

ANTIMICROBIAL POTENTIALS OF *ECLIPTA ALBA* BY WELL DIFFUSION METHOD

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Abstract

The susceptibility of nine microbial species to an antimicrobial extract from *Eclipta alba* was screened using the well diffusion assay. Three different volumes (24, 30 and 36 µl/well) were tested. Analysis of the data revealed that all extracts from *Eclipta alba* showed antimicrobial activities. An N-butanol fraction showed inhibitory activities against all nine microbial species. Samples extracted with petroleum ether, dichloromethane, methanol or water had varying levels of inhibition against some of these microorganisms. The most resistant microbial strain was *Salmonella typhi* when tested against petroleum ether, dichloromethane, methanol and water extracted samples. The most susceptible Gram-positive bacterium was *Bacillus subtilis*, which was inhibited by all six extracts from *Eclipta alba*, while the most resistant Gram-positive bacterium was *Bacillus cereus*. *Erwinia carotovora* was the most susceptible Gram-negative bacterium, while *Salmonella typhi* and *Escherichia coli* were highly resistant among the Gram-negative bacteria.

Introduction

Medicinal plants are a rich source of antimicrobial agents (Kubmarawa *et al.*, 2007; Bakht *et al.*, 2011a). Many plant secondary metabolites are constitutive, existing in healthy plants in their biologically active forms, but others occur as inactive precursors and are activated by tissue damage or pathogen attack (Osbourne, 1996). Currently, majority of the pharmaceutically important secondary metabolites are isolated from wild or cultivated plants as their chemical synthesis is not economically feasible (Caldentey & Inze, 2004). Major groups of antimicrobial compounds from plants include simple phenols and phenolic acids, quinones, flavones, flavonoids and flavonols, tannins, coumarins, alkaloids, terpenoids and essential oils, lectins and polypeptides (Cowan, 1999). Antimicrobial compounds identified have shown promising activity *In vitro* (Zahin *et al.*, 2010). Different *In vitro* methods used for determining antimicrobial susceptibility include broth dilution assay, disc diffusion assay and well diffusion assay.

Eclipta alba (L.) Hassk. belongs family Asteraceae. The synonyms of *Eclipta alba* are *Eclipta erecta*, *Eclipta prostrata*, and *Verbesina alba*, while common names include false daisy, bhringraj, bhringaraja, dodhak, bhangra, and yerba de tago. *Eclipta alba* is adapted to a wide range of environments and found in poorly drained wet areas and canals of irrigated lowlands, rice paddies and upland fields. It is found all over the world including Southeast and South Asia. *Eclipta alba* is one of the important medicinal herbs with a role in the traditional medicine systems of the East. It is reported to possess antiseptic, analgesic, antipyretic, antispasmodic, antimicrobial and antiviral properties. *Eclipta alba* is reported to be effective for the retrieval of memory (Banji *et al.*, 2007). It is hepatoprotective (Tabassum & Agrawal, 2004; Malhotra & Singh, 2007), anti-inflammatory (Arunachalam *et al.*, 2009) and antimalarial (Bapna *et al.*, 2007; Chenniappan & Kadarkarai, 2010). This plant is considered rejuvenative and good for hair, and a blackening dye for hair is obtained from this plant. The

leaves of *Eclipta alba* are used against snake bites and scorpion stings. This plant is an important constituent of the polyherbal cardioprotective drug called abana (Baliga *et al.*, 2004). *Eclipta alba* is also reported to have antianaphylactic (Patel *et al.*, 2010), antihyperglycemic (Ananthi *et al.*, 2003) and antioxidant (Karthikumar *et al.*, 2007; Veeru *et al.*, 2009) properties.

Wedelolactone present in *Eclipta alba* has been reported to be useful for treating hepatitis and cirrhosis (Wagner *et al.*, 1986) and to be antibacterial and antihemorrhagic (Kosuge *et al.*, 1981; Prachayasittikul *et al.*, 2010; Bakht *et al.*, 2011b). The present study was initiated to investigate the antimicrobial effects of different solvent extracts from *E. alba* on different microorganisms.

Materials and Methods

Plant material: Aerial parts of *Eclipta alba* were collected from different locations of Peshawar Khyber Pakhtun Khwa (KPK), including University of Peshawar campus, Hayatabad Peshawar residential area and University Town Peshawar KPK Pakistan. The collected plants were thoroughly washed with tap water to remove dust and dirt. The clean plant material was placed in a shaded room for a period of seven days at 25°C.

Crude extract preparation: Shade dried aerial parts of *Eclipta alba* were chopped and ground with an ordinary grinder to obtain a fine powder. About 700 grams of dried powder were stirred into extraction drums containing five litres of methanol. These extraction drums were kept at room temperature for six days. During this period, the drums were shaken twice daily. The methanol-soluble compounds were filtered using Whatman filter paper No. 1. Fresh methanol (2500 ml) was added to the solid residue and the whole process was repeated three times. The filtered methanolic solution was dried in a rotary evaporator by removing methanol from the solution below 45°C under vacuum pressure. The semisolid extract was removed and dried in a china dish in a water bath at about 45°C.

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Fractionation of crude extract: Crude extract was divided into two parts. One part (10 g) was poured into glass vials to be tested as crude methanol extract for antimicrobial activity while the second part (100 g) was transferred to a glass beaker for fractionation with different solvents. The second portion was dissolved in water and poured into a separation funnel. Distilled petroleum ether (20 ml) was added to the funnel. The separation funnel was shaken to separate the two phases. Compounds soluble in the upper petroleum ether phase were collected and the lower aqueous phase was re-extracted thrice with petroleum ether. All fractions of petroleum ether were combined and dried to a semisolid state with a rotary evaporator. The semisolid petroleum ether fraction was dried in a china dish at about 45°C and stored in glass vials until used. The same process of fractionation was carried out with dichloromethane, ethyl acetate and butanol, respectively, resulting in dichloromethane, ethyl acetate and butanol fractions. The lower aqueous phase at the end of the process was dried via rotary evaporator and water bath. At the end of the process (Fig. 1), six different extracts, i.e. crude methanol extract, petroleum ether fraction, dichloromethane fraction, ethyl acetate fraction, butanol fraction and aqueous fraction, were prepared for antimicrobial testing.

Culture media: Nutrient agar medium was used for the culturing and growth of all microorganisms used in the study. Nutrient broth was used for shaking incubation and standardization of these microorganisms (Anon., 1995; Tassou *et al.*, 2000).

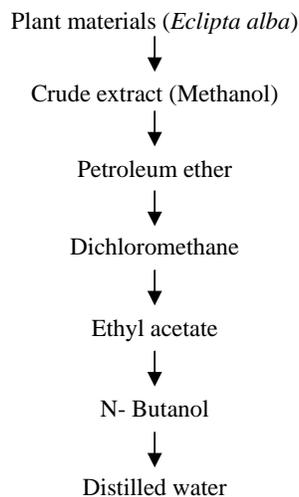


Fig. 1. Flow chart showing crude extract preparation and different fractions by various solvents.

Preparation of media: The required quantities of nutrient agar and nutrient broth were prepared and poured into conical flasks. Some of the nutrient broth (approx. 20 ml/test tube) was also poured into test tubes. All the media flasks and test tubes were plugged with cotton wool and sterilized in an autoclave. After sterilization, nutrient agar medium was poured aseptically into sterilized petri plates. A sterile environment was maintained during pouring to avoid contamination. The medium was allowed to solidify in petri plates for about an hour before the petri plates were placed in an inverted position (to avoid evaporation of water from the medium within the plates) in an incubator at 37°C for 24 hrs. After 24 hrs, uncontaminated plates were used for culturing of bacteria and fungi. The nutrient broth in flasks (approx. 20 ml/flask) was used for shaking incubation of microorganisms while nutrient broth in test tubes was used for standardization of microbial cultures.

Microorganisms used: Antimicrobial activity of different solvent extracted samples of *Eclipta alba* was tested against the following different bacterial and fungal strains (Table 1).

All microbial stock cultures were freshened by streaking using a sterile inoculation loop on nutrient agar medium plates in a laminar flow hood, then incubated at 37°C for 24 hrs. After 24 hrs, the streaked cultures were again subcultured on medium plates and incubated at 37°C for 24 hrs. The second streaked cultures were inoculated into nutrient broth in flasks and subjected to shaking incubation for 18 hrs at 37°C (200 rpm).

Well diffusion susceptibility method: Nutrient agar medium plates were seeded with 18-24-hour-old cultures of microbial inocula (a standardized inoculum of $1-2 \times 10^7$ CFU ml⁻¹ 0.5 McFarland Standard). Four wells (8 mm in diameter) were cut into the agar media with a sterilized cork borer and then plant extracts in 24, 30 and 36 µl volumes containing 4, 5 and 6 mg were poured into the wells. An antibiotic (24 µl per well) and DMSO (24 µl per well) were also poured into one well each as a positive and negative control, respectively. Inoculated plates were then incubated at 37°C for 24 hrs and zones of inhibition were measured in mm. Three replicates were prepared for each microorganism.

Positive controls

For Gram-positive bacteria; Azithromycin 50 µg per 6 µl
 For Gram-negative bacteria; Ciprofloxacin 30 µg per 6 µl
 For *Candida albicans*; Clotrimazole 50 µg per 6 µl

Table 1. Microbial strains tested for susceptibility to *Eclipta alba* extracts.

Microbial species	Gram strain type	Details of the microbial strains used
<i>Bacillus cereus</i>	Positive	Clinical isolate obtained from Microbiology lab. QAU Islamabad Pakistan
<i>Bacillus subtilis</i>	Positive	Clinical isolate obtained from Microbiology lab. QAU Islamabad Pakistan
<i>Candida albicans</i>	Fungus	Clinical isolate obtained from Hayatabad Medical Complex Peshawar KPK Pakistan
<i>Erwinia carotovora</i>	Negative	Plant Pathology Department KPK AUP Pakistan
<i>Escherichia coli</i>	Negative	ATCC # 25922
<i>Klebsiella pneumoniae</i>	Negative	Clinical isolate obtained from Microbiology lab. QAU Islamabad Pakistan
<i>Pseudomonas aeruginosa</i>	Negative	ATCC # 9721
<i>Salmonella typhi</i>	Negative	Clinical isolate obtained from Microbiology lab. QAU Islamabad Pakistan
<i>Staphylococcus aureus</i>	Positive	ATCC # 6538

Results and Discussion

Fig. 2 shows the inhibitory activity of petroleum ether, dichloromethane, ethyl acetate, butanol, methanol and water extracts from *Eclipta alba* against *Bacillus cereus* by the well diffusion susceptibility assay. The data showed that ethyl acetate and butanol extracts were more effective against *Bacillus cereus*. Ethyl acetate and butanol extracts equally inhibited the growth of *Bacillus cereus* at all volumes. The inhibition by the ethyl acetate extract was 37%, 41% and 42% at 24, 30 and 36 $\mu\text{l/well}$ volume, respectively. Reduction in growth by the butanol extract at these volumes was 38%, 40% and 42%, respectively. Petroleum ether, dichloromethane, methanol and water extracts did not inhibit the growth of *Bacillus cereus* at any volume. These results confirm the findings of Rahman & Rashid (2008) and Karthikumar *et al.*, (2007). Figure 3 presents the data concerning different extracts against *Bacillus subtilis*. Petroleum ether and water extracts did not inhibit the growth of *Bacillus subtilis*. Dichloromethane, ethyl acetate, butanol and methanol extracts were found to be effective against *Bacillus subtilis* at all volumes. *Bacillus subtilis* was highly susceptible to ethyl acetate extract, followed by butanol, dichloromethane and methanol extracts, respectively. The highest antibacterial activity was shown by the ethyl acetate extract (49% at 36 $\mu\text{l/well}$), while the lowest activity was seen from the methanol extract (30%) at the same volume. These results confirm the findings of Rahman & Rashid (2008).

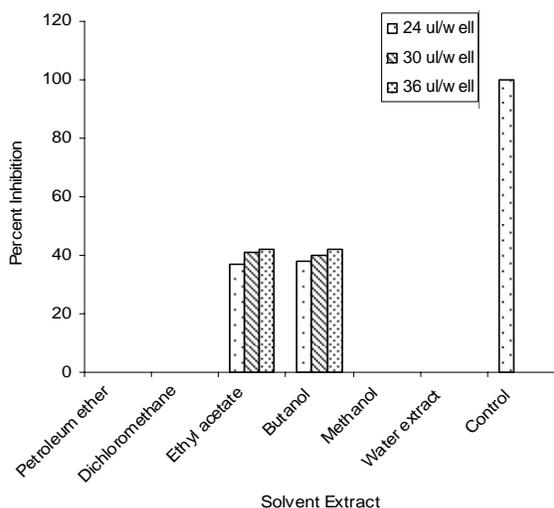


Fig. 2. Antibacterial activity of petroleum ether, dichloromethane, ethyl acetate, butanol, methanol and water extracts from *Eclipta alba* against *Bacillus cereus* by well diffusion assay.

The data showed that dichloromethane, ethyl acetate, butanol and methanol extracts were active against *Candida albicans* at all volumes (Fig. 4). Three fractions, i.e. dichloromethane, ethyl acetate and butanol, exhibited increased antifungal activities against *Candida albicans* at higher concentrations. The highest inhibition of *Candida albicans* growth was recorded for butanol (92%) at 36 $\mu\text{l/well}$ followed by butanol (86%) at 30 $\mu\text{l/well}$ and ethyl acetate (86%) at 36 $\mu\text{l/well}$. The antimicrobial activity of methanol and petroleum ether extracts exhibited varying degrees of inhibition of *Candida albicans* at different

volumes. Petroleum ether extract did not inhibit the growth of *Candida albicans* at 24, 30 and 36 $\mu\text{l/well}$, however, growth reduction of 53% occurred at 36 $\mu\text{l/well}$ concentration. The data further suggested that the petroleum ether fraction was effective only at higher volumes. Methanol extracts, on the other hand, showed the highest antifungal activity (72%) at 30 $\mu\text{l/well}$, compared with 42% and 44% inhibition at 24 or 36 $\mu\text{l/well}$, respectively. Similar results were also reported by Phongpaichit *et al.*, (2005). Our data also indicated that water extracts at lower volumes (24 or 30 $\mu\text{l/well}$) did not inhibit the growth of *Erwinia carotovora*. However, the same extract had 27% inhibition of *Erwinia carotovora* at 36 $\mu\text{l/well}$. Petroleum ether, dichloromethane, ethyl acetate, butanol and methanol extracts exhibited effective inhibitory activity against *Erwinia carotovora* at all concentrations. The butanol extract showed the highest inhibition (43%) at 36 $\mu\text{l/well}$ volume, while ethyl acetate extract demonstrated variable degrees of inhibition (37% at 30 $\mu\text{l/well}$ and 35% at 36 $\mu\text{l/well}$) when compared with their positive controls (Fig. 5).

Fig. 6 shows the antibacterial activity of petroleum ether, dichloromethane, ethyl acetate, butanol, methanol and water extracts from *Eclipta alba* against *Escherichia coli*. Ethyl acetate and butanol extracts were effective against *E. coli*. The butanol fraction was more effective than the ethyl acetate extract to control the growth of *E. coli*. The inhibition of *E. coli* growth by the butanol fraction was 41%, 42% and 46% at 24, 30 and 36 $\mu\text{l/well}$, respectively, while the inhibition by the ethyl acetate fraction was 37%, 38% and 40% at 24, 30 and 36 $\mu\text{l/well}$, respectively. The data further showed that *E. coli* was resistant to dichloromethane, petroleum ether, methanol and water extracts. These extracts did not inhibit the growth of *Escherichia coli* at any concentration. Similar results were also reported by Lenza *et al.*, (2009) and Rahman & Rashid (2008). Data shown in Fig. 7 indicate that *Klebsiella pneumoniae* showed resistance to petroleum ether, dichloromethane, methanol and water extracts of *Eclipta alba*. Ethyl acetate fractions reduced the growth of *Klebsiella pneumoniae* by 45%, 50% and 59% at 24, 30 and 36 $\mu\text{l/well}$, respectively, when compared with their positive controls. The butanol extract showed 53%, 58% and 59% inhibition of *Klebsiella pneumoniae* growth at the above-mentioned volumes (Fig. 7). These results agree with those reported by Lenza *et al.*, (2009).

The data indicated that petroleum ether, dichloromethane, methanol and water extracts did not inhibit the growth of *Pseudomonas aeruginosa* even at higher volumes. Ethyl acetate and butanol extracts were active against the growth of this bacterium. The percentages of inhibition shown by the ethyl acetate extract against *Pseudomonas aeruginosa* were 39%, 45% and 47% at 24, 30 and 36 $\mu\text{l/well}$ volumes, respectively. The butanol extracts showed inhibition of 35%, 36% and 42% against *Pseudomonas aeruginosa* at the above-mentioned volumes (Fig. 8). These results agree with those reported by Karthikumar *et al.*, (2007) and Devi *et al.*, (2009). The data showed that petroleum ether, dichloromethane, methanol and water extracts were not effective against *Salmonella typhi* (Fig. 9). The data further indicated that the ethyl acetate extract reduced the growth of *Salmonella typhi* by 39%, 45% and 47% at 24, 30 and 36 $\mu\text{l/well}$, respectively, while the butanol extract exhibited 40%, 45% and 47% reduction in the growth of *Salmonella typhi* at 24, 30 and 36 $\mu\text{l/well}$, respectively.

These results agree with those reported by Lenza *et al.*, (2009). Figure 10 displays the data concerning the antibacterial activities of petroleum ether, dichloromethane, ethyl acetate, butanol, methanol and water extracts from *Eclipta alba* against Gram-positive *Staphylococcus aureus*. The data indicate that petroleum ether, dichloromethane, methanol and water extracts were ineffective against *Staphylococcus aureus* and did not inhibit its growth. Ethyl acetate and butanol extracts were

highly active in controlling the growth of *Staphylococcus aureus*. Ethyl acetate extract reduced the growth of *S. aureus* by 51%, 53% and 53% at 24, 30 and 36 μ l/well volumes, respectively. Similarly, the butanol fraction reduced the growth of *S. aureus* by 44%, 52% and 53% at the same volumes, respectively. These results confirm the findings of Wiart *et al.*, (2004), Rahman & Rashid (2008) and Dalal *et al.*, (2010).

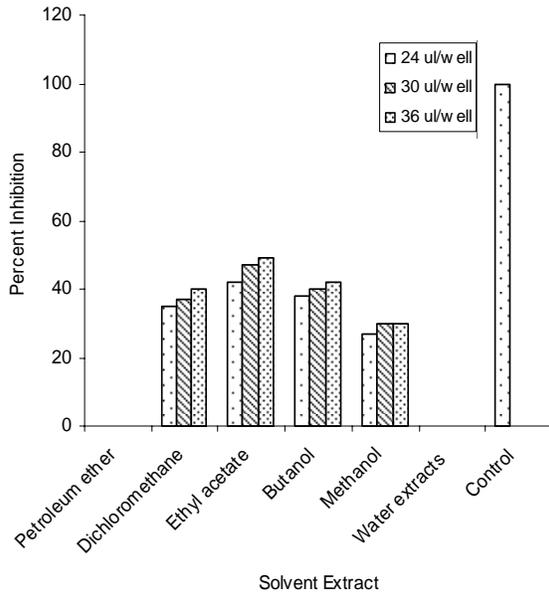


Fig. 3. Antibacterial activity of petroleum ether, dichloromethane, ethyl acetate, butanol, methanol and water extracts from *Eclipta alba* against *Bacillus subtilis* by well diffusion assay.

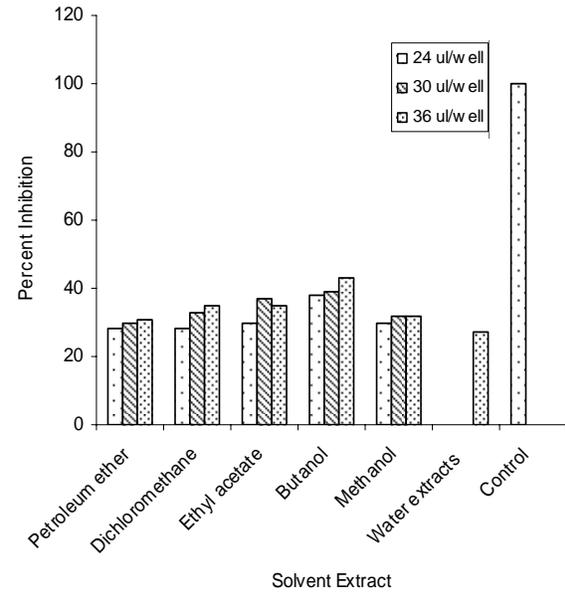


Fig. 5. Antibacterial activity of petroleum ether, dichloromethane, ethyl acetate, butanol, methanol and water extracts from *Eclipta alba* against *Erwinia carotovora* by well diffusion assay.

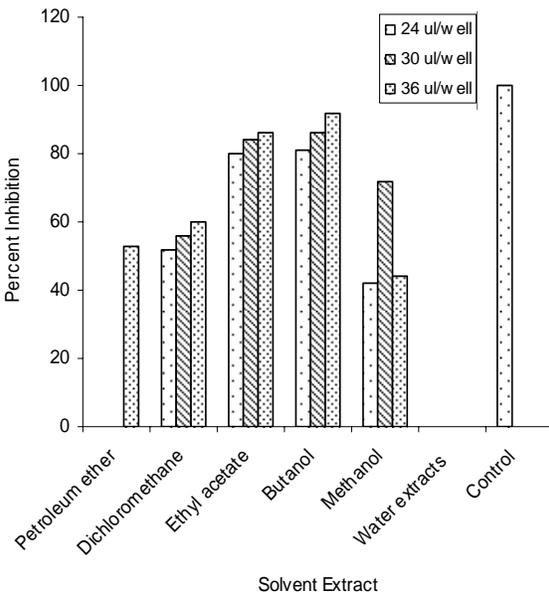


Fig. 4. Antibacterial activity of petroleum ether, dichloromethane, ethyl acetate, butanol, methanol and water extracts from *Eclipta alba* against *Candida albicans* by well diffusion assay.

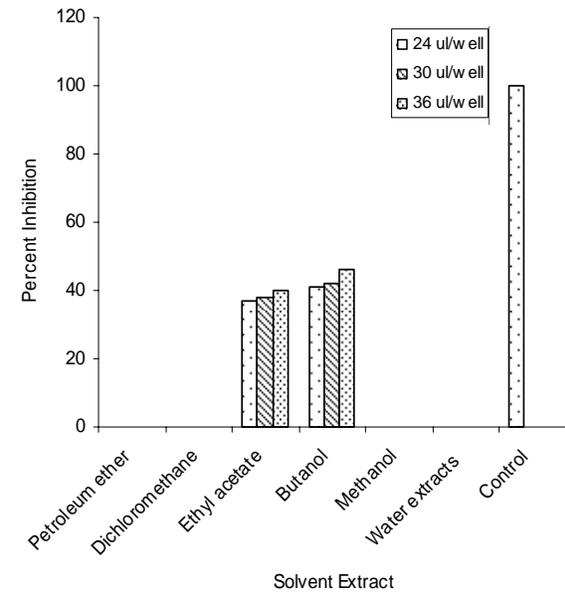


Fig. 6. Antibacterial activity of petroleum ether, dichloromethane, ethyl acetate, butanol, methanol and water extracts from *Eclipta alba* against *Escherichia coli* by well diffusion assay.

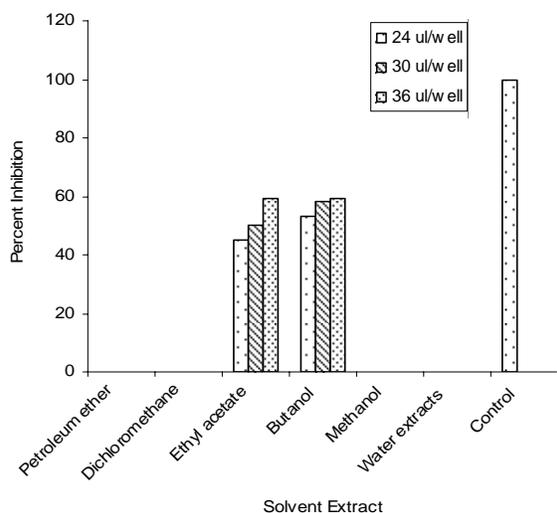


Fig. 7. Antibacterial activity of petroleum ether, dichloromethane, ethyl acetate, butanol, methanol and water extracts from *Eclipta alba* against *Klebsiella pneumoniae* by well diffusion assay.

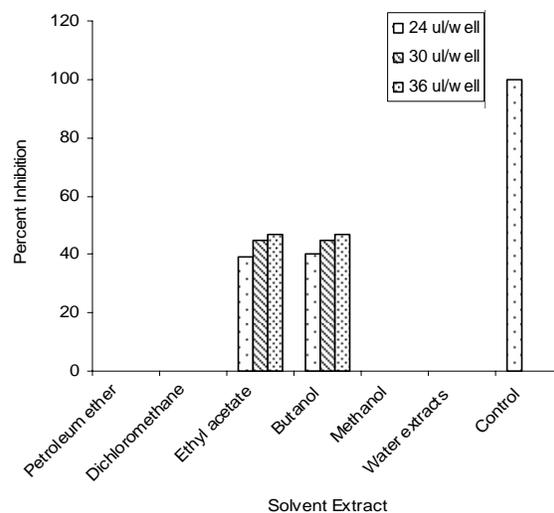


Fig. 9. Antibacterial activity of petroleum ether, dichloromethane, ethyl acetate, butanol, methanol and water extracts from *Eclipta alba* against *Salmonella typhi* by well diffusion assay.

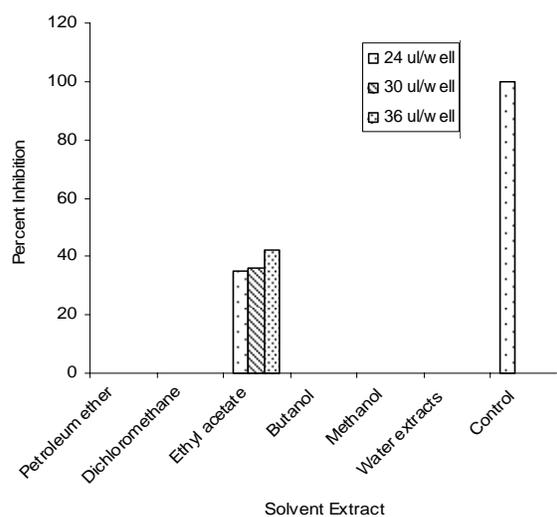


Fig. 8. Antibacterial activity of petroleum ether, dichloromethane, ethyl acetate, butanol, methanol and water extracts from *Eclipta alba* against *Pseudomonas aeruginosa* by well diffusion assay.

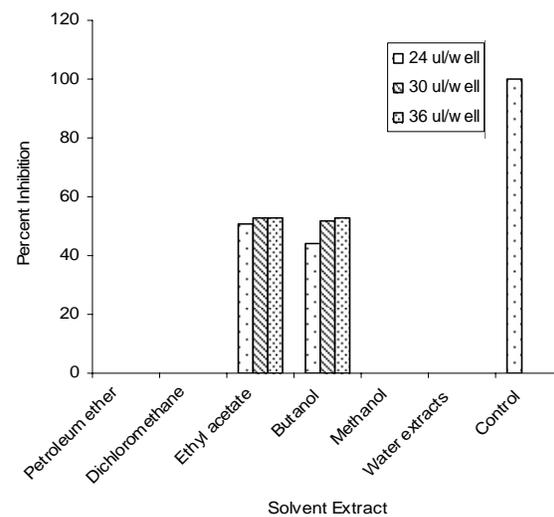


Fig. 10. Antibacterial activity of petroleum ether, dichloromethane, ethyl acetate, butanol, methanol and water extracts from *Eclipta alba* against *Staphylococcus aureus* by well diffusion assay.

Conclusion

Different extracts from the aerial parts of *Eclipta alba* showed antimicrobial activity against nine microbial species. Most of the antimicrobial compounds of *Eclipta alba* are soluble in butanol and ethyl acetate. The crude methanol extract also showed effective antifungal activity, suggesting a potential use of this plant as an antifungal agent. The antimicrobial compounds present in *Eclipta alba* may serve as an affordable and new source for the treatment of infectious diseases.

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