CHAPTER 9

DISCUSSION

Herbal drugs are probably the most common source of samples for evaluation in high-throughput screens of natural products. They yielded many useful compounds and ingredients derived from plant, which are important components of modern phytopharmaceuticals. Certain types of these substances not only help the body in providing sources of energy but also assist in the plastic repairs as well as interfere the complicated chemical reactions of life processes. In many developing countries therapeutic lists commonly contain a number of plant-based drugs, because modern synthetic drugs are beyond the reach of the population in rural areas and heavy financial burden of expensive synthetic drugs.

Phytotherapy provides a complimentary form of medicinal agents and an alternative to modern orthodox medicine. When medicinal plant products are subjected to the rigorous application of science, the experiments reveals the usefulness and clarifies the pharmacodynamic basis for the use of such plants in medical practice. These re-evaluations would provide a rationale for the use of those plants [1].

The herbal drugs can be continually used in chemotherapeutics as (i) the plants drugs provide a desirable therapeutic effect with reduced risk of iatrogerrous diseases associated with allopathic medicine; (ii) the infusion dosages of herbal preparation have advantages of complete bioavailability over conventional dosage forms; (iii) the enhanced solubility and dispersion of active molecules at gastro-intestinal absorption sites reduced the problems encountered in the pharmacodynamic phase of drug therapy; (iv) herbal drugs are most suitable for the diseases require long-term medication such as immunosupression, arthritis, hepato-biliary deficiency, spleen diseases, etc, because most plant extracts are used in repeated low doses; (v) compared to the synthetic compounds herbal drugs have a reduced chance of acute toxicity and secondary drug effects; and (vi) the multiple component feature of phytotherapeutic agents is considered to be a positive attribute as biodynamic agents since the same extract often include compounds that have synergistic activity [2].

It is therefore important to study the phytotherapeutic agents as they play vital roles in clinical medicine. The through study of medicinal plant is very much important because some of the adverse effect occasionally observed with herbal drugs. Hence it is of immense clinical importance to determine the acute and chronic toxicity of therapeutically useful plant products and to know whether they exhibit the expected pharmacological action. This approach to the discovery and development of plant products as medicines holds a lot of promise for people of developing countries. The increase in the acceptance of the 'holistic' philosophy of therapy by medical profession, which the traditional systems exemplify, is a further indication that there will be continually growing demand for drugs based on traditional medicine derived from natural products in future too [3].

We have studied the phytochemicals, their toxicities and pharmacological activities of two well-known plant used in traditional medicines *Colebrookea oppositifolia* Smith of the family Labiatae and *Heracleum nepalense* D.Don. DC of the family Umbelliferae from Sikkim Himalayan region. Both of these plants were extensively used by the Sikkimese for their primary health care such as skin infections, digestive, stimulant, aphrodisiac as described in the literature review. The present study on the plants was undertaken, for the first time, with the following aims (i) to evaluate the bioactivity of *Colebrookea oppositifolia* and *Heracleum nepalense*, both *in vitro* and *in vivo*, for justification of their use as ethnomedicine (ii) to isolate and identify the bioactive principle(s) in pure form. Standard methods were followed for the collection and processing of the plants and their useful parts. Authentication of the plants was made with the help of qualified scientists from the BSI, Gangtok branch, Sikkim, India. The extractions of the plant parts and prescreening of the extracts were done by standard protocols and the universally accepts methodologies, as described in Materials and Methods.

The phytochemical studies of the plants were covered in chapter 3. The extractions of the plant parts were made at room temperature using water and methanol as solvent and the collected extracts were concentrated under reduced pressure. The extracts were then

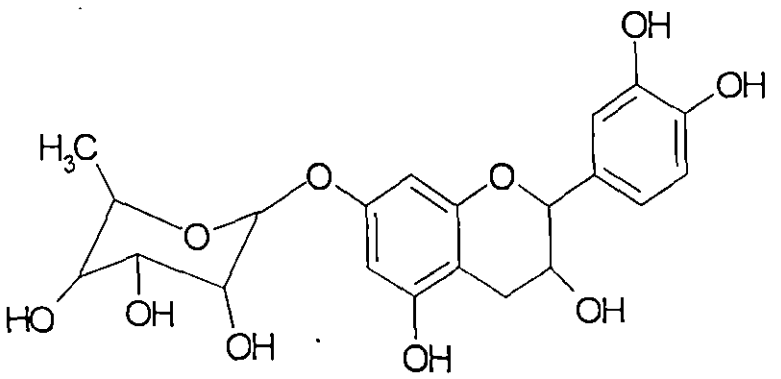
concentrated, suspended in hot distilled water, cooled and the blast precipitate was filtered off. The water-soluble component was fractionated by extracting it successively with petroleum ether, ethyl acetate and acetone. The ethyl acetate soluble fraction was subjected to chromatographic analysis [4,5].

Using multistep coloumn chromatography technique with various developing phases compound I and compound II were isolated from ethyl acetate fractions of *C.oppositifolia* leaf and *H.nepalense* root respectively. Their structures were determined by study of acid hydrolysis products with the help of spectral analysis of their UV, ^{1}HNMR, ^{13}C NMR and MS spectra.

The crystalline material isolated from the leaf of *C.oppositifolia* yielded a flavonoid glycoside (compound I). The UV absorption spectrum confirmed that compound I was a 7-substituted derivative with the presence of phenolic aromatic rings. The IR spectrum confirmed the presence of hydroxyl group and aromatic ring in the compound. Acid hydrolysis of the compound gave rhamnose and is identified by sugar tests [7]. Existence and identification of the sugar moiety is further confirmed by the ^{1}H NMR study. ^{1}H and ^{13}C NMR spectrum confirmed that compound I was identical with + (–) Catechin-7-O-β-rhamnopyranoside [7-11]. In the mass spectrum the presence of fragmentation ion at m/z 291 inferred that the compound should have + (–) Catechin as an aglycone. The structure of the compound was further confirmed by elemental analysis of the available literature of catechin [9, 11]. All the recorded spectral and elemental analysis and evidence discussed in **Chapter 3** (3.3.1) conclusively prove the identity of the isolated compound as + (–) Catechin-7-O-β-rhamnopyranoside. This confirmed that catechin is one of the major bioconstituent of the plant.

Catechin (5, 7, 3′, 4′ - tetrahydroxy – flavan – 3ols) is a polyphenol compound, generally found in green tea, catechu and rarely in other bark and leaf of plant kingdom. The traditional uses of plants containing catechin in folk medicine are multiple. Contemporary research also revealed that catechin isolated from different herbal sources and green teas possess diverse *in vivo* and *in vitro* pharmacological actions like anticancerous [9],

antioxidant, anti-inflammatory and antimicrobial properties [12, 13, 14]. The catechin polyphenols have also been shown to markedly inhibit digestive lipase *in vitro*, resulting in decreased lipolysis of triglycerides, which may translate to reduce fat digestion in humans [15].

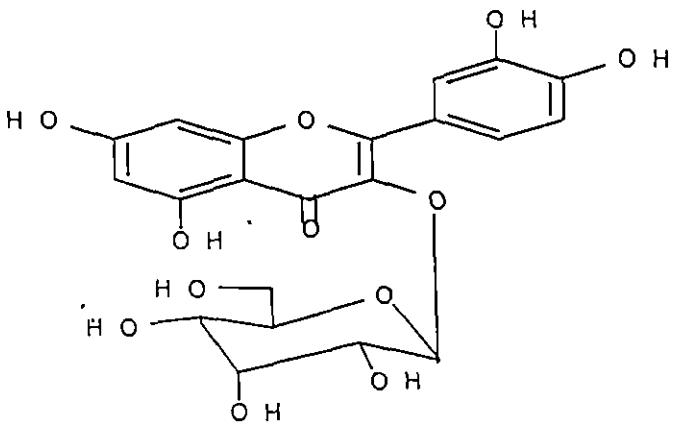


(+) – Catechin-7-O-β-rhamnopyranoside (Compound I).

The crystalline material isolated as compound II from the root of *H.nepalense* demonstrated the positive tests for flavonoid. The chemical nature was characterized by comparison of its physical parameters and UV, IR, ^{1}H NMR, ^{13}C NMR and MS spectral data with that of flavonoid glycoside [16-22]. The complete acid hydrolysis of compound II gave glucose as sugar moiety and is confirmed by sugar tests. The UV analysis proved the substitution of hydroxyl group in the compound by the bathochromic shift of band I in presence of aluminium chloride and hydrochloric acid. Therefore it was indicated that the compound was monodesmoside [23]. All the experimental evidences discussed in **Chapter 3** (3.3.2) suggest that the compound II isolated as quercetin-3-O-β-D-glucopyranoside. The isolation is a mark significance since no flavonoid has previously been isolated from the plant under study.

Quercetin (3, 3', 4', 5, 7-pentahydroxy flavone) is widely distributed in the plant kingdom and is the most abundant in the flavonoid molecules. It is found in many consumed foods, including apple, onion, tea, berries and brassica vegetables as well as many seeds, nuts,

flowers, roots, barks and leaves. It is reported for having many beneficial effects on human including cardiovascular protection, anticancer activity, antiulcer effects, anti-allergy activity, catharact prevention, antimicrobial activity, antioxidant activity and anti-inflammatory effects [24, 25]. It is reported to inhibit metabolic enzyme systems and P-glycoprotein [26]. Recent reports indicate that quercetin and its glycoside stimulate human peripheral blood leukocyte proliferation and significantly increase the helper T cells [27].



Quercetin-3-O-β-D-glucopyranoside (Compound II).

Determination of acute toxicity is the primary step in toxicological investigation of unknown substances which has been included in chapter 4 of the present studies. Fifty percent lethal dose of LD_{50} (acute oral/ i.p toxicity) is performed in Swiss albino mice following standard protocol [6]. The median lethal dose (MLD) of the methanol extract of *Colebrookea oppositifolia* leaf was found to be 3.0 g/kg body weight and 4.5 g/kg body weight in intraperitoneal and oral route respectively. While the MLD of the methanol extract of *Heracleum nepalense* roots was to be 4.5 g/kg body weight and more than 5.5 g/kg body weight in intraperitoneal and oral route respectively. Therefore, the doses of 600 mg/kg and 100 mg/kg p.o. were fixed as the highest and least doses to carry out all pharmacological experiments. These selected doses are well tolerated in the system tested and no untoward effect was observed with the methanol extract of either *C.oppositifolia* leaf or *H.nepalense* root.

We have studied several pharmacological activities of the methanol extracts and isolated compounds of *Colebrookea oppositifolia* leaf and *Heracleum nepalense* root on the basis of their ethnomedicinal uses. The bioactivities studied included enhancement of bioavailability of some antibiotics, immunostimulant, antioxidant and antimicrobial activities, which have been covered in chapter 5 to chapter 8.

Improvement of bioavailability of a large number of poorly biologically available, long-term treatments, toxic and expensive drugs may be proved as having greater clinical value and thus the problems with their therapy can be overcome. In the first part of the present research work (chapter 5) attempt has been made to study the effect of *C.oppositifolia* leaf and *H.nepalense* root with their isolated compounds on bioavailability and pharmacokinetics of some widely used antibiotics in rabbits. The present experimental findings clearly demonstrated that methanol extract of *C.oppositifolia* leaf and compound I enhanced the bioavailability of amoxycillin and rifampicin. On the other hand, methanol extract of *H.nepalense* root failed to show any significant increase in the bioavailability of these antibiotics but compound II isolated from root of the plant has moderately increased the bioavailability of these drugs.

The results of methanol extract of *C.oppositifolia* and compound I on bioavailability of amoxycillin showed that co-administration or pre-administration of methanol extract and compound I increased all the pharmacokinetic parameters of the amoxycillin compared to control group. Based on the literature data the mechanism by which the extract and compound I increases the bioavailability of amoxycillin is due to increased gastro intestinal blood supply, increased gastrointestinal motility or influence on epithelial cell wall modification [28, 29]. However, it cannot be ruled out for their direct inhibitory effect on microsomal enzymes or enzyme systems. The pre-administration of methanol extract of *C.oppositifolia* and compound I shifted the time to reach maximum concentration (t_{max}) 30 min and 1.0 hr respectively earlier than the control group. Similarly the relative bioavailability (RB %) of the pre-administration group of methanol extract and compound I was found to be higher than the co-administration group. This may be due to the effects of the extract and compound I on the permeability of intestinal epithelium to

promote absorption of antibiotic [30]. Intestinal epithelial cells represent the tight junctions and achieve the high transepithelial electrical resistance (> 700 ohm cm^2) in confluent monolayer. The methanol extract and compound I may have direct effect on the fragmentation of the tight junction between the cells and thus increased the permeability of the antibiotic [30].

From the experiments performed, it was observed that the elimination rate constant (k_{el}) is reduced by the extract and compound I and there is corresponding increase in the plasma half-life of the antibiotic. The results were consistent with the reported literature i.e. Choi et al. [31] Scambia et.al. [32], Woo et al. [33], and Zhang et al. [34] in that flavonoids and P-glycoprotein (Pgp) inhibitor KR 30031 increased the bioavailability of different drugs by inhibiting either the Pgp efflux pump or cytochrome P-450. Methanol extract of *C.oppositifolia* and compound I affected the bioavailability of amoxycillin in similar manner to those of quercetin, flavone, which are Cyp3A and Pgp efflux pump inhibitors.

The effect of methanol extract of *C.oppositifolia* and compound I on the bioavailability of cefixime indicated that the methanol leaf extract failed to change the bioavailability of cefixime after preadministration or coadministration of the extract. However the preadministration of compound I 30 min before the administration of cefixime increases all the pharmacokinetic parameters of cefixime including Area under curve (AUC) and relative bioavailability (RB %). This could be attributed to the fact that compound I may alter the permeation coefficient of intestinal epithelium and increases the absorption of cefixime[30].

The results of effect of methanol extract of *C.oppositifolia* and compound I on bioavailability of rifampicin indicated that coadministration or preadministration of methanol extract and compound I enhanced the bioavailability of rifampicin significantly. The analysis of various pharmacokinetic parameters t_{max}, C_{max}, $t_{1/2}$, AUC and RB % revealed that methanol extract and compound I might influence the different physiological process like alternation in gastric milieu or transportation and absorption process [28]. Rifampicin is a hepatic microsomal enzyme inducer and helps its own

metabolism in liver. So the direct inhibitory effect of *C.oppositifolia* and compound I on microsomal metabolizing enzyme systems cannot be ruled out [35]. The presence of high flavonoidal content of *C.oppositifolia* is responsible for the enhancement of bioavailability of rifampicin.

The effect of *H.nepalense* root extract and compound II on bioavailability and pharmacokinetics of amoxycillin, cefixime and rifampicin revealed that the methanol extract of *H.nepalense* failed to produce any effect on the bioavailability of all the antibiotics. All the pharmacokinetic parameters of antibiotics are unchanged with preadministration or coadministration of methanol root extract of the plant. However the coadministration of compound II enhanced the bioavailability of amoxycillin and rifampicin. The analysis of various pharmacokinetic parameters t_{max}, C_{max}, $t_{1/2}$, k_{el}, AUC and RB % indicated that compound II might influence the different physiological process like alternation in gastric milue or transport and absorption process [28]. The results were consistent with the result reported by Choi et al. [31] Scambia et.al. [32], Woo et al. [33], and Zhang et al. [34], in that the flavonoid (quercetin, naringin and flavone) and p-glycoprotein inhibitor KR 30031 increase the bioavailability of different drugs by inhibiting either the Pgp efflux pump or cytochrome P-450. This result was also consistent with the results reported by Bailey *et.al* [36], Rashid *et.al.* [37], Lasker *et. al* [38] that quercetin and their glycoside increased the bioavailability of rifampicin, tetracycline and sulphadiazine. Rifampicin and amoxycillin has been reported to be metabolized by cytochrome P-450 both in the liver and epithelial cells of the small intestine [39]. In addition P-glycoprotein efflux pump inhibited the absorption of these antibiotics in the intestinal mucosa [40]. Compound II might affect the bioavailability of amoxycillin and rifampicin in similar manner to those of other CYP3A4 and Pgp inhibitors.

Immunostimulatory therapy, which relates to stimulation of immune response of the host, is now being recognized as an alternative to conventional chemotherapy for variety of diseased conditions, especially when host's defense mechanisms have to be activated under condition of impaired immune response of the host [41]. The human body is continuously exposed to a series of stress factors, which more or less weaken the function

of the immune system and thereby generate immunosupression. Immunostimulators have been known to support T-cell function, activate macrophages, granulocytes, complement, natural killer cells and also help the production of various effector molecules generated by activated cells (Para immunity) [42].

The results of immunostimulatory activity of the methanol extract of *C.oppositifolia* leaf and compound I, as explained in chapter 6, indicated that the methanol extract at higher dose (1000 mg/kg body weight) moderately increased in the rate of carbon clearance from blood. However, administration of compound I at 50 mg/kg body weight dose resulted significant increased in the rate of carbon clearance compared to the rate of clearance produced by the standard immunostimulating drug levamisole at the same dose level. Hence methanol extract of *C.oppositifolia* and compound I at higher dose found to stimulate the phagocytic activity of macrophages as evidenced by an increase in the rate of carbon clearance.

The results of HA titer and DTH response model showed that the methanol extract increased the antibody titer and mean paw edema in mice at a dose of 1000 mg/kg body weight. Compound I also pronounced significant activity at dose of 50 mg/kg body weight. The HA titer and DTH response are believed to be related to humoral mediated and cell mediated immune response. The presence of flavonoid could be responsible for the stimulation of the immune response [41].

The *in vitro* immunostimulatory activity of methanol extract and its isolated compound I was tested on human polymorphonuclear and mononuclear cells. The phagocytosis and intracellular killing of microorganisms by polymorphonuclear phagocytes was determined by direct measurement of the microbicidal activity [42]. Phagocytosis was expressed as the phagocytic index, in which the percentage of decrease in number of viable extracellular bacteria was determined microbiologically after incubation with polymorphonuclear leukocytes. The phagocytic index of *C.oppositifolia* leaf extract was found to have moderate phagocytic activity as compared with the control group.

However, compound I showed maximum phagocytic index, which is slightly lower than that of the standard drug Interferon-α-2b.

Further, the immunostimulatory effect of extract and its isolated compound I was tested in mitogen activated cultured mononuclear cells. PHA was used for activating mononuclear cells in the culture. The mitogenic PHA is polyclonal activators, in that they activate mononuclear cells including memory type cells, irrespective of their antigenic specificity[43]. The effect of methanol extract of *C.oppositifolia* failed to produce any stimulation of PHA activated mononuclear cells and hence there was no increase of cells viability compared with the control. However, the compound I at 50 µg/ml concentration showed moderate immunostimulatory activity as compared with standard drug.

Prophylactic treatment of *H.nepalense* and its isolated compound enhanced the clearance of carbon from the blood in the rate of (more than 2 fold) when compared with the control group. The result is due to a mechanism related to phagocytosis by macrophages. The process of phagocytosis by macrophages includes opsonisation of the foreign particulate matter with antibodies and complement C3b leading to more rapid clearance of foreign particulate matter from blood [44] *H.nepalense* was found to stimulate the phagocytic activity of the macrophages as evidenced by increase in the rate of carbon clearance.

The results of the HA titer experiment indicated that administration of methanol extract at a dose of 1000 mg/kg body weight increase in the HA titer value almost four times as compared to the untreated control animals. The isolated compound also pronounced significant activity at a dose of 50 mg/kg body weight. This is owing to the augmentation of the humoral response by stimulating the macrophages and B-lymphocytes subsets involved in antibody synthesis [45]. The DTH response, which is a direct correlation of cell-mediated immunity (CMI), was found to be the highest at the maximum dose of root extract tested (1000 mg/kg body weight). The mechanism behind this elevated DTH during the CMI responses is due to sensitized T-lymphocytes, when challenged by the antigen, which are converted to lymphoblasts and secret lymphokines, attracting more

scavenger cells to the site of reaction. The infiltrating cells are thus immobilized to promote defensive (inflammatory) reaction [46]. Increase in DTH response indicates that root extract of *H.nepalense* and isolated compound II has a stimulatory effect on lymphocytes and a type of accessory cell required for the initiation of the reaction. [47].

The phagocytic index of *H. nepalense* root extract has found to be increased in a time and dose dependent manner. The isolated compound II at 50 μg/ml concentration showed significant activity nearly equal to the phagocytic index of root extract at 1000 μg/ml concentration after 120 min incubation as compared to control. The result of cell proliferation model indicated that methanol extract of *H.nepalense* at low concentration failed to show any immunostimulating effect. However, at higher concentrations increased cell stimulation was observed. Similarly, the standard drug and the isolated compound II demonstrated a significant stimulation of the mononuclear cells at 0.5 million IU and 50 μg/ml of tested concentrations respectively. This is attributed to the fact that the methanol extract and isolated compound may stimulate the PHA activated mononuclear cells and induce the release of cell proliferating factors like Interleukin and TNFα [48].

Earlier reports on the phytochemistry of *H. nepalense* indicate the presence of compounds like steroids and coumarins. We have isolated a known flavonoid quercetin glycoside from the plant. However, there are no report on the chemical nature and pharmacological activity of the plant. Contemporary research revealed that quercetin glycoside isolated from different herbal sources, has several pharmacological actions like antioxidant, anticancer, antiulcer, anti-inflammatory and antiviral [49]. Recent reports indicate that several types of flavonol stimulate human peripheral blood leukocyte proliferation, and significantly increase the activity of helper T cells, cytokines, interleukin 2, γ-interferon and macrophages and thereby useful in the therapy of several diseases caused by immune dysfunction [50]. It is thus apparent that the immunostimulatory effect produced by methanol extract of *H.nepalense* containing quercetin glycoside may be due to the cell mediated and humoral antibody mediated immune response.

Oxidative stress has been implicated in the pathology of many diseases and conditions including diabetes, cardiovascular diseases, inflammatory conditions, cancer and ageing[51]. Antioxidants may offer resistance against oxidative stress by scavenging the free radicals, inhibiting the lipid peroxidation and by many other mechanisms and thus prevent diseases [52].

The results of ferrous sulphate induced lipid peroxidation, from the experiments performed in chapter 7, showed that methanol extract of *C.oppositifolia* leaf at 1000 µg/ml concentration produced maximum percentage inhibition (58.67 %) of lipid peroxidation as compared with the standard antioxidant Vitamin E while compound I at 50 µg/ml showed an inhibition of 77.46 % greater than the inhibition produced by Vitamin E i.e.72.94 %. The inhibition could be attributed to the prevention of ferryl-perferryl complex or by changing the ratio of Fe^{3+} / Fe^{2+} or by reducing the rate of conversion of ferrous to ferric or by chelating the iron itself or combination thereof [53].

DPPH is a stable free radical that can accept an electron or hydrogen radical to become a stable dimagnetic molecule. Due to its odd electron, the methanolic solution of DPPH shows a strong absorption at 517 nm. DPPH radical reacts with suitable reducing agents and then by accepting an electron becomes paired off and the solution loses colour stoichometrically with the number of electrons taken up [54]. Such reactivity has been widely used to test the ability of compound to act as free radical scavengers. Reduction of the DPPH radicals can be observed by the decrease in absorbance at 517 nm. The effect of the methanol extract of *C.oppositifolia* and compound I in DPPH scavenging model revealed that the methanol extract of *C.oppositifolia* at 1000 µg/ml concentration showed an inhibition to DPPH reduction by 71.52 % as compared to the inhibition produced by Vitamin E (79.69 %). Compound I at 50 µg/ml showed higher inhibition of DPPH radical, 80.54 % compared to the standard drug Vitamin E i.e. 79.69 % at 5 mM concentration. The percentage of activity was also dependent on time.

Hydroxyl radicals are the major active oxygen species causing lipid peroxidation and enormous biological damage [55]. Ferric-EDTA solution was incubated with H_2O_2 and

ascorbic acid at pH 7.4. Free hydroxyl radicals were formed in the solution and were detected by their ability to degrade 2-deoxy-2-ribose into fragments that formed a pink chromogen upon heating with TBA at low pH [56]. When the test compounds were added to the reaction mixture they removed hydroxyl radicals from the sugar and prevented their degradation. The extract of *C.oppositifolia* significantly inhibited degradation of deoxy-ribose mediated by hydroxyl radical by 79.12 % at the concentration of 1000 µg/ml, compared to that of known scavenger mannitol (88.14 %). Here the compound I at 50 µg/ml concentration exhibited an inhibition of 81.42 % compared to 88.14 % for the standard drug mannitol at a concentration of 50 mM.

Superoxide radical O_2^- is highly toxic specie, which is generated by numerous biological and photochemical reactions. Both aerobic and anaerobic organisms possess superoxide dismutase enzymes, which catalyse the breakdown of superoxide radical [57]. Reduced phenazine methosulfate assay was used to measure the superoxide dismutase activity of methanol extract of *C.oppositifolia* and compound I. The methanol extract showed significant scavenging capacity of superoxide free radical at a concentration of 1000 µg/ml by 58.46 %. The compound I at 50 µg/ml concentration showed maximum inhibition of superoxide radicals (68.23 %) as compared with standard drug Vitamin E (69.23 %) at 5mM concentration.

The results of antioxidant activity of methanol root extract of *H.nepalense* on ferrous sulphate induced lipid peroxidation showed that the extract of *H.nepalense* inhibited the lipid peroxidation in a dose dependent manner. Compound II at 50 µg/ml concentration exhibited 72.38 % inhibition, which is comparable to the inhibition produced by vitamin E (73.42 %) at 5 mM concentration. The DPPH scavenging capacity of the extract was found to be 72.38% at the maximum tested concentration (1000 µg/ml). The compound II at 50 µg/ml, on the other hand, exhibited 76.38 % inhibition compared with 80.46 % for the standard drug vitamin E at 5mM. The extract of *H.nepalense* significantly inhibited 80.38 % of degradation of deoxy-ribose mediated by hydroxyl radicals at the concentration of 1000 µg/ml, compared to that of a known scavenger mannitol at 50mM

(89.64 %). The concentration of the methanol extract needed for 50% inhibition (IC_{50}) was 615.57 μg/ml. The compound II at 50 μg/ml on the other hand, exhibited 79.68 % inhibition compared to 89.64 % inhibition for the standard mannitol at 50mM concentration. Reduced phenazine methosulfate assay was followed to measure the superoxide dismutase activity of *H.nepalense* root extract and compound II. The results indicated that the scavenging capacity of the extract was 60.57 % at 1000 μg/ml concentration as compared with standard drug Vitamin E (68.36 %) at 5 mM concentration. The compound II at 25 μg/ml exhibited equal inhibition of superoxide radicals as compared with the standard drug (68.36 %). IC_{50} was found to be 8.9 mg/ml. Inhibition was proportional to the amount of the extract added.

Several epidemiological studies support that consumption of fruits and vegetables, rich in antioxidant compounds, flavonoids are associated with a lower incidence of diseases induced by oxidative stress [58]. It has been reported that flavonoids prevents injury and cell death caused by oxidative via several mechanisms, such as scavenging oxygen radicals, protecting against lipid peroxidation and chelating metal ions [59]. Here methanol extracts of *C.oppositifolia* leaf and *H.nepalense* root as well as compound I and compound II showed significant antioxidant effects in concentration dependent manner in all the models tested. Thus, the flavonoids present in the extracts are responsible for observed antioxidant activity.

Antimicrobial drugs have received immense importance in the therapeutic list of last century. Many infectious diseases sometimes considered incurable and lethal are now amenable to cure with a few doses of some drugs. The remarkable powerful and specific activity of anti-microbial drugs is due to their selectivity for specific targets that are either unique to microorganisms or much more important in terms of human use [60]. The antimicrobial compounds mainly isolated from microbes are structurally different from the compounds isolated from plant sources. The antimicrobial of plant source include flavonoids, essential oils, alkaloids, anthraquinones, triterpenoids etc. One of the main

approaches for the discovery of antimicrobials from higher plants is the evaluation of the medicinal plant extracts on pathogenic microbes [61, 62].

The methanol leaf extract of *C.oppositifolia* demonstrated significant *in vitro* antimicrobial activity against 257 Gram-positive and Gram-negative bacteria including multi resistance *Staphylococcus* strains (MRSC). The antimicrobial activity spectrum of *C.oppositifolia* leaf extract, as explained in chapter 8, revealed that out of 257 bacterial strains 171 (68 %) isolates were inhibited by the extract at a concentration up to 512 μg/ml. The experiment determining the minimum inhibitory concentration (MIC) of methanol extract revealed that 63 (81 %) out of 78 Gram-positive bacteria were sensitive within 512 μg/ml concentration while 94 (53 %) out of 179 Gram-negative isolates were sensitive within the same concentration. The disc diffusion test also demonstrated significant degree of antibacterial activity as compared with standard drug amoxycillin and gentamicin. The isolated compound I was also screened against several bacterial strains of *Escherichia coli*, *Staphylococcus spp*, *Salmonella spp* and *Vibrio spp* including three MRSC, most of them being inhibited at 128 μg/ml concentration of compound I.

The *in vitro* studies were followed by extensive *in vivo* tests. When methanol extract and compound I were injected to Swiss albino mice infected with a virulent dose of *Salmonella typhimurium* NCTC 74, offered significant protection to the animals at dose of 256 and 100 μg/ml respectively for the test organism. Thus methanol extract and compound I elucidated remarkable *in vivo* antimicrobial activity as evident from the reduction in initial bacterial cfu count in different vital organs determined on 18h post infection.

The methanol leaf extract and compound I were found to be bactericidal against *S.aureus* 8530. When methanol leaf extract & compound I were added to the logarithmic growth phase of the broth cultures of *S.aureus* 8530 at a concentration of their MIC values, the viable counts were sharply reduced from the culture as observed at 2,4,8 and 18 h.

The methanol root extract of *H.nepalense* also exhibited a significant *in vitro* activity against 257 Gram-positive and Gram-negative bacteria including MRSC strains. The antimicrobial activity spectrum of *H.nepalense* root extract revealed that out of 257 bacterial strains 197 (77 %) isolates were inhibited by the extract at a concentration up to 512 µg/ml. The results revealed that the methanol root extract was most effective against Gram-positive bacteria (84 %) than Gram-negative bacteria (67 %). The isolated compound II was also effective within the concentration of 256 µg/ml against several species of *Escherichia coli*, *Staphylococcus spp*, *Salmonella spp* and *Vibrio spp*. All the three MRSC strains were susceptible to compound II at a concentration of 128 µg/ml, while they were resistant to the two standard antibiotics Amoxycillin and Gentamicin.

The *in vitro* studies were followed by extensive *in vivo* tests. When methanol root extract and compound II were injected to Swiss albino mice infected with a virulent dose of *Salmonella typhimurium* NCTC 74, offered significant protection to the animals at dose of 128 and 100 µg/ml respectively for the test organism. Hence, the result demonstrated that the treatment with methanol root extract of *H.nepalense* and compound II successfully obliterate the severity of infection, as evident from the reduction in initial bacterial cfu count in different vital organs determined on 18h post infection.

The methanol root extract and compound II were found to be bactericidal against *S.aureus* 8530. When methanol root extract & compound II were added to the logarithmic growth phase of the broth cultures of *S.aureus* 8530 at a concentration of their MIC values, thee viable counts were sharply reduced from the culture as observed at 2,4,8 and 18h.

The present investigation therefore reveals that the methanol extract of *C.oppositifolia* leaf and *H.nepalense* root have a significant degree of antimicrobial activity, which may be due to the presence of compound I and compound II as evident by the *In vitro* and *in vivo* tests.

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