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STUDY THE CYTOTOXIC EFFECTS OF AQUEOUS (AE) AND ETHANOLIC (EE) EXTRACTS OF *Artemisia herba alba* ON HUMAN LARYNGEAL CARCINOMA (Hep-2) CELL LINE AND MURINE MAMMARY ADENOCARCINOMA (AMN-3) CELL LINE *IN VITRO*

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ABSTRACT
The present study was carried out to evaluate the cytotoxic effects of aqueous (AE) and ethanolic (EE) extracts of *Artemisia herba alba* on human laryngeal carcinoma (Hep-2) cell line and murine mammary adenocarcinoma (AMN-3) cell line *in vitro*. The *in vitro* cell growth assay showed that there were time- and concentration-dependant cytotoxic effects of both extracts on the tested cell lines. The results revealed that high significant effect of all concentrations of both extracts were achieved after 72 hrs of exposure, while the exposure of cell lines for 24 hrs showed significant effect on both cell lines only with highest concentration. The values of cell viability (%) revealed time-dependant significant effects. There was increasing cytotoxic effect proportional to concentrations of both extracts. In conclusion, the results of this study revealed the high cytotoxic effect of *Artemisia herba alba* extracts on Hep-2 and AMN-3 cell lines *in vitro*.

KEYWORDS: cytotoxic effects, aqueous, ethanolic, *Artemisia herba alba*, Hep-2, AMN-3.

INTRODUCTION

The use of wild herbs in folk medicine is old as man himself. Our ancestors started to learn from nature by testing and using what was available. It is well known that old civilians have flourished in the Middle East and used the natural plants for various daily needs, such as food, shelter, clothes and medicine. Traditionally, such habits have been inherited by successive generations, and, thus, some of the plants became well known for their uses especially by herbalists. The use of plants in medicine was best known among Greeks, Arabs, the Chinese in the old world, and the red Indian in the new world (Al-Khazraji, 1991). However, the use of medicinal plants was highly practiced by Arabs during the middle ages and through them it was transferred to Europe (Sa'eed, 2004). Herbal medicines are culturally accepted and widely used in many countries for treatment of disorders and hence are of great importance as a mechanism to increase access to health care services. However, only few countries have some forms of policy/mechanism on traditional/complementary and alternative medicine (TC AM). Other countries need to develop their policy on TCAM to provide a sound basis in defining the role of TCAM in national health care delivery, ensuring that necessary, regulatory and legal mechanisms are created for promoting and maintaining good practices, that access is equitable, affordable and that authenticity, safety and efficacy of therapies are ensured (El-Gendy, 2004).Recently synthesized drugs started to replace natural ones due to many well-known reasons. But after the increase of drug industry and modern technology, man began to test plant products due to some harmful side effects or symptoms caused by some synthesized drugs (Oran and Al- Eisawi, 1998). However, as for cancer, the disease is complicated and heterogeneous, which makes it difficult to be well diagnosed, especially by traditional healers. The ethnomedical information obtained for a plant extract that is used to treat cancer might therefore not be reliable (Cragg *et al.*, 1994).

This study aims to Study the effect of aqueous and ethanolic extracts of *Artemisia herba alba* on the growth of several cancer cell lines *in vitro*.

MATERIALS & METHODS

Extraction of test plant (Plant collection)

Artemisia herba alba was collected from Al-Najaf province, south of Baghdad in December 2003, and was shed and dried at room temperature. A voucher specimen of the plant was deposited to be identified and authenticated at the National Herbarium of Iraq Botany Directorate in Abu-Ghraib (Certificate no. 3522 in 23/12/2003). The dried plant then was separated into: roots and aerial parts, then the aerial(leaves and barks) parts were ground into powder by coffee electrical grinder (mesh no.50), and the powdered parts were kept in a plastic bags in deep freeze (-20˚C) until the time of use (Harborne *et al*., 1975).

FIGURE 1: *Artemisia herba alba*

Preparation of aqueous and ethanolic extracts of *A. herba alba*

According to Harborne *et al*. (1975), aqueous extract of plant was prepared as follows:

1. aliquots of 50g of the powdered plant were suspended in 200 ml of distilled water (D.W.) in Erlyn Myer flask and stirred on a magnetic stirrer over night at 45˚C.
2. After 24 hours, the sediments were filtered by gauze and then by filter papers.
3. Steps (1) and (2) were repeated 4-5 times.
4. The pooled extract was evaporated to dryness (45˚C) under reduced pressure in a rotary evaporator.
5. The weight of crude extract resulted from that amount of powdered plant was measured.
6. The crude extract then was kept at -20˚C until the time of use.
7. The ethanolic extract of *A. herba alba* was prepared in the same manner as that of the aqueous extract except using of 70% ethyl alcohol instead of D.W. For following experiments, 1 g of powdered plant extract was dissolved into 100 ml PBS (as solvent), the suspension then filtered and sterilized by using 0.4 µm sterile millipore filter and kept in deep freeze (-20˚C) until use.

Cell Growth Inhibition Assay

Cell cultures in the microtitration plate were exposed to a range of plant extracts concentrations during the log phase of growth and the effect determined after recovery time.

The following protocol (Freshney, 1994) was performed on aqueous and ethanolic extracts of *A. herba alba*:

a) After trypsinization, cell suspensions seed in a microtitration plates at 50000 cells/ml for all cell lines. Growth medium used for seeding.
b) Plates were then incubated for 24 hrs.
c) Because of lack of knowledge about the effective limits of plant extract on types of cell lines, we first performed an exposure of Hep- 2 cell line to a wide range of dilutions through previously made tenfold serial dilutions of aqueous extract starting from 100000 µg/ml ending 0.01 µg/ml. The time of exposure was 72 hrs.
d) By using maintenance medium, two-fold serial dilutions were prepared starting from 5000 µg/ml, ending with 156.25 µg /ml.
e) After incubation for 24 hrs. Cells were exposed to different extract dilutions. Only 20 µl of each concentration were pipetted into each well. (Six replicates for each tested concentration). 20 µl of maintenance medium added to each well of control group (Twelve wells were used).

   The times of exposure were 24, 48 and 72 hrs. The plates were sealed with self adhesive film then returned to incubator.
f) After exposure to the extract of test, the medium was decanted off and cells in the wells were gently washed by adding and removing 0.1 ml sterile PBS two times. Finally 0.1 ml of maintenance medium was added to each well and incubated for further 24 hrs.
g) At the end of recovery time, 50 µl of 0.01% neutral red dye were added to the wells, the plates were incubated in a CO2-incubator for 2 hrs. at 37˚C.
h) After incubation, excess dye removed by washing the wells three times with PBS and then 50 µl of (PBS and absolute ethanol 1:1 V/V) were added to each well to extract the dye from the viable cells.

The optical density of each well was read by using a micro-ELISA reader at a transmitting wavelength on 492 nm (Mahony *et al*., 1989; Freshney,1994).

RESULTS

The results of *in vitro* studies revealed the cytotoxic, antiproliferative, and lowering activity of the incidence of genotoxicity, when aqueous and ethanolic extracts of *Artemisia herba alba* were tested. Moreover, the *in vivo* study showed inhibitory action of aqueous extract of the plant on tumor growth when compared with untreated groups.

Extraction

Extraction was accomplished on 50 gm for each type of the extraction, aqueous extract (AE) and ethanolic extract (EE).

The end products were obtained in the form of fine brown powder for both extracts. The percentage of AE and EE were 21%and 27.2% respectively.

Cell Growth Inhibition Assay

Cytotoxic assay

With exception of the control group (0 µg/ml), the highest values denotes for minimum response, in contrary the lowest value indicating maximum inhibition. Table(1) demonstrates the results of cytotoxic assay of ten-fold dilutions of AE after 72 hrs of exposure. The results were revealed that the concentrations over 100 µg/ml have significant effect (P<0.05-P<0.0001), therefore, the concentrations used later in cell growth inhibition assay were chosen around these effective values.

TABLE 1: Mean values of optical densities of Hep-2 cell line after treatment with aqueous extract of *A. herba alba* for 72 hrs. in cell growth assay

Conc. µg/ml	(Mean ± S.E.) of optical density	P-value
0	0.368±0.0023	
0.01	0.343±0.0092	N.S
0.1	0.342±0.0171	N.S
1	0.353±0.0035	N.S
10	0.348±0.0065	N.S
100	0.337±0.0069*	0.05
1000	0.233±0.0105*	0.001
10000	0.133±0.0071*	0.0001
100000	0.124±0.0667*	0.0001

* The mean difference is significant in comparison with Conc.=0 µg/ml

TABLE 2: Optical densities of Hep-2 and AMN-3 cell lines after treatment with aqueous extract of *A. herba Alba* for 24 hrs in cell growth assay

Concentration (µg/ml)	Hep-2 (Mean±SE)	P-value	AMN-3 (Mean±SE)	P-value
0	0.325±0.0014		0.274±0.0012	
156.25	0.321±0.0020	N.S	0.274±0.0022	N.S
312.5	0.315±0.0037	N.S	0.265±0.0105	N.S
625.0	0.271±0.0509*	0.001	0.263±0.0105	N.S
1250	0.277±0.0180*	0.001	0.253±0.0150	N.S
2500	0.269±0.0137*	0.001	0.261±0.0779	N.S
5000	0.277±0.0178*	0.001	0.200±0.0226*	0.0001

*The mean difference is significant in comparison with Conc. =0 µg/ml of the same cell line.

Table (2) showed the effect of different concentrations of AE (from 156.25 to 5000 µg/ml) on tumor cell lines after 24 hrs of exposure. The optical densities of different concentrations then compared with control group (0 µg/ml). The results revealed no significant cytotoxic effect for all concentrations except 5000 µg/ml on AMN-3 cell line, while Hep-2 cell line was significantly affected after treatment with concentrations (625, 1250,2500,5000 µg/ml).Table (3) revealed the cytotoxic effect of EE which was almost very similar to that of AE.

With increase the time of exposure to 48 hrs., all concentrations of AE and EE extracts were found to be significantly effective on AMN-3 cell line, while only 156.25 µg/ml treated group showed no significant cytotoxic effect on Hep-2 cell line at level (P<0.05) (Tables 4 and 5). The greatest cytotoxic effect was observed after 72 hrs exposure time. All concentrations of AE exhibited high significant effect (P<0.0001) on AMN-3 and Hep-2 cell lines except (156.25 µg/ml) which showed considerable significant effect (P<0.05) on both cell lines (Table 6).

Ethanolic extract showed high significant effect (P<0.0001) on AMN-3 cell line in all used concentrations after 72 hrs of exposure. While their cytotoxic effect become not significant at concentration (156.25 µg/ml) and moderately significant (P<0.001), when Hep-2 exposed to the concentration of 312.5 µg/ml (Table 7).To compare the values of optical densities which resulted from exposure of both cell lines to AE and EE, these values should be converted to cell viabilities as percentages in proportion with control group. This aimed to evaluate the effect of time on the cytotoxicity of these extracts and to compare the response of the used cell lines after exposure to the same extract as well as comparison between cytotoxic effects of two extracts was carried out.The results revealed time-dependant significant differences. There was increasing cytotoxic effect at concentrations of 625, 1250, 2500 and 5000 µg/ml of AE on both cell lines at high significant levels (P<0.0001). The concentration of 312.5 µg/ml was also highly effective (P<0.0001) on AMN-3 but less effective (P<0.01) on Hep-2 cell line. On other hand, the concentration of 156.25 µg/ml revealed no significant effect on Hep-2 cell line and low significant effect on AMN-3 (P<0.05). All results were documented in table (8), which also revealed other comparison that was carried out to determine the role of cell line type in cytotoxic assay.

TABLE 3: Optical densities of Hep-2 and AMN-3 cell lines after treatment with ethanolic extract of *A.herba alba* for 24 hrs. in cell growth assay

Concentration (µg/ml)	Hep-2 (Mean ± SE)	P-value	AMN-3 (Mean ± SE)	P-value
0	0.325±0.00128		0.274±0.0012	
156.25	0.326±0.00068	N.S	0.265±0.0056	N.S
312.5	0.310±0.00743	N.S	0.255±0.0100	N.S
625.0	0.282±0.01819*	0.001	0.261±0.0102	N.S
1250	0.269±0.01699*	0.001	0.251±0.00862	N.S
2500	0.280±0.01538*	0.001	0.258±0.00661	N.S
5000	0.270±0.01804*	0.0001	0.247±0.01257*	0.001

*The mean difference is significant in comparison with Conc.=0 µg/ml of the same cell line.

TABLE 4: Optical densities of Hep-2 and AMN-3 cell lines after treatment with aqueous extract of *A. herba alba* for 48 hrs. in cell growth assay

Concentration (µg/ml)	Hep-2 (Mean±SE)	P-value	AMN-3 (Mean±SE)	P-value
0	0.342±0.0012		0.261±0.0057	
156.25	0.318±0.0269	N.S	0.234±0.0104*	0.01
312.5	0.309±0.0302*	0.05	0.203±0.0055*	0.001
625.0	0.299±0.0108*	0.01	0.175±0.0160*	0.0001
1250	0.170±0.0097*	0.0001	0.163±0.0205*	0.0001
2500	0.166±0.0031*	0.0001	0.170±0.0286*	0.0001
5000	0.166±0.0035*	0.0001	0.116±0.0205*	0.0001

*The mean difference is significant in comparison with Conc.=0 µg/ml of the same cell line

TABLE 5: Optical densities of Hep-2 and AMN-3 cell lines after treatment with ethanolic extract of *A.herba alba* for 48 hrs. in cell growth assay

Concentration (µg/ml)	Hep-2 (Mean ± SE)	P-value	AMN-3 (Mean ± SE)	P-value
0	0.342±0.0012		0.261±0.0057	
156.25	0.321±0.0143	N.S	0.152±0.0032*	0.0001
312.5	0.284±0.0152*	0.01	0.148±0.0064*	0.0001
625.0	0.219±0.0225*	0.0001	0.152±0.0041*	0.0001
1250	0.216±0.0038*	0.0001	0.149±0.0038*	0.0001
2500	0.226±0.0161*	0.0001	0.149±0.0029*	0.0001
5000	0.203±0.0170*	0.0001	0.094±0.0088*	0.0001

*The mean difference is significant in comparison with Conc.=0 µg/ml of the same cell line

TABLE 6: Optical densities of Hep-2 and AMN-3 cell lines after treatment with aqueous extract of *A.herba alba* for 72 hrs. in cell growth assay

Concentration (µg/ml)	Hep-2 (Mean±SE)	P-value	AMN-3 (Mean±SE)	P-value
0	0.333±0.0023		0.253±0.0055	
156.25	0.309±0.0029*	0.05	0.227±0.0023*	0.05
312.5	0.234±0.0136*	0.0001	0.196±0.0006*	0.0001
625.0	0.185±0.0108*	0.0001	0.186±0.0502*	0.0001
1250	0.168±0.0020*	0.0001	0.147±0.0038*	0.0001
2500	0.132±0.0034*	0.0001	0.134±0.0005*	0.0001
5000	0.128±0.0012*	0.0001	0.116±0.0089*	0.0001

*The mean difference is significant in comparison with Conc.=0 µg/ml of the same cell line.

The AE caused no significant difference in its cytotoxic effect on both cell lines after 24 hrs. Of exposure, while Hep-2 cell line showed high significant difference from AMN-3 cell line after treatment with high concentrations of AE (1250, 2500 and 5000 µg/ml) for 48 hrs. In contrast, the moderate concentrations (312.5 and 625 µg/ml) showed significant effect on AMN-3 cell line. The lowest concentration (156.25µg/ml) didn't give significant difference in the cytotoxic effect on both cell lines. At 72 hrs of exposure time to AE, Hep-2 cell line was showed

significant cytotoxic effect (P<0.05) from the corresponding groups of AMN-3 cell line after exposure to all concentrations except the highest and lowest ones which were showed no significant difference between both types of cell lines. Table (9) exhibited the results of exposure of study cell lines to EE of *A. herba alba*. The significant time-dependant effect of EE on Hep-2 appeared even at low concentrations. After exposure to concentrations of (156.25 and 312.5 µg/ml), the low significant effect (P<0.05) appeared at 48 hrs and 72 hrs. With increase the concentration, the effects became high statistically significant in a time-dependant manner. These results were similar to those obtained after exposure of AMN-3 to EE, which showed high significant time-dependant cytotoxic effect (P<0.0001). The effects of all concentrations of EE on AMN-3 cell line were significantly different from Hep-2 cell line after exposure for 48 and 72 hrs, while the exposure for 24 hrs showed no significant difference between both cell lines except the concentration of (156.25 µg/ml) treated AMN-3 cell line which revealed low significant difference (P<0.05) from corresponding Hep-2 group. The results indicated that the cell growth was reduced in a concentration- and time-dependant manner, in addition to influence of cell line type in response to plant extracts, particularly after exposure for 48 and 72 hrs.

TABLE 7: Optical densities of Hep-2 and AMN-3 cell lines after treatment with ethanolic extract of *A.herba alba* for 72 hrs. in cell growth assay

Concentration (µg/ml)	Hep-2 (Mean ± SE)	P-value	AMN-3 (Mean ± SE)	P-value
0	0.333±0.0023		0.253±0.0055	
156.25	0.316±0.0024	N.S	0.135±0.0227*	0.0001
312.5	0.284±0.0162*	0.001	0.140±0.0261*	0.0001
625.0	0.203±0.0140*	0.0001	0.129±0.0116*	0.0001
1250	0.167±0.0014*	0.0001	0.111±0.0373*	0.0001
2500	0.146±0.0043*	0.0001	0.090±0.0431*	0.0001
5000	0.122±0.0066*	0.0001	0.065±0.0070*	0.0001

*The mean difference is significant in comparison with Conc.=0 µg/ml of the same cell line

TABLE 8: Cell viability (%) of Hep-2 and AMN-3 cell lines after treatment with aqueous extract of *Artemisia herba alba* for 24, 48 and 72 hrs in cell line growth assay.

Concentration	Hep-2 (Mean ± SE)			AMN-3 (Mean ±S E)		
(µg/ml)	24 hrs.	48 hrs.	72 hrs.	24 hrs.	48 hrs.	72 hrs.
0	100.0±0.00	100.0±0.0	100.0±0.00	100.0±0.00	100.0±0.00	100.0±0.00
156.25	98.7±0.63	92.9±3.62	92.7±4.03	100.0±0.00 [a]	89.6±0.34	90.0±3.98
312.50	96.9±1.16	90.3±3.62	70.2±4.03 [a b]	96.8±3.81 [a]	77.7±3.95 [b]	77.8±2.16
625.00	83.3±6.36	87.4±3.34	55.5±3.19 [a b]	96.2±3.75 [a]	67.3±5.94 [b]	73.6±2.47
1250.0	85.2±5.57 [a]	49.7±2.84 [b]	50.4±0.63 [b]	92.5±5.42 [a]	62.4±3.76	58.3±3.27
2500.0	82.7±4.23 [a]	48.5±0.91 [b]	39.6±1.03 [b]	95.2±2.92 [a]	65.5±1.02	53.0±4.55
5000.0	85.2±5.50 [a]	48.5±1.05 [b]	38.4±1.47	73.2±8.22 [a]	44.5±3.93	46.0±3.39

[a] Significantly different in comparison with corresponding groups of the same tested cell line at levels (P<0.05-P<0.0001).
[b] Significantly different in comparison with corresponding group of other cell line at levels (P<0.05- P<0.0001).

TABLE 9: Cell viability (%) of Hep-2 and AMN-3 cell lines after treatment with ethanolic extract of *Artemisia herba alba* for 24, 48 and 72 hrs in cell line growth assay

Concentration	Hep-2(Mean ± SE)			AMN-3(Mean ± SE)		
(µg/ml)	24 hrs.	48 hrs.	72 hrs.	24 hrs.	48 hrs.	72 hrs.
0	100.0±0.00	100.0±0.00	100.0±0.00	100.0±0.00	100.0±0.00	100.0±0.00
156.25	100.3±0.21 [a]	93.8±0.774	94.8±4.088	96.7±2.024 [a b]	58.2±0.341 [b]	53.3±0.494 [b]
312.50	95.3±2.276 [a]	83.0±3.620	85.2±4.895	93.0±3.662 [a]	56.7±4.292 [b]	55.3±1.095 [b]
625.00	86.7±5.606 [a]	64.0±4.217	60.0±6.700	95.2±3.815 [a]	58.2±1.909 [b]	50.9±0.715 [b]
1250.0	82.7±5.202 [a]	63.1±0.421	50.1±1.064	91.6±3.082 [a]	57.4±6.009 [b]	43.8±0.577 [b]
2500.0	86.1±4.169 [a]	66.0±1.282	43.8±4.770	94.1±2.462 [a]	57.0±6.834 [b]	35.5±0.462 [b]
5000.0	83.0±5.540 [a]	59.3±1.983	36.6±5.283	90.1±4.589 [a]	36.3±1.115 [b]	25.6±1.358 [b]

[a] Significantly different in comparison with corresponding groups of the same tested cell line at levels (P<0.05-P<0.0001).
[b] Significantly different in comparison with corresponding group of other cell line at levels (P<0.05- P<0.0001).

DISCUSSION

Extraction

The amount of crude product resulted from the extraction process was consistent to that resulted in the study of Al-Khazraji (1991). Plant extracts containing several different constituents which vary considerably in relative concentration (O'Hara *et al*., 1998; Vickers and Zollman, 1999). No study during literature survey was regarding the variation of constituents in *Artemisia herba alba* could be seen.The relative proportion between the amount of plant used for extraction and crude product was variable depending on several factors, such as the method of extraction and solvent used in extraction process as well as the type of study plant (Henning *et al*., 2003).

Cell growth assay (*In vitro* study)

The results showed that growth inhibition was significantly progressed as the concentrations of extract increased as well as the time of exposure. While the effect of extract type was variable according to type of cell line and the time of exposure. The obvious result obtained in the growth inhibitory assay was the dose- and time- dependant cytotoxic effect of the two extracts on both study cell lines. The present results were consistent with that reported in the study of Campbell *et al.* (2002) in which dose-response curves were obtained for several of the most potent crude extracts. Flow cytometric analysis of breast cancer cells suggest that the herb arrests cells in the G2/M phase of the cell cycle. They are also agree with the results of Al-Atby (2001), Al-Hilli (2004), Al-Qadoori (2004), Sa'eed (2004) and Al-Yaqube (2004) They were mentioned that the plant extracts of their studies have cytotoxic effects on different cell lines such as Hep-2, AMN-3 and RD in a dose- and time-dependant manner. The results were also consistent with the results of the in vitro study which carried out by Jo *et al.* (2004). They were mentioned that the cell proliferation of MCF-7 human breast cancer cell line was reduced in a dose- and time-dependant manner after exposure to licorice root. They also found that the ethyl acetate fraction of black cohosh induced cell cycle arrest at G1 when tested at 30 µg/ml and at G2/M at 60 µg/ml in MCF-7 cells. This suggests that the extract contains a mixture of components with the more active (or more abundant) causing G1 arrest and the less active causing G2/M arrest. In addition, the *in vitro* study done by Tai *et al*. (2004) demonstrated a concentration-dependant antiproliferative effect of Sutherlandia on several tumor cell lines, and they also showed that Essiac and Flor-Essence herbal teas had demonstrated antiproliferative and differentiation inducing properties *in vitro* only at high concentrations. Many studies were using purified constituents isolated from *Artemisia* that showed antiproliferative, cytotoxic activities on various cancer cell lines. Terpenoids and flavonoids derived from *Artemisia annua* have cytotoxic activities on several human tumor cell lines (Zheng, 1994). Two of the components, artemisinin and artesunate have studied in Asia as anticancer treatment (Bensky and Gamble, 1993). Rinner *et al.* (2004) explained that the artesunate has an endoperoxide bridge that reacts with iron in heme to form singlet oxygen and free radicals. It has been shown to have an antiproliferative effect on medullary thyroid carcinoma. Beta-sitosterol isolated from *Artemisia argyi* and *Artemisia absinthium* was considerably acting as antitumor (Zhong and Cui,1992), while a benzoquinone isolated from the extract of *Artemisia stolonifera* showed significant cytotoxicity against five human tumor cell lines *in vitro* (Kwon *et al*., 2001).The triterpene glycoside fraction inhibited growth of MCF-7 human breast cancer cell line, and induced cell cycle arrest at G1 (Einbond *et al*.,2004).The *in vitro* study performed by Shaikenov *et al.* (2001) monitored that the susquiterpene gamma-lactones isolated from *Artemisia glabella* registered as antitumor substance in the Republic of Kazakstan. They propose that compound is suitable for the treatment of ras-related malignancies.

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