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Evaluation of the Anticancer Potential of Medicinal Plant Extracts Against Colorectal Cancer: A study on *Croton megalocarpus, Terminalia brownii, Prunus africana*, and *Warburgia ugandensis*

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Abstract

Colorectal Cancer (CRC) is globally prevalent, ranking second in mortality (9.3%) and third in incidence (9.6%). Chemotherapy has significant side effects, necessitating alternative treatments. Historically, medicinal plants and natural products have been of interest. This study investigates the anticancer activity of various plant extracts: ethanolic extract of *Croton megalocarpus* leaves, *Terminalia brownii* bark, ethyl acetate extract of *Prunus africana* bark, and chloroform and acetone extracts of *Warburgia ugandensis* bark on a colon adenocarcinoma cell line. Additionally, the study also explores mechanistic cell death pathways (apoptosis or necrosis). Cytotoxic effects using a neutral red (NR) uptake assay was assessed on a colon adenocarcinoma cell line. DNA Damage characterized using the Alkaline comet assay. Cytotoxicity and Plasma Membrane Damage evaluated using the lactate dehydrogenase (LDH) Leakage Assay. Mechanistic Pathways explored by measuring mRNA expression levels to distinguish between intrinsic and extrinsic apoptosis pathways (P53, Bax, Bcl-2, Bcl-xl for mitochondrial pathway; caspase-8 for extrinsic pathway; caspase-3 for apoptosis activity; survivin as a marker for apoptosis). IC50 values determined for the extracts: *C. megalocarpus* leaves (45.5 µg/ml), *T. brownii* bark (41 µg/ml), *P. africana* bark (70.2 µg/ml), and *W. ugandensis* bark (4.4 µg/ml for chloroform, 20.7 µg/ml for acetone). Apoptosis: Induced in all treatments except for *C. megalocarpus* leaves ethanol extract due to a significant decrease in survivin expression.

Keywords: Medicinal plant; anti-cancer; Alkaline comet assay;qRT-PCR; apoptosis; necrosis

1. Introduction

With an expected 9.7 million fatalities and 20 million new cases of Cancer in 2022, lung cancer remained the top cause of cancer death for both sexes, accounting for 18.7 percent of all cancer deaths. It was found for mortality colorectal (9.3%), liver (7.8%), and stomach (6.8%), and for incidence lung (12.4%), breast (11.6%), colorectal (9.6%), and prostate (7.3%). So lung cancer takes the first position for incidence in both sexes, followed by colorectum and liver cancer (for mortality) and breast and colorectum cancer (for incidence) [1].

Unfortunately, doctors have determined that no treatment can eradicate cancer cells. Still, some malignancies may be curable with specific treatments, so They combat Cancer by enhancing internal components and the immune system's ability to destroy tumors. Compared to many conventional tumor treatments, medicinal plant treatment has a higher success rate [2-4].

Docetaxel, mitomycin, and cisplatin are all chemotherapy medicines that cause severe side effects or persistent problems [5-8]. Liver damage, loss of hearing, kidney failure, loss of blood cell count, vein damage, and nerve damage are some of the reported side effects of conventional cancer treatments. Other undesired symptoms include bone necrosis, skin devascularization, immune suppression, and lung fibrosis [9,10]. Due to the quick partitioning of mucosa cells during therapy, hemorrhagic cystitis and drug toxicity may damage multiplying cells such as endothelial, fibroblasts, or parenchymal stem cells. And lead to vascular damage and chronic fibrosis [11,12].

For that, using a natural product, like a crude extract of a medicinal plant or active compound, is beneficial in treating cancer [2,13]. Approximately 80% of the global population relies on traditional medicinal systems, and medications derived from plants have considerable therapeutic worth [14]. Nearly a quarter of all newly authorized anti-cancer drugs were sourced from natural substances between 1981 and 2019 [15].

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The two primary ways of cell death are apoptosis and necrosis [16,17]. Two different pathways can activate apoptosis: intrinsic pathway (mitochondria) and extrinsic (death receptor) [18,19]. Therefore, this study aimed to investigate different plant extracts in the cancer cell death pathway on the Caco-2 cell line via an in vitro investigation.

2. Results and Discussion

2.1. Cytotoxicity effects of extracts

The impact of toxicity on the proliferative capacity of Caco2 cells was assessed using the Neutral Red assay. The viability of Caco-2 cells was evaluated after they were treated with varying concentrations for 24 hours. As shown in (Fig. 1), The half maximal inhibitory concentration (IC50) calculated for Ethanolic extracts of *C. megalocarpus* Leaves on Caco2 cells was 45.5 µg/ml, as shown the ethanolic extracts of *T. brownii* bark, and the ethyl acetate extract of *P. africana* bark, were 41 µg/ml, and 70.2 µg/ml respectively, while, The IC_{50} values for the chloroform and acetone extracts of *W. ugandensis* bark were 4.4 µg/mL and 20.7 µg/mL, respectively

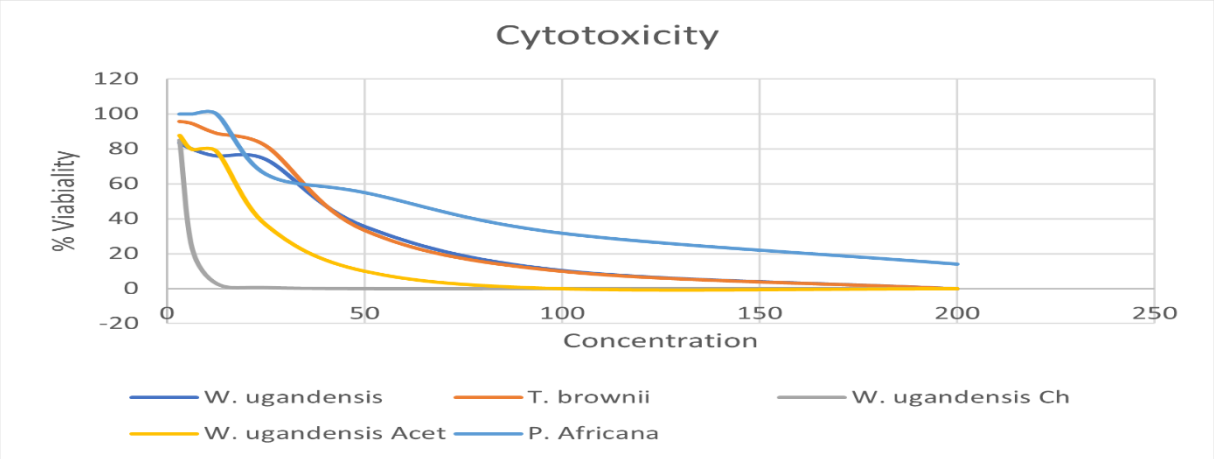

Figure 1. Dose-Response Curve of Different Plant Extracts and the IC50 (µg/ml). All are against the Caco-2 cell line. Cells were treated for 24 hr., and cell viability was determined using NR uptake assay. Display broad efficacy in reducing cell viability. *C. megalocarpus*: ethanol extracts of *C. megalocarpus* Leaves, *T. brownii*: ethanol extracts of *T. brownii* bark, *W. ugandensis*ch: chloroform extract *W. ugandensis* bark, *W. ugandensis* Acet: acetone extract *W. ugandensis* bark, and *P. africana*: ethyl acetate extract of *P. africana* bark

C. megalocarpus exhibits significant cytotoxic activity against multiple cancer cell lines, including Caco-2 cells. Studies demonstrate that extracts from this plant can induce apoptosis in cancer cells, as shown by the activation of caspases, which are essential in the apoptotic pathway [29]. Moreover, it was emphasized that the leaf extracts of *C. megalocarpus* demonstrate significant antiviral efficacy with minimal toxicity, indicating a favourable potential for therapeutic use [30]. The existence of bioactive compounds in Croton species, including alkaloids and terpenoids, contributes to their cytotoxic effects, which warrants further investigation into their mechanisms of action [31].

T. brownii, although not extensively researched about Caco-2 cells, is acknowledged for its traditional medicinal applications. Its extracts exhibit antibacterial properties, which may be advantageous in addressing infections that aggravate cancer treatments [32]. The bark of Terminalia species contains numerous bioactive substances that may improve the effectiveness of cancer treatments by reducing infection risks [33].

P. africana is another notable plant in this setting. The bark extracts have been recorded for their cytotoxic effects on cancer cells, particularly Caco-2 cells, and exhibit anti-inflammatory activities that may help mitigate tumor-associated inflammation [34]. The extractive components of the bark have demonstrated the ability to suppress cancer cell proliferation, positioning it as a possibility for further investigation in cancer therapy [35]. Moreover, the plant's extracts have shown promise in alleviating symptoms related to prostate cancer, underscoring its significance in oncological research [36].

W. ugandensis has been examined for its cytotoxic properties. Research indicates that extracts from this plant demonstrate considerable antibacterial and cytotoxic properties, with IC_{50} values indicating effective concentrations against many infections [37]. The plant's bioactive compounds may interact with cellular pathways involved in cancer progression, making it a valuable subject for further pharmacological studies [38].

2.2. Alkaline comet assay

The comet assay involves several measures, with tail length, % DNA in the tail, tail moment, and Olive tail moment being the most frequently utilized. (Table 1). At relatively modest levels of damage, the tail length increases, and the percentage of DNA in the tail (tail intensity) increases as the damage increases, but it does not affect the tail length. The Tail moment is the sum of the tail length and the percentage of DNA in the tail. Both the tail moment and the tail length do not exhibit a linear dose-response and are more susceptible to effects from background parameters and thresholds [39-41].

Nevertheless, both OTM and %Tail DNA may be employed for scientific purposes. [42]. The COMET assay's % DNA in Tail and OTM measurements have been proven reliable for detecting dose responses in DNA damage. The most frequently recommended and selected measurement is the percentage of DNA in the tail, as some perceive it as being more easily comprehensible and enabling differentiation on a broad scale [43,44].

Table 1: DNA Damage Tail Parameters in CACO-2 Cells Treated with Various Plant IC_{50} Extracts and Negative Control by the Comet Assay

Sample	Tail Length (px)	%DNA in Tail	Tail Moment	Olive tail moment
Negative control	10.5 ± 0.28 ^{ab}	1.52 ± 0.45 ^{cd}	0.097 ± 0.01 ^{cd}	0.23 ± 0.06 ^{d}
*W. ugandensis a*cetone	7.44 ± 0.60 ^{c}	1.18 ± 0.08 ^{d}	0.077 ± 0.01 ^{d}	0.29 ± 0.01 ^{cd}
*T. brownii*ethanol	8.88 ± 0.54 ^{abc}	2.58 ± 0.53 ^{bcd}	0.27 ± 0.07 ^{bc}	0.45 ± 0.03 ^{bc}
P. Africana EtOA	8.52 ± 0.52 ^{bc}	3.41 ± 0.57 ^{b}	0.32 ± 0.07 ^{b}	0.42 ± 0.05 ^{bc}
*C.megalocarpus*ethanol	8.76 ± 0.38 ^{abc}	2.88 ± 0.49 ^{bc}	0.33 ± 0.08 ^{b}	0.54 ± 0.1 ^{b}
*W.ugandensis*chloroform	11.11 ± 0.27 ^{a}	5.62 ± 0.30 ^{a}	0.62 ± 0.04 ^{a}	0.92 ± 0.01 ^{a}

The data is provided as mean ± SD (n=3). The mean values within the same column with different superscript letters are significantly different.

However, the drawback of the % DNA in Tail measurement is that it relies solely on a single measurement derived primarily from the quality of a digital image (Fig. 2). Measurement is highly susceptible to instrumental factors such as variations in camera sensitivity, uniformity and intensity of the light source, and the quality of the microscope lens and/or cleanliness. It is also influenced by the settings used for image analysis [43,45]. In addition, it can distort DNA migration by disregarding crucial aspects such as the distribution and distance of migration [46]. So, the Olive tail moment (OTM) will be considered in this study[42-44]. (Fig. 3).

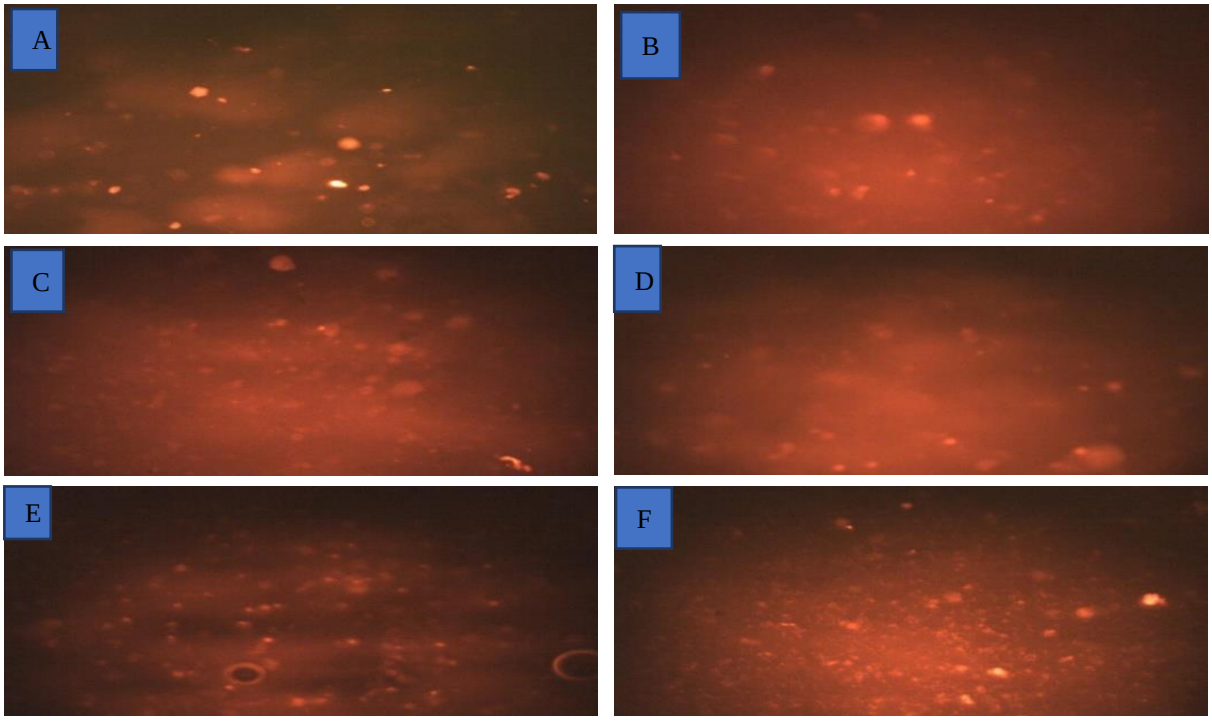

Figure 2. DNA damage following the administration of plant extracts CACO-2 cells were observed with fluorescence microscopy for 24 hours. (A) Negative control (unblemished); (B) 20.7 µg/ml acetone extract treatment of *W. ugandensis* bark (C) The ethyl acetate extract treatment of *P. africana* bark was found to be 70.2 µg/ml. (D) The ethanol extract treatment of *T. brownii* bark was 41 µg/ml. (E) The ethanol extract treatment of *C. megalocarpus* Leaves was 45.5 µg/ml. (F) The chloroform extract treatment of *W. ugandensis* bark was 4.4 µg/ml.

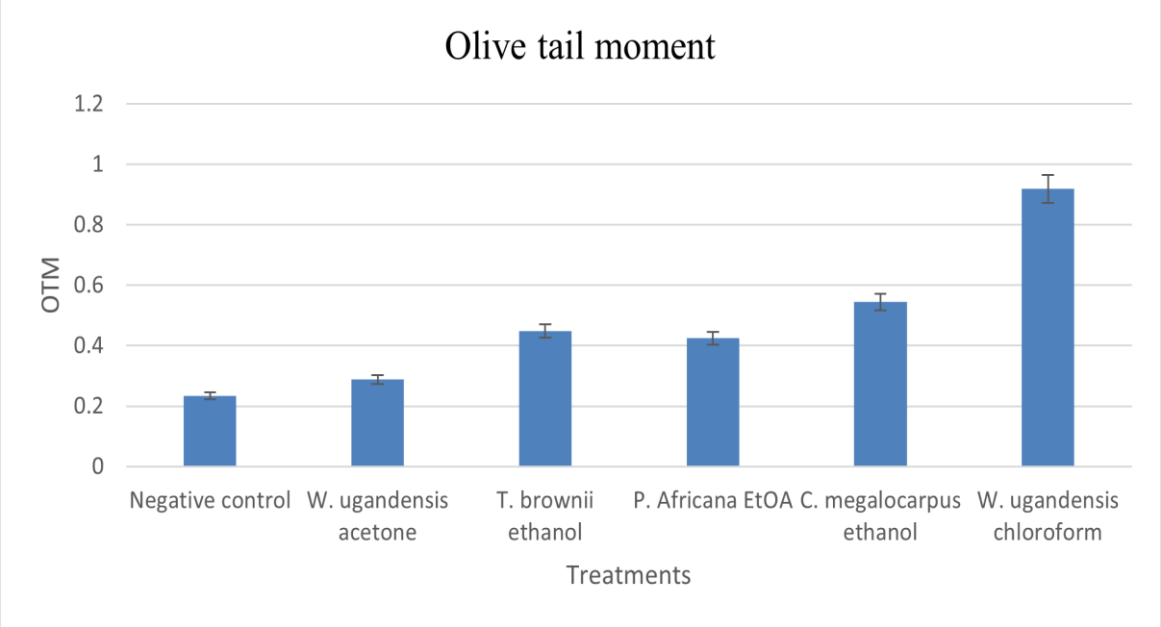

Figure 3. The average median of the Olive tail moment of CACO-2 cells after 24 hours of treatment with plant extracts. EtOA: ethyl acetate. The values are plotted as mean ± S.E.

The highest Olive tail moment (OTM) value was observed for the chloroform extract of *W. ugandensis* (0.92 ± 0.01), followed by the ethanolic extract of *C. megalocarpus* leaves (0.54 ± 0.10).

Moderate responses were recorded for the ethanolic extract of *T. brownii* bark, the ethyl acetate extract of *P. africana* bark, and the acetone extract of *W. ugandensis* bark, with no significant differences observed between them (0.45 ± 0.03, 0.42 ± 0.05, and 0.29 ± 0.01, respectively). The lowest OTM was recorded for the negative control (0.23 ± 0.06), with no significant difference compared to the acetone extract treatment of *W. ugandensis* bark.

2.3. Lactate dehydrogenase leakage measurement

Lactate dehydrogenase leakage(LDH), a stable cytoplasmic enzyme present in cells, is typically retained within the cell; however, it may be discharged into the cell culture supernatant in case of cell membrane damage. [47]. As a result, the evaluation of LDH levels in the supernatant can indicate cellular damage and cytotoxicity. A notable rise in LDH release was observed in negative control cells; nevertheless, treatment with the cell extracts significantly reduced LDH leakage.

The most substantial inhibition, reaching 90%, was achieved with the acetone extract from *W. ugandensis* bark, while the lowest inhibition, at 35%, was seen with the ethanol extract from *T. brownii* bark. These findings highlight the extracts' ability to preserve cell membrane integrity and prevent the release of LDH from Caco-2 cells into the culture supernatant.

As shown in Fig. 4, the level of LDH activities was detected, and the highest value recorded was (279.23 ± 3.4) U/L for the cell line without any treatment.

The lowest was (28.3 ± 0.6) U/L for treatment with acetone extract of *W. ugandensis*bark. Ethanol extract of *C. megalocarpus*leaves had an LDH of 56.6 ± 0.9. In contrast, ethyl acetate extract of *P. africana* bark, chloroform extract of *W. ugandensis*bark, and ethanol extract of *T. brownie* bark *had* 101.13 ± 1.4, 149.73 ± 1.75, and 182.03 ± 2.3 respectively. Differences between all interventions under investigation were statistically significant ($p > 0.05$).

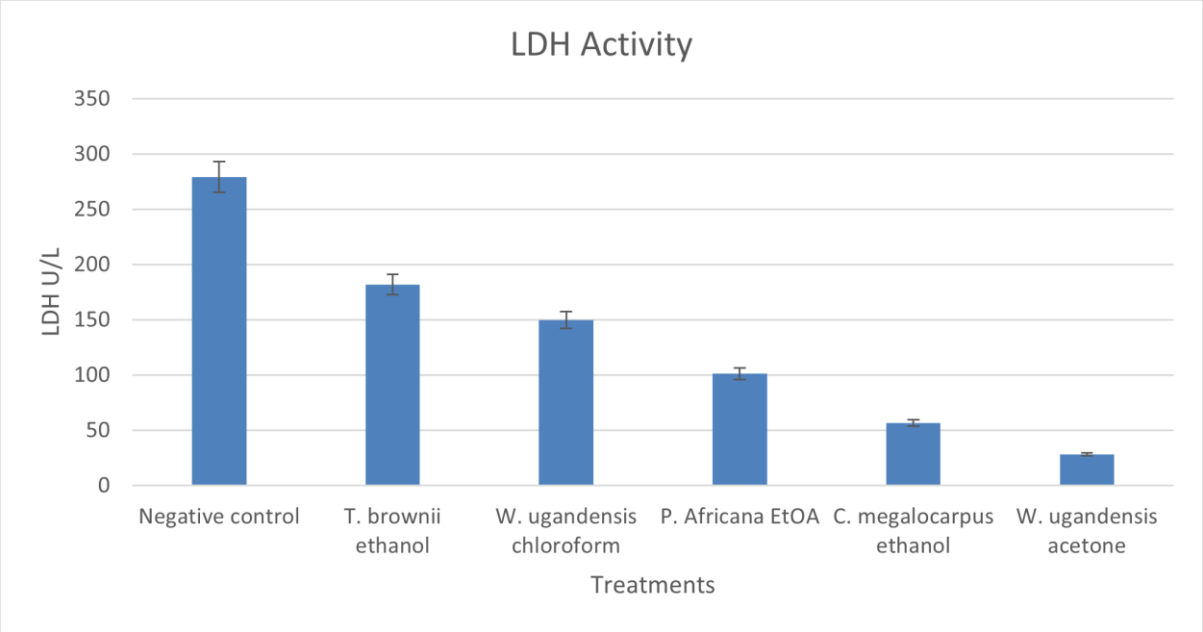

Figure 4. the effects of different plant extracts on LDH activity level compared to negative control results are means ± S.E. (n =3). The levels of lactate were significantly different between colon tumor cells and between treatments (P <0.05).

2.4. Analysis of the expression levels of anti-apoptotic and apoptotic Genes

The expression levels of key apoptotic and anti-apoptotic markers were assessed for extracts obtained from W. ugandensis bark (Wub), T. brownii bark (Tbb), P. Africana bark (Paf), and C. megalocarpus leaves (CML) using various solvents.

W. ugandensis bark (Wub) Chloroform Extract (Wub Chlo) Among all tested extracts, Wub Chlo demonstrated the strongest pro-apoptotic activity, as indicated by the highest activation of CASP-8 (4.08) and CASP-3 (1.02), key executioner caspases responsible for apoptosis initiation. Additionally, the extract showed moderate suppression of BCL-2 (1.81) and BCL-XL (-0.12), suggesting inhibition of anti-apoptotic signaling. These findings highlight Wub Chlo as the most effective apoptosis inducer among tested extracts.

W. ugandensis bark (Wub) Acetone Extract (Wub Acet) Wub Acet exhibited comparable pro-apoptotic effects to Wub Chlo but with slightly lower caspase activation (CASP-8: 3.43). The mild activation of P53 (0.18) suggests a potential DNA damage response, which may contribute to the apoptotic effects. While potent, its effectiveness is slightly weaker than that of Wub Chlo.

T. brownii bark (Tbb) Ethanol Extract (Tbb Ethan) The Tbb Ethan extract showed mixed effects, demonstrating moderate CASP-8 activation (1.81) alongside strong BCL-2 upregulation (2.12). The increase in BCL-2 expression suggests a pro-survival effect that may counteract apoptosis, making this extract less effective as a standalone apoptosis inducer. Further optimization or combination strategies may be required to enhance its therapeutic potential.

P. Africana bark (Paf) Ethyl Acetate Extract (Paf EtoA) Pdf EtoA exhibited notable suppression of Survivin (-0.63), a key anti-apoptotic protein that contributes to tumor survival. Additionally, moderate CASP-8 activation (1.41) was observed, indicating apoptosis induction. These findings suggest that Pdf EtoA may be particularly effective in cancers where survivin overexpression is a key factor.

C. megalocarpus leaves (CML) Ethanol Extract (CML Ethan) CML Ethan displayed the weakest apoptotic response, with minimal activation of CASP-8 (0.35) and slight P53 suppression (-0.76). The limited effect on apoptosis markers suggests that CML Ethan may have low therapeutic relevance as an apoptosis-inducing agent and may require reformulation with a different solvent or complementary treatment strategies.to compare and describe the results we plot fold-change as logarithm value Fig. 5.

The findings of this study highlight the crucial role of solvent selection in modulating apoptotic and anti-apoptotic responses in medicinal plant extracts. The *W. ugandensis* bark chloroform extract (Wub Chlo) demonstrated the strongest pro-apoptotic activity, as evidenced by the highest activation of CASP-8 (4.08) and CASP-3 (1.02). These caspases are central to apoptotic progression, particularly in the extrinsic pathway, suggesting that Wub Chlo contains bioactive compounds that directly initiate programmed cell death. The concurrent suppression of BCL-2 (1.81) and BCL-XL (-0.12) further supports its efficacy, as inhibition of these anti-apoptotic proteins enhances cell death pathways.

The *W. ugandensis* bark acetone extract (Wub Acet) exhibited a similar, albeit slightly weaker, pro-apoptotic effect with CASP-8 activation (3.43). Notably, a mild increase in P53 expression (0.18) suggests that Wub Acet may contribute to DNA damage response and intrinsic apoptotic signaling. This provides additional insight into its mechanism of action and potential as a therapeutic agent.

By contrast, *T. brownii* ethanol extract (Tbb Ethan) demonstrated a mixed response, with CASP-8 activation (1.81) suggesting moderate apoptosis induction. However, BCL-2 upregulation (2.12) indicates a potential resistance mechanism that may reduce overall efficacy. Ethanol-based extracts are known to interact with cellular membranes, potentially leading to oxidative stress-related apoptosis rather than direct caspase activation. The observed BCL-2 increase raises concerns about cytoprotective effects, necessitating further refinement of extraction conditions or combination strategies to optimize apoptosis.

The *P. africana* ethyl acetate extract (Paf EtoA) showed potential in targeting survivin-overexpressing cancers, as evidenced by Survivin suppression (-0.63), alongside moderate CASP-8 activation (1.41). Survivin is a critical regulator of apoptosis resistance in cancer cells, and its suppression enhances cell death. These findings indicate that Paf EtoA may be valuable for specific therapeutic applications, particularly in tumors reliant on survivin expression for survival.

Lastly, *C. megalocarpus* leaves ethanol extract (CML Ethan) exhibited weak apoptotic activation, with CASP-8 expression (0.35) and slight P53 suppression (-0.76). The limited apoptotic response suggests that ethanol may not effectively extract apoptosis-inducing compounds from *C. megalocarpus*. Reformulation with alternative solvents or combinatory approaches may be necessary to enhance its therapeutic potential.
Additionally, *P. africana* ethyl acetate, *W. ugandensis* bark acetone, and *W. ugandensis* bark chloroform extracts downregulated Bcl-xL, reinforcing their pro-apoptotic potential [69,70].

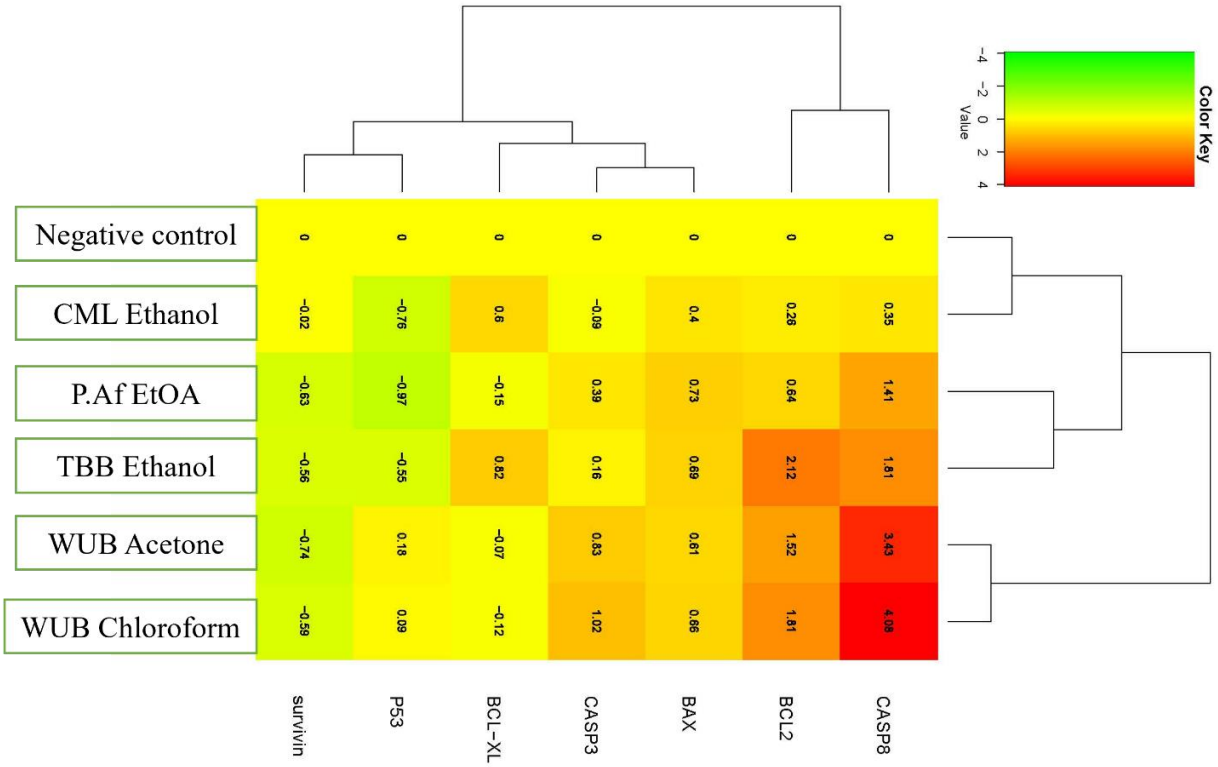

Figure 5. Gene expression heatmap. Up-regulated genes are coded with the red color, while yellow-coded genes are at normal expression levels (like the control). Green-coded genes represent the downregulated genes relative to the control. All values within the heatmap are plotted as Log transformation of mean ± S.E of fold changes. WUB: *W. ugandensis*bark, TBB: *T. brownie* bark, Paf EtOA: ethyl acetate extract of *P. africana* bark, CML: *C. megalocarpus*leaves

Our study aimed to evaluate the cytotoxic activities of five crude extracts of different plant species. The ethanol leaf extracts of *C. megalocarpus* and *T. brownii*bark, ethyl acetate extract of *P. africana*bark, and chloroform and acetone extracts of *W. ugandensis* bark.

Cell death can occur through many processes, but the two major forms are apoptosis and necrosis (Fig. 6) [16,48]. Even though the morphologies and mechanisms of apoptosis and necrosis exhibit differences, they share similarities [16,49].

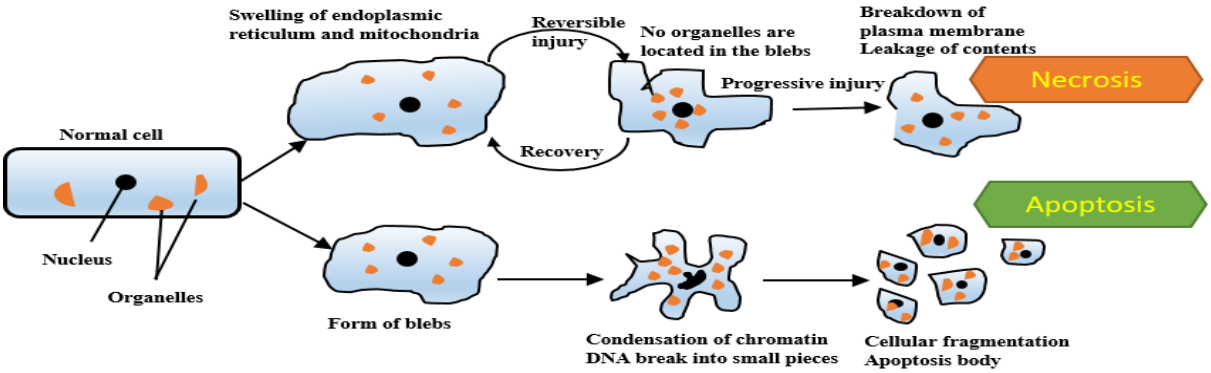

Figure 6. A diagrammatic representation illustrates two primary cellular demise pathways: apoptosis and necrosis. (created by PowerPoint)

Dosages of different plant extracts, with IC_{50} values determined from the Neutral Red uptake assay, were used to treat Caco-2 cells, resulting in inhibition of cell growth. These treatments induced DNA damage, as evidenced by the migration of DNA fragments forming a comet tail. The cell shape is typically described as "clouds," "non-detectable cell nuclei" (NDCN), or "ghost cells." However, this method cannot distinguish between necrotic and apoptotic processes [50-53], as it is associated with both cytotoxic and non-cytotoxic agents [54-58].

Metabolism of cancer cells, unlike a normal cell, the glycolysis end product (pyruvate) is changed over to acetyl coenzyme A by pyruvate dehydrogenase, which, after the Krebs cycle (NADH and FADH2) were formed, that were needed for oxidative phosphorylation process. In contrast, in cancer cells, Lactate dehydrogenase converts pyruvate to lactic acid., which inhibits energy synthesis in the mitochondria and contributes to the acidification of the tumor microenvironment [59]. It is hypothesized that the acidic pH will increase the ability of tumor cells to invade and spread by breaking down extracellular matrix components. [60]. That's why cancer cells overproduce lactate compared to normal cells [61,62].

The necrotic process can be detected by determining plasma membrane damage, which can be identified by detecting LDH levels. Almost all cells contain the soluble cytoplasmic enzyme lactate dehydrogenase, which leaks to the extracellular space from injured cells [63]. The LDH activity decreased significantly after treatment with plant extracts Fig. (4), an observation also made by [64] after crocetin treatment.

Apoptosis can be activated through mitochondrial (intrinsic) or death receptor (extrinsic)pathways Fig. (7). However, there is some evidence that the two routes are connected and that one molecule in one route can impact the other [65].

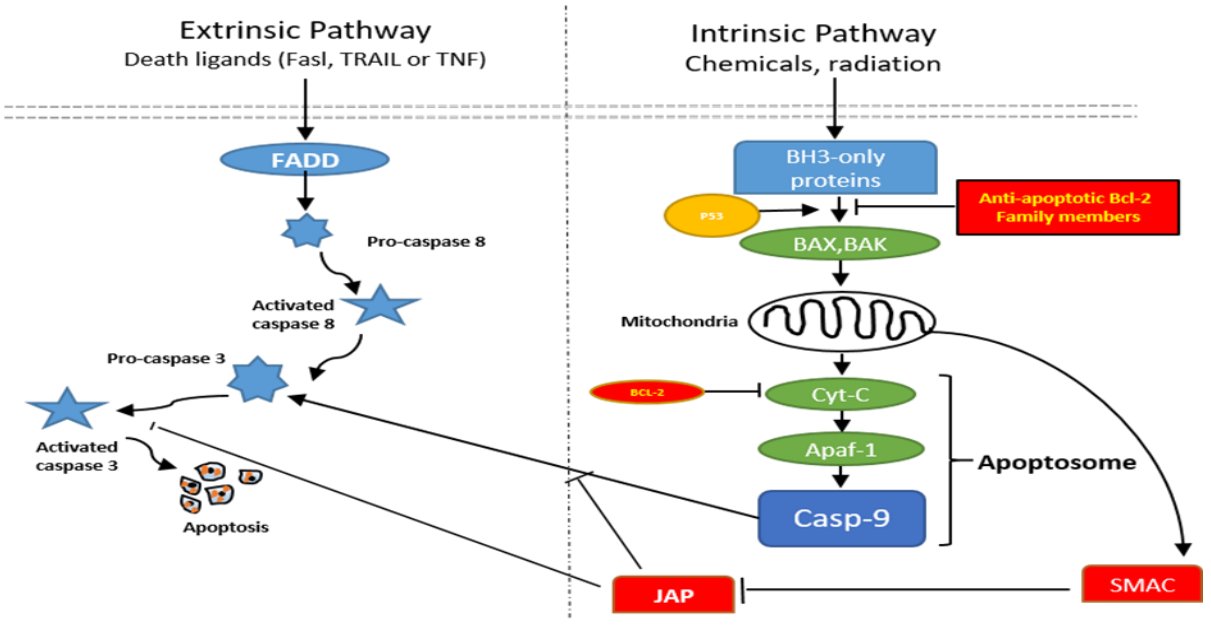

Figure 7. The Schematic Diagram illustrates the Intrinsic and Extrinsic Pathways of Apoptosis. (created by PowerPoint).

The intrinsic apoptotic pathway is regulated by proteins of the Bcl-2 family, which includes both pro- and anti-apoptotic proteins. [66,67].It is well known that the p53 protein negatively regulates Bcl-2 expression and positively regulates Bax expression[68].

Our results in Fig (5) indicate that all the treatments with the extracts that resulted in upregulation of the Bax gene led to up-regulation of the Bcl-2 gene. In contrast, three treatments (P. Afethylacetate, WUB acetone, and WUB chloroform) down-regulated Bcl-xL gene. WUB acetone and WUB chloroform up-regulated the expression of the p53 gene. Bcl-2 up-regulation could be attributed to the presence of inflammatory cells [69]. Also, high levels of Bcl-2 expression alone are insufficient to protect all cells from apoptosis [70].

The caspase family plays a central role in the apoptosis process [71]. caspase-3, also called an apoptotic executor downstream of the Bcl-2 family [72,73]. It cleaves the anti-apoptotic protein Bcl-2, not Bax [71,74]. The activation of caspase-3 can be achieved by three main apoptotic pathways: mitochondrial perturbation, death receptor activation, and endoplasmic reticulum [71,74].

The results also indicate that all plant extracts induce apoptosis partly by activating caspase-8 in Caco-2 except in CML ethanol treatment. However, the mechanism by which different plant extracts activate caspase-8 remains to be elucidated, but it indicates that the apoptosis could occur by the death receptor (extrinsic) pathway that activates Caspase-8 [75,76]. And that is reflected in Caspase-3 activity.

All treatments have a significant decrease in survivin expression except CML ethanol treatment. Survivin belongs to the inhibitor of apoptosis protein (IAP) family member [77]. Survivin blocks apoptosis induced by various apoptotic triggers with other IAP proteins [78]. Reports indicate that survivin binds directly to caspase-3 and -7, the last enzymes in apoptotic protease cascades, and inhibits their activity.[78,79]. Attaining survivin expression using antisense targeting led to apoptosis. [80,81].

3. Experimental

3.1. Chemicals and reagent

Dulbecco's modified Eagle's (DMEM) medium high glucose (1X) was provided by Lonza (North America, USA). Fetal Bovine Serum (FBS) from Seralab(WestSussex UK), (10x) trypsin-EDTA 0.5% and PBS (Phosphate Buffered Saline), pH 7.2 (1x) were purchased from Gibco Invitrogen Company (Scotland, UK). (DMSO) Dimethyl sulfoxide, streptomycin, doxorubicin, penicillin, and Neutral Red were obtained from Sigma Chemical Company (St. Louis, MO, USA). The greatest analytical grade of all other chemicals and reagents utilized in this study was maintained.

3.2. Methods

3.2.1. Plant extract

Crude ethanolic extracts of *C. megalocarpus*Leaves and *T. brownii* bark,ethyl acetate extract of *P. africana* bark, chloroform and acetone extract of *W. ugandensis*bark were obtained from previous work and selected according to the potential of antioxidant activity [20]..Plant samples were gently provided from Jomo Kenyatta university for agriculture and Technology (JKUAT) compound between June and August 2017, The plants were identified using scientific literature and authenticated by a botanist in JKUAT. Voucher specimens were stored in the Botany department.

3.2.2. Cell culture

A human Caucasian colon adenocarcinoma (Caco-2 cells), were obtained from Vacsera (Giza, Egypt) and grown according to [21,22]. The cell suspension was counted using a hemocytometer, and the trypan blue technique checked cell viability. 200 µl of the cell suspension (containing ≈12,000 cells per well) was added to the inner 60 wells of the 96-well plate. The plate was incubated for 24 hours.

3.2.3. Neutral red uptake assay

After the cells were seeded and attached, 200µl of treatment media was distributed into four duplicates for each concentration. Some wells were filled with media alone (to serve as a control), while others were filled with Doxorubicin HCL (4ppm) for a positive control. The plates were then incubated at 37° C for 48 hours.

Once the incubation period had passed, the cultures were analyzed using an inverted microscope to note any cell shape alterations caused by the test extracts' cytotoxic effects. To determine the cytotoxicity of the samples, a neutral red (NR) uptake assay was used according to [23].

The viability % and dose-response curve were calculated for the tested extracts' concentration, reflecting the half-maximum inhibitory concentration of the cell proliferation (IC_{50}).

Caco2 Cells were seeded at 8 x 10^5 cells per T-25 flask overnight in complete medium at 37°C and 5% CO_2 in a humidified atmosphere. After the incubation period, cells were treated with the IC_{50} doses of the extracts along with a negative control set each for 24 hrs., For further investigation.

3.2.4. Alkaline comet assay

The treated and control cell samples were collected in microcentrifuge tubes, and the assay was conducted according to[24]. A computerized image analysis of the images was done to determine the intensity profiles foreach cell and estimate the comet cell components and the range of derived parameters. A method by [25,26] was adopted for the calculation of the tail moment, as shown in Equation 1 below:

$$\boldsymbol{Tail\ moment}\ (\boldsymbol{arbitrary\ unit}) = \boldsymbol{Length\ of\ DNA\ migration}\ (\boldsymbol{um}) \times \boldsymbol{percentage}\ (\%)\ \boldsymbol{of\ migrated\ DNA}.$$

3.2.5. Lactate dehydrogenase leakage Assay

Lactate dehydrogenase (LDH) activities in culture supernatant and cell lysate was measured using an LDH-level estimate using the Kinetic ultraviolet method. Spectrum Diagnostics liquizyme LDH kit (Egyptian Company for Biotechnology, Cairo. Egypt) was used following the manufacturer's guidelines for both untreated and treated Colon cancer cells [27].

3.2.6. Gene expression levels associated with apoptosis and anti-apoptosis

Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) was employed to ascertain the expression levels of widely established apoptotic and anti-apoptotic related genes, including BAX, Bcl-2, Bcl-Xl, P53, survivin, caspase-8, and caspase-3, using the primers enumerated in Table 2. All the primers were primer pairs synthesized by Vivantis Technologies SdnBhd (Vivantis, Malaysia).

QIAGEN's RNeasy Mini kit (New York, USA) was employed to extract total RNA from untreated and treated Colon cancer cells (Caco-2) in accordance with the manufacturer's guidance.

A Nanodrop 2000c spectrophotometer (Thermo Scientific, USA) was employed to assess the purity and quantity of the isolated RNA. The high-capacity cDNA reverse transcription reagent (Applied Biosystems, Foster City, CA, USA) was used to reverse transcribe 1 µg of the total RNA into cDNA following the manufacturer's directives.

Quantitative RT-PCR (qRT-PCR) was conducted in a step one thermocycler (Applied Biosystems, Foster City, CA, USA). A 20ul PCR reaction mixture was prepared, which included 0.3 µl (10 pmol/µl) of each primer (Table 2), four µl of 5x HOT FIREPolEvaGreen qPCR Mix Plus reagent (Solis BioDyne OÜ, Tartu, Estonia), one µl of cDNA template, and 14.4 µl of nuclease-free water.

The PCR cycling parameters included an initial activation step at 95 °C for 12 minutes, followed by 40 cycles of two-step amplification: denaturation at 95 °C for 15 seconds, and annealing/extension at 60 °C for 1 minute. A melting curve analysis was performed to confirm the specificity of the amplification products for each primer pair.

Gene expression in the treated cells was compared to that in the untreated control cells. Relative expression was calculated based on the [28] method with β-actin as reference gene:
by the formula $2^{-\Delta\Delta CT}$

$$\Delta\boldsymbol{CT} = \boldsymbol{CT}(\boldsymbol{a\ target\ gene}) - \boldsymbol{CT}(\boldsymbol{a\ reference\ gene})$$

$$\Delta\Delta\boldsymbol{CT} = \Delta\boldsymbol{CT}(\boldsymbol{a\ target\ sample}) - \Delta\boldsymbol{CT}(\boldsymbol{a\ reference\ sample}$$

Table 2: The reference endogenous control gene and primer sequences of human apoptosis-related genes.

Gene	Human primer sequence (5' – 3')
β-actin	F:5'-AGCGAGCATCCCCCAAAGTT-3' R:5'-GGGCACGAAGGCTCATCATT-3'
Bcl2	F: 5'- TGA AGT CAA CAT GCC TGC CC 3' R: 5'- AAA GCC AGC TTC CCC AAT GA -3'
Bax	F: 5'- AGG ATG CGT CCA CCA AGA AG - 3' R: 5'- TGT CCA GCC CAT GAT GGT TC-3'
BL-XL	F: 5'- AAAAGATCTTCCGGGGGCTG - 3' R: 5'- CCCGGTTGCTCTGAGACATT -3'
P53	F: 5'- TGACACGCTTCCCTGGATTG -3' R: 5'- GTTTTCAGGAAGTAGTTTCCATAGG -3'
Survivin	F: 5'- TTCTCAAGGACCACCGCATC -3' R: 5'- AATGGGGTGCTCATCTGGCT -3'
Caspase-8	F: 5'- GGAACTTCAGACACCAGGCA -3' R: 5'- CCTCCGCCAGAAAGGTACAG -3'
Caspase-3	F: 5'- CGGCGCTCTGGTTTTCGTTA -3 ' R: 5'- CAGAGTCCATTGATTCGCTTCC -3'

3.2.7. Statistical analysis

The mean ± SE was used to express all experimental measurements, which were conducted in triplicate. Statistical significance was defined as $P < 0.05$ when compared to the control group, and the significance levels for the comparison of differences were determined using a one-way ANOVA.

4. Conclusions

The study determined the IC50 values for various plant extracts, with *C. megalocarpus* leaves (45.5 µg/ml), *T. brownii* bark (41 µg/ml), *P. africana* bark (70.2 µg/ml), and *W. ugandensis* bark (4.4 µg/ml for chloroform and 20.7 µg/ml for acetone). Apoptosis was induced in all treatments except for the ethanol extract of *C. megalocarpus* leaves, which displayed a significant decrease in survivin expression, indicating a necrotic mode of action. Treatments with the chloroform and acetone extracts of *W. ugandensis* bark up-regulated P53 and caspase-8, suggesting both intrinsic and extrinsic pathways. The ethanol extract of *T. brownii* bark and the ethyl acetate extract of *P. africana* bark induced apoptosis via the extrinsic pathway, as evidenced by the up-regulation of caspase-8. This finding represents the initial discussion of the cell death pathway associated with these extracts, necessitating further analysis at the protein level to validate the mode of action.

5. Conflicts of interest

The writers herein affirm that they are entirely free from any ties or financial conflicts of interest that may have seemed to impact the results presented in this work.

6. Formatting of funding sources

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