STUDIES ON ANTIOXIDANT AND XANTHINE OXIDASE INHIBITORY ACTIVITIES OF *COCCINIA GRANDIS (L.) VOIGT*, *DATURA METEL (Linn.)* AND *TAGETES PATULA (L.)* FROM RUPANDEHI DISTRICT OF NEPAL

A

DISSERTATION

SUBMITTED FOR THE

PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR

THE MASTER OF SCIENCE DEGREE IN CHEMISTRY

BY

PRABHAT KHAREL

Exam Symbol No.: CHE 756/073

T.U. Regd. No.: 5-2-0448-0021-2012

CENTRAL DEPARTMENT OF CHEMISTRY

INSTITUTE OF SCIENCE AND TECHNOLOGY

TRIBHUVAN UNIVERSITY, KIRTIPUR

KATHMANDU, NEPAL

2020

BOARD OF EXAMINER AND CERTIFICATE OF APPROVAL

This dissertation entitled "STUDIES ON ANTIOXIDANT AND XANTHINE OXIDASE INHIBITORY ACTIVITIES OF *COCCINIA GRANDIS (L.) VOIGT*, *DATURA METEL (L.)* AND *TAGETES PATULA (L.)* FROM RUPANDEHI DISTRICT OF NEPAL", by "Prabhat Kharel*",* under the supervision of Asst. Prof. Dr. Bimala Subba Central Department of Chemistry, Tribhuvan University, Nepal, is hereby submitted for the partial fulfillment of the Master of Science (M. Sc.) Degree in Chemistry. This dissertation has been accepted for the award of a degree.

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I, "**Prabhat Kharel**", hereby declare that the work presented herein is genuine work done original by me and has not been published or submitted in this institute for the requirement of a degree program. Any literature, data or works done by others are cited in this dissertation has been given due acknowledgement and listed in the reference section.

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ACKNOWLEDGEMENTS

The successful completion of this study was possible with the support, assistance, motivation and advice of various personnel. I would like to express my whole hearted gratitude and appreciation to the following:

I would like to express my deepest gratitude and sincere appreciation to my supervisor **Asst. Prof. Dr. Bimala Subba,** Central Department of Chemistry, for her constant support, guidance, invaluable suggestions and regular feedback at all stages of my dissertation work.

I am grateful to the Head of the Central Department of Chemistry, respected Prof. **Dr. Ram Chandra Basnyat** for providing me an opportunity to conduct this dissertation as well as for his support during the work.

My deepest appreciation goes to all my respected teachers of CDC. I would also like to convey my thanks to all the administration staff as well as laboratory staffs of Department for their continuous assistance throughout the dissertation work.

I would especially like to thank **Mr. Karan Khadayat**, for his help and guidance during the lab work.

My special thanks go to all my colleagues whom I have been surrounded, for support and encouragement during the work.

I would also like to convey my thanks to my seniors and my juniors, for their help and suggestion during this work.

Last but not least, my sincere obligation goes to my dearest family for their love, encouragement, inspiration and cooperation that have made this dissertation possible.

Thank you all,

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ABSTRACT

Nepal is great country having all the types of biodiversity which is suitable for the growth of different type plants. Different types of natural products and their biological activities can be obtained from these plants which could have great benefits in cure of different diseases. Phytochemical screening of different plant extracts showed the rich resources of secondary metabolites such as alkaloids, flavonoids, phenols etc. Calculations of TPC and TFC value of plant extracts showed that *T. patula* content highest value TPC (630.839±2.633 mgGAE/g) and TFC (79.605±1.808 mgQE/g) respectively. Antioxidant activities of these plant extracts studied by both DPPH and FRAP both showed *D. metel* with IC_{50} value of 0.039 mg/mL and 357.0365±0.682317 mm Fe (II)/L respectively, as a potent antioxidant among the selected three plant extracts. The antibacterial activity was studied by well diffusion method which showed plant extract of *T. patula* only showed the activity of 10 mm ZOI value at concentration of 50 mg/mL as compared to standard drug against *S. aureus* while other plant extracts did not showed any activity against both gram positive and gram negative bacteria. Xanthine oxidase (XO), an enzyme widely distributed among mammalian tissues, is associated with the oxidation of xanthine and hypoxanthine to form uric acid. This is the serious effect on the human being many people are suffering from this disease. The antigout activity of these plant extracts were studied by xanthine oxidase inhibition assay where methanolic extract of *C. grandis* with IC_{50} value 0.491 mg/mL showed the highest xanthine oxidase inhibition activity among selected plants, refers it as potent antigout. Since *C. grandis* was selected for column and FTIR analysis of different fraction was observed. FTIR analysis showed different carbonyl, phenolic, alkane compounds are present in the extracts. This study may provide scientific basis for the use of selected plants and may provide valuable information for further research.

Keyword: *Xanthine, Xanthine Oxidase, Antioxidant, Natural Products, Antibacterial, TPC, TFC and FTIR.*

LIST OF ACRONYMS AND ABBREVIATIONS

AGE	:	*Apium graveolens*
DMSO	:	Dimethyl Sulphoxide
DPPH	:	1, 1-diphenyl-2-picryl hydrazyl
EtoAC	:	Ethyl Acetate
ETSBA	:	Electron Transfer Signal Blocking Activities
FBX	:	Febuxostat
FCR	:	Folin-Ciocalteau Reagent
Fe(II)	:	Ferrous
FTIR	:	Fourier Transform Infrared
GAE	:	Gallic Acid Equivalent
GCMS	:	Gas Chromatography mass spectrometry
IC_{50}	:	Inhibitory Concentration for 50% inhibition
MHA	:	Muller Hinton Agar
MHB	:	Muller Hinton Broth
MSU	:	Monosodium urate monohydrate
NB	:	Nutrient Broth
QE	:	Quercetin Equivalent
TFC	:	Total Flavonoid Content
TLC	:	Thin Layer Chromatography
TPC	:	Total Phenolic Content
TPTZ	:	2, 4, 6 – tripyridyl – s - triazine
UV	:	Ultraviolet
WHO	:	World Health Organization
XO	:	Xanthine Oxidase
XOI	:	Xanthine Oxidase Inhibition
ZOI	:	Zone of Inhibition

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INTRODUCTION

1.1. General Introduction

Nepal is a landlocked country with rich sources of natural resources situated between India and China. It includes tropical alluvial flat toward south and all time snow covered high mountains in north that show enormous geographic diversity. The country can be divided into five major physiographic zones, namely, from north to south, the High Himal, High Mountains, Middle Mountains, Siwalik Hills and Terai Plains. All five zones extend lengthwise from east to west across the country. The climate varies from alpine cold semi-desert type in the trans-Himalayan zone to tropical humid type in the tropical lowlands in the south. Mountain biodiversity in Nepal is of high importance for a number of ecological functions, including soil retention and slope stability. Those functions are often closely connected with the extent of above the ground and below-ground vegetation. The high functional diversity of plants in mountain ecosystems might have added to the resiliency of those ecosystems that often provides effective barriers to high-energy events such as landslides, rockfalls and avalanche. 39.6 percent of Nepal's land areas are occupied by the shrubs lands along with forests. Forests in the Middle Mountains have been generally better managed, and in many places forest cover has increased in recent years due to community forestry programs.[1]

The economy of Nepal is very much dependent on the use of natural resources. Biodiversity is closely linked to the livelihoods and economic well-being of most Nepalese people. The subject relates to almost every aspect of Nepalese life, including agricultural productivity, food security, human health and nutrition, indigenous knowledge, gender equality, culture, climate, water resources and aesthetic value to the society.[1]

According to an estimate, at least 1,463 species of herbal medicinal plants are used by the rural people in Nepal.[2]

Modern therapeutic medicine is historically based on indigenous therapies and ethnopharmacological uses, which have become recognized tools in the search for new sources of pharmaceuticals. Globalization of herbal medicine

along with uncontrolled exploitative practices and lack of concerted conservation efforts, have pushed many of Nepal's medicinal plants to the verge of extinction. Sustainable utilization and management of medicinal plants, based on traditional knowledge, is therefore necessary.[3]

Nepal is ranked as 9th among the Asian countries for its floral wealth with an estimated 9,000 species of flowering plants. So far, 6,653 species of flowering plants have been reported. Among these, about 50% fall under the rubrics "useful" and "ethnobotanical", and about 25%–50% are ethnomedicinals. 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. These plants are also important for local livelihoods and income generation, and they do fetch higher market prices.[4]

There are many potential drugs that had been formulated from medicinal plants by traditional societies are proving to be an important source of potentially therapeutic drugs.[5] Around the World there are, at least 122 drugs that are being prescribed stand on substances within plants.[6]

It is estimated that about 1700 species of plants are utilized by different ethnic groups in Nepal to meet their primary healthcare needs.[7, 8, 9]

Around 6,500 flowering plants of Nepal 1,624 constitute medicinal value. Various ethnic group of Nepal utilizes about 23% of angiosperms within Nepal for their medicinal properties.[10]

Nepal is rich in all the three levels of biodiversity, namely species diversity, genetic diversity and habitat diversity. In Nepal, thousands of species are known to have medicinal values and the use of different parts of several medicinal plants to cure specific diseases has been in vogue since ancient times. Nepal is blessed with diverse climatic and soil conditions suitable for growth of variety of flora and fauna. The indigenous people are well used with the properties and uses of plants of their surroundings. About 60% of the world population and developing countries rely on traditional remedies for their primary health treatment from plants.[11]

Natural plant products could also prove useful in minimizing the adverse effects of various chemotherapeutic agents as well as in prolonging

longevity. The global interest in the medicinal potential of plants during the last few decades is therefore quite logical.[57]

Rupandehi District, a part of Province Number 5, is one of the seventy seven districts of Nepal and covers an area of 1,360 km^2 (530 sq mi). The district headquarter is Siddharthanagar. As per the national census 2011, the population of Rupandehi was 880,196. The district lies on the southern and western part of Nepal. On the East it shares border with Nawalparasi District, on West with Kapilvastu District, on North with Palpa District and on South with India. The elevation of the district lies between 100m to 1229m from sea level. The total area of the district is 1,360 km^2 with 16.1% in Churia Range and rest in the Terai region.[12]

1.2 Antioxidants

Antioxidants are compounds that inhibit oxidation. Oxidation is a chemical reaction that can produce free radicals, thereby leading to chain reactions that may damage the cells of organisms. Antioxidants such as thiols or ascorbic acid (vitamin C) terminate these chain reactions.

The term "antioxidant" is mostly used for two entirely different groups of substances: industrial chemicals that are added to products to prevent oxidation, and naturally occurring compounds that are present in foods and tissue. The former, industrial antioxidant, have diverse uses: acting as preservatives in food and cosmetics, and being oxidation-inhibitors in fuels. There are different methods to determine the antioxidant activity of different plants and samples. Among them DPPH and FRAP are the important method to calculate the antioxidant activities of samples.

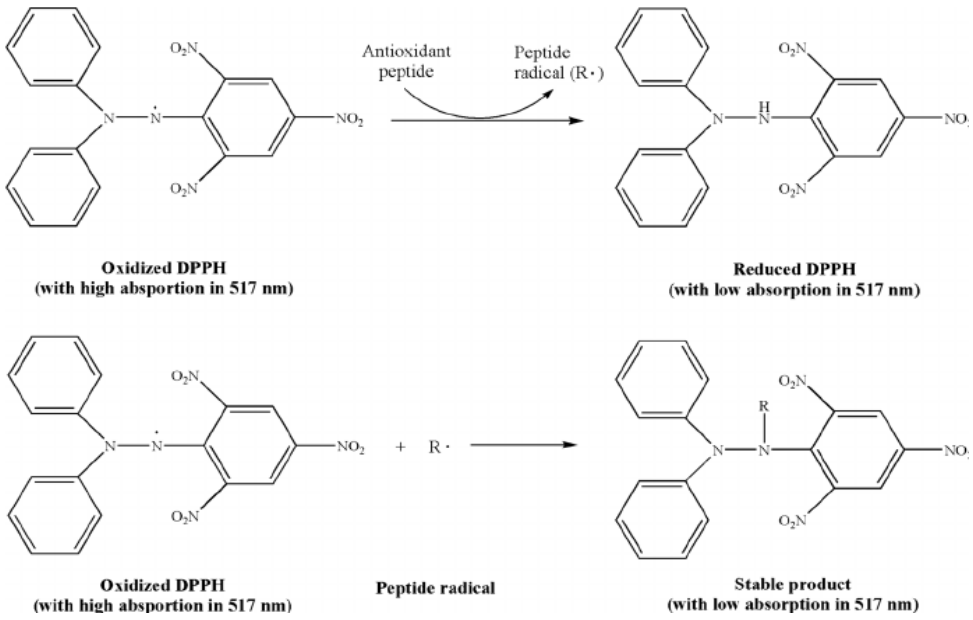
When the enzymatic system is used in the study, the scavenging effect may come directly from the radical-quenching effect or/and the enzyme-inhibiting effect. The structure activity relationship of flavonoids as inhibitors of xanthine oxidase and as scavengers of the superoxide radical.[113]

1.2.1 DPPH free radical scavenging method

The DPPH free radical scavenging assay is a quick, easy, reliable and reproducible parameter measure antioxidant activity of compounds present in sample extract *in vitro*.[13] In this method present antioxidants can reduce and decolorize of DPPH is observed from where particular antioxidant contains high or low antioxidant activity can be evaluated. However, antioxidants either transfer a hydrogen atom or electron to DPPH free radical to neutralize its free radical character being stable diamagnetic molecule.[14]

Mechanism of DPPH with Antioxidant

The proposed mechanism involves the transfer of a hydrogen atom from an antioxidant to the DPPH molecule to form DPPH-H molecule which is stable with loss of its violet color and also its paramagnetic resonance that does not absorb at 517 nm. DPPH solutions show a strong absorbance band at 517 nm due to its odd electron appearing a deep violet color; the absorption vanishes as electron pairs off.[15]



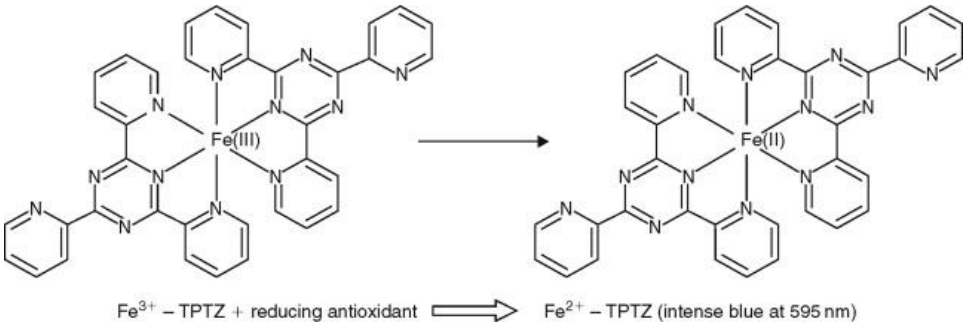
The antioxidant activity of plant extracts or its compounds is evaluated by comparing with standard antioxidant. Some of the standard antioxidants are ascorbic acid, cysteine etc.

1.2.2 FRAP method

The FRAP assay is a novel method for assessing antioxidant power by measuring the ferric reducing ability of plant extracts. The FRAP assay method is inexpensive, reagents are simple to prepare, results are highly reproducible, the procedure is straightforward and speedy and the equipment required is of a type commonly found in biochemical laboratories. Working FRAP reagent contains acetate buffer, TPTZ solution and $FeCl_3$ solution. FRAP assay involves the reduction of ferric to ferrous ion. FRAP values are obtained by comparing the absorbance change at 593 nm in test reaction mixtures with those containing ferrous ions in known concentration.

Mechanism

This assay works on the principle that ferric ion is reduced to ferrous ion in the presence of an antioxidant. In this experiment, an antioxidant is defined as a molecule that can donate one or more electrons to an electron acceptor like ferric ion. The more antioxidant power, the more ferric ion is reduced. Free iron has a relatively low redox potential and is unlikely to take part in the redox reaction therefore, the more oxidizing Fe^{III}-TPTZ complex is provided. In this reaction, at low p^H straw yellow colored Fe^{III}-TPTZ complex is reduced to blue colored Fe^{II}-TPTZ complex with an absorption maximum at 593 nm by the action of antioxidant components of plant extract. The mechanism is given as:



Or,

$$\text{Antioxidant} + Fe^{+3} \rightleftharpoons Fe^{+2} + \text{oxidized antioxidant}$$

$$Fe^{+2} + \{Fe(CN)_6\}^{3-} \rightleftharpoons Fe[Fe(CN)_6]^{-}$$

1.3 Antimicrobial

An **antimicrobial** is an agent that kills microorganism or stops their growth. Antimicrobial medicines can be grouped according to the microorganisms they act primarily against. For example, antibiotics are used against bacteria, and antifungals are used against fungi. They can also be classified according to their function. Agents that kill microbes are microbicidal, while those that merely inhibit their growth are called biostatic. The use of antimicrobial medicines to treat infection is known as antimicrobial chemotherapy, while the use of antimicrobial medicines to prevent infection is known as antimicrobial prophylaxis.[16]

Any substance of natural, synthetic and semi-synthetic origin that kills or inhibits the growth of microorganisms but cause little or no damage to the host is refer as an antimicrobial. Antibiotics are one of the most important weapons to deal with the bacterial infections and have great benefit to health in the human life.[17]

1.4 Phenols and Flavonoids

Phenolic compounds are compounds which commonly possess an aromatic ring bearing one or more hydroxyl groups which are available in plant substances. Phenolic compounds are the most abundant secondary metabolites in the plants and can be classified into non-soluble compound such as tannis, lignins, cell wall bound hygroxycinammic acids, and soluble compound such as phenolic acid, phenylpropanoid, flavonoids and quinones.[18] All these groups are found in plants and animals.

The xanthine oxidase inhibitory activity of natural polyphenols, that includes Fe-ion-catalyzed radical oxidation products from phenolic compounds as xanthine oxidase inhibitors.[118] Flavonoids are the largest group of naturally occurring phenolic compounds, which occurs in different plant parts both in free states and as glycosides. These natural products were known for their beneficial effects on health. More than 4000 varieties of flavonoids have been identified many of them are responsible for attractive color of flowers, fruits and leaves. Flavonoids are abundant in plants, in which they perform several functions. They are essential pigments for

producing the colors needed to attract pollinating insects. In higher order plants, flavonoids are also required for UV filtration, nitrogen fixation, cell cycle inhibition, and as chemical messengers. The flavonoids can be divided into six major subtypes, which include chalcones, flavones, isoflavonoids, flavanones, anthoxanthins and anthocyanins. More generally, planar flavones and flavonols with a 7-hydroxyl group inhibit xanthine oxidase.[109]

Xanthine oxidase (XO) is a versatile molybdoflavoprotein, widely distributed, occurring in milk, kidney, lung, heart, and vascular endothelium. The silico docking studies of various flavonoids (benzopyran) for virtual screening as xanthine oxidase inhibitor.[115]

During the past decade, definite progress has also been achieved in the understanding of the XO enzyme structure, and rational drug development approaches led to the discovery of new powerful XO inhibitors of various classes, including purine analogs, imidazole and triazolederivatives, and flavonoids among many others.[121] The inhibitory ability of twenty flavonoid compounds (with hydroxyl groups at C-5 and C-7 position to structurally mimic the xanthine scaffold) against XO was determined. Moreover to rationalize the binding behavior of the flavonoids within the active site of the XO enzyme, molecular modeling studies were also carried out. Overall the study provided the new insights for flavonoids as potent XO inhibitors. The inhibitory activity which indicated that inhibitory activities generally increased with the increasing affinities of flavones and flavonols.[116]

1.5 Antigout

Gout is a systemic disease that results from the deposition of monosodium urate crystals (MSU) in tissues. Increased serum uric acid (SUA) above a specific threshold is a requirement for the formation of uric acid crystals. Despites the fact that hyperuricemia is the main pathogenic defect in gout, many people with hyperuricemia do not develop gout or even form uric acid crystals. In fact, only 5% of people with hyperuriceamia above 9 mg/dL develop gout. Accordingly, it is thought that other factors such as genetic predisposition share in the incidence of gout.[19] The over-activity of this

enzyme results in a condition, generally called as -0.25" -0.25"gout.[20] Gout is one of the most common metabolic disorders affecting humans.

Gout is caused by a condition known as hyperuricemia, where there is too much uric acid in the body. The body makes uric acid when it breaks down purines, which are found in your body and the foods you eat. When there is too much uric acid in the body, uric acid crystals (monosodium urate) can build up in joints, fluids, and tissues within the body. Hyperuricemia does not always cause gout, and hyperuricemia without gout symptoms does not need to be treated. It is characterized by marked hyperuricemia, leading to the deposition of urate monohydrate crystals in joint and kidney, resulting in gouty arthritis and uric acid nephrolithiasis.[21] There are a number of possible consequences of this buildup of uric acid in the body, including acute and chronic gouty arthritis, kidney stones, and local deposits of uric acid in the skin and other tissues. The selection of natural products such as plants used in ethnomedicine and screening of their extracts for pharmacological activity may provide identification of newer medicaments for the treatment of various ailments.[22]

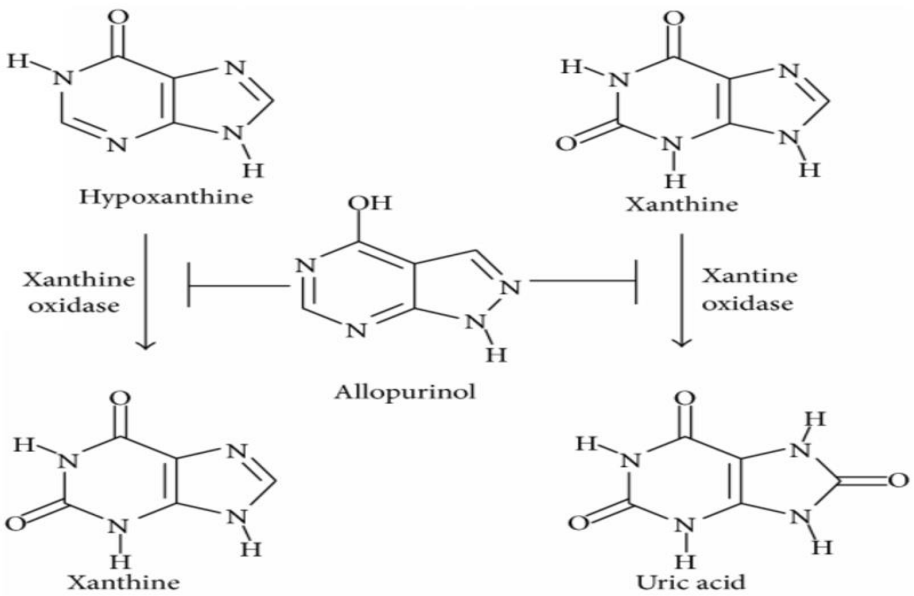
$$\text{Hypoxanthine} + H_2O + O_2 \longrightarrow \text{Xanthine} + H_2O_2$$

$$\text{Xanthine} + H_2O + O_2 \xrightarrow{\text{Xanthine oxidase}} \text{Uric acid} + H_2O_2$$

Xanthine oxidase inhibitors like allopurinol and febuxostat have been commonly used medications to decrease the circulating uric acid levels. Although it has been used as drugs to lower the uric acid level, it has various side effects such as skin rashes and even renal failure.[23]

Some known and novel antioxidants and non-antioxidants phenolic compounds, coumarins, flavonoids, and steroids were found to have relatively high activity and in few instances the initially discovered leads became a subject of more detailed structure/activity studies. Such fact in binding preferences also extends to a wealth of substrates oxidized by XO that includes not only purine derivatives but simple aliphatic, aromatic, and heteroaromatic aldehydes as well.[120]

Mechanism of inhibition of Xanthine oxidase by Allopurinol to form uric acid.



The xanthine oxidase inhibitors are the mainstay of therapy for reducing serum urate levels in patients with gout. The xanthine oxidase inhibitor allopurinol was approved in 1966, and febuxostat, a nonpurine inhibitor of xanthine oxidase, was approved for the management of hyperuricemia in patients with gout in 2009. Allopurinol and its metabolites are structural analogues of both purines and pyrimidine compounds and thus can affect enzymes in both metabolic pathways, whereas febuxostat is a nonpurine inhibitor of only xanthine oxidase.[24]

At low concentrations, allopurinol is a substrate for and competitive inhibitor of the enzyme; at higher concentrations, it is a noncompetitive inhibitor. Oxypurinol is a noncompetitive inhibitor of the enzyme; the formation of this compound, together with its long persistence in tissues, is responsible for much of the pharmacological activity of allopurinol.[119]

1.6 Enzyme inhibition mechanisms

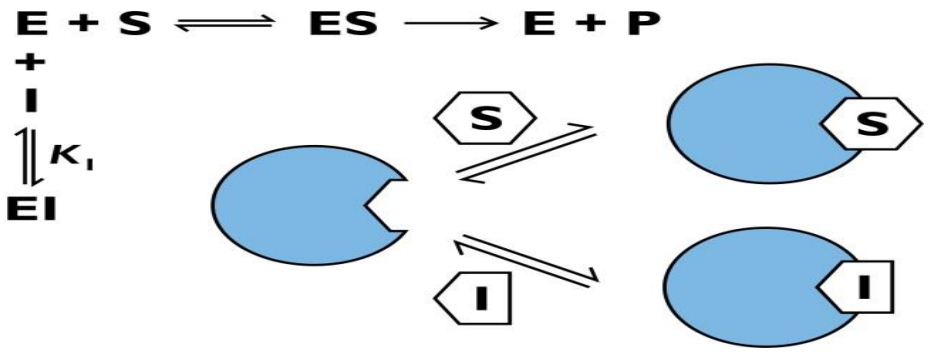
Enzymes play central roles in life processes. Enzymes can be regulated at multiple levels, ranging from transcriptional regulation of the expression of the enzyme-encoding gene through the direct regulation of the activity of the enzyme molecule by effector molecules to the controlled proteolytic decomposition of the enzyme. Inhibitors can be classified into three

mechanistic groups based on their mechanism of action: competitive, uncompetitive and mixed inhibitors. The type of inhibition can be determined through enzyme kinetic measurements.

- **Competitive inhibition**
- **Uncompetitive inhibition**
- **Mixed inhibition**

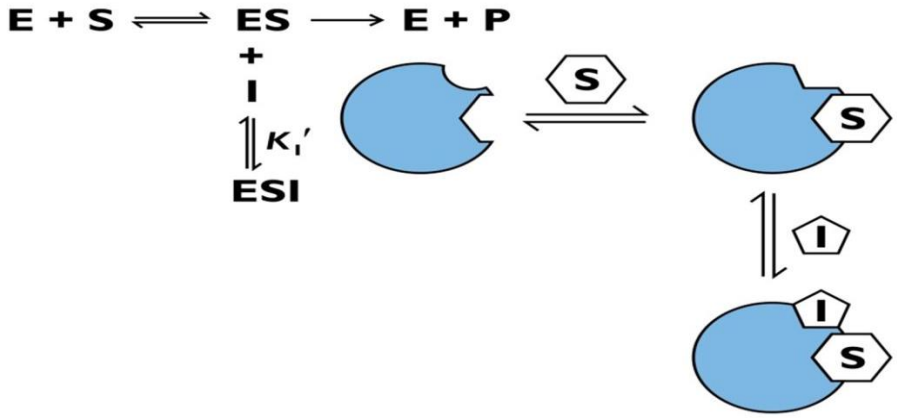
1.6.1 Competitive inhibition

Competitive inhibitors compete for the substrate-binding site of the enzyme with the substrate, because the substrate and the inhibitor bind to identical or overlapping sites. Due to the overlapping nature of the binding sites, a ternary complex in which the substrate and the inhibitor would simultaneously bind to the enzyme. Accordingly, in the enzyme-inhibitor complex, the enzyme is completely inactive.



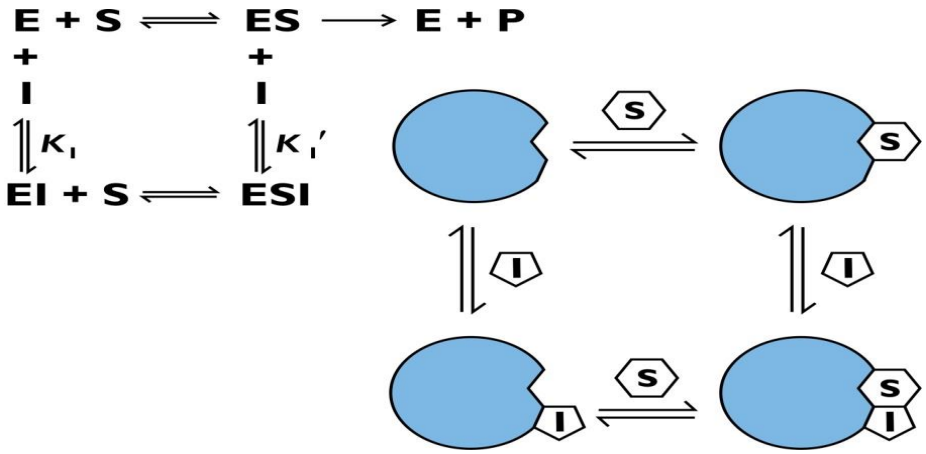
1.6.2 Uncompetitive inhibition

Some inhibitors bind only to the ES complex without binding to the free enzyme. These binding events occur exclusively at a site distinct from the precise active site occupied by substrate.



1.6.3 Mixed inhibition

There are inhibitors that can bind both to the free enzyme as well as to the ES complex. This is known as mixed inhibition. These inhibitors represent a combination of the two already discussed types.



1.7 Column chromatography

Chromatography is an important biophysical technique that enables the separation, identification, and purification of the components of a mixture. Column chromatography is a one of the important paradigm of Chromatography. Column chromatography in chemistry is a chromatography method used to isolate a single chemical compound from a mixture. Many modifications and improvements were made to column chromatography to derive advanced chromatography techniques. The advanced forms of column chromatography are high performance liquid chromatography (HPLC), gas chromatography (GC), Ultra performance liquid chromatography (UPLC), etc. Despite many advanced methods of

chromatography, chromatography is widely used in science research and industry.[25] Column chromatography is a technique in which the substances to be separated are introduced onto the top of a column packed with an adsorbent, passed through the column at different rates that depend on the affinity of each substance for the adsorbent and for the solvent or solvent mixture, and are usually collected in solution as they pass from the column at different times. This chromatography is a type of adsorption chromatography techniques. In this separation of components depends upon the extent of adsorption to stationary phase where the stationary phase is a polar solid material packed in a vertical column. When a mixture of mobile phase and samples to be separated are introduced from top to the column the individual components of mixture move with different rates. Those with lower affinity and adsorption to stationary phase move faster and eluted out first while those with greater adsorption affinity move or travel slower and get eluted out last. The solute molecule adsorb to the column in a reversible manner.[26]

1.8 Fourier-transform infrared spectroscopy (FTIR)

Infrared spectroscopy is an important technique in organic chemistry. It is an easy way to identity the presence of certain functional groups in a molecule. Analysis by infrared spectroscopy is based on the fact that molecules have specific frequencies of internal vibration. These frequencies occur in the infrared region of the electromagnetic spectrum: $4000cm^{-1}$ to $200cm^{-1}$. When a sample is placed in a beam of infrared radiation, the sample will absorb radiation at frequencies corresponding to molecular vibrational frequencies, but will transmit all other frequencies. The frequencies of radiation absorbed are measured by an infrared spectrometer, and the resulting plot of absorbed energy vs. frequency is called infrared spectrum of the material.

It is a non- destructive technique which requires no external calibration. Because all of the frequencies are measured simultaneously, most measurement are made by FTIR are made in matters of seconds rather than several minutes. It is sometimes referred to as the Felgett advantage. The

detectors employed are much more sensitive, the optical throughput is much higher which results in much lower noise levels.[27]

The key structural features for potent activity were: i) hydroxyl groups on ring were quite important for inhibition as well as affinity towards XO, ii) methylation of flavonoids hydroxyl groups enhanced the activity whereas affinity remained uncertain, iii) glycosylation of flavonoids decreases the binding affinity toward XO most probably due to the non-planer structure, iv) hydrogenation of C_2=C_3 double bonds diminishes the inhibitory activity indicating that unsaturation played a prominent role for XO inhibition.[116]

1.9 Objectives of Study

Varieties of the medicinal plants have been reported from the several part of Nepal. Despite of having great significant medicinal plants, not much work has been reported on chemical and biological investigation on varieties of medicinal plants from found in Nepal. Furthermore, various plants and herbs found in Rupandehi district have variety of potential like anti gout properties and different other medicinal properties. The floras found are not well explored for their biological activities in that area. Hence, this work mainly focused on the following general and specific objectives.

1.8.1 General Objective

The main objective of this work is to check the inhibitory activity of xanthine oxidase from the plant extract of selected medicinal plants and also the antioxidant properties of these plants extract and some chemical analysis.

1.8.2 Specific Objectives

Based on the objective mentioned above, following specific objectives are pointed out.

- To collect the plants and carry out the extraction and analysis the extracts for their phytochemical screening.
- To determine the total phenolic and total flavonoid content of selected medicinal plant.
- To evaluate the antioxidant activity of selected plants extract.
- To study the antibacterial activity of medicinal plant extracts against gram positive and gram negative bacterial strains.
- To test xanthine oxidase inhibitory activity of selected plants.
- Isolation and purification of bioactive compound from hexane fraction of methanolic extract of *C. grandis* from silica gel column chromatography.
- FT-IR analysis of fraction collected from column chromatography.

CHAPTER-2

LITERATURE REVIEWS

From the beginning the world is infected with various types of diseases and now the diseases are more advanced that we cannot imagine. Lots of efforts are being carried out to cure these types of diseases from the natural products. Combinatorial chemistry, synthetic chemistry and retrosynthetic analysis techniques have developed many drugs but these drugs are more expensive, more hazardous and allergic and have side effects. Apart from the side effects of chemical treatments, their cost and accessibility causes big problem for the patients in country like Nepal. Thus natural plants and plant extracts could be interesting alternatives in therapeutic treatments. Extensive literature review revealed that many plants had been reported for their medicinal value with scientific support.

2.1 *Tagetes patula L.*

Classification of *T. patula L.* Carl Linnaeus

Kingdom: Plantae

Phylum: Tracheophytes

Class: Angiosperm

Order: Asterales

Family: Asteraceae

Genus: *Tagetes*

Species: *Patula L*.

T. patula, commonly called French marigold is an important flowering plant that belongs to Asteraceae family which includes more than 23600 species and 16200 genera. It includes different plants with various medicational properties,

also as food, cosmetics, ornamental and fodder for cattle. *T. patula*, is about 20-30 cm height. The plant produces fragrance, increases digestion and is used as diuretic and tranquillizer. It is used to treat upset stomach, abdominal cramps, constipation and inflamed and painful eyes .[27, 28]

Among the secondary metabolities, only one alkaloid has been reported (+) jafrine, from the petroleum ether extract of *T. patula* flowers.[30] However, Bano et al. isolated cholesterol, β-sitosterol and stigmasterol from the flower.[31]

Different secondary metabolites are detected in flowers and leaves of *T. patula* were terpenes, alkaloids, carotenoids, thiophenes, fatty acids and flavonoids as constituents, some of which may elicit the biological activities reported to date; these include antimicrobial activities. [37]

Methanol extract of flowers of *T. patula* contained phenols, flavonoide, alkaloids, saponins, and terpenes. This extract showed an inhibitory effect better than essential oil (undiluted) against *Staphylococcus aureus, Pseudomonas aeruginosa, Klebseilla pneumoniae, and Candida albicans.*

Essential oil of *T. patula* (aerial parts) exhibited strong antibacterial action against *Enterococcus faecalis, Klebsiella pneumoniae, Escherichia coli, Staphylococcus aureus* and *Pseudomonas aeruginosa*.[32]

The flower which was extracted in the methanol solvent was found to possess antimicrobial activity against a number of bacteria with inhibition zone diameters ranging from 9 to 20 mm. The methanol and 70% methanol extract showed significant activity against *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*, respectively, *Streptococcus pneumoniae* were significantly sensitive to the methanol extract, whereas 70% methanol extract possessed very weak activity against all the bacteria.[33]

The study reported that Chloroform soluble fractions of *T. patula* contain maximum phenolic content (67.44 mg of GAE/gm of extractives). Various extracts of *T. patula* leaves were taken for antibacterial activity at concentration of 400 µg/disc against *Sarcina lutea, Bacillus megaterium, B. cereus, B. subtilis, Pseudomonas aeruginosa, Salmonella typhi, Salmonella paratyphi, Shigella boydii, Staphylococcus aureus, Sh. dysenteriae, E. coli, Vibrio mimicus and Vibrio parahaemolyticus*. Among the tested fractions, CCl_4 fraction showed maximum zone of inhibition against *E. coli* (zone of inhibition 22 mm). *E. coli* was more sensitive to aqueous fraction (16 mm) than the rest of bacteria. Crude

methanolic extract showed maximum zone of inhibition (15 mm) against *Sarcina lutea*.[34]

However, study had reported the hot aqueous extracts of *T. patula* flower exerted higher antibacterial activity as compared to cold aqueous extract and methanol extract at 40 mg/mL concentration in Agar well diffusion method.[35]

Flowers of *T. patula* are useful against fever and convulsions. The leaves are used to cure hemorrhoids, kidney problems, muscular pain and wounds and swellings. Similarly, their extracts are used to cure earache and sore eyes.[36]

The acetone extract had the highest total flavonoid content, 25.13 ± 1.02% (4.07%); and the best radical scavenging activity, with IC50 of 15.74 µg/mL ± 1.09 (6.92%), but with lower dry residue, 6.62 ± 1.33% (20.10%).[37]

According to study, *T. patula* is an important source of flavonoids. The ethanol extract of flower have higher flavonoids content than leaves, i.e. 124.59 and 72.74 mg of quercetin equivalents per gram of dry extract.[38]

The silver nanoparticles from aqueous leaf extract of *T. patula* has reported fungicidal against *Colletotrichum chlorophyti*.[39]

The study revealed the total phenol content of the alcoholic extracts of leaves and flowers of *T. patula* expressed in term of gallic acid. The yield of gallic acid was found 30 GAE/g for flowers and for leaves it was 80 GAE/g respectively. The flavonoid content was found in range 30- 65 quercetin equivalents per gram of extract respectively in leaves and flowers.[40]

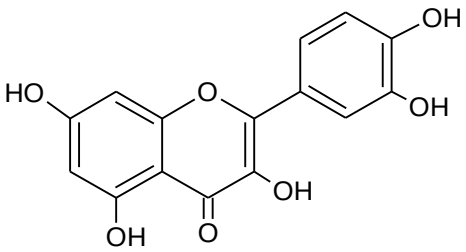
It has been reported that hexane extracts of the roots contain thiophenes as major constituent.[41]

The study showed that antibacterial activity by disc diffusion method (100 µL) of *T. patula* petal extracts (hexane and methanolic) and microdilution (0.03–72.0 mg/mL) against *B. subtilis, Pastrulla multocida, E. coli* and *S. aureus* strains, presenting inhibition zones with values varying from 8.2 mm to 11.4 mm for the hexane and 12.4 mm–20.2 mm for the methanolic extract.[42]

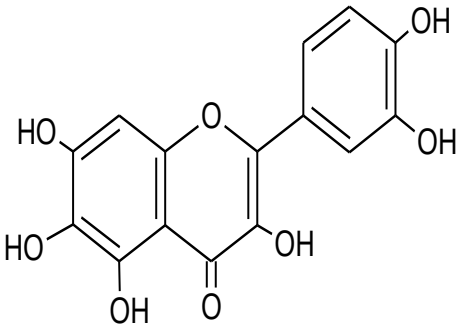
It has been reported that various traditional uses have been reported in Asian countries that need to be scientifically investigated in depth and several pharmacological activities have been reported for the *T. patula* but more detailed and mechanism based studies linked to a particular lead compound need to be targeted in future. *T. patula* also possess the metal-chelating potential, due to specific arrangement of hydroxyls and carbonyl group of the molecule as well

as hydrogen or electron-donating ability to reduce free radicals and delocalize the unpaired electron to form a stable phenoxyl radical.[43]

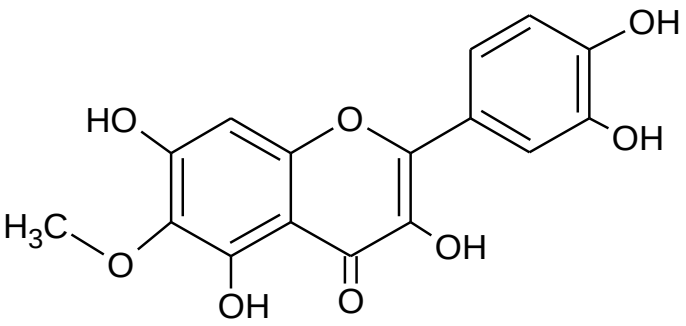
Some of the flavonoid constituent isolated from *T. patula* were Flavonols, quercetin, quercetagetin, patuletin, quercetin-3-glucoside, quercetagetin-7-glucoside, quercetagetin-3,7-diglucoside, Lutein.[122]



Quercetin



Quercetagetin



Patuletin

2.2 *Datura metel (L.)*

Classification of *D. metel* (Linn)

Kingdom: Plantae

Phylum: Tracheophyta

Class: Magnoliopsida

Order: Solanales

Family: Solanaceae

Genus: *Datura*

Species: *D. metel L*.

D. metel Linn is 1.5 m high perennial herbaceous plant with simple alternate, dark green, broadly ovate, shallowly lobed and glabrous leaves. Stem is dichotomously branched, blackish dark to purple coloured fall under the Solanaceae family. Flowers are large, solitary and funnel-shaped with a sweet fragrance. The fruit is in the form of ovoid capsule (about 3 cm in wide) covered with short stout prickles. The yellowish-brown seeds are flat, kidney-shaped, about 5mm long, with bitter and acrid taste Roots are cylindrical with lateral branches and scars.[44, 45]

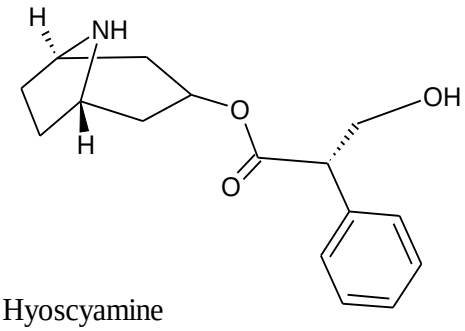
The plant can tolerate average soil but prefers sandy, loamy, well-drained and moist but hardly survives under shade. It prefers a warm temperature and is distributed in warmer regions of the world.[46]

Stem often dark violet. Petiole 2-6 cm; leaf blade ovate or broadly ovate, 5-20 × 4-l5 cm, membranous, glabrescent, base truncate or cuneate, asymmetrical, margin irregularly sinuate-dentate, lobed, or entire, apex acuminate; veins 4-6 pairs. Flowers erect. Pedicel ca. 1 cm. Calyx tubular, 4-9 cm. Corolla white, yellowish, or pale purple, funnel form, sometimes doubled or tripled, 14-20 cm;

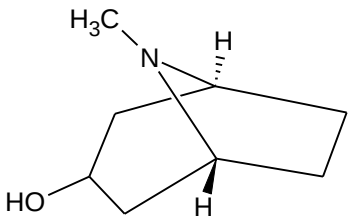
limb 6-10 cm in diam.; lobes elongate. Anthers 1-1.2 cm. Capsules deflexed, subglobose, ca. 3 cm in diam., tuberculate, irregularly 4-valved, subtended by remnants of persistent calyx. Seeds pale brown, reniform-discoid, ca. 3 mm in diameter.[47]

It has been reported that different parts of plant were being used in various countries likewise, crushed leaves are used to relieve pain. In China, the plant is used in the treatment of asthma. In Vietnam, the dried flowers and leaves are cut into small chips and used in antiasthmatic cigarettes. About 3 to 5g of the flower extract can be used as an anesthetic through oral consumption that produces general anesthesia within 5 minutes, which lasted for about 5 to 6 h. The flower of the *D. metel* is used in medicinal purposes as the treatment of pain, chronic bronchitis and asthma.[48, 49]

The study has reported that, the main constituents of the datura plant like tropane alkaloids (hyoscyamine, hyoscine, littorine, acetoxytropine, valtropine, fastusine, fastusinine), a number of withanolides and various trigloyl esters of tropine and pseudotropine. Calystegines, the nortropane alkaloids with glycosidase inhibitory activity, have been found in various *Datura* species.[50]



Hyoscyamine



Tropine

It has been reported that, the aqueous, methanol-water mixture and methanolic extract of *D. metel* were evaluated for xanthine oxidase inhibitory activity at 100 mg/mL. The methanolic extracts of *D. metel* showed more than 50% inhibition compared with the standard antigout drug, allopurinol, which showed 93.21% inhibition at 100 μg/mL concentrations.[51]

Major uses of plant part in different useful purposes. The dried leaves, flowers and roots were used as narcotic, antispasmodic, antitussive, bronchodilator, anti-asthmatic and as hallucinogenic. The plant was also used in diarrhea, skin diseases, epilepsy, hysteria, rheumatic pains, hemorrhoids, painful menstruation, skin ulcers, wounds and burns. In ayurveda, the plant was

considered bitter, acrid, astringent, germicide, anodyne, antiseptic, antiphlogistic, narcotic and sedative.[52]

A new antibacterial agent 51, 71 dimethyl 61 – hydroxy 31, phenyl 3 a - amine b - yne sitosterol was isolated from the plant leaves. It displayed antibacterial activity against *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Proteus mirabis, Solmonella typhi*, *Bacillus subtilis* and *Klebsiella pneumonia* but could not inhibit *Escherichia coli*.[53]

The study reported that, a new antibacterial agent 51, 71 dimethyl 61-hydroxy 31, phenyl 3α-amine β-yne sitosterol has been isolated from leaves of *D. metel*.[54]

This study observed that, the aqueous extracts of leaf, stem bark and roots of *D. metel* showed phytochemical and antioxidant activities between 49.30-23.82% indicating the plant as a natural source of antioxidants. The ethanol extract of *D. metel*(100 mg/mL) showed powerful antibacterial activity with maximum zone of inhibition (26 mm) against *P. aeruginosa*, *E. coli* and *B. subtilis* followed by the ethyl acetate extract of *D. metel* (100 mg/mL) against *E. coli*.[55, 56]

It has been reported that, methanolic seed extracts of *Datura fastuosa* (also known as *D. metel*) were evaluated for antioxidant potential, total antioxidant capacity, total amount of phenolic content, total flavonoid content, total flavonols and total proanthocyanidines contents. The IC_{50} value using DPPH model for methanolic extract of *D. fastuosa* was 28.34 µg/mL. The values of total antioxidant capacity, total amount of phenolic content, total flavonoid content, total flavonols and total proanthocyanidines content for methanolic extract of *D. fastuosa* were found to be 6.83 mg/g, 9.97 mg/g, 6.34 mg/g, 5.37 mg/g and 1.42 mg/g of plant extract respectively.[57]

Similarly, the group of researcher also reported about the various importance of this plant in various sector. The whole plant, especially the leaves and seeds, are widely used in herbal medicine as anesthetic, antispasmodic, bronchodilator, hallucinogenic, antitussive, narcotic, hypnotic and mydriatic and used in asthma, catarrh, epilepsy, hemorrhoids, menstruation, skin ulcers, laryngitis and treacheries, impotence, rheumatic swellings of the joints, lumbago, sciatica, neuralgia, tumors, scabies, eczema, allergy, inflammations, such as mumps, diarrhea, externally for ear ache and as a drug for criminal purposes.[48, 58, 59]

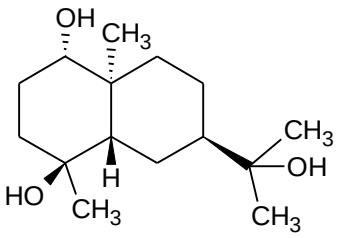
It has been reported that, pronounced anticancer potential against MCF-7 cell lines was exhibited by leaf extract of *D. metel* compared to stem extract of *D. metel*.[60]

In leaves, atropine, hyoscyamine and scopolamine, l-oxo-21, 24S-epoxy-(20S, 22Switha-2,5, 25-trienolide, pyrrole derivative (2'-(3,4-dimethyl-2,5-dihydro-1Hpyrrol-2-yl)-1'- methylethyl pentanoate are found.[61, 62]

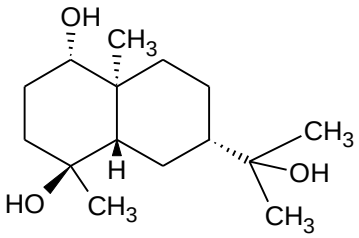
Gout is a disease that results from an overload of uric acid in the body, leads to the formation of tiny crystals of monosodium urate monohydrate that deposit in tissues of the body, especially the joints.[63]

The xanthine oxidase inhibitory activity was assayed for *D. metel* which is traditionally used for the treatment of gout. More than 50% xanthine oxidase inhibitory activity (in vitro) was seen in the methanolic extracts of *D. metel* which was comparable with the standard antigout drug, allopurinol which showed 93.21% inhibition at 100 µg/mL concentration with an IC_{50} value of 6.75 µg/mL. The methanolic extract was also screened for in vivo hypouricaemic activity against potassium oxonate-induced hyperuricaemia in mice and the extract was found effective.[51]

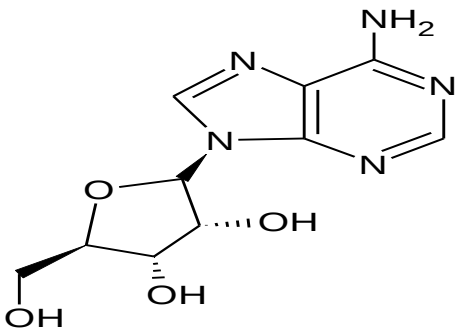
Methanol extract of the whole plant of *D. metel* isolates the seven compounds, including pterodontriol B, disciferitriol, scopolamine, adenosine, thymidine, ilekudinoside C, and dioscoroside D.[123]



Pterodontriol B



Disciferitriol



Adenosine

2.3 *Coccinia grandis*

Classification of *C. grandis (L.) Voigt*

Kingdom: Plantae

Phylum: Tracheophytes

Class: Angiosperm

Order: Cucurbitales

Family: Cucurbitaceae

Genus: *Coccinia*

Species: *C. grandis (L.*)

C. grandis belongs to family Cucurbitaceae, commonly known as Ivy gourd or little gourd also known as baby watermelon, gentleman's toes, and locally known as Kundru, is a tropical plant. *C. grandis* is a perennial, glabrous, climbing herb or trailing vine with glabrous stems and tuberous roots. It is a fast-growing perennial vine that grows several meters long. It can form dense mats that readily cover the shrubs and small trees. Its leaves are arranged alternately along the stems; they vary from heart to pentagon shape and are up to 10 cm wide and long. The upper surface of the leaf is hairless, whereas the lower surface is hairy.

It has been evaluated that, the aqueous extract of leaves of *C. grandis* for antibacterial activity against *Shigella flexneri* NICED, *Bacillus subtilis, E. coli, Salmonella choleraesuis, Shigella dysenteries,* and *Shigella flexneri.*[64]

The antibacterial activity of *C. grandis* leaf extract with solvents such as acetone, ethanol, methanol, aqueous and hexane against five bacterial species. Ethanol leaf extract of *C. grandis* showed high antibacterial activity against *S. pigeons, E. Coli, B. Ceres, K. pneumonia* and *S. aureus*.[65]

C. grandis (Ivy gourd) is occasionally cultivated as a garden vegetable in the tropical and sub-tropical regions of the world. It is believed to be native to

central Africa, India, and Asia. Every part of the plant is beneficial in medicine and also in various preparations that have been mentioned in the indigenous system of medicine like the anti-inflammatory, analgesic and antipyretic activity of fruit and leaves have been studied so far and are found to be noteworthy.[66, 67]

The study reported that the plant contains resins, alkaloids, fatty acids, flavonoids and proteins as chief chemical constituents. The presence of alkaloids, phenols, terpenoids and flavanoids thus corroborates the medicinal appliance of *Coccinia* as reported in literature.[68]

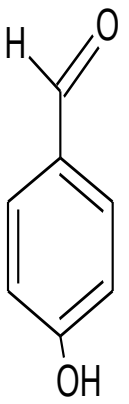
Similarly, the plant contains secondary metabolites such as saponins, flavonoids, sterols, and alkaloids. Prior scientific investigations of *C. grandis* showed that the crude plant extract has hepatoprotective, antioxidant, anti-inflammatory and anti-nociceptive, anti-diabetic, hypolipidemic, anti-bacterial, and anti-tussive activities.[66]

Methanol was observed to be preeminent solvent for polyphenols and flavanoids from Ivy gourd and the land varieties procured from Gujarat and Mumbai.[69]

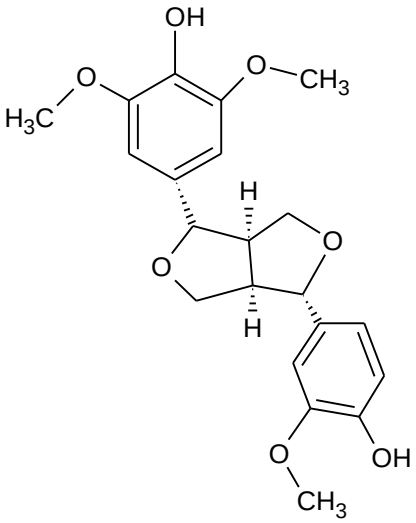
The comparative analysis between the fruit and leaf extracts indicated that the fruit extract showed better antioxidant and phytochemical characteristics than the leaf extract.[70]

The hydroalcoholic extract of *C. grandis Voigt* leaves show the reduction of uric acid with IC_{50} value of 21.25 µg/mL for crude extract.[51]

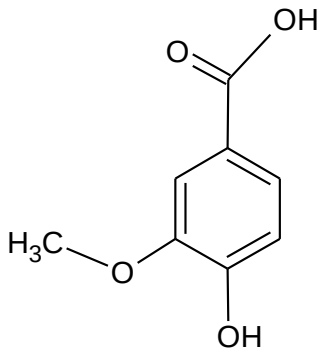
the six compounds were isolated from the ethyl acetate fraction of stem of *C. grandis* which includes 4-hydroxy benzaldehyde; 3,4'-O-dimethylcedrusin 9'-O-glucopyranoside; (+)-medioresinol; syringaldehyde; vanillic acid and (+)-syringaresinol in Vietnam.[108]



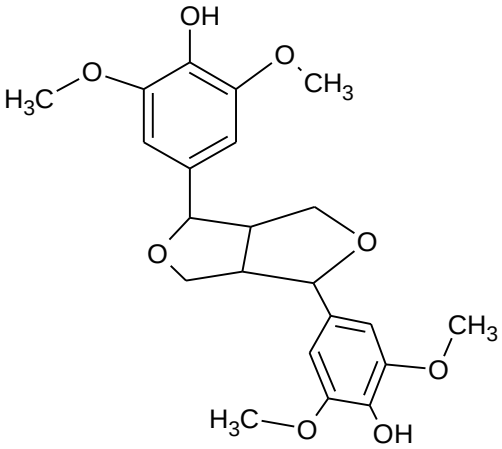
4- hydroxy benzaldehyde



(+)- medioresinol



vanilic acid



(+)- syringaresinol

2.4 Gout

Gout is a common medical problem whose prevalence is increased with increasing age and reaches 9% in men and 6% in women older than 80 years of age. The Rochester Epidemiology Project has indicated that primary gout (that is, patients without diuretic exposure) incidence doubled over the past years.[71] Gout is mediated by the super saturation and crystallization of uric acid within the joints.

Gout is a metabolic disorder associated with abnormal amounts of uric acid in the body, resulting in the deposition of urate crystals in the joints and kidneys, causing inflammation as well as gouty arthritis. Uric acid level is the key factor for the prevention of gout and related disorders.[72]

XO is an enzyme that catalyzes the final two steps in purine catabolism, ultimately generating uric acid, therefore, XO inhibitors are employed as a significantly effective meditator by the suppression of uric acid generation in the treatment of gout.[73]

Microcrystals of monosodium urate monohydrate (MSU) that precipitate in joint tissues from supersaturated body fluids or are shed from preexisting articular deposits result in inflammatory response, namely acute gouty arthritis.[74, 75] Thus, gout can be divided into two types:

chronic gout which is always accompanied by hyperuricemia, and acute gout. There are three stages in the management of gout: (i) treating the acute attack; (ii) lowering excess stores of uric acid to prevent flares of gouty arthritis and to prevent tissue deposition of urate; and (iii) providing prophylaxis to prevent acute flares.[76] The presence of phenolic and flavonoid content in the extract would have contributed towards XO inhibition.[77]

It has been reported that, the test whether a non-nucleoside xanthine oxidase inhibitor, Febuxostat (FBX), can reduce intracellular uric acid levels and inhibit cell death-induced inflammation in two different murine tissue injury models; acid-induced acute lung injury and acetaminophen liver injury. They found that FBX reduced uric acid levels in acid-injured lung tissue and inhibited acute pulmonary inflammation triggered by lung injury. Similarly, FBX reduced uric acid levels in the liver and inhibited inflammation in response to acetaminophen-induced hepatic injury.[78]

The work on Fraxamoside, a macrocyclic secoiridoid glucoside featuring a hydroxytyrosol group, was recently identified as a xanthine oxidase inhibitor (XOI) comparable in potency in vitro to the standard antigout drug allopurinol. Overall, fraxamoside emerged as a lead compound for a new class of XOIs potentially characterized by reduced interference with purine metabolism.[79]

The comprehensive review on the xanthine and aldehyde oxidases pertaining to their structures, functions, pathophysiological role, and a comparative analysis of structural insights of xanthine and aldehyde oxidases' binding domains with endogenous ligands or inhibitors. This review also provides futuristic implications in the design of inhibitors derived from in organic complexes or small organic molecules considering the spatial features and structural insights of both the enzymes.[80]

It has been reported that, inhibition of xanthine oxidase by *Gardenia oudiepe*. Thus, isolated the compounds that were active than the reference inhibitor, a molecular docking study using MOETM tool was carried out to establish the binding mode of the most active flavones with the enzyme, showing important interactions with its catalytic residues. These promising results, suggest the use of these compounds as potential leads for the design and development of novel XO inhibitors.[81]

The study reported the plant *Apium graveolens* for the inhibition of xanthine oxidase. In this study the effect of hydroalcoholic extracts from *A. graveolens* (AGE) against potassium oxonate (PO)-induced hyperuricemia was investigated in mice. This study demonstrated that AGE could reduce the serum uric acid level via inhibition of hepatic XDH/XO and indicated its potential utility as an effective hypouricemic bioactive agent or functional food.[82]

The study reported, the XOD inhibitory effects of seeds of *Plantaginisasiatisca*, and its representative four single compounds, acteoside, 1H-indolo-3-carbaldehyde, isoacteoside, andmyristic acid, were evaluated by electron transfer signal blocking activities (ETSBA), which is based on the electron transfer signal of XOD enzymatic reaction. The results from this study indicated that *Plantaginis* Semen significantly inhibited XOD activities to reduce hyperuricemia and treat gout. The study also proves that measuring the electron transfer signal blocking activities is a simple, sensitive, and accurate method to evaluate the XOD inhibitory effects.[83]

The aqueous, methanol-water mixture and methanolic extract of *D. metel* were evaluated for xanthine oxidase inhibitory activity at 100 mg/mL. The methanolic extracts of *D. metel* showed more than 50% inhibition compared with the standard antigout drug, allopurinol, which showed 93.21% inhibition at 100 μg/mL concentrations.[51]

The antioxidant activity of the plant extracts was assessed on the basis of the free radical scavenging effect, using modified DPPH method. *Datura stramonium* leaf extracts exhibited potent antioxidant property.

CHAPTER-3
MATERIALS AND METHODS

3.1 Chemicals

Xanthine oxidase, xanthine was purchased from Sigma Company and allopurinol and all other chemicals such as methanol was purchased from local traders.

3.2 Collection and identification of plant materials

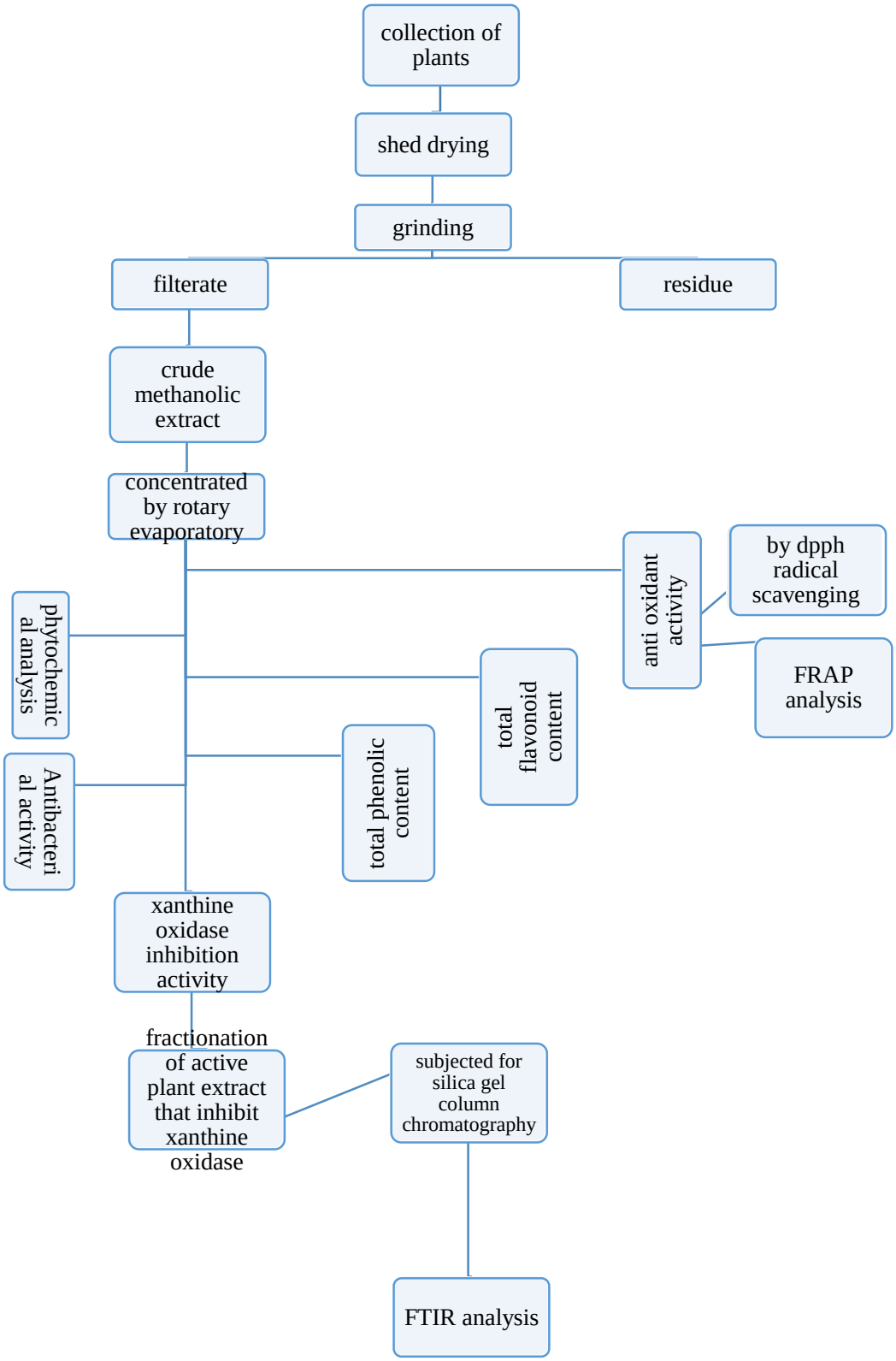
The plant materials were chosen based on their traditional uses by consulting with locals, old persons having indigenous knowledge. Selected plants were collected from Rupandehi District and were identified by "National Herbarium, Godabari". Those plant samples were washed under tap water to remove contaminations like mud, soil, insects, larvae etc. then these plant samples were air dried in shade for about month. Thus obtained materials were grinded to powder and store in labelled bags.

Table 1: List of selected plants

S.N	Scientific name	Nepali name	Used part	Altitude(m)
1.	*C. grandis*	Kundruk	Leaf	100-1210
2.	*D. metel*	Dhaturo	Leaf	100-1210
3.	*T. patula*	Swastini fool	Leaf	100-1210

3.3 Extraction of plant materials

100 gram powder from each sample plant was extracted by cold percolation method in methanol (350 mL) at room temperature for 72 hours. The mixture was filtered through whatsmann filter paper. The process was repeated until the color of mixture was faded from dark green to light color as required for the complete extraction. The instrument Rotatory Evaporator under reduced pressure by maintaining temperature lower than boiling point of methanol was used to concentrate the obtained filtrate which was then transferred to 100 mL beaker and left for further drying. Finally dried extract of plant samples were obtained which was stored in refrigerator at 4 ^{o}C.


collection of plants
shed drying
grinding
filterate
residue
crude methanolic extract
concentrated by rotary evaporatory
anti oxidant activity
by dpph radical scavenging
FRAP analysis
phytochemical analysis
total flavonoid content
total phenolic content
Antibacterial activity
xanthine oxidase inhibition activity
fractionation of active plant extract that inhibit xanthine oxidase
subjected for silica gel column chromatography
FTIR analysis

3.4 Phytochemical screening

The method employed for the phytochemical screening was mainly based on the procedure given by I. Culie (Methods for studying Drugs, personal communication).[84] In this method, the plant material is extracted selectively and successively with the solvent of increasing polarity. The presence of the main groups of natural constituents in these different extractive solutions were analyzed by using different specific reagents.

3.5 Determination of Total phenolic content (TPC)

3.5.1 Preparation of standard Gallic acid stock solutions

1000 µg/mL stock solution of Gallic acid was prepared by dissolving 10 mg of acid in 10 mL of methanol. Dilution of stock solution was done to prepare the concentration of 10, 20, 30, 40, 50, 60, 70 and 80 µg/mL.

3.5.2 Preparation of plant extract

25 mg/mL stock solutions of all the extract was prepared by dissolving 25 mg plant extract in 1 mL 50% DMSO solution. Dilution was carried out to get concentration of 500 µg/mL.

3.5.3 Procedure

Total phenol content of the extracts was measured using Folin-Ciocalteu reagent by 96 well plate methods which was modified from colorimetric method of Ainsworth et al., 2007.[85] At first 20 µL of different concentration of standard 10, 20, 30, 40, 50, 60, 70 and 80 µg/mL Gallic acid was loaded on 96 well plates in triplicate by diluting stock solution of 1 mg/mL with distilled water. Then 20 µL of plant sample of 500 µg/mL was loaded on 96 well plates in triplicate. After that in each well containing standard and sample 100 µL Folin-Ciocalteu followed by 80 µL Na_2CO_3 was added separately. Then it was left in dark for 15 minute and after 15 minute absorbance was taken at 765 nm using micro-plate reader (Synergy, BioTek, Instruments, Inc., USA). Gallic acid was used for constructing the standard curve (10-80 µg/mL) and the total Polyphenolic compound concentration in the extracts was expressed as milligrams of gallic

acid equivalent per gram of dry weight (mgGAE/g) of the extract using gallic acid standard curve.

3.5.4 Calculation of Total Phenolic Content (TPC)

TPC content in extract was expressed as milligrams of gallic acid equivalent per gram (mgGAE/g) which was calculated in all the extract using following formula;

C= cV/m……..... 1

Where, C= total phenolic content in mg GAE (gallic acid equivalent)/g.

c= concentration of gallic acid obtained from calibration curve

V= volume of extract in mL

M= weight of plant extract

3.5.5 Statistical analysis

Mean of triplicate value of absorbance for each concentration was calculated, from which linear correlation coefficient and regression equation were obtained which is given as,

Y= mx + c…….2

Where,

Y= absorbance of extract

m= slope from curve

x= concentration of extract

c= intercept

By the use of this regression equation concentration of extracts were calculated in gallic acid equivalent / gram.

3.6 Determination of Total flavonoid content (TFC)

3.6.1 Preparation of Reagents:

10% Aluminum trichloride was prepared by dissolving 1 gram of $AlCl_3$ into 10 mL distilled water and 1 M potassium acetate was prepared by dissolving 0.98 grams of potassium acetate into 10 mL distilled water.

3.6.2 Preparation Standard quercetin solution

Stock solution was prepared by dissolving 1 mg of quercetin into 10 mL methanol (0.1 mg/mL). Then final concentrations of the standard solution were prepared 10, 20, 30, 40, 50, 60, 70 and 80 µg/mL by diluting the stock solution of 0.1 mg/mL.

3.6.3 Preparation of plant extracts

The plant extracts were prepared 500 µg/mL by diluting the stock solution of 25 mg/mL in 50% DMSO solution.

3.6.4 Procedure

Total flavonoid content of the extracts was determined by 96 well plates method which was modified from colorimetric method of Chang et al., 2002.[86] At first 130 µL of different concentration of standard 10, 20, 30, 40, 50, 60, 70 and 80 µg/mL quercetin was loaded on 96 well plate in triplicate by diluting stock solution of 0.1 mg/mL with distilled water. Then 20 µL of plant sample of 500 µg/mL was loaded on 96 well plate in triplicate, and then 100 µL of distilled water was added in each well containing plant sample maintaining the final volume 120 µL. Then in each well containing standard and plant sample 60 µL ethanol, 10 µL $AlCl_3$ and 10 µL potassium acetate was added separately. Then it was left in dark for 30 minutes and after 30 minutes absorbance was taken at 415 nm using micro-plate reader (BioTek, Instruments, Inc., USA).

3.6.5 Calculation of total flavonoid content

TFC content in extract was expressed as milligrams of Quercetin equivalent per gram (mg QE/g) which was calculated in all the extract using following formula;

$C = cV/m$……..... 1

Where, C= total flavonoid content in mg QE (quercetin equivalent)/g.

c= concentration of quercetin obtained from calibration curve

V= volume of extract in mL

M= weight of plant extract

3.6.6 Statistical analysis

Mean of triplicate value of absorbance for each concentration was calculated, from which linear correlation coefficient and regression equation were obtained which is given as,

Y= mx + c…….2

Where,

Y= absorbance of extract

m= slope from curve

x= concentration of extract

c= intercept

By the use of this regression equation concentration of extracts were calculated in quercetin equivalent / gram.

3.7 Antioxidant activity

Different methods can used to calculate the antioxidant activity of plant samples depending upon the specific free radical being used as reactant. Among them DPPH free radical scavenging and FRAP assay both are the novel methods for assessing antioxidant power by measuring their respective reactant's reducing ability of plants extracts.

3.7.1 DPPH radical scavenging bioassay

3.7.1.1 Preparation of DPPH solution (0.1 mM)

0.1mM DPPH solution was prepared by dissolving 3.9 mg DPPH in 100 mL methanol in volumetric flask covered with aluminum foil.

3.7.1.2 Preparation of Ascorbic acid solution

Stock solution of ascorbic acid was prepared by dissolving 1 mg of ascorbic acid in 1 mL of methanol. Then final concentration at 0.1, 0.05, 0.025, 0.0125 and 0.00625 mg/mL were prepared by diluting the stock solution of 1 mg/mL.

3.7.1.3 Preparation of plant extracts

Stock solution of 50 mg/mL was prepared by dissolving 50 mg of plant extracts in 1 mL of DMSO using vortex machine. The final concentrations of plant extracts were prepared 1000, 500, 250, 125 and 62.5 μg/mL in 50% DMSO solution for *T. patula*, *D. metel* and *C. grandis.*

3.7.1.4 Procedure

Antioxidant activity of the extracts was determined by 96-well plate method which was modified from colorimetric method mentioned by Sabudak *et al.,* 2013; Subedi *et al.,* 2012.[87, 88]

For DPPH test ascorbic acid of 0.1 mg/mL was used as positive control and 50% DMSO was used as negative control. The positive control ascorbic acid, negative control DMSO and plant samples were loaded 100 μL in 96 well plate in triplicate. Then 100 μL of DPPH reagent was added in each well. Then it was incubated for 30 minutes in dark. After 30 minute absorbance was taken at 517 nm using micro-plate reader. The capability to scavenge the DPPH radical was calculated by using the following equation:

$$\% \text{ Inhibition} = \left(\frac{\mathrm{A_{control}} - \mathrm{A_{sample}}}{\mathrm{A_{control}}}\right) \times 100$$

Where, A is the absorbance of the sample and control. The IC_{50} values value was calculated using Graph pad prism program (Graph pad prism).

3.7.2 FRAP method

The antioxidant activity by FRAP assay was conducted according to the procedure given by Benzie and Strain in 1996.[89]

3.7.2.1 Preparation of FRAP reagent

FRAP reagent was freshly prepared by mixing chemicals as below:

1. 200 mL acetate buffer

2. 20 mL FeCl3

3. 20 mL TPTZ solution (2, 4, 6- tripyridyl-s-triazine)

4. 24 mL distilled water

3.7.2.2 Reagents and Samples

The reagents included 300 mM acetate buffer (p^H = 3.6), 10 mM TPTZ in 40 mM HCl, 20 mM $FeCl_3$.$6H_2O$.The working FRAP reagent was prepared, as required, by mixing 25 mL acetate buffer , 2.5 mL TPTZ solution and 2.5 mL $FeCl_3$.$6H_2O$ solution. Further aqueous solution of known Fe II concentration, ranging from 0.1 to 1.0 mM (FeSO4 .$7H_2O$) were used for calibrations. Similarly, stock solution of 0.1 mg/mL in methanol was prepared for each of the methanol extract.

[Thus formed FRAP reagent was straw colored and was kept in a reagent bottle. Its constant temperature was maintained by keeping in water bath at 37 ˚C.]

3.7.2.3 Preparation of standard solutions of Ferrous sulfate

Standard ferrous sulfate (1 mM) solution was prepared by dissolving 0.278 gm FeSO4 .$7H_2O$ into 1 liter distilled water. Five test tubes were set up in a test tube stand to prepare standard solutions of ferrous sulfate having different concentrations by serial dilutions of 1 mM freshly prepared stock solution. The solution was diluted to make a series of standards in each test tube as follows:

Table 2: Dilution of Stock solution of Ferrous sulfate

Test tube	Standard concentration(mM)	Ferrous sulfate solution (mL)	Distilled water (mL)
1	0.1	1	9
2	0.2	2	8
3	0.4	4	6
4	0.6	6	4

5	0.8	8	2
6	1.0	10	0

3.7.2.4 Preparation of Sample Solutions of Extracts

Different plants show different antioxidant power. As a result, some plant extracts may have high antioxidant activity and opposite may be true for others. Since absorption of sample solutions of extracts may vary plant to plant accompanying their antioxidant power, the concentration of solution to be made for each extract was determined by frequent trials by serial dilution method so that the absorption of sample solution could be in the range of spectrometer provided.

3.7.2.5 FRAP Assay

The spectrophotometer was switch on for half an hour and absorbance of standard solutions as well as sample solutions were recorded as follows;

1. Set of blanks was run first. 4 cuvettes were placed in spectrophotometer. 90 µl of distilled water was added to each cuvette and 3 mL of FRAP reagent was added vigorously in each cuvette so that the contents get mixed thoroughly.
2. Spectrometer was set at 593 nm and the temperature strip was set at 37 ˚ C. Stop watch was set for 4 minutes.
3. After 4 minutes, blank was made zero and sample absorbance was recorded.
4. Above method was repeated to all standard solutions thrice to record absorbance, the first of which was always blank.
5. Same method was repeated thrice for sample solutions of plant extracts to record absorbance, the first of which was always blank.

3.7.2.6 Data Analysis

The absorbance of water blank was subtracted from the samples and standard absorbance value and then a linear regression for the standards was constructed from absorbance against concentration. Finally, the regression equation was used to calculate the FRAP values under the unit of mM Fe (II) per liter of samples.

Linear regression equation from graph would obtain in the form of,

$y = mx + c$ ---------- (1)

Here, y = Absorbance (A)

x = concentration of standard ferrous sulfate

m = slope of line

c = constant

From the value of absorbance (y) of sample solution of plant extracts, the equivalent concentration (x) of standard ferrous sulfate was calculated in mM Fe (II) per liter.

3.8 Antibacterial activities

Screening of the various bioactive compounds from the plants has led to the discovery of new medicinal drugs. Antibacterial screening of the plant extracts was performed by agar well diffusion method. Antimicrobial activity was determined by measuring zone of inhibition. Procedure to operate antimicrobial activity was followed from clinical laboratory standard institute 2018 guidelines.

3.8.1 Preparation of stock/working solution

Stock solution of 50 mg/mL was prepared by dissolving 50 mg of plant extracts in 1 mL of DMSO using vortex machine. After making the stock solution, solution were sealed and stored in refrigerator at 4 ^{o}C until use.

3.8.2 Preparation of inoculums

All the strains of bacteria was cultured in Nutrient broth (NB) and incubated at 37 ºC for 18 hours. After incubation each stain were diluted with sterile distilled water. The turbidity of dilution was compared with 0.5 % McFarland standards (approximately 10^8 CFU/mL). Prepared inoculums were incubated for 30 minutes at 37 ºC prior to use.

3.8.3 Preparation of media

The media used in the study were prepared by adding 28 gram of Muller-Hinton Agar (HiMEDIA) in 1000 mL of distilled water. Then it was boiled with continuous shaking for about 15 to 20 minutes. The sterilized media was allowed to cool and the solution was poured in sterilized petri-plates. Plates were then left for solidification and incubation at 37 ^{o}C for 24 hours to make sure there were no contaminations on the plate.

3.8.4 Screening and Evaluation of Antibacterial Activity

Already prepared sterile Muller-Hinton Agar (MHA) plates were dried to remove excess of moisture from the surface of the media. Sterile cotton swab was dipped into the prepared inoculums and the excess of inoculums were removed by pressing and rotating against the upper inside wall of the tube above the liquid level and then swabbed carefully all over the plates. The plate was rotated through an angle of 60^{o} after each swabbing. Finally the swab was passed round the edges of the Agar surface. The inoculated plates were left to dry for minutes by closing with lid.

The wells were made in the incubated media plates with the help of sterile cork borer (4 mm) and labeled properly. Then 20 µl of the working solution of the plant extracts were loaded into the respective wells with the help of micropipette. The solvent (50% DMSO) was tested for its activity as a control at the same time in the separate well. The Neomycin 20 µg/mL was used as a positive control. The plates were then left for half an hour with the lid closed so that extracts diffused to the media. The plates were incubated overnight at 37 °C. After proper incubation (18-24 hours) the plates was observed for the zone of inhibition around well which is suggested by clean zone without growth was noted. The ZOI were measured with the help of the ruler and mean was recorded for the estimation of potency of antibacterial substance.

3.9 Anti-gout activity

Xanthine oxidase inhibition activity was performed using a standard protocol spectrophotometrically in 96 well plates with modifications. The uric acid formation was measured before and after the inhibition of xanthine oxidase enzyme at 290 nm described previously by Noro *et al.* with minor modifications (1983).[90]

3.9.1 Preparation of Plant extracts

25 mg/mL stock solutions of all the extract was prepared by dissolving 25 mg plant extract in 1 mL 50% DMSO.

3.9.2 Preparation of 0.1M HCl solution

11.66M HCl which was available in laboratory was taken and diluted required concentration about 1M by adding distilled water in volumetric flask.

3.9.3 Preparation of stock solution of positive control (allopurinol)

100 mg of allopurinol was dissolved in 100 mL phosphate buffer in volumetric flask to maintain 1mg/mL (1000 ppm) concentration. Thus prepared stock solution was diluted to required concentration i.e., 100 µg/mL. 100 µg/mL solution was further diluted serially.

3.9.4 Xanthine oxidase inhibitory activity assay

A well-known XOI, allopurinol (100 µg/mL) will be used as a positive control for the inhibition test. The reaction mixture consists of 75 µl of 50 mM sodium phosphate buffer (pH 7.5), 20 µl of sample solution will be dissolve in DMSO (final concentration maintain less than 5%), 20 µl of freshly prepared enzyme solution (0.1 units/mL of xanthine oxidase in phosphate buffer). The assay mixture will be pre-incubated at 37 °C for 15 min. Then, 60 µl of substrate solution (0.15 mM of xanthine) was added into the mixture. The mixture was incubated at 37 °C for 30 min. Next, the reaction was stopped with the addition of 25 µl of 0.5 M HCl 1 mol/L solution prior to measuring the absorbance at 290 nm on a Bio Tek microplate spectrophotometer. A blank was prepared in the same way, however the order of adding the substrate and HCl solution was reversed.

Xanthine oxidase inhibitory activity was calculated by using the formula obtaining % inhibition from following,

% inhibition = {(A-B)/A} × 100.

Where, A = control

B = absorbance measure of sample solution.

3.10 Isolation of compounds

On the basis of xanthine oxidase inhibitory activity leaves extract of *C. grandis* was selected as the potent extract for the isolation of active chemical constituents.

3.10.1 Separation of compounds by the use of column chromatography

Compounds from one of the bioactive plant extract that show good activity to xanthine oxidase inhibition was separated on silica gel packed column by eluting with gradients of suitable solvents (hexane, ethyl acetate, methanol etc.). 5 gram of plant extract was mixed with 15 gram of silica in 3:1 ratio and subjected for column for separation. Separated fractions was collected and

characterized by using chromatography and spectroscopy techniques such as TLC, IR.

3.11 Analytical conditions for FTIR

The FTIR instrument measure infrared radiation ranging from 400 to 4000 cm^{-1} by calculating radiation absorbed and transferred through the sample. Thus absorbed radiation is converted into vibrational energy by sample molecule. Each molecule or chemical compound present in the sample will produce a unique spectral fingerprint, making FTIR analysis a great tool for chemical identification.

Active fraction which was obtained from the column chromatography was collected in the separate test tube and dried. Furthermore, obtained fractions were purified by different solvent like alcohol, acetone, Chloroform. TLC was performed, then the fraction which give single spot was taken and subjected to FTIR analysis. Sampling area of IR spectrometer was cleaned by alcohol before subjected the sample on it. Measurement was done, after the FTIR data were obtained which were further analyzed.

CHAPTER-4

RESULTS AND DISCUSSION

4.1 Phytochemical screening

Table 3: Phytochemical screening of methanolic extract of all plants

S.N	Groups of compounds	*C. grandis*	*D. metel*	*T. patula*
1	Alkaloids	+	+	+
2	Flavonoids	+	+	+
3	Coumarins	-	-	-
4	Glycoslides	-	-	-
5	Polyphenol	+	+	+
6	Carbohaydrates	-	-	-
7	Saponins	-	+	-
8	Tannis	-	+	+
9	Terpenes	-	-	-
10	Quinones	-	-	-
11	Volatile oils	-	+	+
12	Phenol	+	+	+
13	Reducing sugar	-	+	+

Where,

(+) represents presence and (–) represents absence

From the Table 3, we can observe that alkaloids, flavonoids, Phenol and polyphenols were present in all of the extract selected whereas only *D. metel* showed saponins. Presence of reducing sugar, volatile oils, tannis were not shown by *C. grandis* but shown by other two extracts. Carbohydrates, Coumarins, glycoslides, terpenes were not shown by each of the extracts. However, it has been reported the presence of resins, fatty acids, terpenoids.[68] Similiary, saponins has also been reported by Kumar *et al.*, 2013 in methanolic extract of leaves of *C. grandis* which might be due to the different biodiversity and environmental conditions where plant has been found.[66] The key chemical constituents of *T. patula* were thiophenes, terpenes, flavonoids, benzofurans, carotenoids and terpenoids.[43]

Main constituents of the *D. metel* plant are tropane alkaloids (hyoscyamine, hyoscine, littorine, acetoxytropine, valtropine, fastusine, fastusinine).[50]

In a recent study, saponins have been shown to possess anti-arthritic effects in vitro because of their ability to interfere with uric acid metabolism by inhibiting xanthine oxidase.[110]

Flavones, coumarins and curcumin represent the class of secondary metabolites possessing xanthine oxidase inhibitory potential. The structure activity relationship for xanthine oxidase inhibition of these secondary metabolites has been extensively explored.[113]

The results presented in the above table might be slightly different then the data present in the some of the literature of some plants which is due to variation in the altitude of plants, different environmental conditions, methods and time of sampling and also due to the chemical grades.[91]

4.2 Determination of Total Phenolic Content (TPC)

Total phenol content of the extracts was determined by using Folin-Ciocalteu reagent by 96 well plate methods in reference with gallic acid equivalent that involves the oxidation of phenols in alkaline solution by yellow colored Folin-Ciocalteu reagent and changes to deep blue molybdotungstophosphate.[92] Here, phenols present in the plant extracts react with FCR to form colored complex that exhibit absorption as to quantitative and qualitative composition of phenol present in plant extracts by UV-visible spectrometry at 760 nm.

The absorbance curve for standard gallic acid demonstrated in Figure 1;

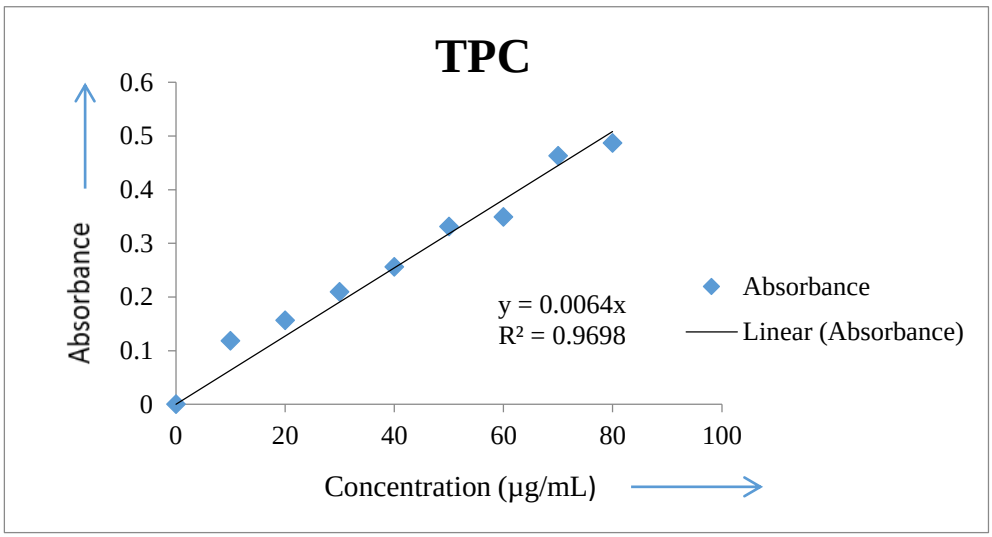


Figure 1: Calibration curve for standard gallic acid

4.2.1 Calculation of TPC in different plant Extracts.

The absorbance value of each extracts was calculated at 500 µg/mL which was recorded at 760 nm by using 96 well plates spectrophotometer. The TPC in the plant extracts taken under study was calculated by using regression equation y = 0.0064x , R^2 = 0.9698 , of the curve obtained from above graph followed by the formula cV/m and expressed as mg GAE per g of extract in dry weight. The TPC of different plant extract (mg gallic acid equivalent per g dry extract) is tabulated in Table 4.

Table 4: Total phenolic content of different plant extracts

S.N	Scientific name	TPC mg GAE/g±SD
1	*D. metel*	110.724±3.325
2	*T. patula*	630.839±2.633
3	*C. grandis*	257.276±1.58

More conveniently TFC is represented in the bar diagram as;

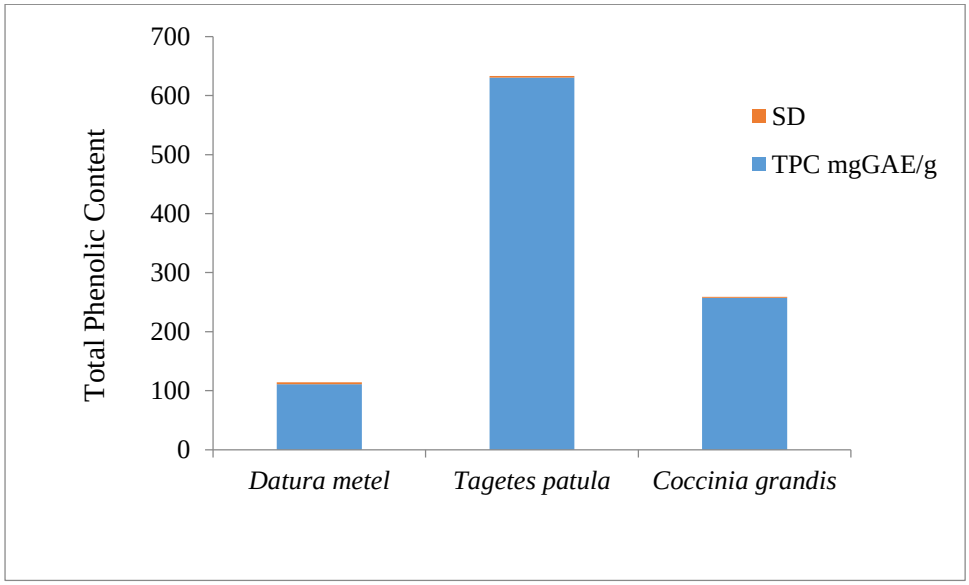


Figure 2: Total phenolic content of different plant extracts

The result shows that the total phenolic content was highest in *T. patula* (630.839±2.633 mgGAE/ g) while, 67.44 mgGAE/ g in chloroform soluble extract was reported by Kuddus et al., 2012. Similarly, in ethanolic extract of leaves of *T. patula*, TPC was 80 GAE/ g that was reported by Kushwala and Verma, 2017.[40]

Among the three selected plant extract minimum amount of TPC was shown by *D. metel* about (110.724±3.325 mgGAE/ g) while, Total amount of phenolic content on methanolic extract of *D. metel* was found to be 9.97 mg/g.[57] Continuously, 1.5 mg/ g amount of TPC was reported from the methanol extract of fruit and leaves of *C. grandis* in Goa, India.[69]

From the above discussion, plant found in Rupandehi show good potent for TPC value. Presence of Phenolic compounds might possess high antioxidant properties. It has been reported that extracts with high yield of TPC possesses greater antioxidant activity. Although quantitative evaluation of phenolic compound in plant extracts are affect by their structural complexity, biodiversity assay method and also due to presence of interfering substance.[93]

4.3 Determination of flavonoid content

The estimation of total flavonoid content was done by Aluminium chloride colorimetric assay in the methanolic extract of every plant extract following standard protocol assuming quercetin as a standard source of flavonoid. The presence of flavonoid in the plant extract forms acid liable complexes with the Aluminium chloride, has an intense yellow fluorescence which was observed under UV spectrophotometer at 415 nm. Concentration of flavonoid presence is proportional to the intensity of absorbed light at that wavelength.

The absorbance curve for standard Quercetin is shown in Figure 3;

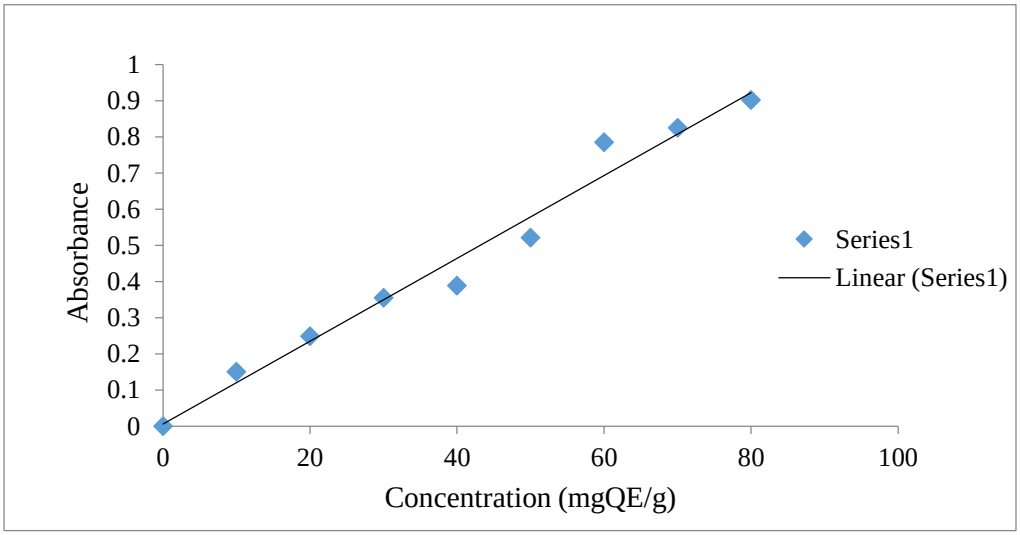


Figure 3: Calibration curve for standard quecretin.

4.3.1 Calculation of TFC in different plant extract

The absorbance values of every plant extract recorded at different concentrations were observed at 415 nm by using spectrophotometer. The presence of TFC in the plant extracts was calculated by using regression equation y = 0.0115x + 0.0056, R^2 = 0.976, of the curve obtained from above graph followed by the formula cV/m and expressed as mgQE/g of extract in dry weight. The TFC of plant extracts are tabulated in Table 5.

Table 5: Total flavonoid content of selected plant extract

S.N	Scientific Name	TFC mgQE/g±STD Error
1	*C. grandis*	6.098±0.380
2	*D. metel*	27.547±0.401
3	*T. patula*	79.605±1.808

More conveniently TFC is represented in the bar diagram as;

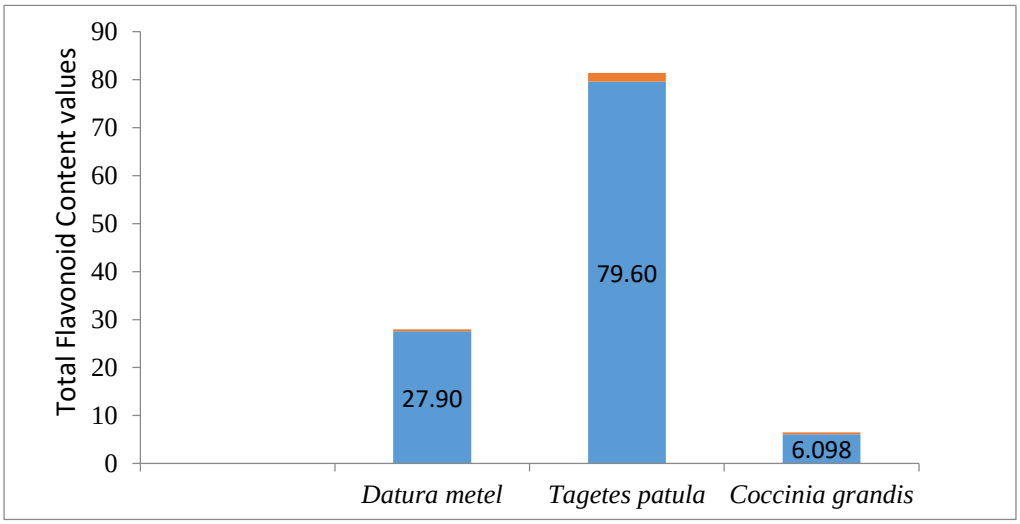


Figure 4: Total flavonoid content in different plant extracts

Among the studied plant extracts, it seems variation in TFC contents ranging from the *C. grandis* (6.098 mgQE/g ± 0.380) to *T. patula* (79.605 mgQE/g ± 1.80). Vanessa et al., 2014 reported 25.13±1.02 mg/ g in the acetone extracts of *T. patula*. Similarly, Politi et al., 2016, reported 72.74mgQE/ g in leaves whereas flower extracts 124.59 mgQE/ g TFC of leaves of ethanol extracts.

Here, it is observed that among the three plant extracts *T. patula* has highest level of flavonoid content and least in the *C. grandis*.

While it has been reported that 8.21 mg/g of Flavonoid contain in the methanol extract of fruits and leaves of *C. grandis* from the Mumbai, India.[69] which is nearly equal to the amount of TFC obtain in this work.

Extracts with high yield of TFC might possess greater antioxidant activity. Although, variation in quantitative evaluation of flavonoid compound in plant extracts are affect by their structural complexity, biodiversity, Assay method, also due to presence of interfering substance and land altitude, climatic change.

4.4 Antioxidant activity

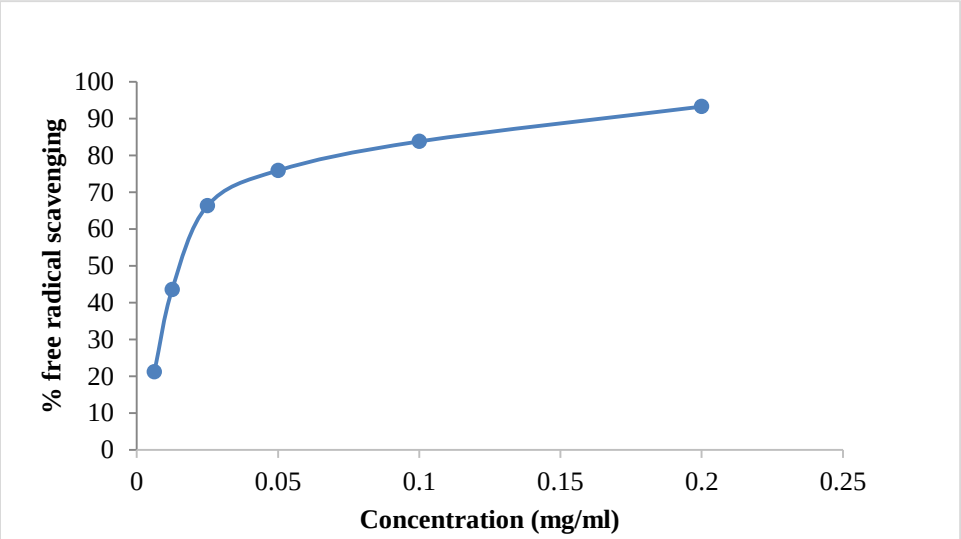
4.4.1 By DPPH free radical scavenging activity

In this study the hydrogen atom or electron donation ability of each plant extract against DPPH free radical was measured from the bleaching of violet colored DPPH solution at 517 nm. DPPH is scavenged by antioxidants through the donation of proton forming the reduced DPPH and the color changes from purple to yellow after reduction, which can be quantified by its decrease of absorbance at wavelength 517 nm. The degree of decolorization indicates the free radical scavenging potentials i.e. antioxidant potentials of the samples.

The DPPH radical assay was performed for each plant extract by using ascorbic acid as standard according to standard procedure. In this assay the mixture of DPPH with different concentrations of extract solution and ascorbic acid were separately incubated at room temperature and absorbance was recorded at 517 nm by spectrophotometer. Antioxidants results on a decrease of absorbance proportional to the concentration and antioxidant activity of the compound itself.

The observed absorbance with the different concentration of ascorbic acid is plotted in the graph as shown in Figure 5.

Figure 5: A plot of percentage radical scavenging activity with concentration of ascorbic acid



Graphical representations of DPPH assay of all extracts present in this study is demonstrated in Figure 6;

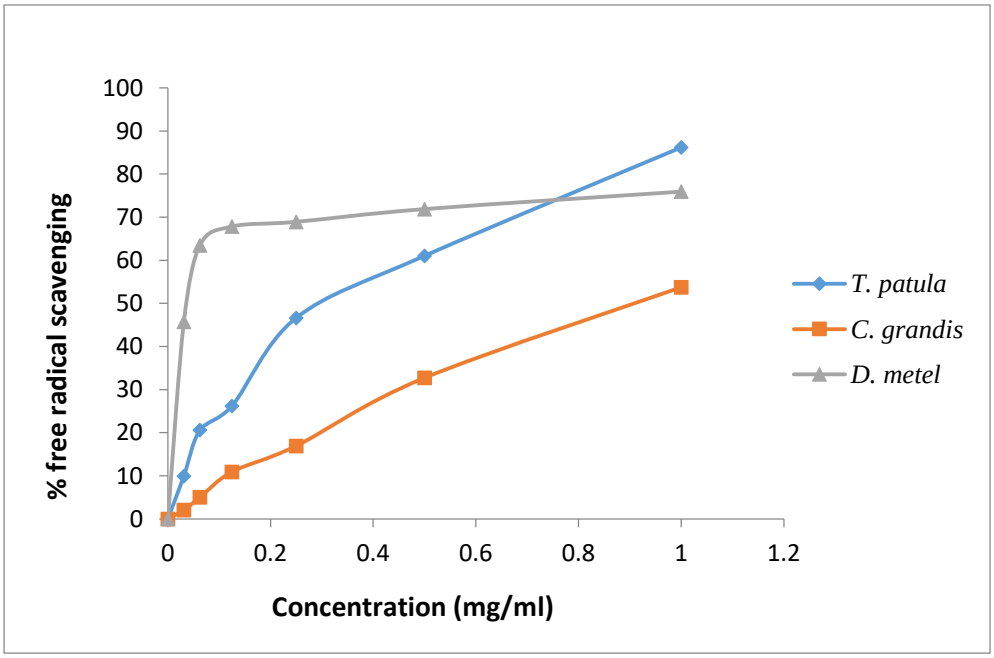


Figure 6: A plot of percentage radical scavenging activity with concentration of different plant extracts

The linear regression of the percentage of radical scavenging versus concentration was used for the calculation of concentration of each plant extract required for 50% inhibition of DPPH activity (IC_{50}). The antioxidant potential is in an inverse relation with IC_{50} value, lower the value of IC_{50} higher is the antioxidant activity. The IC_{50} values of the plant extracts along with the standard ascorbic acid is tabulated below.

Table 6: Comparision of IC_{50} values of different plant extracts with standard ascorbic acid

S.N	Sample	IC_{50} (µg/mL)
1	Standard Ascorbic acid	18
2	*C. grandis*	911
3	*D. metel*	39
4	*T. patula*	309

More conveniently, the Table 6 is represented in the bar diagram below;

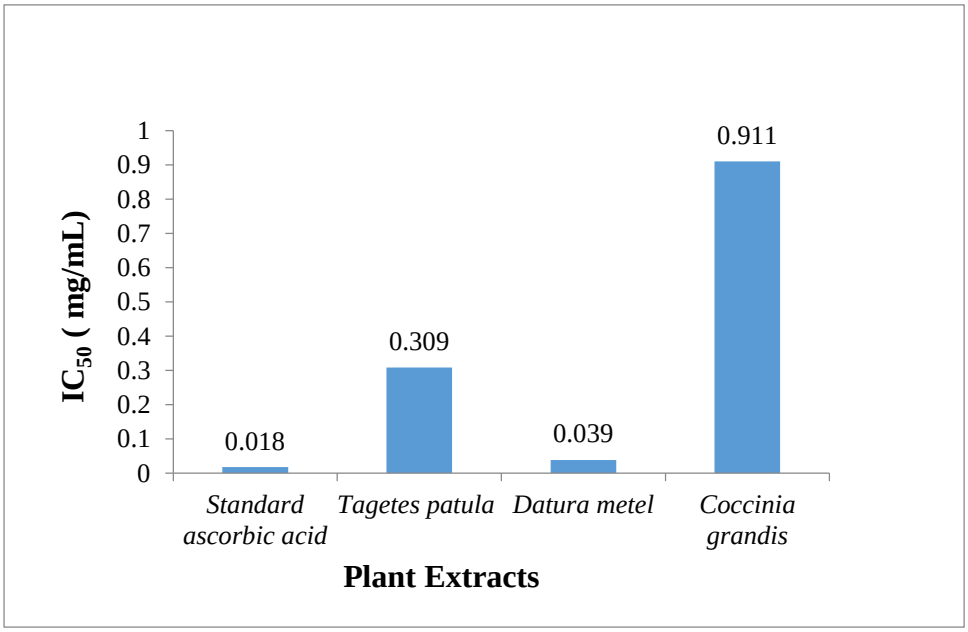


Figure 7: Free radical scavenging activity (IC_{50}) in different concentration of methanolic plant extracts.

The table and bar graph present above showed that the plant extracts have antioxidant activity whereas some of the plant extracts among them showed good potential for antioxidant activity as their IC_{50} values were found close to

the standard ascorbic acid taken. More effective results were obtained from the methanolic extract of *D. metel* (0.039 mg/mL), which showed the strong DPPH radical scavenging activity as its IC_{50} value was close to standard ascorbic acid (0.018 mg/mL). Furthermore, methanolic extract of *T. patula* (0.309 mg/mL) showed the strong DPPH radical scavenging activity then *C. grandis* (0.911 mg/mL) additionally, it showed less activity then *T. patula* and ascorbic acid. Among three selected plants *D. metel* and *T. patula* studied are found good source of antioxidant compound.

Vanessa et al, 2014 reported that acetone extract of flower of *T. patula* showed the IC_{50} value of 15.74 µg/mL which showed more active antioxidant then the methanolic extract of its leaves.

However, Akharaiyi, 2014 reported that ethanolic extract of *D. metel* showed IC_{50} value 49.30 % antioxidant activity which less active than the methanolic extract of *D. metel* as observed on this study.

Although quantitative evaluation of antioxidant activity of plant extracts may be affected due to climatic conditions, biodiversity, assay method, land altitude and also due to presence of interfering substance present in the plant extracts.

4.4.2 By FRAP method

The FRAP assay is a novel method for assessing antioxidant power by measuring the ferric reducing ability of plant extracts. The FRAP assay method is inexpensive, reagents are simple to prepare, results are highly reproducible, the procedure is straightforward and speedy and the equipment required is of a type commonly found in biochemical laboratories. Working FRAP reagent contains acetate buffer, TPTZ solution and $FeCl_3$ solution. FRAP assay involves the reduction of ferric to ferrous ion. FRAP values are obtained by comparing the absorbance change at 593 nm in test reaction mixtures with those containing ferrous ions in known concentration.

4.4.2.1 Ferrous Sulfate as an Antioxidant

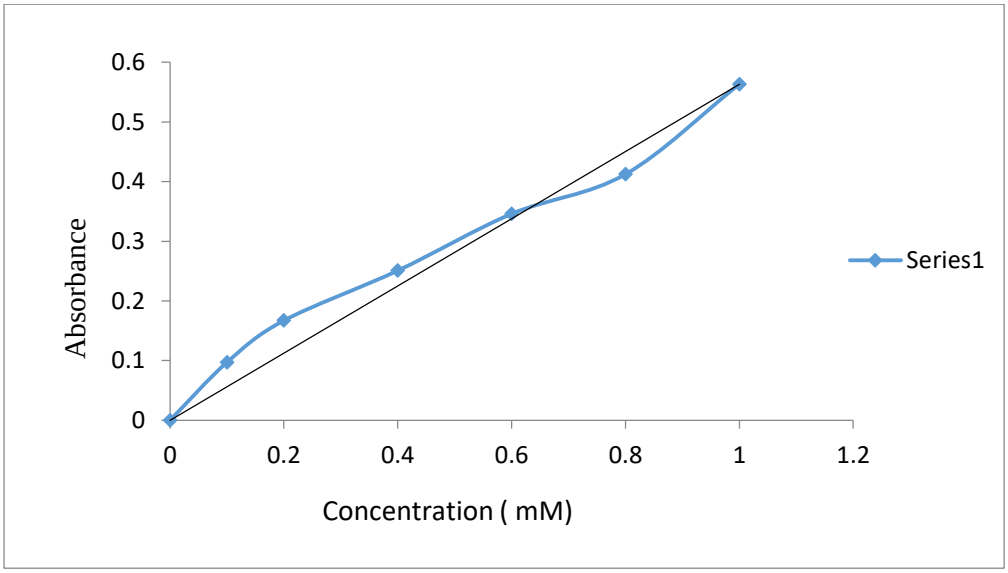
The FRAP assay was conducted for each plant extracts by using ferrous sulfate as a standard. In this method, sample solution and standard solution of ferrous sulfate was mixed with FRAP reagent separately and then absorbance was

recorded by spectrophotometer. The results obtained from the assay are given in Table 7:

Table 7: Standard Ferrous Sulfate as an Antioxidant

S.N	Concentration of $FeSO_4.7H_2O$ (mM)	Absorbance
1	0.1	0.0971
2	0.2	0.16713333
3	0.4	0.25083333
4	0.6	0.34593333
5	0.8	0.4124
6	1	0.56336667

Figure 8: Graph of Absorbance versus Concentration of Ferrous Sulfate



The graph of this data has given a best linear fit of y = 0.5147x + 0.0344 with 0.9838 regression coefficient(R). Where, y = Absorbance (A) x = concentration of ferrous sulfate in mM

The antioxidant powers of plant extracts in ferrous sulfate [Fe (II)] equivalents were calculated by solving the linear equation for “x” by providing “y”.

Table 8: Antioxidant power of Methanol Extracts of different plants

S.N	Plant Extract	Concentration (mg/mL)	Antioxidant Power [mM Fe(II)/L]
1	*D. metel*	0.1	357.0365±0.682
2	*T. patula*	0.1	339.68±0.296
3	*C. grandis*	0.1	255.1±0.194

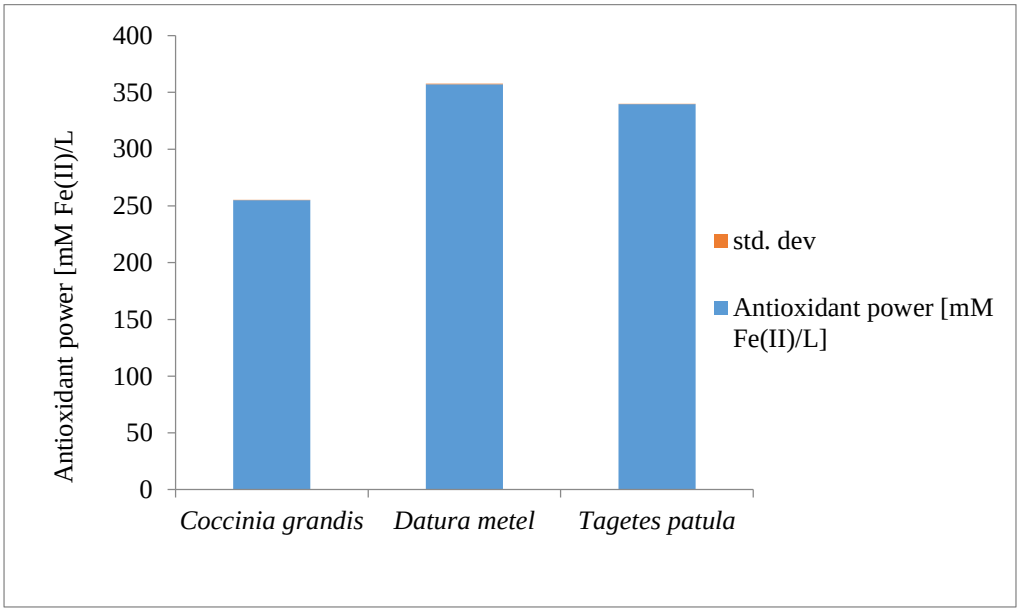


Figure 9: Comparison of Antioxidant Power of plant extracts in 0.1 mg/mL.

From the Table 8, plant extracts of leaves of *D. metel*, *T. patula* and *C. grandis* had shown antioxidant power of 357.036±0.682, 339.68±0.296 and 255.1±0.194 mM Fe(II)/L respectively. Methanolic leaves extract of *D. metel* exhibited maximum antioxidant power out of these above plants. Similar result was also observed in the DPPH assay method which also showed *D. metel* as good antioxidant among three of the selected plant extracts.

4.5 Antibacterial activity

The diameter of zone of inhibition (ZOI) produced by plant extracts on particular bacteria was measured for the estimation of their antibacterial activity. The methanolic extract of *C. grandis* and *D. metel* did not show any

zones of inhibition at 50 mg/mL. But methanolic extract of *T. patula* was found to be resistant against only in gram positive bacteria.

Results observed from antibacterial screening of different extracts are listed in Table 9.

Table 9: Antibacterial screening of different plant extracts

S.N	Bacteria	Plant Extract	ZOI of extracts at 50 mg/mL(mm)	Positive control(mm)
1	*E. coli*	*C. grandis*	-	
		D. metel	-	14
		T. patula	-	
2	*S. typhi*	*C. grandis*	-	
		D. metel	-	18
		T. patula	-	
3	*S. aureus*	*C. grandis*	-	
		D. metel	-	19.3
		T. patula	10	
4	*K. pneumonia*	*C. grandis*	-	
		D. metel	-	16.3
		T. patula	-	

(-) = No effective antibacterial activity

ZOI = Zone of Inhibition

E.coli : Gram-negative bacteria

S. typhi : Gram-negative bacteria

S. aureus : Gram-positive bacteria

K. pneumonia : Gram-negative bacteria

Table 9 illustrate that the 10 mm ZOI was shown by *T. patula* for the inhibition against gram positive bacteria *S. aureus*. Remaining other two plant extract did not show any ZOI on the each of the gram positive and gram negative bacteria. Antibacterial activity by disc diffusion method (100 µL) of *T. patula* petal extracts (hexane and methanolic) and microdilution (0.03–72.0 mg/mL) against

B. subtilis, P. multocida, E. coli and *S. aureus* strains, presenting inhibition zones with values varying from 8.2 mm to 11.4 mm for the hexane and 12.4 mm–20.2 mm for the methanolic extract.[42] *T. patula* leaves were taken for antibacterial activity at concentration of 400 μg/disc, CCl_4 fraction showed maximum zone of inhibition against *E. coli* (zone of inhibition 22 mm). *E. coli* was more sensitive to aqueous fraction (16 mm) than the rest of bacteria.[34]

The ethanol extract of *D. metel*(100 mg/mL) showed powerful antibacterial activity with maximum zone of inhibition (26 mm) against *E. coli.*[55] Antibacterial agent was isolated from leaves of D. metel which displayed antibacterial activity against *S. aureus, P. aeruginosa, P. mirabis, S. typhi, B. subtilis* and *K. pneumonia* but could not inhibit *E. coli*. [53]

The aqueous extract of leaves of *C. grandis* for antibacterial activity against *E. coli, S. choleraesuis, S. dysenteries,* and *S. flexneri*.[64]

The antibacterial activity of *C. grandis* leaf extract with solvents such as acetone, ethanol, methanol, aqueous and hexane against five bacterial species. Ethanol leaf extract of *C. grandis* showed high antibacterial activity against *E. Coli, K. pneumonia* and *S. aureus*. [65]

The variation in results may be due to the change in climatic condition, variation in biodiversity, sources of bacteria sample taken and also due to different laboratory condition.

4.6 Antigout activity/ Xanthine oxidase inhibitory activity:

Gout is a form of arthritis caused by a deposition of uric acid crystals in the joints. Purines are the form of protein which can be obtained from food we consume thus, Uric acid is a breakdown product of purines. Gout has the unique distinction of being one of the most frequently recorded medical illnesses throughout history.

Alcohol is known to have diuretic effects that can contribute to dehydration and precipitate acute gout attacks.

Alcohol can also affect uric acid metabolism and cause hyperuricemia. Xanthine oxidase (XO) is the rate-limiting enzyme in the synthesis of urate, and hence inhibition of this enzyme decreases urate synthesis. Formation of uric acid in the joints can be reduced by inhibiting the xanthine oxidase enzyme which

results in the formation of uric acid by breakdown of purine in purine metabolism.

Xanthine oxidase inhibitory activity of plant extracts was determined by using UV-visible spectrophotometry. The result of xanthine oxidase inhibition activity is tabulated in Table 10;

Table 10: Percentage of xanthine oxidase inhibitory activity with different concentration of plant extracts

% of xanthine oxidase inhibition (mean±SD)					
Plant Extracts	**Concentration (mg/mL)**				
	0.15625	**0.3125**	**0.625**	**1.25**	**2.5**
C. grandis	33.18±0.73	42.76±0.86	55.42±1.20	67.54±0.49	78.39±0.48
T. patula	7.363±0.48	36.26±1.07	47.74±0.71	64.69±0.96	75.99±0.63
D. metel	12.51±0.36	24.07±0.59	41.25±0.60	56.93±0.73	60.25±0.49

Table 11: Percentage of xanthine oxidase inhibition with Allopurinol

% of xanthine oxidase inhibition (mean±SD)					
Sample	**Concentration (µg/mL)**				
	6.25	**12.5**	**25**	**50**	**100**
Allopurinol	35.70±0.59	49.16±0.47	72.21±0.95	89.23±0.59	98.49

In the present study, the % inhibition effect on xanthine oxidase was concomitantly increased with the increasing concentration of methanolic extract. The percentage inhibition of xanthine oxidase by methanolic extract of leaves of *C. grandis* was found to be high among the three selected plant extracts and that of *D. metel* was found to be low. Allopurinol was taken as standard drug for the evaluation of inhibition of xanthine oxidase.

The methanolic extract of leaves of *C. grandis* and *T. patula* showed potential xanthine oxidase inhibition with IC_{50} value nearly equal to standard Allopurinol. Graphical representation of xanthine oxidase inhibition of all plant extracts is given in Figure 10;

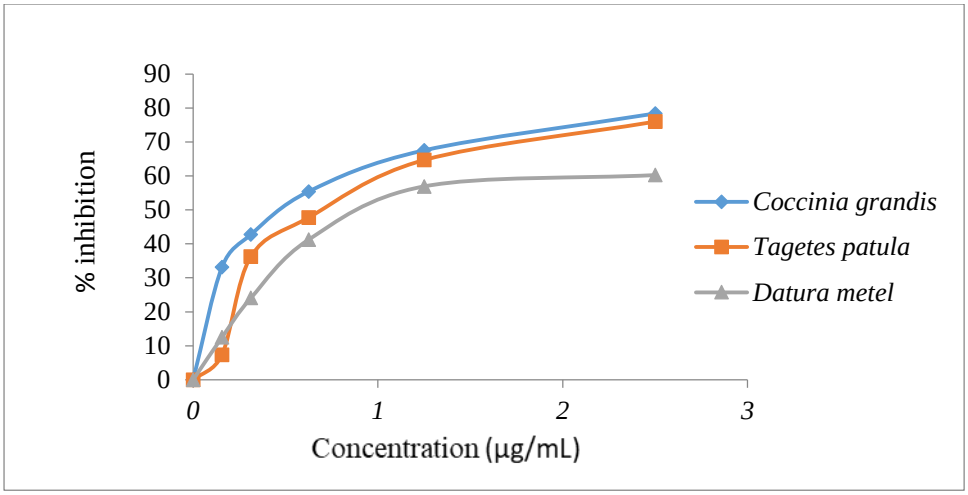


Figure 10: Comparision of % enzyme inhibition of different plant extract

Graphical representation of xanthine oxidase inhibition of Allopurinol is given in Figure 11;

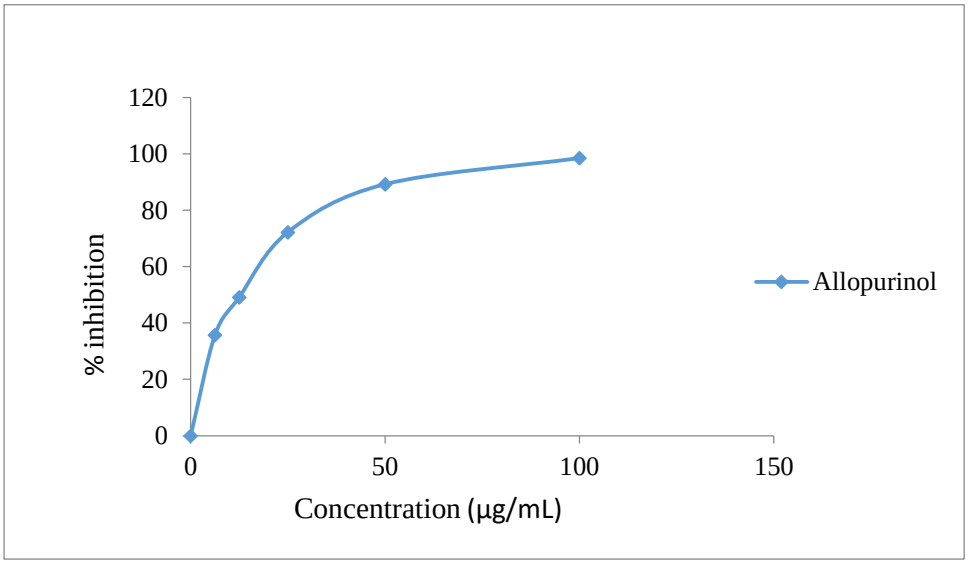


Figure 11: Graphical representation of % enzyme inhibition by Allopurinol

Results obtained are as follow. IC_{50} value is calculated by using graph pad prism software.

Table 12: IC_{50} value for xanthine oxidase inhibition assay

S.N	Plant Extracts	IC_{50} (mg/mL)
1	*C. grandis*	0.491
2	*T. patula*	0.708
3	*D. metel*	0.974
4	Allopurinol	0.0232

The IC_{50} value was calculated for all plant extract which is mentioned in tabulated form as well as in bar diagram.

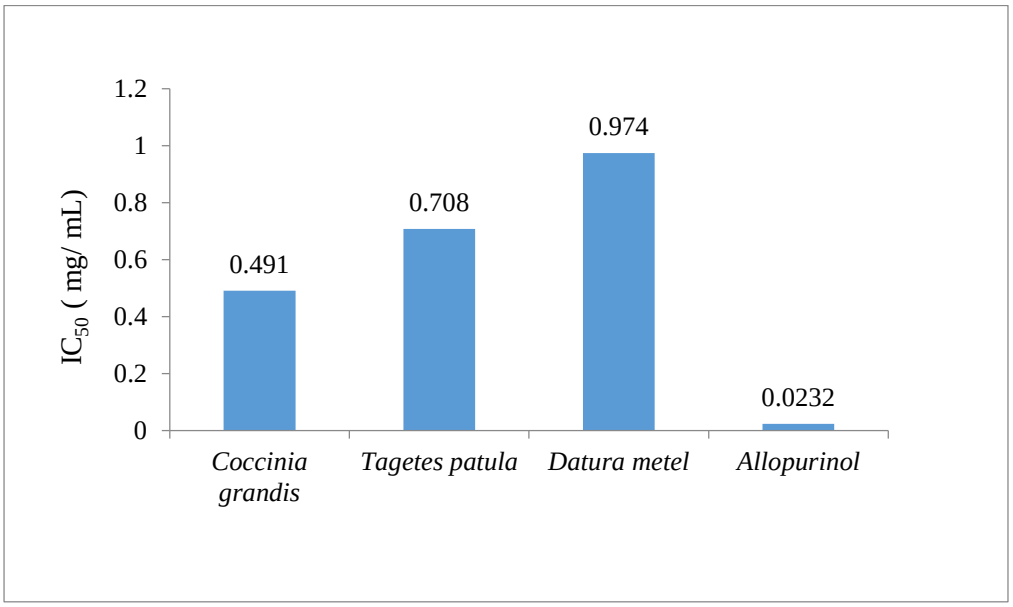


Figure 12: Comparative antigout properties of plant extracts

In the present study methanolic extracts of leaves of plants were observed for their potential to inhibit xanthine oxidase enzyme thus, formation of uric acid could be inhibited. IC_{50} value of standard allopurinol was found to be 0.0232 mg/mL. Among the selected plants, methanolic extract of leaves of *C. grandis* showed the highest xanthine oxidase inhibitory activity with IC_{50} value of 0.491 mg/mL. Along with other methanolic extract of plant also had showed effective result such as *T. patula* (0.708 mg/mL) *and D. metel* (0.974 mg/mL). Umamaheswari et al., 2007 illustrate that, methanol extract of leaves of *D. metel* showed the IC_{50} of 76.75 g/mL for crude extract which showed reduction of uric acid generation activity.

Similiarly, methanol extract of leaves of *C. grandis* show the reduction of uric acid with IC_{50} value 29.75 µg/mL.[51]

Variation in activity in inhibition by plant extracts may be affected due to climatic conditions, biodiversity, assay method, land altitude and also due to presence of interfering substance present in the plant extracts.

4.7 Isolation of compounds

On the basis of various biological activities the leaves extract of *C. grandis* was selected as the potent extract for isolation among the three selected plants. After column chromatography TLC of each was performed and fractions having similar TLC were mixed. Results obtained after column chromatography is illustrated in Table 13.

Table 13: Column and TLC report of different fraction

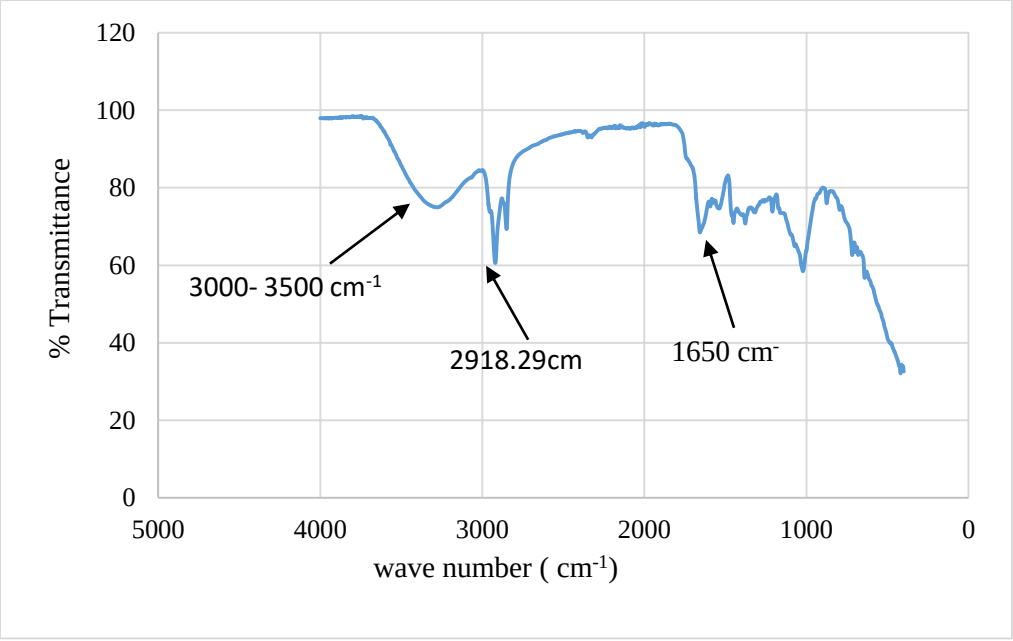
S.N	Elution solvent system	Volume of eluent (mL)	Remarks of TLC spots
1	100% hexane	250	Single spot
2	5% EtoAC in hexane	250	Tailing
3	10% EtoAC in hexane	250	Single spot
4	15% EtoAC in hexane	250	Tailing
5	20% EtoAC in hexane	300	Single spot
6	25% EtoAC in hexane	300	Tailing
7	30% EtoAC in hexane	300	Single spot
8	35% EtoAC in hexane	300	Tailing
9	40% EtoAC in hexane	300	Multiple spot
10	50% EtoAC in hexane	300	Single spot
11	60% EtoAC in hexane	400	Tailing
12	70% EtoAC in hexane	400	Tailing
13	80% EtoAC in hexane	400	Multiple spot
14	90% EtoAC in hexane	400	Tailing
15	100% EtoAC	250	Multiple spot
16	5% methanol in EtoAC	250	Tailing
17	10% methanol in EtoAC	250	Single spot
18	15% methanol in EtoAC	250	Tailing
19	20% methanol in EtoAC	250	Multiple spot
20	25% methanol in EtoAC	250	Tailing
21	30% methanol in EtoAC	250	Single spot
22	100% methanol	200	Tailing

After column chromatography TLC of each fraction was performed. Fractions having similar TLC report were mixed. Fraction of pure hexane 10, 20, 30, 50% ethylacetate in hexane and 10 and 30% methanol in ethylacetate were seen as single spots and the significant yield was obtained so the respected fractions were further analyzed via FTIR technique.

4.8 Chemical analysis of constituents of single spot fraction from Fourier Transform Infrared Spectroscopy (FTIR) analysis

Infrared spectroscopy has always been a powerful tool for the identification of organic materials. FTIR offers quantitative and qualitative analysis for organic and inorganic samples. Fourier Transform Infrared Spectroscopy (FTIR) identifies chemical bonds in a molecule by producing an infrared absorption spectrum. The spectra produce a profile of the sample, a distinctive molecular fingerprint that can be used to screen and scan samples for many different components. FTIR is an effective analytical instrument for detecting functional groups and characterizing covalent bonding information. FTIR analysis of obtained fraction from column chromatography have illustrated in Figure 13;

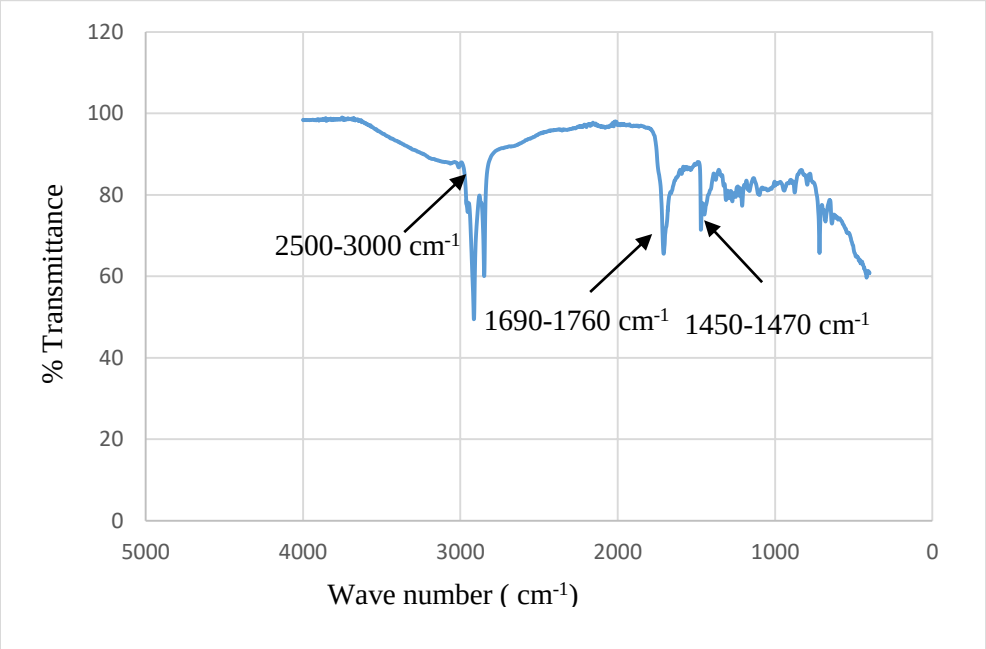
Figure 13: FTIR of fraction of 10% Ethyl acetate in hexane



Active fraction obtained from column was introduced into FTIR spectroscopy for the manipulation of possible functional group present in the sample. A peak at 1650 cm^{-1} may indicate the presence of C=C bond stetching. A sharp peak at 2918.29 cm^{-1} wave number indicate stretching vibration of C-H bond. Similarly

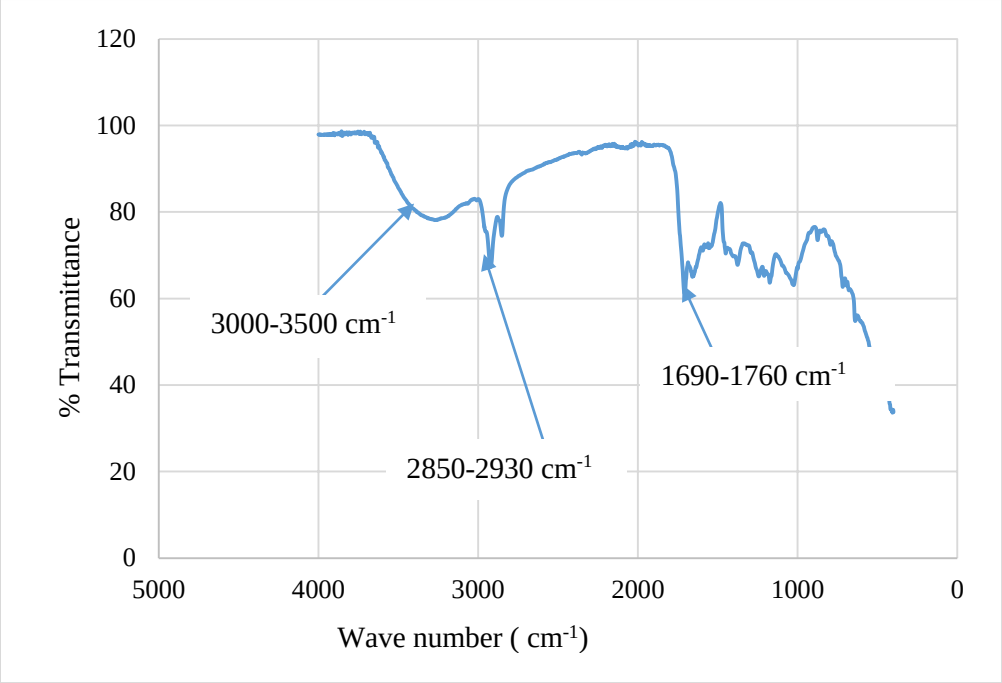
broad peak in the range of 3000-3500 cm^{-1} generally shows the presence of O-H bond may be H bonded alcohol or phenol group in the sample.

Figure 14: FTIR of fraction of 20% Ethyl acetate in hexane



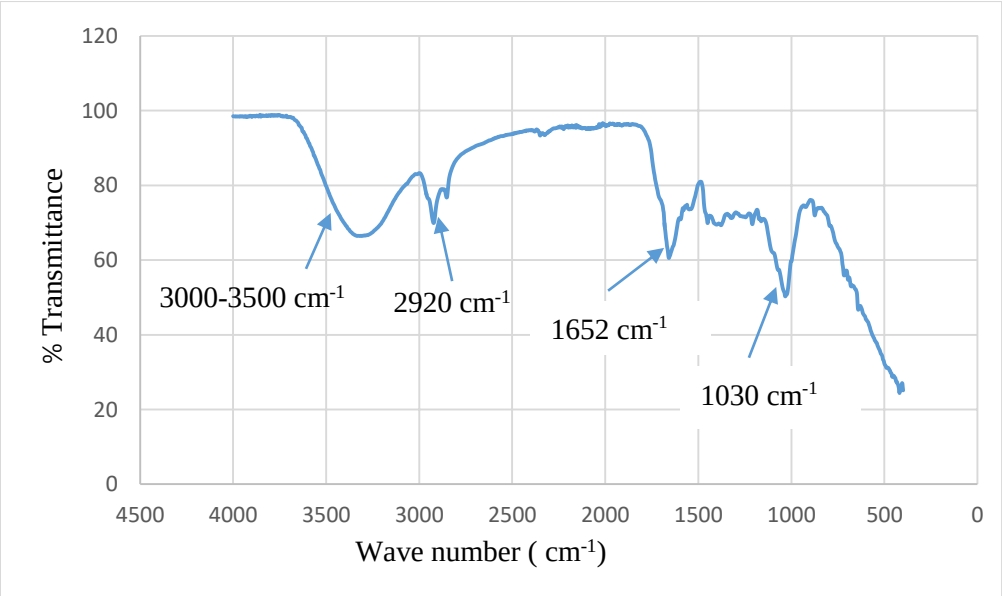
Active fraction obtained in 20% ethyl acetate in hexane was collected and subjected for FTIR spectroscopy for observation of functional group present in the respective sample. A sharp peak at 1450-1470 cm^{-1} wave number might indicate the presence of C-H bending. Simultaneously, a peak at 1707 cm^{-1} falls under the carbonyl group between 1690-1760 cm^{-1} that may be of aldehyde, ketone, oic acid, Ester etc. Similarly, peak at 2848 cm^{-1} and 2914 cm^{-1} may indicate the possibility of presence of stretching of C-H group or may be due to aldehyde =C-H.

Figure 15: FTIR of fraction of 30% Ethyl acetate in hexane



Active fraction obtained from column was introduced into FTIR spectroscopy for the manipulation of possible functional group present in the sample. A peak at 1650 cm^{-1} may indicate the presence of C=C bond stretching. Simultaneously a peak at 1707 cm^{-1} indicate the presence of carbonyl group. A sharp peak at 2920 cm^{-1} wave number indicate stretching vibration of C-H bond. Similarly broad peak in the range of 3000-3500 cm^{-1} generally shows the presence of O-H bond may be H bonded alcohol.

Figure 16: FTIR of fraction of 10% methanol in Ethyl acetate



Active fraction obtained in 10% methanol in ethyl acetate was collected and subjected for FTIR spectroscopy for observation of functional group present in the respective sample. A sharp peak at 1030 cm^{-1} wave number might indicate the presence of C-F bond. Simultaneously, a peak at 1650 cm^{-1} falls under the amide group. Similarly, peak at 2920 cm^{-1} may indicate the possibility of presence of stretching of C-H group or may be due to aldehyde =C-H. Broad bending at the range at 3000-3500 cm^{-1} indicate the presence of O-H bond.

CHAPTER – 5
CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

Phytochemical screening of methanolic extract of the plants showed the alkaloids, flavonoids, Phenol, polyphenols, saponins. Presence of reducing sugar, volatile oils, tannis were not shown by *C. grandis* but shown by *D. metel* and *T. patula* extracts. Carbohydrates, Coumarins, glycoslides, terpenes were not shown by each of the extracts.

T. patula showed the highest amount of total phenolic content (630.839±2.633 mgGAE/ g) on comparison other two plants extract whereas *D. metel* contains (110.724±3.325 mgGAE/ g) and *C. grandis* contains (257.276±1.58 mgGAE/g) which was determined by the 96 well plates spectrophotometer.

Total flavonoids content of *T. patula* was found to be 79.605±1.808 mgQE/g and was found to be highest among three of the plant extracts present in this study. Also *D. metel* (27.547±0.401 mgQE/g) showed the significant total flavonoid content.

The plant extract of *T. patula* showed a significant antibacterial activity against *S. aureus*. But *D. metel* and *C. grandis* did not show any antibacterial activity against both gram negative and gram positive bacteria.

The methanolic extracts of leaves of *D. metel* was found to high antioxidant property with IC_{50} value 0.039 mg/mL, which showed the strong DPPH radical scavenging activity as its IC_{50} value was close to standard ascorbic acid (0.018 mg/mL). Furthermore, methanolic extract of *T. patula* (0.309 mg/mL) showed the strong DPPH radical scavenging activity then *C. grandis* (0.911 mg/mL) additionally, it showed less activity then *T. patula* and ascorbic acid. Among three selected plants *D. metel* and *T. patula* studied are found good source of antioxidant compound.

From the FRAP assay methanolic plant extracts of leaves of *D. metel, T. patula* and *C. grandis* had shown antioxidant power of 357.036±0.682, 339.68±0.296 and 255.1±0.194 mM Fe(II)/L respectively. Methanolic leaves extract of *D. metel* exhibited maximum antioxidant power out of these above plants. Nearly, similar result was obtained from the both of the antioxidant assay method.

Xanthine oxidase inhibition enzyme assay was carried out, among which *C. grandis, T. patula* and *D. metel* showed effective result with IC_{50} values of 0.491, 0.708, 0.974 mg/mL respectively. Whereas, the IC_{50} value of standard allopurinol was 0.0232 mg/mL.

C. grandis was selected as potent extract for the column chromatography and different fraction was separated through column filled with the silica as adsorbent. Various TLC was performed and fraction which showed single spot was separated and carried for FTIR analysis.

FTIR analysis was separated fraction revealed the presence of different functional group. C=O group, O-H group, aromatic C-H, Alkanes was present in the respective fraction.

5.2 Recommendations

The present study strongly recommended the further work to isolate, purify, characterize and standardize the bioactive constituents from the active extract of *C. grandis*. In vitro and in vivo activity of these plant extracts could be performed which may be the part of drug discovery process. The plants extracts are the good sources of bioactive chemical constituents that may be subjected to isolate the target compounds.

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APPENDIX – I

PHYTOCHEMICAL SCREENING

General Procedure

About 100g of air dried plant material was extracted successively with methanol by cold percolation method. Each extracts was concentrated in Rotatory evaporator and was then subjected for phytochemical test as in given procedure below;

1. **Test for Reducing Sugars:**

   Each sample was shaken with distilled water first and then filtered. To each filtrate a few drops of fehling solution A and B were added and boiled for few minutes. The appearance of orange red precipitate confirmed the presence of reducing sugars.

2. **Test for Polyphenols (Ferric Chloride Test):**

   The methanolic extract 1 mL was mixed with water 1 mL. To this solution 5% w/v ferric chloride solution was added about 3 drops. A greenish blue colour was developed indicating the presence of polyphenols.

   Neutral Ferric Chloride Solution was prepared by adding 1 gm of ferric chloride in 100 mL distilled water. To this aqueous solution, sodium carbonate is added with continuous stirring until the slight turbidity persisted. The mixture was filtered and colourless filtrate was used as neutral ferric chloride.

3. **Test for basic alkaloids:**

   The methanol extract about 10 mL was concentrated to yield a residue and dissolved in 2% (v/v) HCl (3mL). This solution was equally divided into two test tubes.

   i) **Meyer's Test**: The first test solution was treated with three drops of Meyer's reagent. White precipitate indicated the presence of basic alkaloids.

   ii) **Dragendrof's Test**: The second test solution was treated with three drops of Dragendrof's reagent. White precipitate was obtained indicating the presence of basic alkaloids.

4. Test for glycosides:

The methanolic extract (8 mL) was concentrated to half the original volume and divided into two tubes.

i) The first test solution (2 mL) was treated with 25% (v/v) ammonium hydroxide solution (2 mL) and was shaken vigorously. A cherry red colour developed indicating the presence of glycosides.

ii) **Molisch's Test:** the second test solution was treated with the Molisch reagent (5 drops) and concentrated sulphuric acid was slowly added drop wise from the sides of the tubes without disturbing the solution. A violet ring at the junction of the two liquids was observed and on shaking the solution turned violet completely indicating the presence of glycosides or free sugars.

5. Test for Quinones:

To the methanolic extract (2 mL) freshly prepared ferrous sulphate solution (1 mL) and ammonium thiocynate (few crystal) were added and treated with conc. Sulphuric acid drop wise. A persistent deep red colour is observed indicating the presence of quinons.

6. Test for Saponins:

The methanolic extract (4 mL) was concentrated and mixed with water and shaken vigorously for 30 seconds in the test tube. A thick froth persistent even after 30 minutes indicated the presence of saponins.

7. Test for Coumarins:

Firstly single pellets of KOH was taken and dissolved in 1 mL ethanol. Now 1 mL of extract solution was added. The presence of precipitate indicates the presence of coumarin.

8. Test for Tannis:

0.5 mL of plant extract with 1 mL of water was prepared in which 2 drops of ferric chloride solution was added. Appearance of blue color indicate the presence of gallic tannis.

9. Detection of diterpenes:

Extracts were dissolved in the water and treated with 3-4 drops of copper acetate solution. Formation of emerald green color indicates the presence of diterpenes.

10. Test for Flavonoids:

i) **Lead Acetate Test:** The test solution was treated with 10% lead acetate solution, yellow precipitate indicate the presence of flavonoids.

ii) **Shinoda Test**: 5 mL of dilute ammonia solution was added to the aqueous filtered solution of each fraction followed by the addition of conc. Sulphuric acid. The appearance of yellow color indicate the presence of flavonoid. The yellow color disappeared after sometimes.

APPENDIX – II

PREPARATION OF REAGENTS

1. Maeyer's Reagent

Mercuric chloride (0.679 g) was weighed in a 50 mL volumetric flask and dissolved in a distilled water. To this solution, potassium iodide (2.5 g) was added. The scarlet red precipitate was dissolved by shaking and then diluted with distilled water up to the mark of volumetric flask.

2. Dragondroff's Reagent

Bismuth nitrate was dissolved in 5 N nitric acid (10 mL) to make solution 'A' next potassium iodide (13.5 g) was dissolved in distilled water (20 mL) to make solution 'B'. These solutions A and B were mixed together to prepare reagent.

3. Molisch's Reagent

5 g of alpha napthol was dissolved in 50 mL of methanol solution.

APPENDIX - III

Appendix 3.1: Absorption value of gallic acid

S.N	Concentration of gallic acid (µg/ mL)	Absorption at 765 nm (avg.)
1.	10	0.118333
2.	20	0.156667
3.	30	0.209333
4.	40	0.256
5.	50	0.331
6.	60	0.349
7.	70	0.462667
8.	80	0.486667

Appendix 3.2: Absorption of plant extract at 500 µg/ mL for TPC

S.N	Plant Extract	Absorption					
		Initial			Final		
		OD1	OD2	OD3	OD1	OD2	OD3
1.	*C. grandis*	0.081	0.076	0.08	0.782	0.773	0.776
2.	*T. patula*	0.241	0.23	0.233	1.867	1.852	1.862
3.	*D. metel*	0.301	0.302	0.31	0.345	0.363	0.348

Appendix 3.3: Absorption value of quercetin

S.N	Concentration of quercetin (µg/ mL)	Absorption at 510 nm (avg.)
1.	10	0.15
2.	20	0.248333
3.	30	0.354333
4.	40	0.388333
5.	50	0.521
6.	60	0.785333
7.	70	0.824667
8.	80	0.902

Appendix 3.4: Absorption of plant extract at 500 µg/ mL for TFC

S.N	Plant Extract	Absorption					
		Initial			Final		
		OD1	OD2	OD3	OD1	OD2	OD3
1.	*C. grandis*	0.124	0.121	0.126	0.162	0.166	0.165
2.	*T. patula*	0.613	0.644	0.669	1.097	1.099	1.12
3.	*D. metel*	1.46	1.455	1.464	1.62	1.619	1.632

Appendix 3.5: % inhibition for standard ascorbic acid (control = 0.337889)

S.N	Concentration (mg/mL)	Mean absorption at 517 nm	% inhibition
1.	0.2	0.022667	93.29168037
2.	0.1	0.054667	83.82111148
3.	0.05	0.081333	75.92897073
4.	0.025	0.113667	66.35975008
5.	0.0125	0.190667	43.57119369
6.	0.00625	0.266	21.27589609

Appendix 3.6: Absorption and inhibition of DPPH by *T. patula* plant extract (control = 0.337889)

S.N	(Final- initial) absorption			% inhibitory conc.			Concentration (Mg/mL)
	od1	od2	od3	od1	od2	od3	
1.	0.055	0.037	0.048	83.72246	89.04965	85.79415	1
2.	0.128	0.133	0.134	62.11772	60.63795	60.34199	0.5
3.	0.185	0.185	0.171	45.24827	45.24827	49.39165	0.25
4.	0.246	0.26	0.242	27.195	23.05163	28.37882	0.125
5.	0.254	0.273	0.278	24.82736	19.20421	17.72443	0.0625
6.	0.303	0.312	0.298	10.32555	7.661953	11.80533	0.03125

Appendix 3.7: Absorption and inhibition of DPPH by *D. metel* plant extract (control = 0.337889)

S. N	(Final- initial) absorption			% inhibitory conc.			Concentration (Mg/mL)
	od1	od2	od3	od1	od2	od3	
1.	0.085	0.081	0.078	74.8438	76.0276 2	76.9154 9	1
2.	0.095	0.093	0.097	71.8842 5	72.4761 6	71.2923 4	0.5
3.	0.104	0.106	0.105	69.2206 5	68.6287 4	68.9247	0.25
4.	0.107	0.109	0.11	68.3327 9	67.7408 7	67.4449 2	0.125
5.	0.123	0.121	0.126	63.5975	64.1894 1	62.7096 3	0.0625
6.	0.19	0.175	0.185	43.7685	48.2078 3	45.2482 7	0.03125

Appendix 3.8: Absorption and inhibition of DPPH by *C. grandis* plant extract (control = 0.337889)

S. N	(Final- initial) absorption			% inhibitory conc.			Concentration (Mg/mL)
	od1	od2	od3	od1	od2	od3	
1.	0.159	0.154	0.156	52.9431 1	54.4228 9	53.8309 8	1
2.	0.228	0.225	0.229	32.5222	33.4100 6	32.2262 4	0.5
3.	0.283	0.283	0.279	17.1325 2	16.2446 6	17.4284 8	0.25
4.	0.303	0.299	0.301	10.3255 5	11.5093 7	10.9174 6	0.125
5.	0.322	0.322	0.319	4.70240 1	4.70240 1	5.59026 6	0.0625

6.	0.33	0.33 1	0.33 2	2.33475 8	2.03880 3	1.74284 8	0.03125

Appendix 3.9: Absorption and inhibition of xanthine oxidase by Allopurinol (control = 0.421)

S. N	(OD- blank) absorption			% inhibitory conc.			Concentration (µg/mL)
	od1	od2	od3	od1	od2	od3	
1.	0.006	0.008	0.005	98.5748 2	98.0997 6	98.8123 5	100
2.	0.043	0.045	0.048	89.7862 2	89.3111 6	88.5985 7	50
3.	0.117	0.113	0.121	72.2090 3	73.1591 4	71.2589 1	25
4.	0.216	0.214	0.212	48.6935 9	49.1686 5	49.6437 1	12.5
5.	0.271	0.268	0.273	35.6294 5	36.3420 4	35.1543 9	6.25

Appendix 3.10: Absorption and inhibition of xanthine oxidase by *C. grandis* (control = 0.421)

S. N	(OD- blank) absorption			% inhibitory conc.			Concentration (mg/mL)
	od1	od2	od3	od1	od2	od3	
1.	0.089	0.091	0.093	78.8598 6	78.3848	77.9097 4	2.5
2.	0.136	0.135	0.139	67.6959 6	67.9334 9	66.9833 7	1.25
3.	0.183	0.193	0.187	56.5320 7	54.1567 7	55.5819 5	0.625
4.	0.24	0.245	0.238	42.9928 7	41.8052 3	43.4679 3	0.3125

5.	0.278	0.284	0.282	33.96675	32.54157	33.01663	0.15625

Appendix 3.11: Absorption and inhibition of xanthine oxidase by *T. patula* (control = 0.421)

S. N	(OD- blank) absorption			% inhibitory conc.			Concentration (mg/mL)
	od1	od2	od3	od1	od2	od3	
1.	0.1031	0.1021	0.0981	75.51069	75.74822	76.69834	2.5
2.	0.145	0.148	0.153	65.5581	64.8456	63.6579	1.25
3.	0.22	0.223	0.217	47.74347	47.03088	48.45606	0.625
4.	0.268	0.264	0.273	36.34204	37.29216	35.15439	0.3125
5.	0.388	0.392	0.39	7.83848	6.888361	7.36342	0.15625

Appendix 3.12: Absorption and inhibition of xanthine oxidase by *T. patula* (control = 0.421)

S. N	(OD- blank) absorption			% inhibitory conc.			Concentration (mg/mL)
	od1	od2	od3	od1	od2	od3	
1.	0.169	0.168	0.165	59.85748	60.09501	60.80761	2.5
2.	0.184	0.178	0.182	56.29454	57.71971	56.76961	1.25
3.	0.247	0.245	0.25	41.33017	41.80523	40.61758	0.625

4.	0.317	0.322	0.32	24.7030 9	23.5154 4	23.9905	0.3125
5.	0.368	0.367	0.37	12.5890 7	12.8266	12.1140 1	0.15625

APPENDIX – IV

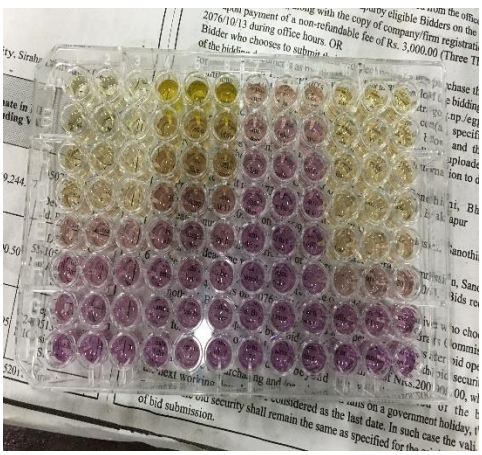
PHOTOGRAPHS

C. grandis

T. patula

D. metel

Photographs during lab work

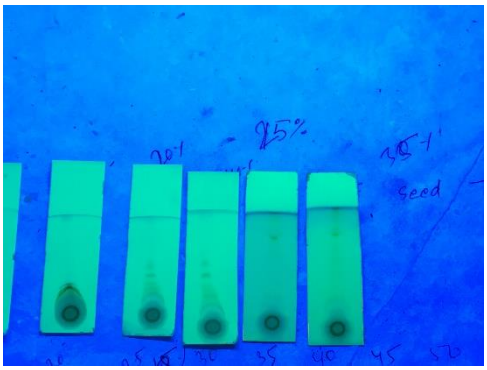


DPPH Scavenging

TPC

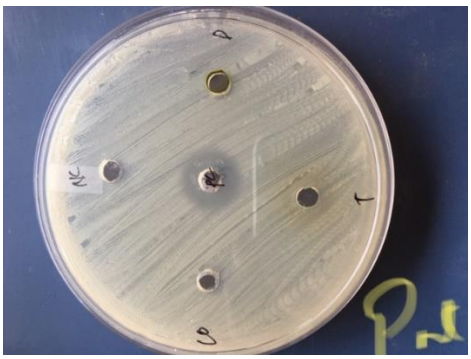
TFC

TLC

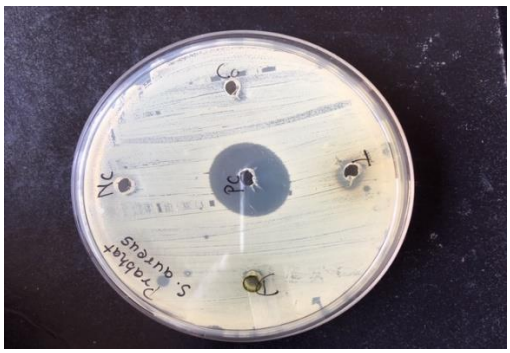


TLC in UV light

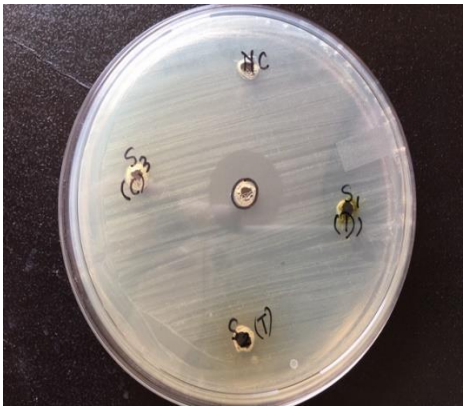
Column chromatography



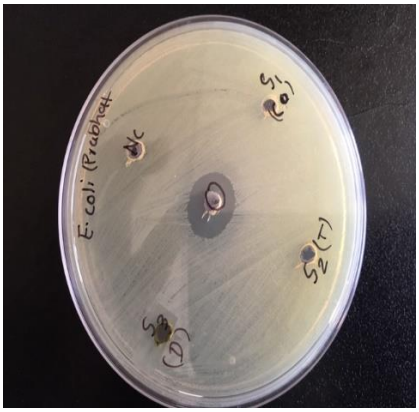
Antibacterial activities



S. aureus



K. pneumoniae



E. Coli

Column chromatography set up