Research Article
AMERICAN JOURNAL OF PHARMACY AND HEALTH RESEARCH
www.ajphr.com
2015, Volume 3, Issue 3
ISSN: 2321–3647(online)

Evaluation of Phytochemical Screening, Anticancer and Antimicrobial Activities of *Robinia Pseudoacacia*

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ABSTRACT

The *Robinia pseudoacacia* (Fabaceae) is one of the medicinal plants, Native to North America, commonly distributed in various regions of Kashmir. The plant is used as an antispasmodium, febrifuge, antioxidant;diuretic, emollient, antitumor etc. The present study was carried out to evaluate the anticancer, antibacterial potentials of *Robinia pseudoacacia* along with the phytochemical screening of the major constituents of the aforesaid plant. The extracts of the *Robinia pseudoacacia* were phytochemically screened for the presence of Alkaloids, Flavonoids Saponins, Tannins, and Phenols. However the positive results for the detection of Flavonoids, Tannins and phenols were obtained. Further, the Methanolic extract of the same plant was evaluated for its anti-microbial activities where 100mg/ml of the extract showed between 12-19mm inhibitory zones on the test organism. The aforementioned plant's ethanolic extract also showed anticancer activity against four cancer cell lines – **C6**, **MFC**, **T47D** and **A549** using MTT assay.

Keywords*:* *Robinia pseudoacacia*, antibacterial, anticancer, MTT.

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Received 03 February 2015, Accepted 21 February 2015

Please cite this article as: Bhat BA *et al*., Evaluation of Phytochemical Screening, Anticancer and Antimicrobial activities of *Robinia Pseudoacacia*. American Journal of Pharmacy & Health Research 2015.

INTRODUCTION

Cancer is a major public health burden in both developed and developing countries, occurs due to some molecular changes within the cell. It was estimated that there were 10.9 million new cases, 6.7 million deaths, and 24.6 million persons living with cancer around the world in 2002[1].It becomes the second major cause of death in the human after cardiovascular disease[2]. The major causes of cancer are smoking, dietary imbalances, hormones and chronic infections leading to chronic inflammation[3].With increase in longevity, and the disease is going to bea problem even in India. Cancers affecting the digestive tract are among the most common of all the cancers associated with aging. About 1 out of every 14 men and women in America is diagnosed with gastrointestinal cancer at some time in his/her life. However, some microorganisms like, *E.coli, P .vulgaris* can cause not only diarrhea but more serious diseases such as hemorrhagic colitis and hemolytic uremic syndrome leads to ulceration and finally in cancer[3]. Because of high death rate associated with cancer and serious side effects of chemotherapy and radiation therapy, many cancer patients seek alternative and complementary methods of treatment. Recent researches revolve round the urgency to evolve suitable chemotherapy consistent with new discovery in cell biology for the treatment of cancer with no toxic effect. In the late 90's, the WHO stated that a big percentage of the world's population depends on plant based therapies to cover the needs of the primary health care (WHO 1999)[4].Moreover, towards the end of the 20thcentury, plant based products, and food supplements comprising the complementary and alternative therapies have gained a big share in the drug market in the developed countries. Considering the vast potentiality of plants as a source of traditional medication a systematic investigation was carried out to evaluate their anticancer and antimicrobial properties. A natural product is a chemical compound or substance produced by a living organism, found in nature that are usually has a pharmacological or biological activity like anticancer, antimicrobial etc. for use in pharmaceutical drug discovery and drug design[5]. Many crude drugs were observed by the local healers to have some medicinal value. Many of these aqueous, ethanolic, distilled, and dried extracts did exhibit a real and beneficial effect[6]. Treating cells with a cytotoxic compounds result in a variety of cell fates. The cell may undergo necrosis, in which they lose membrane integrity and die rapidly as a result of cell lysis. Assessing cell membrane integrity is one of the most common ways to measure cell viability and cytotoxic effects[7]. The antitumor areas have the greatest impact of plant derived drugs and have improved the chemotherapy of some cancers[8]. The continuing search for new anticancer

compounds in plant medicines and traditional foods is a realistic and promising strategy for its prevention[9]. Numerous groups with antitumor properties are plant derived natural products including alkaloids, flavonoids, phenylpropanoids and terpenoids[10]. The spread of drug resistant pathogens is one of the most serious threats to successful treatment of microbial diseases. Down the age the plant extracts have evoked interest as sources of natural products. They have been screened for their potential uses as alternative remedies for the treatment of many infectious diseases [11]. *Robinia pseudoacacia* (black locust) belongs to family Fabaceae is one of the medicinal plants, Native to north America, commonly distributed in various region of Kashmir like Kupwara, Banks of Manasbal Lake, Ganderbal, Pulwama[12] etc. It is a medium sized, melliferous tree which grows upto 6 meters. The plant is used as an antispasmodic, febrifuge, antioxidant, diuretic, emollient, laxative, antitumor and antimicrobial[13]. Four oligomeric flavonoids like robinetinidol-leucorobinetinidins, robinetinidol-dihydrorobinetins, robinetinidol-robinetin and robinetinidol-flavone have been reported. Five flavonoids like acacetin, secundiflorol, mucronulatol isomucronulatol and isovestitol have been isolated from the ethanolic extract of the whole plant. A bioactivity-directed fractionation of the ethanolic extracts of *Robinia pseudoacacia* has yielded robinlin, a novel homo-monoterpene. It has shown strong bioactivity in the brine shrimp lethality test (BST)[14]. All parts of the plant (except flowers) and especially the bark are considered to be toxic[15]. The toxins have been reported to get destroyed by heat.

MATRIALS AND METHOD

Plant collection and Identification

The *Robinia pseudoacacia*plant material was collected from district Pulwama (Puchal), Jammu & Kashmir, India.The plant has been identified by Curator Akhtar H. Malik. The Voucher specimen of *Robinia pseudoacacia* bearing specimen no. 1922 were deposited at KASH herbarium in Centre plant taxonomy, University of Kashmir, Srinagar.

Extraction and Isolation

Dried and powdered plant material was subjected to soxhlet with organic solvents in increasing order of their polarity (petroleum ether, ethyl acetate and methanol). The extracts thus obtained were concentrated by vacuum evaporation using rota-vapor.

Test Micro Organisms

Clinical isolates gram positive bacteria and gram negative bacteria were grown in nutrient broth medium and incubated at 37^0C for 24 hrs, followed by frequent sub culturing (every 24 hrs) to

refresh medium. Bacterial strains were maintained on Muller Hinton Agar Medium. The bacteria used for the analysis were *Proteus vulgaris* (MTCC-321), *Escherichia coli (*SKIMS) and *Klebsiella pneumonia* (SKIMS). Gentamicin (10mg/disc) was used as a standard antibacterial substance. Dimethyl sulphoxide (DMSO) was used as negative control.

Preparation of Microbial cultures

The bacterial strains were inoculated into nutrient broth for 24 hrs. In the Agar well diffusion method, sterile Muller Hinton agar for bacteria was inoculated with the test and then incubated at 35^0c for 24 hrs. At the end of the period. Zones of inhibition were measured in millimeters [16].

Cell culture:

The cell lines used were human mammary carcinoma cell line (MCF), human ductal breast epithelial tumor cell line (T47D), human lung adenocarcinoma epithelial *cell* line (A549) and rat glial cell line (C6). These cell lines were maintained in cell culture as recommended by the ATCC (LGC Promochem GmbH, Wesel Germany). These cell lines were grown in DMEM medium supplement with 10% heat inactivated fetal bovine serum, 1% of 2mmol/l l-glutamine, 50U/ml penicillin, and 50µg/ml streptomycin. Cells were maintained in humidified atmosphere in 5% CO2 at 37^oC.

RESULTS AND DISCUSSION

***In vitro* anti-proliferative assay**

Assay was carried out by MTT (3-(4, 5-Dimethylthiazol-2-yl) - 2, 5 diphenyl tetrazolium bromide, a tetrazole) protocol to evaluate the anti-proliferative effect of methanolic extract of *Robinia pseudoacacia*. For this purpose, a sufficient number of exponentially growing cells were used to avoid confluence of the culture during the treatment. The cell lines C6, MCF, A549, and T47D were seeded at 10^4cells/well and allowed to adhere for 12 hours. Media was replaced with 200µl of fresh medium before treatment with drug. In order to evaluate the optimum concentration at which the extract inhibited the cell proliferation in all the four cell lines, cells were treated with different concentrations (10, 20 and 50µg/ml). Methanol was used as a solvent for the dilution of the drug, which was also used as a experimental control. After 12 hrs treatment, cell growth was evaluated by MTT assay (Alley et al. 1986, 1988). MTT solution of 50µl (5 mg/ml of PBS) was added to each well and the plates were incubated for 3 hrs at 37^oC in dark. The media was aspirated and 150µl of MTT solvent (4 mM HCl, 0.1% Nonidet P-40, all in isopropanol) was added to each well to solubilize the formazan crystals. The absorbance of

plates were measured on ELISA reader (Benchmark, BioRad) at a wavelength of 570 nm. The sample was performed in triplicate, and the experiment was repeated thrice.

Wound healing assay

Cells were plated in 35mm dishes at a density of7.5×10^5 cells per ml and cultured to form confluent cell monolayer and grown to 90% confluency. Then adherent cells were scraped with a 10µl pipette tip and washed with PBS to remove nonadherent cells, followed by incubation in medium. After 24 hours of incubation with the drug or solvent only, the plates were photographed under a phase contrast microscopy and wound areas were calculated using image J software. The experiments were repeated three times and the quantitative data were expressed as mean ± SD.

Phytochemical screening of crude extract

The phytochemical screening of the plant extracts was carried out according to procedure of Farnes worth [17].

Alkaloids:

The Methanolic extract (30ml) was evaporated to dryness in an evaporating dish on water bath. 5ml of 2N HCl were added and stirred while heating on the water bath for 10 minutes. Cooled, filtered and the filtrate was treated with a few drops of Mayer reagent. The samples were then observed for the presence of turbidity or precipitation.

Flavonoids:

The alcoholic extract (75ml) of plant sample was evaporated to dryness on a water bath. Cooled and the residue was defatted by washing several times with petroleum ether. The defatted residue was dissolved in 30ml of 80% ethanol and filtered. The filtrate was treated with a few drops of concentrated HCl and magnesium turnings (0.5g). The presence of flavonoids was indicated if a pink or magneta red colour developed within 3 min.

Tannins:

The alcoholic extract (25ml) was evaporated to dryness on a water bath. The residue was extracted several times with n-hexane & filtered. The insoluble residue was stirred with 10ml of hot saline solution, the mixture was cooled, filtered and the volume of filtrate was adjusted to 10ml with more saline solution. To 5ml of this solution, few drops of ferric chloride test reagent were added. An intense green blue or black colour was taken as an evidence for the presence of tannins.

Saponins:

1g of ethanolic extract was dissolved in 10ml of distilled water in a test tube and shaked vigorously for 1-2 min. The presence of Saponins was indicated by characteristic honeycomb froath at least 1cm in height, which persisted for 30min.

Table 1: Phytochemical screening Methanolic extract of *Robinia pseudoacacia*

S.No	Constituents	Observation
1	Alkaloids	+
2	Flavonoids	+
3	Tannins	+
4	Saponins	-
5	Phenols	+
6	Steroids	+

+ = present - = absent

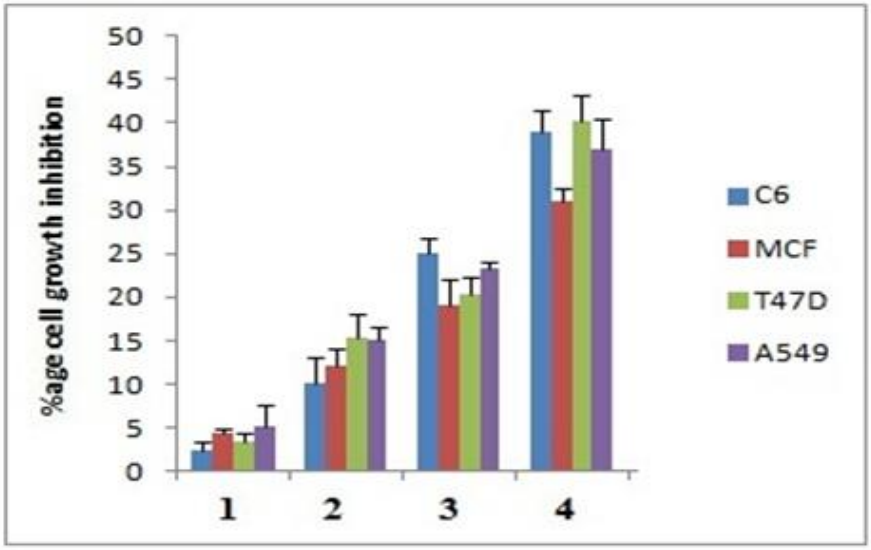

Figure 1: Anti-cancer activity of *Robinia pseudoacacia.* Where (1) shows DMSO control (2) shows drug concentration of 10µg/ml (3) shows drug concentration of 20µg/ml (4) shows drug concentration of 50µg/ml.

Wound healing assay was done using C6 cell lineat 0 and 24 h, in the presence of methanolic extract of *Robinia pseudoacacia* (50µg/ml) at 24 h. A, a representative image of three experiments is shown for each group. Gap size was quantified in the regions from the opposite edges of the migratory cells. The residual gap between the migrating cells from the opposing edges is expressed as a percentage of the initial, scraped area. B, The migration results show that methanolic extract of *Robiniapseudoacacia* significantly inhibits the reduction in gap size caused by cell migration.

Assay for antibacterial activity

Preparation of inoculum

Stock cultures were maintained at 4^0C on nutrient agar (HiMedia) slants. Active cultures for experiments were prepared by transferring loopful of culture to 10 mL of nutrient broth (HiMedia) and incubated at 37 ^{0}C for 24 hours for bacterial proliferation.

Agar-well diffusion method

Agar well bioassay was employed for testing antibacterial activity of *Robinia pseudoacacia* leaves. Each extracts were made to a final concentration of 100 mg/mL. 24 hour old cultures of test organisms (0.05 mL) were seeded onto Mueller Hinton agar (HiMedia) plate and uniformly spread with a spreader. Wells (100mm) were made in the agar plate with a sterile cork borer. The plant extract was introduced into the well and the plates were incubated at 37^0C for 24 hours. The antibacterial activity of the plant extract was determined by measuring the diameter of the inhibition zone. Controls contained only Dimethyl Sulfoxide (DMSO). The antibacterial assay for each of the extracts against all micro organisms tested was performed in triplicates**.**

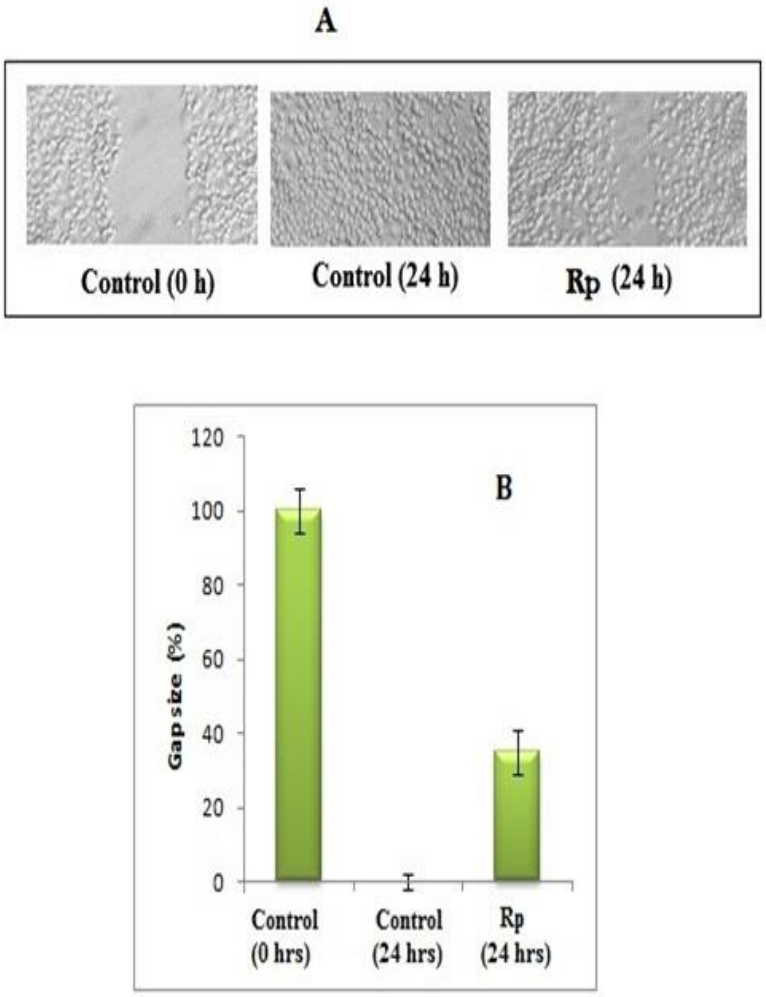

Figure 2: Wound healing assay: Methanolic extract of *Robinia pseudoacacia* inhibits cancer cell migration.

Table 1: Antimicrobial activity of *Robiniapseudoacacia*(RP) in terms of zone of inhibition.

Test organism	Zone of inhibition mm Conc. of RP in mg/ml				Gentamicin sulphate +ve Control	Source
	Bacterial type	**40 µl**	**70 µl**	**100µl**	**10 mg/disc**	
Klebsiella pneumonia	Gram positive	8mm	11mm	18mm	29mm	SKIMS
Escherichia coli	Gram negative	5mm	11mm	17mm	30mm	SKIMS
Proteus vulgaris	Gram negative	7mm	10mm	13mm	30mm	MTCC-321

Evaluation of the antimicrobial activity of Methanolic extract.

The antimicrobial activity was tested using reference and clinically isolated microbial strains, belonging to Gram-positive, (*Klebsiella pneumonia*), Gram-negative (*Escherichia coli, Proteus valgaris)*As it is evident from the table-1 that Methanolic extract of *Robinia pseudoacacia* inhibits the growth of test bacterial strains (*Klebsiella pneumonia, E.coli, Proteus valgaris).* The maximum zone of inhibition (17mm) was observed in *Klebsiella pneumonia*at 100µl and 5mm zone of inhibition was observed against *E.coli* at a concentration of 40µl.

CONCLUSION

From the above investigation it can be conclude that the extract of above mentioned medicinal plant (*Robinia pseudoacacia*) can be considered as a resource for potential anticancer and antimicrobial agents. Also, the selected plant can be further exploited for the discovery of novel anticancer/anti-microbial agents. Nevertheless, the present findings may also supplement and strengthen the process of standardization and validation of herbal drugs containing active ingredients derived from the selected medicinal plants.

ACKNOWLEDGEMENTS

We would like to acknowledge that Dr. M.A. Qureshi, Malik Aijaz, Department of chemistry, university of Kashmir and Dr. Rafiq Bhat for their valuable guidance.

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