Pak. J. Bot., 50(4): 1487-1497, 2018.

AN INSIGHT INTO *IN VITRO* AND *IN VIVO* PHARMACOLOGICAL ACTIVITIES OF *PINUS WALLICHIANA*

NAZISH[1], IBRAR KHAN[1*], KHALID KHAN[2], SADIQ AZAM[1], IKHWAN AHMAD[2]AND INAM ULLAH[1]

[1]*Centre of Biotechnology and Microbiology, University of Peshawar, Pakistan*
[2]*Veterinary Research Institute, Peshawar, Pakistan*
[3]*Department of Biotechnology, Bacha Khan University, Charsadda.*
[*]*Corresponding author's email: ibrarkhan1984@uop.edu.pk*

Abstract

The aim of the present study was to screen the test samples (Cr. Met. Ext and different fractions of stem, leaves and resin) of *Pinus wallichiana* for possible *in-vitro* and *In vivo* pharmacological activities. The aqueous fraction of leaves presented good antibacterial activity (63%) against *Vancomycin Resistant Staphylococcus aureus* (*VRSA*), Ethyl acetate (EtOAc) fraction of resin against *Acinetobacter baumannii* (60.8%) and Cr. Met. Ext of the stem against *Proteus vulgaris* (62%), *S. aureus* (61.5%) and *Escherichia coli* (60.3%). All of the test samples showed low or no activity against test fungal pathogens. The phytotoxic activity of test samples was moderate to low at 1000µg/ml. The results of haemagglutination indicated lack of phytoagglutinins in *P. wallichiana*. The antioxidant activity was good to moderate at 500µg/ml and the activity was concentration dependent. Good and significant insecticidal effect was recorded against the selected test insects. The Cr. Met. Exts were found safe at the tested dose and no lethality was observed in acute toxicity assay. The test samples presented significant decrease in the number of writhes after intervals of 30, 60 and 90 minutes in a dose dependent manner. The percent Gastro Intestinal Tract (GIT) motility observed in test group treated with different doses of the test samples was; 46.35 (stem), 49.93 (leaves) and 42.72% (resin) at 100mg/kg and same decreased was observed at 200 and 300mg/kg. The test samples of the selected plant also possess significant anti-pyretic effect after 1, 2 and 3 hours.

Key words: Antibacterial, Antifungal, Antioxidant, Acute toxicity, GIT motility, Anti-pyretic.

Introduction

One of the goals of human effort, from very beginning, was to find ways to treat various kinds of diseases. In the quest, various solutions; herbal, allopathic and homeopathic medicines were discovered, which for sure had a positive effect in treating different kind of diseases. Different medicinal plants were identified as rich sources for many potentially bioactive compounds; steroids, glycosides, flavonoids, alkaloids, tannins, phenols and volatile oils; that are present in seeds, fruits, bark, flowers, leaves and roots of the plants (Pathumthip *et al.,* 2001). But in the current era, the situation is against worsened by the emergence of antibiotic resistance (Schmidt, 2004). Some available antibiotics are being resisted by pathogenic bacteria and can also cause allergy in certain conditions (Frigas *et al.,* 2008; Scott, 2007). So, there is a need of finding new and innovative antimicrobial agents that can cure diseases without or fewer side effects. An array of medicinal plants has been screened for various biological activities *In vitro*; antimicrobial, insecticidal, phytotoxic and insecticidal and *In vivo* to determine their medicinal importance on scientific grounds (Pathumthip *et al.,* 2001). In recent years, natural drugs, especially of plant origin, are replacing the synthetic one because of fewer side effects (Rozman & Jersek, 2009). Some natural products, produced and isolated from medicinal plants, possess effective antimicrobial properties making it logical to screen these plants for their active constituents. The importance of plants, as a source of new antimicrobial drugs, can be seen from the fact that out of the 25 best-selling pharmaceutical products, 12 are from medicinal plants (Shan *et al.,* 2007).

The regular and misuse of insecticides has not only resulted in development of resistant pests but has also affected the natural environment and its biota in a harmful manner (Champ & Dyte, 1977). Plants contain certain phytochemicals which can serve as larvicides, ovipositor attractant, insects repellents and growth regulators as supported by reported literature (Khalid *et al.,* 1989; Vinayaka *et al.,* 2009). The antioxidants are playing an important role for the maintenance of human health, treatment and diseases. There is an inherent anti-oxidative mechanism in human body that are responsible for anti-carcinogenic, anti-mutagenic and anti-aging responses (Gulcin, 2012). Different synthetic antioxidants; Butylated Hydroxy Toluene (BHT) and Butylated Hydroxy Anisole (BHA) create various toxic and carcinogenic effects when tested on animal models (Amarowicz *et al.,* 2000). The destruction caused by oxidative stress is effectively prevented by natural antioxidants either in crude or pure form (Zengin *et al.,* 2011).

Heterotermes indicola not only cause a huge loss in agriculture and forestry but are also responsible for tremendous losses to wooden structures in buildings (Sen-Serma, 1975). Synthetic germicides are being used to control *H. indicola* growth, but due to their residual effects, it was realized as a great hazard to environment. Therefore, germicides like Dieldrin, Aldrin, Dichloro-Diphenyl-Trichloro-ethane (DDT), heptachlor and Benzene Hexa Chloride (BHC) have been banned by the concerned authorities. The scientific community is, therefore, in a continuous search for environment friendly and effective formulation and plants can be one of the potent sources. A study reported the anti-termite effect of various fractions of *Acacia modesta* against *H. indicola* concluding that Cr. Met. Ext, aqueous and chloroform ($CHCl_3$) fractions have good anti-termite activity (Bashir *et al.,* 2011). It has been reported that majority of medicinal plants possess anti-ulcer, anti-tumor, anti-diarrheal, acetyl cholinesterase inhibitory and anti-

secretory activities (Murad *et al.,* 2011). The medicinal plants contain certain bioactive compounds having blood purifying, laxative and muscular pain relief potential so they can be used in different types of pharmacological investigations (Mthethwa, 2009).

Pinus wallichiana (Peuch/Nakhtar, Shunty), a coniferous evergreen tree, is native to Northern areas of Pakistan, Hindu-Kush, Himalaya and Eastern Afghanistan. It is high altitude Pine, occurring from 1800-4000 meters (Ghimire *et al.,* 2010). Resin of this plant is used as stimulant and for cure of gonorrhoea and stomachic. Externally it is used as a plaster to buboes and blisters for suppuration. Wood of *P. wallichiana* is used for burning sensation of body, cough, ulceration and fainting and is good for re-plantation and soil maintenance (Alya *et al.,* 2011). Keeping in view the traditional medicinal importance of *P. wallichiana*, the current study was designed to screen different parts of *P. wallichiana*for possible *In vivo* and *In vitro* pharmacological / biological activities.

Methodology

Plants collection, extraction and fractionation: The selected parts (leaves, stem and resin) of *P. wallichiana* were collected from Malamjabba, Khyber Pakhtunkhwa, Pakistan) and identified by Dr. LalBadshah, Department of Botany, University of Peshawar (Voucher # COBAM/Bot-13). The dried samples were grounded to powder followed by soaking (twice) in methanol at room temperature for 15 days with proper shaking. The soaked materials were filtered each time and concentrated using rotary evaporator at 40ºC for obtaining Cr. Met. Exts. The Cr. Met. Ext of leaves, suspended in 500ml of distilled water and was partitioned with *n*-hexane (3x500 ml), $CHCl_3$ (3x500 ml) and EtOAc (3x500 ml), respectively, to get the respective fractions. Same procedure was followed to obtain different fractions of stem and resins. Some of the Cr. Met. Ext of each part was preserved for various biological activities (Sukumaran *et al.,* 2012).

In vitro biological activities

Antibacterial activity: The antibacterial activity of test samples was determined against clinical isolates of *E. coli, Pseudomonas aeruginosa, P. vulgaris, S. aureus, A. baumannii, Morganella morganii* and *VRSA.* Nutrient broth and agar media were prepared, autoclaved and for sterility check incubated at 37ºC for 24 hours. One ml of broth containing pure bacterial culture was transferred to sterile nutrient agar plates and using 6mm borers, wells were made in it. Test sample (100µl) was transferred to respective well from the stock solutions (3mg/ml of ≤1% Dimethyl Sulfoxide (DMSO) and incubated at 37ºC for 24 hours. Amoxicillin and DMSO were used as positive and negative control, respectively. After incubation, percent zone of inhibition was measured for each sample in comparison with positive control (Ahmad *et al.,* 2009). For determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC), the procedure of Banso, 2009 was followed.

Antifungal activity*:* The antifungal activity of the test samples was determined against *Aspergillus niger, Hemimycenapseudocrispula, Penicillium notatum, Aspergillus parasiticus, Verticillium longisporum* and *Penicillium chrysogenum*. Sterile *Sabouraud Dextrose Agar (SDA)* plates were prepared to refresh above mentioned fungal cultures. Stock solution of the test samples (24mg/ml) was prepared in DMSO and 66.66µl was transferred to test tubes containing 4ml of the SDA medium before slants preparation. The slants were inoculated with 5-7 days old fungal culture and incubated at 28±1ºC for 7 days. The DMSO and Miconazole were used as negative and positive control, respectively. The percent linear growth inhibition was measured in comparison with control (Ahmad *et al.,* 2009).

Phytotoxic activity: The phytotoxic effect of the test samples was determined against*Lemna minor* (Haroon *et al.,* 2012). From the stock solutions (20mg/ml of methanol) 1000, 100 and 10µl were introduced into the respective flask and methanol was allowed to evaporate. The E-media (20ml) and 10 healthy *L. minor* were introduced into each flask and incubated for seven days at 28ºC in growth chamber. Results were recorded on day seven by observing the number of damaged plants in comparison with Paraquat used as standard growth inhibitor.

Haemagglutination activity: Haemagglutination activity of test samples was carried out against Red Blood Cells (RBCs) of the ABO blood groups in different dilutions (1:16, 1:8, 1:4 and 1:2), prepared in phosphate buffer (pH 7.4) from stock solution (1mg/ml). Blood was collected from healthy volunteers and centrifuged to make 2% RBC's suspension in phosphate buffer on the day of experiment. 1ml of test sample from each dilutions and 1ml of the RBC's suspension was taken in a test tube followed by incubation at 37ºC for 30 minutes. The test tubes were then centrifuged for smooth and rough button formation which indicates the negative and positive results, respectively while extent of deposition determined the intensity of positive result (Haroon *et al.,* 2012).

Anti-oxidant activity: From the stock solution (1mg/1ml of methanol) different dilutions; 100, 200, 300, 400 and 500µg/ml, were made to determine the antioxidant activity of the test samples. 2ml of freshly prepared 2, 2-diphenyl-1-picrylhydrazyl [DPPH] solution was mixed with 1ml of each dilution. The mixture was incubated in dark room for 10 minutes and at 517nm, the absorbance was recorded by Ultra Violet Visible Spectrophotometer (Shimadzu UV-1601) to determine the antioxidant activity of the samples by free radical scavenging effect of DPPH. The control tube used in the current study was 1ml methanol + 2ml DPPH solution. The 50% Effective Concentration (EC_{50}) was determined by plotting a curve of percent scavenging effect against samples concentrations (Kanatt & Sharma, 2007).

Insecticidal activity: The insecticidal activity of the test samples was determined against *Tribolium castaneum* and its larvae, *Callosobruchus chinensis, Rhyzopertha dominica, Chaetocnema pulicaria* and *H. indicola* using

reported procedures (Abbot, 1987; Salihah *et al.,* 1993). The test samples dissolved in methanol were loaded into respective Petri plate. The methanol was allowed to evaporate and slight water spray was done on each plate to meet humidity requirement. 10 healthy insects of uniform age and size were transferred to each labelled plate and control and were incubated for one day at 27°C. The percent mortality was calculated in comparison with Permethrin (235.9µg/cm^2) and Termisolve B-PRO serving as positive control.

***In vivo* biological activities:** Various *In vivo* biological activities were conducted for the test samples after taking approval from the ethical committee of Veterinary Research Institute (VRI), Peshawar, Pakistan.

Acute toxicity assay*:* The acute toxicity assay was performed according to Khan *et al.,* 2010 on Swiss Albino mice *(Balb-C)* of either sex (30±5g) in 3 groups (n=6). The test samples (Cr. Me. Ext.) of *P. wallichiana*leaves, stem and resins were screened at different doses (600, 400 and 200mg/kg, body weight). The test doses were injected through intraperitoneal (i.p) route and the animals were kept under observation for 24 hours with unrestricted access to food and water. After 24 hours, number of survived and dead mice was recorded (Khan *et al.,* 2010).

Antinociceptive assay: Antinociceptive assay was performed by acetic acid induced writhing test on Swiss Albino mice (*Balb-C*) of either sex (18-22g) for possible analgesic effect. For the experiments, the mice were divided into 5 groups (n=6). At least 2 hours before starting the experiment, mice were withdrawn from food. The test samples, Diclofenac Sodium and normal saline were i.p. administered, 30 minutes prior to acetic acid (1%, 10ml/kg). Writhing behaviour was induced in the mice through i.p. administration of acetic acid. Finally, number of writhes was observed for 20 minutes; past 5 minutes of acetic acid induction (Khan *et al.,* 2010). Groups received the treatment as follows:

Group I: Negative control was treated with normal saline (10ml/kg).

Group II: Positive control was treated with Diclofenac Sodium (20mg/kg)

Group III-V: Test samples (stem, leaves and resin); 100, 200 and 300mg/kg

Gastrointestinal tract motility (charcoal meal protocol): The test samples were screened for its possible effect on GIT motility. The selected *Balb-C* mice (25-30g) were distributed into 5 groups (n=6). Group I and II were treated with normal saline (10ml/kg) and castor oil (0.1ml/kg) serving as negative and positive control, respectively. The group III, IV and V were treated with Cr. Me. Ext at a dose of 100, 200 and 300mg/kg, i.p. After 15 minutes, each mouse was treated with 0.3ml of charcoal suspension. The mice were dissected after 30 minutes of charcoal treatment to observe the movement of charcoal in small intestine for calculation of percent GIT motility (Al-Ghamdi, 2001).

Anti-pyretic activity: Anti-pyretic activity of the test samples was determined by Brewer's yeast (Merck, Germany) induced pyrexia in Swiss Albino mice (*Balb-C*) of either sex (25-30g). The mice were kept overnight with unrestricted access to water but without food and were divided into 5 groups (n=6). The normal body temperature of mice was noted before developing hyperpyrexia. The probe of the digital thermometer (Model CA92121, ACON Laboratories, USA) was lubricated with olive oil to measure their body temperature. To create hyperpyrexia conditions, 20% of Brewer's yeast (10ml/kg b.w) was injected below the nape of neck in the back. After 24 hours, changes were observed in the rectal temperature with the help of thermometer. Mice showing a rise of at least 0.3-0.5°C in rectal temperature were selected for further dosing of test samples (Al-Ghamdi, 2001). The groups received treatment as follows:

Group I: Negative control was treated with normal saline at 10ml/kg.

Group II: Positive control was treated with Paracetamol at 50mg/kg.

Group III-V: Test samples (stem, leaves and resin); 100 and 200mg/kg

The temperature was recorded after 1, 2 and 3 hours of test sample injection for possible anti-pyretic activity.

Statistical analysis: The data obtained were analysed by ANOVA followed by Dunnett's post hoc analysis with $p<0.05$ being considered as significant.

Results

In vitro biological activities

Antibacterial activity

Antibacterial activity of leaves: The Cr. Met. Ext of leaves of *P. wallichiana* exhibited moderate activity against *P. vulgaris* (56% with MIC_{50}=2.96 and MBC=5.6), *S. aureus* (53.8% with MIC_{50}=2.4 and MBC=6.6), *VRSA* (52.6% with MIC_{50}=2.52 and MBC=4.04), *A. baumannii* (52.1% with MIC_{50}=2.4 and MBC=4.6), *M. morganii* (45.5% with MIC_{50}=3.12 and MBC=6.72), *E. coli* (44.4% with MIC_{50} = 3.12 and MBC=6.88) and *P. aeruginosa* (40.7% with MIC_{50}=3.52 and MBC=7.2). The *n*-hexane fraction rendered moderate activity against *VRSA* (57.8% with MIC_{50}=2.8 and MBC=5.52), *A. baumannii* (52.1% with MIC_{50}=2.52 and MBC=4.2), *P. aeruginosa* (51.8% with MIC_{50}=3.4 and MBC=9.5), *P. vulgaris* (48% with MIC_{50}=2.2 and MBC=4.92), *M. morganii* (45.5% with MIC_{50}=3.12 and MBC=6.8) and *E. coli* (40.7% with MIC_{50}=3.64 and MBC=7.4), while inactive against *S. aureus*. The $CHCl_3$ fraction displayed moderate activity against *VRSA* (57.8% with MIC_{50}=2.96 and MBC=5.6), *P. aeruginosa* (44.4% with MIC_{50}=3.24 and MBC=6.84), low activity against *E. coli* (37% with MIC_{50}=6.2 and MBC=11.4) while it was inactive against *A. baumannii, P. vulgaris, M. morganii*and *S. aureus*. The EtOAc fraction

showed moderate activity against *P. vulgaris* (56% with MIC_{50}=2.72 and MBC=5.96), *P. aeruginosa* (55.5% with MIC_{50}=2.96 and MBC=5.88), *VRSA* (52.6% with MIC_{50}=4.92 and MBC=7.0), *M. morganii* (50% with MIC_{50}=4.56 and MBC=8.4) and *E. coli* (48.1% with MIC_{50}=2.08 and MBC=4.88), low activity against *A. baumannii* (34.7% with MIC_{50}=4.48 and MBC=9.08), and was inactive against *S. aureus*. The aqueous fraction showed good activity against *VRSA* (63% with MIC_{50}=2.32 and MBC=5.04), moderate activity against *P. vulgaris* (58% with MIC_{50}=3.48 and MBC=6.52), *P. aeruginosa* (51.8% with MIC_{50}=3.44 and MBC=6.12), *S. aureus* (46.1% with MIC_{50}=2.76 and MBC=6.44), low against *E. coli* (37% with MIC_{50}=6.52 and MBC=11.4), *A. baumannii* (30.4% with MIC_{50}=5.92 and MBC=10.6) while was inactive against *M. morganii* (Fig. 1, Tables 1 and 2).

Antibacterial activity of resin: The Cr. Met. Ext of *P. wallichiana* resin revealed moderate activity against *S. aureus* (53.8% with MIC_{50}=4.2 and MBC=6.6), *P. vulgaris* (52% with MIC_{50}=4.92 and MBC=6.88), *M. morganii* (50% with MIC_{50}=4.52 and MBC=8.24) and *E. coli* (44.4% with MIC_{50}=3.04 and MBC=6.8). It showed low activity against *P. aeruginosa* (39.2% with MIC_{50}=3.8 and MBC=7.6), *A. baumannii* (39% with MIC_{50}=3.8 and MBC=7.4) and *VRSA* (31.5% with MIC_{50}=5.48 and MBC=10.4). The *n*-hexane fraction exhibited moderate activity against *M. morganii* (59% with MIC_{50}=2.12 and MBC=4.08), *P. vulgaris* (50% with MIC_{50}=3.96 and MBC=6.0), *A. baumannii* (47.8% with MIC_{50}=3.24 and MBC=5.4) and *E. coli* (44.4% with MIC_{50}=3.68 and MBC=6.52). This fraction presented low activity against *VRSA* (36.8% with MIC_{50}=6.64 and MBC=11.72) and no activity against *S.aureus* and *P. aeruginosa*. The $CHCl_3$ fraction of resin showed moderate activity against *S. aureus* (57.6% with MIC_{50}=2.96 and MBC=5.6), *A. baumannii* (56.5% with MIC_{50}=2.8 and MBC=5.68), *M. morganii* (50% with MIC_{50}=4.52 and MBC=8.36), *VRSA* (42.1% with MIC_{50}=3.64 and MBC=7.0) and *E. coli* (40.7% with MIC_{50}=4.46 and MBC=7.68), low activity against *P. aeruginosa* (37% with MIC_{50}=6.52 and MBC=11.04) and no activity against *P. vulgaris*. The EtOAc fraction showed good activity against *A. baumannii* (60.8% with MIC_{50}=4.48 and MBC=9.08), moderate against *M. morganii* (54.5% with MIC_{50}=4.56 and MBC=8.4), *VRSA* (52.6% with MIC_{50}=2.92 and MBC=5.48) and *P. vulgaris* (48% with MIC_{50}=2.72 and MBC=5.96). The test sample exhibited low activity against *S. aureus* (30.7% with MIC_{50}=2.96 and MBC=5.88) and *E. coli* (30% MIC_{50}=2.08 and MBC=4.88) and no activity against *P. aeruginosa*. The aqueous fraction displayed moderate activity against *M. morganii* (59% with MIC_{50}=2.12 and MBC=4.04), *S. aureus* (52.3% with MIC_{50}=3.92 and MBC=7.69), *P. vulgaris* (52% with MIC_{50}=2.48 and MBC=4.44) and *VRSA* (42.1% with MIC_{50}=3.0 and MBC=5.96). It showed low activity against *A. baumannii* (39% with MIC_{50}=4.92 and MBC=6.96) and *E. coli* (37% with MIC_{50}=2.96 and MBC=6.0) and no activity against *P. aeruginosa* (Fig. 1, Tables 1 and 2).

Antibacterial activity of stem: The Cr. Met. Ext of *P. wallichiana* stem exhibited good activity against *P. vulgaris* (62% with MIC_{50}=2.0 and MBC=3.96), *S. aureus* (61.5% with MIC_{50}=2.04 and MBC=4.44), *E. coli* (60.3% with MIC_{50}=2.12 and MBC=4.52) and *A. baumannii* (60% with MIC_{50}=2.36 and MBC=5.0). The test samples showed moderate inhibition against *VRSA* (57.8% with MIC_{50}=2.88 and MBC=5.68), *M. morganii* (54% with MIC_{50}=2.52 and MBC=4.04) and *P. aeruginosa* (53.7% with MIC_{50}=2.48 and MBC=4.96). The *n*-hexane fraction exhibited moderate activity against *VRSA* (57.8% with MIC_{50}=3.0 and MBC=6.08), *A. baumannii* (52.1% with MIC_{50}=2.56 and MBC=4.24), *P. vulgaris* (52% with MIC_{50}=2.76 and MBC=4.42), *P. aeruginosa* (50% with MIC_{50}=4.76 and MBC=8.88), *S. aureus* (50% with MIC_{50}=5.0 and MBC=8.96), *M. morganii* (45.5% with MIC_{50}=3.12 and MBC=6.16) and *E. coli* (44.4% with MIC_{50}=3.24 and MBC=7.0). Looking at the percent inhibition of the $CHCl_3$ fraction of the stem, the values were; *A. baumannii* (56.9 with MIC_{50}=3.0 and MBC=6.16), *P. vulgaris* (56 with MIC_{50}=3.36 and MBC=6.48), *S. aureus* (56 with MIC_{50}=2.96 and MBC=5.88), *E. coli* (54 with MIC_{50}=2.52 and MBC=6.44), *VRSA* (47.3 with MIC_{50}=2.96 and MBC=6.08), *P. aeruginosa* (45.1 with MIC_{50}=3.76 and MBC=6.32) and *M. morganii* (41 with MIC_{50}=3.44 and MBC=7.12). The EtOAc fraction exhibited moderate activity against *E. coli* (55.5% with MIC_{50}=2.96 and MBC=5.88), *VRSA* (52.6% with MIC_{50}=3.0 and MBC=5.98), *P. vulgaris* (52% with MIC_{50}=2.88 and MBC=5.0), *S. aureus* (46% with MIC_{50}=3.0 and MBC=6.44), *M. morganii* (46.6% with MIC_{50}=3.08 and MBC=6.98) and *P. aeruginosa* (40.7% with MIC_{50}=3.52 and MBC=7.28). Low activity was recorded against *A. baumannii* (39% with MIC_{50}=4.0 and MBC=8.12). The aqueous fraction of the stem exhibited moderate activity against *P. vulgaris* (58% with MIC_{50}=2.36 and MBC=5.08), *S. aureus* (57.6% with MIC_{50}=3.04 and MBC=6.56), *VRSA* (55.7% with MIC_{50}=3.12 and MBC=6.48), *E. coli* (48% with MIC_{50}=2.36 and MBC=4.16), *M. morganii* (47.7% with MIC_{50}=4.04 and MBC=7.52) and *A. baumannii* (43.4% with MIC_{50}=3.96 and MBC=6.72) while it was inactive against *P. aeruginosa*(Fig. 1, Tables 1 and 2).

Antifungal activity

Antifungal activity of leaves: The results of antifungal activity of Cr. Met. Ext and fractions of leaves are presented in figure 2. As can be seen from the figure that in most cases, the test samples showed low inhibitory effect against the test fungi. The Cr. Met. Ext showed low activity against *A. niger* (25%), *P. notatum* (23%), *H. pseudocrispula* (20%) and *P. chrysogenum* (11.6%) while it was inactive against *A. parasiticus* and *V. longisporum*. Similarly, the *n*-hexane fraction presented low activity against *H. pseudocrispula* (18.1%), *P. notatum* (17.4%) and *P. chrysogenum* (10.6%) while was inactive against *A. niger*, *A. parasiticus* and *V. longisporum*. The $CHCl_3$ fraction exhibited low activity against *H. pseudocrispula* (18%) and *A. niger* (10%) while inactive against rest of the test fungal pathogens. The EtOAc fraction showed low activity against *P. notatum* (14%) and *P. chrysogenum* (12%) and aqueous fraction exhibited low activity against *P. chrysogenum* (3.3%) only.

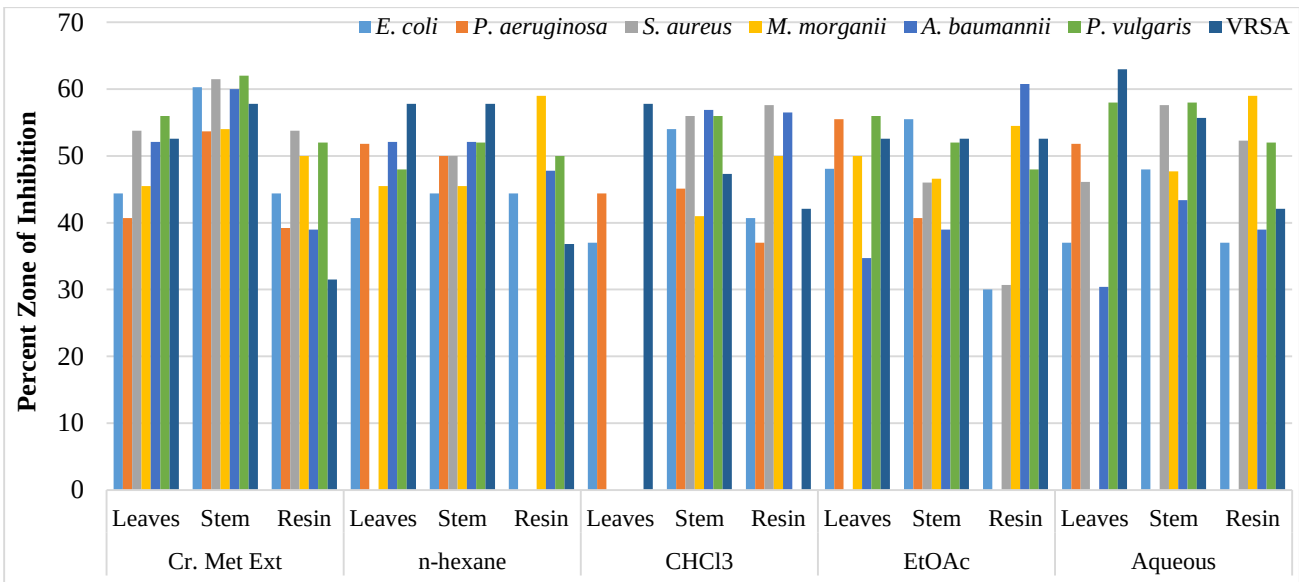

Fig. 1. Antibacterial activity of *Pinus wallichiana* leaves, stem and resin.

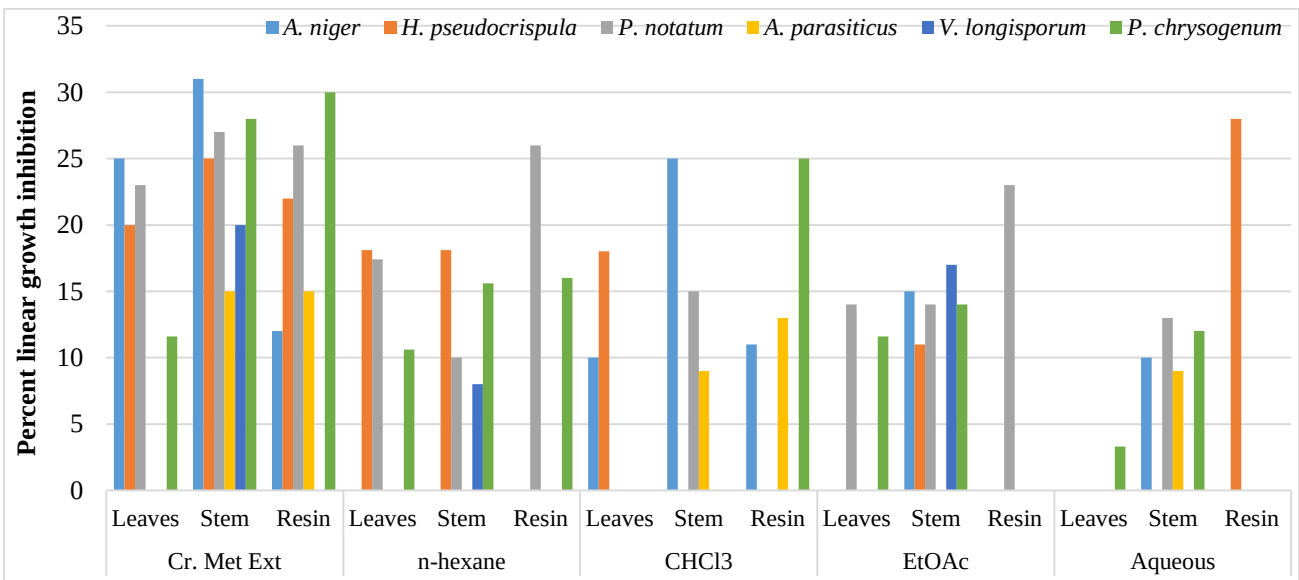

Fig. 2. Antifungal activity of *Pinus wallichiana* leaves, stem and resin.

Table 1. The MIC_{50} (mg/ml) values of *Pinus wallichiana* leaves (L), stem (S) and resin (R).

Bacteria	Cr. Met. Ext			*n*-hexane			$CHCl_3$			EtOAc			Aqueous		
	L	S	R	L	S	R	L	S	R	L	S	R	L	S	R
E. coli	3.12	2.12	3.04	3.64	3.24	3.68	6.2	2.52	4.64	2.08	2.96	2.08	6.52	2.36	2.96
P. aeruginosa	3.52	2.48	3.8	3.4	4.76	---	3.24	3.76	6.32	2.96	3.52	2.96	3.44	---	---
S. aureus	2.4	2.04	4.2	---	5.0	---	---	2.96	2.96	---	3.0	---	2.76	3.04	3.92
M. morganii	3.12	2.52	4.52	3.12	3.12	2.12	---	3.44	4.52	4.56	3.08	4.56	---	4.04	2.12
A. baumannii	2.4	2.36	3.8	2.52	2.56	3.24	---	3.0	2.8	4.48	4.0	4.48	5.92	3.98	4.92
P. vulgaris	2.96	2.0	4.92	2.2	2.76	3.96	---	3.36	---	2.72	2.88	2.72	3.48	2.36	2.48
VRSA	2.52	2.88	5.48	2.8	3.0	6.64	2.96	2.96	3.64	4.92	3.0	2.92	2.32	3.12	3.0

Table 2. The MBC_{50} (mg/ml) values of *Pinus wallichiana* leaves (L), stem (S) and resin (R).

Bacteria	Cr. Met. Ext			*n*-hexane			$CHCl_3$			EtOAc			Aqueous		
	L	S	R	L	S	R	L	S	R	L	S	R	L	S	R
E. coli	6.88	4.52	6.8	7.4	7.0	6.52	11.4	6.44	7.68	4.88	5.88	4.88	11.4	4.16	6.0
P. aeruginosa	7.2	4.96	7.6	9.5	8.88	---	6.84	6.32	11.04	5.88	7.28	5.88	6.12	---	---
S. aureus	6.6	4.44	6.6	---	8.96	---	---	5.88	5.6	---	6.44	---	6.44	6.56	7.69
M. morganii	6.72	4.04	8.24	6.8	6.16	4.08	---	7.12	8.36	8.4	6.98	8.4	---	7.52	4.04
A. baumannii	4.6	5.0	7.4	4.2	4.24	5.4	---	6.16	5.68	9.08	8.12	9.08	10.6	6.72	6.96
P. vulgaris	5.6	3.96	6.88	4.92	4.4	6.0	---	6.48	---	5.96	5.0	5.96	6.52	5.08	4.44
VRSA	4.04	5.68	10.4	5.52	6.08	11.72	5.6	6.08	7.0	7.0	5.98	5.48	5.04	6.48	5.96

Antifungal activity of resin: Against *A. niger*, the Cr. Met. Ext and $CHCl_3$ fraction showed low activity; 12 and 11%, respectively while *n*-hexane, EtOAc and aqueous fractions showed no activity against it. The Cr. Met. Ext and aqueous fraction of resin showed low activity (22 and 28%) against *H. pseudocrispula* while *n*-hexane, $CHCl_3$ and EtOAc fractions were found inactive against it. The Cr. Met. Ext, *n*-hexane and EtOAc fraction showed low activity (26, 26 and 23%) against *P. notatum* while $CHCl_3$ and aqueous fractions were found inactive. Low activity of 15 and 13% was exhibited by Cr. Met. Ext and $CHCl_3$ fraction against *A. parasiticus* while *n*-hexane, EtOAc and aqueous fractions were inactive. The Cr. Met. Ext and all the fractions were inactive against *V. longisporum*. The percent linear growth inhibition against *P. chrysogenum*was: Cr. Met. Ext (30), *n*-hexane (16), $CHCl_3$ (25), EtOAc and aqueous (0) (Fig. 2).

Antifungal activity of stem: Against *A. niger*, the percent linear growth inhibition was: Cr. Met. Ext (31), *n*-hexane (0), $CHCl_3$ (25), EtOAc (15) and aqueous (10). The Cr. Met. Ext, *n*-hexane and EtOAc fraction showed low activity of; 25, 18.1 and 11%, respectively against *H. pseudocrispula* while $CHCl_3$ and aqueous fractions were inactive against it. The Cr. Met. Ext and all other fractions showed low activity against *P. notatum*; Cr. Met. Ext (27), *n*-hexane (10), $CHCl_3$ (15), EtOAc (14) and aqueous (13). The Cr. Met. Ext, $CHCl_3$ and aqueous fractions showed low activity, 15, 9 and 9%, respectively against *A. parasiticus* while *n*-hexane and EtOAc fractions were inactive. Against *V. longisporum*, Cr. Met. Ext, *n*-hexane and EtOAc fractions showed low activity; 20, 8 and 17%, respectively while $CHCl_3$ and aqueous fractions were inactive against it. Against *P. chrysogenum*, Cr. Met. Ext, *n*-hexane, EtOAc and aqueous fractions showed low percent activity; 28, 15.6, 14 and 12, respectively while $CHCl_3$ fraction was found inactive (Fig. 2).

Phytotoxic activity

Phytotoxic activity of stem: The Cr. Met. Ext, $CHCl_3$ and aqueous fractions of the stem presented good phytotoxicity (60, 67.5 and 67.5%, respectively) at 20mg/ml while low growth regulation was recorded for the *n*-hexane (10%) and EtOAc (27.5%) fractions. The Cr. Met. Ext possess moderate phytotoxicity (50%) at 1000µg/ml and low phytotoxicity was observed at 100 and 10µg/ml. The $CHCl_3$ fraction possess good (60%) and moderate (50%) growth inhibition of *L. minor* at higher concentrations while low activity at lower concentration (Figs. 3 and 4).

Phytotoxic activity of resin: The Cr. Met. Ext and $CHCl_3$ fraction of the resin showed good phytotoxicity (65 and 60%, respectively) while moderate growth regulation was recorded for the *aqueous* (55%) and EtOAc (45%) fractions at 20mg/ml. At the same concentration the *n*-hexane fraction presented low phytotoxicity (17.5%). The Cr. Met. Ext, $CHCl_3$ and EtOAc fractions moderately inhibited the growth of *L. minor* at 1000, 100µg/ml. Low phytotoxicity was observed at 10µg/ml for the test samples (Figs. 3 and 4).

Phytotoxic activity of leaves: The Cr. Met. Ext and aqueous fraction of the leaves exhibited good phytotoxicity (62.5 and 70%, respectively) while moderate growth regulation was recorded for the $CHCl_3$ (52.5%) and low for the rest of the fractions at 20mg/ml. A moderate phytotoxic effect (40%) was recorded for the aqueous fraction of the leaves at higher concentration while low activity was observed at low concentrations (Figs. 3 and 4).

Haemagglutination activity: Some plants contain agglutinin in their different parts and they can be isolated just like the agglutinins from animals which can be utilized for the productions blood typing reagents. The agglutinins obtained from plant sources are advantageous due to its availability in large quantities. Therefore, the Cr. Met. Ext and different fractions of the test samples were screened for their haemagglutination activity against the ABO blood groups. The results, as shown in table 3, indicated that all test samples showed no activity against all blood groups, at all dilution, meaning that this plant lack phytoagglutinins.

Anti-oxidant activity: The Cr. Met. Ext of *P. wallichiana* leaves showed good antioxidant potential (60.29%) at 500µg/ml, moderate (55 and 46%) at 400 and 300µg/ml while low activity of 35 and 30%, at 200 and 100µg/ml respectively. The *n*-hexane fraction presented moderate antioxidant activity at 500(57%), 400(50%) and 300µg/ml (40%) while low potential was observed at 200(36%) and 100µg/ml(29%). The $CHCl_3$ fraction showed good antioxidant activity of 60.4% at 500µg/ml, moderate (55.5, 49.6 and 42%) at 400, 300 and 200µg/ml, respectively and low activity of 35% was recorded at lower concentration. The percent antioxidant activity of EtOAc fraction at 500, 400, 300, 200 and 100µg/ml was 66.73, 59.63, 55.53, 45.43 and 36.33, respectively. The aqueous fraction presented good antioxidant activity of 72.21 and 65.21% at 500 and 400µg/ml, moderate (59.21, 49.21%) at 300 and 200µg/ml, respectively, and low activity of 39.21% at 100µg/ml (Fig. 5).

Similarly, the Cr. Met. Ext of *P. wallichiana* resin and stem presented good, moderate and low antioxidant activity and was concentration dependent (Fig. 5).

The EC_{50} values of Cr. Met. Ext and fractions of different parts are presented in table 4. The EC_{50} of aqueous fraction of resin was 136.5µg/ml, which was lower than the standard (169.78µg/ml), giving a signal that resin of this plant may be subjected to isolation of potent antioxidant compounds.

Insecticidal activity: Extensive efforts have been focused on plant derived chemicals which have the potential as commercial insecticides which are environment friendly, convenient to use and economical (Rachid *et al.*, 2006). Adding to the effort, the Cr. Met. Ext of stem showed significant activity against *T. castaneum*and its larvae, *R. dominica* and *C. chinensis* while low activity against *C. pulicaria*. The *n*-hexane fraction showed significant insecticidal potential against *T. castaneum*and its larvae and *R. dominica*, good against *C. pulicaria* while moderate inhibition was recorded against *C. chinensis*.

The $CHCl_3$ fraction showed significant activity against *R. dominica*, *C. chinensis*, *C. pulicaria* and *T. castaneum* larvae while good activity against *T. castaneum*. The EtOAc fraction of leaves expressed significant mortality against all the test insects except *C. chinensis*. The aqueous fraction showed significant activity against *R. dominica*, low against *C. chinensis*, *T. castaneum* larvae and *T. castaneum* while it was inactive against *C. pulicaria*. The Cr. Met. Ext of leaves and resin also presented significant, good, moderate and low insecticidal activity against the test insects (Fig. 6). The Cr. Met. Ext, $CHCl_3$ and aqueous fractions of the leaves showed significant activity (100%) against *H. indicola* while EtOAc and *n*-hexane fractions exhibited moderate activity of 50 and 60%, respectively. The Cr. Met. Ext, $CHCl_3$ and EtOAc fractions of stem exhibited significant activity (100%), the *n*-hexane fraction good (70%) while aqueous fraction presented moderate activity (60%). The Cr. Met. Ext and EtOAc fraction of resin showed significant activity (100 and 90%), the *n*-hexane fraction exhibited good (70%) while the $CHCl_3$ and aqueous fractions presented moderate inhibitory activity (60 and 50%), respectively.

In vivo biological activities

Acute toxicity assay: In this study, Cr. Met. Ext of *P. wallichiana* leaves, resin and stem were screened for possible *In-Vivo* biological efficacy. As the first step towards this approach, acute toxicity assay was performed, using test doses of 200, 400 and 600mg/kg of body weight as per the OECD guidelines (Torrico *et al.*, 2013). All extracts were found safe at the test doses and no lethality was observed (Fig. 7).

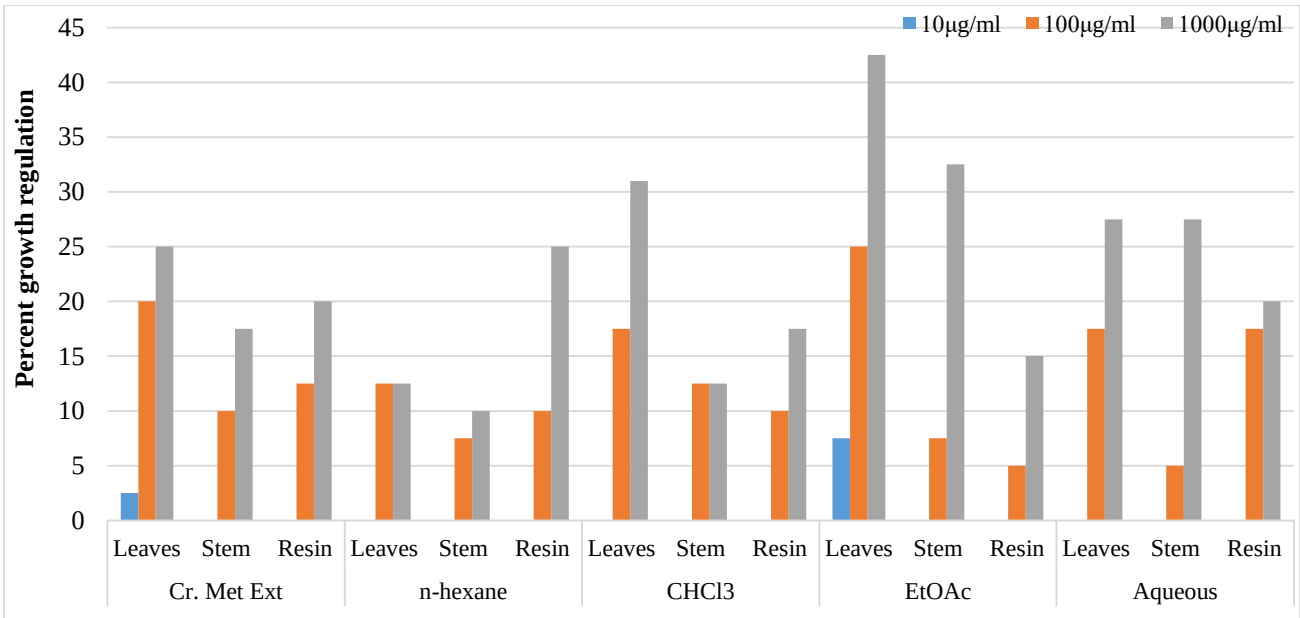

Fig. 3. Phytotoxic activity of Cr. Met. Ext and fractions of leaves, stem and resin.

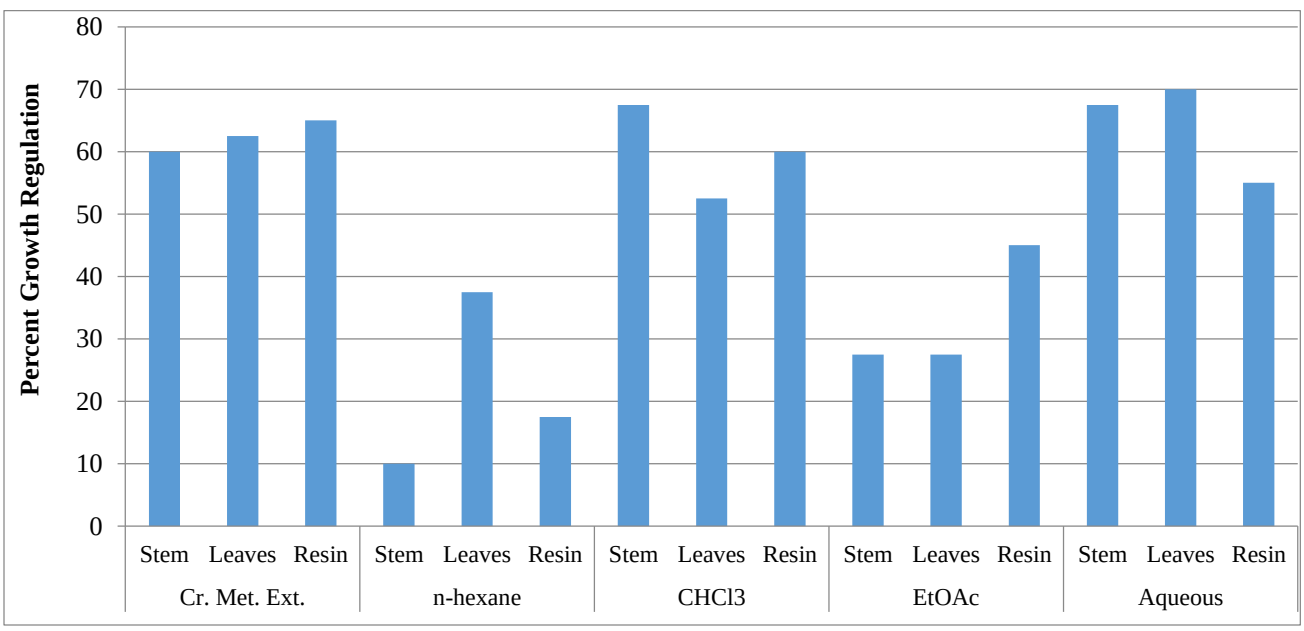

Fig. 4. Phytotoxic activity of *Pinus wallichiana* leaves, stem and resin at 20mg/ml.

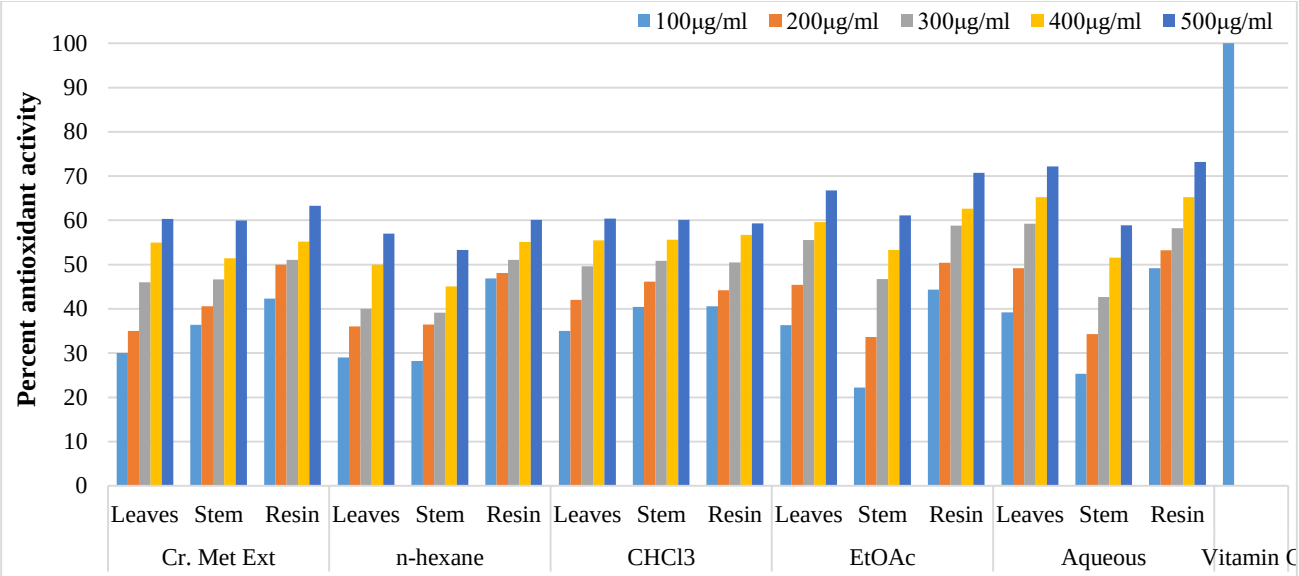

Fig. 5. Antioxidant activity of *P. wallichiana*leaves, resin and stem.

Table 3. Haemagglutination activity of stem, resin and leaves of *Pinus wallichiana*.

Blood groups	**AB^{-ive}, AB $^{+ive}$, O $^{+ive}$, O $^{-ive}$, A^{-ive}, A $^{+ive}$, B^{-ive}, B $^{+ive}$**			
Dilutions	**1:2**	**1:4**	**1:8**	**1:16**
Cr. Met. Ext.	-	-	-	-
n-hexane	-	-	-	-
$CHCl_3$	-	-	-	-
EtOAc	-	-	-	-
Aqueous	-	-	-	-

Table 4. EC_{50} value of *P. wallichiana* leaves, resin and stem.

Test sample	**EC_{50} (µg/ml)**					
	Cr. Met. Ext	***n*-hexane**	**$CHCl_3$**	**EtOAc**	**Aqueous**	**Standard**
Leaves	365	407.24	323.32	263.60	214.15	169.78
Resin	249.87	241.9	294.79	187.63	136.5	
Stem	351.78	462.74	287	367.63	388.21	

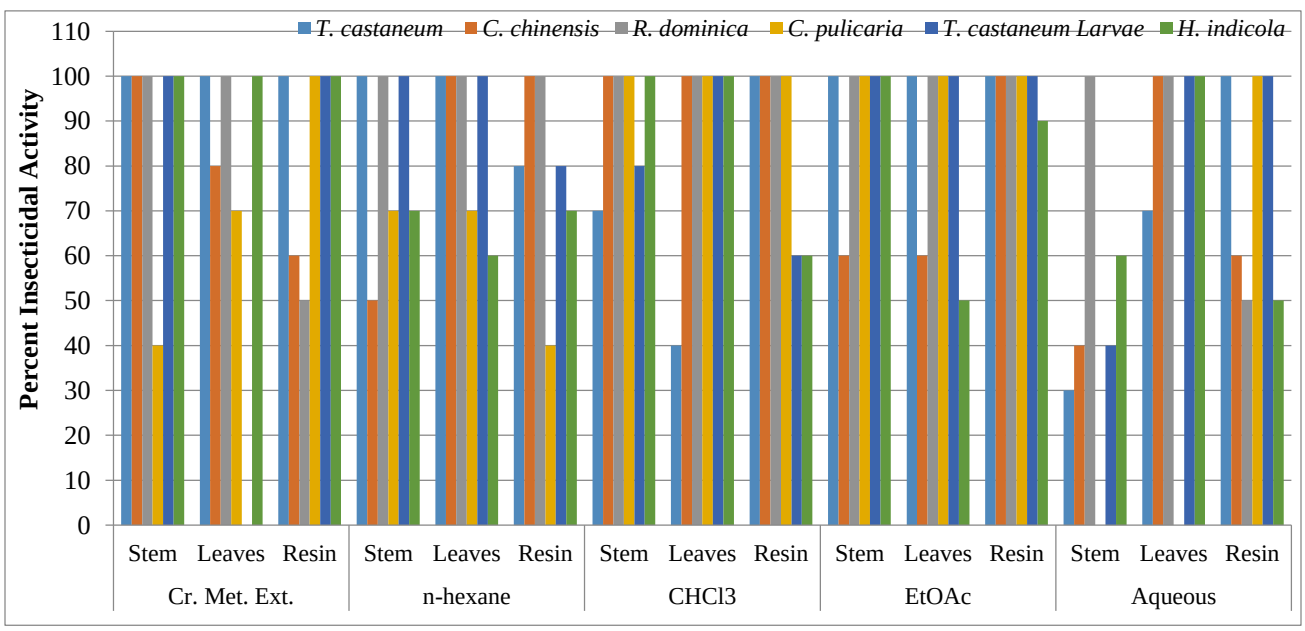

Fig. 6. Insecticidal activity of *Pinus wallichiana* leaves, stem and resin.

Antinociceptive assay (acetic acid induced writhing test): The Cr. Met. Ext of *P. wallichiana* stem, resin and leaves showed significant decrease in the number of writhes as determined via ANOVA and Dunnett's post-hoc analysis after intervals of 30, 60 and 90 minutes. The percent writhing inhibitory effect produced by different test doses of Cr. Met. Ext of stem was 21.9 (100mg/kg), 28.6 (200 mg/kg) and 43.9 (300mg/kg). The percent inhibitory effect produced by different doses of Cr. Met. Ext of leaves was 27.6 (100mg/kg), 31.5 (200mg/kg) and 36 (300mg/kg) while that of Cr. Met. Ext of resin was 35.5 (100mg/kg), 42.7 (200mg/kg) and 58.6 (300mg/kg). In saline treated group writhing was 74.33%, which acted as negative control. As can be seen from the above mentioned results the inhibitory effect was dose dependent and increased with increase in dose of the test samples. The maximum percent inhibition produced by Diclofenac sodium at 10mg/kg dose was 82%, which was greater than the highest dose of Cr. Met. Ext (300mg/kg) as shown in figure 8. The Cr. Met. Ext of *P. wallichiana* stem, leaves and resin were all found to be highly significant at all test doses (***p<0.001).

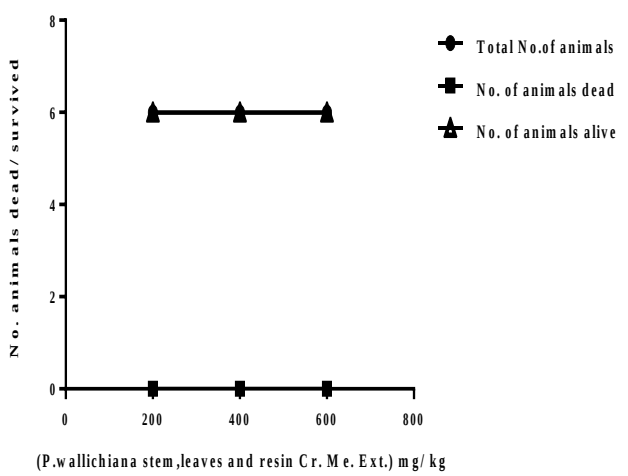

Fig. 7. Acute toxicity of Cr. Met. Ext of *Pinus wallichiana* stem, leaves and resin.

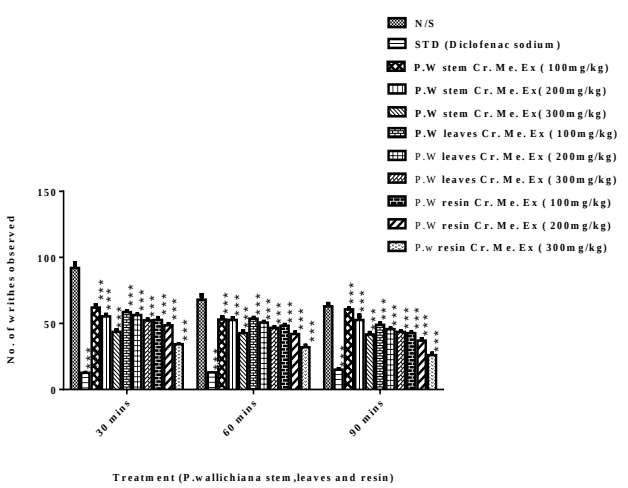

Fig. 8. Antinociceptive activity of Cr. Met. Ext of *Pinus wallichiana* stem, leaves and resin.

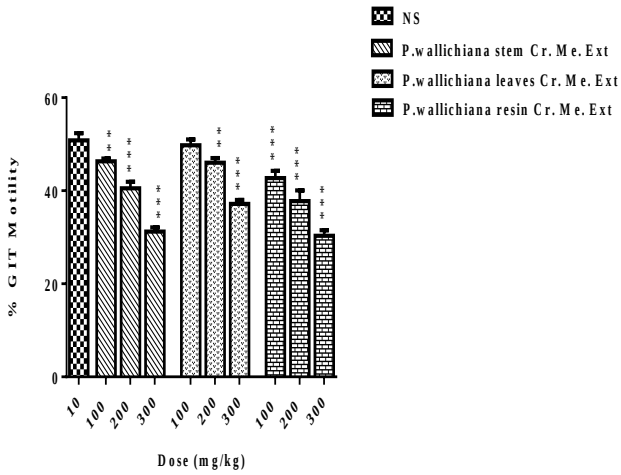

Fig. 9. Effect on GIT motility of Cr. Met. Ext of *Pinus wallichiana* stem, leaves and resins.

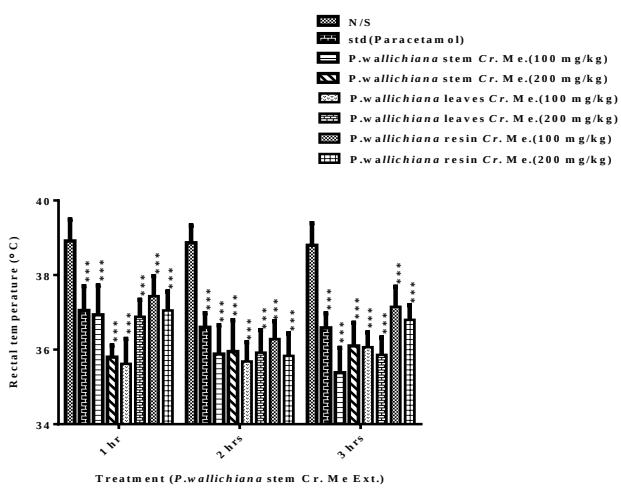

Fig. 10. Anti-pyretic effect of Cr. Met. Ext of *Pinus wallichiana* stem, leaves and resin.
Data were presented as mean±SEM (n = 6). * P b 0.05, ** P b 0.01, *** P b 0.001, all groups were compared with negative control (Normal saline).

Gastrointestinal tract (GIT) motility (charcoal meal protocol): The percent decreased in GIT motility was observed in a dose dependent manner as presented in figure 9. The percent GIT motility observed in the test group treated with 100mg/kg of *P. wallichiana* stem, leaves and resin was 46.35, 49.93 and 42.72, respectively. The same decrease in percent GIT motility was observed at 200mg/kg of stem, leaves and resin; 40.51, 46 and 37.8, respectively. The percent GIT motility observed at 300mg/kg of stem, leaves and resin was; 31.21, 37.17 and 30.32, respectively while for the group treated with normal saline the GIT motility was 50.85. It is evident from the results that Cr. Met. Ext of different parts of *P. wallichiana* effect the motility of GIT in a dose dependent manner. The test samples may cause relaxation of small intestine through M3 receptor (muscarinic) activation by acetylcholine. The antispasmodic agents alleviate the abdominal cramps, so different plant extracts used for the management of diarrhoea and abdominal spasm reduces GIT motility (Naveed *et al.,* 2012).

Anti-pyretic activity: The Cr. Met. Ext of *P. wallichiana* stem, leaves and resin were observed to have significant anti-pyretic effect after intervals of 1, 2 and 3 hours in comparison to negative control (Normal saline) and are presented in figure 10. The temperature recorded for the paracetamol (50mg/kg) was 36.74ºC as compared to the normal saline. All the test samples also showed results comparable to Paracetamol. At 100 mg/kg, the temperature observed was; Cr. Me. Ext of leaves (35.78ºC), stem (36.06ºC) and resin (36.95ºC). The results at 200mg/kg were: Cr. Me. Ext of stem (35.95ºC), resin (36.56ºC) and leaves (36.11ºC). The Cr. Met. Ext stem, leaves and resin showed significant anti-pyretic effects at test doses (***$p<0.001$).

Discussion

Sharma *et al.,* (2009) reported good antibacterial activity for the ethanolic Ext of *Z. officinale*, *P. granatum* and acetone extract of *T. chebula* against *E. coli* making it supportive for our study. Our results could also be supported by konning *et al.,* 2004, where Met. Ext of certain plants were significantly active against Gram positive bacteria but presented low activity against *A. niger*. In the published study, low activity was observed against *P. aeruginosa*, which is in contrast to the current study giving a clue to bioactivity guided isolation against *P. aeruginosa* from the selected plant. The ethanolic extracts of *Alpinia galangal* and *Curcuma longa* exhibited significant (100%) phytotoxic effect against *L. minor* (Khattak *et al*., 2005) making it against our results as our selected plant lacks potent phytotoxic activity. Our laboratory is involved in screening different medicinal plants for its haemagglutination potential but so far we haven't obtained a potent source (Bashir *et al.,* 2012) and the current study is yet evidence that plants usually lack phytoagglutinins. Another study investigated the insecticidal activity of *Zygophyllaceae* and *Euphorbiaceae* members against *T. castaneum* revealing low inhibitory effect, which is in contrast to the current findings (Farrukh & Ghulam, 2013). The acute toxicity of Cr. Met. Ext of root-bark of *Fagarazanth oxyloides* in male albino mice (27-35g) reported that within 6 hours all mice in the test group died at dose of 10g/kg while at 2.0g/kg the test sample was safe (Jasper *et al.,* 2003). All test doses (200, 400 and 600mg/kg) in current study were found safe and no lethality was observed, making it a safe source of bioactive compounds. The antinociception potential of aqueous extract of leaves of *Amanoa almerindae* at 94 and 188mg/kg was comparable to that of acetylsalicylic acid (Torrico *et al.,* 2013). The test doses in the current study showed significant decrease in the number of writhes after intervals of 30, 60 and 90 minutes and the results were highly significant. The anti-pyretic potency of Cr. Me. Ext of *V. Betonicifolia* at 300mg/kg was 78.23%, as compared to Paracetamol (90%) making it in line with the current findings (Naveed *et al.,* 2012). Overall the findings of the current study revealed that the selected plant may be given due research attention.

Conclusion

The results of the current study revealed that *P. wallichiana* can be a potential source for antibacterial, anti-termite, insecticidal and antioxidant compounds. It can be a safe source of different potential drugs as it was not lethal at the test doses and have analgesic and antipyretic effects.

Acknowledgment

The authors are thankful to Higher Education Commission (HEC), Islamabad, Pakistan, for financial support under National Research Program for Universities (NRPU), project No. 4767.

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(Received for publication 17 August 2017)