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2-(4-Chlorobenzylideneamino)-5-Thioxoisothiazolidin-3-One Modified Pomegranate Juice for Anticancer Activity Applications

T. A. Zidan [a]* and N. M. Elbakary [b]

[a] Department of polymers and pigments, National Research Centre, 33 El Buhooth street (former El Tahrir St.), 12622, Dokki, Giza, Egypt.
[b] Radiation Biology Department, National Center for Radiation Research and Technology, Egyptian Atomic Energy Authority, Cairo, Egypt.*

In Loving Memory of Late Professor Doctor ""Mohamed Refaat Hussein Mahran""

Abstract
Pomegranate juice has therapeutic effect observed through in vitro and in vivo studies. It can be used as antiviral, antibacterial, antidiabetic and anticarcinogenic material. This work includes in vitro studies for modified pomegranate as anticancer for liver and breast cancer. N-aminorhodanine is reacted with p-chlorobenzaldehyde to form 2-(4-chlorobenzylideneamino)-5-thioxoisothiazolidin-3-one (CBAT). CBAT was characterized with FTIR and ^{1}HNMR. Pomegranate juice is then modified with CBAT. CBAT is reacted with phenolic –OH groups of pomegranate juice to make modification. The modified pomegranate was characterized using FTIR and ^{1}HNMR spectroscopy. It was examined against both human hepatoma HepG2 cells and breast cancer cells MCF7 through in vitro studies. It was found that modified pomegranate has good synergistic inhibitory and pro apoptotic effects on human hepatoma HepG2 cells and breast cancer cell MCF7. Modified pomegranate has stronger effect on HepG2 cells than on MCF7 cells.
Keywords: Pomegranate; N-Aminorhodanine; Hepatoma HepG2 cells; Breast cancer cells MCF7; FTIR; ^{1}HNMR.

1. Introduction

Cancer is the second leading reason of death in our culture, trailing only heart disease. A certain dosage of anticancer drug can kill the cells of cancer but it can affect negatively on other ordinary tissues. As a result, scientists are inventing and studying new chemicals for the treatment of various types of malignancies that are free of the adverse effects caused by existing antineoplastic medications in order to find new anticancer drugs 1.

Despite significant advancements in cancer therapy over the last decade, support is growing for an alternative approach that uses naturally occurring and dietary substances to prevent and manage cancer 2. Natural products have shown to be the most effective at altering the function of cancer-related proteins. For thousands of years, plants have been used as a source of medicine. Even today, according to the World Health Organization, about 80% of people depend on traditional medicines like herbs as a primary source of medication3. Phenolic chemicals have gotten a lot of interest lately since they have a lot of antioxidant potential, are plentiful in fruits and vegetables, and have been proven to have anticancer properties. Pomegranate fruit is used in traditional remedy, commonly known as Ayurvedic medicine, to treat diseases such as diarrhea, diabetes, ulcers, parasitic infections, and bleeding 4,5. As seen in Fig. 1, pomegranate juice has a variety of chemical structures that are split into two groups; (A) is anthocyanins which are phenolics attached to sugar moieties and (B) is phenolic acids 6. These bioactive compounds give pomegranate juice its therapeutic effects. As pomegranate fruit contains many antioxidants that protect human body from many diseases and its benefit in nutrition and medicinal uses, great interest was directed towards this fruit. Pomegranate juice beverages is an intriguing cause of antioxidants and phytochemicals preventing macromolecules oxidation including, lipids, proteins, and DNA [7].

Cancer is the leading cause of death in the global. Surgery, chemotherapy, and radiation therapy, as well as a combination of these options, are currently available for cancer treatment [8]. Many heterocyclic compounds have been identified as possible anticancer agents in recent decades [9]. It has made a significant contribution to society in the form of a huge number of medications for the treatment of various disorders, and it has a prominent position in medicinal chemistry due to their diverse biological activities [10].

*Corresponding author e-mail: tayseerzidan@yahoo.com; Tel: +2 01007392152
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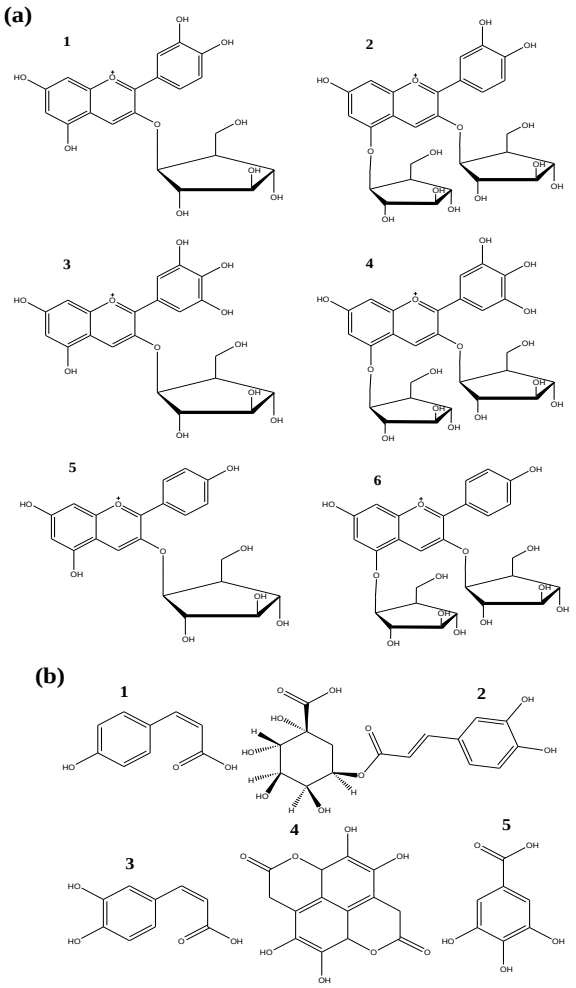


Fig. 1 Chemical structures present in pomegranate juice

In current bioorganic and medicinal chemistry, the thiazolidinone core is one of the most efficient and significant heterocyclic building motifs for developing novel highly active compounds [11, 12]. Among functionally substituted compounds, this is a significant class with a well-established broad pharmacological profile. 4-thiazolidinones are a smaller chemical category of 2-imino derivatives with a wide range of biological actions, including anti-cancer properties [13,14], anti-inflammatory, free-radical scavenging action [15,16], antifungal, antimicrobial etc [17].

Unfortunately, as we all know, anticancer drugs are effective in killing cancer cells but are often destructive to normal tissue and cause a slew of adverse effects, limiting their treatment efficacy [9]. As a result, we're looking for new anticancer treatments by creating and testing new chemicals for the treatment of various types of malignancies that don't have the adverse effects that traditional antineoplastic drugs have.

As a result, the conjugation of 2-imino-4-thiazolidinone template with pomegranate juice might be considered as a promising technique for the development of drug-like compounds, based on the notion of molecular hybridization. So the goal of this study was to synthesize pomegranate modified with 2-(4-chlorobenzylideneamino)-5-thioxoisothiazolidin-3-one and investigate the synergistic effect by screening its anti-cancer efficiency in vitro. FTIR and 1HNMR are used to prove the chemical structures of the prepared compounds. The in vitro studies of the modified pomegranate proves the anticancer activity towards both liver and breast cancer cells.

2. Experimental

2.1 Materials

N-Aminorhodanine, p-chlorobenzaldehde, potassium carbonate, absolute ethanol and dimethyl formamide (DMF) were purchased from Sigma Aldrich, Egypt.

2.2 Instruments

Fourier transforms infrared (FTIR) were recorded using a JASCO FTIR-430 (Japan) Instrument in the range of 400 to 4000 cm-1. 1H NMR was measured in deuterated DMSO using Bruker 400 MHz NMR spectrometer. Pomegranate juice is obtained by squeezing the fresh pomegranate. Human hepatoma cells (HepG2) and breast cancer cells (Mcf7) were purchased from the experimental animal center of Serum and Vaccine Center (Vacsera), Cairo, Egypt. The cells were grown at 37°C in a humidified incubator with a 5 percent CO2 atmosphere in RPMI-1640 media supplemented with 10% FBS, penicillin (100 units/mL), and streptomycin (100 g/mL).

2.3 Synthesis of 2-(4-chlorobenzylideneamino)-5-thioxoisothiazolidin-3-one (CBAT)

N-Aminorhodanine (1.48 g, 0.01 mole) was reacted with p-chlorobenzaldehyde (1.40 g, 0.01 mole) in absolute ethanol at reflux temperature for 4 hrs to form 2-(4-chlorobenzylideneamino)-5-thioxoisothiazolidin-3-one (CBAT). The formed Schiff base was characterized by both FTIR and ^{1}HNMR spectroscopy.

2.4 Modification of phenolic –OH groups of pomegranate juice by CBAT

Modification of the phenolic –OH groups of pomegranate juice was conducted by using CBAT in DMF and in presence of potassium carbonate (K_2CO_3) at room temperature for 24 hrs. The modified pomegranate was characterized by FTIR and ^{1}HNMR spectroscopy.

2.5 Cell proliferation MTT Viability assay

2.5.1 Determination of sample cytotoxicity on cells (MTT protocol)

MTT assay was used to assess the inhibitory effects of modified pomegranate on HepG2 and MCF7 cell growth. Cell viability was assessed in three independent experiments with six doses of modified pomegranate [18]. In 96-well culture plates, cells were planted at a density of 5*106 cells per well. After 24 hours, the cells were rinsed with fresh media and treated with various doses of modified pomegranate as part of a series of tests that lasted varying amounts of time. Following the 24-hours incubation period, 10 mL of MTT solution (5 mg/ml) diluted in PBS was added to each well and incubated for another 4 hours. Finally, 150 L of DMSO was added to each well, and the formazan crystals that formed were dissolved. The absorbance was measured at 570 nm using a microplate reader (Multiskan Go, Thermo Electron Corporation, USA). The cytotoxicity percentage was determined using the following formula:

% cytotoxicity = (Optical density of sample/optical density of control) × 100

The final results were expressed as inhibition concentration (IC50), which was determined visually for each cell proliferation curve as the concentration of sample capable of inhibiting cell proliferation by 50%.

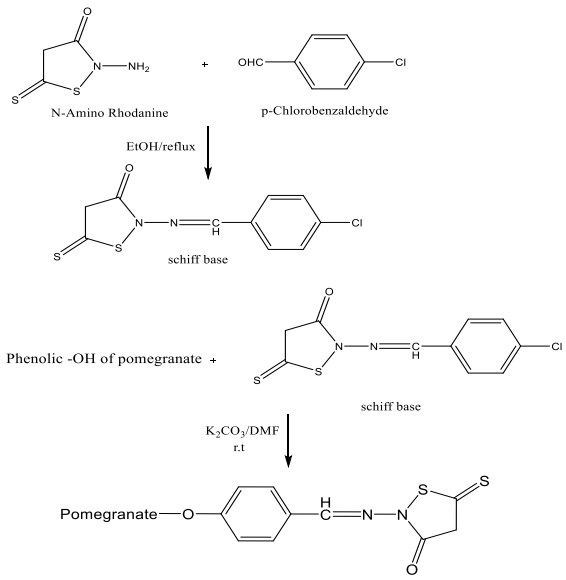
3. Results and discussion

3.1. Synthesis of CBAT

CBAT was obtained by the reaction of N-aminorhodanine with p-chlorobenzaldehyde in absolute ethanol at reflux temperature for 4 hrs according to Scheme 1. The formed Schiff base was characterized by FTIR and ^{1}HNMR spectroscopy.

FTIR of CBAT was illustrated in Fig. 2. It illustrated the presence of a band at 3063 cm^{-1} related to the stretching vibrations of unsaturated =CH groups. The appearance of two bands at 2918 and 2849 cm^{-1} corresponding to the asymmetric and symmetric vibrations of aliphatic –CH_2 group present in rhodanine ring. The stretching vibration of rhodanine carbonyl group appeared at 1703 cm^{-1}. The band at 1626 cm^{-1} is attributed to the stretching vibration of (N=CH) group of Schiff base. The phenyl ring vibration appeared at 1587 cm^{-1}. The vibration of C=S group appeared at 1235 cm^{-1}. The presence of all these bands reveals the correct structure of the formed CBAT and this was proved also by ^{1}HNMR.

Fig. 3 shows ^{1}HNMR of CBAT. It was conducted in dueterated DMSO. The signal appeared at 4.02 ppm is corresponding to the aliphatic –CH2 protons present in rhodanine moiety. The two protons of aromatic ring next to –Cl group appeared at 7.29 ppm. The other two protons of phenyl group next to imino group appeared at 7.57 ppm. The imino group proton appeared at 7.8 ppm. Both FTIR and ^{1}HNMR proves the structure of CBAT.



Scheme 1 Synthesis of CBAT and modified pomegranate (MP)

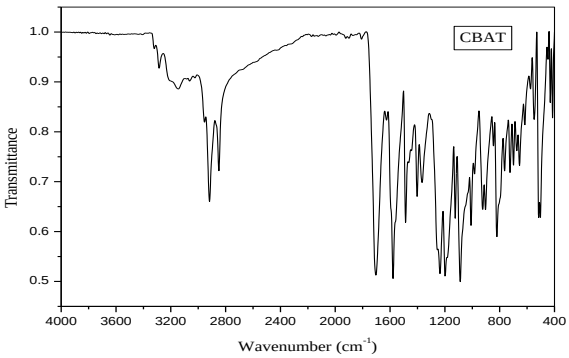


Fig. 2 FTIR of CBAT

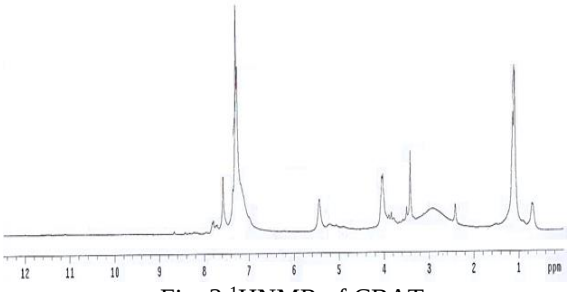


Fig. 3 ^{1}HNMR of CBAT

3.2 Modification of phenolic –OH groups of pomegranate juice by CBAT

Modification of the phenolic –OH groups of pomegranate juice was conducted by CBAT in DMF in presence of K_2CO_3 at room temperature for 24 hrs. The modified pomegranate (MP) was characterized by FTIR and ^{1}HNMR spectroscopy.

FTIR of pure pomegranate (P) juice and modified pomegranate (MP) is represented in Fig. 4. The FTIR of (P) reveals the presence of a broad band at 3385 cm^{-1} corresponding to the stretching vibrations of –OH groups present in pomegranate juice. The band appeared at 2932 cm^{-1} is corresponding to the stretching vibration of aliphatic –CH groups. The band appeared at 1728 cm^{-1} is related to carbonyl group. The C=C vibrations appears at 1632 cm^{-1}. The FTIR of (MP) reveals the absence of the broad band of –OH groups of pomegranate indicating the modification. Also, the bands corresponding to CBAT appears. The bands at 2920 and 2851 cm^{-1} are corresponding to asymmetric and symmetric vibrations of aliphatic –CH group. The stretching vibration at 1710 cm^{-1} is corresponding to the carbonyl group. The band located at 1623 cm^{-1} is related to imino group of CBAT. The aromatic ring stretching vibrations appears at 1580 cm^{-1}. The band located at 1239 cm^{-1} is related to C=S group of rhodanine moiety. The absence of broad band of –OH group of pomegranate and the presence of bands related to CBAT indicates the success of pomegranate juice modification.

^{1}HNMR of (MP) is represented in Fig. 5. It is conducted in deuterated DMSO. The signal appears at 4.8 ppm is related to aliphatic $–CH_2$ group of CBAT rhodanine moiety. The signals of aromatic ring protons appeared at 7.03 ppm and 7.29 ppm. The imino group proton appeared at 8.15 ppm. The appearance of signals of CBAT protons in modified pomegranate indicates the modification occurance.

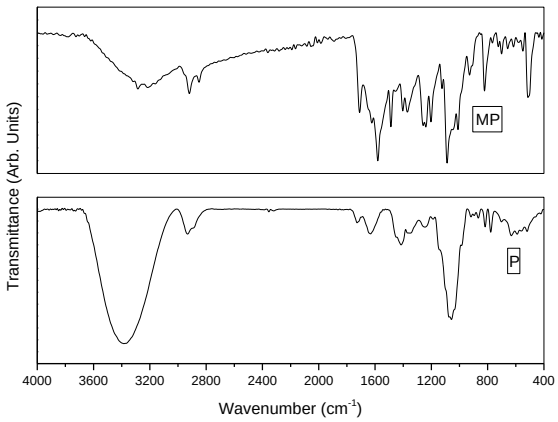


Fig. 4 FTIR of P and MP.

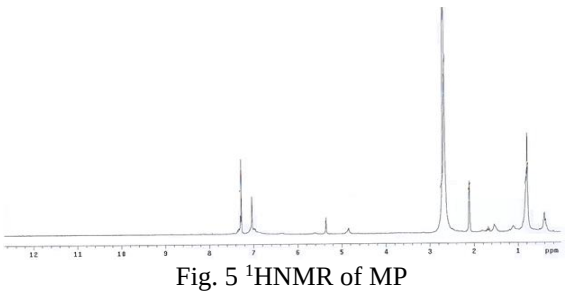


Fig. 5 ^{1}HNMR of MP

3.3 Determination of the Inhibitory concentration required for 50% cytotoxicity (IC50) value

The MTT test is a widely used tool for determining natural product cytotoxicity. The ability of mitochondrial dehydrogenase enzymes from viable cells to cleave the tetrazolium rings of the pale yellow MTT and generate dark blue formazan crystals that are mostly impermeable to cell membranes, resulting in their buildup within healthy cells, is the basis for this assay. As a result, the quantity of living cells in the system determines the absorbance of the solubilized formazan crystal [19].

Modified pomegranate produced a reduction in cell proliferation. The obtained results showed that modified pomegranate inhibited the survival rate of HepG2 cells (hepatoma cells) and MCF7 (breast cancer cells) in a dose dependent manner. It showed different anti proliferative profiles regarding cancer cell type and concentrations. The incubation of modified pomegranate, with HepG2 and MCF7 cancer cell lines induced cytotoxicity with IC50 values of 80.4, and 114.58 µg/mL, respectively as represented in Figs. (6,7).

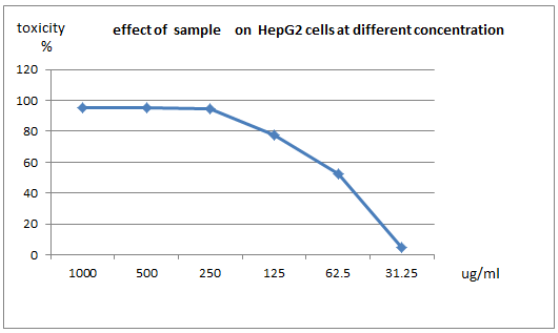


Fig. 6 The anticancer effect of different concentrations of modified pomegranate on hepatic liver cancer cell lines (HepG2).

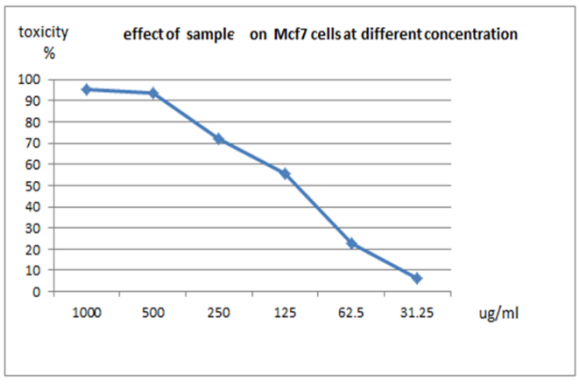


Fig. 7 The anticancer effect of different concentrations of modified pomegranate on Breast cancer cell lines (MCF7).

The lower the IC50 value, the more effective the anticancer potential of the pure compound, so modified pomegranate has strong anticancer effect on

HepG2 than MCF7. Thus, the results demonstrated that the inhibition on HepG2 cell proliferation induced by modified pomegranate is stronger than that in case of its inhibition on MCF7 breast cancer cells.

3.4 Microscopic examinations

Control HepG2 cells which didn't receive any concentration of modified pomegranate showed complete viable cells (100% viability) which appeared shining under microscope. Incubation with different concentrations of modified pomegranate leads to the presence of some dead cells, increasing the concentration of modified pomegranate accompanied by the increase in the percent of dead cells which appear black under microscope as shown in fig. 8. The same observations were noticed in case of breast cancer cell lines MCF7 as shown in fig. 9.

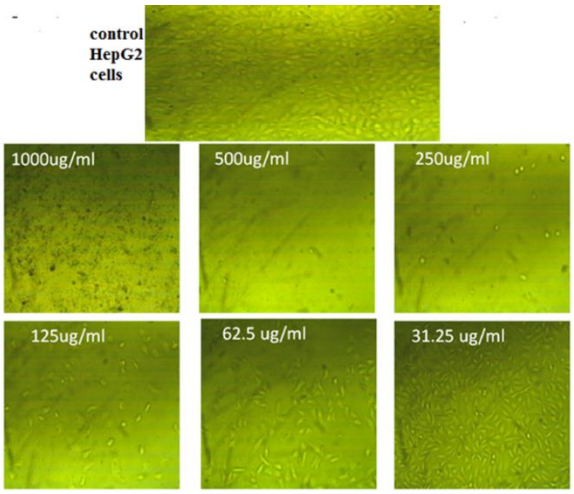


Fig. 8 Microscopic examination on the effect of different concentration of modified pomegranate on the viability of HepG2 cells.

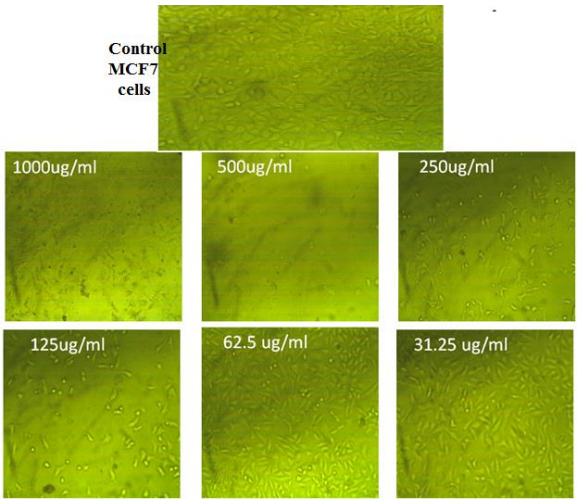


Fig. 9 Microscopic examination on the effect of different concentration of modified pomegranate on the viability of MCF7 cells.

3.5 Discussion

Hepatocellular carcinoma (HCC) is one of the most commonly wide spread disease all over the world. In several nations, like the United States, its prevalence is anticipated to rise through 2030. The most common causes of chronic liver disease and HCC are viral hepatitis B (HBV) and hepatitis C (HCV) 20.

Breast cancer is the secondly spread cancer overall [21], according to the American Cancer Society. Inherited gene mutations are associated to 5–10% of breast cancers (the most frequent are BRCA-1 and BRCA-2 mutations), but 85 percent of cases develop in women with no family history of breast cancer [22]. The MTT assay was used in the current investigation to assess the biosafety of modified pomegranate's cytotoxic effect on HepG2 and MCF7 cell growth. The obtained results are in agreement with many studies conducted to assess the cytotoxic effect of many pomegranate extracts towards several cancer cell lines [3,23,24]. Pomegranate juice's high quantities of polyphenols are responsible for the majority of its biological and pharmacological activities. Polyphenols have a variety of biological effects, including antioxidant, anti-mutagenic, and anti-tumor properties [7,25,26,27]. Liver cancer cell lines inhibition is explained by the existence of phenolic phytochemicals like Ellagic acid (EA) that is mainly distributed in pomegranate. EA is a polyphenolic molecule from the family of ellagitannins (ETs), which has long been thought to be the active ingredient in pomegranate's antioxidant effects (Punica granatum) [28].

EA derivatives were discovered to suppress HCC cell proliferation in a dose- and time-dependent manner, stop the cell cycle at the G1/S phase, and reduce tumor invasiveness [30]. EA was also able to scavenge free radicals, reduce liver injury, exert protective effects against oxidative stress, and induce a significant decrease in serum levels of hepato carcinogenesis markers (i.e., α-fetoprotein, glypican-3, GPC-3, and STAT-3), as well as a negative immunoreaction for VEGF in the liver tissue, all of which reduced hepato carcinogenesis [31,32]. Pomegranate and its primary ingredient EA exhibit pro-apoptotic, antiangiogenic, and antiproliferative properties, through which it may be beneficial in chemoprevention and treatment of HCC.

Pomegranate, on the other hand, has been shown to have a powerful anti-breast cancer impact, with EA decreasing cell viability, inhibiting cell growth, and inducing cell death in a dose-dependent manner in various malignant cell lines, including human MCF-7 and mice S115 cell lines [32]. In estrogen-sensitive human breast cancer MCF-7 cells, EA also behaved as a powerful anti-estrogenic drug, raising insulin-like growth factor-binding protein 3 (IGFBP-3) levels, which are normally blocked by estrogens [33].

Thiazolidinones have potent anti-cancer properties, with heterocyclic moieties accounting for around 60% of cancer treatments due to their unique chemical reactivity [34]. Thiadiazole's bioactive capabilities are linked to the fact that this heterocyclic ring is a bioisostere of pyrimidine, which is the skeleton of three nucleic bases. As a result, 1,3,4-thiadiazole derivatives have the ability to impair DNA replication processes. This allows them to stop both bacterial and cancer cell replication [35]. And this explains the anti-cancer effect of the heterocyclic thiazolidinone, heterocyclic moieties are employed in the treatment of cancer in about 60% of cases. [9,34].

In fact, thiazolidinone and pomegranate were shown to contain several antioxidants. Thus, the combinations of these compounds can be made and have to be further assessed for their synergistic effects.

4. Conclusion

2-(4-Chlorobenzylideneamino)-5-thioxoisothiazol idin-3-one (CBAT) is prepared by the reaction of N-aminorhodanine with p-chlorobenzaldehyde. It is used to modify pomegranate juice to obtained modified pomegranate. Both CBAT and modified pomegranate are characterized by FTIR and 1HNMR that prove the correct structures of the prepared materials. In vitro studies of the prepared modified pomegranate towards both human hepatoma HepG2 cells and breast cancer cells MCF7 are conducted. The results reveal that modified pomegranate has good synergistic inhibitory and pro apoptotic effects on human hepatoma HepG2 cells and breast cancer cell MCF7. It has stronger effect on HepG2 cells than on MCF7 cells.

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