

Triterpenoid Saponins Investigation and Pharmacological (Cytotoxic and Antioxidant) Properties of *Bacopa monnieri* L. Cultivated in Iraq

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Abstract:

Bacopa monnieri L. (Scrophulariaceae), synonyme is *Herpestis monniera* that provides bioactive compounds, especially triterpenoid saponins (Bacosides) which exhibits an important biological activities, like hypothyroidism, anticonvulsant, memory enhancing and antistress. Because there are no researches about *B. monnieri* L. plant that grow in Iraq, and there active compounds especially triterpenoid saponin (TS), and there effects. This study was detected the presence of (TS) in, and examined the cytotoxic and the antioxidant activity of these compounds in vitro. The study was included the extraction and identification of TS from the whole parts of *B. monnieri* L. by using three methods, and the best yield was analyzed by High Performance Liquid Chromatographic (HPLC) to identify bacosides compounds. In vitro, TS was examined the cytotoxic activity against two cancer cell lines, human cervical cancer (Hela), rhabdomyo sarcoma (RD), and rat embryogenic fibroblast (REF) as a normal cell, at concentrations of 62.5, 125, 250, 500 and 1000 µg/ml at 24, 48, and 72-hr. Free radical 1,1Dyphenyl-2-picrylhydrazyl radical (DPPH) was used for testing the ability of the TS as antioxidant at 20, 40, 60, 80, 100 µg/ml concentrations. The results revealed that *B. monnieri* plant had many components of bacosides when detected by HPLC. Cytotoxic study suggested that TS inhibited the growth of cancer cells, and this effect is depending on the extraction of TS concentration. The effect of TS on Hela cell line was more than that for RD, while the highest effect of TS on Hela was at the concentration of 250 µg/ml after 48 hr. The cytotoxic effect has a significant effect at ($P \leq 0.05$). The results revealed that TS has high antioxidant influence 99.37% at 100 µg/ml concentrations, followed by 98.20% in 80 µg/ml concentration.

Keywords: *Bacopa monnieri* L., triterpenoid saponin, HPLC, antioxidant, cytotoxic

Introduction:

Bacopa is a herbaceous genus with about 100 species, it is represented by a solitary species in Iraq. *Bacopa monnieri* (L.) BARBIN BARRI (Arabic name) Brahmi Sak (Indian name) is widely distributed in wet, marshy land and warmer area of the world throughout India, China, Sri Lanka and in Iraq-Basra in AL-Halfaya and Kahajish marshes(1). This plant was used in traditional medicine around the world to treat several types of diseases and complaints, especially in Ayurveda medicinal system for improving memory brain function, teaching and increasing the concentration (2). It has been used in India for five thousand years to treat insomnia, epilepsy and used as a sedative and as anti-anxiety medication. *B. monniera* is considered as a highly valuable medicinal plant and widely used in relation to pharmacological, ethanopharmacological, toxicological, antimicrobial

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and phytochemical studies, it is used in the field of medicine to treat wide spectrum of diseases such as skin disease, also found to be more effective in several neurological disorder such as anxiety and neurosis. it is also used as antimicrobial agent, antioxidant, antipyretic anti-inflammatory, treat enlargement of spleen, asthma, epilepsy leprosy hoarseness and cardio tonic (3,4). The chemical constituents of *B. monnieri* include Alkaloids brahmine, nicotine and herpestine; saponin, hersaponin. The major chemical entity that is responsible for neuropharmacological effects and the nootropic action or anti-amnesic effect are triperpenoid saponins that have biological interest like bacoside A1, A2, A3, A4, bacopasaponins A,B,C; bacopasaponin D; bacoside I, II, III, IV; monnieri, plantioside B and others(5,6,7).

Triterpenoid saponins are triterpenes that belong to the group of saponin compounds, in *B. monnieri* is known as bacosides which are the key components of the plant, It has also been widely used as an anti-cancer and treats some types of lymphoma like

using ethanolic extract of the whole plant as a cytotoxic agent against Daltons lymphoma ascite cells in both *In vitro* and *In vivo* assays. Ethanolic extract and bascocide A are used to inhibit tumor progression in fibrosarcoma bearing rats(5,8).

HPLC technique is very important in the industrial and analytical field, that it helps in structure elucidation and quantitative determination of impurities and degradation compounds in drug, pharmaceutical formulations and herbal medicine (9).

Because there are no studies in Iraq about extraction, isolation and identification of active compounds of *B. monnieri* L. cultivated in Iraq, especially triterpenoid saponin(bacosides), and the interesting medically of bacosides compounds, so this study focuses on the assessment of some bacosides and determined the anticancer and antioxidant activity of these compounds.

Materials and Methods:

Plant materials:

Whole plant of *B. monnieri* was collected from Al-Sindibad land /Basra/Iraq, in May 2017, cleaned gently, identified and authenticated by prof. Dr. Abdul Reda, University of Al- Basra. dried at room temperature at 25-30 °C for 7 days, and coarsely powdered by mechanical grinder.

The methods were carried out on two parts as the following:-

Part one:-extraction and identification of TS

Three methods were tested to extract TS:-

1- Extraction Method No. 1(10)

Thirty grams of powder of *Bacopa monniera* L. were extracted by maceration in (450 ml) of methanol 99.8% for seven days at room temperature and then filtered. The filtrate was evaporated at reduced pressure in the rotary evaporator to a thick residue. This residue was repeatedly washed with petroleum ether (40-60 °C) to remove the chlorophyll and fatty materials. The process was continued till there were no traces of colouring matter in the petroleum ether. A thick residue was obtained and dissolved in (100 ml) of methanol 99.8%. To methanolic solution diethyl ether was added, white-yellowish precipitate was formed. The addition of diethyl ether was continued, until no further precipitate was formed. The precipitate was recovered by decantation, and then air dried at room temperature to yield the dry crude extract (3.9 g).

Extraction method No. 2.

Thirty grams of powdered of *Bacopa monniera* L. were extracted by maceration in (500 ml) of 75 % methanol for 48 hours, then

ultrasonicated at room temperature for 30 minutes according to Shu and *et.al.* (11) with modification, t. The mixture was filtered, then the supernatant was obtained by centrifugation of extract at 2500 rpm for 10 minutes. Supernatants were combined together into a volumetric flask, and repeatedly washed with petroleum ether (40-60°C) to remove the chlorophyll and fatty materials. To methanolic extract, diethyl ether was added, light yellow precipitate was formed, and then, air dried at room temperature to yield the dry crude extract (4.8 g).

Extraction method No.3(12)

Thirty grams of powder of *Bacopa monniera* L. were extracted by 99.9% ethanol three times at room temperature. After concentration in vacuum, the residue (23 g) was suspended in water and then extracted with petroleum ether; the water extracts (combined) were evaporated under reduced pressure to obtain (2.6 g).

By HPLC, bacosides compounds were identified and measured its quantities. The conditions of HPLC were C-18, 3µm partial size, mobile phase: 0.72% w/v anhydrous sodium sulphate: acetonitrile, (65:35, v/v), pH 3.2, flow rate 1.2 ml/min., UV 280nm.

Part two:-cytotoxic and antioxidant activity of TS

Cytotoxic activity:-the cell lines used in this study were supplied by tissue culture unit/ Iraqi Centre for Cancer and Medical Genetics Research (ICCMGR). The procedure for examining TS against cell lines was carried out according to Alwash(13). A 0.03g of TS was dissolved in 10 ml solution (media and dimethyl sulfoxide), then the concentrations (62.5, 125, 250, 500 and 1000) µg/ml of TS were prepared and tested on each cell line, with three replicates for each concentration. Periods of exposure of cell lines were measured at 24, 48, and 72 hr. under complete sterile conditions. Antioxidant Activity: TS assessed the scavenging ability to stable free radical 1,1-Diphenyl-2-picrylhydrazyl (DPPH). Volumes of 0.5 ml of DPPH solution (0.4 µM) and 1ml of each concentration (20, 40, 60, 80, 100 µg/ml) of TS were mixed and allowed to stand for 30 min. at room temperature. The absorbance of samples was recorded at 518 nm, by a spectrophotometer. Ascorbic acid was used as calculator according to the formula(14):

$$\text{Scavenging Activity \%} = \frac{\text{A518 control} - \text{A518} \times 100}{\text{A518 control}}$$

Results and Discussion:

Part one:-extraction and identification of triterpenoid saponin

Extraction of TS from the dried whole *B. monnieri* L. was done by three extraction methods,

the method No.2 showed higher yield of dry TS crud than other methods, as shown in Table (1). So the method No.2 was chosen for identification of some bacosides in plant.

Table 1. Percentage of TS extracts obtained from extraction methods

Extraction method	% yield of TS crud
Method No.1	13
Method No.2	16
Method No.3	8.6

Qualitative and quantitative evaluation of five TS (bicosides) were done by using High Performance Liquid Chromatography (HPLC) in which identifications were made by comparison of

retention times obtained at an identical chromatographic conditions of analyzed sample and authentic standard of bacopaside I, bacoside A3, Bacopaside II, bacopaside III and bacopasaponin C, as shown in Table (2) and Fig(1)

Table 2. Quantitative of TS compounds (bacosides) from *Bacopa monnieri* L. by HPLC

Name of TS (bacosides)	Retention time min.	Concentration $\mu\text{g/ml}$
Bacopaside I	1.44	422.8
Bacoside A ₃	2.955	468.8
Bacopaside II	3.808	112.63
Bacopaside III	5.71	512.82
Bacopasaponin C	6.978	736.77

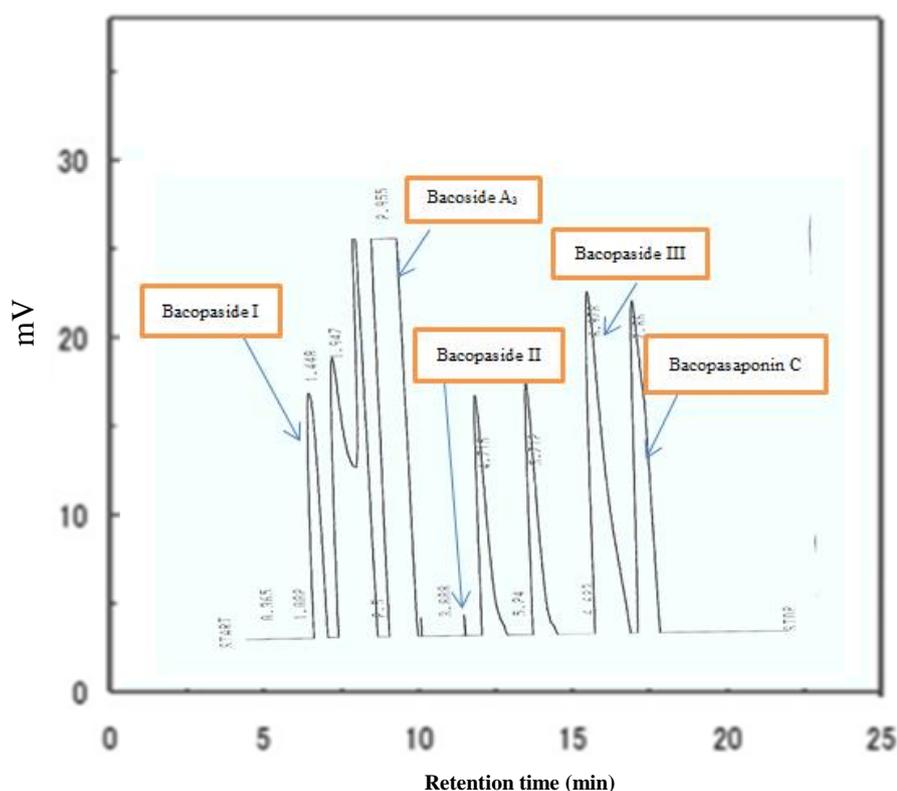


Figure 1. HPLC analysis of isolated TS (bacosides)

Major saponins found in *B. monnieri* in this study are Bacopasaponin C followed by Bacopaside III. Many researchers used HPLC analysis for bacosides detection, because it is suitable for measuring quantity and quality components (15,16).

Part two: cytotoxic and antioxidant activity for TS

Cytotoxic activity

The cytotoxic activity was determined by using five different concentrations of TS from *B.monnieri* plant on two cancer cell lines and one normal cell line after 24, 48 and 72 hour exposure time. The results are shown in Table 3, 4 and 5. The statistical analysis indicated that the concentrations of TS have a significant cytotoxic effect on HeLa and RD after 24, 48 and 72 hour at levels ($P \leq 0.05$) for all concentrations.

Table 3. Cytotoxic effect of concentrations of TS extract on Hela, RD and Ref cell lines after 24 hr.

Concentration ($\mu\text{g/ml}$)	Cell Line			LSD value
	Hela	RD	Ref	
0	0.00 \pm 0.00	0.00 \pm 0.00	0.00 \pm 0.00	0.00 NS
62.5	88.16 \pm 5.62	89.75 \pm 6.31	12.50 \pm 0.54	11.49*
125	90.61 \pm 5.87	87.00 \pm 4.64	23.34 \pm 1.04	10.83*
250	91.63 \pm 4.71	89.25 \pm 6.35	25.00 \pm 1.46	11.69*
500	91.02 \pm 4.07	89.25 \pm 5.22	21.87 \pm 0.89	11.85*
1000	91.42 \pm 4.33	87.75 \pm 5.72	23.43 \pm 1.17	12.38
LSD value	12.07*	10.95*	7.63*	----

*($P \leq 0.05$).

Table 4. Cytotoxic effect of concentrations of TS extract on Hela ,RD and Ref cell lines after 48 hr.

Concentration ($\mu\text{g/ml}$)	Cell Line			LSD value
	Hela	RD	Ref	
0	0.00 \pm 0.00	0.00 \pm 0.00	0.00 \pm 0.00	0.00 NS
62.5	84.71 \pm 4.67	81.71 \pm 3.57	12.90 \pm 0.62	10.65*
125	84.71 \pm 3.92	80.40 \pm 4.76	1.61 \pm 0.004	13.26*
250	.8565 \pm 4.55	81.17 \pm 4.25	16.13 \pm 0.83	.1198*
500	85.41 \pm 4.61	82.47 \pm 3.97	6.45 \pm 0.27	.1154*
1000	86.12 \pm 4.09	79.87 \pm 3.25	9.68 \pm 0.79	10.67*
LSD value	11.09*	.1086*	7.95*	----

*($p \leq 0.05$).

Table 5. Cytotoxic effect of concentrations of TS extract on Hela ,RD and Ref cell lines after 72 hr.

Concentration ($\mu\text{g/ml}$)	Cell Line			LSD value
	Hela	RD	Ref	
0	0.00 \pm 0.00	0.00 \pm 0.00	0.00 \pm 0.00	0.00 NS
62.5	78.44 \pm 3.59	83.48 \pm 4.09	8.06 \pm 0.72	.1093*
125	77.63 \pm 3.41	84.62 \pm 4.33	5.65 \pm 0.35	10.08*
250	87.06 \pm 5.02	85.52 \pm 3.79	12.68 \pm 0.83	.1146*
500	86.79 \pm 4.66	85.75 \pm 4.21	12.90 \pm 0.77	10.95*
1000	80.86 \pm 3.76	83.48 \pm 4.97	11.11 \pm 0.62	10.83*
LSD value	12.69*	12.04*	6.71*	----

*($p \leq 0.05$).

These tables show that TS has an effect on Hela cell line than RD cell line, but the best result was on Hela at concentration of 250 $\mu\text{g/ml}$ after 24 hr., followed by its effect on RD cell line at concentration of 62.5 $\mu\text{g/ml}$ after 24 hr., 91.63 and 89.75, respectively. Table (3) reveals that the inhibitory effect on Hela from 125 to 1000 $\mu\text{g/ml}$ was more than that of RD, while the effect of TS at concentration of 62.5 $\mu\text{g/ml}$ on RD cell line was more than Hela.

Table (4) shows the highest inhibitory effect of the TS on Hela cell lines than RD after 48 hr. that ranging from 62.5 to 1000 $\mu\text{g/ml}$, whereas, Table (5) show the inhibitory effect of TS on Hela only at concentration of 250 $\mu\text{g/ml}$ and 500 $\mu\text{g/ml}$ than RD cell line.

The cytotoxic effect of TS on Ref cell line was lower than Hela and RD cancer cell lines from concentrations 62.5 to 1000 $\mu\text{g/ml}$. The inhibitory effect on cell lines revealed a significant result ($p \leq 0.05$) for all concentrations. The differences in activity of TS on Hela and RD cell lines are in

response to the presence or absence of specific cellular receptors in each type of cell lines.

After treatment with different concentrations of TS of *B. monniera* L. during 24, 48 and 72 hour, the optical densities (OD) of stained cell lines, detected differences of (OD) among concentrations, the high concentration gave the low value of OD, indicating a maximum response because the dead cells were removed by washing during staining procedure and the light colour represented for attached viable cells. The low concentration gave the high value of OD, which indicates minimum response in ratio to high percentage of viable cells (17).

These results indicate that *B. monniera* have many types of TS components like bacopaside I, bacosid A3, bacopaside II and others, that are active against cells. The effect of TS on cancer cell lines explained the highest sensitivity of cell lines by the activation of some glutathione-S- transferase enzymes (GSTs) via several compounds in plant extract, especially the triterpenoid compounds(18,19,20). Those authors also mentioned during their studies, that triterpenoid

saponins are able to inhibit cancer formation and progression by modulating multiple signaling targets related to cellular proliferation, apoptosis, autophagy, metastasis, angiogenesis, inflammation, oxidative stress, multidrug resistance, cancer stem cells, and microRNAs.

The triterpenes strongly induced apoptosis by altering the potential of mitochondria membrane and organizing the expression of proteins Bcl-2, modulating the activation of different caspases. Triterpenes seem to act on multiple signaling pathways and cell surface receptors, the molecular

functional analysis of triterpenes are complicated (21, 22).

Antioxidant activity

When the TS extract was tested for their ability to scavenge DPPH, the activities of TS were significantly higher than those of the control group (ascorbic acid) ($p \leq 0.05$).

The concentrations of TS were exert antioxidant activity which were determined by utilizing scavenging action against DPPH as free radical from 40 to 100 $\mu\text{g/ml}$, compared with ascorbic acid, as shown in Table (6).

Table 6. Antioxidant activity of TS of *B.mommi* plant compared with ascorbic acid

Concentrations ($\mu\text{g/ml}$)	% Antioxidant activity of TS	% Antioxidant activity of Ascorbic acid	LSD value
20	21.40 \pm 0.27 c	36.60 \pm 0.48 d	2.177 *
40	80.60 \pm 2.15 b	79.69 \pm 0.63 b	2.941 NS
60	83.60 \pm 3.49 b	72.53 \pm 0.88 c	2.552 *
80	98.20 \pm 4.07 a	86.38 \pm 0.96 a	2.961 *
100	99.37 \pm 0.17 a	88.60 \pm 1.19 a	3.084 *
LSD value	4.337 *	5.149 *	---

* ($p \leq 0.05$).

Means with the different letters in same column indicated significantly different.

The statistical results shown in Table (6) indicate that TS have higher antioxidant influence by 99.37% in 100 $\mu\text{g/ml}$ concentration followed by 98.20% in 80 $\mu\text{g/ml}$ concentration compared with the standard control ascorbic acid. The results showed that there were significant differences ($P \leq 0.05$) between the concentrations of TS and ascorbic acid, except at 40 $\mu\text{g/ml}$. This study gave evidence that TS have the ability to scavenge free radicals when compared with the reference standard, because TS compounds are donating their hydrogen atom to suppression the free radicals of DPPH, These results are mentioned by (18) and (23).

The (GSTs) is acts as an antioxidant factor, It causes detoxification from cells by increasing their combination with reduced glutathione leading to the cancer cell toward apoptosis program (20).

The results of this study agreed with many researchers which emphasize that the great number of biological activity of triterpenoids is found to have cytotoxicity against a variety of cancer cells (24,25). These results are mentioned to use the TS of *B.monni* L. plant to protect the human body against free radicals and cancer better than the use of crud extract, which may lead to lose some effective compounds during the extraction process.

Conflicts of Interest: None.

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الاستقصاء عن الصابونيات ثلاثية التربين وخصائصها الدوائية(السمية الخلوية، ومضاد للاكسدة) في نبات الـ *Bacopa monnieri* L المزروع في العراق

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الخلاصة:

نبات *Bacopa monnieri* L. (Scrophulariaceae)، الاسم الشائع *Herpestis monniera*، يحتوي على المركبات وخصوصا الصابونيات ثلاثية التربين ذات *bacosides* الفعالية البايولوجية المهمة مثل نقصان افراز الغدة الدرقية و مضاد للاختلاج وتحسين الذاكرة ومضاد للاجهاد. ولان لا توجد بحوث عن نبات *Bacopa monnieri* L. النامي في العراق ومركباته الفعالة وخصوصا الصابونيات ثلاثية التربين وتأثيراتها الطبية، لهذه كشفت هذه الدراسة عن وجود الصابونيات ثلاثية التربين الموجودة في نبات البربين البري النامي في العراق واختبار الفعالية السمية ضد الخلايا السرطانية والمضادة للاكسدة خارج الجسم الحي. تضمنت الدراسة استخلاص وتشخيص بعض المركبات الصابونيات ثلاثية التربين من جميع اجزاء النبات باستعمال ثلاث طرق استخلاص، واختبار الطريقة ذات الناتج الاعلى للتعرف على المركبات باستعمال تقنية الكروماتوغرافي السائل ذات الاداء العالي. خارج الجسم الحي اختبرت الفعالية السمية ضد نوعين من الخطوط السرطانية هي سرطان عنق الانسان *Hela* وسرطان الدماغ *RD* ودراسة تأثيرها على الخط الطبيعي. *REF* عند التراكيز 62.5، 250 و 500 $\mu\text{g/ml}$ عند الفترة الزمنية 24 و 48 ساعة. الجذر الحر (*DPPH*) لاختبار فعالية الصابونيات ثلاثية التربين كالمضادة للاكسدة عند التراكيز 20 و 40 و 60 و 80 و 100 $\mu\text{g/ml}$. اثبتت النتائج وجود العديد من مركبات *bacosides* عند التعرف عليها بجهاز الـ *HPLC* اثبتت الدراسة السمية الصابونيات ثلاثية التربين ان لها تأثيرا مثبتا على الخلايا السرطانية وهذا التأثير يعتمد على اختلاف تركيز المستخلص. ان تأثير الصابونيات ثلاثية التربين على الخط الخلوي *Hela* كان اكثر من تأثيره على الخط الخلوي *RD* وكان التأثير الاكثر على *Hela* عند التركيز 250 $\mu\text{g/ml}$ بعد 48 ساعة. ان التأثير السمي كان عند التأثير المعنوي ($P < 0.05$) اظهرت النتائج ان للصابونيات الثلاثية التربين تأثيرا مرتفع كمضاد للاكسدة 99.37% عند التركيز 100 $\mu\text{g/ml}$ تلاء 98.20% عند التركيز 80 $\mu\text{g/ml}$.

الكلمات المفتاحية: *Bacopa monnieri* L، السمية الخلوية، مضادات الاكسدة، الصابونيات ثلاثية التربين، *HPLC*.