PHYTOCHEMICAL STUDIES AND BIOLOGICAL EVALUATION OF *Roylea cinerea* AS ANTIMALARIAL FOR *Plasmodium vivax*

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(MAY- 2012)

Submitted in partial fulfillment of the Degree of

Bachelor of Pharmacy

DEPARTMENT OF PHARMACY

JAYPEE UNIVERSITY OF INFORMATION TECHNOLOGY, WAKNAGHAT

HIMACHAL PRADESH

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CERTIFICATE

This is to certify that the work entitled **"Phytochemical Studies and Biological Evaluation of *Roylea cinerea* as Antimalarial for *Plasmodium vivax*"** submitted by **"Ms. Jeenu Mary Varghese** and **Mr. Anuj Verma"** in partial fulfillment for the award of Degree of Bachelor of Pharmacy of Jaypee University of Information Technology, Waknaghat (Solan), has been carried out under my supervision. This work has not been submitted partially or wholly to any other University or Institute for the award of this or any other degree or diploma.

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ACKNOWLEDGEMENT

It is our proud privilege and sincere thanks to Dr. R.S. Chauhan, Head, Department of Pharmacy, for providing us with the facilities and encouragement for doing the final year project.

Our sincere gratitude to Dr. Neeraj Mahindroo (Project Supervisor) for his supervision, encouragement and support.

Thanks to Dr. N.K. Kapoor, for the authentication of the plant, Himalayan Forest Research Institute, Conifer Campus, Panthaghatti, Shimla, Himachal Pradesh.

We would like to acknowledge our gratitude towards Department of Biotechnology (SAIF) Panjab University Chandigarh for performing the proton NMR of our isolated compounds.

We also thank Mr. Raj Kumar Tiwari, Ms. Shilpi Chanda, Ms. Sonika Gupta and all our colleagues for their support and help.

Pleasure to acknowledge Jaypee University of Information Technology for providing us the opportunities and encouragement for carrying on the project work.

Last and certainly not the least, we are indebted to our family members for their unflinching support to us from the first day.

Jeenumary

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Anuj Verma

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DATE: 28-05-2012

SUMMARY

Malaria is an infectious diseases caused by the plasmodium species *(Plasmodium falciparum, Plasmodium vivax, Plasmodium malariae, Plasmodium ovale).* Main focus of the research is on *P. vivax* caused malaria. *P. vivax* malaria is not fatal, but relapses often occur months to years after treatment because some of the parasites can become dormant in the liver. *P. vivax* malaria has classically been treated with chloroquine and primaquine. Primaquine acts against the liver stage, decreasing the risk of relapse. The parasite is becoming resistant to chloroquine and primaquine, so alternate drugs need to be explored.

Roylea cinerea (Patkarru) Wall. (Lamiaceae), a shrub of monotypic genus, is found in western Himalayas from Kashmir to Nepal at altitudes from 600-1500 m. The aerial parts of the plant are widely used as folklore medicine for treatment of various liver disorders, especially, jaundice and liver debility and antiprotozoal activity against erythrocytic stages of *P. falciparum* have also been reported. Ethanolic extracts (by hot continuous extraction) of the leaves, stems and roots and aqueous extract of leaves (by hot continuous extraction) were prepared and phytochemical screening, for the detection of various phytoconstituents was carried out. Phytochemical screening showed the presence of alkaloids, phenolic compounds and saponins in the alcoholic extracts and the presence of phenolic compounds and saponins in the aqueous extract of the plant. TLC profile of the ethanolic and aqueous extract was also carried out by observing the developed TLC plate under visible and UV (254 nm and 366 nm) light and after derivatization with iodine and anisaldehyde - sulphuric acid spray reagent. Column chromatography of the ethanolic extract led to isolation of two compounds, the structures for which are currently being elucidated. The extracts have been submitted for evaluation against *P. vivax* at National Malaria Research Institute, New Delhi and results are awaited.

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Date: 28/05/2012

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Date: 28-05-2012

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Chapter 1

INTRODUCTION

1.0 Malaria

Malaria, a disease caused by protozoa, is a major health problem in tropical areas and is responsible for significant morbidity and mortality worldwide. [1]

1.0.1 Cause and transmission of malaria

Malaria is caused by the protozoa of the genus *Plasmodium*. Humans are infected by one (or more) of the following four species: *P. falciparum*, *P. vivax*, *P. ovale*, and *P. malariae*. Plasmodia are primarily transmitted by the bite of an infected female *Anopheles* mosquito, but infections can also occur through exposure to infected blood products, such as transfusion malaria and by congenital transmission. [2]

The malaria parasite is transmitted to the female *Anopheles* mosquito when it takes a blood meal as a prelude to the reproductive process from an infected individual. Climatic features such as rainfall and humidity affect the stability of transmission. The parasite undergoes a lifecycle change within the mosquito before it becomes infectious to other individuals. As the ambient temperature declines the period required for this life-cycle change increase, and given the life span of the mosquito, transmission becomes much less likely when the temperature falls below 18 °C. Moreover, at temperatures below 16 °C malaria parasites cease development completely. [3]

Out of about 400 species of anopheline mosquitoes throughout the world, only 60 species are vectors of malaria. In India 9 species out of 45 anopheline species have been incriminated as malaria vectors. There are four species of human malaria parasites *P. vivax, falciparum, malariae* and *ovale*. In India 35 to 40% due to *P. falciparum* and 60 to 65 % of the infections are due to *P. vivax*. Only few cases of *P. malariae* have been reported from Orissa and Karnataka. [4]

1.0.2 History

Malaria was well known to Hippocrates and to other Greek and Roman physicians. By the experience of centuries the association of this disease with marshes was confirmed and was reflected in the name of 'marsh-fever', although the terms 'intermittent fever' or 'ague' have been used. The name mal'aria - meaning bad or spoiled air - came from Italy in 1740 and indicates the general belief, at that time, that some noxious vapour was responsible for the disease. Meckel in 1847 noted the presence of brown pigment in the organs of persons who died of pernicious fever. Meckel also pointed out that the dark colour of the spleen, liver, brain or kidneys on autopsy of these cases was associated with the accumulation of pigment in the blood. Virchow and Frerichs in Germany confirmed it. Alphonse Laveran, a French army surgeon working in Algeria, in 1880 first identified malaria. He documented the description of parasites in the red blood cells of a man showing symptoms of malaria.

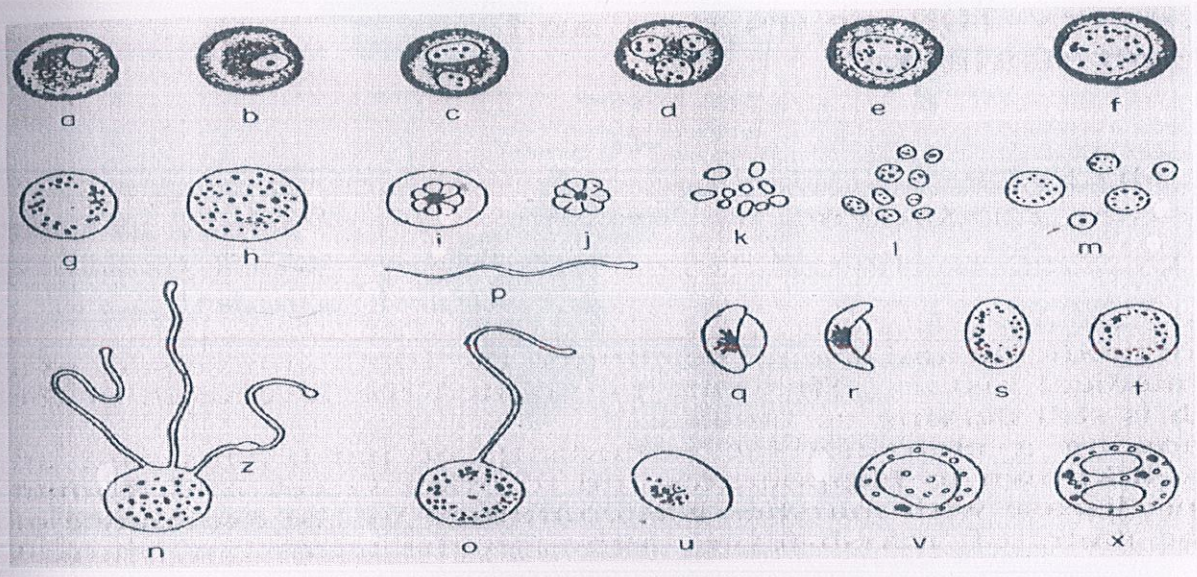


Figure 1: Malaria parasites (Haemamoeba malariae) observed and drawn by Alphonse Laveran; published in Compt'es Rendus de l Academie des Sciences, 24 October 1881. (From Sergent and Sergent 1929).

In 1886; Camillo Golgi established that there were at least two forms of the malaria. Within a decade further discoveries were documented. In 1898, Ronald Ross (a British officer in the Indian Medical Service), demonstrated that patients suffering from malaria could transmit the parasites to mosquitoes during a blood meal. [5]

1.0.3 Malaria Cases Worldwide

In the 1980s and 1990s global malaria death estimates ranged from 800,000 to almost 2·5 million; the range in the 2000s, was from 650,000 to more than 1 million. In 2010, there were 1,238,000 (929,000–1,685,000) malaria deaths, a 32% decrease since 2004. Findings showed that malaria is the underlying cause of death for 1.24 million individuals, including 714,000 children younger than 5 years and 524,000 individuals aged 5 years or older in 2010. Study showed that 433,000 more deaths occurred worldwide in individuals aged 5 years or older in 2010 than was suggested by WHO estimates. [6]

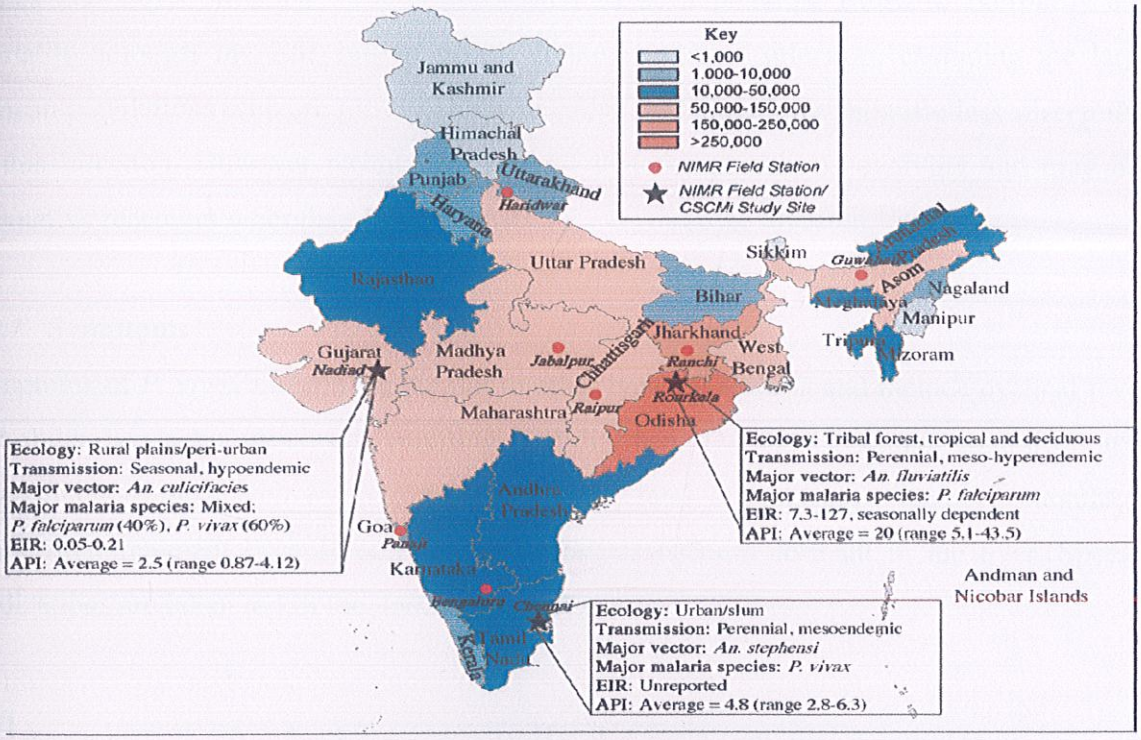


Figure 2: Malaria endemicity in India (http://www.nvbdcp.gov.in/, May 12, 2012)

1.0.4 Malaria in pregnancy

Malaria is one of the major causes of maternal, fetal and neonatal morbidity and mortality worldwide. [7] Malaria poses a severe threat to the pregnancy of any woman and a woman who is pregnant is at greater risk from malaria than one who is not.[8] Non-immune pregnant women are at high risk of fetal loss and of complicated malaria.[9] Miscarriages, stillbirths and premature births are common in pregnant women with malaria.[10]

Chapter 2

REVIEW OF LITERATURE

2.0 Plasmodium vivax

The most common of four human malaria species (*P. falciparum*, *malariae*, *ovale*, and *vivax*) is *Plasmodium vivax*.[11] *P. vivax* is the most geographically widespread species of Plasmodium causing human disease, with most cases reported in Central and Southeast Asia, Africa and Latin America.[12] It is considered to be a potential cause of morbidity and mortality amongst the 2.85 billion people living at risk of infection, excluding the large African populations who are mostly Duffy negative and, therefore, naturally less susceptible to this infection. However recent data suggest that the parasite is evolving and may use alternative receptors other than Duffy (DARC) for erythrocyte invasion. [13]

2.0.1 Symptoms [14]

Symptoms of *P. vivax* malaria are similar to other types of malaria and include cyclical fever and chills, headache, weakness, vomiting, and diarrhea. The most common complication is enlargement of the spleen. *P. vivax* malaria is rarely fatal, but relapses often occur months to years after treatment because some of the parasites become dormant in the liver. Special medications are taken to kill the dormant parasites.

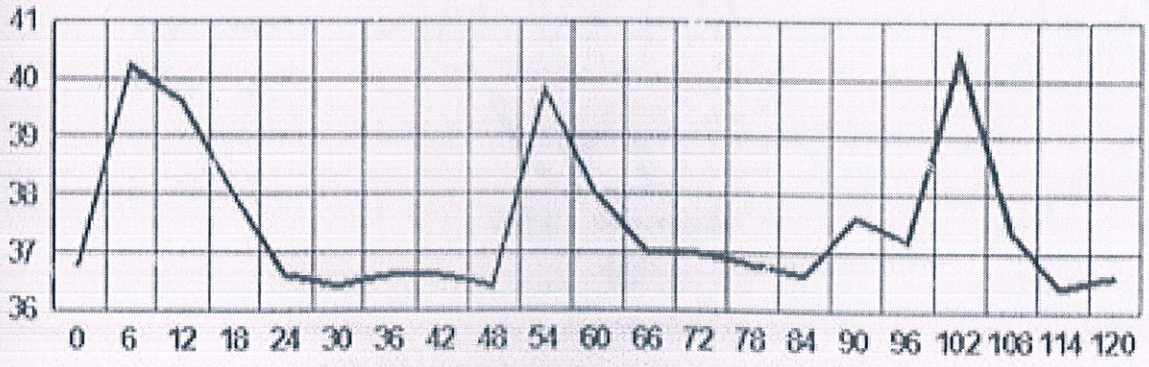


Figure 4: Malaria tertiana: 48h between fevers (*P. vivax*)

2.0.2 Life cycle of *Plasmodium vivax*

Plasmodium vivax has hypnozoites in their life cycles. [15-16] *P. vivax* like *P. falciparum* is introduced into the bloodstream by the *Anopheles* mosquito. The parasites enter the liver cells, where they divide to form schizonts consisting of numerous merozoites. [17] Merozoites invade red blood cells leaving the liver, and reproduce. After 48 hours or so, enough merozoites are produced to burst the red blood cells, which results in fever and chills characteristic of malaria. Some merozoites then develop into male or female forms, which when taken up by a mosquito, can start the cycle over again.

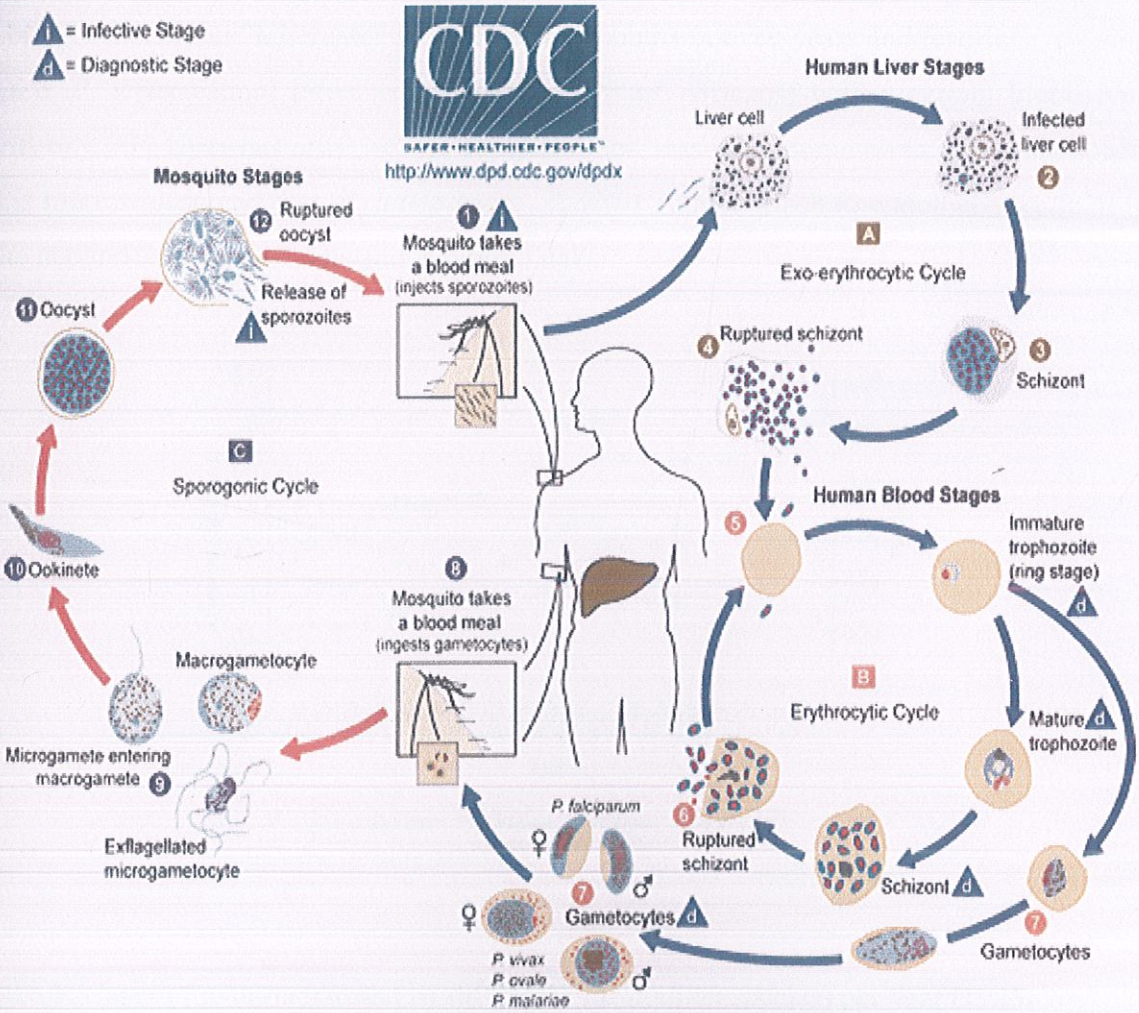


Figure 3: Life Cycle of *P. vivax* (http://dpd.cdc.gov/dpdx/htmL/Malaria.htmL, April 22, 2012)

2.0.3 Comparison to *P. falciparum*

P. falciparum has been used extensively in *in vitro* screens since 1976; techniques to culture the other three human *Plasmodium* species have been less successful. For *P. vivax* and *P. ovale* drug sensitivity testing remains a problem as continuous culture of these species is difficult due to host cell and nutritional requirements; and only short term cultures have been used in drug tests. [18] *In vitro* culture of the exoerythrocytic liver stages of malaria infection have been achieved using hepatomas and hepatocytes to culture *P. berghei*, *P. vivax* and *P. falciparum*, [19] but these models have not been used widely in drug evaluation studies.

P. vivax differs from *P. falciparum* in several ways. The parasite invades younger, smaller red blood cells. It can "hibernate" in the liver for months or even years and resurface, causing disease. *P. vivax* cannot infect people (95% of West Africans) with a certain blood type (Duffy+). [20] *P. vivax* has fewer severe complications, it is more common in temperate zones, and is more widespread than *P. falciparum*. *P. vivax* cannot attach to endothelial cells deep in the network of blood vessels and is rarely fatal.

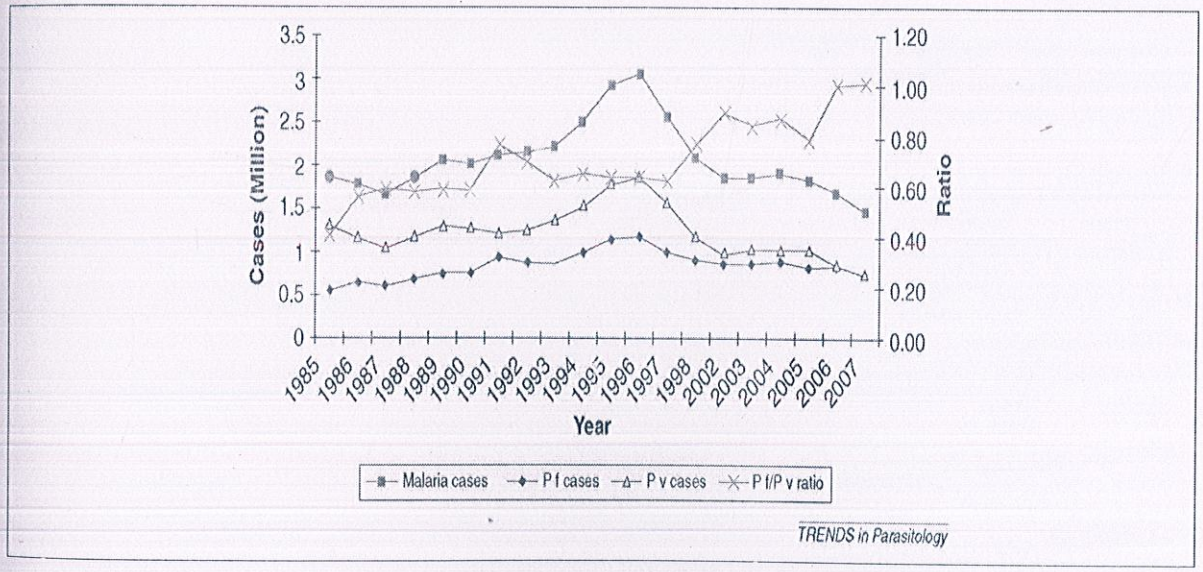


Figure 5: Cases of malaria (caused by different *Plasmodium* species) per year (1985-2007). [21]

2.0.4 Treatment

P. vivax malaria has been treated with chloroquine and primaquine. Primaquine acts against the liver stage, decreasing the risk of relapse. For radical cure, artemisinin combination therapy in combination with primaquine, may be used for *P. vivax* malaria. [22] Alternate drugs are being used and explored as the parasite is becoming resistant to chloroquine and primaquine. [23]

2.0.5 Chloroquine Resistance in *P.vivax*

The first cases of chloroquine-resistant *P. vivax* parasites were reported in 1989 from Papua, New Guinea [23] and Northern Papua, Indonesia.[24-26] More cases have been reported recently from Myanmar, [27] Viet Nam, [28] India, [29] Turkey, [30] and South America.[31] Increasing morbidity and mortality due to the emergence of *P. vivax* resistance to chloroquine (CQ) [32] results in an urgent need to find alternative treatments for *P. vivax* infection.

2.1 Roylea cinerea (D. Don) Baill. [33]

Synonyms: *Ballota cinerea* D. Don., *Phlomis calycina* Roxb., *Roylea calycina* (Roxb.) Briq., *Roylea elegans* Wall. ex Benth.

Folk: Patkarru; Titpaati, Karanoi, Karui (Kumaon); Kaur, Kauri (Punjab).

Roylea cinerea is a pleasantly aromatic shrub. Stems, including older woody parts, densely greyish tomentose with a covering of ad pressed and some spreading glandular hairs. Leaves 2-4 x 0.7-3 cm, ovate, crenate to almost lobed, truncate or broadly cuneate, acute, with few scattered hairs on adaxial side, below with numerous eglandular spreading hairs mainly on veins and densely gland-dotted; petiole to 8 mm. Verticillasters 4-12-flowered, distant, on very short peduncles. Bracts linear-subulate, c. 3.5 mm. Pedicels 0.5 mm. Calyx c. 15 mm with adpressed-spreading eglandular hairs mainly on veins and sessile oil globules; tube c. 6-7 mm; lobes subequal, elegant, oblong-elliptic narrowed at base, obtusely acuminate, reticulately veined, c. 7 x 2 mm, ± rigid; calyx not or scarcely enlarging in fruit. Corolla white or pink, c. 13 mm; tube slender straight, c. 9 mm, annulate; upper lip entire, pilose-villous; lower lip 3-lobed, median slightly larger. Nutlets (scarcely mature) c. 3.5 x 2 mm, apically ± truncate and papillose.

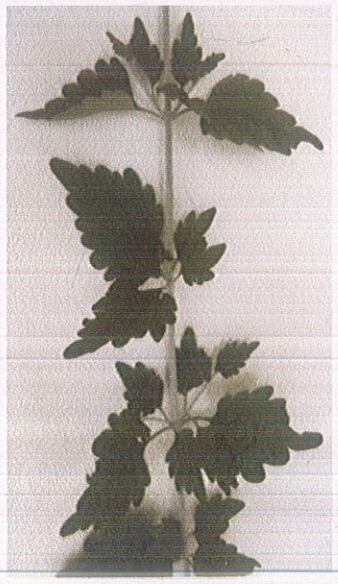


Figure 6: Leaves of *Roylea cinerea*

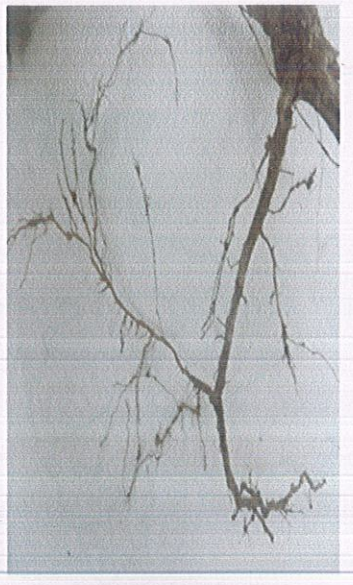


Figure 7: Roots of *Roylea cinerea*

Figure 8: Flowering of *Roylea cinerea*

2.1.1 Taxonomy

Domain: *Eukaryota* - Whittaker and Margulis, 1978 - eukaryotes

Kingdom: *Plantae* - Haeckel, 1866 - Plants

Subkingdom: *Viridaeplantae* - Cavalier-Smith, 1981

Phylum: *Magnoliophyta* - Sinnott, 1935 Ex Cavalier-Smith – Flowering Plants

Subphylum: *Euphyllophytina*

Infraphylum: *Radiatopses* - Kenrick and Crane, 1997

Class: *Magnoliopsida* - Brongniart, 1843 - Dicotyledons

Subclass: *Lamiidae* - Takhtajan Ex Reveal, 1992

Superorder: *Lamianae* - Takhtajan, 1967

Order: *Lamiales* - Bromhead, 1838

Family: *Lamiaceae* - Lindley, 1836 - Mint Family

Genus: *Roylea*

Specific epithet: *cinerea* - Wall.

Botanical name: - *Roylea cinerea* Wall.

2.1.2 Geographical Distribution of *Roylea cinerea*

Roylea cinerea (D. Don) Bailon is widely distributed in the Himalayan region from Kashmir to Nepal. [34]

Figure 8: Geo-Distribution Map for *Roylea cinerea*
(www.envis.frlht.org/distrimaps/?plant_id=3953, May, 16, 2012)

2.1.3 Constituents

The leaves contain β-amyrin, betulin, β-sitosterol, stigmasterol, cetylalcohol, glucose, fructose, arabinose and palmitic, stearic, oleic, gallic, oxalic and tartaric acids. The leaves and stems contain the diterpenes, calyenone, precalyone and calyone and a triterpene, moronic acid.[35]

2.1.4 Traditional Uses

The leaves of *Roylea cinerea* have been used as a bitter tonic and febrifuge, also as a tonic for contusions.[34] Aerial parts of *Roylea cinerea* have also shown spasmolytic and CNS depressant activity.[36] Ethno botanic use against malarial fever has been reported.[37] The juice from the leaves of *Roylea cinerea* is used in fever.[38] In some areas of India shoots are crushed and eaten with salt to strengthen the liver.[39] The whole plant is used in the preparation of *Keem* – the starter of *Soor* (an alcoholic beverage).[40] In disease of throat infusion of leaves is given. Fresh juice of plant are given for syphilis. [41] The plant (aerial parts) has also been traditionally used for its hepatoprotective activity. [42]

Antioxidant Activity

The antioxidant activities of the four extracts (petroleum ether, methanol, aqueous and hexane) of Roylea cinerea (bark) have been determined. The successive methanol and aqueous extracts have shown the maximum antioxidant activity with IC_{50} values of 25 µg/mL and 22.14 µg/mL respectively. The petroleum ether and hexane extracts have also shown antioxidant activity with IC_{50} values of 37 µg/mL and 31 µg/mL, respectively. The values of Total Phenolic Content (TPC) in *Roylea cinerea* (bark) were found to be 50.1, 81.0, 87.5 and 220.6 µg Gallic acid equivalent per gram of the plant material. [43]

Antiplasmodial Activity

The petroleum ether and the chloroform extracts of *Roylea cinerea* have shown good antiplasmodial activity. The chloroform extracts of *Roylea cinerea* exhibited Selectivity Index (SI) values for *Plasmodium falciparum* between 8 and 78, while the petroleum ether extracts of *Roylea cinerea* showed SI values of 8, 4, 10 and 10, respectively. The petroleum ether, chloroform and methanol extracts of *Roylea cinerea* showed antiprotozal activity with IC_{50} values of 4.39, 1.84 and >5 µg/mL respectively. Petroleum ether and chloroform extracts of *Roylea cinerea* showed IC_{50} values >40 mg/mL. [44]

2.2 Natural Products in Drug Discovery

Natural products represent a source of potential new pharmacophores, the warheads that are needed for killing the parasite. For discovering and developing new drugs pharmaceutical research in natural products represents a major strategy. Since ancient times the use of medicinal plants for the treatment of parasitic diseases is well known and documented e.g. by the use of *Cinchona succiruba* (Rubiaceae) as an antimalarial. At the beginning of the 19th century the discovery of pure compounds as active principles in plants, was first described, and the art of exploiting natural products has become part of the molecular sciences since then. The discovery of quinine [45] from *Cinchona succiruba* (Rubiaceae) and its subsequent development as an antimalarial drug has represented a milestone in the history of antiparasitic drugs from nature for the treatment of all parasitic diseases. Plants contain a high variety of constituents and it is often claimed that the use of a whole plant rather than one single purified product may be more effective therapeutically. New classes of medicines to combat malaria, due to infection by *P. falciparum* and *P. vivax* are urgently needed. Natural products based on herbal remedies are widely in use in the community. Quinine, the original natural product used in anti-malarial chemotherapy was identified from cinchona tree bark, and purified in 1820. The attempt to synthesize quinine led to the development of methylene blue. From this came the classical 4-aminoquinolines and amino-alcohols, such as chloroquine, amodiaquine and mefloquine, which have been the mainstay of malarial treatment over the last century. The natural product naphthoquinone lapichol also was identified as the active ingredient in tree bark used to treat malaria. [46]

Chapter 3

WORK DESCRIPTION

3.0 MATERIALS AND METHODS

All chemicals and reagents used in current study were of laboratory grade. The solvents and reagents used were petroleum ether, ethyl acetate, hexane, methanol, ethanol, acetic acid, ferric chloride, sodium hydroxide, chloroform, sulphuric acid, hydrochloric acid, Fehlings solution, Benedicts reagent. All the solvents were used as provided by the manufacturers. Extraction was done using soxhlet apparatus (JSGW). After the extraction the solvents were evaporated using the Equitron rotatory evaporator and the aqueous extract was lyophilized in Alied Frost Lyophilizer. The prepared silica gel GF 254 plates, activated at 110 °C in the oven (Macflow Engineering), and Merck pre-coated silica GF 254 plates were used for thin layer chromatography. Iodine vapor, vanillin and anisaldehyde - sulphuric acid spray reagents were used for detection of compounds. ^{1}H NMR spectra were recorded with Bruker AVANCE II 400 MHz spectrometer using $CDCl_3$ as solvent, and tetramethylsilane was used as internal standard.

3.0.1 Collection of Plant Material

Whole plant of *Roylea cinerea* was collected on 16th August 2011, from Domehar Bani, Waknaghat, Solan (Himachal Pradesh) and authenticated by Dr. N.K. Kapoor (Himalayan Forest Research Institute, Conifer Campus, Panthaghatti, Shimla, Himachal Pradesh). The fresh parts of *Roylea cinerea* were dried at room-temp (25-30 °C, shade) for 15-20 days and powdered using a grinder.

3.0.2 Extraction of the Plant Material

Preparation of ethanolic extracts of leaves, roots and stems of the plant using Hot Continuous Extraction (Soxhlet)

Powdered plant material 100 g (each of leaves, roots and stems) was subjected to hot continuous extraction in a soxhlet apparatus (20 cycles) with 700 mL of 95% ethanol. The extracts were allowed to cool and solvent was removed *in vacuo* using rotatory evaporator at 40°C. Extracts were stored in glass vials at 4°C in a refrigerator. The preliminary phytochemical analysis was carried out.

Table 1: Description of the extraction process (of ethanolic extracts)

Solvent	Weight of the plant material (g)	Vol. of the solvent (mL)	Duration of 1st cycle (mins)	No. of cycles
Ethanol	Leaves – 100	700	60	20
Ethanol	Roots – 100	550	50	21
Ethanol	Stems – 100	550	50	20

Preparation of aqueous extract of leaves using Hot Continuous Extraction (Soxhlet)

Powdered leaves 100 g were subjected to soxhlet extraction (20 cycles) with 700 mL of distilled water. Then 200 mL of the obtained solvent containing the extract was lyophilized and to the rest 500 mL solvent containing the extract, toluene (200 mL) was added, to make an azeotrope, and the solvent was removed *in vacuo* using rotatory evaporator at 50°C. Extracts were stored in glass vials at 4°C in a refrigerator. The preliminary pytochemical analysis was carried out.

Table 2: Description of the extraction process (of aqueous extract)

Solvent	Weight of the plant material (g)	Vol. of the solvent (mL)	Duration of 1st cycle (mins)	No. of cycles
Distilled Water	Leaves – 100	700	80	20

3.0.3 Lyophilization

The first step in the lyophilization process was to freeze the solvent containing the extract (200 mL in the round bottom flask), to solidify all of its water molecules, at -80°C. Once it was frozen, the product was placed in the lyophilizer at -80°C till the product was dry (three days). The product obtained was weighed and stored in closed vials.

3.0.4 TLC PROFILING

Technique

The sample(s) were applied to the layer of adsorbent, near one edge, as a small spot of a solution. After the solvent had evaporated, the adsorbent-coated sheet was placed vertically in a closed chamber, with the edge to which the spot was applied down. The spot on the thin layer plate was positioned above the level of the solvent in the container (Figure 10). The solvent, which is in the bottom of the container, travels up the layer of adsorbent, passes over the spot, and, as it continues up, effects a separation of the compounds in the spot ("develops" the chromatogram). When the solvent front had nearly reached the top of the adsorbent, the thin layer plate was removed from the container (Figure 11).

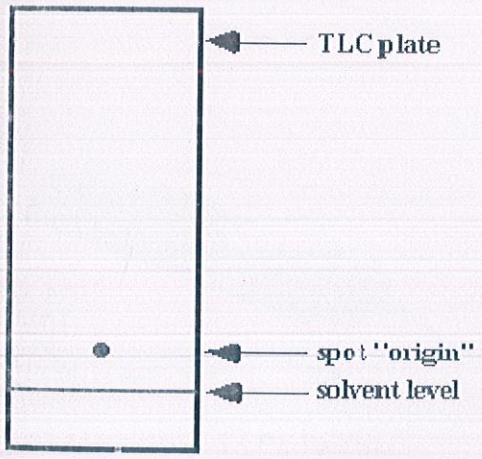


Figure 10: Position of the spot on a thin layer plate.

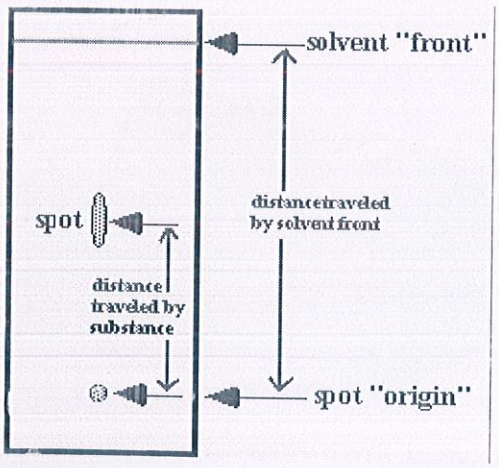


Figure 11: TLC plate showing distances traveled by the spot.

Because the distance traveled by a compound relative to the distance traveled by the solvent front depends upon the molecular structure of the substance. TLC can be used to identify

substances as well as to separate them. The relationship between the distance traveled by the solvent front and the substance is usually expressed as the **R_f value**:

$$R_f \text{ value} = \frac{\text{distance traveled by substance}}{\text{distance traveled by solvent front}}$$

The R_f values are strongly dependent upon the nature of the adsorbent and solvent.

Visualization

When the solvent front had moved to within about 1 cm of the top end of the adsorbent, the plate was removed from the developing chamber, the position of the solvent front marked, and the solvent allowed to evaporate. The components of the sample which were colored, were observed directly. If not, they were visualized by ultraviolet light on the plate (Figure 11) or by allowing the plate to stand for a few minutes in a closed container in which the atmosphere is saturated with iodine vapor. The spots were also visualized by spraying the plate with a reagent.

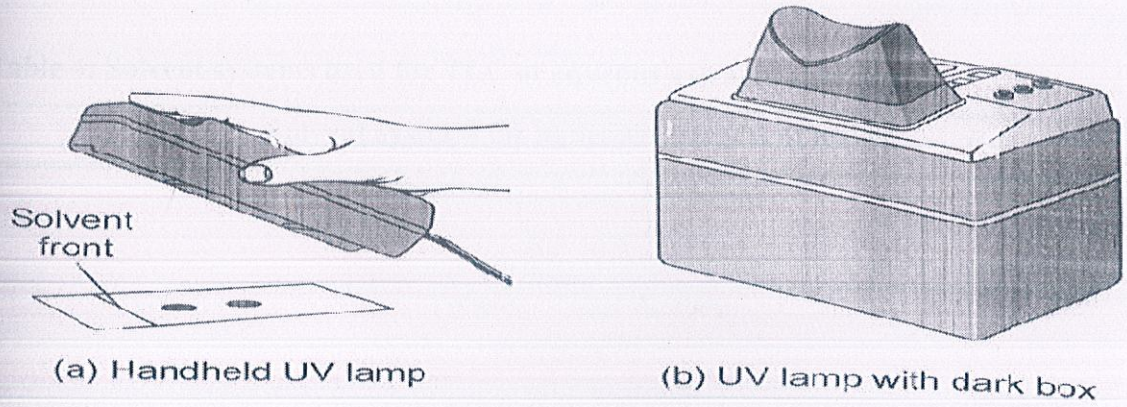


Figure 12: Visualization of spots by UV light.

Different Solvent Systems for TLC Profiling

Table 3: Solvent systems used for TLC of ethanolic extracts (leaves, roots& stems)

Solvent systems for ethanolic extracts		
Leaves	**Roots**	**Stems**
Dichloromethane: Methanol 9.8 : 0.2	Toluene : Ethyl Acetate 7 : 3	Toluene : Ethyl Acetate 9 : 1
Dichloromethane : Methanol 9 : 1	Dichloromethane : Methanol 9.5 : 0.5	Toluene : Dichloromethane : Methanol 4.25 : 5 : 0.75
Dichloromethane : Methanol 9.5 : 0.5	Acetonitrile : Water 4 : 8	
Toluene : Ethyl Acetate 7 : 3		
Toluene : Dichloromethane 6 : 4		

Table 4: Solvent systems used for TLC of aqueous extracts of leaves

Solvent systems for aqueous extracts of leaves
Dichloromethane : Methanol 9.5 : 0.5
Toluene : Ethyl Acetate 7 : 3
Toluene : Dichloromethane: Methanol 4.25 : 5 : 0.75

Preparation of Stains for Developing TLC Plates

I. *p*-Anisaldehyde Stain

To 135 mL of absolute ethanol 5 mL of concentrated sulfuric acid, 1.5 mL of glacial acetic acid and 3.7 mL of *p*-anisaldehyde were added. The solution was then stirred vigorously to ensure homogeneity. The resulting staining solution was stored in a 100 mL bottle.

II. Vanillin

A solution of 15g vanillin in 250 mL ethanol and 2.5 mL conc. sulfuric acid was prepared.

III. Dinitrophenylhydrazine (DNP)

12g of 2, 4-dinitrophenylhydrazine, 60 mL of conc. sulfuric acid, and 80 mL of water were mixed in 200 mL of 95% ethanol.

IV. Ferric Chloride

A solution of 1% ferric (III) chloride in 50% aqueous methanol was prepared.

V. Bromocresol Green Stain

To 100 mL of absolute ethanol 0.04 g of bromocresol green was added. Then a 0.1 M solution of aqueous NaOH was added dropwise until a blue color just appeared in solution (the solution should be colorless prior to addition). Then it was stored in 100 mL bottle.

3.0.5 Phytochemical Screening

Ethanolic extracts were subjected to preliminary phytochemical screening for the detection of various phytoconstituents such as alkaloids, glycosides, tannins and phenolic compounds, steroids and saponins.

I. Test For Alkaloids

Extract (2-3 mL) was added to 5 mL of 1% HCl and was kept in boiling water bath. The 1 mL of filtrate was treated with drops of :

1) Mayer's reagent. Turbidity or precipitate observed was taken as indication for the presence of alkaloids.

2) Wagner's reagent: brick red colouration which indicates for the presence of alkaloids.

II. Test For Saponins

Extract (0.5 g/mL) was stirred with water in a test-tube. Frothing persist on warming was taken as evidence for the presence of saponin.

III. Test for Steroids (Salkowski Test)

About 0.5 mL of the extract was dissolved in 3 mL of $CHCl_3$ and was filtered. To the filtrate conc. H_2SO_4 was added which formed a lower layer. A reddish brown colour was taken as positive for the presence of steroid ring.

IV. Test for tannins

Extract (2-3 mL) was taken in a test tube and 1 mL of 5% ferric chloride test solution was added. A blue-black colouration indicated the presence of tannin.

V. Test for Glycoside (Keller Killani Test)

Extract was taken in acetic acid containing trace of ferric chloride and transferred to surface of concentrated sulphuric acid. At this junction reddish brown colour which gradually becomes blue indicates presence of glycosides.

3.0.6 Column Chromatography

Preparation of the column

The column was plugged with a small amount of cotton and it was tapped down lightly. Then a slurry of silica gel (100 -200 mesh), approximately 75 g, in petroleum ether was prepared and poured into column and allowed to settle while allowing the flow of solvent through the column. The sample was loaded onto the silica gel column. Dry method was used, and first the sample JMVAJUIT 01 (1.4g) was dissolved, in the minimum amount of methanol and about 75 mg of silica (100-200 mesh) was added. The mixture was swirled until the solvent evaporated and only a dry powder remained. The dry powder was placed on a folded piece of weighing paper and carefully transferred to the top of the prepared column. Petroleum ether was added to the top and the elution process began. The column was eluted using hexane: ethyl acetate gradient (1%, 2%, 4%, 8%, 20% and 40%) followed by elution with dichloromethane (400mL) and then dichloromethane : methanol gradient (0.5%, 1%, 2% and 5%). Equal sized fractions were collected sequentially and carefully labeled for later analysis. The fractions were then analyzed by TLC. The fractions with similar TLC patterns were combined and the solvent was evaporated *in vacuo* at 40 °C.

3.0.7 Preparative Thin-Layer Chromatography

Compound JMVAJUIT01-05 obtained after column was purified by preparative thin-layer chromatography. A large developing chamber and thickly coated plates were used.

Preparation of TLC plate

TLC applicator was set up. A pencil line about 1" from the bottom of the plate (20 X 10 cm) was drawn. The applicator was set up so that the tip of the capillary traverses the line as it was drawn along the bench top. The applicator was filled with Silica gel and binder (Calcium

sulphate). The plate was streaked on the line. The thickness of the band was kept 2 mm and kept for drying in oven at 110 °C.

Preparative TLC Procedures

The sample was dissolved in dichloromethane. Solvent system of Hexane: Ethyl Acetate 30% was prepared and added in the TLC developing chamber.

Development of plate

The plate was developed, multiple times, in the developing chamber until the solvent front reached within one inch of the top. Then the plate was removed from the tank and the solvent front was marked with a pencil.

Isolation of the product

When the plate was dry enough, the bands were visualized using UV light and a band was observed at the top. Using a cutter knife the top band was scrapped off onto a lengthwise folded piece of clean, white paper. Then the same TLC plate was run in Hexane: Ethyl Acetate 40%, twice and the bands were separated by scrapping off. The top layer was washed off the silica into a round bottomed flask using 60 mL of Ethyl acetate and TLC was performed in Hexane: Ethyl Acetate 1%, Hexane: Ethyl Acetate 30%, Hexane: Ethyl Acetate 40%. The solvent was removed *in vacuo* using rotatory evaporator at a temperature of 40 °C. The weight of the product was determined. The product was characterized by ^{1}H NMR.

Test For Evaluating Purity

Compound JMVAJUIT01-09 obtained from column chromatography was dissolved in dichloromethane and then evaluated to check purity using, Hexane: Ethyl Acetate 10%, Hexane: Ethyl Acetate 20%, Hexane: Ethyl Acetate 30%, Hexane: Acetone 20%, Toluene: dicholoromethane 40%, Toluene: Ethyl Acetate 30%. The product was characterized by ^{1}H NMR spectral analysis.

CHAPTER 4

RESULTS AND DISCUSSION

Current project involved the collection, identification, extraction and phytochemical evaluation of extracts derived from commonly occurring local plant *Roylea cinerea* growing in Domehar Bani, Waknaghat, Solan (Himachal Pradesh), authenticated by Dr. N.K. Kapoor (Himalayan Forest Research Institute, Conifer Campus, Panthaghatti, Shimla, Himachal Pradesh). The plant has been reported to treat a variety of diseases in traditional system medicine and antiprotozoal activity against erythrocytic stages of *P. falciparum* have also been reported. Using Hot Continuous Extraction (Soxhlet), the percentage yield of ethanolic extracts obtained was 11.72 w/w of dried leaves, 2.11 w/w of roots, and 3.35 w/w of stems (Table 5), and the percentage yield of aqueous extracts of dried leaves was 1.43 w/w (lyophilized product) and 4.67 w/w (product after rotatory evaporation) (Table 6). Phytochemical screening was done (Table 9). TLC profile of the ethanolic extract and aqueous extract was also done by observing the developed TLC plate under visible and UV (254 nm and 366 nm) light and after derivatization with iodine and anisaldehyde - sulphuric acid spray reagent. Column chromatography of the ethanolic extract of leaves of *Roylea cinerea* was performed and the fractions with similar TLC patterns were combined and after evaporation in rotatory evaporator, they were stored in different vials & weighed (Table 8). TLC showing similar R_f values were selected and the purity of the compounds were determined using different solvent systems. Using preparative thin layer chromatography compound JMVAJUIT01-05 was purified to isolate a single compound and top layer was scrapped off. The weight of the product obtained was 18 mg. The product was characterized by ^{1}H NMR. Then compound JMVAJUIT01-09 isolated from column chromatography was evaluated for purity and sub column was done to isolate a single compound. The weight of the product obtained was 22.6 mg. The product was characterized by ^{1}H NMR spectral analysis.

Phytochemical screening of plant materials

In the present investigations, physicochemical characteristics of *Roylea cinerea* were studied. The preliminary phytochemical analysis showed the presence of various phytoconstituents. Ethanolic leaf and shoot extract showed the presence of alkaloids, phenolic compounds,

saponins and steroids. Ethanolic root extract showed the presence of alkaloids and saponins. Aqueous extract of leaves showed presence of phenolic compounds and saponins.

TLC finger printing profile:

The TLC profile of ethanolic extracts of leaves, stem and root and aqueous extract of leaves were established carried out by different solvent system. Amongst various solvent systems, Toluene : Ethyl Acetate (7:3) for ethanolic extract of leaves, Toluene: Dichloromethane : Methanol (4.25: 5: 0.75) for ethanolic extract of stems, Dichloromethane : Methanol (9.5 : 0.5) for ethanolic extract of roots and Dichloromethane : Methanol (9.5 : 0.5) for aqueous extract of leaves showed maximum resolution as depicted in (Table 7 & Figure 13, 14).

Percentage yield of extracts

Table 5: Percentage yield of the ethanolic extracts

Weight of the plant material (g)	% Yield
Leaves – 100	11.72 w/w
Roots – 100	2.11 w/w
Stems – 100	3.35 w/w

Table 6: Percentage yield of aqueous extract

Weight of the plant material (g)	% Yield
Leaves – 100	Lyophilized Product - 1.43w/w
	Product after rotatory evaporation - 4.67w/w

Thin layer chromatography

The TLC profile of ethanolic extract was carried out by using different solvent systems. The visualization of spots was done by observing the plate under UV light (both long and short) and after derivatizing with anisaldehyde - H_2SO_4 reagent.

Table 7: Solvent system in which a better separation of compounds was observed

Plant Parts	Solvent System (v/v)
Leaves (ethanolic extract)	Toluene : Ethyl Acetate (7 : 3)
Roots (ethanolic extract)	Dichloromethane : Methanol (9.5 : 0.5)
Stems (ethanolic extract)	Toluene : Dichloromethane : Methanol (4.25 : 5 : 0.75)
Leaves (aqueous extract)	Dichloromethane: Methanol (9.5 :0.5)

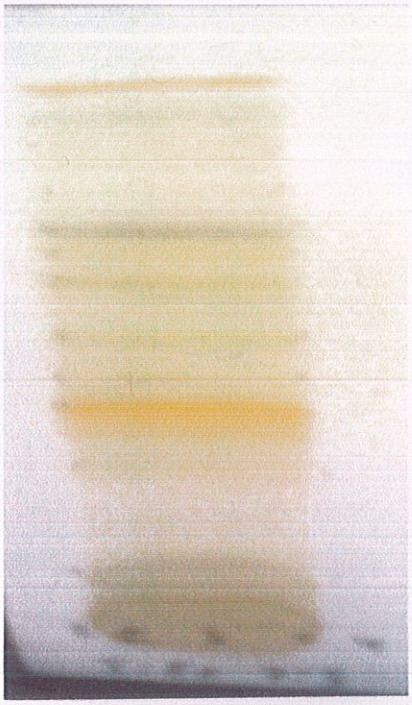


Figure 13: TLC of ethanolic extract of leaves.
Toluene: Ethyl Acetate (7 : 3)

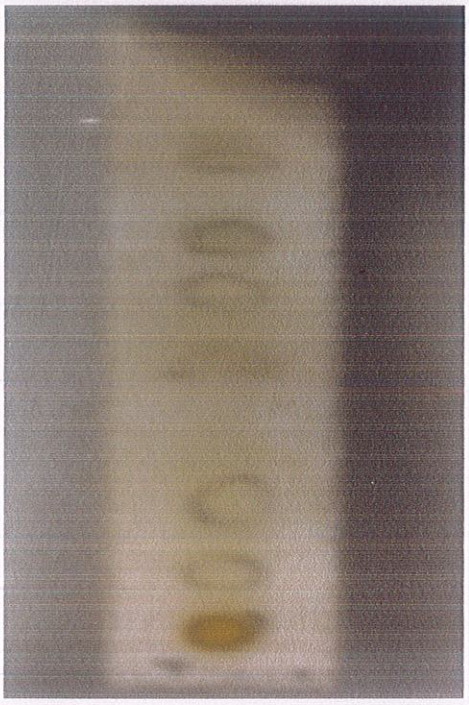


Figure 14: TLC of ethanolic extract of stem.
Toluene : Dichloromethane : Methanol (4.25: 5: 0.75)

Table 8: Fractions of compounds obtained after column chromatography

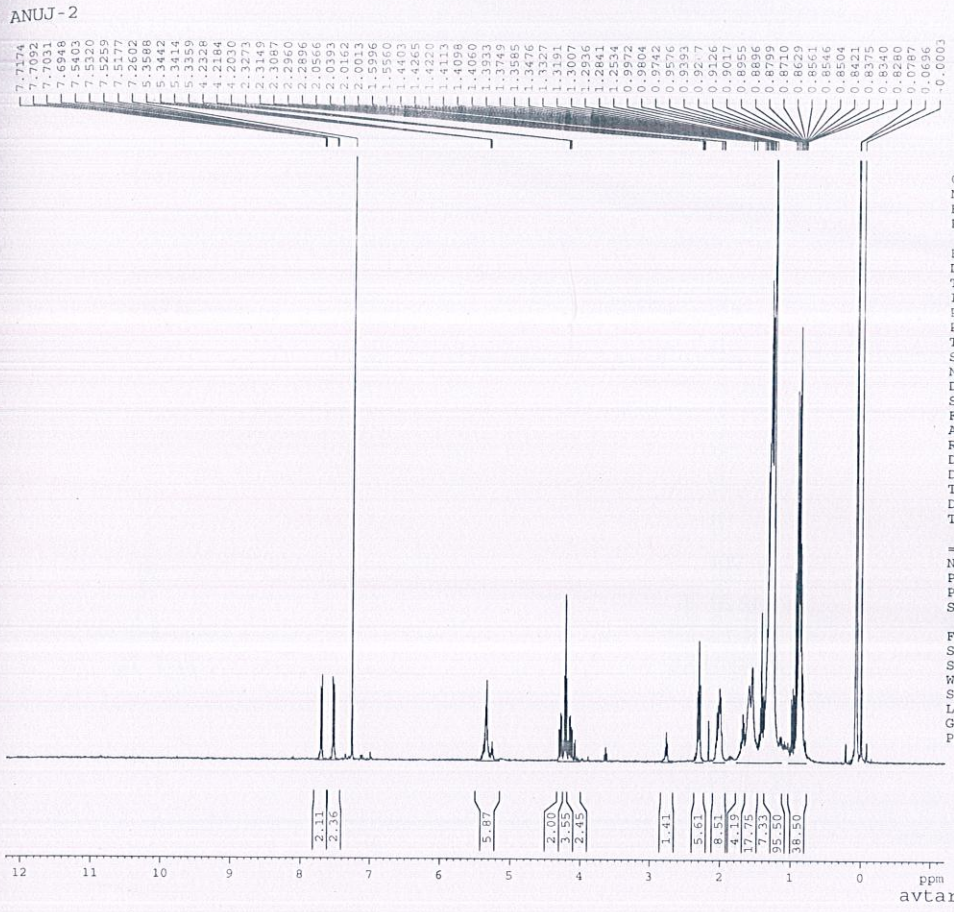
S.No.	Test Tube No.	Weight of compounds (mg)
1.	34-36	80
2.	28-30	100
3.	31-33	90
4.	21-22	50
5.	26-27	40
6.	19-20	130
7.	24-25	30
8.	23	20
9.	38-46	60
10.	47-48	50
11.	37	30
12.	52-54	130
13.	49	20
14.	50	70
15.	55-73	50
16.	78	20
17.	77	10
18.	88-90	70
19.	82-83	40
20.	74-76	40
21.	84-85	60
22.	80-81	30
23.	79	30
24.	91	60
25.	86-87	80

Table 9: Phytochemical screening

Phytochemical Tests	Ethanolic Extracts				Aqueous Extract of leaves
	Leaves	Activated Carbon Treated Leaf Extract	Shoots	Roots	
Glycoside (Keller Killani Test)	-	-	-	-	-
Alkaloids (Wagner's Test, Mayer's Test)	+	-	+	+	-
Phenolic compounds (5% Ferric Chloride Test)	+	-	+	-	+
Saponins	+	-	+	+	+
Steroids (Salkowski Test)	+	-	+	-	-

(+) Present ; (-) Absent

Figure 15: ^{1}H NMR of compound JMVAJUIT01-05



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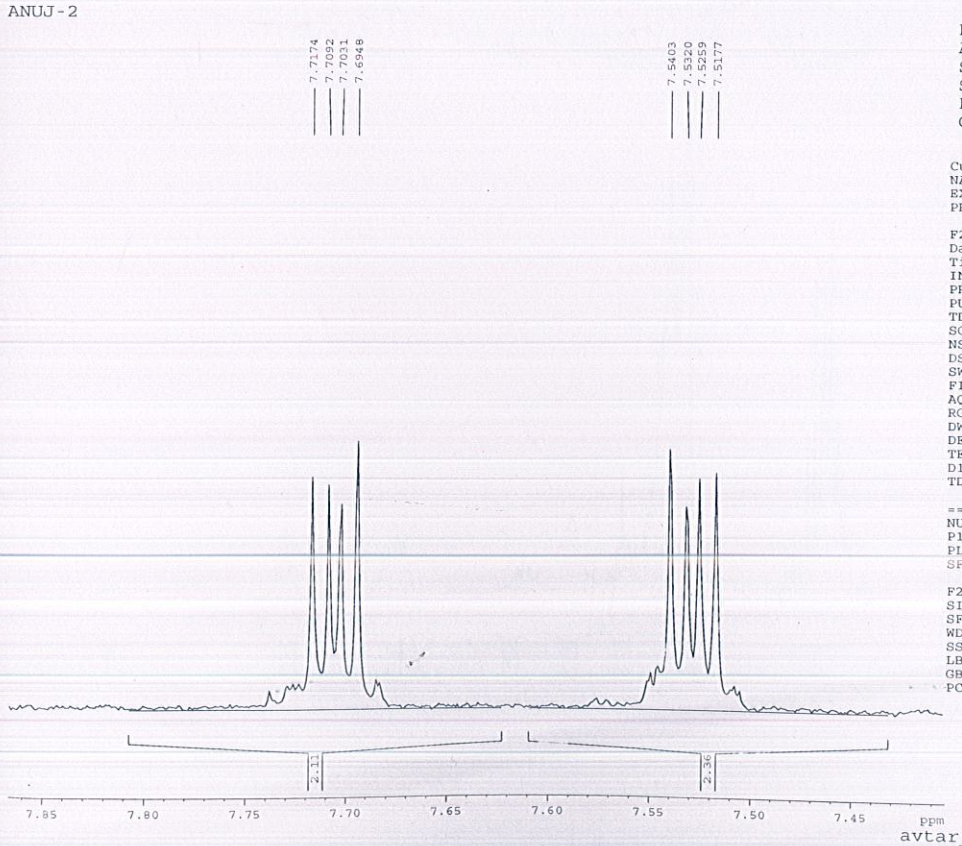

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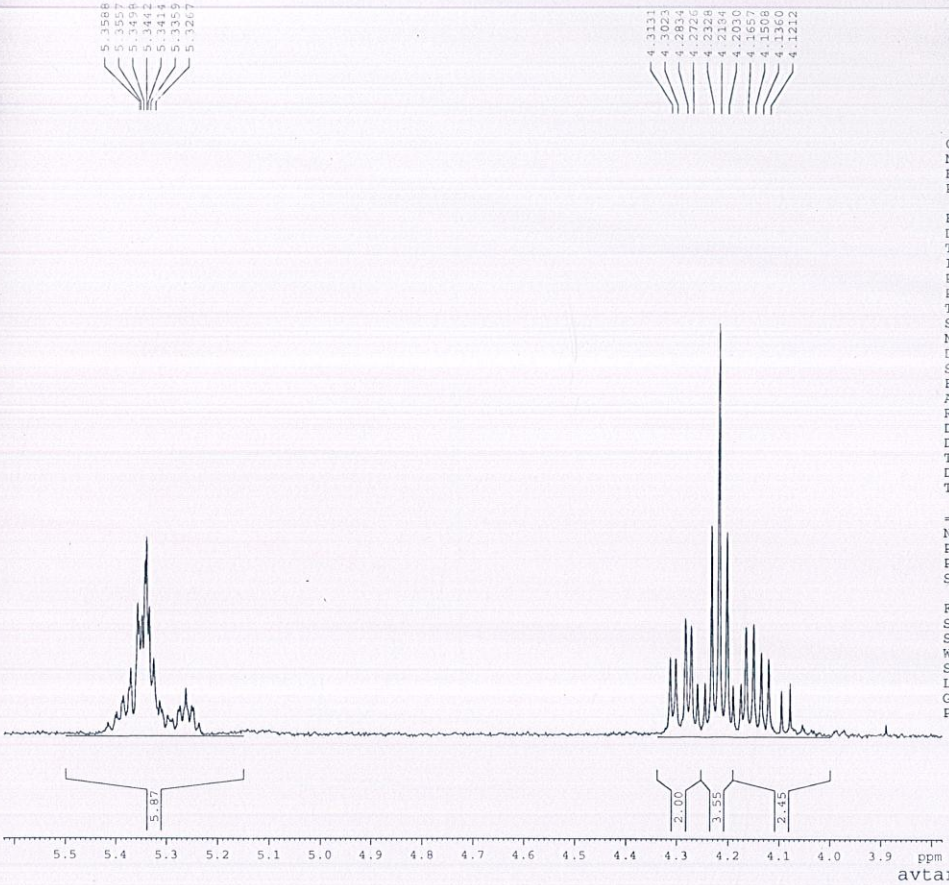

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SAIF
Panjab University
Chandigarh

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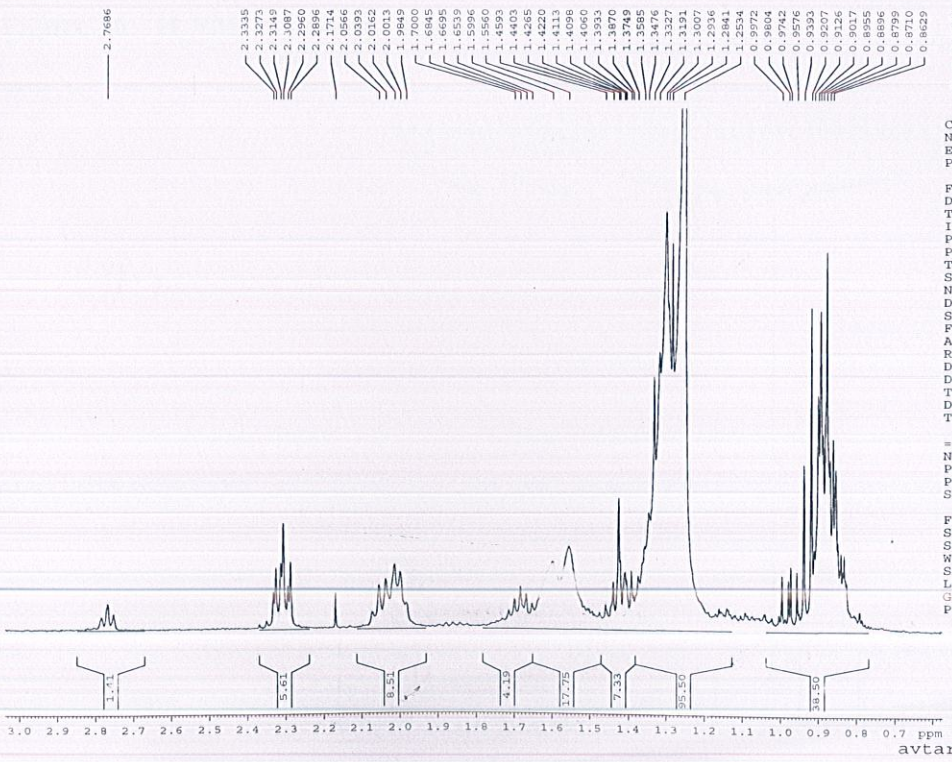

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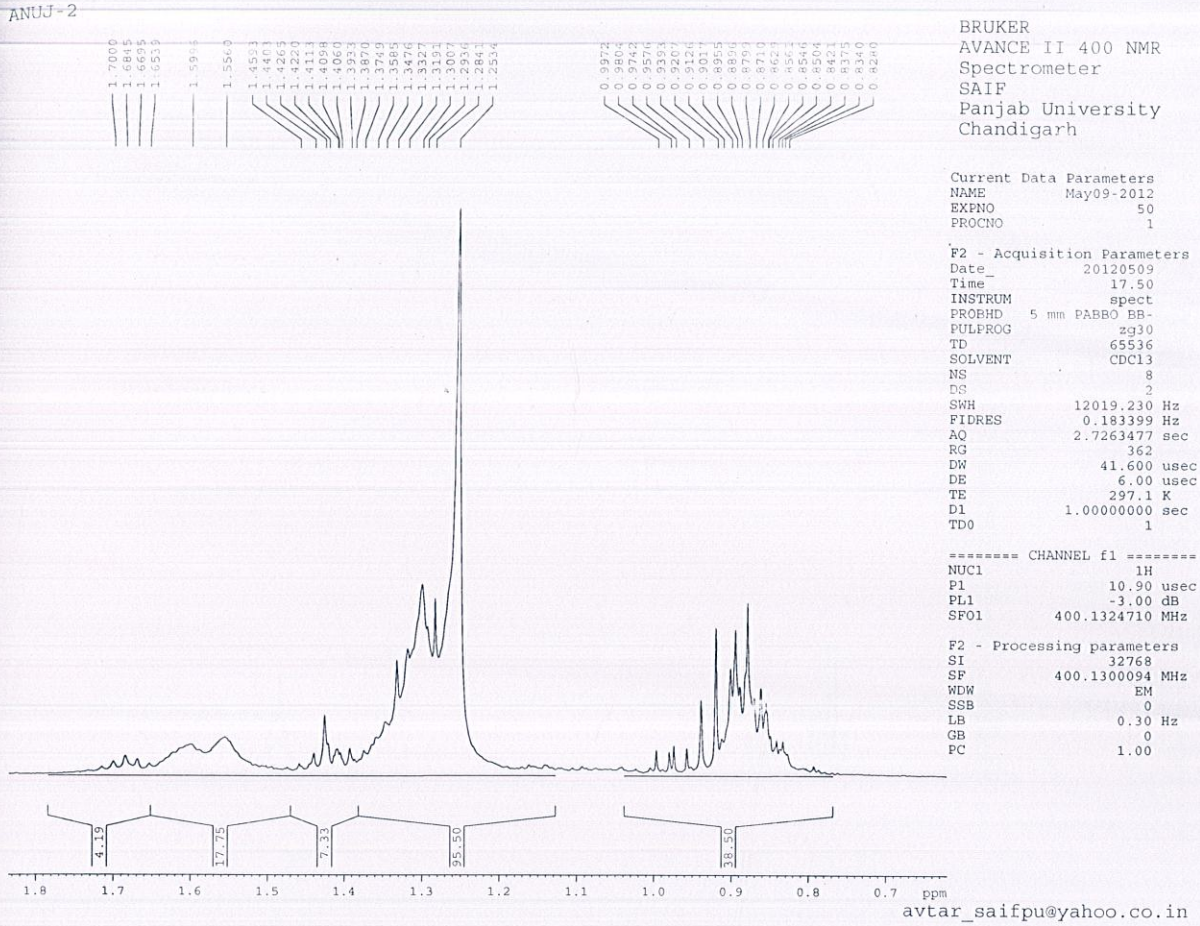
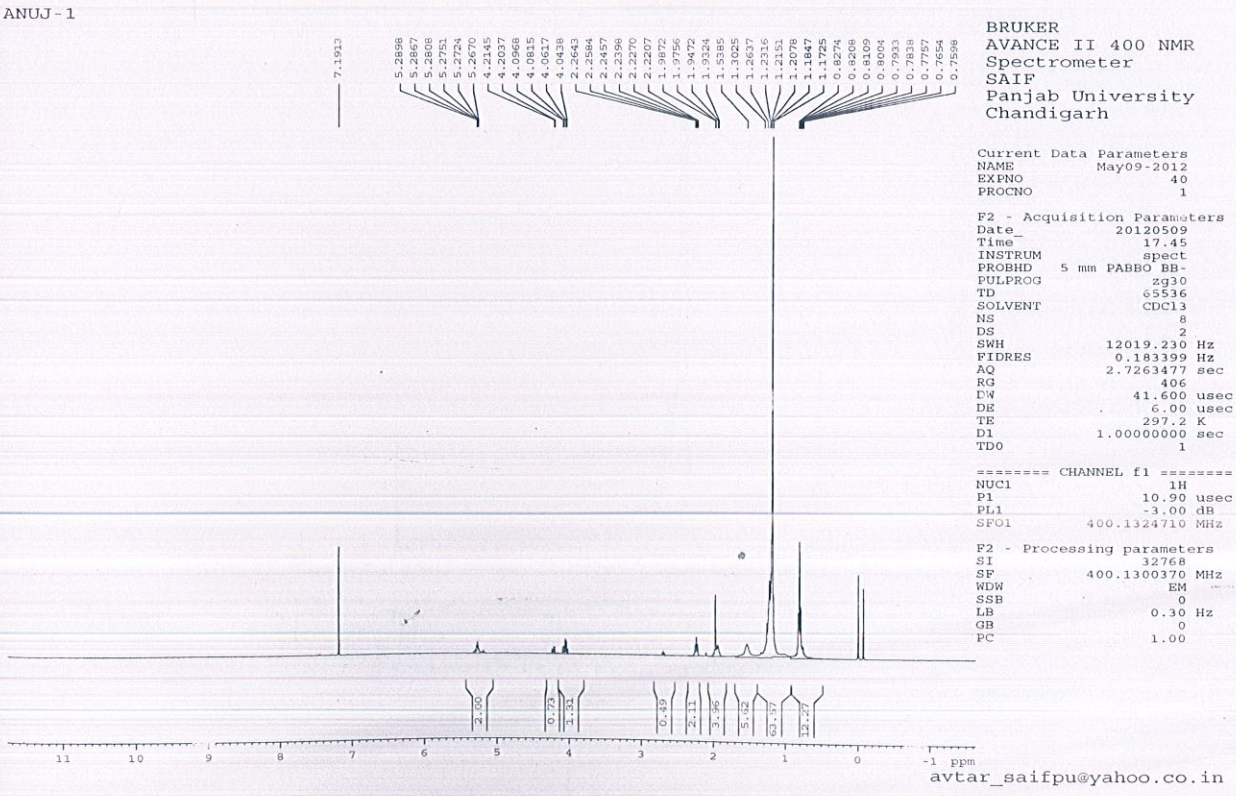


Figure 16: ^{1}H NMR of compound JMVAJUIT01- 09



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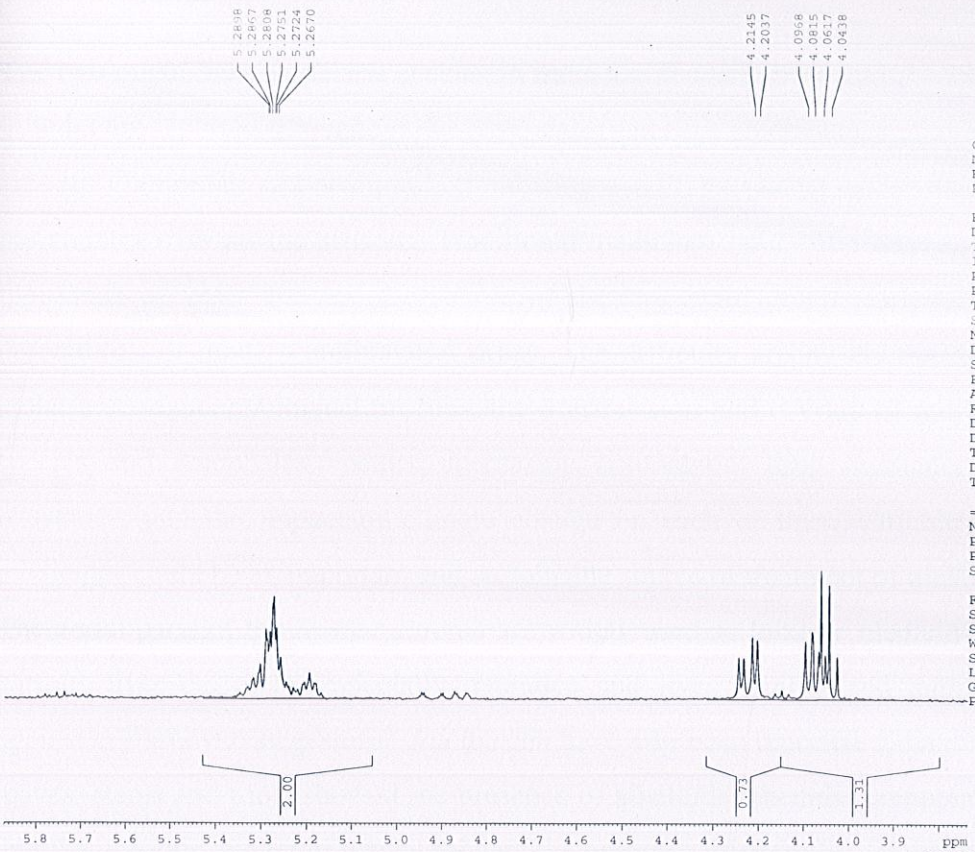

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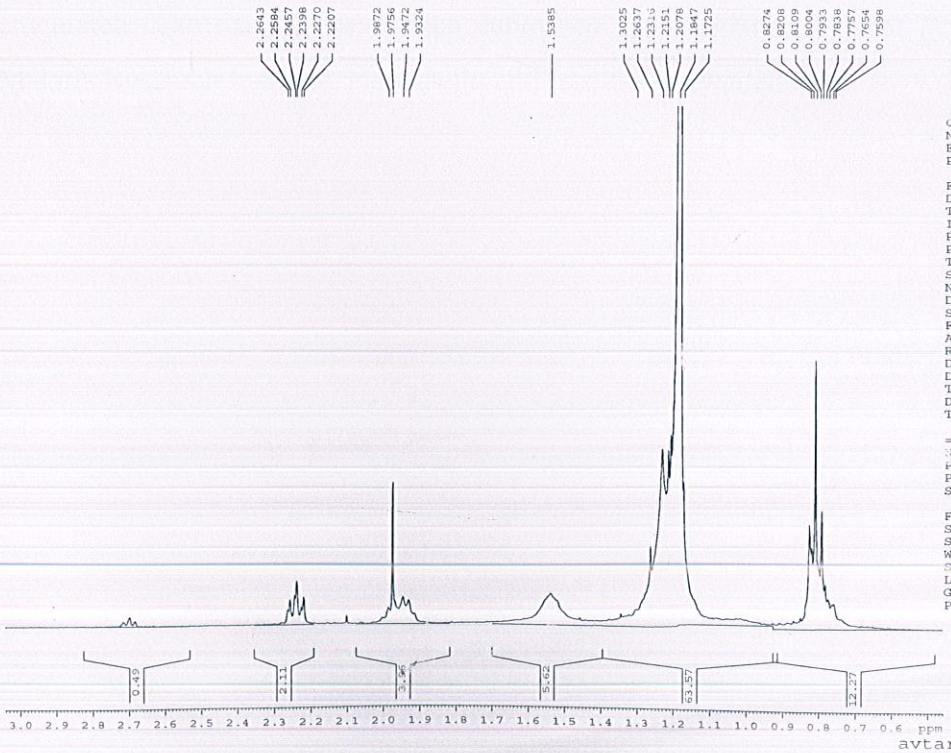

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CONCLUSION

The two major human malaria species in India are *P. falciparum* and *P. vivax*. Malaria cases in India are reported throughout the year, since a perfect combination of average temperature (15–30 °C), rainfall and precipitation-inducing conditions persist across the different parts of the country over all the seasons. Traditional medicines have been used to treat malaria for thousands of years and are the source of the two main groups (artemisinin and quinine derivatives) of modern antimalarial drugs. The difficulty arising are due to highly variable malaria eco-epidemiological profiles, the transmission and overlap of multiple Plasmodium species and Anopheles vectors, increasing antimalarial drug resistance and insecticide resistance, and the impact of climate change on each of these variables. The traditional medicines could be an important and sustainable source of treatment of malaria.

The aerial parts of the *Roylea cinerea* are widely used as folklore medicine for treatment of various liver disorders, especially, jaundice and liver debility and antiprotozoal activity against erythrocytic stages of *P. falciparum* have also been reported. Ethanolic extracts of the leaves, stems and roots showed the presence of alkaloids, phenolic compounds and saponins and the aqueous extract of leaves showed the presence of phenolic compounds and saponins. Isolation and ^{1}H NMR of two compounds have been carried out and the structures are being elucidated. The extracts have been submitted for evaluation against *P. vivax* at National Malaria Research Institute, New Delhi and results are awaited.

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