., *et al*. "Indigenous use and bio-efficacy of medicinal plants in the Rasuwa District, Central Nepal". *Journal of Ethnobiology and Ethnomedicine* 6.3 (2010).

4. Sapkota Shova., *et al*. "Diversity of Flowering Plants in Nubri Valley, Manaslu Conservation Area, Central Nepal". *American Journal of Plant Sciences, Scientific Research Publishing* (2017).

5. Sheng-Ji P. "Ethnobotanical approaches of traditional medicine studies: some experiences from Asia". *Pharmaceutical Biology* 39.1 (2001): 74-79.

6. Tiwari S., *et al*. "In: Proceeding of local experience-based national strategy for organic production and management of MAPs/NTFPs in Nepal.

7. Pyakurel Dipesh., *et al*. "Trade and Conservation of Medicinal and Aromatic Plants in Western Nepal". *Botanica Orientalis: Journal of Plant Science* (2018): 27-37.

8. Singh MP., *et al*. "Medicinal plants of Nepal – retrospects and prospects". *Economic Botany* 33 (1979): 185-198.

9. Olsen CS and Larsen HO. "Alpine medicinal plant trade and Himalayan mountain livelihood strategies". *The Geographical Journal* 169 (2003): 243–254.

10. Kunwar RM., *et al*. "Indigenous use and ethnopharmacology of medicinal plants in far-west Nepal". *Journal of Ethnobotany Research and Applications* 7 (2009): 5-28.

11. Kaur R., *et al*. "Medicinal plants for the treatment of sexual transmitted diseases". *International Journal of Pharmaceutical Innovations* 2 (2012): 13-23.

12. Neeraja PV and Margaret E. "Amruthavalli: Tinospora cordifolia: Multipurpose rejuvenator". *International Journal of Pharmaceutical, Chemical and Biological Sciences* 3 (2013): 233-241.

13. Sachet Hedge and M Jayaraj. "A Review of the Medicinal Properties, Phytochemical and Biological Active Compounds of Tinospora sinensis (Lour.) Merr". *Journal of Biologically Active Product from Nature* (2016).

14. Mitra R and Raghunandan K. "Anonymous, Pharmacognosy of Indigenous Drugs 1; (Central Council for Research in Ayurveda and Siddha, New Delhi)" (1982): 321.

15. Sharma OP and Bhat TK. "DPPH antioxidant assay revisited". *Food Chemistry* 113.4 (2009): 1202-1205.

16. Neeraja PV and Margaret E. "Amruthavalli: Tinospora cordifolia: Multipurpose rejuvenator". *International Journal of Pharmaceutical, Chemical and Biological Sciences* 3 (2013): 233-241.

17. Mazumder Papiya Mitra., *et al*. "Phytopharmacology of Berberis aristata dc: a review". *Journal of Drug Delivery and Therapeutics* 1.2 (2011): 46-50.

18. Saied S., *et al*. "Phytochemical studies of Berberis aristata". *Journal of Basic and Applied Sciences* 3 (2007): 1-3.

19. Parmar C and Kaushal MK. “Berberis aristata: Wild fruits. Kalyani Publishing Ludhiana (1982): 10-14.
20. Saied S., *et al*. “Phytochemical studies of Berberis aristata”. *Journal of Basic and Applied Sciences* 3 (2007):1-3.
21. Blasko G., *et al*. “Taxilamine, a pseudobenzylisoquinoline alkaloid”. *Heterocycles* 19 (1982): 257-259.
22. Chakravarti KK., *et al*. “Alkaloidal constituents of the bark of Berberis aristata”. *Journal of Scientific and Industrial Research* 9.7 (1950): 161-164.
23. Katiyar D., *et al*. “Isolation and characterization of ndocosane from heartwood of Berberis aristata”. *International Journal of Pharmaceutical Sciences and Research* 2 (2011): 331-335.
24. Ambastha SP. “The Wealth of India. Publication and Information Directorate, New Delhi, CSIR 2 (1988): 118.
25. Das K., *et al*. “Techniques for evaluation of medicinal plant products as antimicrobial agent: Current methods and future trends”. *Journal of Medicinal Plants Research* 4.2 (2010): 104-111.
26. Das K., *et al*. “Techniques for evaluation of medicinal plant products as antimicrobial agent: Current methods and future trends”. *Journal of Medicinal Plants Research* 4.2 (2010): 104-111.
27. Nikhal SB., *et al*. “Hydroalcoholic extraction of Mangifera indica (leaves) by Soxhletion”. *International Journal of Pharmaceutical Sciences* 2.1 (2010): 30-32.
28. Ncube NS., *et al*. “Assessment techniques of antimicrobial properties of natural compounds of plant origin: current methods and future trends”. *African Journal of Biotechnology* 7.12 (2008): 1797-1806.
29. Rengganaten V. “*In-vitro* Screening of Cytotoxic Effect and Antioxidant Activity of Pereskia bleo and Centella asiatica Crude Extracts, Department of Biochemical Science, University Tunku Abdul Rahman (2013).
30. Handa SS., *et al*. “Extraction Technologies for Medicinal and Aromatic Plants”. *International Centre for Science and High Technology, Trieste* (2008): 21-25.
31. Abdul Wadood., *et al*. “Phytochemical Analysis of Medicinal Plants Occurring in Local Area of Mardan”. *Department of Biochemistry, Pakistan* (2013).
32. Manjulika Yadav., *et al*. “Preliminary phytochemical screening of Six Medicinal Plants Used In Traditional Medicine”. University of Arahabad (2014).
33. Handa SS., *et al*. “Extraction Technologies for Medicinal and Aromatic Plants”. *International Centre for Science and High Technology, Trieste* (2008): 21-25.
34. Enne VI., *et al*. “Persistence of sulphonamide resistance in Escherichia coli in the UK despite national prescribing restriction”. *The Lancet* 28 (2001): 13251328.
35. Alagesaboopathi C. “Antimicrobial Potential And Phytochemical Screening Of Andrographis Affinis Nees An Endemic Medicinal Plant From India”. *International Journal of Pharmacy and Pharmaceutical Sciences* 3.2 (2011): 157-159.
36. Ahmad I and Beg AZ. “Antimicrobial and phytochemical studies on 45 Indian medicinal plants against multiple drug resistant human pathogens”. *Journal of Ethnopharmacology* 74 (2001): 113-123.
37. Verzelloni E., *et al*. “Relationship between the antioxidant properties and the phenolic and the flavonoid content in traditional balsamic vinegar”. *Food Chemistry* 105.2 (2007): 564-571.
38. Sharma OP and Bhat TK. “DPPH antioxidant assay revisited”. *Food Chemistry* 113.4 (2009): 1202-1205.
39. Mustafa RA., *et al*. “Total phenolic compounds, flavonoids, and radical scavenging activity of 21 selected tropical plants”. *Journal of Food Science* 75.1 (2010): C28-C35.
40. Burits M and Bucar F. “Antioxidant activity of Nigella sativa essential oil”. *Phytotherapy Research* 14.5 (2000): 323-328.
41. Szabo MR., *et al*. “Improved DPPH determination for antioxidant activity spectrophotometric assay”. *Chemical Papers* 61.3 (2007): 214-216.
42. Shukla Abha., *et al*. “Phytochemical Screening, Proximate Analysis and Antioxidant Activity of Dracaena reflexa Lam. Leaves”. *Indian Journal of Pharmaceutical Sciences* 77.5 (2015): 640-644.