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The cytotoxic activity of local *Cyperus rotundus* phenolic extract on human breast cancer cell lines

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Abstract:

Background: *Cyperus rotundus L.* is a herbal plant used worldwide to treat different diseases in traditional medicine, including malignancy.
Methods: Purified phenolic compounds of *C. rotundus* rhizomes had been extracted, and GC-MS analysis of phenolic extract was performed and conducted according to the National Institute of Standard and Technology. The cytotoxic activity of *C. rotundus* phenolic extract investigated on various cell lines of human breast cancer (MCF-7, AMJ13, CAL-51) and normal cell line HBL-100 using MTT assay to evaluate IC50. Acridine orange-propidium iodide (AO-PI) stains have been used to investigated the apoptosis effects.
Results: results of GC-MS analysis revealed that the extraction of *C. rotundus* rhizomes contained six phenolic compounds. *C. rotundus* phenolic extract displayed a significant cytotoxic efficacy against cancer cells MCF-7, CAL -51, and AMJ-13 with (IC50) of (135.3± 2.887, 218.6± 6.009, and 148.4± 4.619) µg /ml, respectively, but it has a negligible effect on HBL-100 at these concentrations with IC50 of 329.6± 5.196 µg/ml.
Conclusion: The presence of anticancer activity in *Cyperus rotundus* phenolic extract suggested a further research in screening these phytochemicals and investigating their cytotoxic activity against various types of cancer cell lines.

Key words: *Cyperus rotundus L., breast cancer, AMJ-13, GC-MS.*

Introduction:

Cancers including breast cancer are the most life threatening problems [1]. Although many available therapies can reduce and inhibit the proliferation and metastasis of cancer cells, they have so far been unable to effectively overcome cancers, and their efficacy is constrained by factors like tumor recurrence and serious therapeutic-induced toxicity [2]. Therefore, with the saving of normal cells, it was necessary to develop new therapeutic anticancer to be effective against cancer cells. Complementary and alternative medicine (CAM) has been widely accepted as a treatment for malignancy, especially in the care and relief of cancer patients [3]. More than 60% of anticancer drugs today come from or are derived from natural compounds, making such bioactive molecules more attractive for drug companies, even as samples of the final anti-cancer formulations [4].

The main source of various secondary metabolites is medicinal plants, and the most new medicines come from medicinal plants indirectly [5]. Approximately 3000 species of plants were reported to have suspected activity against cancer [6]. Nonetheless, due to toxicity issues, side effects and high costs, plant-based bioactive compounds are considered an alternative to prescription cancer drugs [7].

Cyperus rotundus L. is a crop used worldwide in traditional medicine for treating malignancy and other diverse diseases [8]. *C. rotundus*, were mentioned to contain phenols, flavonoids, alkaloids, saponins, glycosides, and tannins [9]. Phenolic compounds, one of the largest groups of plant phytochemicals, have amazing effects based on their toxicity to foreign

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organism cells [10]. This group of compounds has been found to have a wide range of biological functions, in addition to their antioxidant role, especially as carcinogenesis modulation [11].

Previous studies have documented that phenolic agents exhibit multiple mechanisms to inhibit and prevent carcinogenesis. These can generally be divided in two groups, cancer-blocking agents and cancer-suppressing agents. Cancer-blocking prevents carcinogenesis from being initiated by multiple mechanisms: improving carcinogen detox, altering carcinogen absorption and metabolism, sweeping free radicals and promoting repair of DNA. Cancer suppressants inhibit cancer development and propagation after pre-neoplastic lesions have been formed by interfering through one or more of the following : regulation of the cell cycle, signal transduction, regulation of transcription, and apoptosis [12-14].

Many researches has proven cytotoxic impacts of *C. rotundus* rhizome extract against various malegnant cells [15-17]. Mannarreddy et al. (2017), and Abo_Al-temen, (2019) study the cytotoxic activity of methanolic extract of *C. rotundus* rootstock on various cancer cell lines of human, and indicates a significant anticancer effect of MRCr on all examined cell lines with protection of the non-malegnant cells [18, 19].In this study, many of natural polyphenolic compounds extracted from the C Rotundus rhizome have been characterized, and then we evaluated their activity in several cell lines human breast cancer.

Materials and Methods:

2.1. Plant Sample Collection and Preparation

C. rotundus rhizomes were purchased from Iraqi markets and reported in Baghdad's Iraqi herbarium / Science Faculty / University. A dried herb parts have been ground into coarse powder with a coffee grinder. No sieving has been applied.

2.2. Phenolic Compounds Separation

The separation of the polyphenol component was performed according to Harborn (1984) [20]. Approximately 200g of herb powder has been shaken with 1L of 80 percent ethanol for 72 hrs in cool and dark location. The extract, then filtered and dried at a temperature of 30-40 °C via a rotary evaporator. For acid hydrolysis 10 percent concentration HCl was used for 10-30 min in a water bath. This step resulted in the hydrolyse the glycosidic linkage to get aglyconic part, cooled and filtrated. Finally, the mixture was extracted using chloroform 1:1 percent in three times separation fennel. The polypnenolic fraction (chloroform layer) was collected and submitted to rotary evaporator to remove the solvent form. The ferric chloride test was applied to detect phenol compounds in plant extract. In order to determine the chemical composition of the plant extract, GC-MS analysis was performed.

2.3. Ferric Chloride Test to Detecte Phenols

Two grams of plant extract has been added to 10ml of triple distilled water then heated to boil; left cool and filtrated, after that 1 percent aqueous ferric chloride (FeCl3) to filtered solution was added. The result considered positive if dark color appearance referred to the existence of phenol compounds[20]. (Harborne, 1984; Al-Shahaat, 1986).

2.4. GC-MS Technique

Gas Chromatography/Mass Spectroscopy analysis has been carried out at Aglente 5977E Series GC/MSD System, USA, and was performed in Ibn Al-Betar researches center, Iraqi Ministry of Science and Technology. GC-MS was administer according to the National Institute of Standard and Technology (NIST) database.

2.5. Preparation of stock solution of plant extract:

C. rotundus extract has been attended by dissolving 0.01g of extract in 10m1 of solvent (0.1m1 DMSO + 9.9ml TDW, the stock concentration is 1000µg/m1) and filtered by 0.22µ millipore filter [21].

2.6. Cell lines

The cell banking unit of the Iraqi Center for Cancer and Medical Genetics Research (ICCMGR) supplied four cell lines (HBL-100), (CAL-51), (MCF-7), and the Iraqi breast cancer cell line (AMJ13) [22]. MCF-7, CAL-51 and HBL-100 cells were maintained in MEM medium as monolayer cultures supplementes with 10 percent FCS, while AMJ-13 cells were grown in RPMI-1640 supplements with 10 percent FCS and frequently evaluated for normal growth characteristics and were always verified.

2.7. Cytotoxicity assay

Breast cell lines (HBL-100, CAL-51, MCF-7, and AMJ13) were cultured on 96 well microtiter plates until monolayer were formed. From stock, six double fold concentrations of Cyperus extract were made (400,200, 100, 75, 50 and 25) µg / ml. The cell lines were exposed to 0.2ml of each prepared dilution and three replicates were used for each concentration and control (cells with SFM only). The exposure time was 48 hrs. Cell viability was measured after using MTT stain [23]. The IC50 was calculated by GraphPad prism software version 7.0 for Windows.

2.8. Hematoxylin and Eosin staining

Cell sections were fixed for 5 min in 10% formaldehyde and washed three times with tap water. Cells re-hydrated using 100%, 90%, then 70% of ethanol three times for each concentration. Cell sections have been soaked for 2-5 minutes into hematoxylin solution and washed for one minute in tap water. Then eosin solution was added for 1-2 minutes. Dehydration was performed using 95% ethanol for one minute, followed by two modifications were preceded for 2 minutes in pure ethanol. Finally, xylene was added to make clearing.

2.9. Apoptosis assay

The apoptosis features of infected cells were investigated using Acridine orange-propidium iodide (AO-PI) stain. The AO-PI stain was prepared by the addition of acridine orange (1µL) and propidium iodide (1µL) in 1 ml of PBS. In 96-well plates cell lines were cultivated. After 80% progression, cancer cells were treated with 200 µg/ml of *C. rotundus* phenolic extract for 48hs. Control wells left without treating. the media have been discarded at the end time of exposure, followed by the addition of 50µL/well of prepared AO-PI stain. Stain was drained from the wells after 20 seconds. The invert fluorescent microscope is used to investigate and capture morphological

differences in living and apoptotic cells. AO is an important dye and it is stains alive and dead cells. PI was colored cells with ruptured membrane. The living cells are evenly colored in green. PI-integrating apoptotic cells so they take orange color. The percentage of apoptotic cells was calculated using Image J software version 1.47 [24].

Results:

3.1. Detection of phenols in *Cyperus rotundus* extract.

Phenols were purified from the crude extracts of rhizomes and detected chemically by ferric chloride test. The appearance of dark green color considered a positive result.

3.2. GC-MS Technique

GC-MS of phenol extracts revealed several peaks through retention time 24 min. The spectra of the known Phenolic compounds in the NIST Library were correlated with each peak and mass measurement obtained for confirmation of the compounds. The most influential components and greater concentration illustrated in the following (Tab.1, Fig.1):

Table 1. phenol components of *Cyperus rotundus* extracts

Peak	R.T min	Area%	Compound Name
1	**6.614**	**1.61**	Orcinol
2	**9.109**	**16.88**	4- mercaptophenol
3	**9.708**	**5.23**	2-Acetyl-6-methoxynaphthalene
4	**12.493**	**3.16**	1-(4-tert-butylphenyl) propan-2-one
5	**12.974**	**2.09**	2 (1H)Naphthalenone
6	**13.721**	**1.63**	phenanthren-3-ol
7	**14.924**	**3.27**	Furan, 2-[(2-ethoxy-3,4-dimethyl-2 -cyclohexen-1-ylidene)methyl]-
8	**15.606**	**3.08**	Hexadecanoic acid , methyl ester
9	**15.887**	**2.85**	1,8-dimethyl-8,9-epoxy-4-isopropyl-9,19-Cycloergost-24(28)-en-3-ol
10	**16.113**	**1.34**	1,4-Methanoazulen-7-ol
11	**16.254**	**3.87**	Hexadecanoic acid, ethyl ester
12	**17.289**	**1.97**	8-Octadecenoic acid, methyl ester (E)-
13	**18.552**	**1.52**	2-Hydroxyiminomethylquinoline-4-carboxylic acid

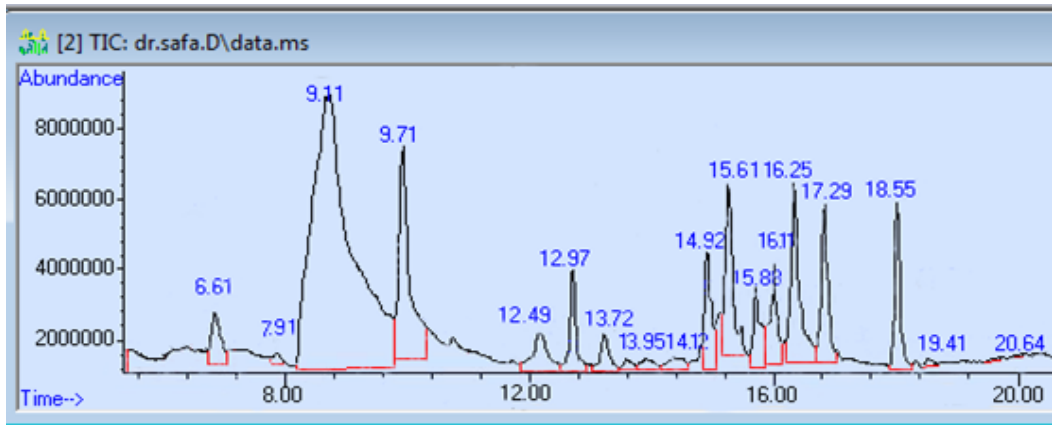

Figure 1: GC-MS technique of the various components of *C. rotundus* extract.

GC-MS results showed that our extract of *Cyperus rotundus* rhizome contains six phenolic compounds (4-Mercaptophenol, 3,3'-dimethoxy-[1,1'-biphenyl]-4,4'-diol, Orcinol, 1,4-Methanoazulen-7-ol, 2H-Benz[e]indene-3,7-diol, and phenanthren-3-ol). In addition to existing non-phenolic organic components and inorganic products, misleading values have been found.

3.3 Cytotoxicity of *C. rotundus* phenolic extract on breast cancer cell lines

The activity of *C. rotundus* phenolic extract on the proliferation of MCF-7, AMJ13, CAL-51 breast cancer cell lines as well as HBL-100 as normal cells was investigated at various concentrations (25-400) µg/ml using MTT assay (Fig. 2).

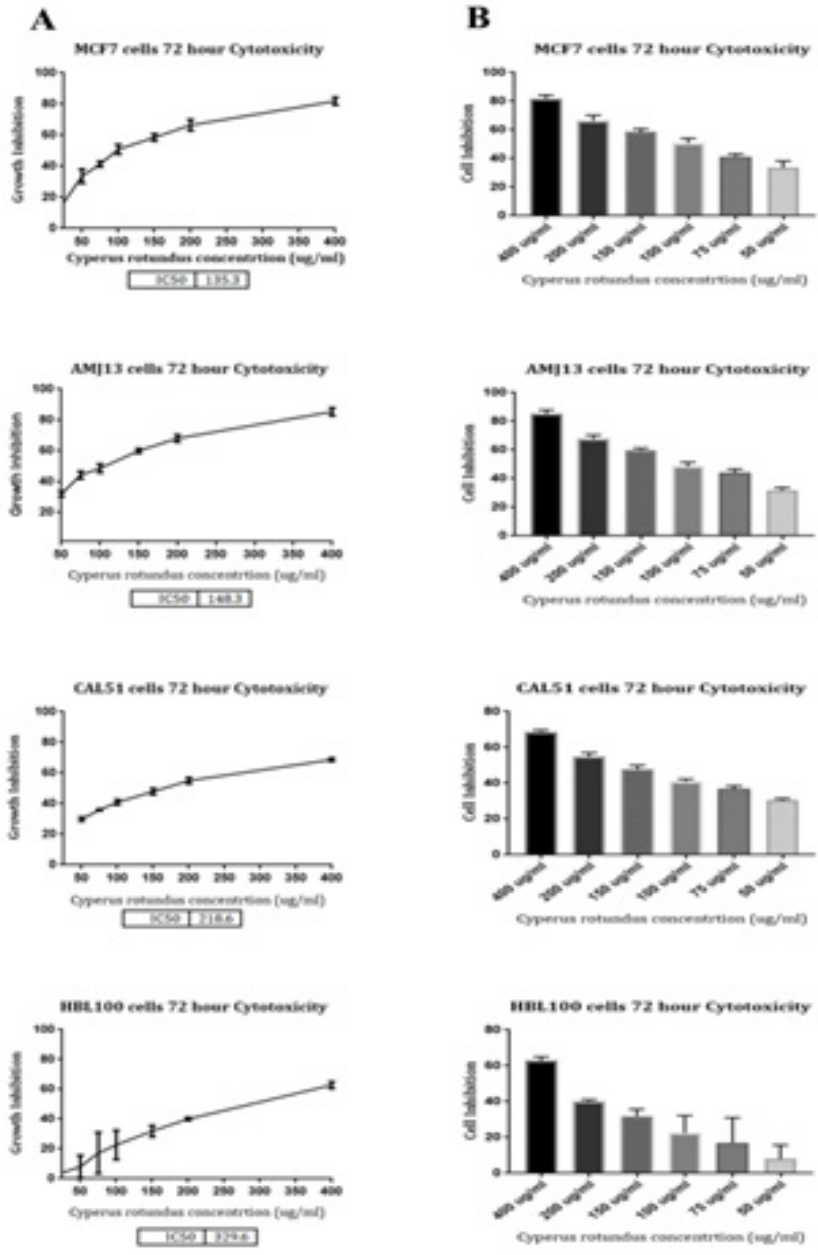

Figure 2: Anti-proliferation activity of of *Cyperus rotundus* phenolic extract on (MCF-7, AMJ13, and CAL-51) breast cancer cells. (A) IC50 of *Cyperus rotundus* phenolic extract. (B) The cell growth inhibition at various concentration.

Results of cytotoxicity demonstrated that the phenolic extract of *C. rotundus* has anti-proliferative effects after 48 hours of treatment and inhibited the growths of MCF-7, CAL-51, and AMJ13 cell lines with inhibitory dose (the concentration required to inhibit 50% of cell growth IC50) of (135.3± 2.887, 218.6± 6.009, and 148.4± 4.619) µg /ml, respectively, but it has a lower effect on HBL-100 at these concentrations with IC50 of 329.6± 5.196 µg /ml when compared to the control group. IC50 was different among the three cell lines. MCF-7 cell line required the less concentration to inhibit 50% of the cell when contrasted with other cell lines. The growth inhibition of cancer cell resulted by *C. rotundus* phenolic extract seemed dose dependent and R2 > 0.979. The *C. rotundus* phenolic extract selectivity index was measured and listed in Tab. 2. Growth inhibition was minor in normal cell line while growth was significantly inhibited in malegnant cell lines, $p<0.05$ (Fig. 3).

Table 2: Cytotoxic effect of *C. rotundus* phenolic extract on different cell lines.

No.	Cell line	IC_{50} Cyperus extract (µg/ml)	R^2 value
1	HBL-100	329.6 ± 5.196	----------
2	CAL-51	218.6 ± 6.009	0.9799
3	AMJ-13	148.3 ± 4.619	0.9942
4	MCF-7	135.3 ± 2.887	0.9963

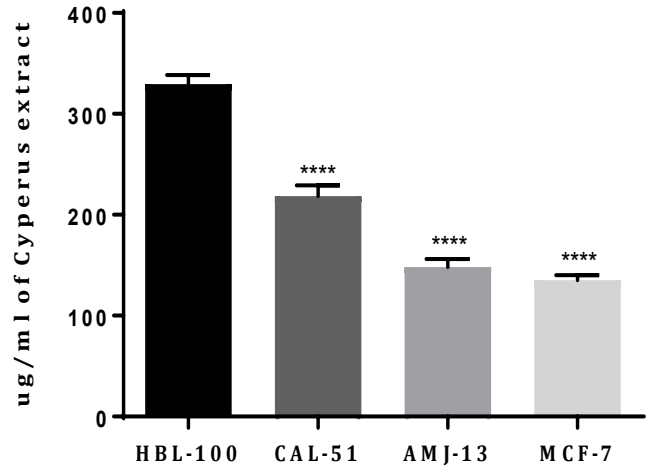

Figure 3: Cytotoxic activity of *C. rotundus phenolic extract* on MCF-7, AMJ13, CAL-51 cancer cell lines and normal cell line (HBL-100) calculated using MTT stain. Values are mean ± SD with five replicate from three independent experiments determinations for (IC50). (***$p < 0.05$ contrasted with HBL100).

The morphology of treated or untreated cultured cells have been documented in microphotography images using hematoxyline-eosin stain (Fig.4). The untreated cells revealed the cellular properties of the related cell lines. The morphological changes in treated cells inclusive of damaged cell membranes and cell shrinkage. Cell damage contributes to the loss of shape and forming of small spherical bodies which are features of apoptosis.

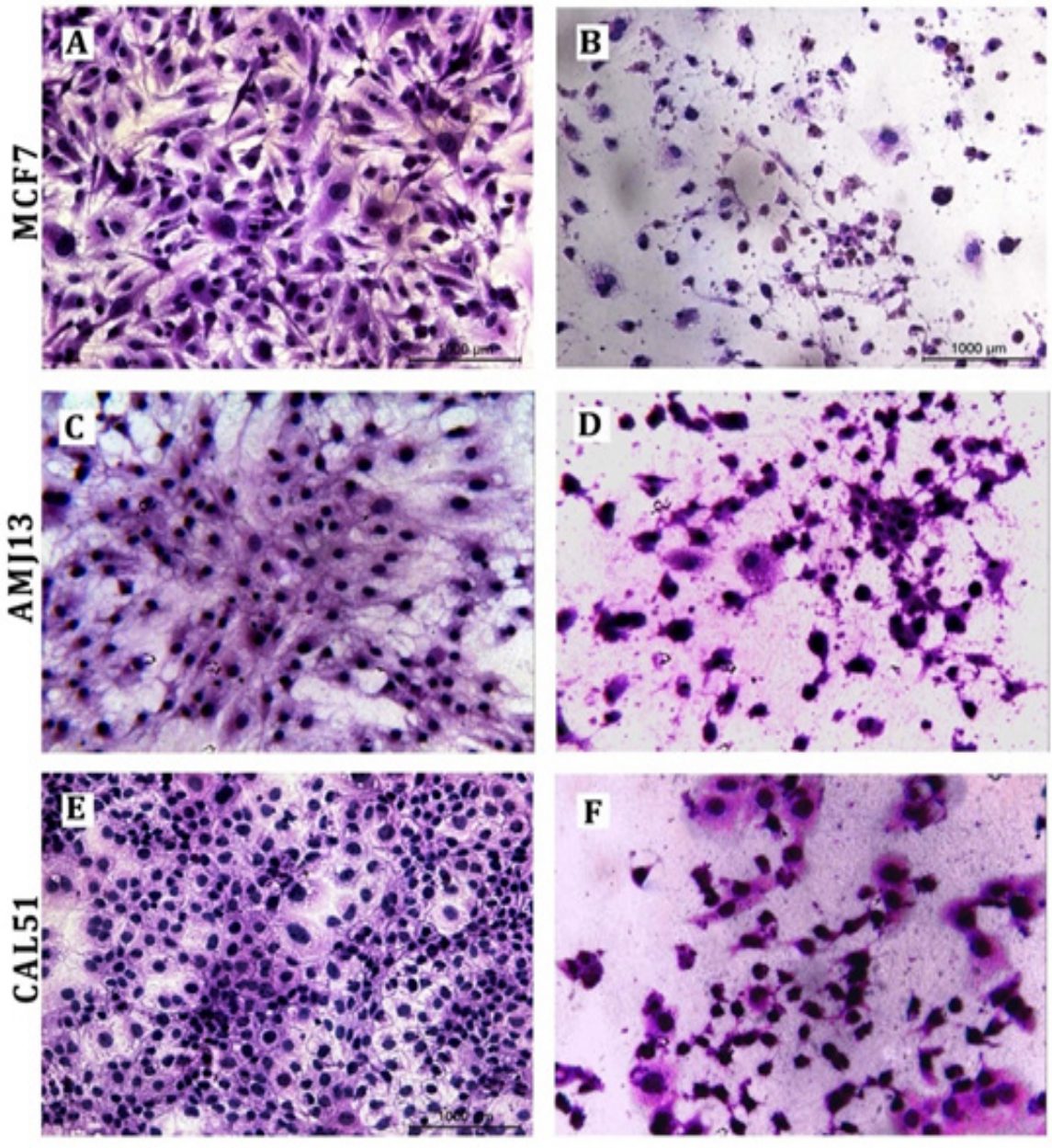

Figure 4: CPE of *C. rotundus* phenolic extract on breast cancer cell lines. (A, C, and C) showing untreated control cells retained their distinct morphology . (B, D, and F) Showing the cytotoxic effect after 48 h of treatment appeared as shrunken forms of agglomerate dead cells and deformed shapes with hollow space and cell debris, (H&E) 20X.

3.4. Apoptosis induction by *Cyperus rotundus* phenolic extract

Staining of acridine orange / Propidium iodide (AO / PI) was used to design apoptosis characteristics which includes nuclear modifications and formation of apoptotic body. Cells have been studied with a fluorescence microscope and the apoptotic cells were quantifiely counted. Green fluorescence was revealed in nucleus and cytoplasm of living cells. Propidium iodide was incorporated in the apoptotic cells and thus appeared red in color, along with exhibited condensed, often fragmented nuclei. Necrotic cells displayed orange color, but have nuclear behavioral properties identical to those of viable cells without condensed chromatin (Fig. 5).

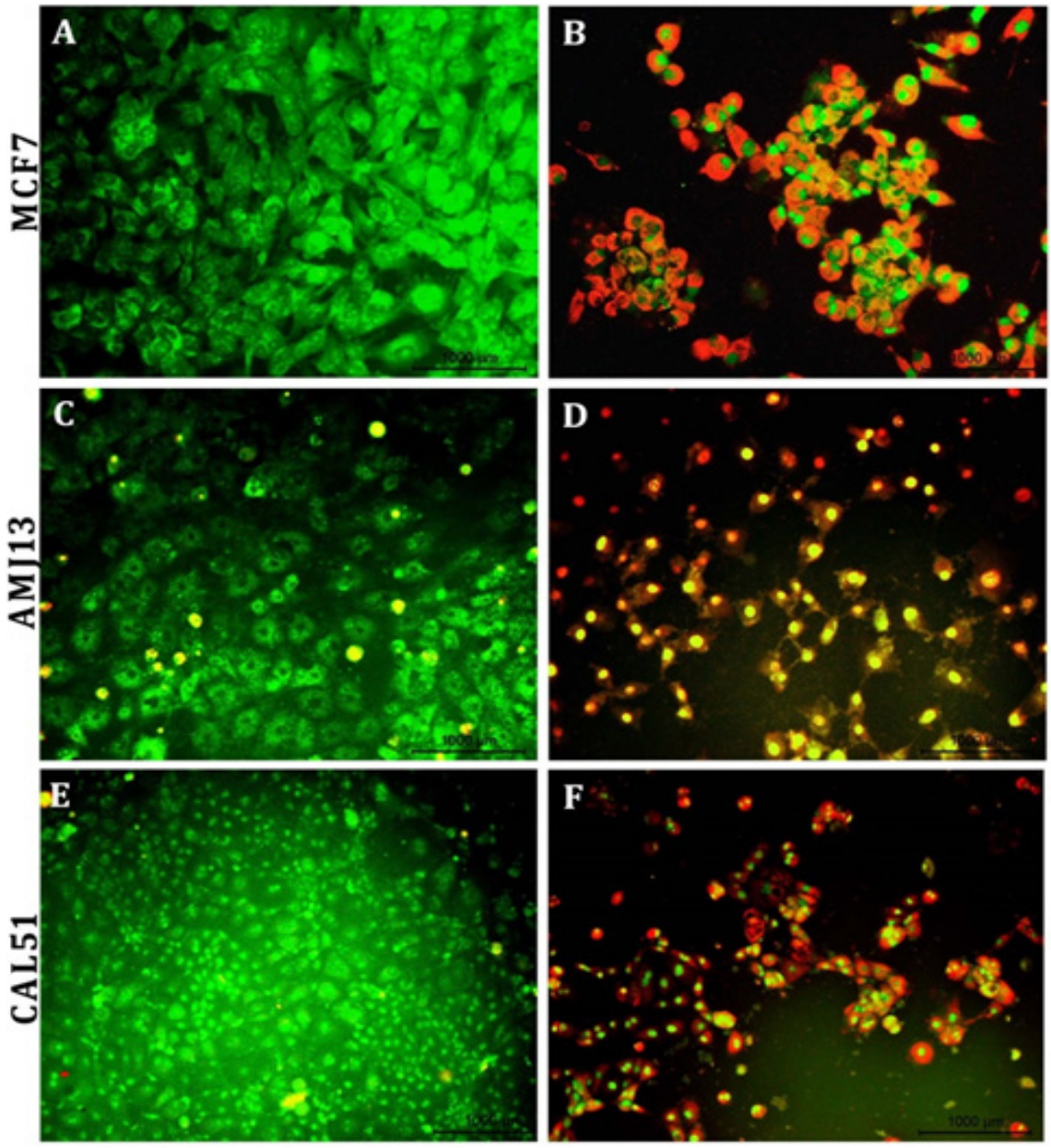

Figure 5: fluorescence microscopy images showed mitochondrial permeability transition apoptosis test for breast cancer cells. (A, C, and E) the non-treated MCF-7, AMJ13, and CAL-51 cells respectively stained green color while (B, D, and F) represent MCF-7, AMJ13, and CAL-51 respectively that treated with *Cyperus rotundus* phenolic extract stained red after 48h, 20X.

Furthermore, the data of this study using ImageJ software to create the Image estimation histogram of (MCF-7, AMJ13, and CAL-51) cell lines apoptosis test (figure 6), showed that the proportion of apoptotic cells were (78.3%, 73.42%, and 57.38%) respectively (fig. 6 and fig. 7).

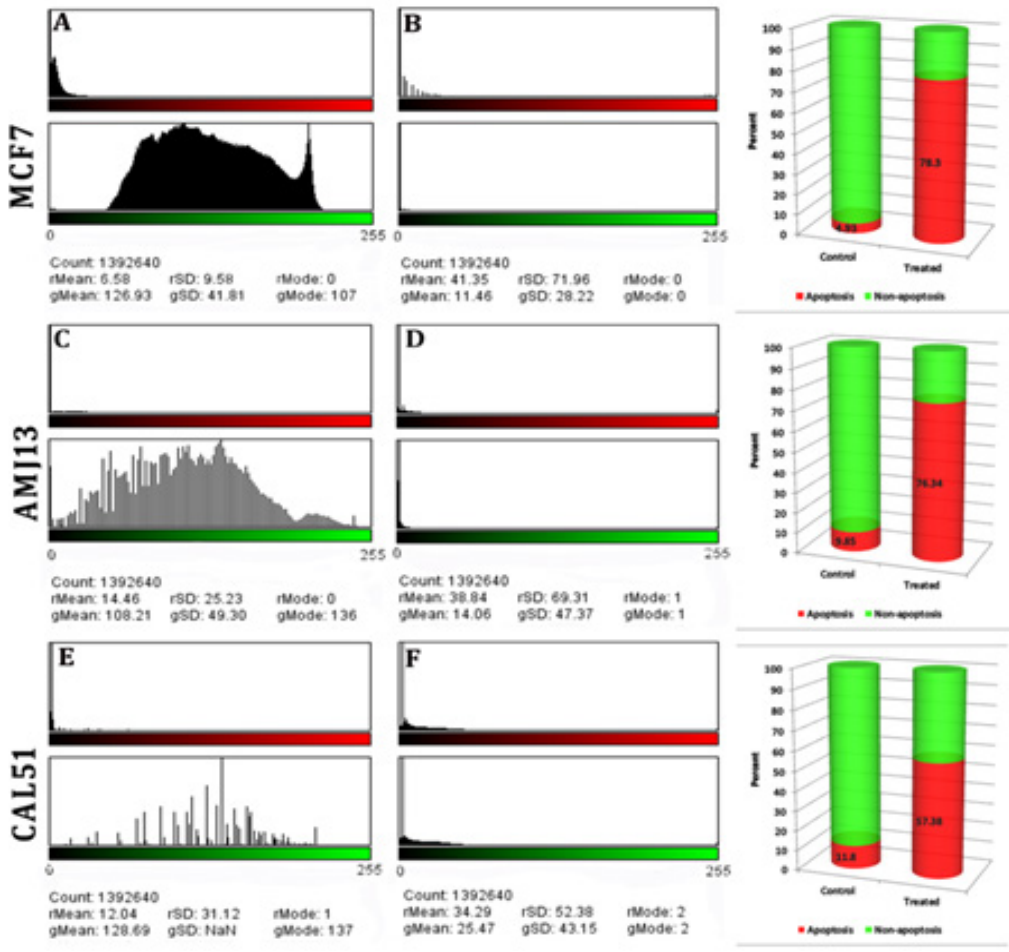

Figure 6: Image estimation histogram of (MCF-7, AMJ13, and CAL-51) breast cell lines apoptosis test after 48h treatment, 20x. Non-apoptotic cells gave green color while apoptotic cells gave red color. (A, C, E) Non-treated MCF-7, AMJ13, and CAL-51 cells; the proportions apoptotic cells were (4.93, 11.8, and 8.56) percent respectively. (B, D, and F) treated MCF-7, AMJ13, and CAL-51 cells; the proportions apoptotic cells were (78.3, 73.42, and57.38) percent respectively.

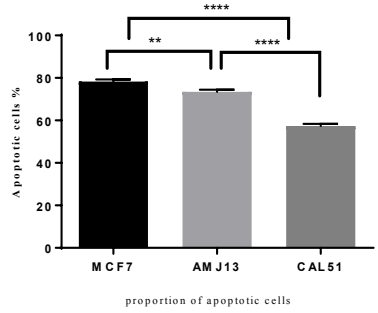

Figure 7: The apoptotic cells proportion after 48h treatement with *Cyperus rotundus* phenolic extract.

Discussion:

Polyphenols are among the plant kingdom's most abundant and widely distributed compound groups. *Cyperus rotundus* has been a possible dietary supplement rich in phenolic bioactive compounds. In this study results of ferric chloride test gave dark green color as a positive result, that was meaning the presence of polyphenolic compounds in *C. rotundus* extract. The dark colour might be resulted in the presence of large quantities of polyphenols and flavonoid [25, 26]. The data for GC-MS analysis showed that our extract of *Cyperus rotundus* rhizome contains six phenolic compounds as well as to some non-phenolic organic and inorganic components at deceptive values. Abo_Al-temen, et al. (2019) found that the chemical composition of the Iraqi *Cyperus rotundus* varies from that of the other plant found in South Africa, Iran, and Tunisia, and they commented that may be related to the employ of other solvents, the quality of the extracts, the part of plant being investigated or possibly due to allelopathic efficacy [18].

Several research in various cell lines, lab animal and human clinical studies indicate a protective effect of dietary phenols against numerous tumor types [27]. Many of human cancers, along with breast cancer, were characterized by the fundamental efficiency of nuclear factor kappa B transcriptional factor [28]. NF-πB is activated and transferred to the nucleus.When the inducer was absent, NF-kB has restricted to the cell cytoplasm where it aligned with IκB (NF-kB inhibitor). Creation of ROS (OH−, O2− and H2O2) stimulates IκB phosphorylation which causes its degradation and ubiquitination via the proteasome. Most phenolic compounds suppress IKK-mediated IκB activation by inducing of NF-κB cytosolic retention leading to its inactivation. Other research indicates the potency of flavonoids as inhibitors of NF-κB. These explain the molecular foundation for polyphenols being able to function as an anticancer [12, 29].

From the six phenolic compound found in our extract the highest concentration 34.8 component in retention time of 9.109 minutes was 4- mercaptophenol. Organotin complexes dependent on 4- mercaptophenol have significant cytotoxic activity against MCF-7 and HeLa malignant cell lines compared to normal MRC-5 cell line [30]. In addition to its antioxidant activity, mrcaptophenol can produce complex compounds which can reduce SH groups in tubulin protein. The ability of these compounds to binding tubulin, allow to recognize these as possible antimytotic agents [31]. In vitro, results of this study appear that the treatment with the *C. rotundus* phenolic extract significantly reduced breast cancer cells viability and triggered apoptosis when compared with the untreated group (fig. 6). In the breast cancer cell lines treated with phenol extract, relative to control, apoptotic cells appear as shrinkage, apoptotic body formation, chromatin condensation as well as DNA fragmentation as a result to endonuclease activation [32].

Many other previous in vitro studies have shown that phenolic compounds can induce apoptosis by activating cell surface receptors via extrinsic pathways and/or by intrinsic pathways that included intracellular signals through mitochondria [33]. Apoptosis can be triggered by activation of proapoptotic or apoptotic proteins like Bcl-xl and caspaces or antiapoptotic proteins like Bc-xL and surviving [34]. A finding of Srinivasan et al., [35] suggested that polyphenols of green tea isolation was inhibited the activation of phosphatidylinositol 3 kinase/ Akt which modulate Bcl-2 protiens, resulting in apoptosis enhancement in T24 oral cavity tumor cells. It was observed from our results that the *Cyperus rotundus* phenolic extract has a significant cytotoxic activity on breast cancer cell lines, that is be due to the presence effective compounds in this extract, as well as the significance was different among the cell types. Moreover, previous studies proved the anticancer effect of these compounds extracted from other plants or herbs [36-38].

Conclusion:

C. rotundus rhizome bioactive products have been extracted using methanol solvent (MRCr) and specified as a potentially phenol compound origin. Cytotoxic efficacy of *C. rotundus* phenolic extract was studied using MCF-7, AMJ13, and CAl-51 breast cancer cell lines. All these cell lines have been responding by apoptosis. The values of IC50 of tested cell lines were different with a minimum value of (135.3 ± 2.887) against MCF-7 and a maximum value of (218.6 ± 6.009) against CAL-51. *Cyperus rotundus* phenolic extract showed minor cytotoxic effect on the non-cancerous cell HBL-100. This study suggested that the *C. rotundus* phenolic extract has apoptosis antiproliferative activity that need more studies to investigate and characterize the purified components against cancerous cells using the rhizome of *C. rotundus*.

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