Fractionation of crude extracts from controlled dried and commercially available stem bark of *Juglans regia* and their antimicrobial effects

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Abstract: The current research investigates the anti-microbial activities of methanol, ethyl acetate, n-hexane , n-butanol and water extracted samples from controlled dried and commercial bark of walnut (*Juglans regia*) against five bacterial (*Staphylococcus aureus*, *Bacilus subtilis*, *Pseudomonas aeruginosa, Escherichia coli, Klebsiella pneumonia* and one fungal pathogenic specie (*Candida albicans*) by discs diffusion susceptibility assay using 0.5 and 1mg disc^{-1} concentrations. Our results revealed that all the extracts from controlled dried and commercial bark of walnut showed varying degrees of antimicrobial activities. Ethyl acetate fraction from both sources exhibited maximum activity against all tested microbial species followed by n-butanol and crude methanolic extraxt. N-hexane and aqueous extracted samples from controlled dried bark reduced the growth of all studied microbes except *Staphylococcus aureus* in case of commercial available bark. Aqueous extracted sample showed inhibitory effects against all tested microbes except *Candida albicans* respectively in case of commercial bark. The most susceptible gram positive bacteria were *S. aureus* while *Bacillus subtilis* was the most resistant one. Among Gram negative bacteria, *Pseudomonas aeruginosa* was the most susceptibility while *Klebsella pneumonia* showed some resistively. Compared to commercial bark samples, controlled dried bark extract and fractions were found to be more active in reducing the growth of all the tested microbes at both concentrations.

Keywords: Antibacterial, antifungal, *Juglans regia*, disc diffusion, controlled dried and commercial bark.

INTRODUCTION

There are around 6000 plant species in Pakistan, and among them 720 are considered to have medicinal importance (Bakht *et al*., 2011 a, b, c and d; 2012; 2013 a,b; 2014 a, b,c; 2015; Nasir *et al.,* 2015; Ullah *et al*., 2015; Zakir *et al*., 2015; Parveen and Bakht, 2015; Chaun *et al*., 2015; Bilal *et al*., 2016; Wajid *et al*., 2016 a, b; Amjad *et al*., 2016; Anwar *et al*., 2016). These medicinal plants have been used for handling several health related problems in the country (Saeed and Rizvi, 1990; Hussain *et al.,* 2009). A huge number of tabibs and a large number of consultants that are not registered are exploiting these medicinal plant ingredients in traditional way. People usually use commercially available plant materials in old-style to make several types of medicines. However, no scientific efforts have been made for the efficient industrial utilization of these important natural resources. In Pakistan herbal medicines are used without any prior testing for their active compounds and quality. Using numerous easily assimilated plant bio-molecules, novel antimicrobial agents and drugs can be prepared and commercialized. In developing countries, where socioeconomic conditions are poor and people cannot meet the expense of standard medicines, uses medicinal plants for different treatments.

Juglans regia also called as walnut belongs to the family Juglandaceae. It is a moderate forestry plant found worldwide. *Juglans regia* mainly used as part of our nutrition and several parts of the same plant are consumed as native traditional remedy (Stamper *et al*., 2006; Oliveira *et al*., 2008). Root bark of *Juglans regia* is mostly used for cleaning and glistening teeth by women in the KPK province of Pakistan. Cleaning the teeth with stem and root bark of *Juglans regia* can improve oral hygiene, inhibiting plaque and caries development, and decrease the occurrence of gingival and periodontal infections (Alkhawajah, 1997). *Juglans regia* consist of ascorbic acid, oxalic acid, globulin, vitamin A and B, juglone, 1,4-napthaquinone and amino acids. The stem and root bark mostly consists of flavonoid, sakuranetetin, juglone, cyclotrijuglone, β-sitosterol, monoterpenes, eugenol. Compared with other nuts like peanuts and almonds, walnuts (particularly in raw form) contain high levels of antioxidants. *Mycobacterium tuberculosis,* a globally health hazard, kills about two and half million people annually. *Juglans regia* is conventionally used to treat different respiration infections including tuberculosis (Delia *et al*., 2008). The current research work was conducted to study and compare the antimicrobial potential of samples from controlled dried and commercially available stem bark of *Juglans regia.*

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MATERIALS AND METHODS

Plant material

The current investigation was carried out at the Institute of Biotechnology and Genetic Engineering, The University of Agriculture Peshawar Pakistan. Plant materials were obtained from different localities of District Dir, Khyber Pakhtunkhwa province of Pakistan. Commercial stem bark was purchased from the local market. The fresh plant materials (stem bark) were washed thoroughly with distilled water to remove any dust particles, dried for seven days and grinded.

Crude extract preparation

One thousand grams of dried plant materials (stems) were mixed in five liters of methanol, kept at 25ºC in dark for one week and the mixture was stirred three times daily. The mixture was filtered through Whatman filter paper No.1. The remaining solid residue was mixed with twenty five hundred ml fresh methanol and the whole procedure was repeated thrice. The different filtrates were pooled together and dried below 45ºC under vacuum pressure in a rotary evaporator. The semisolid extract was divided into two portions, one portion (10g) was used as crude methanolic extract and the second portion (90g) was used for fractionation with different solvents.

Fractionation of crude extract

Ninty grams of crude methanolic extract was dissolved in 500 ml sterile distilled water, mixed with n-hexane (300 ml), shaken gently and allowed to stand for 15minutes to separate the two phases. The upper n-hexane layer was obtained and the lower aqueous layer was re-extracted three times with fresh n-hexane. Different fractions of n-hexane were pooled together and dried at 45°C under vacuum pressure through rotary evaporator. The same procedure was followed to obtain ethyl acetate and n-butanol fractions.

Preparation of media

Nutrient agar medium was used for the culturing and growth of all microorganisms tested in the study. Nutrient broth was used for shaking incubation and standardization of the microorganisms. The required amount of nutrient agar and nutrient broth were poured into conical flasks. Twenty ml of the nutrient broth was also poured into different test tubes. All the media flasks and test tubes were sterilized and nutrient agar medium was poured aseptically into sterilized petri plates in a Laminar flow hood and allowed to solidify in petri plates for about an hour before the petri plates were placed in an inverted position at 37ºC for 24 hrs. After 24 hrs, uncontaminated plates were used for culturing of bacteria and fungi.

Disc diffusion susceptibility assay

The antibacterial activity of different solvent extracted samples from controlled dried and commercially available stem bark of *Juglans regia* was carried by disc diffusion assay as described in Bauer *et al*. (1966) and antifungal activity by Ramdas *et al*. (1998) against different bacterial and fungal strains (table 1). Nutrient agar media plates were inoculated with 18-24 hrs cultures of microbial inoculums (a standardized inoculums 1-2 × 107 CFU ml-1 0.5 McFarland Standard). Three discs of Whatman No. 1 filter paper (6 mm in diameter) were placed on the media in petri plates. Different plant extracts in concentration of 0.5 and 1 mg in 6 and 12µl volume were applied on the discs. Antibiotics (6µl $disc^{-1}$) as positive control and DMSO (6µl $disc^{-1}$) as negative control were also applied on the discs in separate petri plates. Inoculated plates were then incubated at 37ºC for 18-24 hrs. The next day zones of inhibition were recorded in mm around the discs in each plate.

Positive controls

For Gram-positive bacteria; Ciprofloxacin 50µg per 6µl
For Gram negative-bacteria; Ciprofloxacin 50µg per 6µl
For Fungal srains; Fluconazole 50µg per 6µl

STATISTICAL ANALYSIS

Data are presented as mean values of three replications. MSTATC computer software was used for statistical analysis (Russel and Eisensmith, 1983). Least Significant Difference (LSD) test was employed to compare significant difference among means (Steel *et al*., 1997).

RESULTS

Data concerning the antibacterial potential of controlled dried bark of *Juglans regia* against *Bacillus subtilis* is shown in fig. 1. The data suggested that all the samples reduced the growth of *Bacillus subtilis* at both concentrations. High activity was shown by methanol extracted samples at higher and lower concentrations (37 and 50% ZI at 0.5 and 1 mg $disc^{-1}$), while n-butanol and aqueous extracted samples showed the lowest inhibitory activity (26% ZI at 0.5 mg $disc^{-1}$ concentration). Our results revealed that *Staphylococcus aureus* showed high susceptibility in case of n-hexane and ethyl acetate extracted samples of controlled dried bark at both concentrations (47% and 67% ZI at 0.5 and 1 mg $disc^{-1}$). N-butanol and aqueous extracted samples also showed similar activity measuring 53% ZI at concentration of 1 mg $disc^{-1}$ (fig. 2). It is clear from the data shown in fig. 3 that all solvent extracted samples from controlled dried bark inhibited the growth of *E. coli* at higher and lower concentrations. The data also revealed that ethyl acetate and methanol extracted samples showed the highest inhibitory activity (65% ZI at 1 mg $disc^{-1}$) compared to positive controls. N-butanol extracted samples reduced the growth of *E. coli* by 58% followed by aqueous (39% ZI; fig. 3). The data further revealed that all the tested extracted samples from controlled dried stem bark

reduced the activity of *Klebsiella pneumoniae* at both higher and lower concentration (fig. 4). N-hexane, ethyl acetate and n-butanol extracted samples measured highest activity (47% ZI at concentration of 1 mg disc^{-1}). The aqueous extracted samples from controlled dried stem bark on the other hand measured the lowest activity of 24% at concentration of 0.5 mg disc^{-1}.

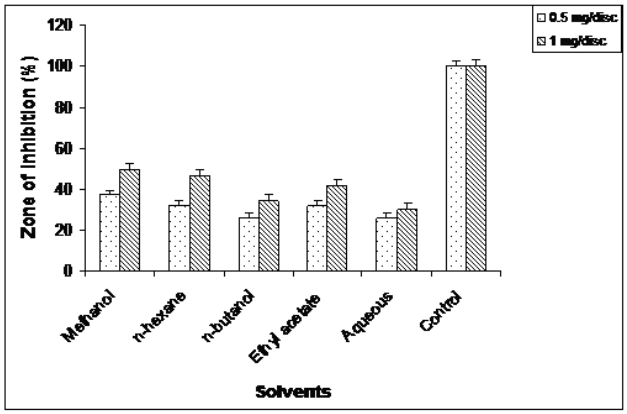

Fig. 1: Antibacterial activity of crude methanol, n-hexane, ethyl acetate, n-butanol and water extracted samples from controlled dried stem bark of *Juglans regia* against *Bacillus subtilis* by disc diffusion assay (Bar shows LSD at p<0.05).

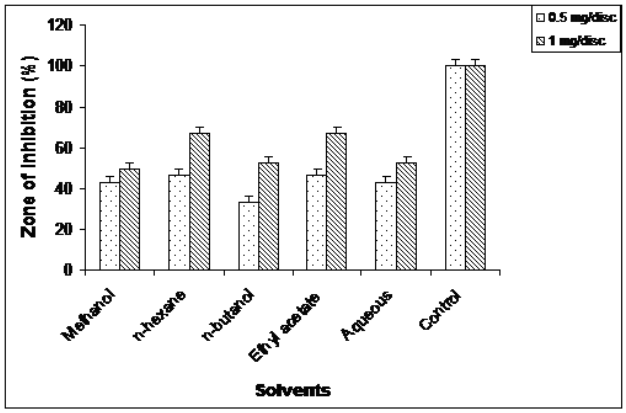

Fig 2: Antibacterial activity of crude methanol, n-hexane, ethyl acetate, n-butanol and water extracted samples from controlled dried stem bark of *Juglans regia* against *Staphylococcus aureus* by disc diffusion assay (Bar shows LSD at p<0.05).

Our results suggested that all tested samples significantly reduced the growth of *Pseudomonas aeruginosa* at both concentrations (fig. 6). *Pseudomonas aeruginosa* was highly inhibited by crude methanol samples measuring 80% inhibitory zone at 1 mg disc^{-1}. Ethyl acetate and n-butanol samples revealed 75% ZI each at higher concentration. N-hexane and n-butanol showed 46% ZI each at 0.5 milligram disc^{-1} concentration when compared with other solvents and positive control (fig. 6). Analysis of the data indicated that highest inhibitory zones were measured by crude methanolic extract and ethyl acetate fraction (60% ZI each) followed by n-butanol fraction (53% ZI) against *Candida albicans* when applied in concentration of 1mg discs^{-1}. Lowest activity was measured by aqueous extracted samples (30% ZI) when applied in 0.5 mg disc^{-1} concentration. The data further showed that n-hexane and aqueous samples showed 47% ZI at concentration of 1mg disc^{-1} when compared with samples and controls.

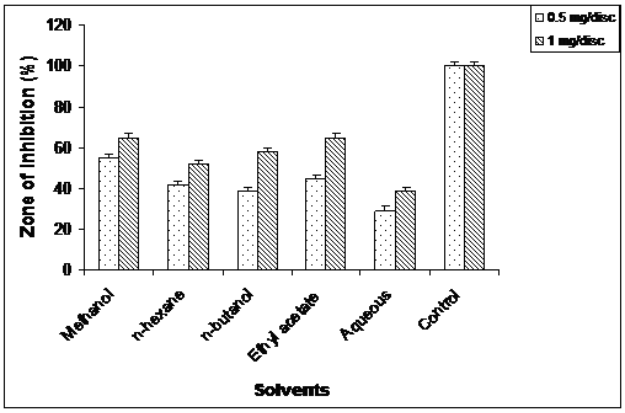

Fig. 3: Antibacterial activity of crude methanol, n-hexane, ethyl acetate, n-butanol and water extracted samples from controlled dried stem bark of *Juglans regia* against *Escherichia coli* by disc diffusion assay (Bar shows LSD at p<0.05).

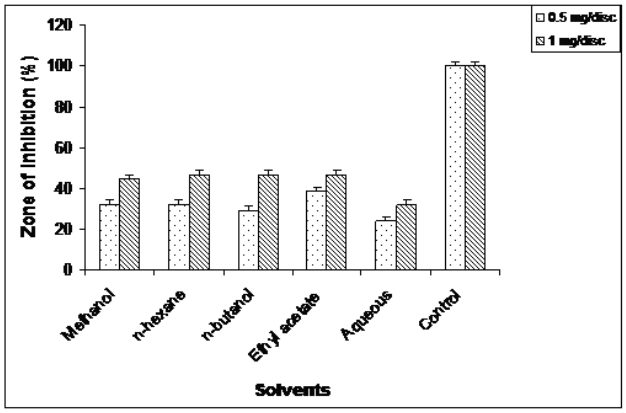

Fig. 4: Antibacterial activity of crude methanol, n-hexane, ethyl acetate, n-butanol and water extracted samples from controlled dried stem bark of *Juglans regia* against *Klebsiella pneumoniae* by disc diffusion assay (Bar shows LSD at p<0.05).

Fig. 7 presents data concerning the antibacterial activity of n-hexane, ethyl-acetate, n-butanol, methanol and aqueous extracted samples from commercially available stem bark of walnut against *Bacillus subtilis*. Highest inhibitory activity was shown by crude methanolic samples and measured 54% ZI at 1mg disc^{-1} when compared with positive controls. The lowest inhibitory zone measured was shown by n-hexane extracted samples at concentration of 0.5 mg disc^{-1}. The data indicated that ethyl acetate extracted samples showed the highest

inhibitory zone of 67% against *Staphylococcus aureus* when applied in concentration of 1mg disc^{-1} compared with positive control (fig. 8). Crude methanolic and aqueous extracted samples inhibited the bacterial growth by 43% at concentration of 1mg disc^{-1}. N-hexane and n-butanol extracted samples were equally effective to reduce the activity of *S. aureus* at concentration of 1mg disc^{-1}.

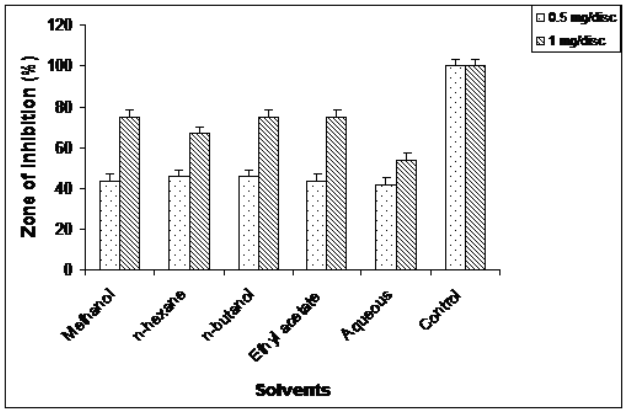

Fig. 5: Antibacterial activity of crude methanol, n-hexane, ethyl acetate, n-butanol and water extracted samples from controlled dried stem bark of *Juglans regia* against *Pseudomonas aeruginosa* by disc diffusion assay (Bar shows LSD at $p<0.05$).

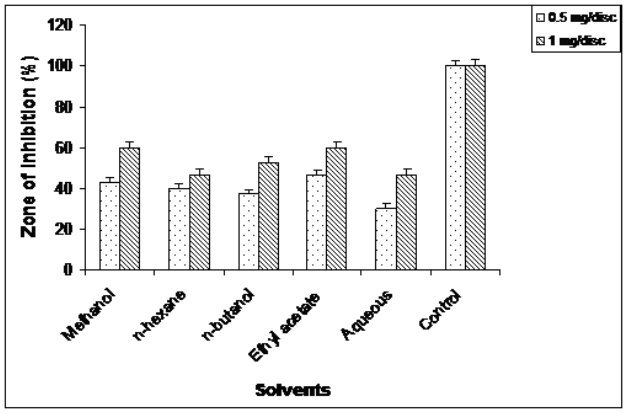

Fig. 6: Antifungal activity of crude methanol, n-hexane, ethyl acetate, n-butanol and water extracted samples from controlled dried stem bark of *Juglans regia* against *Candida albicans* by disc diffusion assay (Bar shows LSD at $p<0.05$).

Analysis of the data indicated that maximum inhibitory activity against *Escherichia coli* was shown by crude methanolic extract of commercially available stem bark measuring 52% and 58% ZI at lower and higher concentrations respectively (fig. 9). Ethyl acetate extracted samples showed inhibitory zone of 48% at higher concentration of 1mg disc^{-1}. The lowest inhibitory zones were measured by n-butanol and aqueous extracted samples (26% ZI) at concentration of 0.5 mg disc^{-1}. *Klebsiella pneumonia* was sensitive to ethyl acetate extracted samples from commercially available stem bark and showed profound inhibitory effect (45% ZI) at higher concentration when compared with Azithromycin (fig. 10). N-butanol extracted fractions reduced the activity of *Klebsiella pneumonia* by 42% at concentration of 1mg disc^{-1}. The data further revealed that n-hexane and crude methanolic extracted samples inhibited the growth of *Klebsiella pneumonia* by 21% compared with other samples under study. fig. 11 presents data regarding the antibacterial activity of different solvents extracted sample from commercial available bark of walnut against *Pseudomonas aeruginosa*. Ethyl acetate extracted samples were more effective against *Pseudomonas aeruginosa* compared with other samples and 58% and 71% at lower and higher concentrations. N-butanol extracted samples also showed good activity against *Pseudomonas aeruginosa* measuring 71% ZI when applied in concentration of 1mg disc^{-1}. The lowest inhibitory zone was measured for n-hexane (29% ZI at concentration of 0.5 milligram disc^{-1}) when compared with activity of Azithromycin (fig. 11).

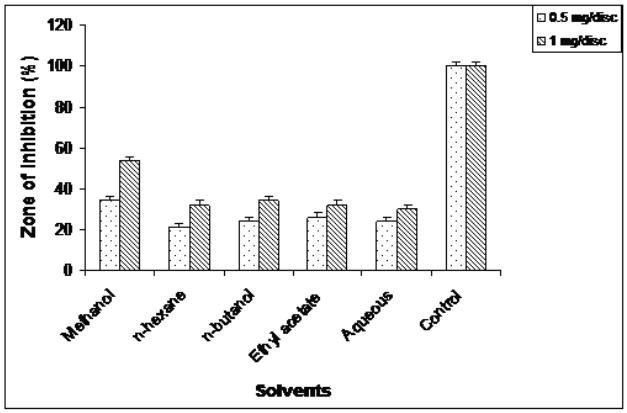

Fig. 7: Antibacterial activity of crude methanol, n-hexane, ethyl acetate, n-butanol and water extracted samples from commercially available stem bark of *Juglans regia* against *Bacillus subtilis* by disc diffusion assay (Bar shows LSD at $p<0.05$).

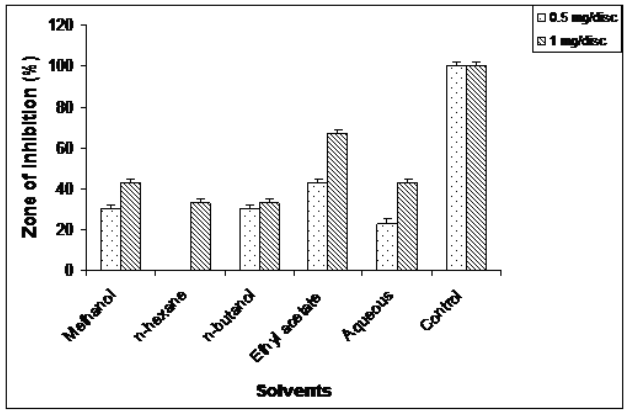

Fig. 8: Antibacterial activity of crude methanol, n-hexane, ethyl acetate, n-butanol and water extracted samples from commercially available stem bark of *Juglans regia* against

Table 1: Microbial strains used during the present study and their source

Microbial Species	Gram strain type	Details of the Microbial strains used
Klebsiella pneumoniae	Negative	Clinical isolate obtained The Department of Microbiology, Microbiology, Quaid-I-Azam University Islamabad, Pakistan
Pseudomonas aeruginosa	Negative	ATCC # 9721
Staphylococcus aureus	Positive	ATCC # 6538
Bacillus subtilis	Positive	Clinical isolate obtained The Department of Microbiology, Microbiology, Quaid-I-Azam University Islamabad, Pakistan
Escherichia coli	Negative	ATCC # 25922
Candida albicans		ATCC # 10231. Plant Pathology Department, The University of Agriculture Peshawar KPK Pakistan

Staphylococcus aureus by disc diffusion assay (Bar shows LSD at $p<0.05$).

The data concerning the antifungal activity of n-hexane, ethyl-acetate, n-butanol, methanol and aqueous extracted samples from commercial available bark of walnut against *Candida albicans* is shown in fig. 12. N-butanol showed the highest inhibitory activity of 50% at concentration of 1mg $disc^{-1}$ when compared with other samples under study. Aqueous extracted sample reduced the growth of *Candida albicans* only at higher concentration by 33% and did not show any activity at lower concentration. Ethyl acetate extracted samples on the other hand inhibited the growth of *Candida albicans* at both 0.5 and 1milligram $disc^{-1}$ concentration measuring 37 and 47% ZI respectively when compared with activity of Azithromycin (fig. 12).

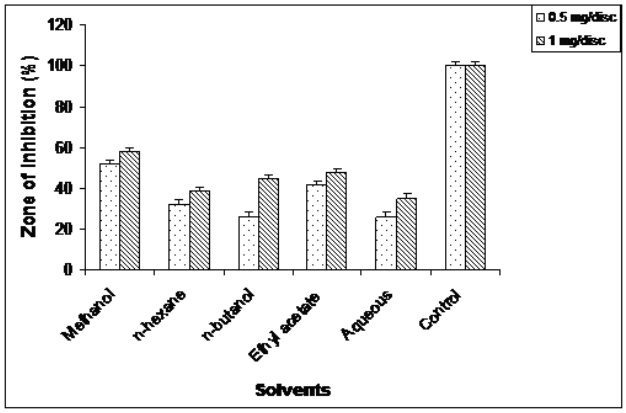

Fig. 9: Antibacterial activity of crude methanol, n-hexane, ethyl acetate, n-butanol and water extracted samples from commercially available stem bark of *Juglans regia* against *Escherichia coli* by disc diffusion assay (Bar shows LSD at $p<0.05$).

DISCUSSION

The data revealed maximum activity was shown by methanol extracted samples from controlled dried samples at higher and lower concentrations while n-butanol and aqueous extracted samples showed the lowest inhibitory activity against *Bacillus subtilis*. These results are in agreement with Hughes and Lawson (1991), Bekenblia (2004), Santas *et al.* (2009) and Ara *et al.* (2013). Our results also showed that *Staphylococcus aureus* showed high susceptibility in case of n-hexane and ethyl acetate extracted samples of controlled dried bark at both concentrations. N-butanol and aqueous extracted samples also showed similar activity against *Staphylococcus aureus* at highest concentration. It has been reported that *Staphylococcus aureus* can produce different types of enterotoxins that causes gastroenteritis, a major food borne disease in majority of the countries (Halpin-Dohnalek and Marth, 1989). Natural products found in *Juglans regia* may be a rich source of anti-infective agents (Cushnie and Lambs, 2005). Similar results are also reported by Pereier *et al.* (2007a and b), Oliveira *et al.* (2008) and Sivasankaridevi *et al.* (2013).

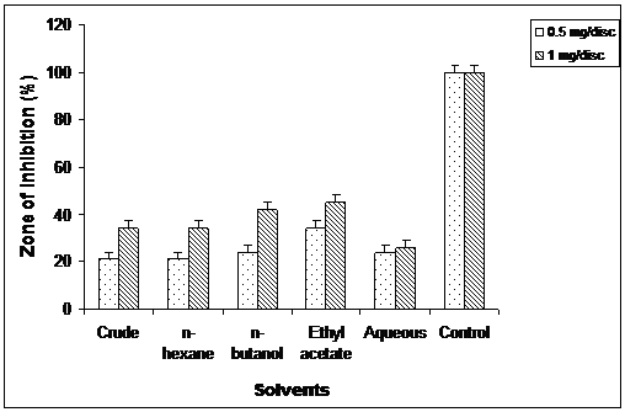

Fig. 10: Antibacterial activity of crude methanol, n-hexane, ethyl acetate, n-butanol and water extracted samples from commercially available stem bark of *Juglans regia* against *Klebsiella pneumoniae* by disc diffusion assay (Bar shows LSD at $p<0.05$).

It is clear from the data that all solvent extracted samples from controlled dried bark reduced the growth of *E. coli* at higher and lower concentrations. Ethyl acetate and methanol extracted samples measured the highest inhibitory activity followed by n-butanol extracted samples. Our results agree with Dimayuga and Garcia (1991) and Udayasankar *et al.* (2012). The data further suggested that all the tested extracted samples from controlled dried stem bark reduced antimicrobial activity of *Klebsiella pneumoniae* at both concentrations. N-

hexane, ethyl acetate and n-butanol extracted samples measured highest activity at concentration of 1 mg disc^{-1}). These results are in agreement with Chathradhyunthi *et al.* (2009) and Rauf *et al*. (2012). The aqueous extracted samples from controlled dried stem bark showed the lowest activity. Our results also suggested that all tested samples significantly reduced the growth of *Pseudomonas aeruginosa* at both concentrations. *Pseudomonas aeruginosa* was highly inhibited by crude methanol samples followed by ethyl acetate and n-butanol samples. These results agree with Pereier *et al.* (2007b), Oliveira *et al.* (2008) and Sharafati-Chaleshfati *et al.* (2011). Analysis of the data revealed that highest inhibitory zones were measured by crude methanolic extract and ethyl acetate fraction from controlled dried stem bark fractions followed by n-butanol fraction against *Candida albicans* when applied in highest concentration. Lowest activity was shown by aqueous extracted samples against the same fungi when applied in 0.5 mg disc^{-1} concentration. Our results agree with Noumi *et al*. (2010).

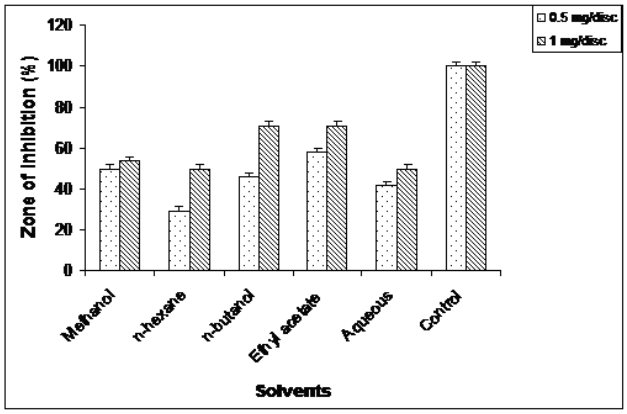

Fig. 11: Antibacterial activity of crude methanol, n-hexane, ethyl acetate, n-butanol and water extracted samples from commercially available stem bark of *Juglans regia* against *Pseudomonas aeruginosa* by disc diffusion assay (Bar shows LSD at $p<0.05$).

Data regarding the antibacterial activity of n-hexane, ethyl-acetate, n-butanol, methanol and aqueous extracted samples from commercially available stem bark of walnut against *Bacillus subtilis* revealed that highest inhibitory activity was shown by crude methanolic samples at 1mg disc^{-1} lowest by n-hexane extracted samples at lower concentration of 0.5mg disc^{-1}. The data indicated that ethyl acetate extracted samples commercially available stem bark was very effective to control the growth of *Staphylococcus aureus* when applied in concentration of 1mg disc^{-1} compared with positive control. Crude methanolic and aqueous extracted samples inhibited activity of the same bacteria at concentration of 1mg disc^{-1}. N-hexane and n-butanol extracted samples were equally effective to reduce the activity of *S. aureus* at highest concentration. Maximum inhibitory activity against *Escherichia coli* was shown by crude methanolic extract of commercially available stem bark higher concentrations respectively followed by ethyl acetate extracted samples at the same concentration and lowest inhibitory zones were measured by n-butanol and aqueous extracted samples at concentration of 0.5 mg disc^{-1}. *Klebsiella pneumonia* was sensitive to ethyl acetate extracted samples from commercially available stem bark at higher concentration when compared with controls. N-butanol extracted fractions reduced the growth of *Klebsiella pneumonia* at concentration of 1mg disc^{-1}. The antibacterial activity of different solvents extracted sample from commercial available bark of walnut against *Pseudomonas aeruginosa* showed that ethyl acetate extracted samples were more effective against *Pseudomonas aeruginosa* compared with other samples at lower and higher concentrations. N-butanol extracted samples also showed good activity against *Pseudomonas aeruginosa* when applied in concentration of 1mg disc^{-1}. The lowest inhibitory zone was measured for n-hexane at concentration of 0.5 milligram disc^{-1}. Data concerning the antifungal activity of n-hexane, ethyl-acetate, n-butanol, methanol and aqueous extracted samples from commercial available bark of walnut against *Candida albicans* suggested that n-butanol measured the highest inhibitory activity at concentration of 1mg disc^{-1} when compared with other samples under study. Aqueous extracted sample reduced the growth of *Candida albicans* only at higher concentration and did not reduce its growth at lower concentration. Ethyl acetate extracted samples inhibited the growth of *Candida albicans* at both concentrations.

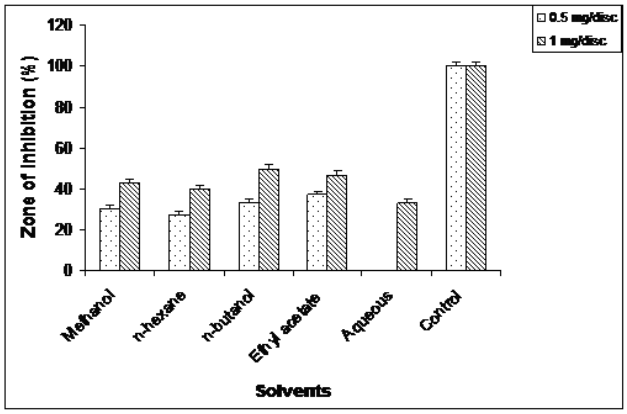

Fig. 12: Antifungal activity of crude methanol, n-hexane, ethyl acetate, n-butanol and water extracted samples from commercially available stem bark of *Juglans regia* against *Candida albicans* by disc diffusion assay (Bar shows LSD at $p<0.05$).

When different solvent extracted samples from controlled dried and commercially available stem bark against the tested microbes were compared, our results revealed that controlled dried samples were more effective than commercially available stem bark. The probable reason could that in commercial samples proper care and

procedures are not adopted in drying the samples resulting in the loss of active ingredients which reveals poor activity against different microbes. On the other hand in controlled drying conditions, proper care is taken not to expose the plant material to strong light and high temperature which preserve the active bio-molecular in the plant materials resulting good antimicrobial activity.

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