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Original Article

## Evaluation of *Zanthoxylum armatum* Roxb for in vitro biological activities



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### ABSTRACT

*Zanthoxylum armatum* fruits are used traditionally as a spice in various food preparations. The aim of this study was analysis of antimicrobial, cytotoxic, phytotoxic, insecticidal, and anti-leishmanial activity. The crude extract showed  $86 \pm 10\%$  antifungal activity (Agar tube dilution method) against *Trichophyton longifusus* while n-hexane, chloroform, and aqueous-methanol fractions inhibited this pathogen by  $90 \pm 7$ ,  $85 \pm 10$  and  $70 \pm 9\%$  respectively. The n-hexane and aqueous-methanol fraction also, respectively, showed  $40 \pm 10$  and  $87 \pm 9\%$  inhibition of *Microsporium canis*. Chloroform fraction also displayed antifungal activity against *Aspergillus flavus* ( $60 \pm 10\%$ ) and aqueous-methanol fraction against *F. solani* ( $40 \pm 8\%$ ). The crude ethanolic extract and its chloroform and aqueous-methanol fraction exhibited significant toxicity (Brine shrimps lethality assay) against brine shrimps having  $LC_{50}$  value of  $6.66 \pm 1.1$ ,  $21.4 \pm 3.3$  and  $29.6 \pm 3.9 \mu\text{g/ml}$ , respectively. The crude ethanolic extract and its n-hexane soluble portion exhibited good anti-leishmanial activity (well serial dilution method) each having  $IC_{50}$  values of  $50 \pm 5 \mu\text{g/ml}$ . The crude extract and various fractions possessed excellent herbicidal activity (*Lemna minor* assay), and caused more than 90% inhibition of the plant growth at  $1000 \mu\text{g/mL}$ . The ethanolic extract, n-hexane and chloroform soluble portions caused 90% mortality in insecticidal activity (direct contact method) of *Rhyzopertha dominica*. The ethanolic extract and its n-hexane soluble portion, respectively, caused 80 and 90% mortality of *Callosobruchus analis*. The present study showed that the tested fruit extracts of *Z. armatum* exhibited strong antifungal, cytotoxic, phytotoxic, insecticidal, and anti-leishmanial effects.

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### 1. Introduction

The foods obtained from plants are valuable source of vitamins, minerals, fibers and so on and are therefore provide many important component of a healthy diet. In addition to diet component, plants are chemo preventive agents. There is always need to search for natural cytotoxic, antimicrobial, and other chemotherapeutic agents due to overwhelming toxicity reports of synthetic compounds.<sup>1</sup>

*Zanthoxylum armatum* (Roxb.) (Rutaceae) is a common plant in Southeast Asia. It grows wildy in Dir, Hazara, and Muree hills of

Pakistan.<sup>2</sup> The seeds of this plants are particularly part of many food ingredients and also used in many traditional medicine. The seeds and the bark of this plant are used as in the treatment of fevers, toothache, indigestion/heartburn, stomachic, carminative, cholera and as tonic.<sup>3</sup> *Z. armatum branchlets* is used as miswak (tooth brush) (Tejbal) for cleaning the teeth while fruit powder is applied in toothache.<sup>4</sup> Various species of *Zanthoxylum* genus possess antimicrobial, larvicidal and cytotoxic activity.<sup>5–8</sup> The different classes of chemical constituents reported from *Z. armatum* include terpenes, sterols, flavonoids, alkaloids and coumarins.<sup>2,9–11</sup>

The current study was designed to extend the search for potential novel agents that may be of therapeutic applications. Here, we report the in vitro study of this plant which include antifungal, antibacterial, cytotoxic, phytotoxic, insecticidal and anti-leishmanial activities in order to rationalize some traditional uses and to explore the presence of these bioactivities in *Z. armatum*.

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## 2. Materials and methods

### 2.1. Plant material

*Z. armatum* berries were collected from the hilly area of Tanawal, District Haripur, and N.W.F.P. Pakistan in the month of June–July. The identification of the plant was confirmed by plant taxonomist Manzoor Hussain and specimen voucher (PG/B/ZA, 2014) was deposited in the herbarium of Botany Department, Government Postgraduate College, Abbottabad.

### 2.2. Extraction and fractionation

The fresh berries (800 g) were ground and macerated with ethanol for 72 h. It was filtered and the filtrate was evaporated under reduced pressure at 40 °C which yielded the crude extract (81 g). A portion of the crude extract (60 g) was successively fractionated to yield n-hexane (16 g), chloroform (20.2 g), and aqueous-methanol (23 g) fractions.

### 2.3. Antifungal activity

Antifungal activity of the ethanolic extract and subsequent fractions was measured by employing the agar tube dilution method against the selected human, animal and plant pathogens.<sup>12</sup> The molds and yeasts used in this study included *Trichophyton longifusus* (clinical isolate, human pathogen), *Microsporum canis* (ATCC11622, human/animal pathogen), *Aspergillus flavus* (ATCC32611, human pathogen), *Fusarium solani* (ATCC11712, plant pathogen), *Candida albicans* (ATCC2091, human pathogen) and *Candida glabrata* (ATCC90030, human pathogen).

Dilutions were made as to make the final concentration of the extracts, subsequent fractions and the standard drug to 200 µg/ml of SDA (Sabouraud Dextrose Agar). Miconazole was used as the standard drug and the experiments were run in triplicates.

### 2.4. Antibacterial activity

Antibacterial activity of the crude extract and subsequent fractions was assessed against various Gram positive and Gram negative human pathogens by the agar well diffusion protocol.<sup>12</sup> Gram positive bacteria included *Bacillus subtilis* (ATCC 6633), *Staphylococcus aureus* (ATCC 25923) while Gram negative organisms included *Escherichia coli* (ATCC 25922), *Shigella flexneri* (clinical isolate), *Pseudomonas aeruginosa* (ATCC 27853) and *Salmonella typhi* (ATCC 19430). Imipenem was used as the standard antibacterial agent.

### 2.5. Cytotoxicity

Brine shrimp lethality of the ethanolic extract and subsequent fractions of *Z. armatum* was assessed against *Artemia salina* (brine shrimp larvae) in three different concentrations (5, 50, and 500 µg/mL).<sup>12</sup> The data were analyzed using with the SPSS (Version 19) statistics software to calculate LD<sub>50</sub> values with a 95% CI (confidence interval). The etoposide was used as standard cytotoxic drug. Prior to experiment approval was taken from the institute ethical committee for use of brine shrimps in bioassay, the approval number allotted was; PHM 0028/EC/B-4-5-15.

### 2.6. Phytotoxicity

Phytotoxic activity of the crude extract and subsequent fractions were evaluated against *Lemna minor* L.<sup>13</sup> in different concentrations (1000, 100 and 10 µg/mL). Paraquat (0.9025 µg/mL) was used as positive control. The results were interpreted as percentages by analyzing the growth regulation calculated with reference to negative control by using the following expression:

$$\text{Growth Regulation (\%)} = 100 - \frac{\text{Number of fronds in test flasks}}{\text{Number of fronds in negative control}} \times 100$$

### 2.7. Insecticidal activity

Insecticidal activity of the ethanolic extract and various fractions was evaluated against *Trogoderma granarium*, *Rhyzopertha dominica* and *Callosobruchus analis* by direct contact method using filter paper (90 mm diameter).<sup>14</sup> Permethrin (235.71 µg/cm<sup>2</sup>) was used as the standard insecticidal agent. The percent mortality was calculated after 36 h using the following formula:

$$\text{Mortality (\%)} = 100 - \frac{\text{Number of survival in samples}}{\text{Number of survival in control}} \times 100$$

### 2.8. Anti-leishmanial activity

Anti-leishmanial activity of the ethanolic extract and subsequent fractions was assessed against *Leishmania major* using well serial dilution protocol as reported previously.<sup>12</sup> The results were

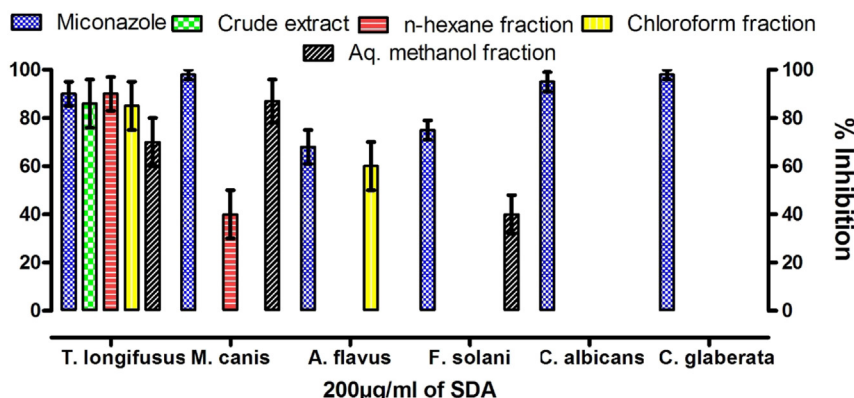


Fig. 1. Antifungal activity as % inhibition of crude extract and subsequent fractions of *Z. armatum* berries against various pathogens fungi.

calculated with the SPSS (Version 19) statistics software as the concentrations inhibiting parasite growth by 50% (IC<sub>50</sub>) after a three-day incubation period. Amphotericin B was used as the reference.

### 3. Results

The results obtained for the antifungal activity of the extract and subsequent fractions derived from *Z. armatum* against the tested pathogens are summarized in Fig. 1. The most prominent antifungal activity was observed against *T. longifusus*. The crude extract showed 86% inhibition against this pathogen while n-hexane, chloroform and aqueous-methanol fractions inhibited the growth of *T. longifusus* by 90 ± 10, 85 ± 10 and 70 ± 9% respectively. The n-hexane and aqueous-methanol fraction showed antifungal activity against *M. canis* and inhibited its growth by 40 ± 10 and 87 ± 9%, respectively. The other active fractions in this bioactivity were chloroform against *A. flavus* (60 ± 10%) and aqueous-methanol against *F. solani* (40 ± 8%).

The extract and subsequent fractions derived from *Z. armatum* displayed a weak antibacterial activity against the tested organisms. The crude extract, n-hexane, and chloroform soluble portions inhibited the growth of *B. subtilis* only by 30% as compared to standard drug (Imipenem). The crude extract and subsequent fractions from *Z. armatum* were screened for brine shrimp lethality studies and the LC<sub>50</sub> values are reported in Fig. 2. The crude extract derived from the berries of *Z. armatum* exhibited significant toxicity against brine shrimps having LC<sub>50</sub> value of 6.66 ± 1.1 µg/mL, which probably indicates the distribution of synergistic activity of compounds within these two fractions. Similarly, the chloroform and aqueous-methanol fraction also displayed promising cytotoxic activity showing LC<sub>50</sub> values of 21.4 ± 3.3 and 29.6 ± 3.9 µg/ml. The n-hexane fraction, however, showed LC<sub>50</sub> values greater than 1000 µg/mL, and thus could be considered to have a low general toxicity. The brine shrimp toxicity bioassay is a useful indicator of general toxicity and also a guide for the detection of antitumor and pesticidal constituents.

The herbicidal potentials of the ethanolic extract and subsequent fractions obtained from *Z. armatum* were evaluated against *L. minor* L. and the results are exhibited in Fig. 3. The crude extract obtained from *Z. armatum* and various fractions have excellent herbicidal activity at the highest concentration (1000 µg/mL) and caused complete inhibition of the plant growth. At 100 µg/mL, the n-hexane fraction caused a complete inhibition of *L. minor* while a moderate phytotoxic activity was observed for the crude extract

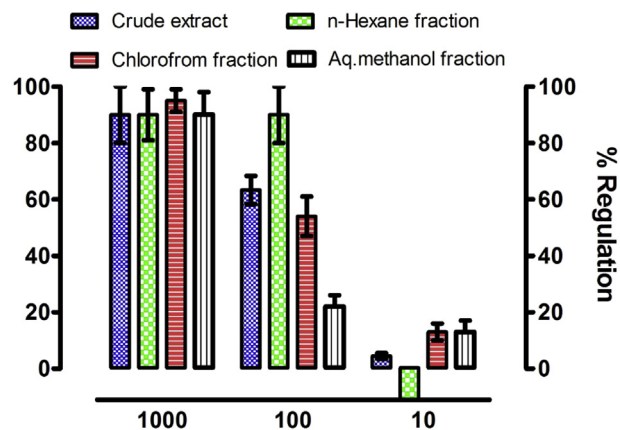


Fig. 3. Phytotoxic activity as % regulation of crude extract and subsequent fractions of *Z. armatum* against *Lemna minor* fronds with dose of 1000, 100 and 10 µg/mL.

and chloroform fraction and exhibited 63.3 ± 5 and 54.54 ± 7% inhibition, respectively. At this concentration, however, only a weak inhibitory activity (22.72 ± 4%) was observed for the aqueous-methanol fraction. Neither the crude extract nor the fractions exhibited any significant herbicide activity at further lower concentration (10 µg/mL). Rather, the n-hexane fraction acted as a plant growth promoter at this concentration Fig. 3.

The results obtained for the insecticidal activity of the ethanolic extract and subsequent fractions obtained from *Z. armatum* against *T. granarium*, *R. dominica* and *C. analis* are given in Fig. 4. Crude extract, n-hexane and chloroform fractions showed excellent insecticidal activity against *R. dominica* and caused 90 ± 10% mortality of this species after 36 h of exposure. Crude extract and n-hexane fraction also displayed significant inhibitory activity against *C. analis*. These caused, respectively, 80 ± 5 and 90 ± 10% mortality of this specie. A 20 ± 5% insecticidal activity was also found in n-hexane fraction against *T. granarium*.

The results for the effects of the ethanolic extract and fractions of *Z. armatum* on the viability of *L. major* promastigote forms are shown in Fig. 5. The ethanolic extract and its hexane soluble portion showed activity against *L. major* with IC<sub>50</sub> values of 50 ± 5 µg/ml each. The n-hexane, chloroform and aqueous-methanol fractions, however, did not show any significant anti-leishmanial activity against *L. major* as their IC<sub>50</sub> values were higher than 50 µg/ml.

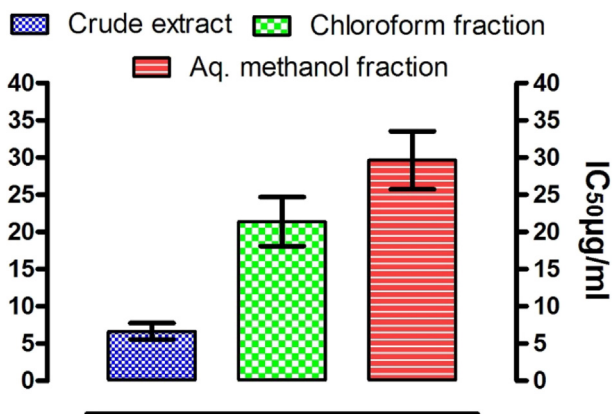


Fig. 2. Cytotoxic effects of crude extract and subsequent fraction of *Z. armatum* berries against brine shrimps larvae.

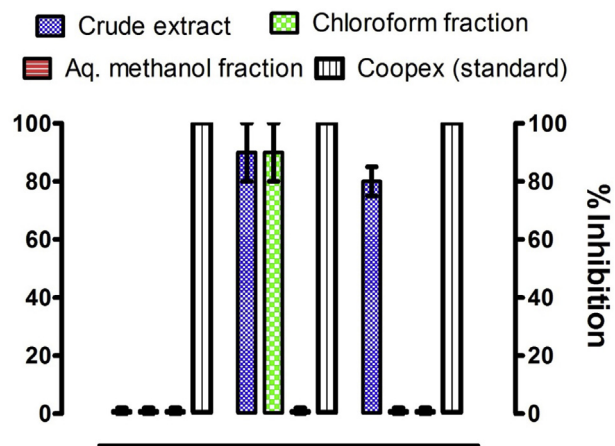


Fig. 4. Insecticidal activity as % inhibition of crude extract and subsequent fractions of *Z. armatum* against different common stored grain pests.

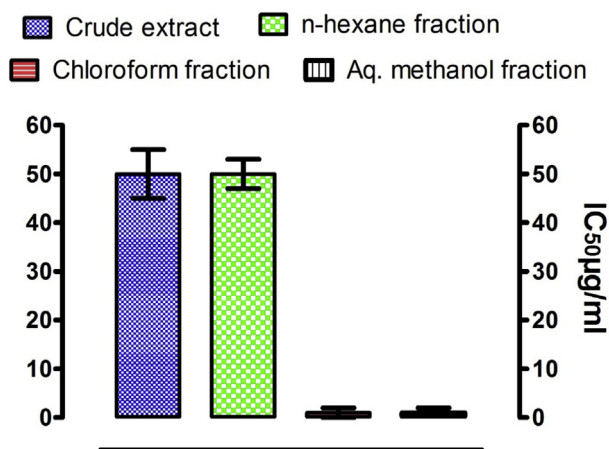


Fig. 5. Anti-leishmanial activity of *Z. armatum* crude extract and subsequent fractions against *L. major*. Values are given as IC<sub>50</sub> (µg/mL).

#### 4. Discussions

It is evident from this study that *Z. armatum* and subsequent fractions could be a rich source of antifungal agents especially against *T. longifusus*, *M. canis*, *A. flavus* and *F. solani*. As the literature reveals, this is first report on the antifungal potentials of *Z. armatum*. Several other species of *Zanthoxylum* have previously been reported to possess antifungal activity against a range of strains.<sup>7,15,16</sup> The previous reports suggest that the presence of alkaloids in the plant may possibly responsible for antifungal activity.<sup>17,18</sup> This study also indicated that the extract and fractions from the berries of *Z. armatum* could be rich sources of valuable anti-tumor and pesticides constituents. A variety of compounds including alkaloids, terpenoids and coumarins are reported to have such cytotoxic effects.<sup>18</sup> On the basis of these experimental and literature evidence the same plant's crude extract and its fractions are under study for anticancer activity on different cell lines in the author's laboratory.

Leishmaniosis, a parasitic infection, is prevalent throughout the world mostly in underdeveloped countries.<sup>19</sup> Due to some drawbacks with synthetic drugs used against leishmaniosis the attention have been shifted to the active phytochemicals isolated from plant for the effective treatment along with minimal side effects.<sup>20</sup> This study revealed that the ethanolic extract and its hexane fraction could be a valuable source of anti-leishmanial compounds as also evidenced from previous studies on *Zanthoxylum*.<sup>21</sup> It is well known that the weeds are developing resistant and the efficiency of the conventional synthetic herbicides is decreasing against these resistant biotypes.<sup>22</sup> These facts necessitate the need for the discovery of new herbicides. Natural herbicides are gaining more importance because they have non-toxic effect and are more suitable as compare to synthetic agrochemicals in use. The results of this study showed that the extract and fractions from *Z. armatum* possessed significant phytotoxic activity against *L. minor* and could be useful as bio-herbicides.

The synthetic insecticides possess well-known adverse effects on agro ecological systems and thus the interest in the insecticides from botanical origin has been increased during the past few decades. Consequently, the studies on plant-insect chemical interactions has increased to explore the potential of plants' secondary metabolites as pest control agents<sup>23</sup> and the use of plant allelochemicals has been accepted as a leading strategy for pest management. In the present study, the ethanolic extract, n-hexane and chloroform soluble portions have shown considerable

insecticidal activity against the most common insects which cause serious damage to wheat, rice and pulses in store condition. This activity may be due to contribution of some alkaloid components for e.g. pellitorine reported from the same genus.<sup>24</sup>

In conclusion, it can be stated that the tested *Z. armatum* extracts appear to be good and safe natural antimicrobial and cytotoxic agent and could also be of significance in the food industry and in the control of various human, animal and plant diseases. Further studies should be done to search for new biological active compounds from *Z. armatum* fruit extracts.

#### Conflict of interest

Author declares that there is no conflict of interest to disclose.

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