

***In vitro* antibacterial activity and phytochemical studies of methanolic extract of leaves of *Hypericum perforatum* L. growing wild in Kashmir Himalaya**

**Mudasir Yousuf¹, Khaleefa Aslam¹, Bilal A. Wani², Nida Aslam¹, Nazir A. Dar² and
*Irshad A. Nawchoo¹**

¹Department of Botany, University of Kashmir, Srinagar- 190006, J&K, India

²Department of Biochemistry, University of Kashmir, Srinagar- 190006, J&K, India

ABSTRACT

To evaluate *in vitro* antibacterial activity and phytochemical analysis of methanolic extract of aerial parts of *Hypericum perforatum* L. growing wild in Kashmir Himalaya. Antibacterial activity was determined by agar well diffusion method and broth dilution assay against six bacterial strains comprising both standard laboratory and clinical strains. Phytochemical study was performed by using standard phytochemical methods. The extract showed pronounced activity against all the bacterial strains tested. *Bacillus subtilis* MTCC- 441 exhibited the maximum antibacterial activity against with mean zone of inhibition of 19.33 mm at concentration of 50 mg/ml. Minimum inhibitory concentration (MIC) ranged from 0.39 mg/ml for *Bacillus subtilis* MTCC- 441 to 3.12 mg/ml for *Salmonella typhi* and *E. coli*. The extract showed higher sensitivity against Gram positive bacterial strains than Gram negative bacterial strains. Quantitative estimation of bioactive phytoconstituents showed that the plant contains alkaloids (19.10 ± 0.4 mg/g plant sample), phenolics (21.90 ± 0.9 mg/g sample), flavanoids (17.10 ± 0.02 mg/g sample), tannins (1.60 ± 0.08 mg/g sample) and carbohydrates (4.0 mg/g sample). Our study clearly indicates that the methanolic extract of *Hypericum perforatum* from high altitude of Kashmir Himalaya possess significant antibacterial activity against diverse bacterial strains due to higher concentration of bioactive phytoconstituents like alkaloids, phenolics, flavanoids and tanins.

Key words: *Hypericum perforatum*, Kashmir Himalaya, antibacterial activity, MIC, phytoconstituents.

INTRODUCTION

Current advancements in drug discovery technology and search for new drugs from plants has always been of great interest for the scientists working in this field. The increasing prevalence of multidrug resistant (MDR) strains of bacteria and the recent appearance of strains with reduced susceptibility to antibiotics raises the specter of untreatable bacterial infections and adds urgency to the search for new infection-fighting strategies [1]. Plants are important source of potentially useful structures for the development of new chemotherapeutic agents. The first step towards this goal is the *in vitro* antibacterial activity assay [2]. The usage of plants as medicine still presents a very important phenomenon in the traditional medicine which is imbedded in the culture of people of developing countries [3, 4].

Hypericum perforatum L. (Hypericaceae) is perennial herb commonly known as St. John's wort. The genus *Hypericum* has about 400 species worldwide [5]. It bears extensive creeping rhizome with stem up to 1m high. The flowers appear in broad cymes at the ends of the upper branches. *Hypericum perforatum* is the hybrid apomictic

species, widespread in Europe, Asia, Northern Africa, as well as in North America, New Zealand and Australia where it was introduced as a very resistant weed [6]. The aerial parts of the plant contains many biologically active compounds the most important are hypericin, a strong photosensitizer fluorescent red plant pigment [7], which causes apoptosis in tumor cells [8] and Hyperforin, the most abundant lipophilic compound is likely to account for the antidepressant action of the plant. Hyperforin has significant effects on serotonergic, noradrenergic, dopaminergic, cholinergic and opioid system activities *in vitro*, as well as in animal models [9]. Hyperforin also was found to have anti-bacterial activity [10]. Previously research on *Hypericum perforatum* reports that the plant has antidepressant, antiviral, antibacterial and anticancer properties [11-14]. Hexane and Ethanol extraction of *H. perforatum* collected from Yatika and Bonera regions of J&K yielded 38% Hyperforin and 2.5% Hypericin respectively [15].

The floristic diversity of Kashmir Himalaya is considerably rich [16]. The medicinal flora of Kashmir has not received its due attention [17]. In Kashmir Himalaya only limited work has been done in this field. All the plants reported to be of value in folklore remedies in this region, need detailed chemical analysis so as to uncover their therapeutic agents. So the present study has been carried out to study the *in vitro* antibacterial activity and phytochemical analysis of *Hypericum perforatum*, a folklore medicinal plant used by Kashmiri people.

MATERIALS AND METHODS

1.1. Plant Material

Fresh harvestable leaves of *Hypericum perforatum* were collected from higher reaches of Gulmarg (J&K, India) at an altitude of 2600 m (asl) during the month of July- 2011. The plant material was properly identified by Dr. Irshad Nawchoo, Associate Professor, Department of Botany, University of Kashmir (Srinagar, India). A voucher specimen was deposited in Kashmir University Herbaria (KASH) for further reference.

2.2. Preparation of extract

The collected leaves were cleaned and cut into small pieces before being dried under shade at room temperature. The dried material were ground to fine powder using a mechanical blender and passed through 24 mesh sieve. Dry leaf powder (100 g) was packed in Soxhlet apparatus and extracted with methanol at 60-65°C. The extract was filtered through Whatmann filter paper No.1 and the solvent was removed under reduced pressure at 35-45°C using rotavapor. The dried extract was labelled and stored at 4°C in storage vials for experimental use [18].

2.3. ANTIBACTERIAL ACTIVITY

2.3.1. Test microorganisms:

Microbial cultures of eight different strains of both Gram positive and Gram negative bacteria were used for determination of antibacterial activity. Four bacterial strains viz. *Proteus vulgaris* (MTCC- 321), *Staphylococcus epidermidis* (MTCC- 435), *Pseudomonas aeruginosa* (MTCC- 1688) and *Bacillus subtilis* (MTCC-441) were standard laboratory isolates obtained from Microbial Type Culture Collection, (Chandigarh, India). The rest four bacterial strains were clinical isolates obtained from Department of Microbiology, Sheri Kashmir Institute of Medical Sciences, (Srinagar, India). All the bacterial strains were sub-cultured at 37°C on Mueller-Hinton agar (Himedia) slants every fifteen days and stored at 4°C.

2.3.2. Antimicrobial activity assay:

Antibacterial activity of *Hypericum perforatum* was determined by agar well diffusion method [19]. Each micro-organisms were grown overnight at 37°C in Mueller-Hinton Broth. 100µl of standardized inoculum (0.5 MacFarland) of each test bacterium were inoculated on molten Mueller-Hinton agar, homogenized and poured into sterile Petri dishes. The Petri dishes were allowed to solidify inside the laminar hood. Standard cork borer of 5mm in diameter were used to make uniform wells into which was added 30µl plant extract dissolved in sterile DMSO. Standard antibiotic gentamicin (30µg/disc) was used as positive control and DMSO alone as negative control. The plates were then incubated at 37 ± 1°C for 24h. The zone of inhibition was measured with the help of standard scale. The experiments were carried out in triplicates and results were calculated as mean ± SD.

2.3.3. Determination of minimum inhibitory concentrations (MICs)

The MIC of *Hypericum perforatum* was determined by macro dilution method [20]. Dilution ranges (50 - 0.012 mg/ml) of plant extracts and standard antibiotic were prepared from stock solution by serial dilution technique. Each sample dilution were mixed properly with 20 ml of sterile molten Muller Hinton agar and poured into 90 mm Petri plates and allowed it to cool under laminar air flow before streaking with 10 µl of 0.5 McFarland standard

inoculums of tested bacterial strains. Plates were incubated at $37 \pm 1^\circ\text{C}$ for 24 hours. The lowest concentration of the extract at which there was no visible growth of microorganisms was considered as minimum inhibitory concentration (MIC).

2.4. PHYTOCHEMICAL STUDY

2.4.1. Phytochemical screening

Phytochemical screening for major bioactive constituents like alkaloids, phenolics, flavonoids, tannins, carbohydrates and lipids were determined by using standard phytochemical methods [21, 22].

2.4.2. Determination of total phenolics

Total phenolic content of methanolic leaf extract of *Hypericum perforatum* was determined by Folin–Ciocalteu method [23] with slight modifications. 200 μl of sample (1mg/ml) was added to 100 μl diluted (1:10) Folin–Ciocalteu reagent and equilibrated for few minute. Then 800 μl of 2.5 % aqueous Na_2CO_3 was added and mixture was allowed to stand for 60 minutes at room temperature with intermittent shaking. The absorbance of the blue colour solution was measured at 765 nm on UV–visible spectrophotometer [Shimadzu UVPC-1650 (Japan)]. Gallic acid (50 mg %) was used as standard. The absorbance of solution was compared with gallic acid calibration curve. The total phenolic content was expressed as gallic acid equivalents (GAE mg/g dry weight of extract), and the values were presented as mean \pm SD of triplicate analysis.

2.4.3. Determination of total flavonoids

Total flavonoid content was determined by aluminum chloride colorimetric method [24]. This method is based on the formation of a complex flavonoid–aluminium, having the absorbance maximum at 430 nm. 0.5 ml of plant extract (1mg/ml) was mixed with 1.5 ml of methanol, 0.1 ml of 10% AlCl_3 , 0.1 ml of 1 M potassium acetate and 2.8 ml of distilled water. After incubation at room temperature for 15 minutes, the absorbance of the reaction mixture was measured at 430 nm on UV–visible spectrophotometer [Shimadzu UVPC-1650 (Japan)]. Total flavonoid contents of leaf sample were expressed as rutin equivalents (RE mg /g dry weight of extract) through the calibration curve with rutin as standard.

2.4.4. Alkaloid estimation

2.5g of the plant powder was extracted using 100ml of 20% acetic acid in ethanol. The solution was covered for almost 4 hours. Filtrate was concentrated to 25ml. Concentrated ammonium chloride was added stepwise for precipitation. The whole solution was kept as such so that precipitate will settle. Collected precipitate was washed with dilute ammonium hydroxide and finally filtered. Filtrate was discarded and pellet obtained was dried and weighed [25, 26].

2.4.5. Tannins estimation

The tannin content in samples was estimated by the method of Price and Butler [27]. Different aliquots of sample were taken and final volume to 3 ml was adjusted by distilled water. The solution after vortexing were mixed with 1ml of 0.016M $\text{K}_3\text{Fe}(\text{CN})_6$, followed by 1 ml of 0.02M FeCl_3 in 0.10 M HCl. Vortexing was repeated and the tubes were kept as such for 15 min. 5 ml of stabilizer (3:1:1 ratio of water, H_3PO_4 and 1% gum arabic) was added followed by revortexing. Absorbance was measured at 700 nm against blank. Standard curve was plotted using various concentrations of 0.001M gallic acid.

2.4.6. Carbohydrate estimation

Phenol sulphuric acid method [28] was used for estimate of total carbohydrate content, which is based on principle that in hot acidic medium glucose is dehydrated to hydroxymethyl furfural. This forms a green coloured product with phenol and has absorption maximum at 490nm. Different aliquots of sample were prepared and final volume was made to 1ml by water. 5ml of 96% of concentrated H_2SO_4 was added followed by shaking and incubation for 40 minutes at room temperature. Then 1ml of 5% phenol was added to each tube and absorbance was read at 490 nm. Standard curve was plotted using different concentrations of 25mg % glucose.

RESULTS AND DISCUSSION

The *in vitro* antibacterial activity of methanolic extract of leaves of *Hypericum perforatum* was evaluated by agar well diffusion method and broth dilution assay. The antibacterial activity was screened against six bacterial strains including three standard laboratory isolates: *Bacillus subtilis* (MTCC-441), *Staphylococcus epidermidis* (MTCC-

435), *Proteus vulgaris* (MTCC- 321) and three clinical isolates: *Staphylococcus aureus*, *Salmonella typhi*, *Escherichia coli* of both Gram positive and Gram negative categories. The plant extract exhibited pronounced activity against all the bacterial strains tested. Results of antibacterial activity of methanolic extract of *H. perforatum* are presented in Table-1 and Table-2. *Bacillus subtilis* (MTCC- 441) and *Staphylococcus aureus* showed maximum sensitivity to methanolic extract of *H. perforatum* with mean zone of inhibition of 19.33 mm and 18.00 mm respectively at the test concentration of 50 mg/ml, which was comparable to standard antibiotic (gentamycin 30µg/disc). The extract showed least activity against *Salmonella typhi* with mean zone of inhibition of 14.66 mm at the same concentration. The minimum inhibitory concentration (MIC) ranged between 0.78 mg/ml to 3.12 mg/ml. Also the MIC of gentamycin ranged between 0.024 and 0.195 mg/ml (Table-2). Gram positive bacterial strains were found to be slightly more sensitive than Gram negative bacterial strains. It may be due to the absence of lipopolysaccharide layer in Gram positive bacteria that might function as a barrier to the phytochemical substances that are responsible for antibacterial activity [29, 30]. Our results confirm broad-spectrum antimicrobial activity of methanolic extract of *H. perforatum* which is also in accordance with the findings of Meral [31] and Dulger [32]. Generally the methanol extract shows the highest activity against both bacterial and fungal isolates, which is due to that the active ingredients of the plant parts are better extracted with methanol than other solvents. The methanol extracts contain alkaloids, coumarins and tannins [33]. Coumarins and tannins have antibacterial and antihelminthic properties [34]. These secondary metabolites exert antimicrobial activity through different mechanisms. Tannins have been found to form irreversible complexes with proline-rich proteins [35] resulting in the inhibition of the cell protein synthesis.

Table 1: shows *In vitro* antibacterial activity of methanolic extract of leaves of *H. perforatum*.

S. No	Microorganism	Mean Zone of inhibition (mm)	
		<i>H. perforatum</i> (50 mg/ml)	Standard Antibiotic (Gentamicin 30 µg/disc)
1.	<i>Staphylococcus epidermidis</i> MTCC- 435.	17.00± 1.00	29.33±0.57
2.	<i>Bacillus subtilis</i> MTCC- 441.	19.33± 1.52	30.33± 1.52
3.	<i>Proteus vulgaris</i> MTCC- 321.	15.33± 1.52	28.00± 1.52
4.	<i>Staphylococcus aureus</i>	18.00± 1.00	28.66± 1.15
5.	<i>Salmonella typhi</i>	14.66± 0.57	27.66± 0.57
6.	<i>Escherichia coli</i>	15.33± 0.57	29.00± 1.00

Results are mean ± SD of three experiments

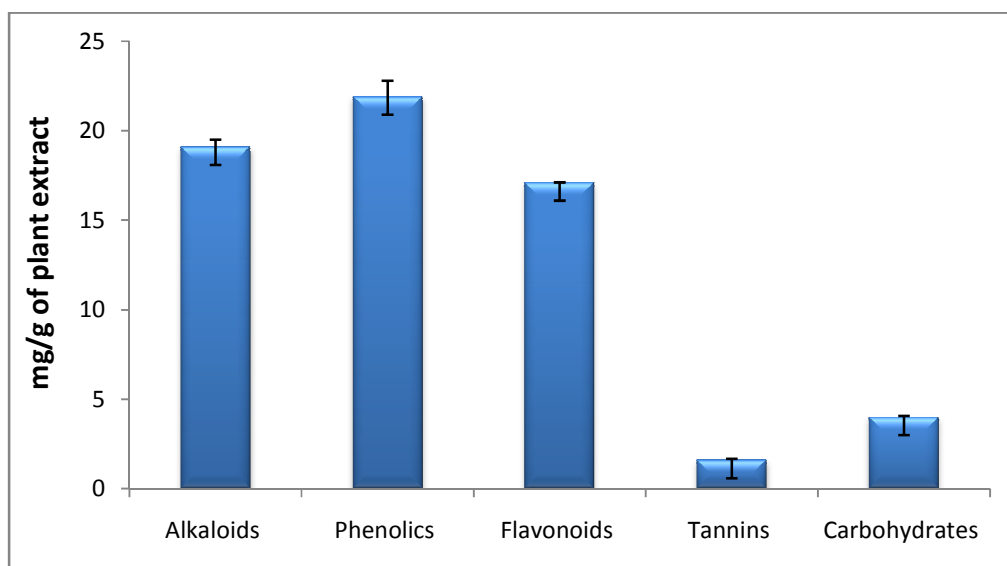
Table 2: Shows minimum inhibitory concentrations (MICs) of methanolic extract of leaves of *Hypericum perforatum*.

S. No	Microorganism.	MIC value (mg/ml)	
		<i>H. perforatum</i>	Standard antibiotic
1.	<i>Staphylococcus epidermidis</i> MTCC- 435.	0.78	0.024
2.	<i>Bacillus subtilis</i> MTCC- 441.	0.39	0.024
3.	<i>Proteus vulgaris</i> MTCC- 321.	1.56	0.048
4.	<i>Staphylococcus aureus</i> .	0.78	0.024
5.	<i>Salmonella typhi</i> .	3.12	0.195
6.	<i>Escherichia coli</i>	3.12	0.195

Table 3: Qualitative phytochemical screening of *Hypericum perforatum*.

Phytoconstituents	Test	Result
Alkaloids	Wagner's/ Dragendrof's test	+
Phenolics	phenol test	+
Tannins	Ferric chloride test	+
Flavonoids	Shinoda test	+
Carbohydrates	Benedict's/ Fehling's test	+

(+) indicates presence.

Figure 1: *Hypericum perforatum* L. in flowering stage.Figure-2: Quantity of biochemical constituents of *Hypericum perforatum*

The phytochemical investigation in the present study revealed the presence of phenolics, alkaloids, flavonoids, tannins and carbohydrates as indicated in table-3. The results of phytochemical quantification analysis of alcoholic extract of *H. perforatum* are shown in table-3 and figure-1. The plant contains alkaloids (19.10 ± 0.4 mg/g plant sample), phenolics (21.90 ± 0.9 mg (GAE)/g plant sample), flavanoids (17.10 ± 0.02 mg (RE)/g sample), tannins (1.60 ± 0.08 mg/g sample) and carbohydrates (4.00 mg/g sample). Different phytochemicals have been found to have a broad range of activities, which may help in protection against chronic diseases [36]. These compounds are known to be biologically active and therefore aid the antimicrobial activities. Alkaloids which are one of the largest groups of phytochemicals in plants have amazing effects on humans and this has led to the development of powerful

pain killer medications [37]. Flavonoids exhibit a wide range of biological activities like antimicrobial, anti-inflammatory, analgesic, anti-allergic, cytotoxic and antioxidant properties [38]. The effective antibacterial activity of methanolic extract of aerial parts of *H. perforatum* from high altitude Kashmir Himalaya is due to the higher concentration of bioactive phytoconstituents when compared to earlier reports [39]. The plant extract being active against both clinical and laboratory isolates is also an indication that it can be a source of very potent antibiotic substances that can be used against multidrug resistant bacterial strains.

Table 4: Quantitative estimation of phytoconstituents present in methanol extract of *H. perforatum*.

Phytoconstituents	Quantity (mg/g plant extract)
Alkaloids	19.10 ± 0.4
Phenolics	21.90 ± 0.9
Flavonoids	17.10 ± 0.02
Tannins	1.60 ± 0.08
Carbohydrates	4.00 ± 0.08

Acknowledgment

The authors are very thankful to the Head Department of Biochemistry, University of Kashmir Srinagar (Jammu and Kashmir) for providing the necessary lab facilities for the current research work.

REFERENCES

- [1] Sieradzki K, Roberts RB, Haber SW, Tomasz A. *N Engl J Med* **1999**; **340**: 517–523.
- [2] Tona L, Kambu K, Ngimbi N, Cimanga K, Vlietinck AJ. *J Ethnopharmacol* **1998**; **61**: 57–65.
- [3] Kloucek P, Polesny Z, Svobodova B, Vlkova E, Kokoska L. *J Ethnopharmacol* **2005**; **99**: 309–312.
- [4] Duraipandiyan V, Auyana M, Ignacimuthu S. *BMC Complementary and Alternative Medicine* **2006**; **20**: 6–35.
- [5] Hickey M, Adamski R, Styp-Rekowska E. *Farm Pol* **1971**; **27**: 237–41.
- [6] Martonfi P, Brutovska R, Ellarova E and Repak M. *Folia Geobot Phytotax* **1996**; **31**: 389–396.
- [7] Campbell MH, Delfosse ES. *The J of Austral Inst of Agric Sci* **1984**; 63–73.
- [8] Piette J, Volanti C, Vantiegheim A, Matroue J, Hbaraken Y, Agonistis P. *Biochemical Pharmacology* **2003**; **66**: 1651–1659.
- [9] Hunt EJ, Lester CE, Lester EA, Tackett RL. *Life Sciences* **2001**; **69**: 181–90.
- [10] Greeson JM, Sanford B, Monti DA. *Psychopharmacology* **2001**; **153**: 402–414.
- [11] Guevich AI. Hyperforin, *Antibiotiki* **1971**; **16**: 510–12.
- [12] Barnes J, Anderson L, Phillipson JD. *J Phar Pharmacol* **2001**; **53**: 583–600.
- [13] Tonk FA, Giachino RRA, Sonmez C, Yuce C, Bayram E, Telci I, Furan *Int J Agric Bio* **2011**; **13**(1): 31–37.
- [14] Hahn G. *J Naturopathic Med* **1992**; **3**: 94–96.
- [15] Jellin JM. *St. John's wort*. Natural Medicines Comprehensive Database **2000**: <http://www.naturaldatabase.com>.
- [16] Vir Jee, Dhar U, Kachroo P. *Proc Indian* 1989; **6**(4) 293–296.
- [17] Koul, VK, Koul S. *Natural product radiance* **2007**; **6**(4) :293–296.
- [18] Kaul MK. Medicinal plants of Kashmir and Ladakh, temperate and cold arid Himalaya. Indus publishing Co. New Delhi, India **1997**.
- [19] Jonathan SG, Fasidi IO. *African Journal. of Biomedical Research* **2003**; **6**: 85 – 90.
- [20] Perez C, Pauli M, Bazerque P. *Acta Biologiae et Medicine Experimentalis* **1990**; **15**: 113–115.
- [21] Jennifer MA. *Journal of antimicrobial chemotherapy* **2001**; **48**: 5–16.
- [22] Harborne IB. Phytochemical methods: A guide to modern techniques of plant analysis. 2nd Ed., *Chapman and Hall, New York* **1973**; 88–185.
- [23] Trease EG, Evan WC. Text book of pharmacognosy. 12th Ed., *Oxford, Alden Press* **2007**; pp. 343–383.
- [23] Kahkonen MP, Hopia AI, Vuorela HJ, Rauha JP, Pihlaja K, Kujala TS, Heinonen M. *Journal of Agriculture and Food Chemistry* **1999**; **48**: 1485–1490.
- [24] Chang C, Yang M, Wen H, Chem J. *J Food Drug Anal* **2002**; **10**: 178–182.
- [25] Edeoga HO, Okwu DE, Mbaebie BO. *Afr J Biotechnol* **2005**; **4**: 685–688.
- [26] Okwu DE, Josiah C. *African J Biotechnology* **2006**; **5**(4): 357–361.
- [27] Price ML, Butler LG. *J Agric Food Chem* **1977**; **25**: 1268–1273.
- [28] Krishnaveni S, Theymoli B, Sadasivam S. *Food Chem* **1984**; **15**: 229.
- [29] Inouye S, Takizawa T, Yamaguchi H. *J Antimicrob Chemoth* **2001**; **47**: 565–573.
- [30] Delaquis PJ, Stanich K, Girard B, Mazza G. *Int J Food Microbiol* **2002**; **74**: 101–109.

-
- [31] Meral GE, Karabay, NU. *Turkish Electronic Journal of Biotechnology special issue* **2002**; 6-10.
 - [32] Dulger B, Gonuz A. *Fitoterapia* **2005**; **76**: 237-239.
 - [33] Okemo PO. Antimicrobial efficacy of selected medicinal plants used by Kenyan Herbal doctors. Ph.D. thesis, Kenyatta University of Nairobi., 173-90.
 - [34] Hedberg I, Hedberg O, Madati P, Mshigeni KE, Mshiu EN, Samuelsson G. *J Ethnopharm.* **1983**; **9**: 105-128.
 - [35] Hagerman AE, Butler IG. *J Biol Chem* 198; **256**: 4494-4497.
 - [36] Liu RH. *Am J Clin Nutr* **2003**; **78**: 517-520.
 - [37] Maskovic PZ, Mladenovic JD, Milica SC, Gordana AD, Slavica RS and Marija MR. *Hem. Ind* **2011**; **65** (2) 159–164.
 - [38] Hodek P, Trefil P, Stiborova M. *Chemico-Biol Intern* **2002**; **139**(1): 1-21.
 - [39] Kam PCA, Liew. *Anaesthesia* **2002**; 57(11): 1083-1089.