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EFFECT OF AQUEOUS EXTRACT OF *COLEBROOKEA OPPOSITIFOLIA* ON BIOAVAILABILITY OF AMOXYCILLINE IN RABBITS

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(Received 23 July 2003) (Accepted 27 October 2003)

ABSTRACT

This study was aimed to evaluate the effect of *Colebrookea oppositifolia* on bioavailability of amoxycilline. A sensitive, rapid and precise High Performance Liquid Chromatography (HPLC) method was used to measure the concentration of amoxycilline in plasma samples collected for 24 hours following different administration of drugs in rabbits. Pharmacokinetic parameters, including E_{max}, C_{max}, K_{el}, AUC, $t_{1/2}$ were calculated as per one compartment model. Analysis of data revealed that the variations in all pharmacokinetic parameters in different administration were statistically significant ($P<0.05$). This concludes that co-administrations of aqueous extract of *C. oppositifolia* enhance the bioavailability of amoxycilline. The enhanced bioavailability could be attributed to the effect of the extract on microsomal enzymes or enzymes systems.

Keywords: *Colebrookea oppositifolia*, Bioavailability, Cyp_s, PgP, Flavonoids.

INTRODUCTION

Colebrookea oppositifolia (Family: *Lamiaceae*) commonly known as 'Bansa' or 'Dosul' is a monotypic genus of evergreen shrubs or small tree, 1.2 to 3.6m tall growing widely in Northern and Southern slopes of the Himalayan range of Sikkim at elevation ranging between 3000-5000 ft. It has been reported to be useful in the treatment of various diseases like, asthma[1], epilepsy[2] tumor[3], and CNS disorders[4]. Various substances are present in the plant e.g. flavonoids, which have recently attracted interest due to their various beneficial pharmacological effects and additional abilities to modulate the bioavailability of different xenobiotics. In the present study we have made an attempt to investigate the effect of high flavonoidal content of aqueous extract of *C. oppositifolia* on bioavailability of amoxycilline in rabbit.

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MATERIALS AND METHODS

Preparation of Extract

The fresh dried leaves (3.0 kg) of *C. oppositifolia* were collected from the southern district of the Sikkim. It was authenticated by Botanical survey of India, Gangtok, Sikkim. After collection, the leaves were washed thoroughly in tap water and dried in shade for about 3-4 weeks. The dried leaves were powdered and stored in a well-closed container. A suspension of 10g of dried leaf powder in 100ml of distilled water was stirred magnetically for 4 hr at room temperature. The residue was removed by filtration and the filtrate was used as such for oral feeding or dried by freeze-drying. The yield of the water extract was about 9g from 10g dried leaves. Chemical tests were performed to confirm the flavonoid in the extract.[5]

Animals

Albino rabbits, (Himalayan Pharmacy Institute) weighing between 1.5 - 2 kg of either sex were used in the bioavalability studies and albino mice, (Himalayan Pharmacy Institute) between 20 - 25g for toxicity studies. All the animals were housed under standard conditions of temperature (27°C ± 3°C), 12hr/12hr light/dark cycles and fed with standard pellets diet (Chakan oil mill) and tap water. The

experiments were performed after getting the experimental protocols approved by the institutional Animal Ethics Committees of the Jadavpur University, Kolkatta.

Drugs and Chemicals

Amoxycilline from Libra Drugs (India), Pune. Sodium hydrogen phosphate from S.D. Fine Chemicals Ltd., Boisar, HPLC grade methanol and water from Qualigens Fine Chemicals, Mumbai, orthophosphoric acid and acetonitrile from E.merck, Mumbai were used during present investigation.

Acute Toxicity Studies

The extract was administered orally to different groups (5mice/group) of mice in doses ranging from 300mg/kg. No lethality was observed in any of the groups. Mice, which received extract of doses above 6000 mg/kg exhibited ptosis (dropping of upper eyelids) and were found lethargic. One tenth of the maximum dose of the extracts tested for toxicity study (800 mg/kg p.o) was selected for evaluation of activity.[3]

Acute toxicity studies in rabbits were also carried out by administering *C. oppositifolia* 30 min prior to amoxycilline treatment to find out whether *C.oppositifolia* potentiate the toxicity of the antibiotic.

Preparation of Test Drugs and Treatment Schedule

Amoxycilline was prepared as suspension in carboxy methyl cellulose (0.5%w/v) using pestle and mortar. Rabbits were randomly divided into 3 groups (6 rabbits/group). The first group served as control and received only amoxycilline (100mg/kg) orally. The second group received Extract of *C. oppositifolia* (800 mg/kg) orally with amoxycilline. The third group received *C. oppositifolia* (800mg/kg) extract orally and 30min later amoxycilline (100mg/kg).

Blood Collection and Determination of Blood Levels of Amoxycilline

Blood samples of rabbits treated with antibiotic alone and with *C. oppositifolia* were collected from the marginal ear vein by 1ml syringe with 26$^{1/2}$ gauge needle (Disposable) at different time intervals (0, 0.1, 1, 1.5, 2, 2.5, 3, 4, 5, 6, 8, 10, 12 and 24 hrs) following the antibiotic treatment. Blood samples were centrifuged at 2000 rpm for 20min. Thereafter the samples were separated and transferred to clean, dry vials and kept at 20°C until analyzed by developed HPLC method. All the samples were coded and labeled properly.

Pharmacokinetic Analysis

C_{max} and t_{max} for antibiotic were determined directly from the raw data. The area under curve (AUC) was calculated using the trapezoidal rule method. The apparent elimination rate constant (Kel) was determined by the regression analysis of the serum concentration verses time data of the terminal phase. The apparent half-life for the drug in plasma was calculated from relationship $t_{½}$=0.693/Kel equation. The significance between the respective treatment groups was calculated by paired student's 't' test.

RESULTS

The serum concentration versus time profile of amoxycilline administered with or without aqueous extract of *C. oppositifolia* is outlined in Table I. The study was taken in single dose, cross over design. The data was fitted to a one compartment open model with first order kinetics. Comparative pharmacokinetic parameters of three sets of the experiments have been shown in the Table II. In this interactive study, in all three treatment categories the absorption process was completed with median t_{max} of 2.58 (S.D. ± 0.08), 1.98 (S.D ± 0.15) and 1.75 (S.D. ± 0.10) hrs for amoxycilline, aqueous extract of *C. oppositifolia* + amoxycilline and *C. oppositifolia* + amoxycilline (30minutes later) respectively.

Aqueous extract of *C. oppositifolia* pretreatment induced a significant ($P<0.05$) shift in t_{max} of amoxycilline. This indicates that the prior treatment of the aqueous extract of *C. oppositifolia* has altered the t_{max} significantly as compared to *C. oppositifolia* + amoxycilline together. Thus the aqueous extract

Table I: Effect of Co-administration of Aqueous Extract of *Colebrookea oppositifolia* on Serum Concentration of Amoxycilline [Values expressed as μg/ml, are mean ± SD]

Sl. No.	Time of collection (hrs)	Amoxycilline (100mg/kg)	*C.oppositifolia* (800mg/kg)+ Amoxycilline (100mg/kg)	*C.oppositifolia* (800mg/kg) + Amoxycilline (100mg/kg) 30 mins later
1.	0	0	0	0
2.	0.5	8.85±0.195	18.15±0.194	28.70±0.701
3.	1.0	18.26±0.477	28.81±0.391	48.69±0.963
4.	1.5	26.28±0.410	45.50±0.267	62.34±0.591
5.	2.0	32.39±0.607	58.21±0.237	58.41±0.656
6.	2.5	37.08±0.394	54.47±0.227	55.35±0.408
7.	3.0	34.22±0. 390	50.57±0.293	51.61±0.516
8.	4.0	28.48±0.226	43.69±o.360	44.48±0.489
9.	5.0	22.81±0.153	36.68±0.575	36.67±0.409
10.	6.0	18.52±0.301	29.35±0.277	30.77±0.486
11.	8.0	12.81±0.162	21.62±0.265	23.18±0.294
12.	10.0	8.39±0.159	14.56±0.121	15.63±0.282
13.	12.0	5.05±0.115	10.80±0.270	11.68±0.443
14.	24.0	0.48±0.058	1.30±0.112	1.67±0.136

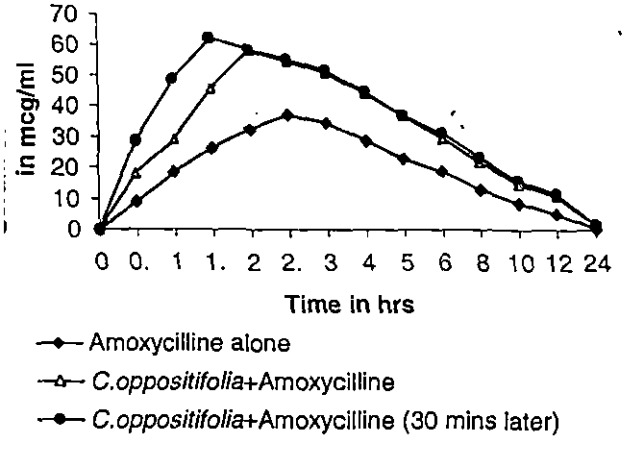
The data based on three separate experiments with six animals in each group. P values. <0.05.

Table II: Comparison of Pharmacokinetic Parameters (Values expressed as μg/ml, are mean ± SD)

Sl. No	Treatment	t_{max} (hr)	c_{max} (μg/ml)	$t_{1/2}$ (hr)	AUC (μg.hr/ml)	k_{el}
1.	Amoxycilline (100mg/kg)	2.58±0.08	37.08±0.34	3.39±0.08	248.69±1.723	0.2044± 0.008
2.	*C.oppositifolia* (800mg/kg) + Amoxycilline (100mg/kg)	1.98±0.15	58.38±0.316	4.015±0.139	429.29±1.968	0.1727± 0.006
3.	*C.oppositifolia* (800mg/kg) + Amoxycilline (100mg/kg) 30minutes later	1.58±0.04	62.34±0.591	4.29±0.140	475.15±3.28	0.1616± 0.005

The data based on three separate experiments with six animals in each group. P values. <0.05.

C. oppositifolia shows the time dependent effect the gastrointestinal absorption of the drug. The ribution phase of the amoxycilline in all three s of the experiments was fairly short i.e. centration decay being evident within 2 hr. of the g administration. Comparison of other rmacokinetic parameters (Table II) revealed t there were significant differences in peak ibiotic levels attained (c_{max}) or mean elimination f life ($t_{½}$). Comparison of AUC showed that higher um levels of amoxycilline were achieved in the up treated with *C. oppositifolia* + amoxycilline. mparison of the serum concentration time curve g.1) of each administration shows significant ferences of amoxycilline present in the blood.



g. 1 : Comparative Mean Plasma Amoxycilline (100mg/g) Concentration versus Time Profile for Rabbits treated ith Amoxycilline (100mg/kg)and Aqueous Extracts of *olebrookea oppositifolia* at various Time Intervals

ISCUSSION

The present experimental findings in animal xperiments were clearly indicated that concurrent dministration of aqueous extract of *C. oppositifolia* ignificantly enhances the bioavailability of moxycilline. The analysis of various harmacokinetic parameters tmax, Cmax, K_{el}, $t_{½}$ and AUC point out that aqueous extract of *C. oppositifolia* might influence the different hysiological process like alteration in gastric ransport or absorption process. Again it was reported hat aqueous extract of *C. oppositifolia* has flavonoid as the major chemical constituent[6-8]. In recent years, flavonoids have attracted increasing interest due to their various pharmacological effects and additional abilities to modulate cyp_s and P-glycoprotien (PgP)[9-12]. Therefore direct inhibitory effect of high flavonoidal content of *C. oppositifolia* on Cyp_s and PgP can not be ruled out.

The studies conclude that aqueous extract of *C. oppositifolia* co-administration with amoxycilline improves the bioavailability. This is reflected from various pharmacokinetic parameters studied. Since the increased bioavailability of amoxycilline has great implication in clinical medicine, co-administration of isolated products like flavonoids from *C.oppositifolia* reduce the amount of the dose of amoxycilline and its dosing frequency. It is directly related with patient compliance and adverse effect of the drug. However careful bioavailability studies using human volunteers and specific isolated flavonoid from the plant need to be under taken to confirm the results obtained.

ACKNOWLEDGEMENT

Thanks are due to all of my colleagues for technical assistance.

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Tropical Journal of Pharmaceutical Research, June 2005; 4 (1): 341-347

Available online at http://www.tjpr.freehosting.net

Research Article

Antioxidant and antimicrobial activities of *Heracleum nepalense* D Don root

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Abstract

Purpose: The aim of the present study was to investigate antioxidant and antimicrobial effects of the methanol extract of *Heracleum nepalense* D.Don roots.
Method: The antimicrobial effect was determined by agar dilution and disc diffusion method. The free radical scavenging potential was studied by using different antioxidants models of screening using vitamin E (5mM) as standard.
Results: The crude methanol extract of *H.nepalense* root was found to be active against both Gram-positive and Gram-negative organisms. The ethyl acetate soluble fraction of the extract showed similar activity against these organisms. Similarly, the methanol extract at 1000 μg. ml^{-1} and the ethyl acetate fraction at 50 μg. ml^{-1} exhibited significant antioxidant activity in ferrous sulphate induced lipid peroxidation, 1,1- diphenyl- 2-picryl hydrazyl (DPPH), Hydroxyl radical and Superoxide scavenging models.
Conclusions: The study confirms the possible antioxidant and antimicrobial potentiality of the plant extract. Presence of flavonoid alone or in combination with its other components could be responsible for the activity.

Keywords: *Heracleum nepalense*, Lipid peroxidation, Superoxide scavenging, DPPH assay, Antimicrobial effect, Flavonoid.

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Introduction

Reactive oxygen species (ROS) such as superoxides, peroxides and hydroxyl radicals are known to play an important role and have been identified as major contributors to all cell and tissue damage in many disease conditions[1]. In living organisms, various ROS can form in different ways. Normal aerobic respiration stimulates polymorphonuclear leukocytes and peroxisomes appear to be the main endogenous sources of most of the oxidants produced by cells. Exogenous sources of ROS include tobacco smoke, alcohol, pesticides, certain pollutants and microbial infections [2-3].

In recent years, there has been increased incidence of antibiotic resistance in pathogenic organisms and the persistence of pathogens in immune compromised individuals is of great concern[4]. Therefore, actions must be taken to reduce this problem such as controlling the use of antibiotics, carrying out research to better understand the genetic mechanisms of resistance and continuing investigations aimed at the development of drugs from natural sources.

The recent years have witnessed resurgence of interest in herbal drugs globally as more people are turning to the use of herbal medicinal products in health care. About 80% of individuals from developing countries use traditional medicine, which involves compounds derived from medicinal plants. It is high time the hidden wonders of plant molecules were revived with the modern tools of target-based screening to develop newer advanced generation antioxidants and antimicrobials with novel modes of action. Antioxidants play an important role in inhibiting and scavenging free radicals, thus providing protection to human body against infection and degenerative diseases.

Heracleum nepalense D.Don (Apiaceae) is a small shrub occurring in Nepal and Sikkim [5]. The plant is used in veterinary medicine. It exhibits stimulant property and increases blood pressure in goats [6]. The roots of the plant are used in folk medicine as digestive, carminative and antidiarrhoeal (Authors personal experience). The roots of the plant are reported to have coumarins [7] and steroids [8]. The present study was aimed at evaluating antioxidant and antimicrobial properties of *Heracleum nepalense*.

Experimental

Plant materials and phytochemical screening

The fresh dried roots of *Heracleum nepalense* (HNSE) were collected from the southern district of Sikkim. The plant was authenticated by Botanical Survey of India, Gangtok, Sikkim. The voucher specimen was preserved in our laboratory for future reference. The dried roots were powdered and stored in a well-closed container. 1 kg of powder (40 mesh size) was extracted by cold percolation with 3 liters of 70% v/v methanol in a percolator for 72 h at room temperature [9] .The residue was removed by filtration. The extract was then evaporated to dryness under reduced pressure in a rotary evaporator at 42-45°C. The concentrated extract of leaves was kept in a dessicator for further use.

The preliminary phytochemical tests of the root extract were done by Pollock and Stevense method [10]. Concentrated methanol extract was suspended in hot distilled water, cooled and the blast precipitate was filtered off. The filtrate (aqueous solution) was fractioned by extracting it successively with petroleum ether, ethyl acetate and acetone. The ethyl acetate fraction of the crude extract on purification yielded one major fraction A (flavonoid) with some fatty substances.

Chemicals

Thiobarbituric acid was obtained from Loba Chemie, India. 1,1-Diphynyl-2-picryl hydrazyl (DPPH), NADH and nitroblue tetrazolium (NBT) were obtained from Sigma chemicals, St. Louis, USA. Deoxy ribose was obtained from Merck India. Dimethyl sulphoxide, ethylene diamine tetra acetic acid, ferrous sulphate, trichloroacetic acid, hydrogen peroxide, ascorbic acid, mannitol, potassium dihydrogen phosphate, potassium

hydroxide, deoxy ribose, phenazine methosulphate were of analytical grade and were obtained from Ranbaxy fine chemicals.

Determination of antioxidant activity

Assay of lipid peroxidation

The extent of lipid peroxidation in goat liver homogenate was measured *in vitro* in terms of formation of thiobarbituric acid reactive substances (TBARS) by using standard method [11] with minor modifications [12]. Goat liver was purchased from local slutter house. Its lobes were dried between blotting papers (to remove excess blood) and were cut into small pieces with a heavy-duty blade. They were then homogenized in glass-Teflon homogenizing tube in cold phosphate buffer saline (pH 7.4). It was centrifuged at 2000 rpm for 10 min and supernatant was diluted with phosphate buffer saline up to final concentration of protein 0.8-1.5 mg/0.1 ml. Protein concentration was measured by using standard method of Lowery et.al [13]. To study the comparative response, the experiment was divided into nine groups. Liver homogenate (5%, 3ml) was aliquoted to nine different 35mm glass petri dishes. The first two groups were treated as control and standard where buffer and vitamin E were added. In the third to seventh group, different concentrations of methanol extract (200-1000 $\mu g.ml^{-1}$) and fraction A (25, 50 $\mu g.ml^{-1}$) were added. Lipid peroxidation was initiated by adding 100µl of 15mM ferrous sulphate solution to 3ml of liver homogenate [14]. After 30 minutes, 100 µl of this reaction mixture was taken in a tube containing 1.5 ml of 10% Trichloro acetic acid. After 10 minutes, tubes were centrifuged and supernatant was separated and mixed with 1.5ml of 0.67% thiobarbituric acid. The mixture was heated in a water bath at 85°C for 30 min and in a boiling water bath to complete the reaction. The intensity of pink colored complex formed was measured at 535nm in a spectrophotometer (Shimadzu model 1601). The percentage of inhibition of lipid peroxidation was calculated by comparing the results of the test with those of controls as per the following formula:

Inhibition (%) = [(control – test) ×100] / control---- --- Eqn 1.

DPPH radical scavenging activity

DPPH scavenging activity was measured by spectrophotometric method[14]. To a methanolic solution of DPPH (100 µM, 2.95ml), 0.05 ml of methanol extract as well as fraction A (25, 50 $\mu g.ml^{-1}$) and Standard compound (vitamin E) were added at different concentrations. Equal amount (0.05 ml) of methanol was added to a control. Absorbance was recorded at 517nm at regular intervals of 1 to 5 min. The percentage of scavenging was calculated by comparing the control and test samples with the Eqn 1.

Hydroxyl radical scavenging activity

Hydroxyl radical scavenging activity was measured by studying the competition between deoxy ribose and test compounds for hydroxyl radical generated by the Fe^{3+}– ascorbate – EDTA – H_2O_2 system (Fenton reaction) according to the method of Kunchandy & Rao [15]. The reaction mixture containing, a final volume of 1.0 ml, 100 µl 2-deoxy-ribose, 500 µl of the various concentrations of the methanol extract as well as fraction A (25, 50 $\mu g.ml^{-1}$) and standard compound (Mannitol 50 mM) in KH_2PO_4- KOH buffer (20mM, pH 7.4), 200 µl 1.04 mM H_2O_2 and 100 µl 1.0mM ascorbic acid was incubated at 37°C for 1 hour. One milliliter 1% trichloroacetic acid was added to each test tube and incubated at 100°C for 20 min. After cooling to room temperature, absorbance was measured at 532nm against a control preparation containing deoxyribose and buffer. Percent inhibition was determined by comparing the results of the test and control samples with the above mentioned Eqn 1.

Superoxide scavenging activity

The superoxide scavenging activity of methanol extract was determined by the method described by Nishimik et al [16], with slight modification. About 1.0 ml NBT solution containing 156 µM NBT dissolved in 1.0 ml 100 mM phosphate buffer, pH 7.4, 1.0 ml NADH solution containing 468 µM NADH dissolved in 1.0 ml 100mM phosphate buffer, pH 7.4, and 0.1 ml of various concentration of the methanol extract as well as fraction A (25, 50 $\mu g.ml^{-1}$) and standard compound (vitamin E) were mixed and the reaction was started by adding 100 µl of

phenazine methosulfate solution containing 60μM phenazine methosulphate in 100 mM phosphate buffer, pH 7.4. The reaction mixture was incubated at 25 °C for 5 min and absorbance at 560 nm was measured against control sample. Percent inhibition was determined by comparing the results of the test and control samples with the above mentioned Eqn 1.

Antimicrobial activity

Bacteria

A total of 257 bacterial strains belonging to different genera were tested in this study. The test organisms were obtained from Department of Bacteriology, Calcutta School of Tropical Medicine and Institute of Microbial Technology (IMTECH), Chandigarh, India. . Three multiresistant *Staphylococcus* strains (*Staphylococcus aureus* ML 275, *Staphylococcus aureus*', NCTC 8530 and *Staphylococcus epidermidis* 865) were kindly provided by Prof. (Mrs.) Sujata Ghosh Dastidar, Department of Pharmacy, Jadavpur University, and Kolkata, India. They were aseptically isolated and identified by the Barrow and Feltham's method and preserved in the freeze-dried state [17]. Gram-positive strains were grown in nutrient broth (NB, Oxoid brand) and Gram-negative bacteria were grown in peptone water (PW, Oxoid brand, bacteriological peptone plus Nacl 0.5%) for 18 h before use.

Determination of antimicrobial activity

Sensitivity tests were performed by disc diffusion method[18]. The nutrient agar plates (Oxoid brand), containing an inoculum size of 10^5-10^6 cfu.ml^{-1} of bacteria were used. Previously prepared crude methanol extract (Concentration 128-2000 μg.ml^{-1}) and fraction A (Concentration 128-2000 μg.ml^{-1}) discs were placed aseptically on sensitivity plates. The discs containing methanol and known antibiotics (Amoxycillin and Gentamicin) served as negative and positive controls. All the plates were then incubated at 37°C±2°C for 18 h. The sensitivity was recorded by measuring the clear zone of inhibition on agar plate around the discs.

The MICs were determined by the standard agar dilution method [19]. All the test compounds were dissolved in methanol. These were then individually added at each final concentrations of 0-2000 μg.ml^{-1}, to molten agar (Oxoid brand), mixed thoroughly, pH adjusted to 7.2 to 7.4 and poured into sterile Petri dishes. Bacterial cell suspensions were spot inoculated on the plates using a bacterial planter (10 μl). The final number of cfu inoculated onto the agar plates was 10^4 for all strains. The inoculated plates were then incubated at 37°C±2°C for 18 h. The lowest concentration of the plate, which did not show any visible growth after incubation, was considered as MIC. The agar plate containing only methanol and Amoxycillin was served as negative and positive control.

Statistical analysis

Data are reported as the mean ± SD of three measurements. Statistical analysis was performed by the student *t*-test and by ANOVA. IC_{50} values for all the above experiments were determined by linear regression method. A p-value less than 0.05 was considered as indicative of significance.

Results and Discussion

Antioxidant activity

Assay of lipid peroxidation

The results presented in Table-1 showed that the methanol extract of the HNSE inhibited $FeSO_4$ induced lipid peroxidation in a dose dependent manner. The extract at 1000 μg.ml^{-1} exhibited maximum inhibition (69.25 ± 1.21%) of lipid peroxidation, on the other hand fraction A at 50 μg.ml^{-1} concentrations showed (72.38 ±1.9%) inhibition, nearly equal to the inhibition produced by vitamin E (Fig 1). The IC_{50} value was found to be 747.5 ±3.16 μg.ml^{-1}. The inhibition could be caused by the absence 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 [20].

DPPH scavenging activity

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

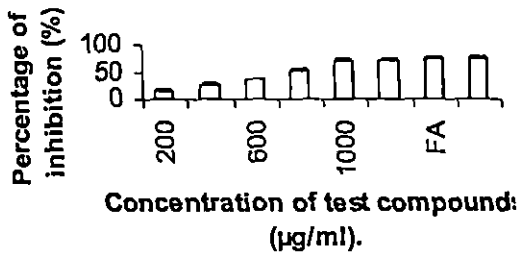

Fig 1: Inhibition (%) of lipid peroxidation by different concentrations of methanol extract of *H. nepalense*, Ethyl acetate fraction (FA) and Vitamin E (Vit E).

absorption at 517 nm. DPPH radical reacts with suitable reducing agents and then electrons become paired off and the solution loses colour stoichometrically with the number of electrons taken up [21]. Such reactivity has been widely used to test the ability of compound/plant extracts to act as free radical scavengers. Reduction of the DPPH radicals can be observed by the decrease in absorbance at 517 nm. The DPPH scavenging capacity of the extract was found to be 72.38 ± 3.92 % at 1000 µg.ml^{-1}. The fraction A at 50 µg.ml^{-1} doses, on the other hand, exhibited 76.38 ± 5.12 % inhibition compared with 80.46 ± 4.62 % for the standard drug vitamin E at 5mM (Table 1). The IC_{50} value was found to be 6.5 mg.ml^{-1}. The activity was also dependent on time (Fig 2).

Hydroxyl radical scavenging activity

Hydroxyl radicals are the major active oxygen species causing lipid peroxidation and enormous biological damage [22]. Ferric-EDTA was incubated with H_2O_2 and ascorbic acid at pH 7.4. Hydroxyl radicals were formed in free 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 [23]. When the test compounds were added to the reaction mixture they removed hydroxyl radicals from the sugar and prevented their degradation. The extract of HNSE significantly inhibited (80.38 ± 2.28 %) degradation of deoxy-ribose mediated by hydroxyl radicals at the dose of 1000 µg.ml^{-1} (Table 1), compared to that of a known scavenger mannitol (50mM). The concentration of the methanol extract needed for 50% inhibition was 615.57 µg.ml^{-1}. The fraction A at 50 µg.ml^{-1} on the other hand, exhibited (78.68 ± 2.62 %) inhibition compared with (89.64 ± 2.84 %) for the standard mannitol.

Superoxide scavenging activity

Superoxide radical O_2 is a highly toxic specie, which is generated by numerous biological and photochemical reactions. Both aerobic and anaerobic organisms possess superoxide dismutase enzymes, which catalyse the

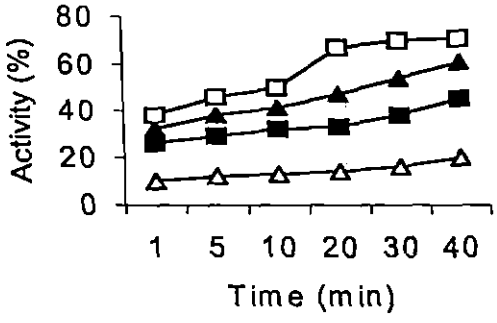

Figure 2: DPPH scavenging activity of *H.nepalense*. 10mg/ml (□), 7.5mg/ml (▲), 5mg/ml (■) and 2.5 mg/ml (Δ).

breakdown of superoxide radical [24]. Reduced phenazine methosulfate assay was used to measure the superoxide dismutase activity of HNSE. The results presented in Table 1 showed that the scavenging capacity of the extract was 60.57 ± 2.34% at 1000 µg.ml^{-1}. The fraction at 25 µg.ml^{-1} exhibited 68.36 ± 2.73 % inhibition of superoxide radicals. IC_{50} was found to be 8.9 mg.ml^{-1}. Inhibition was proportional to the amount of the extract added.

Antimicrobial activity

The methanol extract of HNSE roots exhibited a significant *in vitro* antimicrobial activity against 257 Gram-positive and Gram-negative bacteria including multiresistant *Staphylococcus* (MRSC)

strains. All the three reference strains of bacteria were found to be sensitive within 1000 μg.ml^{-1}. The preliminary biological screening of the fractions showed that the ethyl acetate part was more active than other fractions (Data not shown). The results of the antimicrobial spectrum of the roots extract presented in Table 2 showed that out of 257 bacteria, the growth of 197 isolates were inhibited at a concentration of 128 – 512 μg.ml^{-1}. 57 isolates were resistant up to 1000μg.ml^{-1}, while the remaining 03 isolates where resistant up to >2000 μg.ml^{-1}, the highest concentration tested. The MICs tests revealed that 63 out of 75 Gram-positive bacteria were sensitive between 128 and 256 μg/ml (zone diameter 10–16 mm); while out of 179 Gram-negative isolates, 120 were sensitive between 256-512 μg.ml^{-1} (zone diameter 10-14 mm). Hence, it appears that the antimicrobial activity of the methanol extracts was directed both against Gram-positive and Gram-negative bacteria. The ethyl acetate fraction (fraction A) was also tested for antimicrobial activity. The result revealed that all the isolates were sensitive at 128-256 μg.ml^{-1} (Table 3). It was interesting to note that all the MRSC were susceptible to fraction A at a concentration of 128 μg.ml^{-1}, while they were resistant to the two test antibiotics. Thus, ethyl acetate fraction may become clinically relevant, particularly for antibiotic-resistant strains. However, the activity has to be studied using isolated pure compounds from the fraction to which the more resistant strains were susceptible in order to confirm these findings.

Table: 1 Effect of *H.nepalense* on different antioxidant models.

Concentration (μg/ml)	Percentage of Inhibition (%)			
	Lipid Peroxidation	DPPH	Hydroxyl radical	Superoxide radical
1000	69.25 ± 1.21	72.38 ± 3.92	80.38 ± 2.28	60.57 ± 2.34
800	51.74 ± 1.92	69.42 ± 3.86	79.54 ± 1.24	52.27 ± 3.18
600	36.36 ± 1.84	45.52 ± 3.73	65.37 ± 2.26	40.26 ± 2.16
400	26.57 ± 3.8	36.46 ± 2.32	46.28 ± 1.89	24.12 ± 1.38
200	16.08 ± 4.3	8.47 ± 1.83	32.14 ± 2.24	12.23 ± 1.42
Vitamin E (5mM)	73.42 ± 2.3	80.46 ± 4.62	NT	68.36 ± 2.73
Mannitol (50mM)	NT	NT	89.64 ± 4.62	NT
Fraction A				
	70.15 ± 1.64	71.32 ± 1.85	72.35 ± 2.93	68.36 ± 2.73
25	72.38 ± 1.9	76.38 ± 5.12	79.68 ± 2.62	68.24 ± 1.86
50				
IC_{50} (μg/ml)	747.5 ± 3.16	600.52 ± 3.46	615.57 ± 2.16	891.9 ± 14.42

Values are mean ± S.E.M of 3 replicates. NT: Not tested.

Acknowledgement

The authors are thankful to the All India Council for Technical Education, Government of India, New Delhi and Prof (Dr) S.G.Dastidar, Division of Microbiology, Jadavpur University, Kolkata, India for providing the research facility.

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Asian Journal of Chemistry Vol. 18, No. 2 (2006), 1581–1582

NOTE

A Flavonoid from the Roots of *Heracleum nepalense* D. Don

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A known flavonol glycoside, namely, quercetin-3-O-β-D-glucopyranoside, was isolated from the roots of *Heracleum nepalense* D. Don. The structure was determined on the basis of UV, IR, FAB$^+$, MS, ^{1}H & ^{13}C NMR spectral data. The isolation is significant since a flavonoid has not been previously reported from the plant.

Key Words: Isolation, Quercetin, *Heracleum nepalense*.

Heracleum nepalense D. Don[1-3] (Apiaceae) is a small shrub occurring in Nepal and Sikkim. The plant is used in veterinary medicine. It exhibits stimulant property and increases the rate of respiration and blood pressure in goats. The roots of the plant are used in folk medicine as digestive, carminative and antidiarrhoeal. In our earlier work we have reported the plant having antimicrobial property. The roots of the plant reported for having coumarins[4, 5] and steroids[6]. Flavonoids are more common throughout the family Apiaceae than other constituents[7]. Very little research has been conducted on the roots of *H. nepalense* and nothing has been found concerning the flavonoids, it was of interest to examine the flavonoid patterns of the species. In this report, we describe the isolation and structure elucidation of a flavonoidal glycoside from the roots of *H. nepalense*. It was identified by spectroscopic techniques[8].

The air-dried roots (1 kg) were extracted with methanol (70%) and the extract was concentrated, treated with hot distilled H_2O and filtered. The water-soluble component was fractionated by extracting it successively with petroleum ether, ethyl acetate and acetone. The ethyl acetate soluble fraction was concentrated and then on TLC over silica gel showed three spots. This fraction was submitted to column chromatography on sephadex LH-20 column using benzene : ethyl acetate as eluent with increasing polarity. Fractions 32–46 were combined, evaporated under reduced pressure, dissolved in MeOH and purified on a silica

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gel (60–120) column. From the dry residue of the flavonoid fraction compound 1 (75 mg) was obtained by recrystallization from MeOH.

Compound 1 (Quercetin 3-O-β-D-glucopyranoside)

It was obtained as a pale yellow amorphous powder, m.p 210–214°C; UV $_{max}\lambda^{MeOH}$ nm: 251, 374; +NaOMe 246, 328, 425; +NaOAc-H_3BO_3 229, 268, 320 sh, 383; IR (KBr, cm^{-1}) ν_{max}: 3648–3611 ν(OH), 2904 ν(C-H), 1653 ν(C=O in flavone), 1615–1507 ν(aromatic rings), 1456, 1304, 1263; ^1H NMR (DMSO-d_6), δ (ppm): 12.48 (1H, S, OH-5), 7.56 (2H, dd, J = 2.2 Hz, H-2' and H-6'), 6.87 (1H, d, J = 8.5 Hz, H-2, H-5'), 6.41 (1H, d, J = 1.9 Hz, H-2, H-8), 6.18 (1H, d, J = 1.9Hz, H-6), 4.92 (1H, d, J = 7.3Hz, H-1''), 3.98–3.06 (m, the remaining protons of glucose); ^{13}C NMR (DMSO-d_6), δ (ppm): 176.05 (C-4), 164.14 (C-7), 160.95 (C-5), 156.4 (C-2), 147.9 (C-4'), 147.01 (C-3'), 135.9 (C-3), 125.8 (C-1'), 122.2 (C-6'), 115.3 (C-2), 103.2 (C-10), 98.48 (C-1''), 76.97 (C-5''), 76.92 (C-3''), 75.0 (C-2''), 69.97 (C-4''), 60.47 (C-6''); MS data m/z (rel. int.): 459 (10.2), 415 (15.8), 371 (20.0), 327 (19.2), 303 (98.8), 301 (29.7), 287 (10.2), 277 (12.0), 239 (8.2), 207 (8.0).

The structure of quercetin was established by comparison of measured UV, NMR and mass spectral data with spectroscopic data available from literature[9, 10]. The quercetin 3-O-β-D-glucopyranoside showed ^1H and ^{13}C NMR data in full agreement with those given by Olszewska *et al.*[11] and Irena *et al.*[12]

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ACKNOWLEDGEMENT

The authors are thankful to AICTE, New Delhi, India for financial supports under RPS scheme with file no. 8022/RID/NPROJ/RPS-77/2003-04.

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(*Received*: 4 August 2005; *Accepted*: 31 December 2005) AJC-4625

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of Ethnomedicinal Research (2006) 9, 161-163

JER

: Access publication 12 March 2006.

Isolation and Characterization of a Catechin Glycoside from the Leaves of *Colebrookea oppositifolia* Smith

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Received 17 November 2005; returned 20 January 2006; revised 16 February 2006; accepted 1 march 2006

Abstract

Phytochemical investigation on methanol extract of leaves of *Colebrookea oppositifolia* led to the isolation of a known flavan-3-ol. Ethyl acetate fraction of the extract was subjected to coloumn chromatography and monitored by TLC, resulted a single compound. The compound was established on the basis of UV, IR, FAB$^+$MS, ^{1}H, ^{13}C NMR spectral and elemental analysis. The isolated compound was identified as (+)– Catechin-7-O-β-rhamnopyranoside. This confirmed that Catechin is one of the major bioconstituent of the plant.

Key Words: Isolation, Catechin, Flavan-3-ol, *Colebrookea oppositifolia.*

Introduction

Colebrookea oppositifolia Smith. (Lamiaceae) locally known as "Bansa" or "Dosul" is a monotypic genus of ever green shrubs or small trees, 1.2-3.6m tall growing widely in northern and southern slopes of the Himalayan range of Sikkim at elevation ranging between 3000 to 5000 ft. Ethnobotanical studies indicate that the decoctions of *C.oppositifolia* leaves and stem bark is widely used among the tribal populations of Sikkim to treat skin infections, indigestion (Gurung G, 1999), diarrohea (Jain SP, 1984) wounds and cuts (Manadhar NP, 1995). Alcoholic extracts of leaves is reported to be useful in asthma (Singh V, 1995), epilepsy (Paul SR, 1997) and helminthes (Ansari S, 1982). Earlier reports on the phytochemistry of *C. oppositifolia* indicate the presence of compounds like β-Sitosterol, Stearic acid (Ansari S, 1982), Quercetin (Mukherjee PK, 2001), and Baicalein (Ahmad SA, 1974). The present phytochemical studies for the first time showed the presence of (+) – Catechin in the ethyl acetate fraction of the methanol extract of *C. oppositifolia* leaves.

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Materials and Methods

UV-Visible spectral analysis was carried out on a Shimadzu model 1601 Spectrophotometer. TLC was carried out on polyamide (E.merck & Co) coloumn using n-Butanol: Acetic acid: Water (4:1:5), Phenol:Water (3:1) and Concentrated Hcl: Acetic acid: Water (3:30:10) . For coloumn chromatography Sephadex LH-20 and Silica gel 60-120 (SRL, India) were used. Sugar analysis was carried out according to standard procedure on Whatman paper No.1 along with authentic standard sugar samples (Markham KR, 1982). FAB^+MS was recorded on a JEOL JMS 600 Spectrophotometer. ^1H and ^{13}C NMR spectra were recorded at 400 MHz on Brucker WM 400 Spectrophotometer. The Infrared absorption spectra of the isolated compound were taken with Perkin Elmer FTIR spectrophotometer. Elemental analysis was made with a Carlo Erba EA 1110 apparatus. The structure of catechin was established by comparison of measured UV, NMR and Mass spectral data with spectroscopic data available from the literature (Markham KR, 1982, Ta-Chen Lin, 1999).

Extraction and fractionation

The fresh dried leaves of *C. oppositifolia* were collected from the southern district of Sikkim. It was authenticated by Botanical survey of India, Gangtok, Sikkim. The voucher specimen was preserved in our laboratory for future reference. The dried leaves was powdered and stored in a well-closed container.1 kg of powdered plant (40 mesh size) was extracted by cold percolation with 3 liters of 70% methanol in a percolator for 72 h at room temperature. The residue was removed by filtration. The solvents were then evaporated to dryness under reduced pressure in a rotary evaporator at 42-45°C. The concentrated extract of leaves was kept in dessicator for further use. The methanol extracts were 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 coloumn chromatography on Sephadex LH-20 coloumn using benzene: ethyl acetate as eluent with increasing polarity. Fifty-eight fractions were collected. Identical fractions were combined, evaporated under reduced pressure, dissolved in methanol and purified on a Silica gel 60-120 coloumn. From the dry residue of the fractions compound-I was obtained by recrystallisation from methanol.

Compound I was obtained as yellow amorphous solid; m.p. 181-185°C; UV (MeOH) λ_{max} 241, 281; +NaOMe 250, 293; +$AlCl_3$ 248, 282; +$AlCl_3$+HCl 280, 314sh, 361sh; +NaOAc 243, 283; +NaOAc 281; IR (KBr) γ_{max} cm^{-1}. 3480, 1771, 1632, 1606, 1519, 1456, 1029; ^1H NMR (DMSO-d_6 400 MHz) δppm 2.506 (1H, d, J= 2.0 Hz, 4H), 2.699 (1H,dd, J=16 Hz, 4H), 3.476 (2H, m, 5"), 3.787- 3.872 (1H, m, 4"H), 4.393 (1H, d, J=3.0 Hz, 2"H), 5.698 (1H, d, J=3.0 Hz, 1"H), 6.212 (1H, d, J=2.0 Hz, 8H), 6.226 (1H, d, J=2.0 Hz, 6H), 6.591 (1H, dd, J=2.0 Hz, J=7.6 Hz, 6'H), 6.681 (d, J=7.6Hz, 5'H), 6.731 (d, J=2.0 Hz, 2'H); ^{13}C NMR (DMSO, d_6, 400 MHz): 145.1 (C-2), 130.95 (C-3), 81.23 (C-4), 155.68 (C-5), 94.28 (C-6), 156.75 (C-7), 92.56 (C-8), 156.52 (C-9), 99.46 (C-10), 118.88 (C-1'), 95.51 (C-2'), 115.52 (C-3'), 114.78 (C-4'), 92.56 (C-5'), 91.23 (C-6'), 72.67 (C-1"), 72.25 (C-2"), 70.84 (C-3"), 61.51 (C-4"), 66.63 (C-5"), 28.51 (C-6"); FAB^+MS data (m/z) 581 (3.2), 383 (4.5), 303 (10.0), 291 (100), 274 (18.0), 255 (4.2), 231 (3.3), 207 (5.0). Elemental analysis (Found: C-54.12; H-4.24. $C_{24}H_{22}O_8$. Calc. for C-54.13; H-4.23 %)

Results and Discussion

Compound I was isolated by repeated coloumn chromatography on a Sephadex LH-20 and silica gel 60-120 from ethyl acetate fraction of *C.oppositifolia* leaves. It was

obtained as yellow amorphous solid. The complete acid hydrolysis gave Catechin and glucose (co- TLC). The compound showed strong absorption at 281 nm in its spectrum, which implied the presence of phenolic aromatic rings. The UV spectrum showed absorption bands reagents shifts of the compound to be a 7-substituted derivatives. The absence of free 7-hydroxyl group in the compound was observed in lack of shift of band II in the presence of NaOAc. The IR spectrum confirmed the presence of aromatic ring (1632 cm^{-1}) and hydroxyl group (3480 cm^{-1}). The doublets at 6.212 ppm (J=2.0 MHz) and 6.226 ppm (J=2.0 MHz) in its ^{1}H NMR spectrum were suggestive of two aromatic protons existing at the *meta* positions. The signals at 6.591 ppm (J=2.0 Hz, J=7.6 Hz), 6.681 ppm (J=7.6Hz) and 6.731 ppm (J=2.0 Hz) inferred the presence of another set of aromatic protons existing at 1,3,4 positions. The signals at 2.506 ppm and 2.699 ppm and the double double doublet at 3.476 ppm suggested that the compound should be a flavan-3-ol derivative. Existence of a sugar in its structure was evidenced by the anomeric proton signal, which appeared at a 5.698 ppm (J= 3.0 Hz). The coupling constant (J= 3.0 Hz) of the anomeric proton sugar suggested that the phenoxy group be attached to the anomeric carbon atom by β-configuration. The fragmentation ion at m/z 291 in its mass spectrum inferred that the compound should have (+) – Catechin as an aglycone. The structure of the compound was further confirmed by $^{13}CNMR$ and elemental analysis of the available literature. The signals of C-5, C-7 and C-9 were observed at higher value than 150 ppm, the signals at 156.75 ppm only showed correlations with these of H-6 and H-8 (at 6.212 ppm and 6.226 ppm respectively), we assigned it to C-7. The signals at 155.68 ppm and 156.52 ppm showed correlations with those of H-6 and H-8 respectively, so we assigned them to C-5 and C-9 respectively. The correlation of the anomeric proton signals at 5.698 ppm with that of C-7 at 156.75 ppm suggested that the sugar should be attached at the C-7 position. The sugar analysis was carried out along with authentic standard sugar sample. The result is corresponding to the molecular formula $C_{24}H_{22}O_8$ (confirmed by elemental analysis). From these data we concluded that the structure of the isolated flavonoid was (+)-catechin-7-O-β-rhamnopyranoside.

Acknowledgement

The authors are thankful to the All India Council for Technical Education, Government of India, New Delhi for financial assistance under RPS with File no: 8022/RID/NPROJ/RPS-77/2003-04.

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Research Paper

Stimulation of immune function activity by the alcoholic root extract of *Heracleum nepalense* D. Don

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Received: 15.2.2006
Revised: 15.5.2006
Accepted: 23.6.2006

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ABSTRACT

Objective: This study was designed to assess the immunostimulatory activity of *H. nepalense*, using different *in vitro* and *in vivo* experimental models.

Materials and Methods: The immunostimulatory potential of the test compound was investigated by *in vitro*, phagocytic index and lymphocyte viability tests, using interferon-α-2b, a known immunostimulant drug, as the standard. Other tests such as carbon clearance, antibody titer and delayed type hypersensitivity were studied in mice, using levimasole as the standard.

Results: The dried root extract (1000 µg/ml) and isolated quercetin glycoside (50 µg/ml) significantly increased the *in vitro* phagocytic index and lymphocyte viability in all assays. They also showed a significant increase in antibody titer, carbon clearance and delayed type hypersensitivity in mice.

Conclusion: *H. nepalense* exhibited a dose-dependent immunostimulant effect, which could be attributed to the flavonoid content or due to the combination with other component(s).

KEY WORDS: Cell proliferation, immunostimulation, HIV.

Introduction

Heracleum nepalense D.Don (Apiaceae) is a small shrub which grows in Nepal and Sikkim. It is used in veterinary medicine. It exhibits stimulant property and increases the rate of respiration and blood pressure in goats.[1] The root of the plant is used as a digestive, an aphrodisiac, a carminative and an antidiarrheal in folk medicine.[2] We have earlier reported the antioxidant and antimicrobial properties of the plant.[3] *H. nepalense* was studied for its potential immunomodulatory activity, driven by the presence of its antimicrobial property, and its usage in folk medicine.

Immunostimulatory therapy is now being recognised as an alternative to conventional chemotherapy for a variety of disease conditions, involving the impaired immune response of the host.[4] Immunostimulators have been known to support T-cell function, activate macrophages and granulocytes, and complement natural killer cells apart from affecting the production of various effector molecules generated by activated cells (Paraimmunity).[5] It is expected that these non-specific effects offer protection against different pathogens, including bacteria, fungi, viruses and so on, and constitute an alternative to conventional chemotherapy.[6] In view of the above, the present investigation was undertaken to evaluate the immunostimulatory potential of *H. nepalense* roots, using *in vitro* and *in vivo* models.

Materials and Methods

Plant material and preparation of extract

The fresh roots (9 kg) of *H. nepalense* were collected from the southern district of Sikkim between September-November, 2003. The roots were authenticated by the Botanical Survey of India, Gangtok, Sikkim. A voucher specimen was preserved in our laboratory for future reference. The shade-dried root was ground and yielded 2.5 kg of powder. It was stored in an air-tight, hard polyethylene container with silica pouch up to 10-12 days. One kg of powdered plant (40 mesh size) was extracted by cold percolation with 3 liters of 70% methanol in a percolator for 72 h, at room temperature. The residue was removed by filtration. The solvent was then evaporated to dryness, under reduced pressure, in a rotary evaporator at 42-45°C and yielded 400 mg of extract. The concentrated methanol extract was kept in a dessicator for further use.

Phytochemical studies

The chemical constituents of the methanol extract were identified by qualitative chemical tests for the presence of flavones, tannins, sterols, triterpenoids and saponins (Data

presented).[7] The methanol extract was concentrated, spended in hot distilled water, cooled and the blast cipitate was filtered. The water-soluble component was tionated by extracting it successively with petroleum ether, yl acetate and acetone. The ethyl acetate soluble fraction s subjected to column chromatography on Sephadex LH- column using benzene:ethyl acetate as eluent with reasing polarity. Fractions 32-46 were combined, aporated under reduced pressure, dissolved in MeOH and rified, on a silica gel (60-120) column. A known flavonol coside, namely quercetin-3-O-β-D-glucopyranoside, was lated along with some minor compounds.[8]

st compound formulations

Oral suspensions of the extract and isolated compound ercetin-3-O-β-D-glucopyranoside were prepared by spending them separately in 1% solution of sodium carboxy ethyl cellulose to prepare suitable dosage forms.

imals used

Swiss Albino mice of either sex, weighing 17–25 g each, re used. They were housed under standard conditions of mperature (23±10°C) and relative humidity (55±10%), 2/12 h light/dark cycle, and fed with standard pellets and ater *ad libitum*. The Institutional Animal Ethics Committee viewed the animal protocols prior to the experiments.

rugs and chemicals

EDTA, RPMI-1640, Hank's balanced salt solution (HBSS), extran, phosphate buffered saline, fetal calf serum, reptomycin, penicillin, amphotericin, and Trypan blue were urchased from Himedia. Phytohaemagglutinin, ficoll ypaque and L-glutamine were purchased from Sigma iagnostic, USA. Interferon α-2b and levimasole were btained as gift samples from Fulford (I) Ltd. and Khandelwal aboratories Ltd., Mumbai, respectively.

ntigen

Fresh blood was collected from a healthy sheep from the ocal slaughter house. Sheep red blood cells (SRBCs) were vashed thrice with normal saline and adjusted to a oncentration of 0.1 ml containing $1X10^8$ cells for mmunisation and challenge.

n vivo carbon clearance test

The mice were divided into 8 groups, each consisting of 10 animals. Group I (Control) was given 1% sodium carboxy methyl cellulose in water (0.3 ml/mouse, i.p.) for 7 days, Group II-VIII were given different concentrations of methanol extract (250-1000 mg/kg, p.o.), isolated compound (25, 50 mg/kg, p.o.) and standard drug (Levimasole 50 mg/kg, p.o.) for 7 days. At the end of 7 days, the mice were injected, via the tail vein, with carbon ink suspension (10 μl/g, body weight). Blood samples were drawn (in EDTA solution 5 μl), from the retroorbital vein, at intervals of 0 and 15 min, a 25 μl sample was mixed with 0.1% sodium carbonate solution (2 ml) and the absorbance measured at 660 nm. The carbon clearance was calculated using the following equation: $(Log_e OD_1 - Log_e OD_2)/15$, where OD_1 and OD_2 are the optical densities at 0 and 15 min, respectively.[9]

In vivo humoral antibody (HA) titer and delayed type hypersensitivity (DTH) response

Humoral antibody (HA)

The mice were divided into 8 groups, each consisting of 6 mice. Group I (Control) was given 1% sodium carboxy methyl cellulose in water (0.3 ml/mouse) for 7 days. Group II-VIII were given drug treatment which was exactly the same as with the carbon clearance test.

The animals were immunised by injecting 0.1 ml of SRBCs suspension, containing $1X\ 10^8$ cells, intraperitoneally, on day 0. Blood samples were collected in microcentrifuge tubes from individual animals of all the groups by retroorbital vein puncture on day 8. The blood samples were centrifuged and the serum separated. Antibody levels were determined by the haemagglutination technique.[9] Briefly, equal volumes of 50 μl individual serum samples of each group were pooled. Serial two-fold dilutions of pooled serum samples were made in 50 μl volumes of RPMI-1640 in microtitration plates. To this 50 μl of 1% suspension of SRBC in RPMI-1640 was added. After mixing, the plates were incubated at 37°C for 1 h and examined for haemagglutination under the microscope (button formation). The reciprocal of highest dilution, just before the button formation, was observed and titre values were calculated.

Delayed type hypersensitivity test (DTH)

On Day 8, the thickness of the right hind footpad was measured using a Vernier calliper. The mice were then challenged by injection of $1\ X\ 10^8$ SRBCs in the right hind footpad. The footpad thickness was measured again after 24 h of challenge. The difference between the pre- and post challenge footpad thickness, expressed in mm, was taken as a measure of the DTH response.[10]

In vitro phagocytic index

Preparation of microorganism

Escherichia coli 832 (*E. coli*) was grown and kept on a slope of solid agar medium. Before use, the microorganism was cultured in 100 ml of 2.5% nutrient broth (oxoid) for about 18 h at 37°C. The culture was then washed twice with phosphate buffer saline and re-suspended in gelatin-HBSS to a concentration of $1\ X\ 10^7$ cells/ml. During each experiment, the number of viable microorganisms was determined microbiologically by counting colony forming units (cfu), using nutrient agar plates after incubation, at 37°C for 18 h.[11]

Preparation of human polymorphonuclear leukocytes (PMNCs)

Human blood was collected from a local blood bank and the RBCs removed by sedimentation in 5% (w/v) solution of dextran in buffered saline (m.w. 200,000; 3 ml of solution to 10 ml of blood) for 30 min at 37°C. The PMNC-rich supernatant layer was washed twice with heparin-saline, concentrated by centrifugation (10 min at 110 g), counted with a hemocytometer, and suspended in gelatin-HBSS to make up a concentration of $1\ X\ 10^7$ cells/ml.

Microbiological assay for the phagocytosis

To assess phagocytosis, different concentrations of methanol extract (250-1000 μg/ml), its isolated compound (25, 50 μg/ml) and the standard drug, Interferon α-2b (0.5 million IU), in the final volume of 0.1ml, were incubated respectively with 2 ml of the PMNCs suspension ($1\ X\ 10^7$ cells/ml), 2 ml of the suspended microorganisms ($1\ X\ 10^7$ cells/ml) and 0.4 ml of fetal calf serum at 37°C for 1 h in 5% CO_2 atmosphere in a slanting position. At 30 min intervals up to 120 min, 0.5 ml aliquot of the suspension was removed

and added to 1.5 ml of the ice-cooled gelatin-HBSS to stop phagocytosis. The control was run using gelatin-HBSS in place of the test compounds. These samples were centrifuged at 110 g for 4 min. Under this condition, the non-ingested microorganisms remained in the supernatant fluid. The viable count of the microorganisms was undertaken using the colony counter[11]. Phagocytosis was expressed as the percentage decrease in the initial number of viable extracellular bacteria according to the formula: $P(t) = (1 - N_t/N_0) \times 100$, where P (t) is the phagocytic index at time t = t, N_0 and N_t are the number of viable extra cellular bacteria at time t = 0 and t = 30, 60, 90 and 120 min, respectively.[12]

In vitro cell proliferation assay

This test was performed with peripheral mononuclear blood cells, following their separation from the blood by using ficoll-hypaque gradient centrifugation, according to manufacture's instructions (Sigma Diagnostic, USA). The rate of proliferation of mononuclear cells, under the influence of mitogens, was measured by the method of Sriwanthana,[13] with minor modification. Briefly, under sterile conditions, the cells were diluted to 1 X 10^7 cells/ml with RPMI-1640 (supplemented with 20% fetal calf serum). The cell suspension (2 ml) was transferred into a sterile culture tube and to each sample. Different concentrations of the plant extract (250-1000 μg/ml, filtered through 0.22 μ pore size filter), isolated compound (25, 50 μg/ml) and standard drug Interferon α-2b (0.5 million IU), in the final volume of 0.1 ml, were added, respectively. The proliferation of cells was induced by 50 μl phythaemagglutinin (PHA, 0.1 mg/ml). The prepared samples were incubated for 72 h at 37°C in a CO_2 atmosphere, supplemented with 2 mM L-glutamine, 100 μg/ml streptomycin, 100 units/ml penicillin and 0.25 μg/ml amphotericin. The control incubated with cells minus the plant extract. The viability of the cells was assessed after incubation with test compounds, using the Trypan blue dye exclusion method.[14] Briefly, 20 μl of the incubation mixture was mixed with 20 μl of Trypan blue dye. The total number of mononuclear cells and mononuclear stained blue (dead cells) were counted under an inverted microscope (Olympus, Japan), using the hemocytometer. The percentage of cell viability was taken as a measure of cell proliferation and calculated as per the following formula:[15]

$$\% \text{ of cell viability} = \frac{\text{Total number of cells} - \text{Total number of dead cells}}{\text{Total number of cells}} \times 100$$

Similarly, the percentage of cell stimulation was calculated as per the following formula:[15]

$$\% \text{ of cell stimulation} = \frac{\text{Viability of test} - \text{Viability of control}}{\text{Viability of control}} \times 100$$

Statistical analysis

Statistical analysis was performed using one-way ANOVA, followed by Dunnett's test. The significance in difference was accepted at $P < .05$.

Results

The methanol extract, 250-1000 mg/kg, p.o. and its isolated compound, 25 and 50 mg/kg, p.o. exhibited a significant increase in carbon clearance from the blood in a dose-dependent manner. [Table 1] The doses of test drugs, for which maximum carbon clearance were seen, are methanol extract (1000 mg/kg) and its isolated compound (50 mg/kg). The results (Table 1) also indicate that animals treated with 250, 750 and 1000 mg/kg of methanol root extract produced a significant increase in HA titer (humoral immunity) as evident from hemagglutination after incubation of serum with SRBCs, while the isolated compound at 50 mg/kg and levimasole (50 mg/kg) showed 328.6±10.4 and 430.06±8.3 HA titer, respectively. In the DTH response (cell mediated immunity) test, the methanol extract at higher doses (750 and 1000 mg/kg) showed a statistically significant increase in mean paw edema in mice. The isolated compound at 50 mg/kg, on the other hand, exhibited the maximum DTH response of 0.50±0.22 compared with 0.57±0.21 for the standard drug, levimasole.

The effects of methanol extract (250–1000 μg/ml) and its isolated compound (25–50 μg/ml) on the phagocytic index model are presented in Table 2. The phagocytic index was significantly increased in a dose-dependent manner after 30, 60, 90 and 120 min intervals with methanol extract as well as its isolated compound. Maximum phagocytic index was observed at 1000 μg/ml (97.86±1.67) of methanol extract and 50 μg/ml (97.24±1.23) of its isolated compound after 120 min of incubation; whereas the standard compound Interferon α-2b exhibited maximum phagocytic index (99.23±1.11) after 120 min. The results of the proliferative response, on the basis of the cell viability of mononuclear cells to the PHA mitogen, are presented in Table 3. The percentage of viability of PH- activated mononuclear cells was significantly increased at 1000 μg/ml of the root extract, compared with the control. The maximum viability (60.4±4.58 %) was noticed at standard drug Interferon α-2b; whereas

Table1

Effect of methanol root extract of *Heracleum nepalense* and isolated compound on immunostimulatory activity in mice

Treatment (mg/kg)	*Carbon clearance*	*HA titer*	*DTH response*
Control	0.068±0.012	80.43 ±2.21	0.28 ±0.10
Extract 250	0.122±0.016*	185.3 ±3.31*	0.39 ±0.13
500	0.132±0.014*	172.7 ±0.1*	0.28 ±0.13
750	0.146±0.016*	282.6 ±8.1*	0.43 ±0.10*
1000	0.158±0.018*	320.8 ±10.6*	0.48 ±0.21*
Levimasole (50)	0.164±0.016*	430.06 ±8.3*	0.57 ±0.21*
IC (25)	0.156±0.016*	324.2 ±8.3	0.48 ±0.22
IC (50)	0.160±0.018*	328.6 ±10.4*	0.50 ±0.22*
One-way F	230	5231	6.432
ANOVA P	0.05	0.05	0.05

Values are mean±SEM; n=6 in each group; df=7,40. *P<0.05 in comparison with control. IC: Isolated compound.

Table 2

Effect of methanol root extract of *Heracleum nepalense* and isolated compound on phagocytic index of polymorphonuclear leukocyte

Concentration	Phagocytic index (%)			
(μg/ml)	30 min	60 min	90 min	120 min
Control	61.14±1.26	97.26±1.52	84.32±1.69	92.25±1.83
Extract 250	63.53±1.52*	78.54±1.18*	85.74±1.43*	94.27±1.86*
500	64.12±1.18*	79.23±1.21*	86.23±1.24*	95.67±2.33*
750	67.38±1.24*	81.52±1.42*	88.15±1.86*	96.55±1.83*
1000	69.54±1.12*	83.26±1.37*	91.18±2.14*	97.86±1.67*
Interferon α-2b	70.27±1.64*	84.14±1.32*	94.52±1.65*	99.23±1.11*
IC 25	69.54±1.12*	82.86±1.45*	90.96±1.14*	96.42±1.42*
50	70.27±1.64*	83.09±1.51*	91.14±1.62*	97.24±1.23*
One-way ANOVA F	1.123	1.154	1.272	1.526
One-way ANOVA P	0.05	0.05	0.05	0.05

Values are mean±SEM; n=6 in each group; df=7,40. *P<0.05 in comparison with control. IC:Isolated compound.

Table 3

Effect of methanol root extract of *Heracleum nepalense* and isolated compound on mononuclear cell proliferation

Treatment (μg/ml)	Percentage viability of cells (%)	Stimulation (%)
Control	50.7±10.42	0
Extract 250	47.6±4.42*	(– 6.11)
500	49.8±5.42*	(– 1.77)
750	49.0±4.45*	(– 3.35)
1000	56.3±8.94*	(+11.04)
Interferon α-2b	60.4±4.58*	(+19.13)
IC 25	56.9±6.62*	(+12.22)
50	58.2±4.53*	(+14.79)
One-way ANOVA F	5.630	
One-way ANOVA P	0.05	

Values are mean±SEM; n=6 in each group; df=7,40. *P<0.05 in comparison with control. IC:Isolated compound. "+" indicates increase and "–" indicates decrease in cell stimulation.

the isolated compound at 50 μg/ml concentration showed 58.2±4.53 % viability. Further, the methanol extract at 1000 μg/ml and its isolated compound at 50 μg/ml concentration showed 11.04 % and 14.79% cell stimulations, respectively, as compared with Interferon α-2b, for which it was observed as 19.13%.

Discussion

The present study established the immunostimulatory activity of the methanol extract and its isolated compound. Prophylactic treatment of *H.nepalense* and its isolated compound enhanced the rate of carbon clearance from the blood (more than a two-fold increase) when compared with the control group. The result is owing 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 a more rapid clearance of foreign particulate matter from the blood[12]. *H.nepalense* was found to stimulate the phagocytic activity of the macrophages as evidenced by an increase in the rate of carbon clearance.

The methanol extract, at a dose of 1000 mg/kg, body weight showed almost a four-fold increase in HA titer, compared to untreated controls. The isolated compound also pronounced significant activity at a dose of 50 mg/kg body weight. This could be due to the presence of flavonoids which augment the humoral response, by stimulating the macrophages and B-lymphocytes subsets involved in antibody synthesis.[10] The DTH response, which directly correlates with cell-mediated immunity (CMI), was found to be the highest at the maximum dose tested in the root extract (1000 mg/kg). The mechanism behind this elevated DTH during the CMI responses could be due to sensitised T-lymphocytes. When challenged by the antigen, they are converted to lymphoblasts and secrete a variety of molecules including proinflammatory lymphokines, attracting more scavenger cells to the site of reaction.[16] The infiltrating cells are probably immobilised to promote defensive (inflammatory) reaction.[17] An increase in DTH response indicates that the root extract of *H.nepalense* and its isolated compound have a stimulatory effect on lymphocytes and accessory cell types required for the expression of the reaction.[18]

The *in vitro* immunostimulatory activity of the methanol extract and its isolated compound was tested on human polymorphonuclear and mononuclear cells. The phagocytosis and intracellular killing of microorganisms by polymorphonuclear phagocytes was determined by the direct measurement of the microbicidal activity.[12] Phagocytosis was expressed as the phagocytic index, in which the percentage decrease in the initial number of viable extracellular bacteria was determined microbiologically after incubation with polymorphonuclear leukocytes. In our study, the phagocytic index of *H. nepalense* root extract was found to be increased in a time- and dose-dependent manner. The isolated compound (50 μg/ml) and root extract (1000 μg/ml) showed significant phagocytic index as compared to control.

Further, the immunostimulatory effect of the extract and its isolated compound was tested in mitogen-activated cultured mononuclear cells. PHA was used to activate the mononuclear cells in the culture. The mitogenic PHA are polyclonal activators, in that they activate mononuclear cells including memory type cells, irrespective of their antigenic specificity.[19] The root extract (higher concentrations) and its isolated compound caused a significant stimulation of the mononuclear cells. This is attributed to the fact that the methanol extract and its isolated compound may stimulate the PHA-activated mononuclear cells and induce the release of cell proliferating factors such as interleukin and TNFα.[1,3]

Earlier reports on the phytochemistry of *H. nepalense*

indicate the presence of compounds such as steroids and coumarins. We have isolated a known flavonoid, quercetin glycoside, from the plant.[18] However, there is no report on the pharmacological activity of the plant. Contemporary research revealed that quercetin glycoside, isolated from different herbal sources, has several pharmacological actions such as antioxidant, anticancer, antiulcer, antiinflammatory and antiviral.[20] Recent reports indicate that several types of flavonols stimulate human peripheral blood leukocyte proliferation. They significantly increase the activity of helper T cells, cytokines, interleukin 2, γ-interferon and macrophages and are thereby useful in the treatment of several diseases caused by immune dysfunction.[21] It is thus apparent that the immunostimulatory effect produced by the methanol extract of *H.nepalense*, containing quercetin glycoside, may be due to cell mediated and humoral antibody mediated immune responses.

The present finding provides scientific evidence to the ethnomedicinal use of this plant by tribals in Sikkim. The plant *H.nepalense* has the potential for new therapeutic applications in the future.

Acknowledgement

The authors are thankful to the All India Council for Technical Education, Government of India, New Delhi for financial assistance under RPS with File no: 8022/RID/NPROJ/RPS-77/2003-04.

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