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Full Length Article

# Evaluation of antioxidant and cytotoxic activities of different extracts of folk medicinal plant *Hapllophyllum tuberculatum*

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ABSTRACT

*Hapllophyllum tuberculatum* (*H. tuberculatum*) is a folk medicine used traditionally in Oman for the treatment of arthritis, nausea, fever, gastric pains, intestinal worms and malaria. The design of this study is to prepare different polarity extracts of *H. tuberculatum* and to evaluate antioxidant and cytotoxic activities by using 2,2-diphenyl-1-picrylhydrazyl (DPPH) and brine shrimp lethality (BSL) methods. The coarse leave samples were extracted with methanol by using a Soxhlet method and the obtained methanol extract was defatted and fractionated by different polarity of solvents with increasing polarity to give hexane, chloroform, ethyl acetate, butanol, and water extracts, respectively. The high antioxidant activity was obtained in the ethyl acetate extract and the lowest was in the methanol extract and the order of activity was ethyl acetate > butanol > water > chloroform > hexane > methanol extract. The cytotoxic activity results showed that the hexane, chloroform and ethyl acetate extracts have killed all the shrimp larvae at the concentration of 500 μg/ml. The highest $IC_{50}$ was found in the chloroform extract and the lowest $IC_{50}$ was found in the butanol extract and the order of activity was chloroform > ethyl acetate > hexane > water > methanol > butanol extract. Significant antioxidant and cytotoxic activities results were found first time of Omani *H. tuberculatum* species which is traditionally used as folk medicine all over the world, including Oman. Therefore, the highest activity ethyl acetate extract could be used as a natural antioxidant. The present study is the first report on the evaluation of antioxidant and cytotoxic activities of different polarity extracts of Omani *H. tuberculatum* species.



## 1. Introduction

*Haplophyllum* is one of the most available genus belonging to the Rutaceae family. More than 68 species are available all over the world [1,2]. Its scientific name is *Haplophyllum tuberculatum* [3]. *Haplophyllum tuberculatum* (*H. tuberculatum*) species have originated from Iran-Turanian and it is available now in eastern Anatolia, Gobi desert, Sinai Peninsula, Tien Shan, Altai mountain ranges, Lebanon, Jordan, Israel, Palestine, Syria, Iran, Northern Iraq, Afghanistan, Pakistan, India, and Central Asia [1] Locally, it is called Tafar tase; however, in Muscat, Al-Sharqiya and other Governorates, it is known as Senan tase [3]. The Arabian common names of this plant are Sazab, Zeita, Kheisa and Mesaika. It has many synonyms such *as Haplophyllum arabicum, Haplophyllum candolleanum, Haplophyllum chesneyanum* and *Haplophyllum etremophilum* [4]. *H. tuberculatum* is a medium herb about 40–60 cm of height. All stems are branched from the base. Its color is yellowish green to white color. It has many glands on all parts of this plant. Leaves are leaner, lobed or sometimes deeply cut into 3 lobes. The size of the leaves is 9–50 mm (Fig. 1). It has a special and an unpleasant odor, which makes it unattractive for animals to eat. *H. tuberculatum* is a flowering plant, which start flowering from May to July [4]. There are many flowers on the top with green color, and they are small and separated from each other. The size of fruits is about 2.5–4.5 mm, and the seeds are about 1.5 mm long. The seed's color is dark brown to brownish-black [2]. The essential oil has been collected from several parts of *H. tuberculatum.* It contains several chemical components which are different from country to country. In Iran, the collected essential oil contained 40 chemical components which are responsible for different biological activities. The main components in the Iranian volatile oils are linalool, α-pinene and limonene [5]. Similarly, in Oman, the collected essential oil contains 30 compounds and the main chemical components are β-phellandrene, limonene, β-ocimene, α-caryophyllene, myrcene and α-phellandrene [6,7]. In Saudi Arabia, the oil contains 37 chemical compounds, and in Egypt, contains 88 chemical components [8]. In addition, *H. tuberculatum* also contains several secondary metabolites such as alkaloids, flavonoids, terpenoids, lignins and their oxygenated derivatives [4]. The aerial parts of *H.*

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**Fig. 1.** Plant picture of *H. tuberculatum.*

*tuberculatum* are used traditionally for the treatment of fever, carminative and decongestant. The leaves and stems are externally applied for the treatment of ear and eye problems and the extract of the stem is rubbed onto the skin to protect animals from biting insects and flies. Also, it is used as an antispasmodic, antiflatulent and to treat allergic rhinitis [3]. In Oman, it is used traditionally for the treatment of fever, gastric pains, intestinal worms, malaria and fractures [7]. Due to its medical importance, now this plant is commercially cultivated worldwide. Several biological studies have been conducted on this plant worldwide [6,8]. However, there is not even single extensively research available on cytotoxic and antioxidant activities of the leaves of Omani *H. tuberculatum* species. Therefore, the major purpose of this present study is to prepare different polarities extracts of the leaves of selected plant and to evaluate their antioxidant, and cytotoxic activities by 2,2-diphenyl-1-picrylhydrazyl (DPPH) and brine shrimp lethality (BSL).

## 2. Material and methods

### 2.1. Chemicals and reagents

Several chemicals and glassware have been used to performed this study. The methanol solvent was obtained from Nalar Normapur, France. Chloroform was obtained from Daejung, Korea. DPPH (2, 2-diphynyl-1-picryl-hydrazyl), gallic acid, shrimp egg, butanol and ethyl acetate were obtained from Sigma-Aldrich Company, Germany. Dimethyl sulphoxide (DMSO, purity 99%) was obtained from Sigma, St. Louis, USA. Acetone obtained from Nalar Normapur, EC. Sodium chloride and other chemicals were obtained from Sigma-Aldrich Company, USA.

### 2.2. Instrument for sample analysis

The absorbance of different concentrations of each polarities extract of *H. tuberculatum* was measured by Shimadzu UV–visible spectrophotometer (Model Shimadzu 1800, Japan).

### 2.3. Sample collection

The leaves sample of *H. tuberculatum* was collected from Farq, Al-Dakhiliya, Nizwa. It is about 25 km away from the University of Nizwa Campus. The samples were collected on January 24, 2016 around ~~at~~ 4 to 6 pm. Then the leaves were separated immediately from the stems and kept in a plastic bag for transportation to the Research Laboratory (Room 29 K), Universaity of Nizwa. The separated leave samples were kept at room temperature for wash and drying.

### 2.4. Sample preparation and extraction

The separated leaves samples were washed with water and dried at room temperature under shade for several days until it completely dry. The dried samples were ground into coarse powder by using a kitchen blender machine. The dry coarse powder sample (134.43 gm) was extracted with methanol (550 gm) by using a Soxhlet extraction method for 72 h. Rotary evaporator was used for the evaporation of methanol solvent. After evaporation of methanol solvent, the extract (54.01 gm) was dissolved in 200 ml of water for fractionation. The dissolved extract was transferred into a separatory funnel. Finally, it was fractionated by different solvents with increasing polarities. The mother solvent such as hexane, chloroform, ethyl acetate, and butanol were evaporated by using rotary evaporator under pressure at 24 °C to give hexane (13.73 gm), chloroform (15.17 gm), ethyl acetate (0.88 gm), butanol (1.67 gm) and water (11.41 gm) extracts, respectively [9–12]. The remaining water solvent also evaporated by the same way to give water extract (3.75 gm).

### 2.5. Antioxidant activity

The antioxidant activity of different polarities extracts of *H. tuberculatum* was determined by free radical scavenging method as described by Alabri et al. [12,13] with modification. Five different concentrations 12.5, 25, 50, 100 and 200 μg/ml were used for each extract such as hexane, chloroform, ethyl acetate, butanol, methanol, and water extracts. Each concentration from each extract (4 ml) was placed in a clean test tube and added 1 ml of DPPH (2,2-diphenyl-1-picrylhydrazyl) solution to the same test tube and shaken vigorously by hand. Finally, all the test tubes were kept at room temperature in a dark place for 45 min for complete reaction. The gallic acid standard was prepared to follow the same procedure without adding any plant extract. After incubation, the absorbance was measured in all tested samples at a fixed wavelength 517 nm by using a UV spectrophotometer [10]. The $EC_{50}$ value of each extract was calculated by log and antilog method.

**Table 1**
Antioxidant activity of different leaves organic crude extract of *H. tuberculatum*.

| Crude extracts | Concentration (μg/ml) | Inhibition (%) | $EC_{50}$ (μg/ml) |
|---|---|---|---|
| Hexane | 12.5 | 71.88 | |
| | 25 | 71.88 | |
| | 50 | 71.88 | 17.46 |
| | 100 | 72.91 | |
| | 200 | 73.81 | |
| Chloroform | 12.5 | 72.52 | |
| | 25 | 72.78 | |
| | 50 | 72.86 | 16.80 |
| | 100 | 72.86 | |
| | 200 | 76.12 | |
| Ethyl acetate | 12.5 | 72.14 | |
| | 25 | 74.32 | |
| | 50 | 75.60 | 14.14 |
| | 100 | 79.07 | |
| | 200 | 85.62 | |
| Butanol | 12.5 | 73.55 | |
| | 25 | 76.50 | |
| | 50 | 77.79 | 13.64 |
| | 100 | 79.97 | |
| | 200 | 85.23 | |
| Methanol | 12.5 | 70.98 | |
| | 25 | 71.37 | |
| | 50 | 71.75 | 17.72 |
| | 100 | 72.77 | |
| | 200 | 73.55 | |
| Water | 12.5 | 72.40 | |
| | 25 | 74.58 | |
| | 50 | 74.58 | 15.11 |
| | 100 | 76.50 | |
| | 200 | 81.25 | |
| Gallic Acid | 12.5 | 80.90 | |
| | 25 | 82.44 | |
| | 50 | 84.75 | 11.66 |
| | 100 | 85.39 | |
| | 200 | 87.00 | |

The values are means ± SD of three replicates. $P < 0.05$ when compared with gallic acid. Data are expressed as $EC_{50}$ in μg/ml which is the concentration of extract requires to inhibit growth by 50%.

The percentage of inhibition of each concentration of plant extract was calculated by using the following formula,

$$\%\text{Inhibition} = \frac{A_{\text{control}} - A_{\text{extract}}}{A_{\text{control}}} \times 100 \quad (1)$$

### 2.6. Cytotoxic activity

The cytotoxic activity of each prepared extract of *H. tuberculatum* was determined by the brine shrimp lethality method [14,15]. The brine shrimp eggs were hatched at the covered chamber of the duo compartment plastic container with sea water for 24 h. After hatching, the active nauplii were separated from the eggs, and used for cytotoxic activity. Six concentrations such as 500, 250, 125, 62.5, 31.25 and 15.62 μg/mg were prepared by using dimethyl sulfoxide (DMSO). From each of extract solutions, 50 μl were added to pre-marked test tubes containing 5 ml of sea water. 10 nauplii were added each test tube. After 24 h, the number of surviving nauplii in each test tube was counted using magnifying glass and recorded the surviving nauplii. The percentage of lethality of brine shrimps was calculated for each concentration of the sample. The $IC_{50}$ value of each extract was calculated by log and antilog method.

### 2.7. Statistical analysis

All experiments were performed in triplicate and the results were presented as mean ± SD. The concentration that killed 50% of the nauplii ($LC_{50}$) was determined for each polarity extract by Statistical Analysis Systems (SAS) computer programme [16]. It was determined by plotting a graph of percentage mortality of shrimp larvae against the logarithmic concentrations of extracts tested (Log and Anti Log).

## 3. Results and discussion

Polyphenols, including phenolic and flavonoid compounds occur widely in food of plant origin and are highly diversified. All

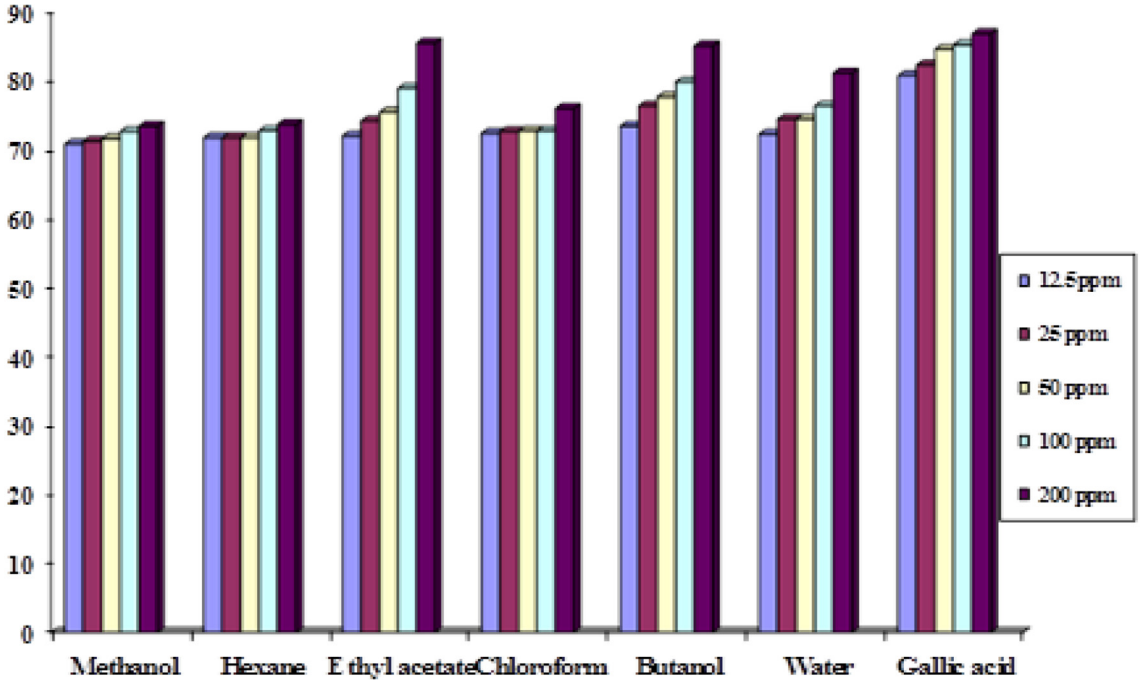


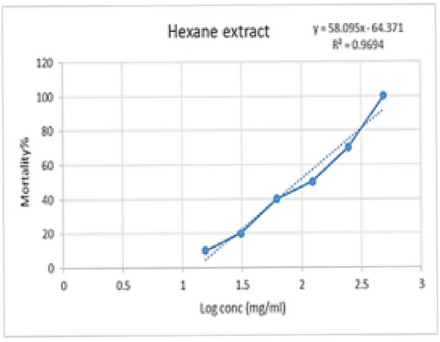

**Fig. 2.** Comparison antioxidant activity of different crude extracts of *H. tuberculatum*.

of them have played a vital role in the successful medical treatments since ancient times. Recently, some polyphenol compounds have gained interest because they exhibit beneficial health effects due to their potential antioxidant, *anti*-inflammatory and cancer-preventive activities [17–21]. They are present widely in the body cells and fluids as a result of ingestion of fruit, vegetables, and plant-derived food such as tea and chocolate [22]. Now-a-days, so many antioxidants based formulations drug are used for the prevention as well as treatment to cure some incurable diseases like arthritis, different stroke, diabetes mellitus, Alzheimer's disease and cancer [23]. More recently, interest has increased significantly in finding natural antioxidants from natural sources to replace pharmaceutical antioxidants drugs due to their toxicity/carcinogenicity [24,25]. The selected Omani plant species are used extremely as a folk medicine by the local communities for the treatment of fever, gastric pains, intestinal worms, malaria, carminative and decongestant. However, there is not a single study available on the Omani species. Therefore, the present study was conducted on the screening of antioxidant and cytotoxic activities of locally grown *H. tuberculatum*. The collected dried leaves powder samples were extracted with methanol and fractioned by different organic solvents with increasing polarities. The prepared organic extracts were used for the evaluation of antioxidant and cytotoxic activities by using DPPH and BSL methods [13,16].

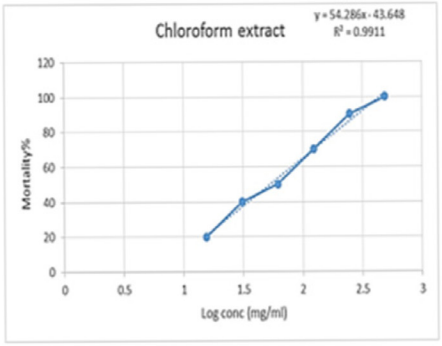
The antioxidant activity of organic extracts was determined by the DPPH method with modification [10]. The highest antioxidant activity was in the ethyl acetate extract and the lowest was in the methanol extract and followed by the order of ethyl acetate > butanol > water > chloroform > hexane > methanol extracts. (Table 1 and Fig. 2). The experimental findings showed that different polarities leave extracts at different concentrations exhibited significant free radical scavenging activity (Table 1 and Fig. 2). The antioxidant activity of different organic extracts of leaves of *H. tuberculatum* was determined through DPPH and the experimental results are presented in the Table 1 and Fig. 2. In this experiment, the role of stable free radical of DPPH is to react with antioxitive free radicals of organic extracts of *H. tuberculatum*. The deep violet color of stable free radical (DPPH) is converting to pale color with the progress of reaction of antioxitive free radicals of the leaves organic extracts. The rate of decolouration of

**Table 2**
Cytotoxic activity of different leaves crude extract of *H. tuberculatum*.

| Hexane extract | | |
|---|---|---|
| Concentration µg/ml | Log Concentration µg/ml | Mortality% |
| 500 | 2.69 | 100 |
| 250 | 2.39 | 70 |
| 125 | 2.09 | 50 |
| 62.5 | 1.79 | 40 |
| 31.25 | 1.49 | 20 |
| 15.62 | 1.19 | 10 |




| Chloroform extract | | |
|---|---|---|
| Concentration µg/ml | Log Concentration µg/ml | Mortality% |
| 500 | 2.69 | 100 |
| 250 | 2.39 | 90 |
| 125 | 2.09 | 70 |
| 62.5 | 1.79 | 50 |
| 31.25 | 1.49 | 40 |
| 15.62 | 1.19 | 20 |




| Ethyl acetate extract | | |
|---|---|---|
| Concentration µg/ml | Log Concentration µg/ml | mortality% |
| 500 | 2.69 | 100 |
| 250 | 2.39 | 80 |
| 125 | 2.09 | 60 |
| 62.5 | 1.79 | 50 |
| 31.25 | 1.49 | 30 |
| 15.62 | 1.19 | 30 |

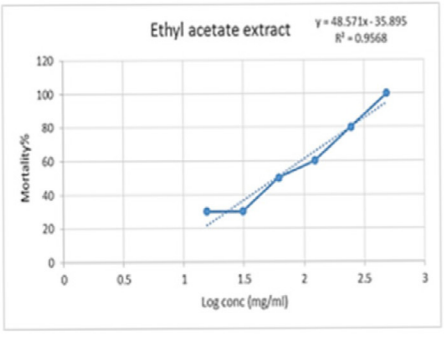
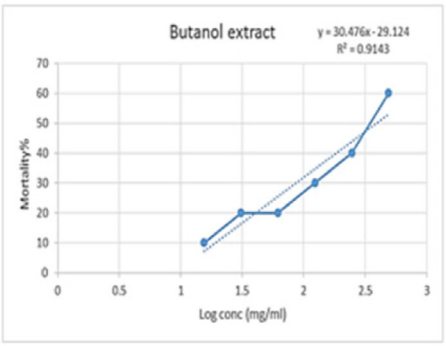
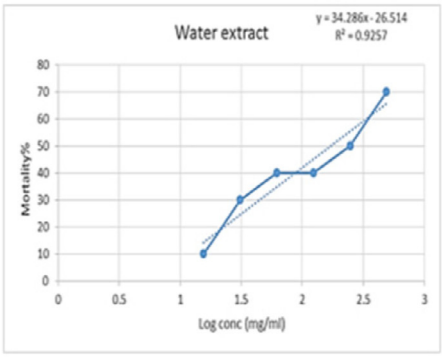


**Table 2** (*continued*)

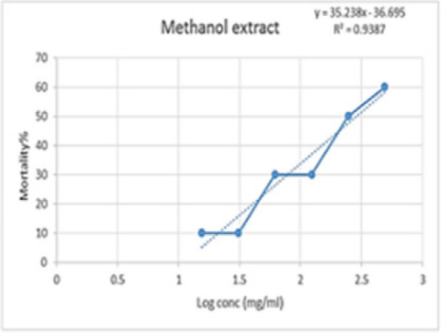
| Butanol extract | | |
|---|---|---|
| Concentration µg/ml | Log Concentration µg/ml | mortality% |
| 500 | 2.69 | 60 |
| 250 | 2.39 | 40 |
| 125 | 2.09 | 30 |
| 62.5 | 1.79 | 20 |
| 31.25 | 1.49 | 20 |
| 15.62 | 1.19 | 10 |




| Water extract | | |
|---|---|---|
| Concentration µg/ml | Log Concentration µg/ml | mortality% |
| 500 | 2.69 | 70 |
| 250 | 2.39 | 50 |
| 125 | 2.09 | 40 |
| 62.5 | 1.79 | 40 |
| 31.25 | 1.49 | 30 |
| 15.62 | 1.19 | 10 |




| Methanol extract | | |
|---|---|---|
| Concentration µg/ml | Log Concentration µg/ml | mortality% |
| 500 | 2.69 | 60 |
| 250 | 2.39 | 50 |
| 125 | 2.09 | 30 |
| 62.5 | 1.79 | 30 |
| 31.25 | 1.49 | 10 |
| 15.62 | 1.19 | 10 |




organic extracts represents the strength of antioxidant activity. In our experiment, all the extracts *H. tuberculatum* were capable to decolourise of DPPH. The antioxidant activity of the organic extracts was determined to be in the order of ethyl acetate > butanol > water > chloroform > hexane > methanol extracts. The literature search reveals that some bioactive organic compounds such as gallic acid, glutathione, ascorbic acid, tocopherol, flavonoids, phenols, amines are decolorizing DPPH gradually by the hydrogen donating capability [24,25]. The above mentioned statement, it was confirmed that the organic extracts of *H. tuberculatum* possess hydrogen donating capabilities to act as antioxidants. In our experiment, the highest antioxidant activity was in the ethyl acetate extract and the lowest was in the methanol extract and followed by the order of ethyl acetate > butanol > water > chloroform > hexane > methanol extracts. The findings showed that different polarities leave extracts at different concentrations exhibited significant free radical scavenging activity. The determination of antioxidant activity of *H. tuberculatum* was done in comparison with that of gallic acid in Table 1. Gallic acid showed a high activity with $EC_{50}$ values of 11.66 µg/ml. In our present experiment, the highest $EC_{50}$ was found in the buatnol extract and the lowest $EC_{50}$ was found in the methanol extract with and in the order of $EC_{50}$ values butanol > ethyl acetate > water > chloroform > hexane > methanol extracts. Our experimental results are not similar to what has been reported for antioxidant activity of *H. tuberculatum* extract [5–9]. It can be concluded that the butanol extract contains the maximum number of bioactive chemicals which could be responsible for its antioxidant and total antioxidant capacity. The significant antioxidant activity of extracts might be due to the high number of polyphenolic compounds or high concentration of bioactive compounds present in this plant sample. This present study highlights that the extracts of *H. tuberculatum* is a good potential source of natural antioxidants to prevent free radical oxidative damage.

The selected plant species is used by Omani people for the treatment of different aliments. However, nobody works on antioxidant and cytotoxic activities of this Omani plant species. The cytotoxic activity of organic extracts was determined by brine shrimp larvae (BSL) method with modification [14]. In our experiment, the hexane, chloroform, ethyl acetate, butanol, methanol and water extracts of leaves of *H. tuberculatum* displayed significant cytotoxic activity against the brine shrimp larvae. The mortalities as a per-

**Table 3**
$IC_{50}$ values of different leaves crude extract of *H. tuberculatum*.

| Exteract | $IC_{50}$ (μg/ml) |
|---|---|
| Hexane | 1.96 |
| Chloroform | 1.72 |
| Ethyl acetate | 1.76 |
| Butanol | 2.59 |
| Water | 2.20 |
| Methanol | 2.46 |

Each value is expressed as mean ± standard deviation of triplicate measurements. Data are expressed as $IC_{50}$ in μg/ml which is the concentration of extract requires to inhibit growth by 50%.

centage (%) of shrimp larvae of different extracts of leaves are shown in Table 2. The cytotoxicity results showed that hexane, chloroform and ethyl acetate extracts from leaves of *H. tuberculatum* have killed all the shrimp larvae at the concentration of 500 μg/ml. However, butanol, methanol and water extracts did not kill all the shrimp larvae at 500 μg/ml. In the present experiment, the highest $IC_{50}$ was found in the chloroform extract and the lowest $IC_{50}$ was found in the butanol extract and in the order of $IC_{50}$ values chloroform > ethyl acetate > hexane > water > methanol > butanol extracts. As shown in Table 3, the leaves extracts displayed significant toxicity against the brine shrimp larvae. The chloroform extract was the most active, exhibiting $LC_{50}$ value of 1.72 ug/ml. These results are not similar to what has been reported for cytotoxic activity of *H. tuberculatum* extract [26,27]. Based on the cytotoxic results of different organic extracts of *H. tuberculatum*, it is probable that the highest toxicity shown by the chloroform extract may be due to the presence of semi polar bioactive compounds [28]. This difference in $LC_{50}$ value could be due to differences in methodologies; while the present study used the BST assay other investigations used the *in vitro* and *in vivo* based assay [29].

## 4. Conclusion

In this study, the determination of antioxidant and cytotoxic activities of leaves extracts of *H. tuberculatum* by DPPH and brine shrimp method has been reported. All the extracts from the leaves showed significant antioxidant and cytotoxic activities. In our findings through this graduation project revealed that the leaves of *H. tuberculatum* species grown in Oman contain a significant number or amount of bioactive compounds which might be responsible for its biological activities. Further, more *in vitro* and *in vivo* studies are needed of the active selected extracts of leaves of *H. tuberculatum* to determine their potential for therapeutic uses of this plant to prevent some chronic diseases.

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