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Original Article

Artemetin, A Dietary Flavonoid Inhibits the Proliferation and Induces Apoptosis in Human Gastric Carcinoma (AGS) Cells

Yuxing Lv[1, 2], Yinquan Wang[1,2,*], Gaopeng Hao[1, 2]

[1]Department of General Surgery, Shanxi Bethune Hospital, Shanxi Academy of Medical Sciences, Tongji Shanxi Hospital, Third Hospital of Shanxi Medical University, Taiyuan, CHINA.
[2]Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, CHINA.

ABSTRACT

Background: One of the most common types of recurrent carcinoma cases worldwide is gastric cancer. Even advances in treatment methods and early discoveries have not been able to significantly lower the death and morbidity rates associated with gastric cancer. On account of their multiple health benefits, apparent lack of toxicity and side effects, and the limitations of chemotherapeutic drugs, natural products have attracted a lot of interest in recent years with the aim of preventing cancer. **Aim:** A flavonoid class phytochemical known as Artemetin, has been discovered to be present in numerous medicinal plants. Anti-bacterial, antioxidant, and anti-inflammatory, hepatoprotective and cardioprotective actions are only a few of its many pharmacological effects. **Materials and Methods:** In the current investigation, the potential of Artemetin as an anticancer agent for gastric cancer was evaluated in AGS cell lines. The influence of Artemetin on the cell viability, apoptotic induction, mitochondrial ATPase activity, ROS generation, and cell cycle progression has been examined. Cisplatin was selected as the positive control for the experiments. **Results:** The impact of Artemetin on the cell viability revealed that its cytotoxic potential increased in a dose-dependent pattern and IC_{50} concentrations were chosen for further experiments. The results showed that apoptosis was induced by Artemetin in AGS cells by triggering ROS pathway and DNA damage. The cell viability of AGS cells was reduced with increased concentration of Artemetin. **Conclusion:** Artemtin's cytotoxic potential in cells is mediated by inducing apoptosis, which is supported by significant levels of ROS and mitochondrial ATPase as well as the findings of the AO/EB staining. All these results suggest that Artemetin can be used as a potent anticancer drug for human gastric carcinoma.

Keywords: Gastric cancer, Artemetin, AGS cells, Reactive oxygen species, Apoptosis.

Correspondence:
Yinquan Wang
[1]Department of General Surgery, Shanxi Bethune Hospital, Shanxi Academy of Medical Sciences, Tongji Shanxi Hospital, Third Hospital of Shanxi Medical University, Taiyuan-030032, CHINA.
[2]Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan-430030, CHINA.
Email: 13834046996@163.com

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INTRODUCTION

Gastric cancers have a significant impact on world health, with Gastric Adenocarcinoma (GAC) being the most prevalent histological form. The primary emphasis of this primer is Gastric Adenocarcinoma (GAC), which accounts for 95% of all stomach-related cancers by histological type.[1] It does rank as the second most widespread cause of cancer-related mortality globally. It is the seventh most frequent cancer in women and the fourth most frequent cancer in men.[2] Age, sex, and race/ethnicity are only a few examples of the many non-modifiable risk factors for gastric cancer. Smoking, consuming a diet high in nitrates and nitrites, and *Helicobacter pylori* infection are among other risk factors that may be addressed. Several additional extremely rare risk factors exist, including pernicious anaemia, prior stomach surgery, and mucosa-associated lymphoid tissue lymphoma.[3]

Intestinal and diffuse gastric cancers are the two main histologic subtypes according to the Lauren classification. The WHO method rates adenocarcinomas according to how closely they resemble metaplastic intestinal tissue. It divides the histologic patterns into five sub-types: intestinal and diffuse adenocarcinoma; mucinous; tubular; papillary; and signet-ring cell.[4] Gastric cancer continues to be a major global problem despite recent improvements in clinical therapy, medicines, the staging procedure, and imaging technology. In terms of treatment synthetic drugs are available and chemotherapy is required. Chemotherapy and synthetic medications often cause negative effects. Chemical drugs or substances have been used to simulate the growth of tumour cells during the induction of apoptosis. Studies show that drugs derived from herbs or other naturally occurring substances can be employed as anti-tumor medications.[5]

Flavonoids are a class of organic compounds with varied phenolic structures that are present in a variety of foods, including

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fruits, vegetables, cereals, flowers, tea, and wine. The health benefits of these natural compounds are widely recognized, and attempts are being undertaken to separate the so-called flavonoids from the other constituents.[6] In a wide range of nutraceutical, pharmacological, therapeutic, and cosmetic uses flavonoids are increasingly seen as an essential component.[7] This is explained by their ability to influence the activity of essential cellular enzymes as well as their anti-inflammatory, anti-oxidative, anti-mutagenic, and anti-carcinogenic capabilities.[6] Artemetin, found in many different medicinal plants such as *Achillea millefolium L., Artemisia absinthium, Artemisia gorgonum, Cordia verbenacea, Vitex trifolia,* and *Vitex negundo.*[8,9] The structural properties of Artemetin, a rare polymethoxylated lipophilic flavone with just a free hydroxyl group participating in a hydrogen connection with the ketone moiety, might result in significantly different biological activity. Artemetin, has excellent oral bioavailability and drug-likeness values,[10] and it possesses numerous bioactivities, such as anti-inflammatory.[9] Various studies suggest that Artemetin exhibit the following activity including anticancer, antioxidant, anti-inflammatory, antihypertensive, and antiparasitic.[11] It has been recently that Artemetin, Hypotensive mechanism of the extracts and artemetin isolated from *Achillea millefolium* L. (Asteraceae) in rats.[12] The current study's objective was to determine the effect of Artemetin on reactive oxygen species, cell viability, apoptosis, the amount of antioxidant indicators, cell cycle, ATPase activity in AGS cells.

MATERIALS AND METHODS

Materials

Artemetin, AGS (gastric adenocarcinoma) cell line, DMEM, FBS, chemicals, and other materials were purchased from Standard vendors.

Cell culture

Dulbecco's Modified Eagle's Medium (DMEM) were used for maintaining AGS adenocarcinoma cells. The mediums were incubated at room temperature in an environment of humidified air while being supplemented with 10% heat-inactivated Foetal Bovine Serum (FBS) and 1% antibiotics (10 µg/mL streptomycin and 100 IU/mL penicillin).

Cell viability by WST-1 assay

All the cells were plated in 96-well plates at a final density of $1*10^6$ cells per well for the cell viability experiment, and they were then incubated for 24 hr. All of the cells were then exposed to Artemetin at a range of doses (5, 10, 20, 40, 60, 80 µg/mL) and incubated at room temperature for an additional 24 hr. Fresh medium was used after the treatment period of 24 hr, and 10 µL of WST-1® solution was added to each well. This was followed by further 3 hr of incubation at room temperature. Cisplatin (10µM) was selected as the positive control for the experiments. The percentages of inhibition were estimated after scanning the absorbance using an ELISA microplate reader at 460 nm to evaluate the viability of the cells.

Determination of Cell apoptosis by AO/EtBr dual staining

The technique of AO/EtBr labelling was used to identify apoptotic cells. AGS cells were seeded in wells at a density of $1*10^6$ cells and given 24 hr to proliferate. Artemetin was applied to AGS cells for 24 hr at IC_{50} concentrations. The cells were incubated for 24 hr before being washed with PBS and fixed for 20 min at 4°C in a 3:1 solution of methanol and glacial acetic acid. The cells were washed with PBS before being stained with a 1:1 AO/EtBr dye for 30 min at room temperature. Before stained cells were studied under a fluorescence microscope, they were cleaned in PBS.

Intracellular Oxygen Species (ROS)

In a 6 well plate, the AGS cells were seeded at a density of $1*10^6$ cells/well and left to incubate for 24 hr. Following incubation, cells were treated with various doses of Artemetin (IC_{50} and Cisplatin) whereas control cells received only the medium and were not treated with Artemetin. Cells were then maintained at room temperature in 5% CO_2 and 95% air. Cells from each well were then obtained, cleaned twice with PBS, and reinstated in 500 µL of 2′,7′dichlorodihydrofluorescein diacetate (H2DCFDA) stain for images under fluorescence microscopy.

Assessment of Mitochondrial ATPase

It was feasible to determine the ATPase activity of the solubilized mitochondrial component of Artemetin by comparing the NADH oxidation and the production of ADP through the previously stated lactate dehydrogenase process. The reaction mixture contained 100 mM Tris, 2 mM MgCl2, 4.0 mM MgATP, 50 mM KCl, 0.23 mM NADH, 0.2 mM EDTA, 1 mM phosphoenol pyruvate, 1.4 units of lactate dehydrogenase, 1.4 units of pyruvate kinase, and approximately 0.8-1.2 mg for the purified preparation. To gauge the F-type ATPase activity either oligomycin, an inhibitor of F0-targeting, or efrapeptin, an efficient and specific inhibitor of F1-targeting, were used.

Estimation of oxidative stress markers

The commercial SOD assay kit was utilized to assess SOD activity after Artemetin was added to the cells in accordance with the instructors. Utilizing a plate reader, the samples' absorbance at 450 nm was calculated. The CAT test kit was used as directed to measure the CAT enzyme activity. The kit measures the enzyme in accordance with the way hydrogen peroxide breaks down. After Artemetin was given to the cells as directed by the instructor, SOD activity was determined using a commercial SOD assay kit. Using a plate reader, the samples' absorbance at 450 nm was calculated. Additionally, a kit was employed to measure the GSH and Gpx activity levels. By continually reducing

5,5′- dithiobis(2-nitrobenzoic acid) in the presence of catalytic amounts (nmoles) of GSH, a kinetic assay is utilized to quantify the quantity of GSH.

Cell cycle analysis by PI staining

Using propidium iodide staining, the cell cycle was studied. Cells were trypsinized, then washed in PBS and stored in 90% ethanol after being treated with Artemetin at IC_{50} concentration and common medication positive control Cisplatin. After two PBS rinses, fixed cells were stained for an hour with 5 µg/mL DNase-free RNase and 50 µM propidium iodide. After that, the outcomes were evaluated with software and flow cytometry.

Statistical Analysis

The average and standard deviation of three distinct samples were used to represent the results. The statistical analysis was conducted using a one-way analysis of variance. The Duncan's Multiple Range Test was used as the measurement to evaluate the variation between the variable groups. p <0.05 was considered to be noteworthy.

RESULTS

Anticancer activity of Artemetin by cytotoxicity assay

The cytotoxic potential of Artemetin toward AGS cells was evaluated via WST-1 assay, and the findings are presented in Figure 1. Our results demonstrated that the Artemetin treatment considerably reduced the growth of AGS cancer cells dose dependently. At the highest concentration of Artemetin, the viability of cells was reduced to 20%. This finding demonstrates the remarkable cytotoxicity of Artemetin toward AGS cells. The IC_{50} value for the cells was determined to be 16.98 µg/mL in 24 hr; therefore, this concentration of Artemetin was applied for further experiments. Cisplatin (10 µM) was selected as the positive control for the experiments.

Assessment of apoptosis by AO/EtBr staining

ROS accumulation within the cells is the primary cause of the cell membrane changes linked to apoptosis. In AGS cells subjected to Artemetin, dual AO/EtBr labeling was used to examine the membrane distortion connected to apoptosis (Figure 2). To correctly identify the apoptosis-related alterations that take place in cell membranes throughout the apoptotic process, dual labeling has been employed. The nuclear DNA of cells that maintain their whole cell membranes can be stained with the AO dye, but the EB dye can only stain cells that have lost their entire cell membrane. AO positive, yellow and greenish colored cells appeared following the Artemetin treatment IC_{50} value indicating early apoptosis even though EtBr positive cells were also found in the test groups and displayed late apoptosis or dead cells after being treated with Artemetin. Cisplatin was selected as the positive control for the experiments.

Detection of ROS by DCFH–DA staining

The ROS can destroy cells in a number of ways and affect both apoptosis and cell development. We investigated the ROS-inducing capacity of Artemetin using DCFH-DA labeling in order to determine the cause of the cytotoxicity noticed in AGS cells. As Artemetin concentration increased, more green fluorescence was seen, indicating that the treated cells developed more ROS (Figure 3). As a result, it was discovered that Artemetin generated ROS in AGS cells, indicating that Artemetin caused oxidative stress-mediated cell death in these cells.

Mitochondrial ATPase assay

Figure 4 displays the mitochondrial ATPase activity of AGS cancer cells treated with artemetin and cisplatin. When cells were subjected to Artemetin 16.98 µg/mL and cisplatin (10µM) treatment, their activity increased compared to control cells, suggesting that these compounds may have the ability to promote tumors.

Estimation of SOD, CAT, GSH, and Gpx levels

In the AGS cancer cells, the influence of Artemetin administration on SOD, CAT, Gpx and GSH levels was assessed, and the findings are illustrated in Figure 5. The Artemetin and cisplatin positive control treated cells demonstrated a significant decrease in the activity of all antioxidants. These results indicated that administering Artemetin to AGS cells significantly reduced the antioxidant levels. Through the production of ROS in human gastric tumor cells, oxidative stress has been shown to contribute to Artemetin-induced cytotoxicity.

Determination of Cell cycle arrest by PI staining

The impact of Artemetin on cell cycle analysis using PI labeling is shown in Figure 6. In order to assess the effects of the Artemetin, we employed flow cytometry to investigate at the DNA content of untreated and treated AGS cells. Comparing DNA content between the treated and untreated cells revealed that the cells exposed to Artemetin were in a sub-G1 cell cycle. Our results showed that the selected IC_{50} concentration effectively inhibited the cell line during a 24 hr treatment period. Flow cytometry was used to determine the stages of cell cycle arrest, and cisplatin-treated cells accumulated more sub-G1 cells than untreated cells could. The Artemetin treatment resulted in a significant proportion of cells dying in the G2/M stage. Thus, cell cycle arrest into the G2/M phase occurs, which is comparable to the effect of cisplatin (10µM) on cells.

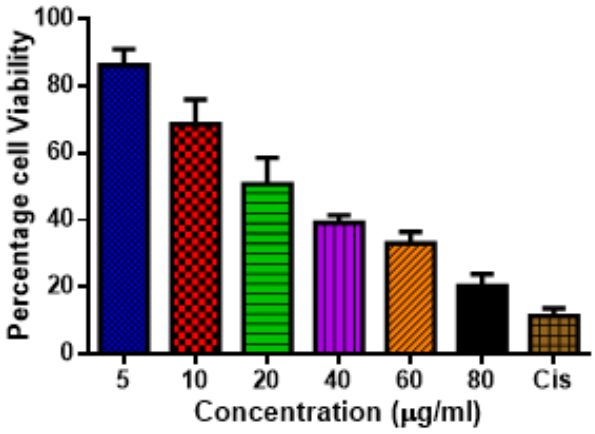

Figure 1: Cell cytotoxicity assay using WST-1 assay and Artemetin at different concentrations in AGS cells in 96-well plates with Cisplatin as the positive control. Experiments were performed in triplicate to determine the IC_{50} value and representative data are shown here for the dose resulting in 50% inhibition of growth.

DISCUSSION

One of the most prevalent tumors, gastric cancer has the second-highest cancer-related death rate worldwide.[13] It still has a poor prognosis and low survival rates in spite of numerous efficient therapeutic options available, including surgical excision, chemotherapy, radiation, targeted therapy, and an amalgamation of these procedures.[14] Therefore, it is highly suggested that new and extremely effective therapy approaches be developed for gastric cancer.[15] Flavonoids are often used as antioxidants because of their ability to scavenge oxygen– free radicals.[16] Furthermore studies also flavonoid Isoorientin can reduce the expression of Bcl–2 and increase the expression of Bax by blocking the PI3K/Akt pathway, thus inducing the apoptosis of HepG2 cells.[17] Additionally, A Mechanism of flavonoid Isoorientin-Induced Apoptosis and Migration Inhibition in Gastric Cancer cells.[18] Among the most potent flavonoids is Artemetin, which has been found to combat malignancies of the cell lines such as K562, Lu1, HepG2, KB, MCF-7, and LNCaP.[19] Therefore, using AGS cells as an *in vitro* model, the current work aims to give a

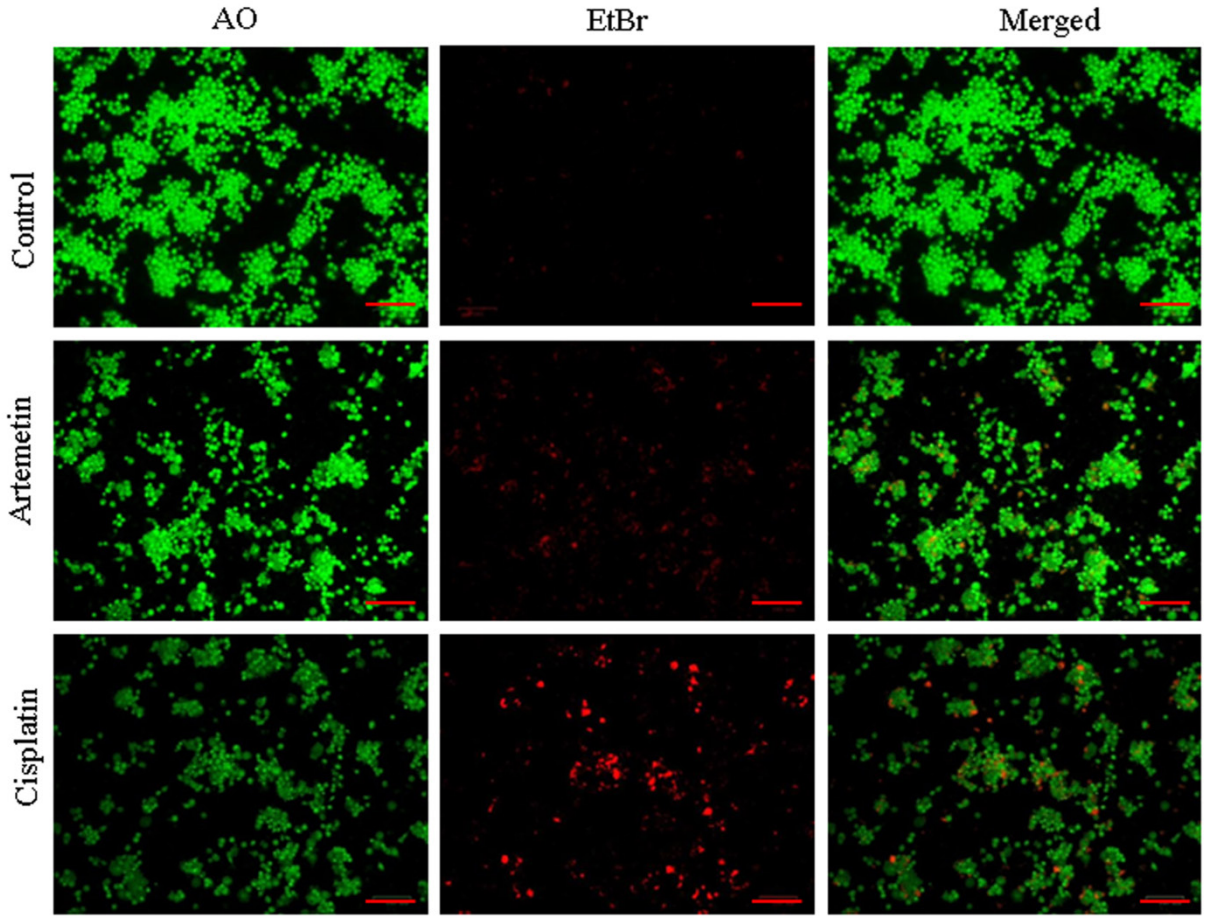

Figure 2: The cells were treated within control and Artemetin 16.98 µg/mL value for 24 hr, stained with dual dye AO/EtBr and then analyzed by fluorescence microscopy. No significant apoptosis was determined in the control group. Green fluorescence indicates healthy cells; orange fluorescence indicates apoptotic cells; red colour stained cells show early phase of apoptosis. Values are expressed as mean ± SD of three experiments.

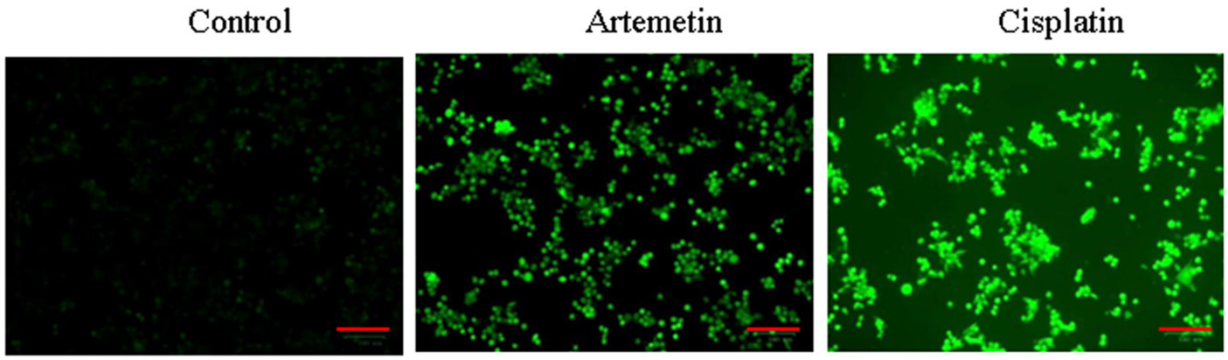

Figure 3: A fluorescence microscope image of intracellular ROS generation induced by Artemetin treated cells stained with DCF-DA. Control cells, treated 16.98µg/mL of Artemetin and cisplatin (10 µM) positive control. This is a representative image of the experiment performed in triplicate with magnification at 20X.

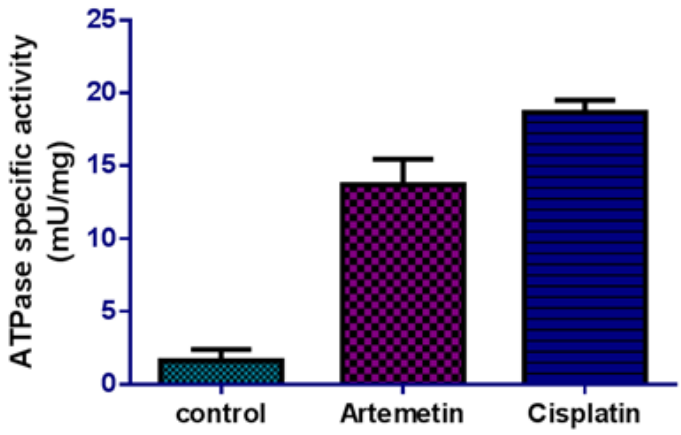

Figure 4: Effect of Artemetin on the mitochondrial ATPase activity at concentrations 16.98µg/mL in AGS cell lines. Cisplatin was employed as the positive control. Results were computed using the mean ± standard deviation of three distinct investigations.

deeper understanding of cell death and the molecular mechanism through which Artemetin mitigates apoptosis.

Cell viability has been extensively assessed using WST-1 (Water Soluble Tetrazolium-1), a tetrazolium salt. MTT is frequently replaced with WST-1 in cell viability experiments. The reduction of WST-1 results in the production of a water-soluble formazan product (4-[1-(4-Iodophenyl)-5-(4-nitrophenyl)formaz-3-yl]-1,3-benzene disulfonate, disodium salt), whose optical density at 450 nm is evaluated to assess cell viability.[20] It was discovered that Artemetin declined the viability of AGS cells in a concentration-based trend. A similar dose-based growth reduction has been reported when Artemetin was tested against HepG2 cancer cells and MCF-7 breast cancer cells.[19]

Apoptosis has been found to be necessary for a variety of activities, including the development of the immune system, normal cell homeostasis, and drug-induced cell death.[21] Research demonstrates that some chemo-preventive treatments prevent cancer by regulating apoptosis, raising the prospect of innovative drugs that may be beneficial in treating cancer.[22] Our understanding of the Artemetin-induced cell death pathway was improved by using the AO/EB staining approach to examine morphological changes in connection to apoptosis. Live cells fluoresced green as a consequence of acridine orange permeating their cell membranes, while apoptotic cells fluoresced orange as a consequence of nuclear shrinkage, indicating that the apoptotic process had been initiated.[23] The mitochondrial ATP synthase, also known as ATPase, which is found in the inner membranes of mitochondrial cells, is responsible for producing the bulk of the ATP required to power cells. Because it is essential for the growth of malignancies, ATP synthase is thought to be a target for cancer treatment.[24] The mitochondrial ATPase activity of Artemetin-treated cells was found to be higher than those of normal cells, suggesting greater tumor progression of cancer cells.

In the mitochondrial-mediated cascade and apoptosis, ROS serve as significant participants.[25] DCFH-DA, a probe that is deacetylated by the enzyme cell esterase and converted into the fluorescent compound 20,70-dichlorofluorescein (DCF) by ROS, was employed to monitor ROS production in greater depth.[26,27] Elevated green fluorescence in the cells treated with the flavonoid Artemetin indicated an augmented intracellular production of ROS. The results are in concordance with an earlier study where Artemetin enhanced the ROS production in liver and breast cancer cells.[27]

Oxidative stress in cancer cells is triggered on by an elevation in ROS, which limits the generation of antioxidants. SOD prevents lipid peroxidation by facilitating the catalytic conversion of superoxide anions to oxygen and hydrogen peroxide. Glutathione (GSH) is one of the most widely distributed thiol antioxidants in cells. It protects cells against internal and external toxins, such as reactive oxygen and nitrogen species.[28] Our findings indicated that the activities of antioxidant enzymes SOD, CAT, Gpx, and GSH were decreased upon Artemetin treatment. Collectively, the current work investigated the cytotoxic potential of Artemetin in AGS cells and discovered that it significantly increased the ROS levels, disrupted cell membrane integrity, reduced antioxidant levels and induced apoptosis, leading to cell death.

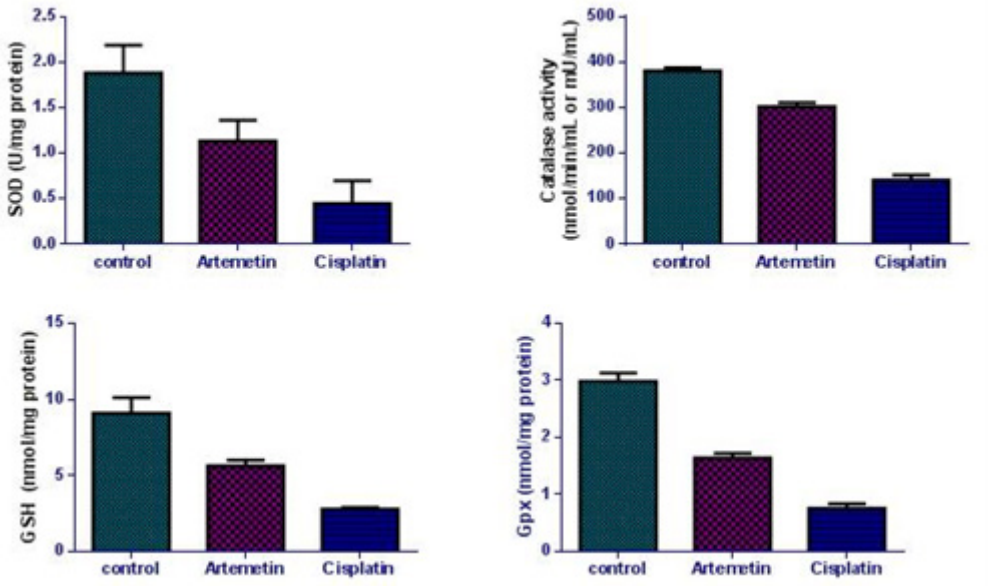

Figure 5: Effect of Artemetin on oxidative stress level in AGS cells. ELISA method was to analyze the oxidative stress parameters (SOD, CAT, GSH, Gpx). Results are statistically significance at $p < 0.05$.

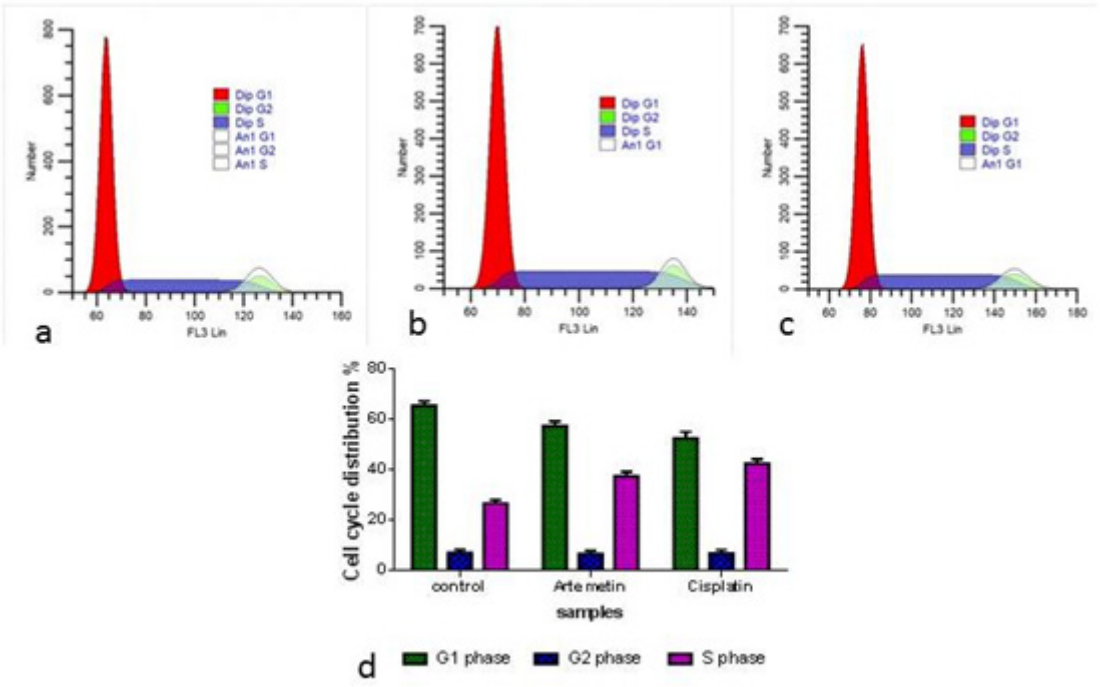

Figure 6: Cell cycle analysis using flow cytometry after staining with Propidium Iodide (PI). AGS cells were treated with Artemetin for 24 hr and standard drug cispaltin with the concentration of 10 µM/mL compared to the control. (a,b,c) Cell cycle pattern and apoptosis distribution; Percentage of cell cycle distribution (d).

Numerous anticancer drugs function through the essential mechanism of cell cycle arrest. It is thought that slowing down the cell cycle of cancer cells may be an effective way for preventing the formation of tumors. The majority of common cancers, including gastric carcinoma, have altered cell cycle regulators, based on their molecular characteristics.[29] Cell cycle studies conducted in the current study showed that the Artemetin suppression of cell viability was brought on by cell cycle arrest in the G2/M phase, which was also accompanied by a reduction in the total number of cells in the G1 phase.

CONCLUSION

This study aimed to investigate the flavonoid Artemetin's anti-tumor potential in relation to AGS gastric cancer cells. It has been examined whether Artemetin affects cell viability, apoptosis induction, ROS production, levels of oxidative stress markers, and cell cycle progression. According to our study findings, Artemtin's cytotoxic potential in cells is mediated by inducing apoptosis, which is supported by significant levels of ROS and mitochondrial ATPase as well as the findings of the AO/EB staining. Apoptosis occurs when the cell membranes are damaged by an increase in ROS levels. Overall, our results are consistent with Artemetin's significant anticancer characteristics, and more research into this concept might advance the development of a robust anticancer drug for the treatment of gastric cancer.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest

ABBREVIATIONS

GAC: Gastric Adenocarcinoma; **DMEM:** Dulbecco's modified Eagle's medium; **FBS:** Foetal Bovine Serum; **AO/EtBr:** Acridine Orange and Ethidium Bromide; **ATP:** Adenosine Triphosphate; **DAPI:** 4',6-diamidino-2-phenylindole; **DCFH-DA:** Dichloro-dihydro-fluorescein diacetate; **SOD:** Superoxide dismutase; **CAT:** Catalase: **PI:** Propidium Iodide.

SUMMARY

Gastric cancer continues to be a major global problem despite recent improvements in clinical therapy, medicines, the staging procedure, and imaging technology. Flavonoids are a class of organic compounds with varied phenolic structures that are present in a variety of foods, including fruits, vegetables, cereals, flowers, tea, and wine. The health benefits of these natural compounds are widely recognized, and attempts are being undertaken to separate the so-called flavonoids from the other constituents. Artemetin, found in many different medicinal plants such as *Achillea millefolium* L., *Artemisia absinthium, Artemisia gorgonum, Cordia verbenacea, Vitex trifolia,* and *Vitex negundo.* Various studies suggest that Artemetin exhibit the following activity including anti-cancer, antioxidant, anti-inflammatory, anti-hypertensive, and anti-parasitic. The current study's objective was to determine the effect of Artemetin on reactive oxygen species, cell viability, apoptosis, the amount of antioxidant indicators, cell cycle, ATPase activity in AGS cells. It has been examined whether Artemetin affects cell viability, apoptosis induction, ROS production, levels of oxidative stress markers, and cell cycle progression. Overall, our results are consistent with Artemetin's significant anticancer characteristics, and more research into this concept might advance the development of a robust anticancer drug for the treatment of gastric cancer.

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