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Antioxidant and Anticancer Activity of *Opuntia elatior* Mill. Ethanol Extract and the Fractions

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ABSTRACT

Opuntia elatior Mill. (OpE)) is a cactus plant and has been empirically used to treat cancer and diseases caused by oxidative stress. Several studies showed that the secondary metabolites in this plant could also be developed into a lead compound to produce new single-compound drug candidates. Therefore, this study aimed to analyze the effect of phenolic and flavonoid levels in OpE on antioxidant and anticancer activity *in vitro*. OpE Ethanol Extract (EE) was fractionated using various solvents, including n-hexane (HF), Ethyl Acetate Fraction (EAF), and Methanol Fraction (MF). The extract and fractions were then tested for Total Phenolic Content (TPC) and Total Flavonoid Content (TFC) as well as antioxidant activity using radical cation (ABTS) and Ferric Reducing Antioxidant Potential (FRAP). Anticancer testing was performed using MTT reagent against cervical cancer cells (HeLa), breast cancer cells (T47D), and colon cancer cells (WiDr). The results showed that EAF had the highest TPC levels (9.84 ± 0.55 mg GAE/g) compared to other fractions and had the second highest TFC levels (11.99 ± 0.79 mg QE/g) after HF. The high TPC and TFC in EAF were directly proportional to antioxidant activity tested using FRAP with IC_{50} value of 106.72 ± 1.60 µg/mL. Furthermore, it had the best anticancer activity against HeLa (IC_{50} 693.61 ± 50.63 µg/mL) and WiDr (IC_{50} 627.69 ± 36.27 µg/mL) compared to EE, HF, and MF. Based on the results, phenolic and flavonoid compounds in EAF could increase antioxidant and anticancer activities. These compounds were also believed to play a major role as anticancer agents for WiDr and HeLa.

Keywords: antioxidant, anticancer, *Opuntia elatior*, fractions

Introduction

Plants are a promising source of medicinal raw materials and play a significant role in the treatment of various diseases.[1] Furthermore, several single compounds derived from secondary metabolites found in plants have undergone successful clinical trials and gained approval for therapeutic use.[2] These compounds have been reported to exhibit diverse pharmacological activities, including antioxidant, anticancer, antiaging, antidiabetic, and anti-inflammatory, either in the pure form or as components in extracts or fractions.[3] According to previous studies, these secondary metabolites include alkaloids, phenolics, flavonoids, terpenoids, tannins, saponins, and steroids.[4] A recent review reported that approximately 49 % of drugs are natural products or derivatives used in cancer treatment.[5]

Several species of cactus plants, particularly the prickly pear (*Opuntia ficus-indica*), belong to the Cactaceae family, originating from Central America (Mexico). This species has been reported to be widely distributed in semi-arid regions, such as Egypt. Furthermore, approximately 1500 cactus plants belong to the genus Opuntia, which is known for the production of edible and favorable fruit. In Mexican traditional medicine, Opuntia leaves and fruit have been used for treating various diseases, including arteriosclerosis, diabetes, and gastritis.[6]

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Several studies showed the analgesic and anti-inflammatory effects of fruit extracts from Opuntia genus. The fruit and young stems have also been traditionally used to treat diabetes, hypertension, asthma, burns, edema, and digestive disorders.[7]

Cactus pears are known for the rich array of bioactive components, and phenolic compounds, betasianin, and betaxanthin pigments possessing strong antioxidant properties are identified as the most abundant. Phenolic compounds are substances with an aromatic ring carrying one or more hydroxyl groups, including functional derivatives. Several studies showed that cactaceae plants and fruits contained glycosylated flavonols, dihydroflavonols, flavonones, and flavonols. The antioxidant properties of phenolic compounds in the cactus pear plant make the fruit an essential source for preventing degenerative diseases, such as cancer, diabetes, and hyperglycemia.[8]

Opuntia, a sprawling genus of succulent shrubs, has gained popularity in warmer regions across the world due to its unique appearance and attractive flowers. These shrubs are commonly known as Prickly pears due to the edible fruit, and *Opuntia elatior* Mill. (OpE) has been reported to possess secondary metabolites, such as phenolic and flavonoid compounds.[9] Phenolic compounds, such as delphinidin, petunidin, cyanidin, malvidin, gallic acid, ferulic acid, sinapic acid, p-coumaric acid, hydroxycinnamic acid, vanillic acid, syringic acid, eucomic acid, and chlorogenic acid, have previously been identified in *Opuntia spp* extracts using LC-MS.[10-12] The extract also contains flavonoids, such as apigenin, myricetin, quercetin, kaempferol, isorhamnetin, and cathecin.[13] Phenolic compounds have also been reported to have antioxidant activity and anticancer potential *in vitro*. According to chemical characterization, these properties can be attributed to the presence of galloyl derivatives, flavan-3-ols, phenolic acids, and flavones/ols.[14]

Phenolic compounds are natural antioxidants contained in plant foods, which are usually biosynthesized from phenylalanine or tyrosine through the shikimate acid pathway.[15] Furthermore, these components have the ability to neutralize free radicals by donating electrons or

hydrogen atoms to various reactive oxygen, nitrogen, and chlorine species, including O_2, OH, peroxyl radicals RO_2, hypochlorous acid (HOCl), and peroxynitrous acid (ONOOH).[16] The configuration, substitution, and number of –OH groups substantially influence several mechanisms of antioxidant activity, including radical scavenging, activation of antioxidant enzymes, reduction of α-tocopheryl radicals, inhibition of oxidase, mitigation of oxidative stress caused by nitric oxide, metal ion chelation ability, and enhancement of low molecular antioxidant properties. In flavonoids, the -OH ring configuration of the core of flavan B is the most significant determinant in the scavenging of ROS and RNS. This is because it donates hydrogen and electrons to hydroxyl, peroxyl, and peroxynitrite radicals, stabilizing them and producing relatively stable flavonoid radicals.[17]

Phenols and flavonoids are natural secondary metabolites extracted from medicinal plants to treat various diseases related to oxidative stress caused by free radicals.[18] Phenolic compounds, such as p-coumaric acid, ferulic acid, sinapinic acid, and resveratrol have been reported to have antiproliferative effects on HeLa cervical cancer cells in a dose-dependent manner. This activity can be caused by cell cycle arrest and induction of apoptosis. The antiproliferative properties of phenolics with histone deacetylase (HDAC) inhibitory activity have been associated with the modulation of various apoptosis and/or cell cycle-related proteins, including Bcl2, p53, p21, CDK4, and pERK1/2.[19] Furthermore, the treatment of T47D human breast cancer cell cultures using curcumin suppresses the activity of NF-κB signaling pathway both by inhibiting the transcription factor and downregulating the expression of NF-κB and IKK.[20-21] Sinapic acid, one of the two phenolic compounds abundant in the dimethyl sulfoxide extract of *Dianthus carmelitarum*, has a selective cytotoxic effect on human colon cancer cells (WiDr) compared with normal variant. This activity is associated with S-phase cell cycle arrest and induction of apoptosis mediated through reduction of MMP.[22] Therefore, this study aims to examine the effect of phenols and flavonoids in OpE extracts and fractions on antioxidant and anticancer activity. At present, there are no reports on the effect of OpE fractions on *in vitro* pharmacological activity. Furthermore, this research wants to prove whether the compounds contained in fractions with different polarities influence the morphology of cancer cells.

Materials and Methods

Plant Collection and Identification

OpE was collected from Balongmulyo Village, Krajan District, Rembang Regency, Central Java, Indonesia, in January 2023. Furthermore, it was identified by Eling Purwantoyo from the Plant Taxonomy Laboratory, Universitas Negeri Semarang, Indonesia. The specimen was then stored in the Semarangense Herbarium (voucher number 530). OpE was dried using indirect sunlight and ground to produce powder.

Extraction and Fractionation

A total of 700 g OpE powder was macerated in this study using 96% ethanol (1:10 w/v). Maceration was carried out for 1 x 24 hours in a closed container and stirred occasionally, and a new solvent replacement for extraction was performed every day for two days. Subsequently, the filtrate obtained from the process was collected and evaporated using a vacuum rotary evaporator at a temperature of 50ºC to obtain ethanol extract (EE). For every gram of EE, 10 mL n-hexane was added and mixed using a vortex mixer for 5 minutes, then centrifuged at 3000 rpm for 5 minutes. The supernatant was separated by pipetting, collected in a cup, and left to dry to obtain hexane fraction (HF). The insoluble part was fractionated in stages using the same procedure with ethyl acetate and methanol as solvents to produce ethyl acetate fraction (EAF) and methanol fraction (MF).

Total Phenolic Content (TPC)

TPC was determined using the Folin-Ciocalteu method with some modifications.[23] In 96-well plates, 12 µL sample in DMSO or standard gallic acid solution was added, followed by 50 µL aquabidest and 13 µL Folin-Ciocalteu (50%, v/v in aquabidest). A total of 125 µL of 7% Na_2CO_3 and 100 µL aquabidest were added after 10 min. Furthermore, the mixture was left for 15 min at 45°C, and the absorbance was determined at 765 nm. TPC was calculated from a standard curve of gallic acid (2-11 µg/mL) with a linear relationship r^2. The data obtained were expressed as mg of gallic acid equivalent (GAE) per gram of sample.

Total Flavonoid Content (TFC)

TFC was determined using a colorimetric method.[24] In a 96-well plate, 100 µL of sample (100 µg/mL in DMSO) or standard quercetin solution and 100 µL of 2% $AlCl_3$ in methanol were added and mixed thoroughly. The reaction mixture was kept at room temperature for 15 min, and the absorbance was recorded at 435 nm. TFC was then calculated using a quercetin standard curve (5-15 µg/mL) with a linear relationship r^2. Furthermore, data were expressed as mg of quercetin equivalent (QE) per gram of sample.

ABTS Free Radical-Scavenging Activity

ABTS free radical test adopted Eden *et al*.[25] method with several modifications. A fresh ABTS reagent solution was prepared by mixing two mM ABTS solution with 70 mM potassium persulfate. The solution was stored in the dark at room temperature for 16 hours before use and diluted with 80% methanol. Furthermore, 100 µL sample solutions of various concentrations was added to 100µL ABTS solution. Absorbance was measured at 734 nm 1 minute after mixing, and all measurements were carried out three times. Standard curves were obtained using sample solutions at various concentrations, and the radical scavenging activity of different sample concentrations against ABTS radicals was also measured to calculate IC_{50} value.

Ferric Reducing Antioxidant Potential (FRAP) Assay

FRAP was measured using a spectrophotometric test based on the method proposed by Azam et al.[26] A total of 100 µL samples with different concentrations, 100 µL phosphate buffer (0.2 M, pH 6.6), and 100 µL potassium ferricyanide $K_3Fe(CN)_6$ (1%) were mixed and incubated at 50°C for 20 minutes. The reaction was stopped by adding 100 µL of 10% (w/v) trichloroacetic acid, followed by centrifugation at 3000 rpm for 10 minutes. Subsequently, 100 µL top layer was mixed with 100 µL distilled water and 25 µL of 0.1% $FeCl_3$ solution. The absorbance was measured at 710 nm, and IC_{50} value was calculated by plotting the absorbance against the corresponding sample concentration. In this study, all tests were carried out in triplicates.[27]

In vitro Anticancer Assay

HeLa, T47D, and WiDr cells, which were 80-90% confluent for harvest, were taken from CO_2 incubator. Furthermore, the number of cells was counted and diluted with a complete medium. A total of 100 µl cells, each with a specific density, were transferred into the wells. The condition of the samples was observed with an inverted microscope to assess their distribution. The samples were incubated in CO_2 incubator overnight, and after returning to normal condition for 1 x 24 hours, a sample concentration series was made (15.625 - 500 µg/mL) for the treatment (including cell control and media control). The plate containing the cells was taken from CO_2 incubator, then the medium was discarded. A series of sample concentrations of 100 µL were put into wells (triplo) and incubated in CO_2 incubator for 24 hours. MTT reagent was placed in PBS (0.5 mg/ml) and diluted with complete medium to 10.0 ml. The medium was removed, and 100 µl MTT reagent was added to each well.[28-29] Cells were incubated for 4 hours in CO_2 incubator and examined with an inverted microscope. When formazan was formed, 100 µl DMSO was added, and the plate was wrapped in aluminum foil, shaken, and incubated in a dark place at room temperature for 15 minutes. The absorbance of each well was read with a plate reader at a wavelength of 595 nm.[30]

Data Analysis

All assays were carried out in triplicate with a sample of EE, HF, EAF, and MF. The results were expressed as mean values ± standard deviation (SD) using SPSS version 20. The statistical differences were represented through a one-way analysis of variance (ANOVA) followed by Tukey's test.

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Results and Discussion

Total Phenolic and Total Flavonoid Contents

Secondary metabolites, such as phenolics and flavonoids played a major role in antioxidant and anticancer activities.[31] Furthermore, the presence of phenolics and flavonoids in OpE was in line with previous studies where *Opuntia dillenii* contained phenolic compounds qualitatively using the ellagic acid test. Furthermore, the test was carried out by reacting the extract with a few drops of 5% glacial acetic acid and 5% sodium nitrite to form a cloudy solution and a brown precipitate.[32] Flavonoid content was also found in *Opuntia ficus-indica* qualitatively by reacting the extract with a few drops of sodium hydroxide solution until a dark yellow color was formed, which turned colorless.[33]

TPC and TFC in extracts and fractions are presented in Figure 1. TPC of OpE in EAF was 9.84 ± 0.55 mg GAE/g but was not significantly different from the levels produced by HF at 9.70 ± 2.88 mg GAE/g. Based on the literature, the results were very relevant to TPC in *Dendrophthoe pentandra* plants, which were divided into three fractions, namely the hexane, ethyl acetate, and water fractions. The highest total polyphenol content was in EAF (67.40 ± 0.82 mg GAE/g), followed by HF (21.35 ± 1.86 mg GAE/g). The high TPC in EAF was due to the presence of polyphenols, which had the same molecular weight as compounds dissolved in the ethyl acetate solvent, such as tannins and flavonols. The high total phenolic compounds in HF compared to other more polar fractions was due to the presence of low polarity phenolic components in the soluble extract, which tended to dissolve in nonpolar solvents. Meanwhile, most of the semipolar phenolic compounds were more soluble in ethyl acetate compared to other organic solvents.[34]

HF had the highest flavonoid content with a value of 67.05 ± 9.94 mg QE/g, which was six times greater compared to EAF. The results were consistent with previous studies, where *Opuntia ficus-indica* extracted using n-hexane solvent had the highest total flavonoid content compared to other solvents.[35] Similar results regarding the highest content in the nonpolar hexane fraction were reported in the *Corydyline terminalis* (68.02 ± 2.15 w/w)[36], *Clinacanthus nutans* (434.98 ± 0.59 mg QE/g)[37] and *Nasturtium officinale* (64.52 ± 2.69 mg of RE/g of dry extract)[38]. The high flavonoid content in HF was caused by the presence of flavonoid compounds dissolved in nonpolar solvents. Several types of flavonoids could dissolve in nonpolar solvents, including polymethoxy aglycones or isoflavones whose sugar groups or glycoside forms had been removed, leading to the dissolution in nonpolar solvents.[39]

Antioxidant Activities

EE had the best antioxidant activity with ABTS method (339.48 ± 4.22 µg/mL) compared to HF and MF. These results were confirmed by a study on *Opuntia ficus-indica* bark using DPPH radical scavenging activity method (78.20 ± 2.72% at a concentration of 100 µg/mL), while an activity equivalent to 1.20 ± 0.07 µg GAE/100 g dry weight was obtained when measured using reducing power activity at 700 nm.[40] Previous studies showed that the antioxidant effect of this plant was due to the presence of phenolics and flavonoids.[41] These constituent compounds had been identified as more effective antioxidants compared to vitamins due to the ability to delay pro-oxidative effects on proteins, DNA, and lipids by forming stable radicals.[41]

EAF had the best antioxidant activity compared to HF and MF, using ABTS and FRAP methods, as shown in Table 1. The high activity in EAF was caused by the presence of certain secondary metabolite compounds soluble in ethyl acetate. These compounds included phenolics, chlorogenic acid derivatives, quercetin O-glycosides, kaempferol O-glycosides, and isorhamnetin O-glycosides.[11] Similar results were reported by Das *et al.*[43] which examined EE and fraction of *Crescentia cujete*. EAF in the leaves and bark of this plant had the greatest radical scavenging activity with IC_{50} of 8.78 and 18.34 µg/mL, respectively. High phenol levels were shown in EAF, namely 371.23 ± 15.77 mg of GAE/g (leaves) and 326.75 ± 4.66 mg of GAE/g (bark) compared to the chloroform and water fractions. A positive correlation (r^2) of 0.9268 indicated an increase in phenolic compounds, which was directly proportional to an increment in antioxidant activity.

The mechanism of action of EAF as an antioxidant was related to redox compared to scavenging free radicals. This was proven by IC_{50} value of FRAP of 106.72 ± 1.60 µg/mL, which was smaller compared to ABTS with IC_{50} of 313.65 ± 1.94 µg/mL. The reduction of ferric cyanide ion $[Fe(CN)_6]^{3-}$ to ferrous cyanide $[Fe(CN)_6]^{4-}$ occurred due to direct electron donation by compounds in EAF. This compound also had a dose-dependent effect on preventing cation radicals from ABTS, but its mechanism as a reducing agent was more dominant.[43]

Relationship between TPC and TFC and Antioxidant Activity

The highest phenol content was found in EAF, which was directly proportional to antioxidant activity tested using FRAP. Several studies also reported that the higher the phenol content, the greater the antioxidant activity. Based on the results, phenolic compounds in EAF were suspected to significantly contribute to warding off oxidants by reducing the oxidation number. The antioxidant effect of phenols (polyphenols) was associated with the free radical scavenging activity, metal-chelating ability, and inhibition of enzymes related to oxidative processes. These properties were primarily due to the phenolic hydroxyl groups attached to the ring structure.[44-45] The flavonoid content in EAF (11.99 ± 0.79 mg QE/g sample) was higher compared to the phenol content. This strengthened the prediction that flavonoid and phenolic compounds influenced antioxidant activity by inhibiting ABTS radicals and redox mechanisms. The antioxidant activity of flavonoids in reducing free radicals was due to the presence of hydroxyl groups in these compounds, where the antioxidant power depended on the number of hydroxyl groups bound to ring B. The more hydroxyl groups bound to ring B, the stronger the flavonoid compound was in warding off free radicals. This was because the hydroxyl group on ring B played a role in stabilizing the aryloxy radical compound. The presence of hydroxyl group donors in radical compounds made previously unpaired and unstable electrons become repaired and stable. The process often transformed previous radical compounds into non-radical due to the contribution of hydroxyl groups in antioxidant compounds. Furthermore, ortho-hydroxyl substitution in ring B or A played an essential role in radical inhibition.[46]

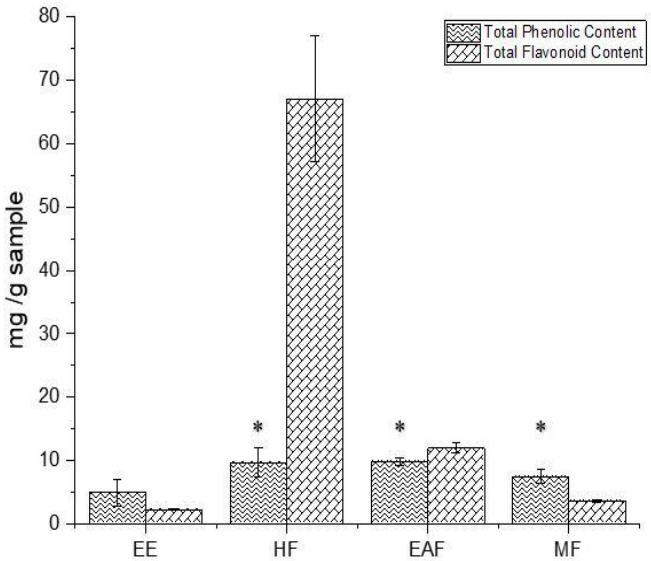

Figure 1: Total phenolic and total flavonoid contents of *Opuntia elatior* Mill. (OpE) extract and fraction (mg/g sample). EE: Ethanolic Extract, HF: n-Hexane Fraction, EAF: Ethyl Acetate Fraction, MF: Methanol Fraction. Data were analyzed using Tukey test $p \leq 0.05$, n=3. (*) no significant differences.

Anticancer Activities

EE had the best anticancer activity against WiDr compared to HF, EAF, and MF with IC_{50} value of 612.90 ± 1.02 µg/mL. This was in line with a study on EE of *Opuntia ficus-indica*, containing phenolics, flavonoids, and tannins, which had the highest anticancer activity. Furthermore, the anticancer effect of the extract was likely due to the presence of Betanin, which effectively inhibited lipid peroxidation and heme decomposition. This suggested that the pigment could protect the body against certain oxidative stress-related disorders. The highest anticancer activity in EE was due to a higher percentage of polyphenols, and this played an important role in antioxidant activity. This result was supported by previous studies regarding the effect of EE on Ehrlich ascites carcinoma cells, where the results showed cell death of 24 ± 0.31% at a concentration of 100 µg/mL.[40]

The results of this study were consistent with Rohmah et al.[47], where EE of mangosteen peel provided cytotoxic activity against WiDr with IC_{50} value of 25 µg/mL. With IC_{50} value of EE of mangosteen peel, the sample could be classified as an effective chemotherapy agent. The anticancer activity of EE and EAF had IC_{50} values that were not significantly different (Table 2) in WiDr, strengthening the conclusion that the secondary metabolite compounds acting as anticancer agents were phenolics with the semipolar nature. Natural phenolic compounds were often considered more soluble semipolar solvents.[48] The results were in line with Wang et al.[49], that ethyl acetate, a semipolar solvent, produced a better cytotoxic effect on cancer cells compared to polar and nonpolar solvents due to the ability to induce ROS-dependent apoptosis and autophagy. This was supported by previous studies, where EAF showed the best cytotoxic activity against WiDr.[50]

Anticancer activity tests were carried out to study the best anticancer potential of various OpE fractions. The best cytotoxic activity of HeLa cells was shown in EAF with IC_{50} value of 693.61 ± 50.63 µg/mL, as shown in Table 2. The results were in line with Baker[51], that EAF of *Achillea millefolium* L had the highest potential against HeLa. Another study regarding the cytotoxicity study of *Pseudocedrela kotschyi* in cervical cancer (HeLa) showed that it had the highest activity.[52] EAF also showed the best cytotoxic activity against WiDr with IC_{50} value of 627.69 ± 36.27 µg/mL. The results were in line with Pasaribu et al.[53], that EAF from *Saurauia vulcani* leaves showed strong cytotoxic activity against WiDr cancer cells. Other studies regarding the activity of *Vernonia amygdalina* Del. on WiDr showed that EAF had the lowest IC_{50} value in the test results. Based on the results, it had powerful cytotoxic activity against WiDr.[54]

The cytotoxic activity of OpE was thought to originate from its phenolic content. This was supported by the highest TFC value in EAF (9.84 ± 0.55 mg GAE/g) compared to other extracts and fractions, as shown in Figure 1. EAF had the highest phenol content because ethyl acetate was a semipolar solvent that could attract polar and nonpolar compounds and tended to be acidic, such as phenolic compounds.[55-56] Previous studies showed that the highest content of phenolic compounds was found in the ethyl acetate extract of *Opuntia littoralis*[57] and EAF of *Opuntia humifusa*.[58] The potential of phenolic compounds as anticancer agents was due to antioxidant activity, namely as free radical scavengers, metal chelators, and modifiers of endogenous defense mechanisms, such as superoxide dismutase. (SOD), catalase (CAT), glutathione peroxidase (GPx), and glutathione redox enhancer (GSH). Furthermore, the anticarcinogenic effects were related to the ability to inhibit cell proliferation (extracellular signal-regulated kinase (Erk)1/2, D-type cyclin, and cyclin-dependent kinase (CDK)), angiogenic factors (endothelial growth factor vascular (VEGF) and MIC-1), oncogenic signaling cascades (phosphoinositide 3-kinase (PI3K) and protein kinase B (Akt)), induction of apoptosis, and prevention of cellular migration and metastasis.[22]

Based on morphological differences, cell death could be divided into two, including reproductive and interphase types. Reproductive cell death was related to the mitotic cycle, while the interphase type often occurred before the mitotic phase and after cells divided and differentiated. Cell death was differentiated based on morphology and various methods or processes, such as apoptosis, autophagy, necrosis, senescence, and mitotic death. Apoptosis was a method or process of programmed cell death caused by conditions in the cell itself, such as DNA damage or external stimuli. Furthermore, it was a normal condition of various physiological processes needed to maintain homeostasis. This showed that organisms could also use the process to eliminate inactive and unneeded cells from the body. Several studies showed that failure to control apoptosis often led to various types of diseases, including cancer.[59]

Apoptosis could occur in several pathways, including the extrinsic pathway, which began from the binding process with cell death ligands. This process could also proceed through the intrinsic pathway, where changes in the mitochondrial membrane potential through the release of cytochrome-c from the mitochondria into the cytoplasm were observed. These pathways had the ability to cause the activation of the caspase enzyme. The apoptosis process began in the mitochondria, through DNA damage or due to a response from the cell membrane.[60] DNA damage could induce this process through p-53 due to increased activation of the pro-apoptotic protein BAX. However, without p-53, the alternative apoptosis pathway could also occur by increasing energy only in the cell membrane.[61]

Morphological differences occurred in WiDr after adding EAF at a concentration of 500 µg/mL, as shown in Figure 2. This study found changes in the morphology of WiDr, which was caused by natural compounds in plants with the ability to induce the apoptotic pathway.[62] According to previous studies, apoptosis was a type of programmed cell death (PCD). Cytomorphological features of apoptotic cells included shrinkage, chromosome condensation, and DNA fragmentation.[63] During this process, the cell cytoplasm became electron-dense, apparently in response to shrinkage. Furthermore, translocation of damage-associated molecular patterns to the bleb cytoplasm and apoptotic functional bleb maturation often occurred.[64-65] The bleb expansion and retraction processes were highly dependent on the organized actin cytoskeleton beneath the plasma membrane. Blebs were formed when the plasma membrane detached from the actin cytoskeleton. This was caused by a local increase in intracellular pressure or local rupture of the actin cytoskeleton.[66]

Relationship between TPC and TFC and Anticancer Activity

The highest anticancer activity of OpE was shown in WiDr at 612.90 ± 1.02 µg/mL in EE and 627.69 ± 36.27 µg/mL in EAF. This showed that all compounds acting as anticancer were attracted to the ethyl acetate solvent. Furthermore, the increase in WiDr anticancer activity was in line with the increment in phenol content in EAF, as shown in Figure 1. The results regarding phenolics role as WiDr anticancer were strengthened by Turan *et al.*[67], where *Dianthus carmelitaru* extract was cytotoxic to WiDr with IC_{50} value of 37 ± 1.5 µg/ml. The identification results of *Dianthus carmelitaru* extract showed that sinapic acid and benzoic acid were found as principal phenolic compounds.

Another observed phenomenon was that EAF had the highest activity on cervical cancer cells (HeLa) with IC_{50} of 693.61 ± 50.63 µg/mL. The role of phenolic compounds as cytotoxic agents in cervical cancer cells had been reported by Soumya *et al.*[68] Furthermore, this study showed moderate cytotoxic activity of gallic acid compounds isolated from *Terminalia chebula* with IC_{50} value of 72.68 ± 2.08 µg/ml. Teniente et al. strengthened the role of phenolics as antineoplastics in in-vitro cervical cancer testing by regulating the induction of apoptosis, cell cycle arrest, and modulating different signaling pathways.[69] Long et al. explained that these compounds played a role in inhibiting the activity of WiDr by modulating the production of inflammatory cytokines through several signaling pathways both *in vitro* and *in vivo*, such as the NF-κB, MAPK, PI3K/AKT, Wnt/β-catenin, and c-Jun N-terminal kinase (JNK) pathways.[70]

Flavonoid compounds were also thought to have a role in increasing the anticancer activity of EAF on HeLa and WiDr. This was confirmed by the second-highest OpE content in EAF at 11.99 ± 0.79 mg QE/g. The compound Hibiscetin-3-glucoside, a group of flavonoids isolated from *Hibiscus rosa sinensis* flower petals, had been proven to have antioxidant activity with an IC_{50} value of 2.82 µg/ml. The antioxidant mechanism was caused by glycosides, which effectively produced hydrogen radicals due to the presence of free hydroxyl groups and could reduce free radicals.

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Table 1: IC_{50} values (µg/mL) of *Opuntia elatior* Mill. (OpE) extract and fraction for antioxidant activity

Assays	EE	HF	EAF	MF
ABTS	339.48 ± 4.22	424.78 ± 5.12	313.65 ± 1.94	452.09 ± 3.37
FRAP	526.53 ± 35.76	210.38 ± 17.33	106.72 ± 1.60	302.46 ± 11.80

Note: Values represent means ± SD for triplicate experiments

Table 2: IC_{50} values (µg/mL) of *Opuntia elatior* Mill. (OpE) extract and fraction for anticancer activity

Cell Lines	EE	HF	EAF	MF
HeLa	1839.21 ± 575.05	1144.19 ± 290.74	693.61 ± 50.63	Not Active
T47D	1145.19 ± 324.61	2399.75 ± 482.38	1551.62 ± 305.03	1497.03 ± 340.49
WiDr	612.90 ± 1.02	1213.86 ± 123.62	627.69 ± 36.27	2236.25 ± 885.00

Note: Values represent means ± SD for triplicate experiments. Not active: no cell death at sample concentration of 500 µg/mL.

This study suggested that the structure of Hibiscetin-3-glucoside from *Hibiscus rosa sinensis* flower petals could also be used as an effective anticancer drug.[71] Flavonoid compounds acting as cytotoxic agents against WiDr had been reported by Kristiani *et al.*[51] in the *Artemisia cina*. The study showed that the best antiproliferative activity was found in *Artemisia cina* ethyl acetate extract with IC_{50} value of 229.5 ± 19.9 µg/ml. The flavonoid compounds found in *Artemisia china* were quercetin and kaempferol.[72] This explained the mechanism of the cytotoxic activity of the flavonoid quercetin against WiDr, including the induction of apoptosis through activation of p53 and inhibition of NFκB, cell cycle arrest due to downregulation of cell cycle genes, and suppression of inflammation through downregulation of COX2, which was generally upregulated in colon cancer.

Relationship between Antioxidant and Anticancer Activity

Oxidant/antioxidant balance had been suggested to be an important factor in the initiation and progression of cancer.[73] ROS were involved in all stages of cancer development, including initiation, promotion, and progression. At the initiation stage, ROS-induced DNA mutations could accumulate if not repaired in cancer tissues. Excessive ROS production could also cause oncogenic DNA mutations, potentially contributing to the emergence of cancer.[74] Antioxidants were molecules that could prevent or slow down the oxidation of macromolecules.[75] The human body had several mechanisms to fight oxidative stress, including the natural production of antioxidants. These compounds were considered to be naturally occurring in the body (endogenous) or supplied externally through food and/or supplements (exogenous). Endogenous and exogenous antioxidants served as "free radical scavengers" by preventing and repairing damage caused by ROS to increase immune defense and reduce the risk of cancer.[73] Antioxidants neutralized free radicals by donating one of their electrons and ending the electron "stealing" reaction, thereby preventing cell and tissue damage mediated by ROS. These compounds were often described as free radical "scavengers," due to the ability to neutralize electrical charges and prevent free radicals from taking electrons from other molecules.[76]

The results showed that EAF had the best cytotoxicity against WiDr, as shown in Table 2. This study showed that the best OpE antioxidant activity was in EAF, with the highest TPC results. Furthermore, the best cytotoxic activity against cancer cells was tested in EAF, and this was supported by previous studies regarding EE of *Angiopteris ferox*. The results found that EAF had the highest antioxidant activity with IC_{50} of 19.55 µg/ml. The best anticancer activity was also obtained from EAF against WiDr with IC_{50} of 94.27 µg/ml.[77] In another study regarding the cytotoxic effect of EE and *Calotropis gigantea* fraction, the samples showed high cytotoxicity against WiDr with IC_{50} of 41.79 µg/ml.[78]

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Conclusion

In conclusion, this study showed that TPC and TFC in EAF could increase antioxidant and anticancer activity. Furthermore, the presence of these compounds in EAF contributed primarily to anticancer activity against WiDr.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.\

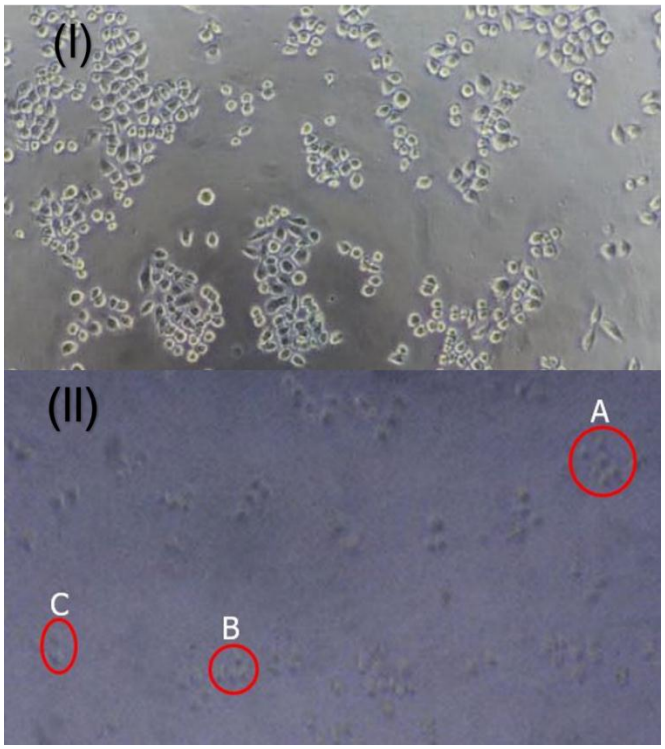

Figure 2: Morphology of WiDr (I) and WiDr apoptotic cell after treatment EAF 500 µg/mL (II). Blebs (A), Spikes (B), and Blisters (C).

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