Investigation of *in-vitro* antioxidant and cytotoxic potential of methanolic extract of *Oroxylum indicum*

A project submitted

by

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This work is dedicated to my parents and brother for their love and constant support…

Certification Statement

This is to certify that this project titled "**Investigation of *in-vitro* antioxidant and cytotoxic potential of methanolic extract of *Oroxylum indicum*"** submitted for the partial fulfillment of the requirements for the degree of Bachelor of Pharmacy (Hons.) from the Department of Pharmacy, BRAC University constitutes my own work under the supervision of Dr. Raushanara Akter, Assistant Professor, Department of Pharmacy, BRAC University and that appropriate credit is given where I have used the language, ideas or writings of another.

Signed,

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Countersigned by the supervisor,

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Abstract

Oroxylum indicum (*O. indicum*) is a local herbal medicinal plant which is commonly known as Sonapatha or Shyonaka. Literature review revealed that the roots, stem, leaf of this plant have been used as drug or component of drug for the treatment of various disorders. Different parts of this plant extract has therapeutic effects for example antitumor, anti-arthritic, antibacterial, anti-mutagenic and many more. In this study, the methanolic extract of the leaf and stem of *O. indicum* was screened for the evaluation of different phytochemicals, antioxidant and cytotoxic activity by standard methods. Several antioxidant screening methods have been used in this study for example DPPH test, total phenolic content, total flavonoid content and total antioxidant capacity for the determination of antioxidant activity. Along with this, MTT assay has been used for investigating cytotoxic activity. The present study has shown that the leaf and stem contain sugars, flavonoids, saponins, steroids and alkaloids. The antioxidant potential of this plant has been studied with stem extract and the findings have showed that with the increase of concentration the total phenolic content, total flavonoid content and total antioxidant capacity increased. In case of DPPH test at the lowest concentration of 50 µg/mL, the extract showed notable % of inhibition which was 84.87%. Extract showed good antioxidant capacity which was expressed as 67.25 mg of ascorbic acid per gram of dried extract which was at the lowest concentration of 200 µg/mL and in case of total phenolic content, with the highest concentration extract showed 98.075 mg of gallic acid per gram of dried extract. The cytotoxic activity was assessed by introducing the leaf extract on MOLT-4 (human acute lymphoblastic leukemia, T cell) of concentration of 0.025 mg/mL, 0.25 mg/mL, 2.5 mg/mL, and 25 mg/mL. At the highest concentration of 25 mg/mL it showed 90 % of the cell death where the cell survival was only 10%. Other concentration did not show remarkable % of cell death. So, antioxidant potential was significant in two methods and cytotoxic activity was not that significant. However, further study can be done on this plant for such as *in-vivo* antioxidant potential assessment and other bioactivities screening.

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Chapter 1

Introduction

The utilization of medicinal plants for help from sicknesses can be followed back over live millennia to composed records of the early civic establishments in China, India and the Near East, yet it is surely a craftsmanship as old as humankind. Indeed, even today, plants are the practically selective wellspring of drugs for most of the total population. After a number of experimental utilization of medicinal plants, the very first finding of active principles- alkaloids, for example, morphine, strychnine, quinine and so more. In the mid nineteenth century denoted another time in the utilization of restorative plants and the start of current restorative plant examine (Hamburger & Hostettmann,1991).

Therapeutic plants are utilized fundamentally in two diverse shapes: 1) as unpredictable blends containing a wide range of constituents such as implantations, fundamental oils, tinctures and extracts. 2) as unadulterated, synthetically characterized dynamic standards (Hamburger & Hostettmann,1991).

1.1 Medicinal plants as alternative of synthetic drugs

Plants are the biggest biochemical and pharmaceutical stores at any point known on our planet. These living stores can create interminable biochemical mixes. In their living, human what's more, creatures are utilizing just a little portion (1 to10%) of plants accessible on Earth (250,000 to 500,000 species) (Borris, 1996).

The collected confirmation got from hundreds of years of use in conventional prescription or from information of how types of plants have advanced and adjusted to their condition can significantly lessen the opportunity to identify advancement hopefuls. A large number of plant- inferred items have been recommended for patients by specialists of conventional pharmaceutical for quite a long time, giving supporting, albeit frequently just narrative, proof of potential adequacy and absence of plain lethality (Gwynn & Hylands, 2000).

Table 1.1: Name of the medicinal plants used in the treatment of common diseases

Scientific Name (Family)	**Part used**	**Traditional uses**	**References**
Curcuma longa L	Rhizome	Used in irritation, inflammation, erythema, burns, itching, sunburn and skin diseases	Zari, S. T., &Zari, T.A. (2015).
Plumbago zeylanica L.	Root	Treatment of paralysis, secondary syphilis, leprosy and ophthalmics	Chopra, R.N., Nayer, & Chopra, I.C. (1992).
Acanthus ilicifolius L.	Roots	Leucorrhoea	Hossan et al. (2010).
Holarrhena antidysenterica Wall.	Bark	Used in the treatment of dysentery, dropsy, fever, diarrhea and intestinal worm infections	Chopra, R.N., Nayer, & Chopra, I.C. (1992).
Aloe vera L.	Leaf gel	Used in the treatment of psoriasis, cold sores, sun burns, microbial skin diseases	Zari, S. T., &Zari, T.A. (2015).

Table 1.2: List of some drugs which are isolated from natural sources

Drug	Chemical class	Source	Medical use	Mechanism of action
Morphine	Alkaloid	Dried latex of Opium poppy, *Papaver somniferum* (1804)	Potent Painkiller/Analgesic, Sertürner (1805).	Opioid agonist by binding to opiate receptors (mu, delta, and kappa)
Paclitaxel (Taxol TM)	Terpenoid	Pacific yew tree, *Taxusbrevifolia*	Tubulin polymerization stabilizer, Sneader (2005).	Tubulin polymerization stabilizer
Penicillin	Penicillin	Fungus, *Penicilliumniger*	Antibiotic	Inhibition of Peptidoglycan synthesis
Salicylic acid	Beta-hydroxy acid	Barks of willow tree	Aspirin is used as an analgesic, anti-inflammatory and antipyretic (NSAID)	Inhibition of COX enzymes in the COX pathway
Quinine	Alkaloid	Cinchona bark, *Cinchona officinalis* (1820)	Anti-malarial agent (Meshnick& Dobson, 2001, p. 396).	Protein synthesis inhibitor
Digoxin	Cardiac glycoside	*Digitalis purpurea*	Atrial fibrillation and Congestive heart failure	Inhibition of the Na + /K + - ATPase membrane pump
Mevastatin	Polyketide	Fungus, *Penicillum citrinum*	Cholesterol lowering drug, (Li, 2009, p. 71-96).	Inhibition of the HMG-coenzyme-A

1.2 Significance of antioxidant potential of plants

Oxidative stress has been distinguished as the underlying cause of the advancement and movement of a few diseases. Supplementation of exogenous cancer prevention agents or boosting endogenous cell reinforcement guards of the body is a promising method for battling the undesirable impacts of reactive oxygen species (ROS) prompted oxidative harm. Plants have an intrinsic capacity to biosynthesize an extensive variety of non-enzymatic cancer prevention agents equipped for lessening ROS-actuated oxidative harm. Several *in vitro* techniques have been utilized to screen plants for their cancer prevention agent potential, and in a large portion of these measures they uncovered intense cell reinforcement action (Kasote et al.,2015).

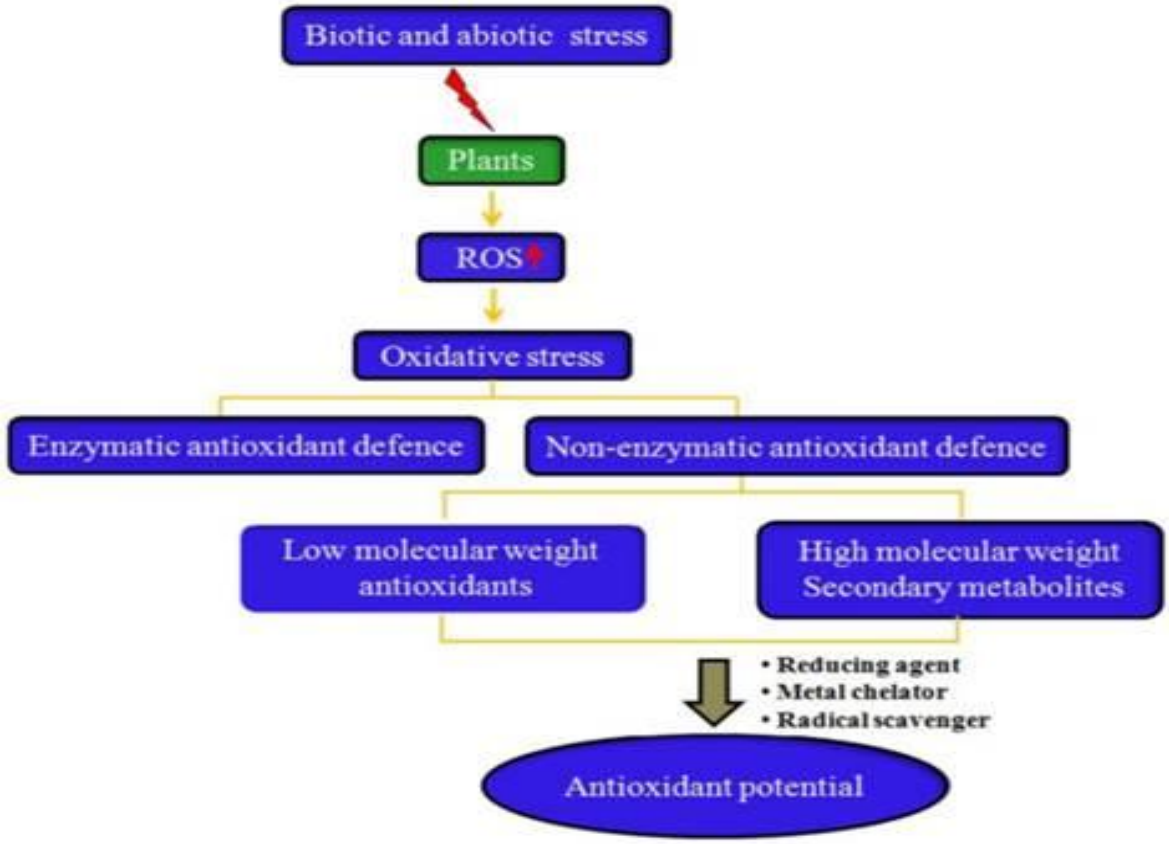


Figure 1.1: Plants have antioxidant activity

Plants have an intrinsic capacity to synthesize non-enzymatic cancer prevention agents. Be that as it may, under biotic and abiotic stretch conditions, the creation of reactive oxygen species (ROS) increments in the plants, bringing about acceptance of oxidative anxiety. In light of expanded oxidative anxiety, plants Augment the creation and aggregation of a few low sub-atomic weight cancer prevention agents (e.g., vitamin C, vitamin E, phenolic acids, and so forth.) and high sub-atomic cancer prevention agent auxiliary metabolites, for example, tannins, which present antioxidants to most plants under in vitro studies by working as free radical scavengers, reducing agents, and metal chelators (Kasote et al., 2015).

To avoid the toxic effects of the free radicals, plants have efficient complex enzymatic and non-enzymatic antioxidant defense systems. Catalase(CAT), glutathione peroxidase(GPx), and glutathione reductase (GR) are enzymatic systems. On the other hand low molecular weight antioxidants for instance, ascorbic acid, glutathione, pro-line, carotenoids, phenolic acids, flavonoids, etc. and high molecular weight secondary metabolites such as tannins are non enzymatic systems (Kasote et al., 2015).

Two reasons behind the synthesis and accumulation of non- enzymatic antioxidants by plants. One of them is the innate ability to perform their normal physiological function because of their capability of synthesizing a wide variety of phytochemicals which is the result of their genetic makeup. It also helps in giving protection from animal herbivores and microbial pathogens. The natural tendency of plants to respond to environmental stress conditions could be the another reason for the synthesis of the reductant phytochemicals (Kasote et al., 2015).

1.3 Medicinal plant as anticancer agent

Medicinal plants as a characteristic asset have gotten impressive consideration lately as potential chemotherapeutic specialists (Dwivedi et al., 2011). The utilization of plants as pharmaceuticals is most likely as old as Human kind itself. More than 150 000 plant species have been examined furthermore, huge numbers of them contain helpful substances (Loc and Kiet, 2011). Around 80% of the total populace in third world nations depends solely on plant items for their essential social insurance (Mans et al., 2000). A few known metabolites

having anticancer properties, such as flavonoids, terpenoids, alkaloids and phenylpropanoids were separated from characteristic sources (Kumar et al., 2012).

Cytotoxic phytochemicals, for example, vinca alkaloids or paclitaxel (Taxol) are frequently utilized as a part of oncology as exceedingly strong medications and additionally fill in as model for engineered mixes (Pandi et al., 2011; Huang *et al*., 2012). Medications, for example, these have been usually segregated as single plant concentrates or divisions thereof or have been blends of divisions/concentrates from various plants and utilized consequent to their assessment of wellbeing and viability in model frameworks and people (Dahiru et al., 2006). Tests in trial frameworks (*in-vitro* and *in-vivo*) have exhibited that a large portion of the phytochemicals demonstration by meddling with a few cell flagging pathways and prompt cell cycle capture or potentially separation enlistment (Chathoth et al., 2008) aside from their apoptosis-inciting potential (Kumar et al., 2012).

Apoptosis is a focal occasion fundamental to keep up tissue homeostasis for all organ frameworks in the human body (Thongrakard & Tencomnao, 2010). Concealment of apoptosis in carcinogenesis assumes a focal part in the advancement and movement of malignancy. Tumor cells utilize an assortment of atomic instruments to subdue apoptosis (Elmore, 2007). Consequently, acceptance of apoptosis in tumor cells is a particular remedial approach towards malignancy chemotherapy (Kumar et al, 2012).

There have been a plenty of reports in the logical writing archiving the chemopreventive potential of phytochemicals, for example, lupulone, hesperidin and blueberry phytochemicals in different tumor cell lines like those from tumors in the colon and the bosom (Park et al., 2008; Lamy et al., 2009; Adams et al., 2010).

Utilization of restorative plants as an approach in counteractive action also, treatment of growth is being taken after since thousands of years. Out of 92 anticancer medications which were accessible economically preceding 1983 in the US and among overall affirmed anticancer medications between 1983 furthermore, 1994, 60% are of common starting point (Cragg, et al., 1997). Worried to the deals for year 2000, normal items or its subsidiaries secured 14 of the main 35 drugs that retailed internationally (Butlet, 2004). Plant-chemotherapeutics was likewise perceived by the National Cancer Institute, where it

gathered around 35,000 plant tests from 20 nations furthermore, screened 1,14,000 concentrates for their anticancer movement (Shoeb, et al., 2005). A portion of the outstanding phytochemicals being used for growth treatment are the vinca alkaloids, the taxanes, and the camptothecins (Kumar, et al., 2012).

The only property of cytotoxicity solely may not be an satisfactory basis for a concentrate to have antineoplastic potential. Exhibit of apoptosis inductivity is important as a proof-of-idea approach for creating operators for chemoprevention, as this has dependably been the acknowledged technique for particularly annihilating malignancy cells. The greater part of the accessible anticancer medications take after this vital method of activity (Alshatwi et al., 2011; Hasan et al., 2011).

1.4 Overview of Leukemia:

Leukemia can be defined as the cancer of the blood cells which begins in a cell in the bone marrow is a cancer of the blood cells. If any leukemic change occurs in the marrow cell, the growth and survive of the leukemic cells is better than the normal cells. With time, the growth of leukemic cells suppress the development of the normal cells. The rate of leukemia progresses and the replacement of normal blood cells with marrow cells differ with different type of leukemia. Leukemia is the blood cancer which is the most common type. It affects 10 times as many adults as children. Mostly over 50 years old people diagnosed with leukemia.

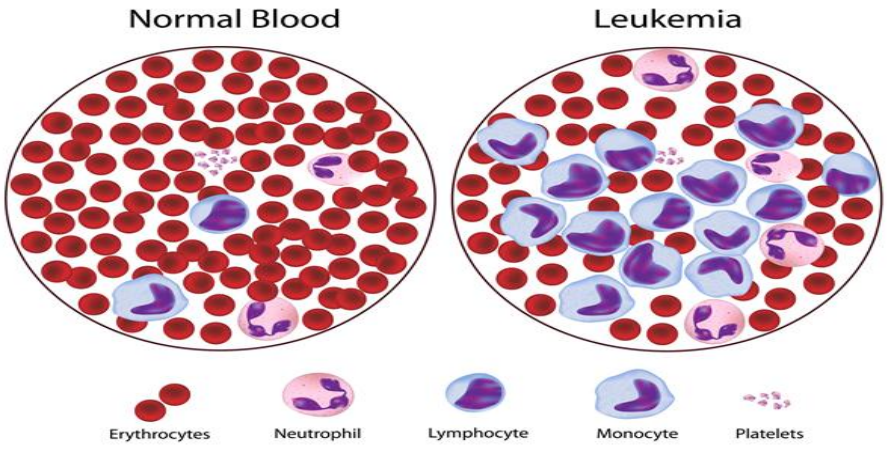


Figure 1.2: Leukemic cells

Types of leukemia

- Acute Lymphoblastic Leukemia
- Acute Myleoid Leukemia
- Chronic Myleloid Leukemia
- Hairy Cell Leukemia
- Pediatric Leukemia
- Plasma Cell Leukemia
- Mast Cell Leukemia
- Lymphoma Cancer
- Acute Myelomonocytic Leukemia
- Aleukemic Leukemia
- Acute Megakaryocytic Leukemia
- Feline Leukemia Complex

Diagnosis and treatment

The disease can be diagnosed by using different methods. They are physical exam, blood tests, biopsy, peripheral blood smear, cytogenic analysis etc. Leukemia can be both acute and chronic form which vary in their response to treatment. Four major treatment approaches are surgery, radiation therapy, immunotherapy, stem cell transplantation. Different types of antimetabolites drugs which are used in the chemotherapy for example, mercaptopurine, methotrexate. There are also genotoxic drugs used in genotoxic chemotherapy. Alkylating agents for instance busalfin, carbopltin are used as the genotoxic chemotherapy. Also, vincaalkaloids, docitaxel, paclitaxel etc are used as spindle inhibitors. They interfere with the cytoskeletal components and help to prevent the proper cell division.

Mechanism of action

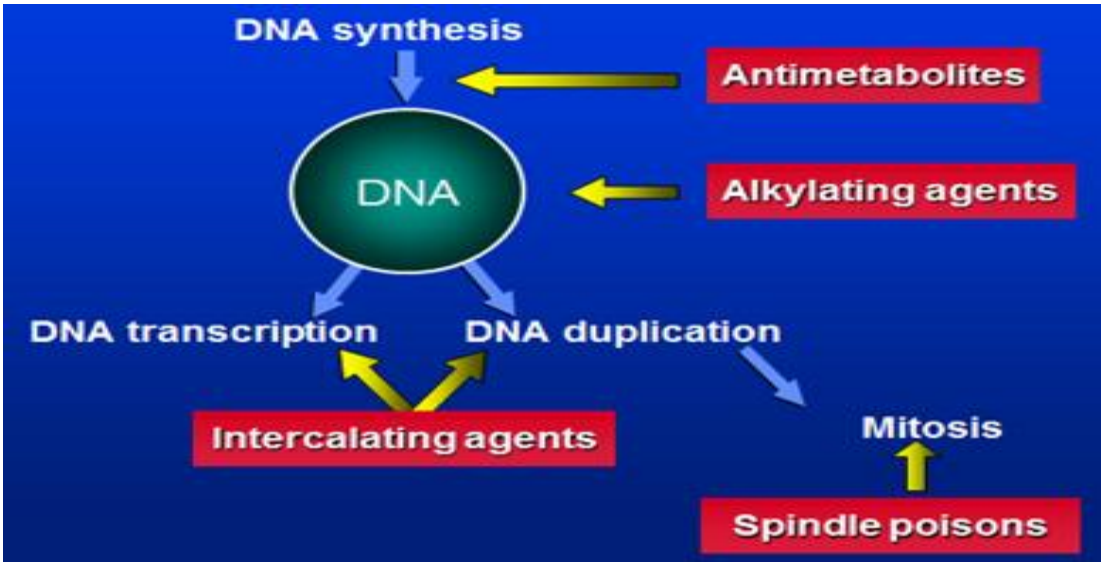


Figure 1.3: Mechanism action of anticancer agents

1.5 Selection of *O. indicum* for the current study

Going through a broad review of the literatures of different activities of this plant, it was confirmed that the antioxidant potential was not studied on stem extract and cytotoxic activity was not studied on MoLT4 cell using leaf extract. Through the online search it was concluded that *O. indicum* can be used for the analysis of antioxidant and cytotoxic potential.

1.6 Introduction to the plant

O. indicum (Bignoniaceae), otherwise called Sonapatha or Shyonaka is generally utilized herbal medicine in Ayurvedic framework. *O. indicum* is a flowering plant which belongs to the genus Oroxylum and the family Bignoniaceae, it is commonly called midnight horror, oroxylum, or Indian trumpet flower. Roots, leaves and stems of *O. indicum* have been utilized as a single medication or as a part of certain compound medication arrangements in the Indian Ayurvedic arrangement of drug for treatment of different issue and in addition utilized as a tonic and Rasayana drug. It is also wellknown for Ayurvedic formulations like Chyavanprash, Dashmularisthaetc (Dev, Anurag & Rajiv, 2010).

1.7 Geographical distribution

O. indicum is commonly native to the Indian subcontinent, in the Himalayan foothills with a part extending to Bhutan and southern China, in Indo-China and the Malaysia ecozone. Also it is diversely available in the forest of National Park in Assam, India, reported from Sri Lanka (Ceylon) (Dev, Anurag & Rajiv, 2010).

It is also found in the forests of Chittagong, Chittagong Hill Tracts, Cox's Bazar, Dhaka, Tangail and in village shrubberies and homestead throughout the country.

1.8 Botanical description

The height of this tree can be of 12 meter (40 feet).The wither and fall off the large leaf and the collection near the base of the trunk appears like a piles of broken limb bones, what's more, flowers are adjusted to normal fertilization by bats. Bark is off dark colored in shading. Leaves are 2 to 4 inch long, wide which has sharp edges. The flowers are purple in color and the flower stalk is one feet long. It has 1 to 3 foot long fruit which is 2 to 4 inch broad. Seeds are level and are 3 inch long and 2 inch in width. The flowers choose rainy season to born and December to March is the time for fruit to appear (Dev, Anurag & Rajiv, 2010).

Figure 1.4: Leaves of *O. indicum*

Figure 1.5: Fruit of *O. indicum*

Figure 1.6: Flowers of *O. indicum*

1.9 Taxonomical classification

Kingdom: Plantae

Division: Magnoliophyta

Class: Magnoliopsida

Order: Lamiales

Family: Bignoniaceae

Genus: Oroxylum

Species: indicum

1.10 Traditional use

According to (Joshi, Shukla & Nailwal, 2014),*O. indicum* or shyonaka is a remarkable plant as every part of this plant like leaves, roots bark, stem bark possess medicinal properties. It has several uses in traditional Ayurvedic medicines.

- The root bark of this plant is used as an important ingredient in prominent tonic formulation for instance Chyawanpurush, Bharma, Dashmularisht etc. The root bark of this plant is administered as bitter tonic, astringent, stomachic etc. It is also used in somatitistuberculosis and nasopharyngeal cancer. The dedoction of bark cure gastric ulcer and bark powder paste is applied for mouth cancer, scabies and other skin diseases.
- The mature fruits of *O. indicum* are acrid, tastes sweet, anthelmintic and stomachic. Mostly useful in various disorders like cadiac disorder, pharyngodynia, cholera, jaundice, dyspepsia, piles, leucoderma, cough etc.
- Seeds of this plant is used as purgative and women use the dried seed powder for conception.
- Another important part of this plant is the root which is sweet ,acrid, diuretic,anthelmintic, carminative, expectorant, anti-diabetic etc (Joshi, Shukla & Nailwal, 2014).

1.11 Previously found chemical constituents of *O. indicum* (Deca, et al., 2013)

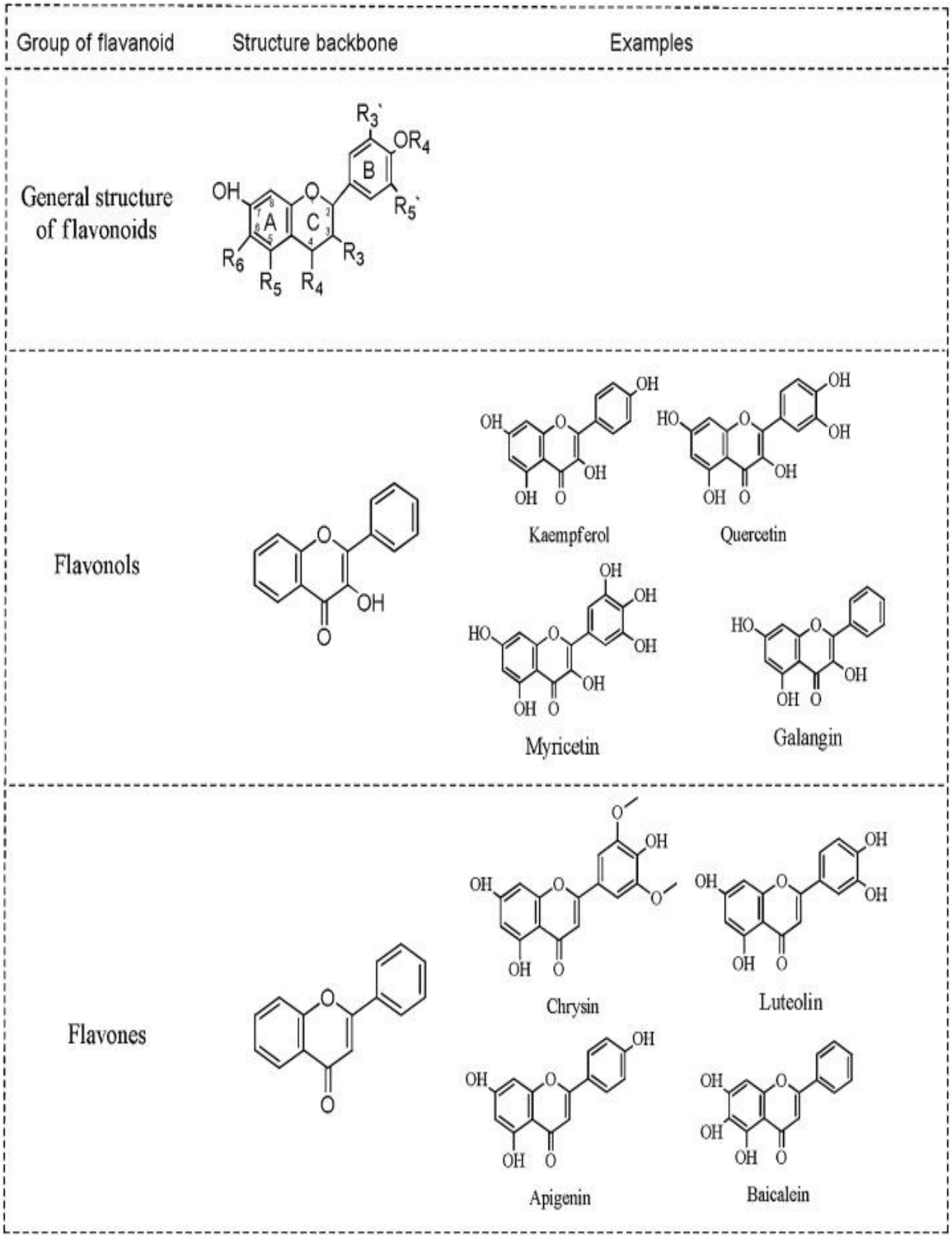


Figure 1.7: Constituents of *O. indicum* (Deca, et al., 2013)

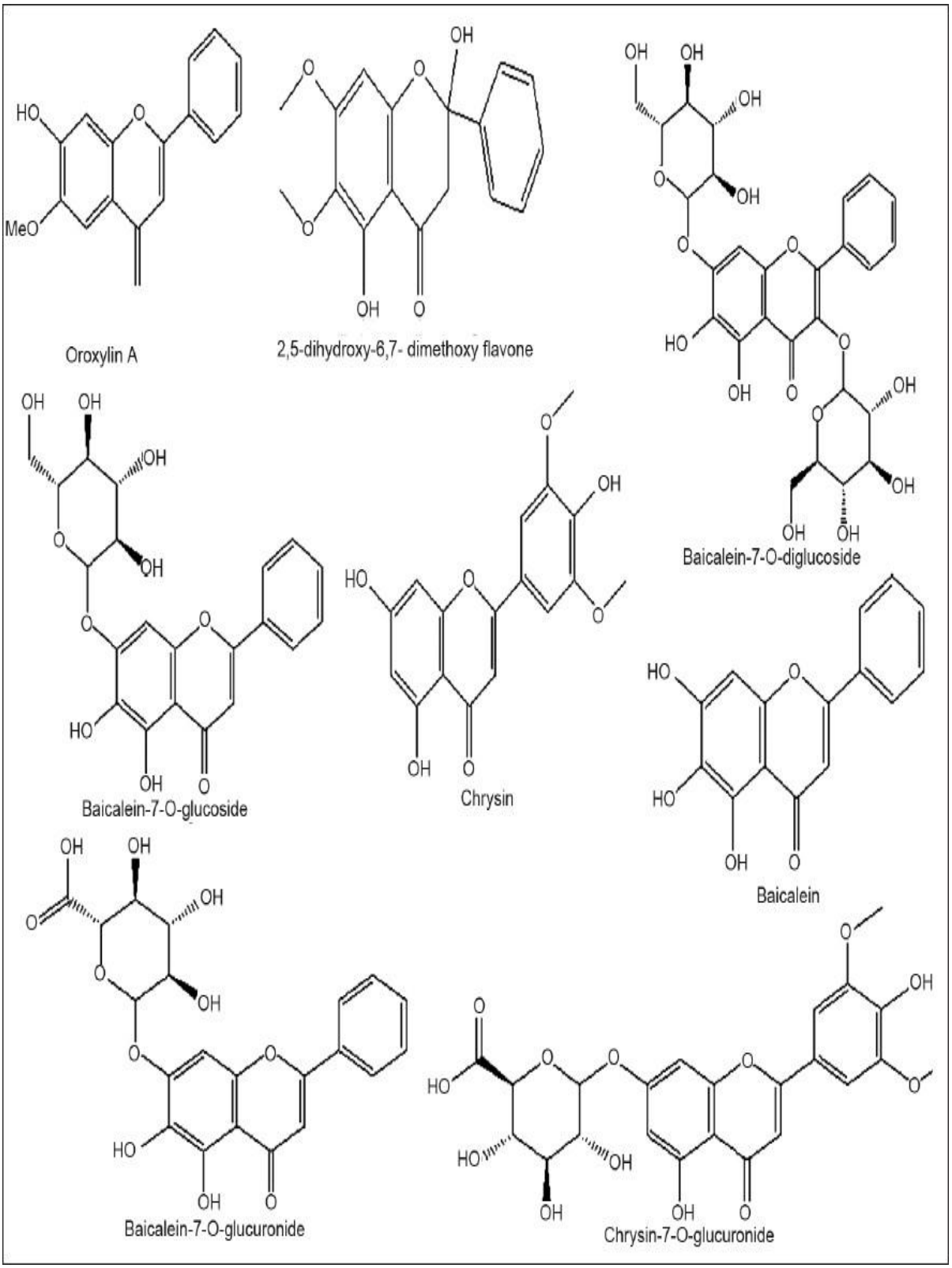


Figure 1.7: Constituents of *O. indicum* (Deca, *et al.*, 2013)

1.12 Previous studies in this field

Several researches have been conducted on *O. indicum*. Previously, different parts of this plant exhibit anti-inflammatory, antiulcer, antioxidant, anticancer, antimutagenic, photocytotoxic, antiarthritic, immunostimulant, antiproliferative, hepatoprotective activities in various *in vivo* and *in vitro* test models (Harminder, Singh & Chaudhary,2011).

1.13 Anti-inflammatory activity

Using diclofenac sodium as standard drug, the antiinflammatory activity was evaluated. It was done by carageenan induced rat model. Two different doses of aqueous extract of *O. indicum* which is 150 mg/kg and 300 mg/kg were used. Maximum antiinflmmatory activity was found at a dose of 300 mg/kg of extract. Although both the doses produced less activity than the reference standard. Be that as it may, the movement created by both the measurements was not as much as the reference standard. Separate at both measurements demonstrated critical ($P<0.05$) mitigating action at 5 h proposing that the concentrate prevalently hinder the arrival of prostaglandin like substances (Harminder, Singh & Chaudhary, 2011).

1.14 Antiulcer activity

The half alcohol concentrate of root bark of *O. indicum* also, its oil ether, chloroform, ethyl acetic acid derivation furthermore, n-butanol divisions were contemplated against ethanol- prompted gastric mucosal harm. The alcohol extract (300 mg/kg, p.o.) and its distinctive parts (100 and 300 mg/kg, p.o.) indicated decrease in gastric ulceration. The oil ether and n - butanol portions indicated most extreme hindrance of gastric sores against ethanol-initiated gastric mucosal harm. The outcomes were similar with omeprazole (reference standard). In the ethanol- prompted gastric ulcer show, treatment with both the dynamic divisions and omeprazole demonstrated critical cell reinforcement movement as clear from the diminishment in the degree of lipid peroxidation. The impact of dynamic portion of root bark on the ulcer record, add up to causticity, add up to corrosive yield, pepsin movement, pepsin yield and add up to sugar to protein proportion in pyloric-ligated rodent was considered. The dynamic

portion of root bark at a dosage level of 100 mg/kg p.o. indicated noteworthy diminishment (P <0.05) in the ulcer record, add up to corrosiveness, add up to corrosive yield, pepsin movement and pepsin yield alongside a noteworthy ascent in complete starch to protein proportion. The instrument of antiulcer action could be credited to an abatement in gastric corrosive secretory and cancer prevention agent exercises prompting gastric cytoprotection. This action could be connected to the nearness of baicalein in the root bark of the plant (Harminder, Singh & Chaudhary, 2011).

1.15 Antimicrobial activity

The antimicrobial movement of unrefined concentrate of *O. indicum* (oil ether, ethyl acetic acid derivation and methanol), compound 1 (2,5-dihydroxy-6,7-dimethoxy flavone) and compound 2 (3,7,3 " ,5 " - tetramethoxy- 2-hydroxy flavone) was tried against fourteen pathogenic microbes (5 Gram-positive and 9 Gram- negative) and seven pathogenic growths. Supplement agar also, supplement juices were utilized as bacteriological media and potato dextrose agar (PDA) was utilized for parasitic development. In antibacterial screening, each test was broken up in methanol at a fixation of 200 µg/10 µl. The action of these examples was contrasted and standard kanamycin circle (K-30 µg/circle) utilizing the standard plate dissemination strategy. The discoveries bolster the utilization of *O. indicum* in conventional solution for the treatment of bacterial and parasitic disease (Harminder, Singh & Chaudhary, 2011).

1.16 Antioxidant activity

The cancer prevention agent movement of ethanol and watery concentrate of *O. indicum* leaves was studied in two in vitro models viz. radical scavenging movement by 1,1-diphenyl-2-picrylhydrazyl (DPPH) diminishment and nitric oxide radical scavenging movement in Griess reagent framework. Ethanol remove had huge antioxidant movement in both the models. In rummaging DPPH radical, removes activity was IC_{50} =24.22 µg/ml while in rummaging nitric oxide (NO) radical, the action was IC_{50} =129.81 µg/ml. The outcome appeared that ethanol concentrate of *O.*

indicum leaves has free radical scavenging action (Harminder, Singh & Chaudhary, 2011).

1.17 Anticancer activity

Methanol concentrate of the fruits of *O. indicum* hindered *in vitro* multiplication of HL-60 cells. The flavonoid baicalein was found as a dynamic part in the concentrate. The *in-vitro* impacts of baicalein on the reasonability and acceptance of apoptosis in the HL-60 cell line was additionally explored. The cell practicality after treating with baicalein for 24 h was evaluated by checking practical cells utilizing tryptan blue recoloring. The result demonstrated that baicalein brought about a half hindrance of HL-60 cells at centralization of 25-30 µM. The hindrance of expansion of HL-60 cells due to 36-48 h introduction with 10 or 20 µM baicalein was related with the collection of cells at S or G2M stages. The outcomes demonstrated that baicalein has antitumors consequences for human tumor cells (Harminder, Singh & Chaudhary, 2011).

1.18 Antimutagenic activity

Methanol concentrate of *O. indicum* emphatically repressed the mutagenicity of Trp-P-1 in an Ames pre-incubation strategy within the sight of S9 blend utilizing *Salmonella typhimurium*. Only 5 µl of the unrefined concentrate repressed 91±5% of the mutagenesis instigated by 50 ng of Trp-P-1. The major antimutagenic constituent was recognized as baicalein with an IC_{50} estimation of 2.78±0.15 µM. The powerful antimutagenicity of the concentrate was associated with the high substance (3.95±0.43%, dry weight) of baicalein. Baicalein go about as desmutagen since it represses N-hydroxylation of Trp-P-2. The antimutagenic impact of baicalein was discovered for the most part because of the hindrance of N-hydroxylation catalyzed by P450monooxygenases in S-9 (Harminder, Singh & Chaudhary, 2011).

1.19 Photocytotoxic activity

The photocytotoxic movement of methanol concentrate of leaves of *O. indicum* was examined against promyelocytic leukemia cell line, HL-60. The HL-60 was brooded with 21 µg/ml of unrefined concentrates for 2 h and lighted with 9.6 J/cm of a wide range light source in four repeats. Survival of cells was evaluated 24 h later after the colorimetric MTT convention. Pheophorbide-an, an industrially accessible furthermore, all around described photosensitizer was utilized as the positive control. To decide tests that have general cytotoxicity, a parallel test without illumination was additionally completed. The outcome appeared that methanol concentrate of leaves of *O. indicum* have photocytotoxic movement at fixation 21 µg/m (Harminder, Singh & Chaudhary, 2011).

1.20 Antiarthritic activity

Aqueous and ethanol concentrate of *O. indicum* were tried for *in vitro* arrival of myeloperoxidase (MPO) from rodent peritoneal leukocytes. The outcomes showed that aqueous concentrate had a critical impact i.e. 64 % hindrance of arrival of MPO (Harminder, Singh & Chaudhary, 2011).

1.21 Immunostimulant activity

n-Butanol concentrate of root bark of *O. indicum* (100 mg/kg, once every day for 22 days) was examined for immunomodulatory movement in rats utilizing measures of insusceptible reactions to sheep red platelets (SRBC haemagglutinating immunizer [HA] titer) and postponed sort extreme touchiness (DTH) responses. In light of SRBC, treatment with the n-butanol part brought on a huge ascent in coursing HA titers amid optional immunizer reactions, demonstrating a potentiation of certain parts of thehumoral reaction. The treatment moreover brought about a huge ascent in paw edema development, showing expanded host DTH reaction. Histopathologic examination of lymphoid tissues in the treatment gathering demonstrated an expansion in cellularity, e.g., T-lymphocytes what's more, sinusoids. Interestingly, dexamethasone treatment brought on critical lessening in the HA titer, DTH reactions, and cancer prevention agent action. In a triple antigen- intervened immunological

edema display, the degree of edema brought up in medication treated rats was more noteworthy looked at to that in charge rats, along these lines affirming upgraded DTH responses because of the medication treatment. Movement of the *O. indicum* may be ascribed to its capacity to improve particular resistant reaction (both humoraland cell-intervened) (Harminder, Singh & Chaudhary, 2011).

1.22 Antiproliferative activity

The antiprolifirative activity of *O. indicum* was studies on human breast tumor cell lines. Results indicated that *O. indicum* have antiproliferative activity against MCF7and MDA-MB-231 breast cancer cell lines (Harminder, Singh & Chaudhary, 2011).

1.23 Hepatoprotective activity

The hepatoprotective movement of *O. indicum* was examined against carbon tetrachloride (CCl4) prompted hepatotoxicity in mice and rats. Biochemical review shown that alcoholic (300 mg/kg), oil ether (300 mg/kg) and n-butanol (100 and 300 mg/kg) removes fundamentally ($P<0.05$) brought down the lifted serum glutamic oxaloacetic transaminase (SGOT), serum glutamic pyruvate transaminase (SGPT), soluble phosphatase (ALP) and aggregate bilirubin (TB) levels when contrasted with the control gathering. The expanded lipid peroxide (LPO) arrangement, decreased glutathione (GSH) and diminished cell reinforcement chemical exercises of superoxide dismutase (SOD), catalase (CAT) in the tissues of CCl_4 - treated animals were altogether standardized by *O. indicum* treatment. Histopathological concentrate likewise uncovered that pretreatment with *O. indicum* reestablished CCl_4 - prompted change in cancer prevention agent status of the tissues. It is proposed that root bark appeared huge cell reinforcement action, which may be thusly in charge of its hepatoprotective movement (Harminder, Singh & Chaudhary, 2011).

1.24 Rationale of the project

- It is a traditional medicinal plant and has been used for years after years for treating different kinds of diseases such as skin disease, jaundice, dyspepsia etc.
- This plant contains various chemical constituents and they are used to treat various diseases for instance inflammation, fever, asthma, malaria, infection etc.
- This study was focused on this particular plant as antioxidant activity using stem and cytotoxic activity on MOLT4 has not been conducted.
- This project was focused on antioxidant activity and cytotoxic activity. These activities are evaluated using traditional plant as traditional herbal medicines are naturally occurring, plant-derived substances with minimal or no industrial processing that have been used to treat illness within local or regional healing practices.
- Phytochemicals or compounds which are attributing to particular bioactivity properties were identified using particular method.

1.25 Aim of the study

The study was performed with the aim of investigating *in-vitro* antioxidant as well as cytotoxic potential of *O. indicum*.

1.26 Objective of the project

- To identify and select the parts of a medicinal plant that have medicinal value and have been using to treat different diseases.
- To determine the presence of chemical constituents that attribute to different bioactivities, phytochemical screening was performed.
- To determine the antioxidant and cytotoxic activity of the selected plant parts using *in-vitro* methods.

Chapter 2

Methodology

Chapter 2: Methodology

2.1 Collection and authentication of the plant material:

For the investigation *O. indicum* was chosen. Although some previous study was conducted on its antioxidant and cytotoxic activity, it was chosen for investigation by alternating the plant parts and cell line which was previously studied. It was decided to be chosen to compare with the previous study and for its availability.

2.1.1 Chemical investigation of *O. indicum*

Name of the plant	Family	Plant part
Oroxylum indicum	Bignoniaceae	Leaves and stem

The plant *O. indicum* was collected from Sylhet, Bangladesh in the month of July 2016. The whole plant was collected. After that the plant was submitted to the National Herbarium of Bangladesh (NHB) for the authentication of the plant. Thereafter, the plant was identified (ACCESSION NO.: DACB- 43774) and its voucher specimen was collected and the taxonomist of National Herbarium of Bangladesh, Mirpur, Dhaka authenticated it.

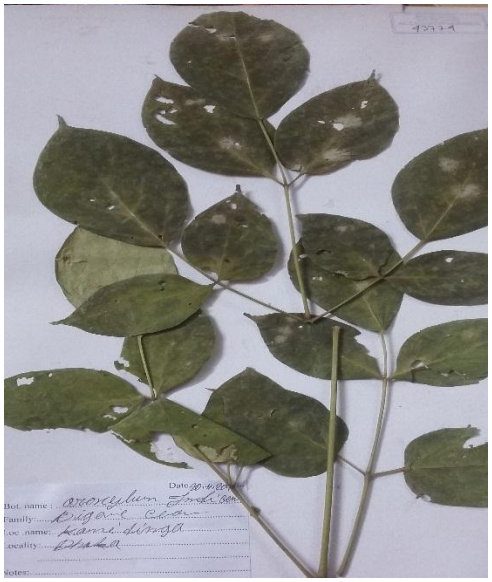


Figure 2.1: *O. indicum* leaves obtained from the National Herbarium

2.2 Extraction process

Several steps are involved in the process of extraction of medicinal plant:

The total extraction process can be alienated in two parts virtually which is shown by flow chart.

a. Plant material is prepared and dried

b. Extraction procedure

2.2 (A) Plant material preparation and drying

The leaves and stem were separated and washed thoroughly with clean water so that any dirt or dust can be removed. Thereafter, the washed plant parts were shed dried for some days until they were properly dried and ready for size reduction.

2.2 (B) Extraction Process

A. Preparation of the plant material (Leaves)

Crude plant was collected

⇩

Leaves were washed

⇩

Leaves were shed dried

B. Extraction process (leaves)

Size of dried leaves were reduced

⇩

Extraction (maceration)

⇩

Filtration

⇩

Concentration

⇩

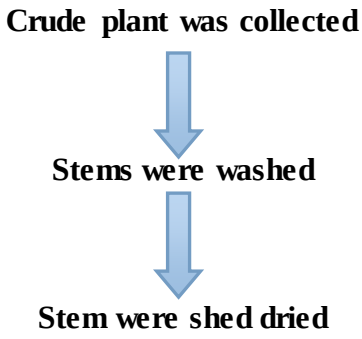
The plant concentrate was dried

⇩

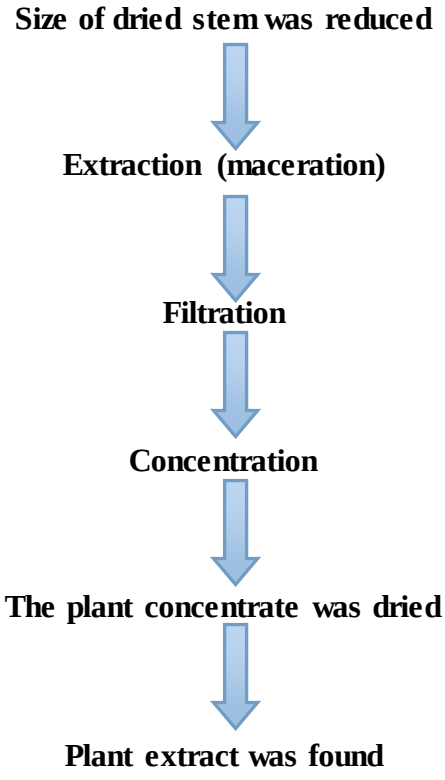
Plant extract was found

The same procedure was followed for the extraction of stem.

a. Preparation of the plant material (stem)



b. Extraction process (stem)



2.3 Size reduction and weighing

The clean, crispy leaves and stems were taken and grounded in a high capacity grinding machine separately to turn them in to coarse powder. The powder was then kept safely in a air tight plastic container which ensures no risk of contamination. Then two plastic container was labeled with necessary information for the leaves and stem and then they were left in a cool and dark place awaiting the necessity of additional research. Then total amount of the powder of leaves and stems were determined by weighing them and the amount was recorded.

2.4 Extraction

The maceration of extraction process was done by using methanol as organic solvent. The powdered leaves and stems were soaked in methanol in separate beaker for three days at normal room temperature (22-25 ˚C) and occasional agitation was performed.

Table 2.4.1: The weight of powdered leaves and stems along with the volume of methanol used for the maceration process:

	Leaf	**Stem**
Weight of plant powder (g)	74	79.26
Volume of methanol (mL)	500	900

Two layer phase was noticed as the outcome of the maceration process. The superior phase with the methanolic solution is alienated by filtration with a unclean deferral of plant parts and sediment was seen in the lowermost phase.

2.5 Filtration:

Using whatman filter the contents were first filtered before the contents of the two separate beaker was decanted after three days of maceration.

2.6 Concentration

After the filtration the filtrate were collected and using rotary evaporator (heidolph) at 100 rpm at 30 ºC, the collected filtrate was concentrated. After that, for the purpose of drying under LAF the mixture was transferred onto the petri-dishes. For stem, same procedure was followed and it was collected in another petri-dish.

2.7 Drying

Laminar Air Flow (LAF) was used to evaporate solvent to stay away from any chance of microbial augmentation at the time of drying the extract. Right following the concentration process the petri-dishes were positioned below Laminar Air flow so as to disperse the solvent from the extract. When the extract is successfully dried, the petri-dishes were safely enclosed with aluminum foil and then chilled them for more investigation.

2.8 Phytochemical screening of leaf and stem extract

With a view to access the qualitative chemical composition for instance, alkaloids, carbohydrates, tannin, flavonoids, glycosides etc. Phytochemical screening was done on the crude extort of *O. indicum*.

The following qualitative tests were performed:

2.8.1 Finding of alkaloid

Three tests were performed for the qualitative determination of alkaloids. For the test, methanolic leaf and stem extract of 0.5 g of *O. indicum* was dissolved in 5 mL of 1 % hydrochloric acid separately. After that it was boiled in a water bath and then filtration was done. The filtrate was used to perform the following tests.

Hager's test

A little drop of Hager's reagent (1% picric solution) was added to 2 mL of the filtrate. The presence of alkaloid would be confirmed by the formation of yellow precipitate. But in this leaf extract there was no yellow precipitation (Waldi, 1965).

Mayer's test

Mayer's reagent (10 mL) was arranged by dissolving 0.1358 g of mercuric (2) chloride and 0.5g of Potassium Iodide in 10 mL distilled water. For the detection of the presence of alkaloid in a 2 mL filtrate of the leaf extract, only some drops of mayer's reagent were given and there was no white or creamy precipitation which indicates the absence of alkaloid in the leaf extract. But in the stem extract precipitation was observed (Evans, 1997).

Wagner's test

By dissolving 0.2 g of iodide crystals and 0.6g of potassium iodide in10 mL distilled water, wagner's reagent (10 mL) were prepared.

The test is performed by adding a few drops of Wagner's reagent to the 2mL filtrate. Absence of brownish-black precipitation confirmed the absence of alkaloid in the leaf extract.

2.8.2 Detection of carbohydrates

0.5 g of methanolic extract of *O. indicum* was dissolved in 5mL of distilled water and the mixture was filtered. In this way carbohydrate is qualitatively detected. The following two tests were performed in the filtrate (Ramkrishnan, Prasannan & Rajan, 1994).

Molisch's test

To a 2 mL filtrate, 2 drops of molisch's reagent was added. After that 2 mL of concentrated sulfuric acid was pipetted along the sides of the test tube in the alcoholic solution of alpha napthol. The presence of carbohydrates was indicated by the formation of carbohydrates.

Fehling's test

In a 1:1 ratio one mL of each A and B fehling's solution were added to 2 mL of the filtrate. After that it was boiled for a few minutes. The presence of reducing sugar was indicated by the formation of brick-red precipitate in both leaf and stem extract.

2.8.3 Detection of flavanoids

Lead acetate test

A few drops of lead acetate were added to the methanolic leaf and stem extract. The yellow color precipitation signifies the presence of flavonoids.

2.8.4 Detection of phenols or phenolic compounds

Ferric chloride test

Soni and Sosa (2013), stated that 2 mL of extract is measured in a test tube and 3-4 drops of 15% (w\v) Ferric chloride solution was added in it. Presence of phenols is identified by the formation of bluish-black precipitate in both leaf and stem extract.

2.8.5 Detection of phytosterols

Libermannurchard's test

According to Soni and Sosa,(2013), 1 mL of chloroform was added to a small amount of leaf and stem extract. After that 2 mL of acetic anhydride was added to the filtrate. Then the filtrate was boiled and cooled. Thereafter, in the solution 1 mL of concentrated sulfuric acid was added. A brown ring at the connection is formed at the junction and presence of phytosterol is confirmed.

2.8.6 Finding of steroids

Salkwaski test

Ghani (2003), stated that 2 mL of chloroform and 1 mL of sulfuric acid were added to 1 mL of extract. The existence of steroid was confirmed by the appearance of red color. But in stem there was no red color. So, in stem it was absent.

2.8.7 Detection of tannins

Lead Acetate test

According to Tiwari and Bimlesh (2011), a few drops of 1% Lead acetate solution were added to 1 mL of the leaf and stem extract. The existence of tannin was established by the creation of yellow color precipitation

Potassium dichromate test

First of all 10 % potassium dichromate solution was arranged when 1 g of potassium dichromate was dissolved in 10 mL purified water. After that crude extract of leaf was converted into 5 ml aqueous solution and in 1 mL of 5 % ferric chloride solution, it was dissolved. The presence of tannin was indicated by the formation of yellow precipitation.

Ferric chloride test

0.5 g of ferric chloride was dissolved in 10 mL distilled water and thus 5% ferric chloride solution was prepared. For the test 5 ml of basic extract was dissolved in 1 mL of 5 % ferric chloride solution. After dissolving greenish black form of precipitation detects the existence of tannin.

2.8.8 Detection of resins:

Soni and Sosa (2013), stated that addition of 5-10 drops of acetic anhydride to 2 mL extract and followed by heating the solution gently helps in identifying the presence of resin. After that, 0.5 mL of sulfuric acid was added to the solution. There was no bright purple color formed. So, resin is absent in both stem and leaf.

2.8.9 Detection of glycosides

Mariaappansenthilkumar, (2013), stated that before subjecting to Borntrager's test the methanolic extract of *O. indicum* was hydrolyzed with dilute Hydrochloric acid.

Borntrager's test

According to kamalakar, Prabhakar &Shailaja (2014), 5 mL of 5 % Ferric (3) chloride and 5 m of dilute hydrochloric acid were added to the 5 mL of filtrate. The mixture was then heated in a boiling water-bath for 5 minutes which was followed by cooling the mixture. Thereafter, 5 mL of benzene was added to the mixture and it was shaken thoroughly. Thereafter, using a separating funnel the organic layer was separated. After that, a color of pinkish-red is formed and signifies the presence of glycosides.

2.8.10 Detection of saponin

Froth test

According to Kokate (1999), the extract was diluted with distilled water and 20mL of the volume was made. Then the contents of the /of the cylinder were shaken for about 15 minute in a graduated cylinder. A 2cm in height foam layer was observed which indicated the presence of saponins. In case of stem there was no foam layer. So, absence of saponin in stem extract.

2.9 In-vitro antioxidant activities

For the determination of anti-oxidant activities of a stem extract, there are various in-vitro methods. There are several methods, among them to determine the antioxidant activity in stem extract, 4 methods were selected. The methods are

1. DPPH (1,1-diphenyl-2-picryl hydrazyl)Free radical scavenging assay
2. Total phenolic content (TPC)
3. Total flavonoid content (TFC)
4. Total antioxidant capacity (TAC)

2.9.1 DPPH (1, 1-diphenyl-2-picryl hydrazyl) Free radical scavenging assay

According to Braca et al (2001), the DPPH free radical scavenging assay of the stem extract of *O. indicum* was determined by using this method.

Reagent and chemicals

Name of Reagent/Chemical Suppliers

1. DPPH Sigma Aldrich, U.S.A.
2. Methanol, Active Fine Chemicals Ltd., Bangladesh
3. L-ascorbic acid, Merck, German

Reagent preparation

2 mg DPPH was dissolved in 50 mL methanol and 0.004% (w/v) DPPH solution was prepared. The solution was then kept at -4°C in the refrigerator till before use.

Sample and standard preparation

The sample stock solution was prepared by dissolving 120 mg of stem extract in 10 mL of methanol to produce 12 mg/mL of concentration.

By serial dilution of the sample stock solution so that 6 serially diluted concentrations of the sample concentrations were prepared as 1200, 800, 400, 200, 100 and 50 µg/mL

Using L-ascorbic acid, the standard was prepared in the same manner. The extract was made by 6 serially diluted concentrations which ranges from 1200-50 µg/mL.

Preparation of blank solution

3 mL methanol was used to prepare the blank solution.

Experimental procedure

a) In test tubes 1 mL of each of the fractions of sample and standard (L-ascorbic acid) were taken.

b) 2mL of 0.004% (w/v) DPPH solution was added to each of the test tube.

c) After that, at room temperature for 30 minutes the test tubes were incubated. Then, the absorbance of the resulting solutions and control (DPPH and methanol was measured at 517nm against blank (methanol) using a spectrophotometer (U-2910 UV-Vis Spectrophotometer).

d) Thereafter, the percentage of free radical scavenging activity (%FRS) was calculated from equation given below :

$$\% \text{ Inhibition of free radical scavengers} = (A_0 - A_1) \times 100/A_0$$

Where, A_0 = The absorbance of the control

A_1 = The absorbance of the sample/standard

2.9.2 Determination of total phenolic content (TPC)

According to Wolfe, Wu and Liu (2003), the TPC of the stem extract of *O. indicum* was determined by using this method.

Reagents and chemicals

Name of Reagent and the chemical Suppliers

1. Folin-Ciocalteu Reagent (FCR),LOBAChemie Pvt. Ltd., India
2. Gallic acid monohydrate (Standard), Sigma Aldrich, USA
3. Sodium carbonate,MerckSpecialities Pvt. Ltd., Mumbai
4. Methanol, Active Fine Chemicals Ltd., Bangladesh

Reagent preparation

25 mL of FCR was taken in a 250 mL volumetric flask and then it was diluted with distilled water to 250 mL mark. In this way 250 mL of 10 % FCR solution was prepared.

7.5 g of Sodium Carbonate was taken in a 100 mL volumetric flask and diluted with distilled water to 100 mL mark. This makes 100 mL of 7.5 % (w/v) Sodium Carbonate.

Sample and standard preparation

The sample stock solution was made by dissolving 120 mg of stem extract in 10 mL of methanol to produce 12 mg/mL of concentration.

By serial dilution of the sample stock solution so that 4 serially diluted concentrations of the sample concentrations were prepared as 1200, 800, 400 and 200 µg/mL.

Using Gallic acid, the standard was prepared in the same manner. The extract was made by 4 serially diluted concentrations which ranges from 1200 to 200 µg/mL.

Preparation of the blank

The solution of blank was prepared by 5 mL FCR solution and 4 mL Sodium Carbonate and 1 mL methanol to make the volume up to 10 mL.

Experimental procedure

a) In test tubes 1 mL of each of the fractions of sample and standard (gallic acid) were taken.
b) 5mL of FCR solution was added in each test tube.
c) Following that 4ml sodium carbonate was added.
d) Each of the mixture was vortexed in a vortex machine for 15s and followed by stand for 30 min at 40 °C in a water bath.
e) After that, the standard and sample absorbance was measured against blank measured at 765 nm using spectrophotometer(U-2910 UV-vis spectrophotometer).
f) The total phenolic content, C, for each of the fractions were expressed as Gallic
g) Acid Equivalents using the following equation:

$$C = (c \times V)/m$$

Where, C = Total content of phenolic compounds, milligram of gallic acid per gram of dried plant extract, expressed as gallic acid equivalent (GAE)

c = concentration of gallic acid obtained from calibration curve (mg/mL)

V = Volume of sample solution (ml)

m = weight of the sample (g)

2.9.3 Determination of total flavonoid content (TFC)

According to Kumaran and Karunakaran (2007), the TFC of the stem extract of *O. indicum* was determined by using this method.

Reagents and Chemical

Name of Reagent/ Chemical Suppliers

1. Quercetin (Standard) Sigma Aldrich Co., Germany
2. Methanol Active Fine Chemicals Ltd., Bangladesh
3. Potassium Acetate, Merck KGaA, Germany
4. Aluminium Chloride, Merck Specialities Pvt. Ltd., Mumbai

Reagent Preparation

By taking 10 g of aluminium chloride in a 100 mL volumetric flask and diluting it to 100 mL mark with distilled water, 100 mL 10 % aluminium chloride solution was prepared.

By taking 9.815 g of potassium acetate in a 100 mL volumetric flask and diluting it to 100 mL mark with distilled water, 100 mL of 1 M potassium acetate solution was prepared.

Sample and standard preparation

The sample stock solution was made by dissolving 120 mg of stem extract in 10 mL of methanol to produce 12 mg/mL of concentration.

By serial dilution of the sample stock solution so that 4 serially diluted concentrations of the sample concentrations were prepared as 1200, 800, 400, and 200 µg/mL .

Using quercetin, the standard was prepared in the same manner. The extract was made by 4 serially diluted concentrations which ranges from 1200 to 200 µg/mL.

Preparation of the blank

The blank solution was prepared by 200 µL of 10 % aluminium chloride solution, 200 µL of 1 M potassium acetate solution, 5.6 mL distilled water and 4 mL methanol to make the volume up to 10 mL.

Experimental procedure

a) In test tubes 1 mL of each of the fractions of sample and standard (quercetin) were taken.

b) 3 mL of methanol was added in each test tube.

c) Using 1000 µL micropipette, 200 µL of 10 % aluminium chloride solution and 200 µL of 1M Potassium acetate solution was added to each test tube.

d) After that, to make the final volume up to 10mL, 5.6mL of distilled water was added to the test tubes.

e) At room temperature for 30 min, the test tubes were incubated.

f) Finally, the standard and sample absorbance was measured against blank measured at 415 nm using spectrophotometer (U-2910 UV-vis spectrophotometer).

g) The total flavonoid content, C, for each of the fractions were expressed as quercetin Equivalents using the following equation:

$$C = (c \times V)/m$$

Where, C = Total content of flavonoid compounds, milligram of quercetin per gram of dried plant extract, expressed as quercetin equivalent (QE)

c = concentration of quercetin obtained from calibration curve (mg/mL)

V = Volume of sample solution (mL)

m = weight of the sample (g)

2.9.4 Determination of total antioxidant capacity (TAC)

According to Prieto, Pineda and Aguilar (1999), the TFC of the stem extract of *O. indicum* was determined by using this method.

Reagents and Chemicals

Name of Reagent/ Chemical Suppliers

1. Ammonium Molybdate, Active Fine Chemicals Ltd., Bangladesh
2. L-Ascorbic Acid (Standard), Merck, Germany
3. Sodium Triphosphate ($Na_3PO_4.12H_2O$), MerckKGaA, Germany
4. Concentrated sulfuric acid (98%) Merck, Germany
5. Methanol Active Fine Chemicals Ltd., Bangladesh

Reagent Preparation

By taking 3.28 mL of 98 % concentrated sulfuric acid in a 100 mL volumetric flask and diluting it to 100 mL mark with distilled water, 100 mL of 0.6 M sulfuric acid solution was prepared.

By taking 4.494 g of potassium acetate in a 100 mL volumetric flask and diluting it to 100 mL mark with distilled water, 100 mL of 0.004 M ammonium molybdate solution was prepared.

By taking 1.0645 g of aluminium chloride in a 100mL volumetric flask and diluting it to 100 mL mark with distilled water, 100 mL of 0.028 M sodium phosphate solution was prepared.

Sample and standard preparation

The sample stock solution was made by dissolving 120 mg of stem extract in 10mL of methanol to produce 12 mg/mL of concentration.

By serial dilution of the sample stock solution so that 7 serially diluted concentrations of the sample concentrations were prepared as 1200, 800, 400, and 200 µg/mL .

Using ascorbic acid, the standard was prepared in the same manner. The extract was made by 7 serially diluted concentrations which ranges from 1200 to 200 µg/mL.

Preparation of the blank

For preparing 3 mL of reagent solution of the blank solution and 300 µl of methanol were used.

Experimental procedure

a) In test tubes 300 µL of each of the fractions of sample and standard (L-ascorbic acid) were taken.

b) Then, 3 mL of Reagent solution (0.6 M sulfuric acid, 0.028 M Sodium phosphate and0.004 M ammonium molybdate) was added into the test tubes.

c) For 90 min in a water bath at 95 °C the test tubes (sample, standard, blank) were incubated.

d) Finally, the standard and sample absorbance was calculated against blank measured at 695 nm using spectrophotometer (U-2910 UV-vis spectrophotometer).

e) The total antioxidant capacity, A, for each of the fractions were expressed as ascorbic acid Equivalents (AAE) using the equation as follows:

$$A = (c \times V)/m$$

Where, A = Total antioxidant capacity, milligram of ascorbic acid per gram of dried plant extract, expressed as ascorbic acid equivalent (AAE)

c = concentration of ascorbic acid obtained from calibration curve (mg/mL)

V = Volume of sample solution (mL)

m = weight of the sample (g)

2.10 Cytotoxicity activity study by MTT assay

Methodology

MOLT-4 (human acute lymphoblastic leukemia, T cell)

Description: MOLT - 4 cell line (human: T lymphoblast; acute lymphoblastic leukemia)

Background: Human MOLT-4 cells were cultured in RPMI 1640 medium with 2 mM L-glutamine and harvested at the log phase of growth. The MOLT-4 cells were fixed in 4% paraformaldehyde and arrayed on a 12-well (5 mm) adhesive coated slide, with each well is surface specifically treated to enhance cellular attachment and to minimize background staining. Each well was loaded with approximately 7.5 x 103 MOLT-4 cells. A follow-up heat dehydration process was done to ensure the attachment of MOLT-4 cells and the stability of cellular proteins.

Tissue Species: Human

Cell Lines: Molt4

Applications: ICC/IF

Application Note: MOLT-4 cell slide is provided for immunocytochemical analysis of protein and protein-protein interaction. Procedures for immunohistochemistry can be directly applied.

Storage instruction: MOLT-4 cell slide can be stored for up to 6 months at 4°C under desiccate conditions.

Notes: For *in-vitro* laboratory use only. Not for any clinical, therapeutic, or diagnostic use in humans or animals. Not for animal or human consumption

Different solutions preparation

Solution of 1% penicillin-streptomycin

Solution of penicillin streptomycin which is usually acknowledged as pen-strep and used in MTT assay to have power over the bacterial infectivity and preserve the hygienic condition during the method. The solution is a combination of 10000 component of penicillin for every mL and 10 mg of streptomycin for each mL.

10 % fetal bovine serum

To prepare 10 % FBS, 50 ml Fetal Bovine Serum was added to the 500 mL of DMEM

Trypsin

In the medium 0.25% trypsin was used.

2% DMSO solution

By adding 60 µl in 2940 ml of distilled water,2% DMSO solution was madefor negative control.

Celltiter 96 assay kit

Celltiter 96 analyze kit is an growth of competent reagents that provide a fast also advantageous method with resolve of the quantity of propagation and cytotoxicity (Ifere et al., 2010). It is a vision of alter of a cell separation with tetrazolium salt beneath a formazan invention that is unquestionably notable utilizing a 96-well platter. A blender of stain solution was put in 96 well plate to produce cells to carry out this assay.

Analysis report

Nature of samples: plant extract

Study design: Cytotoxic effect analysis after 48h

Used instruments: Biological Bio Safety Cabinet (Model: NU-400E, Nuaire, USA), CO_2 Incubator (Nuaire, USA), Trinocular microscope with camera 9Optika, Italy), Hemocytomter

Used consumables: 96-well plate, 15-mL tubes, Tips, Gloves, Cultureflask, Cell cuturemedia, Antibiotics (P+S), Gentamycin, Serological pipette, Trypsin etc.

Procedure

Cytotoxicity effect was checked in Centre for Advanced Research in Sciences by means of their viable services. In short, Molt-4, a human lymphoblastic leukemia cell line, was kept in RPMI having 1% penicillin-streptomycin(1:1) and 0.2% gentamycin and 10% fetal bovine Serum (FBS). Cells (2.5×10^4/100 µl) were sowed onto 96-well plate and hatched at 37°C+5% CO_2. Next day, 25µl of sample (filtered) was added each well. Cytotoxicity was studied after 48h of incubation applying CellTiter 96 Non-Radioactive Cell Propagation Assay Kit (Promega USA). Replacement wells were utilized for every sample.

Preparation of the different concentrations of plant extract

The assay was executed by utilizing 4 concentration 25 mg/mL, 2.5 mg/mL, 0.25 mg/mL and 0.025 mg/mL of the *O. indicum* leaves extract. 25 mg/mL concentration was ready by put in 25 mg of leaves extract in 1 mL DMSO and it was the store solution. 2.5 mg/mL concentration was completed by diluting 25 mg/mL solution 10 times by DMSO. 10µL of sample 1 is put in to the 90 µL of DMSO to make 2.5 mg/mL concentration. Like this 0.25 mg/mL and 0.025 mg/mL concentrations were completed by sequential dilution with. Later the samples were sieved through 0.45µm syringe filter preceding to test.

% of cytotoxic activity=

100-Absorbance of test sample/Absorbance of negative control ×100%

Chapter 3
Results and calculation

Chapter 3: Results and Calculation

The formula mentioned in the methodology was used for the calculation and determination of the results.

Table 3.1: Phytochemical screening of methanolic extract of *Oroxylum indicum*

The phytochemical screening of both leaf and stem extract of *Oroxylum indicum* are done separately.

Class of compounds	Leaf	Stem
Alkaloids	---	+++
Flavonoids	+	+
Phenols/phenolic	+	+
compounds	+	+
Glycosides	+++	+++
Tannins	++	++
Carbohydrates	+	+
Phytosterols	-	-
Resins	+	-
Steroids	+	-
Saponins		

(+) means presence in a single method test, (++) means presence experimented in two methods, (+++) means presence experimented in three methods, and (-) means absence

3.2 DPPH (1,1-diphenyl-2-picryl hydrazyl) free radical scavenging assay

Table 3.2.1: DPPH free radical scavenging assay (Absorbance vs. concentration) (Standard L- ascorbic acid)

Standard (L-ascorbic acid) concentration(µg/ml)	Absorbance	% of inhibition
50	0.006	94.95 %
100	0.005	95.79%
200	0.004	96.64%
400	0.003	97.48%
800	0.002	98.31%
1200	0.001	99.16%

3.2.1 Graphical presentation of DPPH % of inhibition vs. concentration

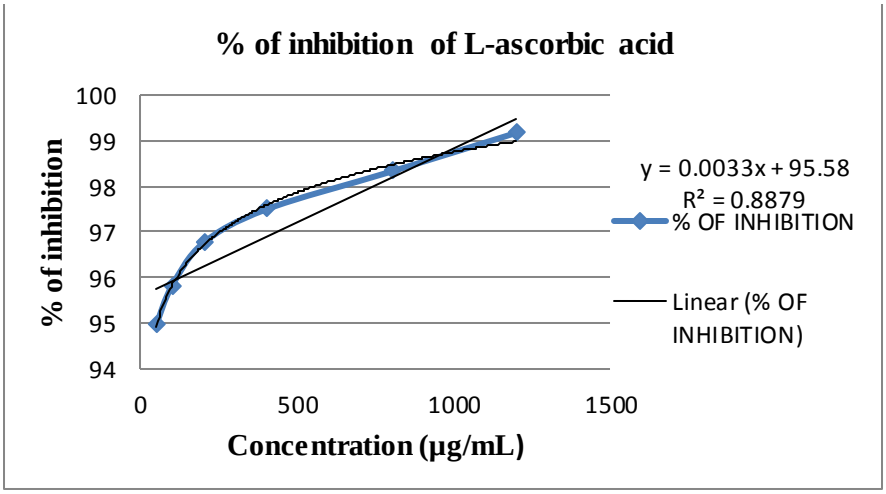


Figure 3.2.1: Graphical presentation of DPPH % of inhibition vs. concentration (µg/mL) for and ascorbic acid (AA).

Table 3.2.2: DPPH free radical scavenging assay of *O. indicum* (Absorbance vs. concentration)

Sample concentration of *O. indicum* (µg/ml)	Absorbance	% of inhibition
50	0.018	84.87%
100	0.017	85.71%
200	0.016	86.55%
400	0.014	88.24%
800	0.013	89.08%
1200	0.012	89.92%

3.2.2 Graphical presentation of DPPH % of inhibition vs. concentration (sample *O. indicum)*

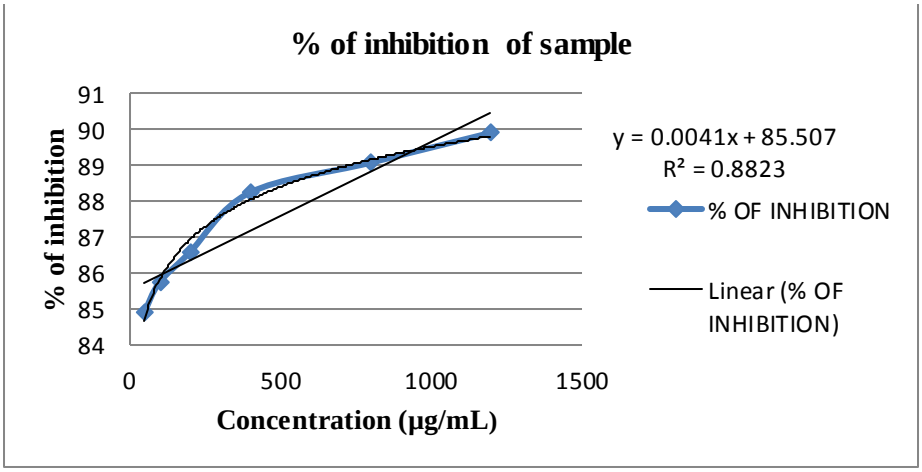


Figure 3.2.2: Graphical presentation of DPPH % of inhibition vs. concentration (µg/mL) for sample

Interpretation: From the table and figure, it was observed that with an increase in concentration of *O. indicum* from 50 to 1200 µg/mL, the absorbance decreased slowly from (0.018 to 0.012) in comparison to that of ascorbic acid (0.006-0.001). Therefore, with an decrease of absorbance the % free radical of inhibition increases. The highest % of inhibition was shown at the highest concentration of 1200 µg/mL. At the lowest concentration of 50 µg/mL, the extract showed notable % of inhibition which was 84.87 %.

3.3 Determination of total phenolic content (TPC)

3.3.1 Calibration curve of gallic acid

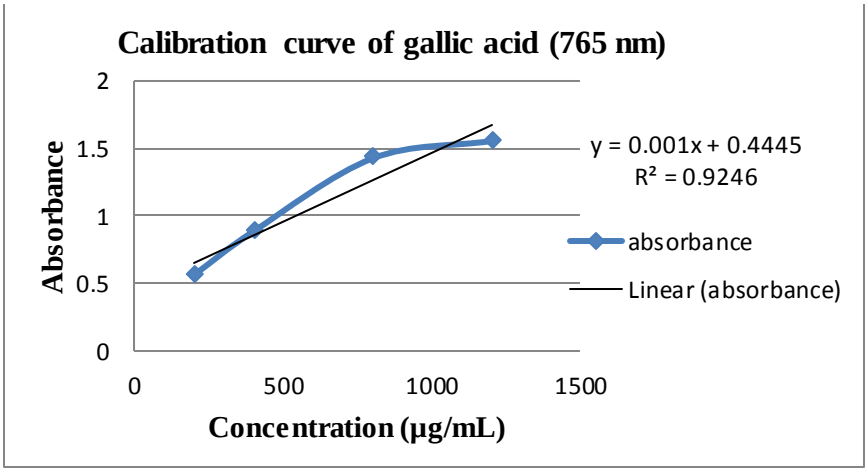


Figure 3.3.1: Calibration curve of gallic acid (GA) at 765 nm for determining TPC in *O. indicum*

Table 3.3.1: Total phenolic content (TPC) in *O. indicum*

Concentration of *O. indicum* (µg/mL)	**TPC (GAE) mg of gallic acid per gram of dried extract**
200	41.67
400	64.08
800	90.92
1200	98.75

Interpretation: It is observed from the figure (4.2.1) & table (4.2.1) that because the concentration of *O. indicum* was raised from 200 to 1200 µg/mL, the total phenolic substance also raised from 41.67 to 98.75 mg of gallic acid per gram of dried extract. Therefore, it indicates that with increases in total phenolic content, its antioxidant activity also increases. At the lowest concentration extract showed 41.67 mg of gallic acid per gram of dried extract. With the highest concentration extract showed 98.075 mg of gallic acid per gram of dried extract.

3.4 Determination of total flavonoid content (TFC)

3.4.1 Calibration curve of quercetin

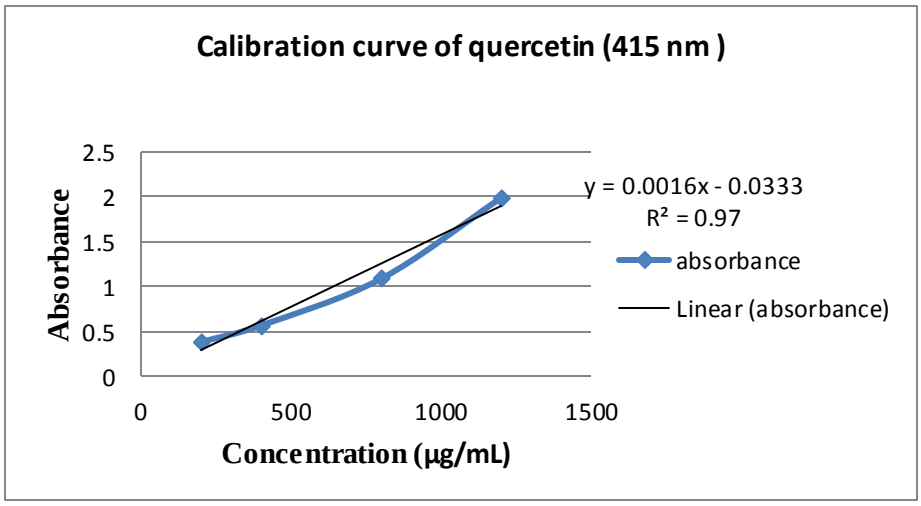


Figure 3.4.1: Calibration curve of quercetin at 415 nm for determining TFC in *O. indicum*

Table 3.4.1: Total flavonoid content (TFC) in *O. indicum*.

Concentration of *O. indicum* (µg/mL)	**TFC(QE) mg of quercetin per gram of dried extract**
200	24.67
400	27.5
800	35.83
1200	41.17

Interpretation: It is detected that as the concentration of *O. indicum* was raised from 200 to 1200 µg/mL, the whole flavonoid content also raised from 24.67 to 41.17mg of quercetin per gram of dried extract. Therefore, it indicates that there is as well, a positive correlation between total flavonoid content of *O. indicum* and its antioxidant activity. With the highest concentration extract showed only 41.17 mg of quercetin per gram of dried extract.

3.5 Determination of total antioxidant capacity (TAC)

3.5.1 Calibration curve of ascorbic acid

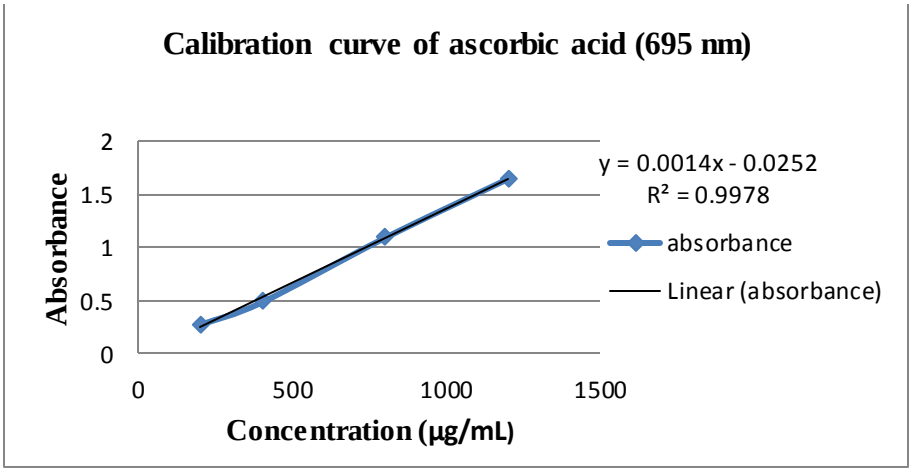


Figure 3.5.1: Calibration curve of ascorbic acid (AA) at 695 nm for determining TAC in *O. indicum*

Table 3.5.1: Total antioxidant capacity (TAC) in *O. indicum*

Concentration of *O. indicum* (µg/ml)	**TAC (AAE)mg of ascorbic acid per gram of dried extract**
200	67.25
400	68.17
800	68.33
1200	68.67

Interpretation: It is observed that as the concentration of *O. indicum* was increased from 200 µg/mL to 1200 µg/mL, the total antioxidant capacity also raised from 67.25 to 68.67mg of ascorbic acid per gram of dried extract. This indicates therefore, that a positive correlation exists between total antioxidant capacity of *O. indicum* and its antioxidant activity. With the highest concentration extract showed only 68.67 mg of ascorbic acid per gram of dried extract.

3.6 Result of cytotoxic activity shown by *O. indicum* leaf extract

At diverse concentration cell viability of methanolic extract of *O. indicum* and 2% DMSO as a control after incubating 48 hours.

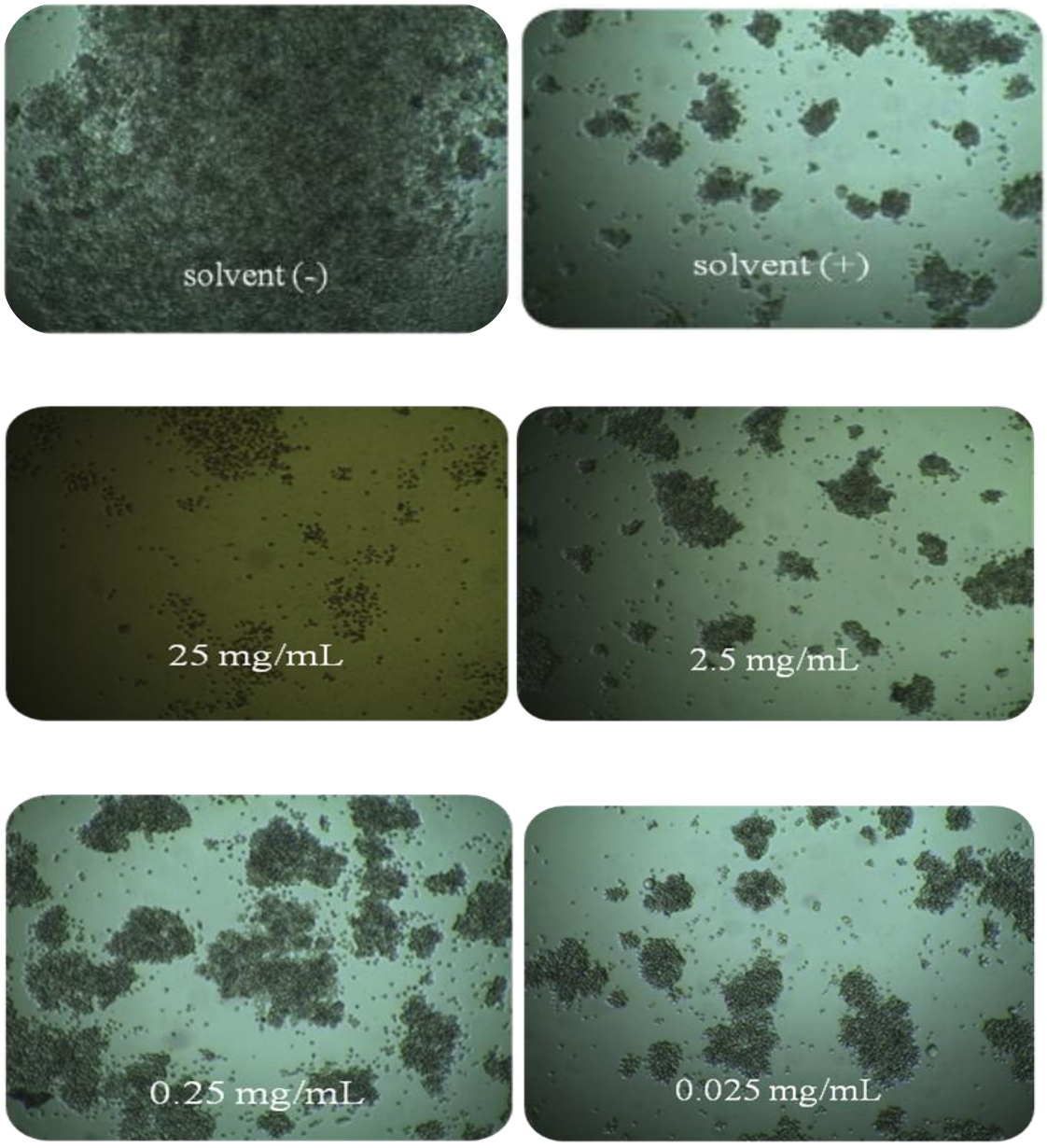


Figure 3.6.1: At diverse concentration cell viability of methanolic leaf extract of *O. indicum* and 2% DMSO as a control after incubating 48 hours.

Table 3.6.1: Cytotoxicity analysis result

Sample ID	Absorbance @ 570nm		Remarks
Solvent (-)	1.494	1.617	Sample color has interfered the reading of absorbance therefore result did not match with microscopic observation.
Solvent (+)	1.251	1.122	
25	3.072	3.829	
Background	3.530	3.633	
2.5	1.744	1.920	
Background	1.427	1.427	
0.25	1.784	1.547	
Background	0.375	0.375	
0.025	1.299	1.394	
Background	0.175	0.175	

Background: only sample

Solvent (-): only cell

Solvent (+): solvent (DMSO)

25 mg/mL: sample+ cell

2.5 mg/mL: sample+ cell

0.25 mg/mL: sample+ cell

0.025 mg/mL: sample+ cell

Table 3.6.2: % of cell survival

Sample Conc. (mg/mL)	% of survival of cells	% of inhibition of cells
0.025	95	5
0.25	95	5
2.5	95	5
25	10	90
Control 2% DMSO	95	5

Interpretation: MOLT4 cell was treated by 0.025 mg/mL, 0.25 mg/mL, 2.5 mg/mL, and 25 mg/mL concentration of *O. indicum* leaf extract in the medium and it was hatched for 48 hours. When the cells studied below a microscope it was monitored that survival of MOLT4 cell was 95 % that means very few cell death was happened for 0.025 mg/mL, 0.25 mg/mL and 2.5 mg/mL concentrations. On the other hand, at the highest concentration 25 mg/ml of *O. indicum* leaf extract survival of MOLT4 cell was 10% that means 90% of cell death was observed in this concentration after 48 hours of incubation.

Chapter 4
Discussion

Chapter 4: Discussion

The project began with the selection of the plant by literature review and online search. Based on that *O. indicum* was chosen for this study. For the determination of antioxidant potential methanolic stem extract was chosen as previous study was done on leaf extract. Cytotoxic activity was never done before on MOLT-4 cell with leaf extract which was the reason behind selecting MOLT-4 as the cell line. Phytochemical screening of methanolic extract of leaves and stem extract of *O. indicum* showed the presence of carbohydrates, flavonoids, tannins, saponins, steroids, alkaloids. These compounds have various significant activities against many diseases. For example, alkaloid has addictive or pain killing or toxic impact and here and there help in imperative cure. Saponins may anticipate colon malignancy. Flavonoids have antiallergic, antiviral, anti-inflammatory and cancer prevention activities. Steroids are utilized to suppress different hypersensitive, provocative and immune system issue Literature data revealed that it has anti-inflammatory, antiulcer, antimutagenic, antiproliferative and some other activities.

In this study, qualitative study determined the existence of flavonoids as well as polyphenolic composites of dried extract of *O. indicum*, then various *in-vitro* antioxidant assays were performed on the extract. The antioxidant potential of this plant had been studied by using four different tests on its stem extract.

From the obtained result of DPPH test, it was observed that with an increase in concentration of *O. indicum* from 50 to 1200 µg/mL, the absorbance decreased slowly from (0.018 to 0.012) in comparison to that of ascorbic acid (0.006-0.001).Therefore, with an decrease of absorbance the % of inhibition increases. At the lowest concentration 50 µg/mL the extract showed highest inhibition 84.87 % which signified that it has antioxidant potential. Other concentrations also showed notable % of inhibition and by comparing them with the standard it was observed that the sample showed notable inhibition which was close to standard.

From the figure 4.2.1 and table 4.2.1 it was observed that with the increase of concentration from 200 to 1200 µg/mL, the total phenolic content also raised from 41.67 to 98.75 mg of gallic acid per gram of dried extract.

Also, from figure 4.3.1 and table 4.3.1 it was observed that from concentration 200 µg/mL to 1200 µg/mL, the total flavonoid content raised from 24.67 to 41.17 mg of quercetin per gram of dried extract. This indicates that a positive correlation exists between total antioxidant capacity of *O. indicum* and its antioxidant activity.

Again from figure 4.4.1 and table 4.4.1 it was noticed that since the concentration of *O. indicum* was increased from 200 to 1200 µg/mL, the total antioxidant capacity also increased from 67.25 to 68.67 mg of ascorbic acid per gram of dried extract. Therefore, it indicates that there is as well, a positive correlation between total antioxidant content of *O. indicum* and its antioxidant activity. At the lowest concentration 200 µg/mL it showed notable result 67.25 mg of ascorbic acid per gram of dried extract.

Therefore, the higher DPPH, TPC and TAC of *O. indicum* can be attributed to the fact that it possesses higher antioxidant potential (Keen et al., 2005).

Moreover, cytotoxic activity is studied on MOLT-4 (human acute lymphoblastic leukemia, T cell) which has not been done previously. Though cytotoxic study has been done on other cell line such as Hela cell line but there is no cytotoxic study on MOLT4 was performed on *O. indicum* before. In this study, *in-vitro* cytotoxic activity on cancer cell lines were examined to determine the cell viability where different concentrations of leaves extracts were used. As positive control 2% DMSO was used and after incubating the medium for 48 hours viable cells was determined using micro plate reader. Analyzing the result it is showed that at low concentration there was no cell death however at high concentration remarkable amount of death of cell has been observed. At higher concentration 25 mg/mL it showed 90 % of cell death where the cell survival is only 10 %. So, cytotoxic activity was not that much significant as antioxidant activity.

This study finding is the scientific proof of the presence of medicinal value in *O. indicum*.

Chapter 5

Conclusion

Chapter 5: Conclusion

O. indicum is a profoundly used in the treatment of cholera, jaundice dyspepsia, leucoderma, cough. Crude extracts from different parts of *O. indicum* have been allocated for different therapeutic applications. Four different examinations were done uncommonly to decide the *in vitro* antioxidant activity on leaf extract and cytotoxic activity of stem extract of *O. indicum* through MTT assay. To evaluate the free radical scavenging capacity DPPH test was performed which showed that at the lowest concentration 50 µg/mL the extract showed highest inhibition 84.87 % which signified that it has antioxidant potential. Total phenolic content showed that at the highest concentration extract showed 98.075 mg of gallic acid per gram of dried extract. The total antioxidant content showed that at the lowest concentration 200 µg/mL it showed notable result 67.25 mg of ascorbic acid per gram of dried extract.

To conclude, after performing the *in-vitro* cytotoxic test on MOLT4 cell it is observed that at higher concentration 25 mg/mL it showed 90 % of cell death where the cell survival is only 10 % which is not that much significant to be used as an anticancer agent. On the other hand, it is established that *O. indicum* has antioxidant potential after performing four individual tests.

Future direction

In-vivo pharmacological evaluation of antioxidant potential should be done of *O. indicum* as it has antioxidant potential. Moreover other compound isolation can be done in future and other bioactivities can be investigated in future of this plant which has not done previously.

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