

Effects of Methanol Extract of *Wedelia chinensis* Osbeck (Asteraceae) Leaves against Pathogenic Bacteria with Emphasis on *Bacillus cereus*

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Darah, *et al.*: Methanol Extract of *Wedelia chinensis* Leaves Against Bacteria

The antibacterial activity of the methanol extract of *Wedelia chinensis* leave was studied and tested against three pathogenic Gram positive bacteria (*Bacillus cereus*, *B. subtilis* and *Staphylococcus aureus*) and three pathogenic Gram negative bacteria (*Escherichia coli*, *Proteus rettgeri* and *Pseudomonas aeruginosa*) by the disk diffusion assay and broth dilution methods. The extract exhibited favourable antibacterial activity against the bacterial cells but was more potent against Gram positive bacteria with the minimum inhibition concentration of 3.12 to 6.25 mg/ml compared to the Gram negative bacteria which had minimum inhibition concentration values of 25 mg/ml. The time-kill study suggested that the extract possessed bactericidal properties at higher concentrations and eradicated the growth of bacterial cells. The major abnormalities occurred to the bacterial cells after exposed to the extract were complete alterations in their morphology and collapsed of the cells beyond repair. The methanol extract of *W. chinensis* may be an effective antibacterial agent to treat bacterial infections.

Key words: Antimicrobial activity, minimum inhibition concentration, pathogenic bacterial, *Wedelia chinensis*

Various therapeutic benefits available in plants are becoming of interest amongst researchers to search for alternatives for combating the rising prevalence of global antimicrobial resistance problems. Moreover, concerns on the safety of some chemical in drugs have prompted an increased interest in natural additives.

Furthermore, the spread of drug resistant pathogens is one of the most serious threats to successful treatment of microbial diseases^[1]. Bacteria for example have shown a remarkable ability to endure and adapt to their environment including the development of different mechanisms of resistance to most old and new antibacterial agents^[2]. Bacterial adaptation to antibiotics has been very successful, and over the years, the increase in antibiotic resistance has generated a considerable worldwide public health problem^[3]. In addition, it was found that the synthetic antibiotics not only costs, but also have caused some side effects in the treatment of infectious disease^[4]. Thus, scientists are forced to search new antimicrobial

substances from various sources and there is a need to develop alternative antimicrobial drugs for the treatment of infectious diseases from medicinal plants^[5].

W. chinensis is a traditionally used medicinal herb in Ayurveda, Siddha and Unani system of medicines^[6]. Traditionally, the fruits, leaves and stem are used in child birth and in the treatment of bites and stings, fever and infection. The leaves are used in the treatment of kidney dysfunction, cold, wounds and amenorrhea^[7]. The leaves are also used for dyeing hair and for promoting their growth besides been used in treating elephantiasis, toothache, headache and cancer^[8,9]. The tonic of the leaves is used in cough and cephalgia. Decoction of the plant is used in menorrhagia and skin diseases^[10]. The plant has also found its use in inflammations, helminthic diseases and liver disorders^[11]. The plant has been used as astringent, bitter, acrid, antiinflammatory, cardiogenic, treatment of wounds, seminal weakness and viral hepatitis^[12-14]. The plant is scientifically reported to possess antioxidant property which indicates its usefulness in reducing anxiety and stress in emotional conditions^[15].

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Since *W. chinensis* exhibited various medicinal properties, this study further evaluated the antimicrobial activity of the crude methanol extract from these leaves against some bacteria species. Its effect on the selected bacterial growth and structure degeneration were studied and evaluated.

MATERIALS AND METHODS

Methanol (Fisher Scientific, United Kingdom), chloramphenicol (Sigma-Aldrich, St. Louis, USA), nutrient agar and nutrient broth (Merck, Germany), filter paper No. 1 (Whatman plc, Kent, UK), 6 mm antibiotic disk (GF A, Whatman, Kent, UK), Shaker (Infors HT Ecotron, Switzerland), rotary evaporator (Eyela, China), UV spectrophotometer Genesys 10 uv (Spectronic Unicam, USA), scanning electron microscope Leica Cambridge S-360 (Leica Cambridge, UK) and transmission electron microscope Philips CM12 (Philips, Eindhoven, Netherland) were used in our study.

Collection, processing and extraction of plant sample:

The fresh sample of *W. chinensis* leaves was collected around the Penang Island, Malaysia. The leaves were rinsed thoroughly under running tap water and the clean samples were then dried in an oven at 45° for 4-7 days until they were completely dried before grinding them into powder form. The dried and finely ground (0.5 mm) of sample was extracted with methanol by using the modified method of Darah and Annie-Clara^[16]. Approximately, 40 g of dried powdered plant sample was soaked in 400 ml of methanol at room temperature (30±2°) for 3 consecutive days with frequent agitation. The mixture was filtered using a muslin cloth and followed by Whatman No. 1 filter paper. The filtrate was then concentrated in a rotary evaporator under reduced pressure until oily paste formed and kept at cool dry place until further used.

Microorganisms and cultural maintenance:

Six pathogenic bacterial species which consisted of three Gram positive (*B. cereus*, *B. subtilis* and *S. aureus*) and three Gram negative (*E. coli*, *P. rettgeri* and *P. aeruginosa*) bacteria were obtained from the Industrial Biotechnology Research Laboratory (IBRL) Culture Collection, School of Biological Sciences, Universiti Sains Malaysia were used throughout the study. The bacterial cultures were

maintained on nutrient agar slants at 37° for 24 h. All the cultures were kept at 4° until further used. Subculturing was done at every 4 weeks to maintain their viability.

Antibacterial activity:

The antibacterial activity of the extract against the test bacteria were determined following the method described by Tong *et al.*^[17] with slight modifications. Test bacteria were cultured on nutrient agar plates and incubated at 37° for 24 h. Bacterial suspensions were prepared by inoculating one loopful of a pure colony into 5.0 ml of sterile distilled water. Sufficient inoculums were added until the turbidity equal to 0.5 McFarland standards which approximately equivalent to 1.5×10⁵ cells/ml.

One milliliter of the suspension was added into 15.0 ml of sterilized molten nutrient agar aseptically. The mixtures were mixed well by swirling the plates left and right and then they were left on the bench to solidify. The commercial antibiotic disk GF A with 6.0 mm diameter was used to screen the antibacterial activity. Each of the sterile disks was then impregnated with 20 µl of the extracts of 100.0 mg/ml of extract stock. Chloramphenicol at the concentration of 30 µg/ml was used as a positive control and methanol was used as a negative control. All the impregnated disks were air dried before placing them on the agar surface. The plates were incubated at 37° for 24 h and the antibacterial activity was determined by measuring the diameter of the inhibition zones formed around the disks.

Determination of minimum inhibitory concentrations:

The determination of minimum inhibitory concentration (MIC) was carried out by the liquid dilution method^[16]. The complete protocol of the MIC test is found in the M7-T2 publication of the National Committee for Clinical Laboratory Standards^[18]. Briefly, different extract preparations were subjected to a serial dilution using sterile nutrient broth medium as a diluents to give final crude extract concentrations between 1.275 and 200 mg/ml. The tubes were inoculated with the bacterial suspension (20 µl/ml broth), homogenized, and incubated at 37° for 24 h. The lowest dilution of the extract that retained its inhibitory effect resulting in no growth (absence of turbidity) of a microorganism was recorded as the MIC value of the extract. The bacterial growth was indicated by the turbidity. Each test was performed

in triplicate and repeated twice. A control experiment was run in parallel to study the impact of the solvent (without plant component) on growth of the test bacteria.

Time-kill study of *B. cereus*:

Bacterial suspension of *B. cereus* was prepared as described previously. Methanol (1 ml) extract stock was added into conical flasks containing 23.0 ml of sterilized nutrient broth and 1.0 ml of inoculum. The final concentrations of the extracts in the flasks were at 1.56 mg/ml (half MIC), 3.13 mg/ml (MIC) and 6.25 mg/ml (2MIC). Control with 1.0 ml of methanol was used in this experiment^[19]. The experiments were conducted in triplicate and all the flasks were incubated in a shaker (Infors HT Ecotron, Switzerland) incubator at 37° with agitation of 150 rpm. One milliliter of the mixture from each flask was withdrawn at every 4 h intervals starting from 0 h until 48 h of cultivation and the bacterial cell growth was monitored by measuring optical density at 540 nm.

Scanning and transmission electron microscope observations:

The *B. cereus* suspension was prepared as described previously. To each sample, 1.0 ml of the 24 h old bacterial suspension was inoculated in a 50.0 ml conical flask containing 30.0 ml of sterilized nutrient broth and incubated in a shaker at 37°, 150 rpm for 18 h. The bacterial suspension was then added to the extract stock solution (the final concentration in each flask was at the MIC value) and incubated at the required incubation time (12, 24 and 36 h). As for a negative control, a conical flask containing bacterial suspension was added with 1.0 ml of methanol. The sample preparation for Scanning (SEM) and transmission (TEM) electron microscopy were done following the method describes by Marez^[20] and Yogalatha *et al.*^[19] respectively. The prepared samples were then viewed under a scanning (Leica Cambridge, S-360, United Kingdom) and transmission (Philips CM12, Eindhoven, Netherland) electron microscopes.

RESULTS AND DISCUSSION

The present study has shown that methanol extract of *W. chinensis* leaves has promising antibacterial activity, and this is probably the reason for its wide use as traditional medicine. Table 1 shows that all

the six test bacteria exhibited zone of inhibition, which were smaller when compared to zones of inhibition produced by the commercial antibiotic, chloramphenicol (30 µg/ml). The results also showed that Gram positive bacteria, *B. cereus*, *B. subtilis* and *S. aureus* produced bigger zone of inhibition of 14.0, 12.0 and 15.0 mm, respectively compared to Gram negative bacteria *E. coli*, *P. rettgeri* and *P. aeruginosa* which produced 7.0, 8.0 and 7.0 mm of inhibition zones, respectively.

Gram positive bacteria, *B. cereus*, *B. subtilis* and *S. aureus* exhibited lower MIC values of 3.12, 6.25 and 6.25 mg/ml, respectively compared to the Gram negative bacteria, *E. coli*, *P. rettgeri* and *P. aeruginosa* which exhibited MIC values of 25 mg/ml extract (Table 1). The results indicated that Gram positive bacteria were more susceptible to the extract compared to Gram negative bacteria.

The activity of the plant against both Gram positive and Gram negative bacteria can be indicative of the presence of broad spectrum antibiotic compounds or simply general metabolic toxins in the plant. Generally, Gram negative bacteria are more resistant than Gram positive bacteria^[21-24]. The same characteristics were observed in other antimicrobial studies of plant extract against pathogenic bacteria^[22,23].

Time-kill studies were performed over a period of 48 h with the *B. cereus* cells being exposed to MIC (3.13 mg/ml), 1/2MIC (1.65 mg/ml) and 2MIC (6.25 mg/ml) values of the extract and the results are shown in fig. 1. At 1/2MIC (1.56 mg/ml) the extract demonstrated a drastic drop in OD after

TABLE 1: ANTIMICROBIAL ACTIVITY

Microorganism	Diameter zone of inhibition (mm)		Minimum inhibitory concentration (mg/ml)
	Methanol extract	Chloramphenicol	
<i>Gram-positive bacteria</i>			
<i>Bacillus cereus</i>	14.0±0.3	20.00±0.3	3.13
<i>Bacillus subtilis</i>	12.0±0.4	21.00±0.2	6.25
<i>Staphylococcus aureus</i>	15.0±0.3	21.00±0.3	6.25
<i>Gram-negative bacteria</i>			
<i>Escherichia coli</i>	7.0±0.3	22.00±0.2	25.00
<i>Proteus rettgeri</i>	8.0±0.5	20.00±0.3	25.00
<i>Pseudomonas aeruginosa</i>	7.0±0.2	20.00±0.4	25.00

16 h, which leads to the stationary phase of the bacterial growth compared to the control. At the values of MIC (3.13 mg/ml) and 2MIC (6.25 mg/ml), the extract produced cell eradication after 12 h. Based on the results obtained from the time-kill studies, it was obviously seen the potency of the methanol extract of *W. chinensis* leaves as antibacterial agents against pathogenic bacteria.

Fig. 2 represents the morphological changes of the nontreated and treated *B. cereus*. Fig. 2a shows the SEM micrographs of bacterial cells without the methanol extract treatment. The figure revealed the normal rod shape cell structure without any shrinkage or cavity formation as the surface was smooth and regular. Fig. 2b shows the morphology of the cell after 12 h of treatment with the extract. The bacterial cells started to show multiple defects with many of cells exhibited crumpled or shrunken cell surface. Fig. 2c revealed more formation of crumpled cells and some the cells formed cavities. After 36 h of exposure (fig. 2d), the bacterial cells were seemed to be totally deformed and many collapsed cells were seen. At this stage, the cells had lost their original rod shape as compared to the control cells in fig. 2a.

These conditions can be seen clearly in fig. 3 where TEM studies were conducted in the extract treated cells. Fig. 3a shows the untreated cells, with typical rod shaped cells of *Bacillus* sp. Fig. 3b shows the cells after 12 h exposure to the extract. The cells showed some alteration in the internal structures with shrunken cytoplasm and the cells seems to start

losing its rod shaped structure. The worst condition occurred on the cells after 24 h of exposure to the extract (fig. 3c) and finally the cells leakage occurred (fig. 3d) where the internal structures of cells including cytoplasm and organelles were found outside the cells. There were some holes formed on the cell wall which reflect of the cell leakages. The sequences exhibited in fig. 3 acted as proves of what happening to the cells in fig. 2, which caused by the effect of *W. chinensis* leaves methanol extract.

Hyde *et al.*^[25], suggested that the morphological changes of the antibiotic-treated bacteria occur when the antimicrobial agent attacked the cell membrane. In this case, the bioactive compound of the methanol extract of *W. chinensis* leaves that locked on the cell surface structure had permeabilized the bacterial membranes. Any disruption in cell wall integrity will have a great influence in bacterial growth. This prediction was coincided well with the findings of Sasidharan *et al.*^[26], who reported the methanol extract of macroalgae *Gracilaria changii* exerted its inhibitory effect on the cell wall of the bacterial cells which led to the complete damage of the cells. Various studies were reported to investigate the mechanism of actions involved in bacterial killing process. Among them are the interactions of antibacterial compound with the cell membrane^[27]. As shown by the SEM and TEM micrographs where the cells became crumpled and exhibited the formation of holes, these damages may indicate the lost of cellular materials and organelles from the cell cytoplasm^[28].

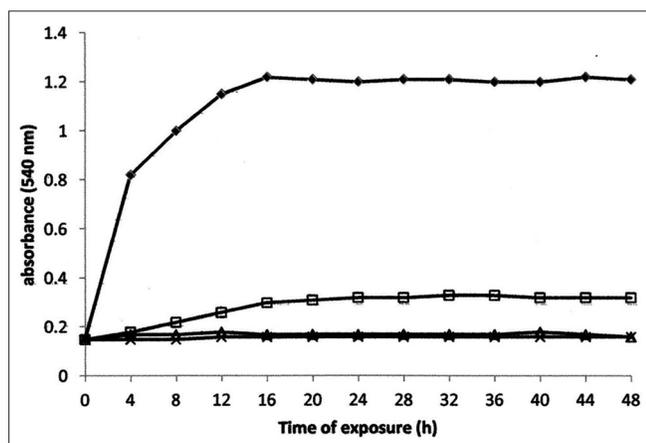


Fig. 1: Effects on the growth of *Bacillus cereus* at different extract concentration.

Effects of *Wedelia chinensis* leaves methanol extract on the growth of *Bacillus cereus* at different concentration of the extract. The control suspension (◆), 1/2 MIC (□), MIC (△), 2MIC (×).

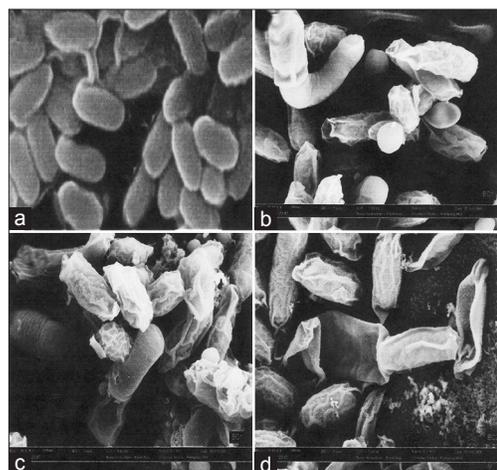


Fig. 2: SEM micrographs.

SEM micrographs of the *Bacillus cereus* cells after treatment with the methanol extract of *Wedelia chinensis*. (a) control at 0 h ($\times 5000$), (b) at 12 h ($\times 6000$), (c) at 24 h ($\times 6000$) and (d) at 36 h of treatment times ($\times 6000$).

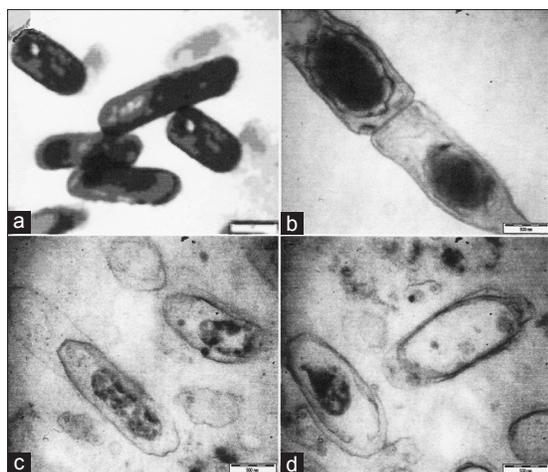


Fig. 3: TEM micrographs.

TEM micrographs of the *Bacillus cereus* cells after treatment with the methanol extract of *Wedelia chinensis*. (a) control at 0 h, (b) at 12 h, (c) at 24 h and (d) at 36 h of treatment times.

These unstable and altered cells were collapsed beyond repair and finally led to cell death.

The ability of the plant bioactive compounds to cause disintegration of bacterial colonies, probably results from their interference with the bacterial cell wall^[29]. Majority of plant extracts have been reported to be more active against Gram-positive bacteria than the Gram-negative bacterial strains^[30]. Theoretically, the Gram-negative bacteria bear an extra outer membrane (OM) which includes the asymmetric distribution of the lipids with phospholipids and lipopolysaccharide (LPS) located in the inner and outer leaflets, respectively can act as additional barrier which hinders the movement of foreign substance into the cell^[31]. This characteristic is absent in the Gram-positive bacteria and the cell wall of Gram-positive bacteria contains lipoteichoic acids (LTA) that represent unique and essential structural components to the cells and should be good drug targets to the bioactive compounds of *W. chinensis*.

The results obtained from this study proved that *W. chinensis* leaves methanol extract can be used to treat bacterial infections topically as it acted directly to the target bacteria cell wall. In order to assure the right time of using the extract against the bacteria, we conducted a study on the effect of addition the extract to the bacterial growth profile. Fig. 4 shows that the control cells grew well and achieved its logarithmic profile at 8 h of cultivation time and then it entered the initial stationary phase at 12 h of cultivation time, followed by a stationary phase from 20 h onward.

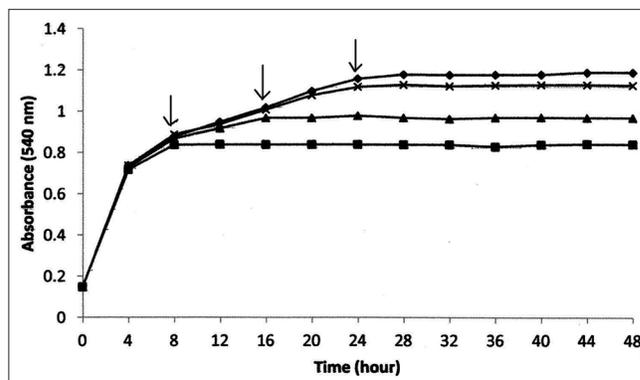


Fig. 4: Effects on the growth of *Bacillus cereus* at MIC value.

Effect of addition the methanol extract of *Wedelia chinensis* leaves (at MIC value of 3.13 mg/ml) on the growth profile of *Bacillus cereus*. Arrows indicated the time (h) of extract addition to the bacterial culture. Control (◆), 8 h (■), 16 h (▲), 24 h (X).

Therefore, the additions of the extract were done at 8, 16 and 24 h of cultivation time in response to those phases. The results revealed that the growth of the treated cells decreased once the extract was added without having any exception on the time of addition. This condition is good in the sense that the extract can be used at any time of the bacterial growth phase to treat its infection.

Generally, the methanol extract was more active than other extracts^[32]. This may be attributed to the presence of soluble phenolic and polyphenolic compounds. Even though there were reports that methanol extract demonstrated inhibitory effects to *B. subtilis*, *P. aeruginosa* and *S. aureus* but not *E. coli*^[33], yet recent research activities on antibacterial activities of crude extracts have implicated the methanol extract for being more active than the other solvents extracts^[34,35]. In this study three Gram positive (*B. cereus*, *B. subtilis* and *S. aureus*) and three Gram negative (*E. coli*, *P. rettgeri* and *P. aeruginosa*) were used as test microorganisms. All these are pathogenic bacteria that are known to cause several diseases and infections in humans and animals. For instance, *S. aureus* and *P. aeruginosa* are most common pathogens causing serious infections while *E. coli* is an opportunistic pathogen at the site of cut wound.

The MIC values of the extract against all the test bacteria were determined using broth dilution methods and the results showed that MIC values for Gram-positive bacteria were between 3.12 to 6.25 mg/ml which were more susceptible than Gram-negative bacteria which exhibited the MIC

values of 25.00 mg/ml. The broader spectrum of activities could be due to the synergic effects of the various components in the *W. chinensis* leaves extract. The exact antibacterial mechanism of the extract is not known, but it can be attributed to the presence of the major phytochemicals such as flavonoids, terbinoids and tannins that were detected in the studies that were reported previously by several researchers^[6,36,37].

Based on these results, it can be concluded that *W. chinensis* leaves methanol extracts have a great potential as antibacterial agent to treat infectious diseases caused by a range of pathogenic bacteria. The study provides support for the use of these plants in the management of infectious diseases. The findings can form the basis for further studies to prepare and optimise preparation of the herbal extract to further evaluate them against a wide range of bacterial strains.

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