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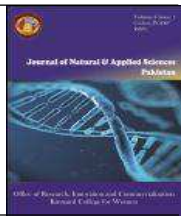

# IN VITRO ANTHELMINTIC ACTIVITY OF EXTRACTS OF WITHANIA SOMNIFERA

Zeb Saddiqe [1*], Saba Khalid [2], Alya Maimoona [3]
[1] Department of Botany, Lahore College for Women University, Lahore, Pakistan.
[2] Department of Botany, Lahore College for Women University, Lahore, Pakistan.
[3] Department of Botany, Postgraduate College for Women, Wahdat Colony, Lahore, Pakistan.

**Article Info**

*Corresponding Author
Email Id: zeb_rukhsana@yahoo.com

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Anthelmintic activity, Haemonchus contortus, Levamisole, Withania somnifera.

**Abstract**

Haemonchus contortus is the most important gastrointestinal parasite of grazing animals especially sheep and goats widely occurring in developing countries like Pakistan. The parasite is responsible for high mortality rate among young animals causing economic losses in agriculture and poultry. The organism has also developed resistance against commonly used anthelmintics. In the present study the crude methanol extract and n-hexane, dichloromethane, ethyl acetate, acetone and aqueous fractions of aerial parts of Withania somnifera were tested for anthelmintic activity using worms H. contortus isolated from sheep intestine at a concentration of 0.05 mg/ml. The results are expressed in terms of number of worms surviving after hourly interval and time for death of 100% worms. The acetone fractions exhibited the strongest anthelmintic activity with an increased death of worms at hourly interval and death of all the worms during 6 hrs period. The reference anthelmintic drug Levamisole caused 100% death after four hrs at 0.5 mg/ml. All the extracts were analyzed phytochemically to test the presence or absence of biologically active components. Total phenolic and flavonoid contents were also determined in each case. The study showed that the polar extracts of the plant can be used as source of anthelmintic drugs.

## 1. Introduction

Helminthiasis is one of the most widespread infections of man posing a serious threat to public health causing anemia, eosinophilia, malnutrition and pneumonia (Bundy, 1994). The disease is also prevalent among grazing animals causing anorexia, anaemia, diarrhea and oedema resulting in poor performance and mortality decreasing meat and milk production (Perry *et al*., 2002; Eysker and Pleoger, 2003; Githigia *et al*. 2005). The disease is of worldwide occurrence especially in developing countries due to poor sanitary conditions, poor management practices and inadequate control measures (Dhar *et al*., 1982). The infection is caused by infestation of the host body with the parasitic worm. The helminth parasites are mainly present in the intestinal tract but their larvae are also found invading the tissues. These worms make their host deprived of food, cause blood loss and also secrete toxins thus producing harmful effects inside the host (Tripathi, 2003).

Anthelmintics are drugs used to expel parasitic worms from gastrointestinal tract and other organs or tissues in which the developmental forms invade. These drugs may also act as vermicides killing these worms (Sharma, 2007). Many anthelmintics are available for the treatment of the infection. However, the gastro-intestinal helminthes have developed resistance against most of these drugs (Coles, 1997; Geert and Dorny, 1995; Melo *et al*., 2003). This demands for alternative strategies against these parasites which should be cost effective and without side effects.

Plants are an authentic source of natural anthelmintics and insecticides. Several medicinal plants have been used to treat parasitic infections in man and animals (Savioli *et al*., 2003; Alawa *et al*., 2003). This potential of plants can be exploited for isolation of natural anthelmintics (Akhtar, 2000; Ajaiyeoba *et al*., 2001; Pratap *et al*., 2012). In a number of studies many plants have shown *in vitro* anthelmintic activity against many helminths including earthworm, *Pheritima posthuma* (Purwal *et al*., 2010; Kumar and Partap, 2012) tapeworms and/or *Ascaris lumbricoides* (El Garhy and Mahmoud, 2002) and hookworms, *Haemonchus contortus* (Alawa *et al*., 2003; Iqbal *et al.,* 2005; Iqbal *et al.,* 2005).

*W. somnifera* is an important member of family Solanaceae commonly used in Ayurvedic system of medicine. Traditionally, the plant has been used in the treatment of inflammation, fever and protection against infections (Satyavati *et al*., 1976). The plant possesses anti-inflammatory, antioxidant, antistress, immunomodulatory and anticancer properties (Asthana and Raina, 1989; Mishra *et al*., 2000). Various species of genus *Withania* have shown *in vitro* anthelmintic activity against different helminths (Gaind and Budhiraja, 1967; Purwal *et al*., 2010; Kirtiman, 2012; Patidar *et al*., 2012).

In the present study the crude methanol extract and subsequent solvent fractions of aerial parts of *W. somnifera* were evaluated for *in vitro* anthelmintic activity against sheep intestinal worm *Haemonchus contortus*.

## EXPERIMENTAL

### Collection and extraction of plant material

Aerial parts of the plant were collected from Lahore College for Women University, Lahore. The plant was identified using authentic sources and consulting Flora of Pakistan. The fresh plant material was dried, grounded and extracted thrice in methanol at room temperature by maceration technique. The combined extracts were concentrated in rotary evaporator under reduced pressure to give crude methanol extract. The crude extract was dissolved and suspended in double distilled water and subjected to liquid-liquid partitioning using *n*-hexane, dichloromethane, ethyl acetate and acetone sequentially. The organic fractions were concentrated in rotary evaporator while the water fraction was freeze-dried. All the extracts were weighed and stored in tightly sealed dark glass containers at 4ºC for further analysis.

### Phytochemical Analysis

All the extracts were analyzed to test the presence or absence of selected phytochemical constituents using standard methods (Harborne, 1973).

***Tannins*:** To detect the presence of tannins 1 mL of freshly prepared 10% KOH was mixed with 1 mL of plant extract. The appearance of dirty white precipitates indicated the presence of tannins.

***Glycosides:*** For glycosides 1 mL of freshly prepared 10% KOH was added to 1 mL of extract. The formation of brick red precipitates confirmed the presence of glycosides.

***Saponins:*** Saponins were detected by frothing test. 2 ml of the extract was vigorously shaken in the test tube for 2 minutes. Presence of frothing indicated saponins.

***Steroids*:** For identification of steroids 5 drops of concentrated $H_2SO_4$ were added to 1 mL of the extract in a test tube. Appearance of red color indicated the presence of steroids.

***Triterpenes:*** For triterpenes, 5 drops of concentrated $H_2SO_4$ were added to 1 mL of extract. Blue green coloration indicated the presence of triterpenes.

***Flavonoids:*** Presence of flavonoids was tested by adding 1 mL of freshly prepared 5% $AlCl_3$ solution to 1 mL of extract. Yellow coloration indicated the presence of flavonoids.

***Phenolics:*** For phenolics, two drops of 5% $FeCl_3$ were added to 1 mL of the extract in a test tube. Appearance of greenish precipitate indicated the presence of phenolics.

***Alkaloids:*** To detect the presence of alkaloids 0.2 gm of plant extract was warmed with 2% sulphuric acid in a test tube for 2 minutes. The mixture was filtered in a separate test tube and few drops of Dragendroff reagent were added and observed for the presence of orange red precipitates for the presence of alkaloids.

**Quantitative Analysis**

**Determination of total phenolic content (TPC)**

For determination of TPC 20 µL of plant extract, 1.58 mL of deionized water and 100 µL of Folin-Ciocalteu (FC) reagent were mixed together. After incubation for 10 min at room temperature 300 µL of 25% $Na_2CO_3$ solution (w/v) was added to the mixture and again incubated at 40ºC. After cooling to room temperature, absorbance was measured at 765 nm against blank containing 20 µL of respective solvent instead of plant sample (Cliffe *et al*., 1994). TPC was calculated using a linear equation based on the standard curve obtained using tannic acid as standard (Figure 1). The results were expressed as mg tannic acid equivalent (TAE)/g dry extract (dE).

$Y = 2.807x + 0.026$; $r^2 = 0.914$

Where *Y* is the absorbance and *x* is the concentration of tannic acid (mg $mL^{-1}$).

**Determination of total flavonoids (TFC)**

TFC was determined using colorimetric method of Dewanto *et al*. (2002). For analysis 250 µL of plant extract, 500 µL of deionized water and 90 µL of 5% $NaNO_2$ solution were mixed and left to stand for 6 min. Then 180 µL of 10% $AlCl_3$ solution was added to the mixture and allowed to stand for 5 min followed by the addition of 600 µL of 1 M NaOH solution. Final volume was made up to 3 mL using deionized water. Absorbance was measured at 510 nm against blank (250 µL of plant extract replaced by 250 µL of extracting solvent). TFC was calculated with linear equation based on the standard curve of quercetin used as standard (Figure 2). The results were expressed as mg quercetin equivalent (QE)/g dE.

$Y = 0.131 x + 0.016$; $r^2 = 0.918$

Where *Y* is the absorbance and *x* is the concentration of quercetin (mg $mL^{-1}$).

**Anthelmintic activity**

**Experimental animal**

The anthelmintic activity assay was carried out using intestinal parasite of sheep *Haemonchus contortus*. The organism was selected because of its resemblance with intestinal worms of human beings. The worms were collected from intestine (abomasum) of freshly slaughtered sheep provided by a local slaughter house. The abomasi, thoroughly washed with distilled water, were opened with scissors in a dish. The contents of the abomasum were poured in the dish and the tissues were washed with normal saline solution to remove all the faecal matter. The worms were carefully collected without damaging and kept in normal saline solution. The average size of these worms was 1-2 cm.

**Preparation of test solutions**

Test solutions were prepared by dissolving 0.5 mg of each plant extract in 0.1 ml of DMSO and 9.9 ml of phosphate buffer saline (PBS) giving a final concentration of 0.05 mg/mL. Reference drug Levamisole was used as the standard anthelmintic drug and was tested at same concentration prepared in PBS.

**Control treatment**

The control set-up was run by using PBS+DMSO in the petri dish to determine the effect of DMSO on the test organism.

**Anthelmintic activity assay**

Anthelmintic activity assay was carried out using the method of Ajaiyeoba *et al*. (2001). Eight groups of ten worms each with approximately equal size were kept in 10 ml of respective solution. Group 1 was the negative control in which the test animals were kept in saline phosphate buffer + DMSO. Group 2 was the positive control in which worms were kept in 0.05 mg/ml solution of Levamisole used as

reference standard. Group 3 to 8 were the test groups in which animals were kept in 10 ml solution of respective plant extract. All the experiments were carried out in triplicates at room temperature. The motility was recorded with hourly interval for 6 h to determine the rate of mortality.

**Statistical analysis**

Mean and standard deviation (SD) of the data was determined with the help of statistical software SPSS 19.

## RESULTS

**Qualitative Phytochemical Analysis**

The preliminary analysis of different plant extracts evidenced the presence of multiple components in the extracts including flavonoids, glycosides, tannins, steroids, saponins, terpenes and phenolic compounds (Table 2). The non-polar fractions *n*-hexane and dichloromethane gave positive results for non-polar compounds such as terpenes and saponins while polar fractions acetone and ethyl acetate were rich in polar compounds i.e. flavonoids, steroids, and phenolics. Glycosides were not detected in any extract.

**Total phenolic and flavonoid content**

The results of TPC and TFC determined in different extracts of the plant are presented in Figure 3. TPC in the crude methanolic extract was 342 ± 2.84 mg TAE/ g dE. After fractionation the highest concentration of phenols was measured in ethyl acetate fraction (426 ± 3.87 mg TAE/g dE) followed by dichloromethane fraction (416 ± 3.52 mg TAE/ g dE). The minimum amount of phenolics was determined in *n*-hexane fraction (228 ± 2.64 mg TAE/g dE).

The results of TFC gave similar trend as for TPC. TFC in the crude methanolic extract was 128 ± 2.34 mg QE/g dE. After fractionation highest concentration of flavonoids was again measured in ethyl acetate fraction (180 ± 2.51 mg QE/g dE) followed by dichloromethane fraction (140 ± 2.30 mg QE/g dE). The *n*-hexane fraction had the lowest flavonoid contents (44 ± 1.00 mg QE/g dE). High solubility of flavonoids in polar solvents provides high concentration of these compounds in the extracts obtained using polar solvents for the extraction.

**Anthelmintic Activity**

The results of anthelmintic activity of crude methanol extract and fractions of aerial parts of *W. somnifera* are summarized in Table 3 and Figure 4. All the extracts showed different anthelmintic activity in terms of mortality rate at the same concentration during the six hour time period. Among the extracts the ethyl acetate, acetone and aqueous fractions showed significant anthelmintic effect with high death rate in the given time interval at the tested concentration (0.05 mg/mL). The rate of death of worms after each hour was different for each fraction which reflects the nature of compounds present in each fraction. The effect of extracts on the death of the worms, according to the result may be indicated as acetone > crude methanolic > aqueous > ethyl acetate > dichloromethane > *n*-hexane. In particular acetone fraction exhibited an increased death of worms at hourly interval killing 100% parasites after 6 hrs.

## DISCUSSION

The emergence of resistance to anthelmintic drugs among helminth parasites has become a worldwide phenomenon (Jackson and Coop, 2000). *H. contortus* has been reported to develop resistance against widely used broad spectrum anthelmintics such as benzimidazole, imidazothiazole and ivermectin (Richard, 1990; Singh *et al*., 2002) as well as against drugs with narrow spectrum such as salicylanilides (Singh *et al*., 1996; Swarhkar *et al*., 1999). A number of medicinal plants have been shown to possess anthelmintic activity against *H. contortus* (Iqbal *et al*., 2001; Alawa *et al*., 2003; Chandrawathani *et al*., 2006; Swarnkar *et al*., 2008). In the present study all the extracts showed activity against the parasite at a very low concentration i.e., 0.05 mg/ml. The results were comparable to the anthelmintic drug Levamisole used as standard which killed all the parasites (100% anthelmintic activity) at 4$^{th}$ hour of exposure at a concentration of 0.5 mg/ml. The acetone fraction showed 100% anthelmintic effect after 6 hrs of exposure while the crude methanol extract and aqueous fraction showed 97% inhibition after 6 hrs. Since the extracts are a mixture of thousands of molecules, the extracts could be a source of anthelmintics which may be active at submolar concentrations.

Many plant phenolics such as tannins and flavonoids are known to possess anthelmintic activity (Anthnasiadou *et al*., 2001; da Silva *et al*., 2008). These compounds kill the parasite by inhibiting ATP synthesis and by binding with the cuticle of the organism making it immobile leading to paralysis and ultimately death of the organism (Martin, 1997; Thompson & Geary, 1995). The difference in anthelmintic activity of different plant extracts may also be due the difference in total phenolic and flavonoid contents in these extracts. Since the polar fractions contained a high phenolic and flavonoid content than the non-polar fractions this may explain the observed difference in the anthelmintic activity of these extracts.

**Conclusion**

The study concludes that the polar extracts of *W. somnifera* possess significant anthelmintic activity and can be used for the treatment of parasitic diseases such as helminthiasis. The extracts can serve as a cheap source of plant derived natural anthelmintics, however, *in vivo* efficacy of the extracts needs to be tested to establish the pharmacological bases for the use of this plant as an anthelmintic drug.

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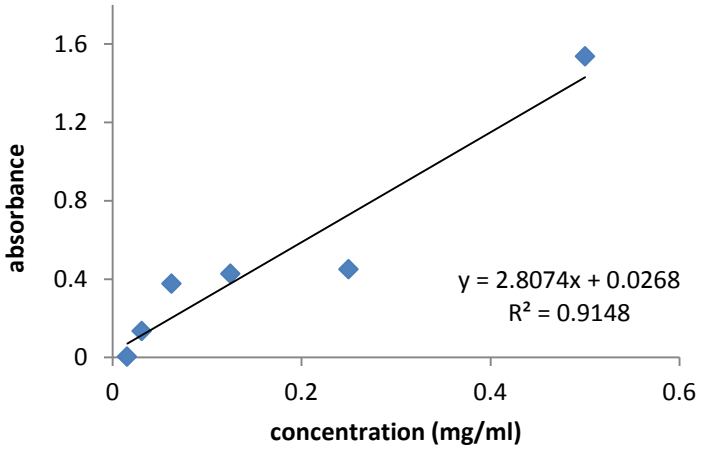


**Figure 1** Callibration curve for tannic acid

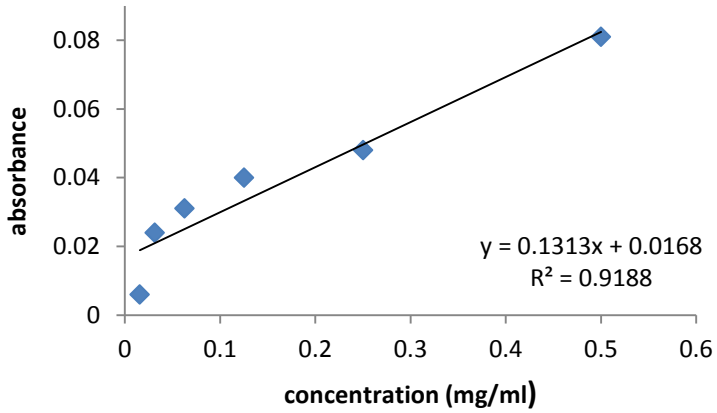


**Figure 2** Callibration curve for quercetin

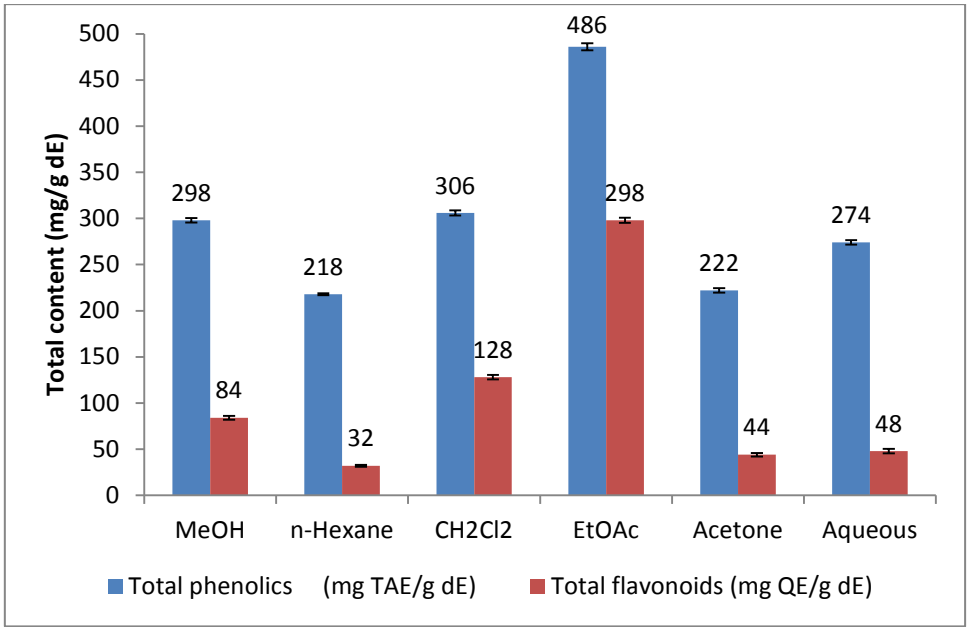


**Figure 3** Total phenolics and total flavonoid content in different solvent fractions of aerial parts of *W. somnifera*

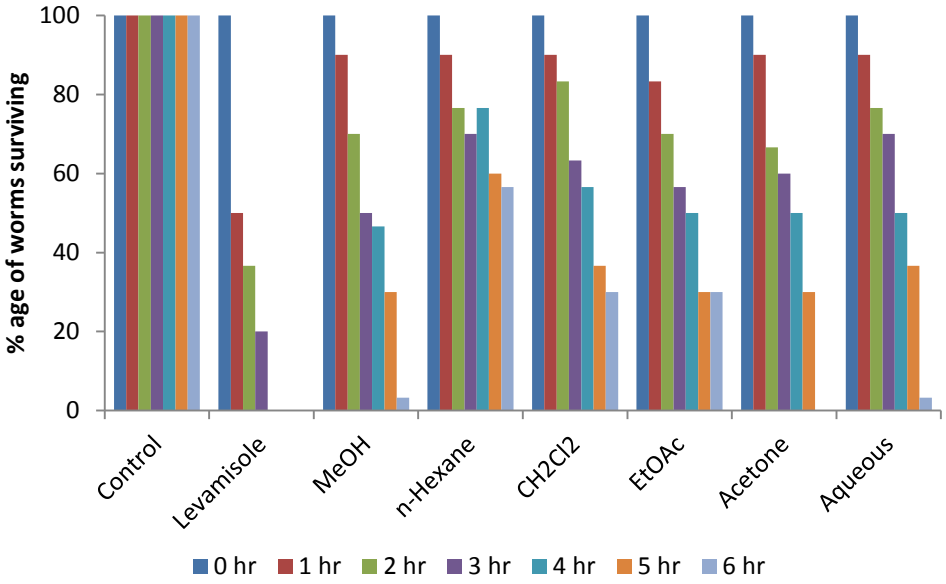

**Figure 4** Anthelmintic activity of crude extract and fractions of *W. somnifera*

**Table 1** Amount (g) and % yield of different solvent fractions of *W. somnifera*

| Fresh wt. | Dry wt. | MeOH | | *n*-Hexane | | $CH_2Cl_2$ | | EtOAc | | Acetone | | Aqueous | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| (g) | (g) | wt. (g) | % age | wt. (g) | % age | wt. (g) | % age | wt. (g) | % age | wt. (g) | % age | wt. (g) | % age |
| 300 | 40 | 5.8 | 14.50 | 0.1 | 1.72 | 0.1 | 1.72 | 1.0 | 17.24 | 1.1 | 18.96 | 3.5 | 60.34 |

**Table 2** Qualitative phytochemical analysis of extracts of *W. somnifera*

| Solvent | Flavonoids | Glycosides | Tannins | Steroids | Saponins | Terpenes | Phenolics |
|---|---|---|---|---|---|---|---|
| MeOH | – | – | + | – | + | – | + |
| *n*-hexane | + | – | + | – | – | + | + |
| $CH_2Cl_2$ | + | – | + | + | + | – | + |
| EtOAc | + | – | + | + | – | – | + |
| Acetone | – | – | + | + | – | + | + |
| Aqueous | + | – | + | + | + | – | – |

(+) = indicates presence, (–) = indicates absence

**Table 3** *In vitro* anthelmintic activity of extracts of *W. somnifera* and standard drug (Levamisole)

| Treatments | Number of worms surviving | | | | | | |
|---|---|---|---|---|---|---|---|
| | 0 hr | 1 hr | 2 hr | 3 hr | 4 hr | 5 hr | 6 hr |
| **Control (PBS+DMSO)** | 10.00 ± 0.00 | 10.00 ± 0.00 | 10.00 ± 0.00 | 10.00 ± 0.00 | 10.00 ± 0.00 | 10.00 ± 0.00 | 10.00 ± 0.00 |
| **Levamisole** | 10.00 ± 0.00 | 5.00 ± 1.00 | 3.66 ± 0.57 | 2.00 ± 1.00 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 |
| **MeOH** | 10.00 ± 0.00 | 9.00 ± 1.00 | 7.00 ± 1.00 | 5.00 ± 1.00 | 4.66 ± 0.57 | 3.00 ± 1.00 | 0.33 ± 0.57 |
| ***n*-Hexane** | 10.00 ± 0.00 | 9.00 ± 1.00 | 7.66 ± 0.57 | 7.00± 1.00 | 7.66 ± 0.57 | 6.00 ± 1.00 | 5.66 ± 0.57 |
| **$CH_2Cl_2$** | 10.00 ± 0.00 | 9.00 ± 1.00 | 8.33 ± 1.52 | 6.33 ± 0.57 | 5.66 ± 0.57 | 3.66 ± 0.57 | 3.00 ± 1.00 |
| **EtOAc** | 10.00 ± 0.00 | 8.33 ± 1.52 | 7.00 ± 1.00 | 5.66 ± 0.57 | 5.00 ± 1.00 | 3.00 ± 1.00 | 3.00 ± 1.00 |
| **Acetone** | 10.00 ± 0.00 | 9.00 ± 1.00 | 6.66 ± 1.52 | 6.00 ± 2.00 | 5.00 ± 1.00 | 3.00 ± 1.00 | 0.00 ± 0.00 |
| **Aqueous** | 10.00 ± 0.00 | 9.00 ± 1.00 | 7.66 ± 0.57 | 7.00 ± 1.00 | 5.00 ± 1.00 | 3.66 ± 0.57 | 0.33 ± 0.57 |