

RESEARCH PAPER

Inhibitory and anti-Cancer Effects of *Crataegus azarolus* Extracts on Gastric Cancer Cell Line (AGS)

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ABSTRACT:

Gastric cancer (GC) is one of the most frequent types of cancer around the world and most of the drugs which are used currently as anti-cancer chemotherapy hold adverse side effects on normal cells and tissues, that calls for a strong need to find new agents to prevent and treat GC. Extracts from certain plants contain compounds with health potentials with minimal side effects; one such plant is the *Crataegus azarolus* commonly called hawthorn. Extracts of hawthorn have been found to exert negative effects on the viability of many types of cancer via a number of pathways both *in vivo* and *in vitro*. Herein, we aimed to investigate the possible anti-proliferative effects of methanolic and acetone extracts of *C. azarolus* leaves on human adenocarcinoma gastric cell line (AGS). Standard phytochemical analysis were used to qualitatively characterize leaf extracts contents. AGS cells were treated for 48 h with different concentrations of both extracts (10, 50, 100, 250, and 500 µg/ml). Our results demonstrated that the methanolic crude extract showed significantly higher anti-proliferative effects than those of acetone, at both 250 and 500 µg/ml concentrations respectively, when estimated by MTT assay at ($p < 0.001$) with an IC_{50} values 293.7 µg/ml. On the other hand, acetone extract treatment showed that 500 µg/ml had a high significant effect at ($p < 0.001$) with an IC_{50} values 576.6 µg/ml. In conclusion, the results of the present study indicated that leaf extracts of *C. azarolus* limited the growth of AGS.

KEY WORDS: Gastric cancer; anti-cancer; *Crataegus azarolus*; AGS; MTT assay.

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1. INTRODUCTION :

Gastric cancer (GC) is a major health concern all over the world. GC is counted as the fifth most prevalence type of cancers and the third cause of mortality worldwide. Geographically GC occurrence is heterogeneous because it is influenced by genetic and environmental factors (Eom et al., 2022, Xia and Aadam, 2022). Every year, more than one million people are diagnosed with GC worldwide, where approximately seven hundred thousand of them die (Wong et al., 2021). In Iraq, studies have shown that the second gastrointestinal malignancy type after colorectal carcinoma is GC (Al-Bahrani and Al-Bahrani, 2013, Shahid et al., 2017)

In Erbil and Duhok governorates, between the years 2013 and 2019 an increase by over two folds was recorded in the total incidence of patients with cancer, where the spread of registered GC among males were 455 cases and 328 in females (M-Amen et al., 2022).

Since most of the current anti-cancer drugs which are used nowadays in chemotherapy have adverse side effects on normal cells and tissues, the emergence of adjuvant therapies is needed (Fouzat et al., 2022). Recently, plant extract compounds have shown considerable promise as substances with potential advantageous health properties due to the minimal expected negative side effects. This has led to an increase in the search for identification and quantification of these compounds by conducting more research in this field (Rastogi et al., 2016). One of such plants is the *Crataegus azarolus* commonly called

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hawthorn (Żurek et al., 2021). Extracts of the genus *Crataegus* have been found to suppress proliferation of several types of cancer cell lines through a number of mechanisms. The most targeted mechanisms is programmed cell death (PCD) type one (apoptosis), PCD type two, autophagic programmed cell death, and necrosis, the third type of PCD mechanism (Shimizu et al., 2014). Apoptosis implicates only target cells without influencing ambient cells via generating inflammations. Thus, triggering the apoptotic pathways have become mechanisms of interest for revealing and improving the potential medicinal effectiveness of novel drugs, mainly as cancer inhibitor therapies (Indran et al., 2011).

Conventional applications of hawthorn have been confirmed and extended to include stomach protection (Niu et al., 2020), anti-inflammatory, anti-oxidant (Liu et al., 2019b), anti-ageing, anti-depressant, anti-bacterial, anti-fungal, anti-viral, and anti-atherosclerotic effects (Żurek et al., 2021). Given these facts, several groups sought to understand the mechanism by which *Crataegus sp.* extracts play a role in regards to inducing cytotoxic activities toward cancer cells and those studies revealed that they triggers apoptotic pathways and that remarkable cleavage of poly (ADP-ribose) polymerase (PARP), DNA fragmentation, induction of cell cycle arrest (anti-proliferative) were noticed (Mustapha et al., 2016).

Thus, *Crataegus sp.* have high pro-treatment potentials, which could be applied in the prevention or treatment of many diseases, including cancers (Guo et al., 2018). These health-promoting properties of hawthorn are mainly attributed to the high content of antioxidant and bioactive components, including polyphenolic compounds which are the most important ones and naturally present in varieties of plants (Żurek et al., 2021, Rajasekaran et al., 2021). The most essential polyphenolic components identified in hawthorn are tannins (Rajasekaran et al., 2021), flavonoids (Sharma et al., 2021), phenolic acids, anthocyanidins, and proanthocyanidins (Edwards et al., 2012, Liu et al., 2019a, Żurek et al., 2021). Alkaloids also have been shown to possess cytotoxic effects including cell proliferation toward cancer cells (Ortiz et al., 2014).

Although many research works have been done in order to elucidate the anti-cancer effects of

hawthorn components and their mechanisms of action, little is known about its effect on stomach. Hence, this current study was aimed at evaluating the effects of different concentrations of *C. azarolus* leaves extracts on gastric cancer cell line viability.

2. MATERIALS and METHODS

2.1 Plant Collection and Powder Preparation

Fresh leaves of *C. azarolus* were collected from Erbil/Shaqalawa, in the Kurdistan region/Iraq, in autumn season in August 2021, the collected plant was identified and authenticated by Mrs. Bnar Khalid Shkar, Lecturer at Biology department, Salahaddin University, Kurdistan/Iraq. After harvesting, leaves were well washed, cut into small pieces, and left to dry in dark place at room temperature, for one week. Then, the dried leaves were crushed and ground to obtain a homogeneous fine powder by electrical grinder. The powder was kept in shaded glass in deep freezer -20°C until time of use.

2.2 Preparation of Plant Crude Plant Extracts

Leaves powder (20 g) were suspended in 600 ml of the selected solvent, methanol. The powder was macerated and stirred for 48 h at room temperature. After that, the suspension was collected and filtered using Whatmann no.1 filter paper. The filtrate was then condensed by using a rotary evaporator at 45°C under reduced pressure. Then 0.5 gram of the obtained sample of the concentrated extract was dissolved into 10 ml of 1% phosphate buffer saline (PBS) as solvent. Then the suspension was sterilized by filtration using Millipore filter paper (0.45 µ.) and (0.22 µ.) and kept in deep freeze -20 C.

While in case of using acetone as a solvent, leaves powder was suspended in 300 mL of acetone (50%; v/v) and kept in a shaded flask at room temperature for 24 h. After that, suspension was filtered by gauze. Later, the filtered solution was centrifuged at 12,000g for 10 min. Then the supernatant was collected, and the remained pellet was re-suspended with acetone (70%; v/v) by using ultrasound bath (TELSONIC TPC-25, Switzerland) at 30°C for 30 min. The supernatant was again centrifuged at 12,000g for 10 min, and was combined with the previous suspension

together and evaporated by a rotary evaporator at 45°C. Finally, 0.5 gram of the extract was dissolved into 10 ml of 1% PBS as solvent. Then the suspension was filtered and sterilized by using Millipore filter paper (0.45 µ.) and (0.22 µ.). The sterile suspensions were kept in deep freeze -20°C as a stock solution until use.

2.3 Phytochemical Screening

Phytochemical screenings are considered as identification methods for a plant constituents. Once the extraction was done, qualitative phytochemical screenings were done to determine the class of compounds within the extracts of *C. azarolus*. This screening is a group of chemical methods used for the detection of chemical compounds produced by plants, where different classes of compounds respond differently to color tests based on their solubilities and reaction with the different types of reagents for each tests and this color changes were detected by naked eye (Harborne, 1984). Phytochemical screening for *C. azarolus* leaves was performed in order to detect chemical compositions such as alkaloids, polyphenol (tannins), flavonoids, triterpenoids, peptides and free amino group, carbohydrate, glycosides, biuret, and saponins. Table (1) shows all the chemical compounds that have been screened, the required reagent with its composition and preparation and the expected results expressed by the color produced as a result of reaction between the compound and the reagent, the intensity of colors referred to the concentration of the compound within the extracts in *C. azarolus*, all the phytochemical tests were done in the presence of a control.

2.4 Cell Lines and Culture Conditions

Human gastric adenocarcinoma cell line, (AGS), was purchased from Pasture Institute, Iran. Cells were cultured in F-12 medium supplemented with 10% fetal bovine serum (FBS), and antibiotics (100 U/mL penicillin, 100 U/mL streptomycins; Gibco 15140122, Gibco, Waltham, MA, USA). Cells were grown at 37°C in a humidified incubator with 5% CO₂. The culture medium was renewed every 2–3 days. For the sub-culturing and passaging, cells were washed with 1% PBS and trypsinized (0.25% trypsin EDTA; Gibco 25200056, Gibco, Waltham, MA, USA).

2.5 Cell Viability Assay

The effect of different concentrations of *C. azarolus* leaves extracts on AGS cells proliferation (anti-proliferative effects) was performed by MTT assay. The principle of the assay depends on the reduction of a tetrazolium salt by interacting with NADPH-dependent enzyme which is produced only by living cells, to produce purple insoluble formazan. The absorbance of the solubilized formazan is taken as a measure of the number of living cells (Mustapha et al., 2015).

Growth inhibition was detected using MTT assay (Sigma Aldrich, USA). Human gastric adenocarcinoma cells, (AGS), were seeded at 10*10³ per well in a 96 well plate, incubated for 24 h. Then cells were treated with different range of concentrations of *C. azarolus* extracts (10, 50, 100, 250, and 500 µg/ml). After 48 h medium was discarded, then 100 µl of MTT reagent was added to each well, and cells were incubated for another 2-4 h. Then medium was removed and 100 µl DMSO was added to the wells. Cell viability was measured at 540 nm by microplate ELIZA reader (DA3200, Iran).

2.6 Statistical Analysis

Data are expressed as the arithmetic means ± SD of three independent experiments. The statistical significance of results obtained from in vitro studies was evaluated by the GraphPad Prism software, one way ANOVA, with probability values P<0.05, <0.01, and <0.001, being considered as significant.

Table 1. Phytochemical Screening for *C. azarolus*

No.	Chemical composition	Reagent	Reagent preparation	Expected result	Reference
1	Alkaloids	Wagner	1.30 gm of iodine and 2.0 gm of potassium iodide in 30 ml DW.	Brown red precipitate	(Harborne, 1984, Tyagi, 2017)
2	Polyphenol (Tannins)	Lead Acetate Solution	1 gm lead acetate into 100 ml DW.	White jelly precipitate	(Harborne, 1984)
3	Flavonoids	Sulphuric Acid	Sulphuric acid (H ₂ SO ₄)	Yellow color	(Velavan, 2015)
4	Triterpenoids	Liebermann-Burchard	4 ml of acetic anhydride with 1 ml concentrated H ₂ SO ₄	Blue-green color appear	(Harborne, 1984)
5	Peptides and free amino group	Ninhydrine 1%	0.5 g ninhydrine into 50 ml DW.	Blue or violet color	(Harborne, 1984, Velavan, 2015)
6	Carbohydrate	Molish	1% alpha-naphthol and ethanol	Violet ring	(Velavan, 2015)
7	Glycosides	Benedict	Benedict (CuSO ₄ and NaOH)	Color change or orange precipitate appear	(Al-Shahaat, 1986)
8	Protein	Biuret Reagent	1.5 gm (CuSO ₄ .5H ₂ O) and 6 gm of Sodium potassium tartarate in 500 ml of DW. 375 ml of 2 molar Sodium hydroxide. Mix both solutions in final volume 1000 ml DW.	Violet color	(Tyagi, 2017)
9	Saponins	-	Agitation	Formation foam above the liquid surface	(Lakache et al., 2016)

3. RESULTS

3.1 Phytochemical Screening of *C. azarolus* Leaf Extracts

Phytochemical screening tests of *C. azarolus* leaves crude extracts elucidated the existence of some significant bioactive components, which are shown in Table 2, this phytochemical study was qualitatively expressed as positive (+) or negative (-) to denote to the presence, intensity and absence of each phytochemical compound, where +, low in concentration; ++, moderate in concentration; +++, high in concentration; and -, absent. The methanolic crude extract showed high concentrations of alkaloids, polyphenol (tannin), flavonoids, carbohydrates, and glycosides; low concentrations of saponins; and absence of triterpenoids, peptides and free amino group, and proteins. While the acetone crude extract on the other hand, showed high concentrations of polyphenol (tannin) and flavonoids; moderate concentrations of carbohydrates and glycosides; low concentrations of alkaloids and saponins; and absence of triterpenoids, peptides and free amino group, and proteins.

3.2 Anti-proliferative Effect of Methanolic and Acetone Extracts of *C. azarolus* Leaves on Human Adenocarcinoma Gastric Cell Line (AGS).

3.2.1 Anti-proliferative Effect of Methanolic Extract of *C. azarolus* Leaves on AGS.

Gastric cancer cell line (AGS) was treated with different concentrations of methanolic extract of *C. azarolus* leaves (10, 50, 100, 250, and 500 µg/ml) respectively. Our results in figure 1 show that methanolic extracts have significant inhibitory (cytotoxic) effects on cell proliferation in a dose-dependent manner after 48 h, where IC₅₀ value (the dose required to inhibit cell growth by 50%) was 293.7 µg/ml. Actually, after exposure to increasing concentration of *C. azarolus* leaves methanolic extracts (100 µg/ml) there was significant ($p < 0.05$) decrease in AGS cell proliferation. Then with the increasing doses (250 and 500 µg/ml) respectively, we see a much higher fold-change and the number of AGS viable cells significantly ($p < 0.001$) decreased relative to untreated control cells. Whereas, lower

concentrations (10 and 50 µg/ml) had no significant effects on AGS cells viability.

3.2.2 Anti-proliferative Effect of Acetone Extract of *C. azarolus* Leaves on AGS.

Gastric cancer cells (AGS) were treated with different concentrations of acetone extract of *C. azarolus* leaves (10, 50, 100, 250, and 500 µg/ml) for 48 h. Our results in figure 2 show that with the acetone leaves extract, only the (500 µg/ml) have a significant ($p < 0.001$) effects in reducing AGS cell viability relative to untreated control and even the reduction is not drastic as with methanolic extract. IC₅₀ value for acetone extract on AGS cell proliferation after 48 hours of incubation was 576.6 µg/ml.

4. DISCUSSION

Gastric cancer still comprises one of the major contributor to cancer prevalence and death globally (Ajani et al., 2022, Yeoh and Tan, 2022). Plants have been consumed as substitutional or complementary source in pharmaceutical applications since long time for their potential and safe alternative controlling of diseases like cancer (El-Saber Batiha et al., 2020), and this has been increased mainly when most of the anti-tumor chemotherapies exert counter effects against normal cells and tissues (Prakash et al., 2013, Fouzat et al., 2022).

Crataegus sp. is counted as one of the most known medicinal plants with a high content of biologically active components, the most important of which are polyphenols (Žurek et al., 2021). These compounds have gained value in recent years due to their high cytotoxic potential against many cancer cells and anti-oxidant activity both *in vivo* and *in vitro* (Mustapha et al., 2015).

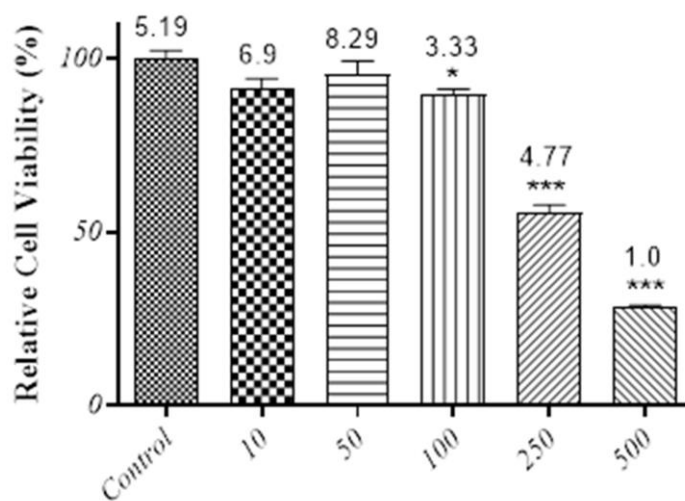
Our results showed that both methanolic and acetone extracts of *C. azarolus* leaves contain most of the valuable ingredients which might have cytotoxic effects on AGS cells. Here, our phytochemical analysis revealed that methanolic extract showed stronger component contents quantitatively.

In this study, our results revealed that the methanol leaves extract significantly ($p < 0.001$) inhibited cell proliferation in a dose-dependent manner; especially at concentrations 250 and 500 µg/ml respectively relative to untreated control. Same

Table 2. Phytochemical Screening of *C. azarolus* Leaves Extracts.

No.	Chemical Components	Methanol Extract	Acetone Extract
1	Alkaloids	+++	+
2	Polyphenol (Tannin)	+++	+++
3	Flavonoid	+++	+++
4	Triterpenoid	-	-
5	Peptides and Free Amino Group	-	-
6	Carbohydrates	+++	++
7	Glycosides	+++	++
8	Proteins	-	-
9	Saponins	+	+

Key: +, low in concentration; ++, moderate in concentration; +++, high in concentration; -, absent.



Methanol Extract of *Crataegus azarolus* Concentrations (µg/ml)

Figure 1. The viability of AGS cells after 48 h of treatment with different concentrations of *C. azarolus* methanolic leaves extract relative to untreated control; measured by MTT assay. The obtained values are presented as mean \pm SD for at least three independent experiments each with three replications (*= $p < 0.05$, **= $p < 0.001$ and ***= $p < 0.001$).

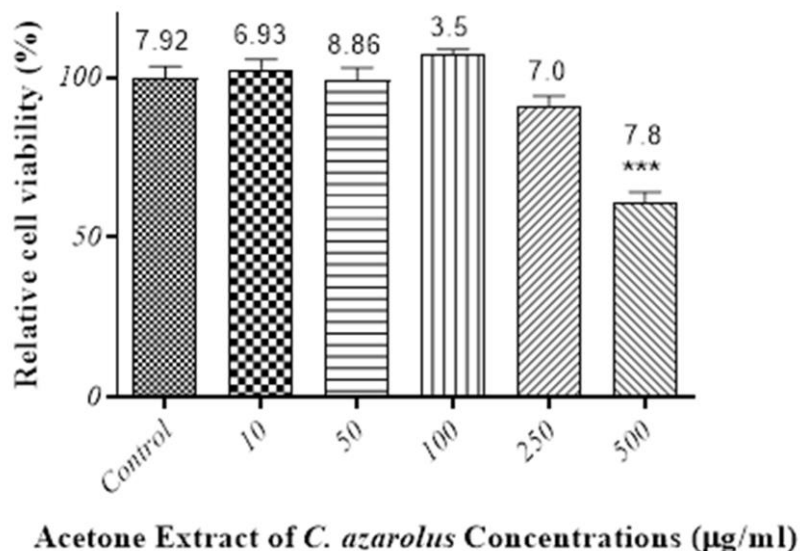


Figure 2. The viability of AGS cells after 48 h of treatment with different concentrations of *C. azarolus* acetone leaves extract relative to untreated control; measured by MTT assay. The obtained values are presented as mean \pm SD for at least three independent experiments each with three replications (***= $p < 0.001$).

effects were observed on other cancer cell lines by Mustafa *et al.*, 2016; (Ma *et al.*, 2020), they reported that different extracts and concentrations of *Crataegus sp.* reduced proliferation of colorectal cancer cell lines, (HT-29) and (HCT-116). Whereas (Zhao *et al.*, 2019) recorded the effects of different components isolated from *C. pinnatifida* on human hepatoma cancer cell lines, (HepG2) and (Hep3B), also (Kallassy *et al.*, 2017) found that *C. azarolus* methanolic extract had time and dose dependent inhibitory effects on Jurkat cancer cell line. Our results also agreed with the potential anti-tumor effects of berries, leaves and flowers extracts of *Crataegus sp.* on human glioblastoma cells, U87MG, which was recorded by Zurek *et al.*, (2021). Furthermore, (Kombiyil and Sivasithamparam, 2022), uncovered that methanolic extracts of *C. oxyacantha* berries had anti-tumor effects on both breast cancer cell line, (MCF-7) and (MDA-MB-231).

We also demonstrated that the methanolic extract had more potent and high significant cytotoxic activities on AGS cell line compared to the acetone one, and this was in agreement with previous observations, where Kallassy *et al.*, (2017) found that methanolic extract was more efficient than aqueous one in exerting suppressive ability towards Jurkat cells. This methanol effect was true for other

medicinal plants as well (AL-Asady *et al.*, 2012, Kaur *et al.*, 2018, Nguyen *et al.*, 2020). The differences in anti-proliferative efficiency might be attributed to the higher solubility capacity of methanol in extracting the bioactive ingredients. Furthermore, the variation in bioactive components extractions and yields from methanol or acetone could be referred to the solvent polarities differences, indicating that highly polar solvents are more effective in extracting materials (Hashim *et al.*, 2016, Toudert *et al.*, 2017, Felhi *et al.*, 2017, Truong *et al.*, 2019).

5. CONCLUSION

In the current investigation, we concluded that the crude methanolic and acetone extracts of *C. azarolus* leaves could significantly exhibit attractive anti-proliferative capabilities against AGS cell line. Importantly, the obtained results elucidated that the methanolic extract contained the highest amount of bioactive components and showed great growth inhibitory activities when compared to the acetone extract. It can also be concluded that *C. azarolus* leaves extract can be utilized as a good source of a natural product and it might offer a novel promising anticancer drug in therapeutic applications for cancer patients and

general health problems, either as an alternative to chemotherapies or along with it. Moreover, these results suggest that in future further studies are required to seek for the effect of each components of the plant's leaves on AGS. Also additional experiments are needed to be done to seek and understand the mechanisms by which *C. azarolus* exerts this growth reducing characteristic and also the necessity for identifications of the active components.

Conflicts of Interests

The authors declare that they have no conflict of interests.

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