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Evaluation of the Total Content of Phenols and Flavonoids in Two Different Extracts of *Orbea wissmannii* O. Schwart and their Toxic Effect on WI38 and HepG2 Cell Lines.

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Abstract

This study aimed at estimating the total phenolic content, total flavonoid content and evaluation of toxic effect of chloroform and aqueous methanol extracts of *Orbea wissmannii* against WI38 and HepG2 cell lines. *Orbea wissmannii* was collected from Qaren region near Amran governorate, Yemen. Chloroform and methanol extracts of whole parts of *Orbea wissmannii* were prepared by maceration and decantation. The total polyphenol content was estimated using Folin–Ciocalteau method. Aluminum chloride colorimetric method was applied for the determination of total flavonoid content. The cytotoxic activity was measured using the MTT assay. The total phenolic content of chloroform and methanol extracts was 70.21 mg GAE/g, 122 mg GAE/g respectively (milligrams of gallic acid equivalents per gram), while the flavonoid content was 21.80 mg QE/g and 54.24 mg QE/g respectively (milligrams of quercetin dihydrate equivalents per gram). Dependently, the two extracts significantly and concentratedly reduced the viability of lung tissue-derived fibroblasts (wi38) and human hepatoma cells (HepG2). The two extracts showed effective effect on HepG2 at 250, 500 and 1000 µg/ml, while the same extracts showed their effect on the WI38 at 500 and 1000 µg/ml. The toxicological properties shown by the extracts of this plant require further study to isolate and purify the active substances responsible for this high anti-cancer efficacy. Phytochemical investigation on chloroform/methanol extracts and their structures is recommended.

Keywords: Orbea wissmannii, Total phenolic, Total flavonoid, a cytotoxic, HepG2, wi38, Amran, Yemen

الملخص: هدفت هذه الدراسة إلى تقدير المحتوى الفينولي الكلي ومحتوى الفلافونويد الكلي لمستخلصات الكلوروفورم والميثانول المائي لنبات السمع وتقييم التأثير السام للمستخلصات ضد سلالات خلايا wi38 و HepG2. تم جمع النبات من منطقة قارن – محافظة عمران – اليمن. تم تحضير مستخلصات الكلوروفورم والميثانول للنبات كاملا بواسطة النقع والترويق وتم تقدير المحتوى الكلي للفينولات باستخدام طريقة Folin–Ciocalteau. وتم تقدير المحتوى الكلي للفلافونيدات باستخدام طريقة القياس اللوني لكلوريد الألومنيوم. تم قياس النشاط السام للخلايا باستخدام فحص MTT. كان المحتوى الفينولي الكلي لمستخلصات الكلوروفورم والميثانول 70.21 مجم GAE / جم ، 122 مجم GAE / جم على التوالي (ملليغرام من مكافئات حمض الغاليك لكل جرام) ، بينما كان محتوى الفلافونويد 21.80 مجم QE / جم و 54.24 مجم QE / جم على التوالي (ملليغرام من مكافئات كيرسيتين ديهيدرات لكل جرام). بشكل مستقل ، قلل المستخلصان بشكل كبير ومركّز من قابلية نمو الخلايا الليفية المشتقة من أنسجة الرئة (wi38) وخلايا الورم الكبدي البشري (HepG2). أظهر المستخلصان تأثيراً فعالاً على HepG2 عند 250 و 500 و 1000 ميكروغرام / مل ، بينما أظهرت نفس المستخلصات تأثيرها على wi38 عند 500 و 1000 ميكروغرام / مل. الخصائص الطبية لهذا النبات تتطلب المزيد من البحث وطرق التنقية للحصول على المواد الفعالة المضادة للسرطان.

1. Introduction

Traditional medicine is widely used in Yemen's primary healthcare system. In Yemen, traditional medicinal plant information is still passed down orally among the indigenous population. Thus, there are only a few research papers, and most of them have been relatively limited in this field [1]. Since

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the cytotoxic effects of phytochemicals (such as flavonoids and phenols) have been proven, such plants have been considered an acceptable source for drug synthesis [2, 3].

The genus *Orbea* belongs to the Asclepiadaceae family that are known to contain cytotoxic and tumoricidal polyoxy pregnane esters and glycosides [4]. Phenols as secondary metabolites are found mostly in the higher plants in the plant kingdom [5]. Indeed, phenols are among the most active anticancer chemicals that have already been described, among hydroxycinnamates, flavonoids, coumarins, hydroxybenzoates, xanthones, stilbenes, chalcones, and lignins [6].

Orbea genus have been used for a variety of therapeutic purposes including treatment of diabetes, wounds, burns, eczema [1] and as an appetite suppressant or appetite curbing [7,8].

Orbea wissmannii (Syn.=*Caralluma wissmannii*) [9] is a leafless, succulent, and angular plant that grows in the wild areas in Yemen (local name is "khusmaa"), and the stems of the plant are eaten (raw) [10] as well as the plant used in the treatment of different disorders such as stomach ulcers, constipation, food poisoning (antidote), diabetes [1], and inhibition of Escherichia coli growth, and antioxidant activities [11].

Our previous study revealed that the *Orbea wissmannii* contains 12-tigiloyl-tayloron-3β-D-thevetopyranosyl-(1→4)-β-D-cymaropyranosyl-(1→4)-β-D cymaropyranoside, 12-tigiloyl-tayloron 3β-D-thevetopyranosyl-(1→4)-β-D cymaropyranoside, 3,4-seco-lup-20(29)-en-3-oic acid methyl ester, lupeol, stigmasterol, β-sitosterol, and luteolin 3',4'–di-O-β-D-glucopyranoside [12]. A spectrum of the biological activities of the *Orbea* can be expected due to the existence of pregnane glycosides, stigmasterol, and other phytochemicals in them [13, 14].

Increasing cancer cell sensitivity to chemotherapy by modifying abnormal metabolism using plant extracts is a potential technique for lowering chemotherapy dosages while maintaining therapeutic outcomes [15]. The *Orbea wissmannii* is a widespread species in Yemen [10], but there are few literature reports concerning its biological activities and phytochemical composition. Thus, the current work aimed to estimate the total phenolic content, total flavonoid content and evaluation of cytotoxic effect of chloroform and methanol extracts of *Orbea wissmannii* against the wi38 and the HepG2 cell lines. The study will be of value in highlighting that the *Orbea wissmannii* in Yemen could be considered as a promising source for several compounds that can be used as anticancer agents.

2. Materials and Methods

2.1 Collection of plant material

The whole parts of the *Orbea wissmannii* was collected from Qaren region, Amran governorate, Yemen in April 2021 and was identified by Dr. Abdulwali A. Alkhulaidi, Plant Ecology and Geography, Agricultural Research Authority, Taiz, Yemen.

2.2 Preparation of extracts

200 g of whole parts of *Orbea wissmannii* dried in the air was extracted by the maceration method using 80% aqueous methanol. The methanol extracts were evaporated under reduced pressure to give a dark-greenish residue (extract). The obtained extract was separated by decantation three times with chloroform and recycled with a rotary evaporator.

2.3 Determination of total polyphenol content of investigated samples

Total polyphenol content was estimated using Folin–Ciocalteau reagent as mentioned by [16].The reaction mixture consists of 0.5 ml of aqueous MeOH extract and its $CHCl_3$ fraction for each sample, 0.1 ml of Folin reagent and 0.5 ml of 7.5% Na_2CO_3 solution. After incubation at room temperature in darkness for one hour, the absorbance was measured at 740 nm. The polyphenol content was determined from the following equation: (Y= 0.0248 ±0.0591), (R2 = 0.9979) using gallic acid as a standard, and results were expressed as mg GAE/g D.W.

2.4 Determination of total flavonoid content of investigated samples

Total flavonoid content (TFC) was determined using a modified aluminum chloride colorimetric method [17]. Briefly, 2 ml of MeOH, 0.2 ml of 1M CH_3COOK (w/v), 0.3 ml of 10% $AlCl_3.6H_2O$ solution, and finally 2 ml of distilled water were added to 0.5 ml of aqueous MeOH extract and its $CHCl_3$ extract for each sample. After 30 minutes of incubation at room temperature, the absorbance at 430 nm was measured. The flavonoid content was calculated from the following equation: (Y= 0.0046 ±0.0585), (R2 = 0.9995) using quercetin dihydrate as a reference, and results were expressed as mg QE/g D.W.

2.5 Preparation of stock solution of extracts

To prepare stock solutions, the two extracts of the *Orbea wissmannii* were dissolved separately in the DMSO, and then the proper amount of distilled water was added. The maximum amount of the DMSO was 10% at stock and 1% at the final concentration in the wells.

2.6 Cell Viability Assay

The anticancer activity of the extracts of *Orbea wissmannii* was done at the Science Way for Scientific Researches and Consultations, Egypt.

In order to avoid a cytotoxic effect of *the Orbea wissmannii* extracts on the HepG2 and wi38 cells proliferation, the cytotoxicity study was carried out using the MTT assay is based on the ability of functional mitochondria to catalyze the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to insoluble formazan, the concentration of which can be measured spectrophotometrically [18]. The HepG2 and wi38 cells were first cultured in 96-well plates at a density of (1 x 10^5 cells / ml (100 µl / well) for 24 hours at 37°C in a CO_2 incubator, washed twice with media. After incubation, the medium was aspirated and different concentrations (31.25, 62.5, 125, 250, 500, and 1000 µg/ml) of extracts were added to the wells, and the cells were reincubated. After 24 hrs. of incubation, MTT reagent 20 µl was added to each wells, placed on a shaking table at 150 rpm for 5 minutes to thoroughly mix the MTT into the media, and the plate was incubated at 37 °C for an additional 4 h to allow the MTT to be metabolized. The media were then removed, and the intracellular formazan product was dissolved in 200ul DMSO. The absorbency of each well was then measured at 560 nm and subtracted from the background at 620 nm, and the percentage viability was calculated.

3. Statistical analysis

Data were obtained from triplicate experiments and presented as the mean ± standard deviation. A T-test was used for comparison of results using Excel software, where $p < 0.05$ was considered statistically significant.

4. Results

4.1 Total phenol and flavonoid Contents

The aqueous methanolic extract of the *Orbea wissmannii* exhibited a higher total phenolic content (122 mg GAE/g) than the chloroform extract (70.21 mg GAE/g). On the other hand, the aqueous methanolic extract of *Orbea wissmannii* exhibited a higher total flavonoid contents (54.24 mg QE/g) than the chloroform extract (21.80 mg QE/g) results are shown in Table 1. The total phenolic content and the total flavonoid content of *Orbea wissmannii* extracts varied by solvent (Table 1). The methanol extract had significantly higher phenolic content and flavonoid content than the chloroform extract.

Table.1 Results of total phenol and flavonoid content for methanol and chloroform extracts.

Extracts	Total Phenol Content mg GAE/g *	Total Flavonoid Content mg QE/g **
MeOH	122	54.24

$CHCl_3$	70.21	21.80

* **GAE**: Gallic acid equivalents **** QE**: Quercetin dihydrate equivalents

4.2 Cytotoxicity effect of *Orbea wissmannii* extracts against WI38 and HepG2 cells

In the present study, the cytotoxic effect of the two extracts from the *Orbea wissmannii* was determined. All the extracts were tested against the WI38 and HepG2 cells at different concentrations using MTT.

Data in Table 2 show the values of cell toxicity percentages after the exposure of WI38 cultured cells to two extracts. It was found that the cytotoxicity (%) increased with increasing concentrations of *Orbea wissmannii* extracts 500-1000 µg/mL (figure 1). Oppositely, the cell viability (%) of Wi38 gradually decreased with increasing concentrations of the plant extracts. The Chloroform extract showed better toxicity.

The Chloroform extract increases toxicity better than the aqueous methanol extract at 500 (14.75%), 1000 (71.2%) and 500 (7.67%), 1000 (61.42%) with IC50 of 798.02 µg/ml and 883.72 µg/ml, respectively, of the WI38 cells.

Table.2: Viability and toxicity of Wi38 cells in the different concentrations of two extracts of *Orbea wissmannii*

WI38	$CHCl_3$			MeOH		
Conc. **µg/ml**	**Viability %**	**Toxicity %**	**IC50**	**Viability %**	**Toxicity %**	**IC50**
--------	100	0	µg	100	0	µg
1000	28.81500426	71.18499574		38.57630009	61.42369991	
500	85.2514919	14.7485081		92.32736573	7.672634271	
250	99.70161978	0.298380222	798.02	99.82949702	0.170502984	883.72 ±
125	99.87212276	0.127877238	± 8.64	99.95737425	0.042625746	15.07
62.5	99.78687127	0.21312873		99.61636829	0.383631714	
31.25	99.70161978	0.298380222		99.87212276	0.127877238	

Values are expressed in mean ± SE of 3 times repeated for each set of CS extract

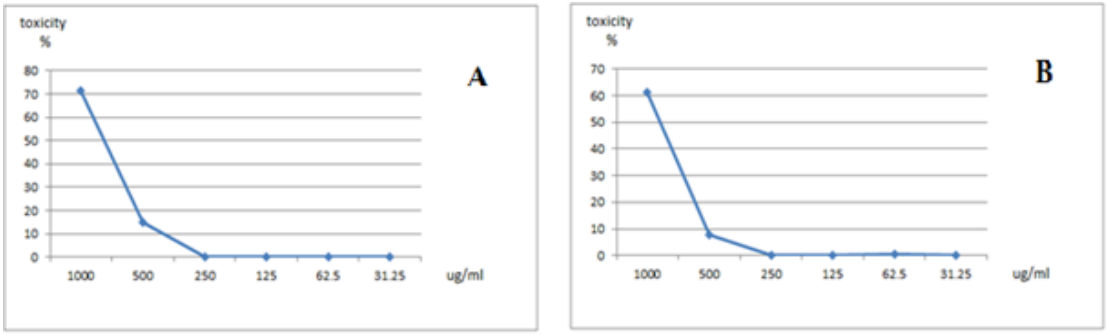

Fig.1: Relationship between toxicity and different concentrations of **(A)** chloroform extract **(B)** methanol extract of *Orbea wissmannii* against WI38 cell line

The two extracts of the *Orbea wissmannii* significantly and concentrations dependently decreased the viability of the HepG2 cells at concentration above 250 µg/ml (Table 3 and Figure 2).

Chloroform extract increases toxicity at 250 (60.85%), 500 (89.19%), and 1000 (93.73%) with IC50 of 222.34 µg/ml better than the methanol extract at 250 (58.56%), 500 (76.83%), and 1000 (95.375%) with IC50 of 228.97 µg/ml of HepG2 cells.

Table 3: Viability and toxicity of HepG2 cells in the different concentrations of two extracts of *Orbea wissmannii*

HepG2	$CHCl_3$			MeOH		
Conc. **µg/ml**	**Viability %**	**Toxicity %**	**IC50**	**Viability %**	**Toxicity %**	**IC50**
--------	100	0	µg	100	0	µg
1000	6.272893773	93.72710623	222.34 ± 3.13	4.624542125	95.37545788	228.97 ± 13.79
500	10.80586081	89.19413919		23.16849817	76.83150183	
250	39.14835165	60.85164835		41.43772894	58.56227106	
125	94.18498168	5.815018315		90.10989011	9.89010989	
62.5	99.77106227	0.228937729		100	0	
31.25	99.86263736	0.137362637		100	0	

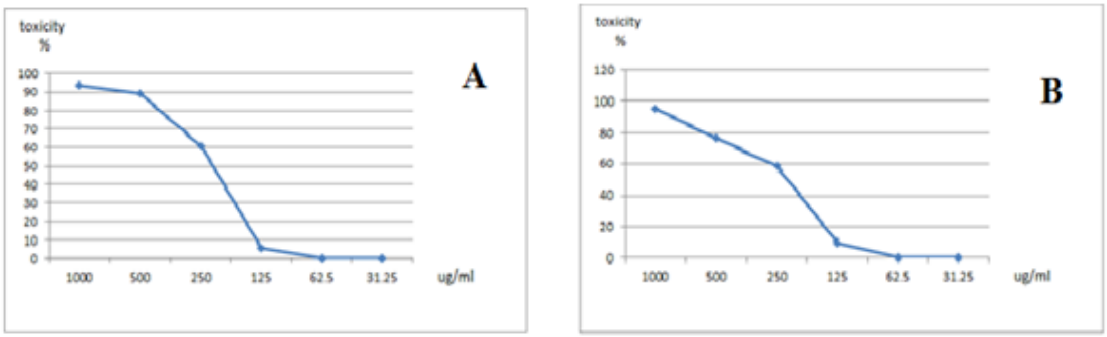

Fig.2: Relationship between toxicity and different concentrations of **(A)** chloroform extract **(B)** methanol extract of *Orbea wissmannii* against HepG2 cell line

The effect of toxicity of the chloroform and methanol extracts on the WI38 and HepG2 cells showed more effect starting at 125 µg/ml on both wi38 and HepG2 cells (tables 2, 3).
HepG2 cells were more susceptible to the chloroform and methanol extracts of *the Orbea wissmannii* compared to the WI38 cells.

5. Discussion

The *Orbea wissmannii* methanolic extract contains total phenolic of 122 mg GAE/g, and the chloroform extract contains total phenolic of 70.22 mg GAE/g. The total phenolic content in chloroform extract is much lower than in methanol extract. While the total content of flavonoids for methanolic extract and chloroform extract were 54.24 mg QE/g and 21.80 mg QE/g respectively, a chloroform extract contains fewer flavonoids than that of the aqueous methanolic extract.
The total phenolic content and the total content of flavonoids depend on the type of solvent used. A comparative investigation of the phenol content in different types of the various solvents concluded that the highest concentration of phenolic compounds was obtained in the plant extracts with the highest polarity of solvent [19].
Some members of the Asclepiadaceae family are rich in the polyhydroxypregnane glycosides and their esters, which are strong cytotoxic agents and have anti-cancer and anti-tumor properties [20]. Furthermore, flavones, flavanols, isoflavones, catechins, and taxanes are phytochemicals with different pharmacological properties that shown responses for the prevention or treatment of different tumors [21, 22].

The two different extracts of the $CHCl_3$ and MeOH from the *Orbea wissmannii* have protective effects on tacrine-induced cytotoxicity in liver-derived HepG2, with IC50 of 222.34 ± 3.13 and 228.97 ± 13.79 µg, respectively. On the other hand, Lung-derived WI38 has IC50 of 798.02 ± 8.64 and 883.72 ± 15.07 which indicate that two extracts are more effective on the HepG2 cell lines. These results indicate that the cytotoxic effect increases with increasing extract concentrations.

Polyphenol compounds might inhibit cancer cells by xenobiotic metabolizing enzymes that change the metabolic activation of potential carcinogens, while some flavonoids could also prevent the development of cancer cells by changing hormone production and inhibiting aromatase [23,24], Lupeol is thought to be a potent inducer of apoptosis in cancer cells, with additional modulatory effects on the drug resistance pathways in cancer cells [25], and some flavonoids induce apoptosis via activation of caspase-3 [26].

6. Conclusions:

According to the above reports, it is suggested that the cytotoxic effect of the two *Orbea wissmannii* extracts in the HepG2 and Wi38 cells could be due to the presence of the pregnane glycosides, polyphenolic and polyflavonoid compounds. Further studies on this plant are necessary and should seek to determine the pharmacokinetic properties of the selected plant.

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Conflicts of interest

There are no conflicts of interest.

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