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In vitro Antioxidant and Antitumor Activity of Rooibos (Aspalathus linearis) Extract on Colon, Prostate and Liver Cancer Cells

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Abstract

Rooibos tea (*Aspalathus linearis*) is recognized for its antioxidant properties, due to distinctive compounds such as aspalathin. This study examined the antioxidant potential of rooibos extract on three cancer cell lines (PC-3, HCT-116, and $HepG_2$) to assess its overall antioxidant potential. Additionally, the inhibitory effect of aqueous rooibos extract at a concentration of 1,000 µg/ml was assessed on the three cancer cell lines. The cytotoxic effects of aqueous rooibos extract and cisplatin were compared for the cancer cell lines, with $HepG_2$ cells showing the highest sensitivity to rooibos extract (IC_{50} = 1,399.41 ± 62.73 µg/ml) and PC-3 cells displaying the highest resistance (I_{50} = 2,431.51 ± 98.71 µg/ml). Cisplatin consistently exhibited lower IC_{50} values across all cell lines, indicating a stronger inhibitory effect on cancer cell proliferation, notably for HCT-116 (IC_{50} = 2.53 ± 0.21 µg/ml) and $HepG_2$ cells (IC_{50}= 3.67 ± 0.25 µg/ml). However, the total antioxidant capacity of rooibos extract (68.91 ± 4.07 mg GAE/g) is comparable to that of the ascorbic acid standard (74.19 ± 3.97 mg GAE/g), showing no statistically significant difference. Moreover, rooibos extract is a potent antioxidant, with lower IC_{50} values in the DPPH (3.62 ± 0.10 µg/ml), ABTS (2.13 ± 0.13 µg/ml), and H_2O_2 assays (4.97 ± 0.01 µg/ml) compared to ascorbic acid and EDTA. However, in the metal chelating activity assay, rooibos extract showed a similar performance (IC_{50} 20.89 ± 0.09 µg/ml) to EDTA (IC_{50} 21.16 ± 1.67 µg/ml), suggesting comparable efficacy in this measure. These results demonstrate the strong antioxidant and anticancer potential of rooibos extract, suggesting it as a promising complement in cancer treatment.

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Introduction

Cancer ranks as the second leading cause of death globally, posing a substantial public health challenge. In 2022, approximately 20 million people received a cancer diagnosis, and 9.7 million died from it.[1] Despite the significant development of cancer therapies that have enhanced patient survival and quality of life, the overall number of cancer deaths is still rising.[2] Due to their unique chemical compositions and minimal side effects, medicinal plants are increasingly being investigated as cancer treatment options.[3] In addition a number of commonly consumed foods are also known for their anticancer properties.[4,5]

Rooibos (*Aspalathus linearis*) is a native herb of South Africa traditionally consumed for its flavor and potential health benefits. Rooibos has high concentrations of polyphenol antioxidants and low levels of tannins and caffeine.[6,7] Aspalathin, a C-glucosyl dihydrochalcone, is the primary antioxidant in rooibos.[8] The total phenolic content of rooibos is associated with its antioxidant capacity, as determined by these antioxidant assays. Moreover, 1,1-diphenyl-2-picrylhydrazyl (DPPH), Ferric Reducing Antioxidant Power (FRAP), and ABTS are commonly used to assess antioxidant quality *In Vitro.*[9-12] Notably, both DPPH and FRAP are directly correlated with total phenolic content.[13-15] Rooibos supplements often include a 20% aspalathin-rich extract and micronutrients such as vitamins A, C, E, and selenium, which work together to increase the health-promoting effects of rooibos.[16]

Rooibos can be brewed hot or cold by steeping the leaves in water. Previous research reported that the brewing temperature influences the tea's polyphenol content and antioxidant activity. Specifically, cold brews consistently had higher polyphenol levels and greater antioxidant capacity compared to their hot-brewed counterparts, with this effect being most notable for white tea.[17]

Rooibos extracts exhibit anticancer effects on liver and prostate cancer cells.[18] Recent studies have shown that rooibos extract inhibits the growth of various cancer cells, including liver ($HepG_2$) and colon (HT-29) cells. However, unlike other cancers, rooibos extract does not induce the apoptosis of HT-29 colon cancer cells.[19] Studies on the HCT-116 colon cancer cell line have demonstrated the potential anticancer properties of natural extracts like rooibos. This highlights the need for more research on colon cancer due to its high prevalence and resistance to current therapies.[20] Moreover, green rooibos extract shows the potential to inhibit enzalutamide-resistant prostate cancer (PCa) cell growth.[21]

Green rooibos is often used instead of the traditional oxidized version to maintain its high flavonoid content. The main antioxidant found in rooibos is aspalathin, a type of C-glucosyl dihydrochalcone. Evidence from recent studies highlighted the potential of green rooibos extract, particularly its active compound aspalathin, in altering gene expression associated with antioxidant defense and oxidative stress. These findings suggest that green rooibos may serve as a effective dietary supplement for stimulating cellular resilience to oxidative damage and promoting overall health.[22] Aspalathin-rich rooibos extract reduces cell survival and proliferation in enzalutamide-resistant prostate cancer cells. The study found that rooibos extract suppresses the c-Myc oncogene and stabilises the androgen receptor, both of which are important factors in prostate cancer growth.[23] Additonally, rooibos flavonoids such as aspalathin, iso-orientin, and orientin improve mitochondrial activity and bioenergetics in cultured skeletal muscle cells that may regulate cellular metabolism, which is frequently changed in cancer cells.[24] Furthermore, bioactive components in rooibos can protect against oxidative stress and suppress cancer cell development via a variety of pathways, including regulation of oxidative stress and inflammation-related gene expression.[25] Moreover, aqueous extracts of rooibos and honeybush (Cyclopia intermedia) have been shown to protect human skin fibroblasts and keratinocytes from the cytotoxic effects of diesel exhaust particles, which are well-recognized to induce oxidative stress and inflammation.[26] Furthermore, rooibos extract inhibits adipocyte activity and has been proven to reduce obesity-related inflammation, principally by inhibiting pro-inflammatory cytokines. This anti-inflammatory action further supports rooibos' potential role in cancer prevention, as obesity-related inflammation is increasingly recognized as a significant risk factor for cancer.[27] In addition, aspalathin and other flavonoids present in rooibos possess the ability to trap α-dicarbonyl compounds and inhibit the formation of advanced glycation end

products (AGEs), which have been implicated in cancer pathogenesis. The capacity of rooibos to modulate these pathways suggests its potential utility as a dietary intervention for reducing cancer risk.[28] Molecular docking studies have demonstrated significant interactions between the components of the extract and the enzyme encoded by the Epidermal Growth Factor Receptor (EGFR) gene. Overexpression of EGFR is a hallmark of various cancer types. Despite the development of anti-EGFR treatments, many cancer patients with EGFR overexpression have not yet benefited from these therapies. Acquired and innate resistance are common outcomes of EGFR kinase inhibition.[29,30]

This study aims to comprehensively evaluate the biological properties of rooibos extract through three primary objectives. The first objective involves comparing its antioxidant activity with established antioxidants such as EDTA and ascorbic acid to demonstrate its potential efficacy and practical applications. Another focus is on assessing the extract's effects on cell survival and proliferation in liver, colon, and prostate cell lines to gain insights into its broader biological impact. Additionally, the study examines its anticancer potential by comparing the IC_{50} values of aqueous rooibos extract to cisplatin across various cancer cell lines, providing valuable information on its largely unexplored anticancer properties.

Materials & Methods

The overall study design is summarized in Figure 1. This diagram provides a visual representation of the experimental workflow.

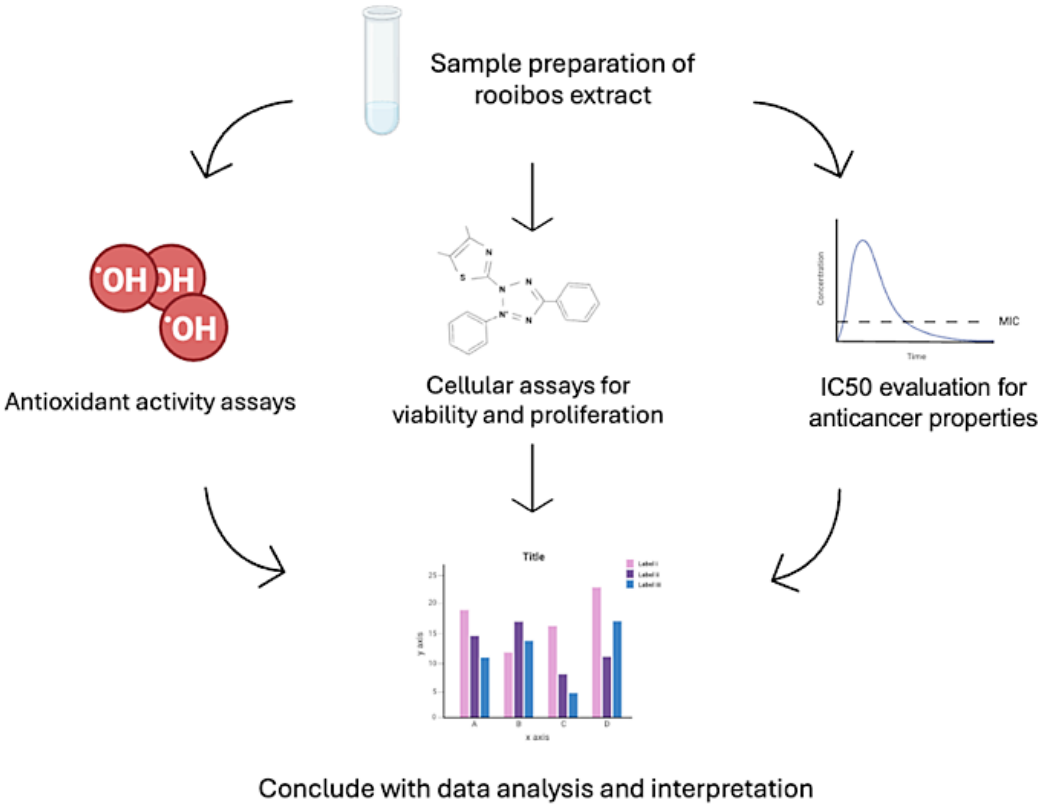


Fig. 1: Graphical research design. The research branches into three key experimental areas: antioxidant activity assays to evaluate the extract's antioxidant capacity, cellular assays to assess cell viability and proliferation, and IC50 evaluation to determine anticancer properties. All branches converge into data analysis and interpretation, providing a comprehensive understanding of the extract's biological effects

Sample Preparation

Commercial fermented rooibos was purchased from Imtenan in Cairo, Egypt. Approximately 2 g of dried rooibos was diluted in 100 mL of boiling tap water, consistent with conventional brewing practices that reflect consumer preparation. Following a 30-minute steeping period at a regulated room temperature of around 25°C, the dilution was subjected to filtration using 0.22-µm MILLEX®GP filters (Millipore, Bedford, MA, USA) to eliminate all particle matter, therefore standardising the extract's purity and consistency. The filtrate was then transferred into sterilized water bottles and stored at 20°C to maintain the bioactive compound integrity.[31]

Cell Culture

The PC-3 (prostate cancer, catalog #CRL-1435), HCT-116 (colorectal carcinoma, catalog #CCL-247), and HepG2 (hepatocellular carcinoma, catalog #HB-8065) cell lines were obtained from the American Type Culture Collection (Rockville, MD, USA). The cells were cultured at a concentration of 1.6×10^5 cells/mL in Eagle's Minimum Essential Medium (Gibco BRL, Rockville, MD, USA), supplemented with sodium pyruvate, nonessential amino acids (Lonza, Houston, TX, USA), 10% fetal bovine serum (Gibco BRL), and a 1% solution of L-glutamine and penicillin-streptomycin (Gibco BRL). Then, cells were maintained at 37°C in a 5% CO_2 atmosphere, with medium changes every two days.

For antitumor evaluation, cells were exposed to varying rooibos extract concentrations (0, 200, 400, 600, 800, and 1,000 µg/ml) for 24 h. Cell viability was then measered using an MTT (3,4,5-dimethylthiazol-2-yl)−2-5-diphenyltetrazolium bromide) kit ThermoFisher Scientific, Waltham, USA). Cells were seeded into 96-well plates at a density of 10,000 cells per well and allowed to adhere overnight. The following day, the cells received treatments with rooibos extract at concentrations of 0, 200, 400, 600, 800, and 1,000 µg/mL for a 24-hour period. Cisplatin used as a positive control. Treated cells were incubated for 4 hours with MTT solution at concentration of 5 mg/mL The formazan crystals were solubilised in DMSO, and absorbance was measured at 560 nm utilising a Tecan GENios plate reader (Tecan, Männedorf, Switzerland). The experimental readings were adjusted by subtracting the blank measurements from negative control wells. This procedure was repeated to ensure accuracy. The correlation between the surviving cell population and the concentration of rooibos extract was analyzed to construct survival curves for each tumor cell line. GraphPad Prism software (San Diego, CA, USA) was utilized to graphically represent the dose-response curve for each concentration. The concentration necessary to inhibit 50% (IC_{50}) of the cell population was derived from these curves.

Antioxidant Assays

DPPH Free Radical Scavenging Activity

To measure DPPH free radical scavenging activity, a 24 mg of DPPH solution was dissolving in 100 mL of methanol. Then, 100 µL rooibos leaf extract was mixed with 3 mL DPPH working solution, and the mixture was stored at room temperature in the dark for 30 minutes to enable the reaction to occurred. After incubation, the absorbance of the solution was measured at 517 nm using a UV-Vis spectrophotometer, and the absorbance of the DPPH solution without the sample was used as a control. To calculate the percentage of DPPH scavenging activity, the formula; DPPH % = (A0 -A1) ÷ A0 ×100, was used, where A0 is the absorbance of the control sample, and A1 is the absorbance of the extract. All measurements were performed in triplicate for accuracy, A higher scavenging activity percentage was indicated as reflecting a more effective sample at neutralizing free radicals.

Scavenging Activity of ABTS Radicals

The scavenging activity of ABTS+ radical cation was measured by using decolorization assay 32. A stock sollution of 7.4 mM was prepared by dissolving 0.0306 g of ABTS+ in 10 mL of distilled water. A 2.6mM potassium persulfate solution was created by dissolving 0.0578 g of K2S2O8 in 10 mL of distilled water. The ABTS+ soloution was mixed with the potassium persulphate solution in a 1:1 volume ratio and the mixture was incubated in the dark at room temperature for 12 hours at 25°C to generate the ABTS radical cation. Before use, the ABTS+ solution was diluted in methanol to achieve an absorbance of 0.70 ± 0.02 at 734 nm. After 7 minutes, the absorbance at 734 nm was measured from a reaction between 1 ml of the ABTS solution and plant extracts at concentrations of 1000 µg/mL using a UV–visible spectrophotometer (Thermo Scientific Technologies, Madison, WI, USA). The percentage ABTS scavenging activity was calculated using the absorbance values obtained from the

control and sample mixtures using the following formula: ABTS radical scavenging activity (%) = ($Abs_{control}$ − Abs_{sample}) ÷ Abs control × 100.

$Abs_{control}$ refers to the absorbance of the ABTS radicals in methanol, whereas Abs_{sample} represents the absorbance of the ABTS radical solution containing the sample extract or standard.

Hydrogen Peroxide (H_2O_2)

Hydrogen peroxide solution was prepared in concentration of 2 mM by diluting it in a 0.2 M phosphate buffer at pH 7.4. The phosphate buffer was prepared by mixing equal volumes of 0.2 M potassium dihydrogen phosphate and 0.2 M sodium hydroxide solutions. 50 mL of 0.2 M potassium dihydrogen phosphate was mixed with 39.1 mL of 0.2 M sodium hydroxide in a 200 mL volumetric flask, after which distilled water was added to reach a total volume of 200 mL.

50 mL of hydrogen peroxide solution was combined with 50 mL of phosphate buffer and incubated at room temperature for five minutes to produce free radicals. A dilution of rooibos extract was created in distilled water and then included into the hydrogen peroxide solution, yielding a concentration of 0.0298 mg/mL. The solution's absorbance was determined at 230 nm with a UV-Vis spectrophotometer following a 30-minute incubation in darkness. A reference stock solution of ascorbic acid was prepared by dissolving 2 mg of ascorbic acid in 2.5 mL of distilled water. A reference stock solution were prepared using methanol (MeOH), ethanol (EtOH), and water (H_2O), with each measurement performed in triplicate to assure statistical validity.

The inhibition of the Ferrozine-Fe^{2+} complex formation was calculated in percentage by using this formula: I = (Abs control - Abs sample)/Abs control × 100

Ferric Reducing Antioxidant Power (FRAP)

A Cobas Fara centrifugal analyzer was employed for the FRAP test. 300 µL of newly made FRAP reagent was heated to 37°C, and a reagent blank measurement was recorded (M1) at 593 nm. Subsequently, 10 µL of the sample was combined with 30 µL of H_2O, yielding a final dilution of 1/34 in the reaction mixture. Absorbance (A) measurements were recorded at 0.5 seconds and subsequently every 15 seconds throughout the observation period. The variation in absorbance (ΔA_{593}nm) between the final measurement and the M1 reading was computed for each sample. The ΔA_{593}nm value was compared to that obtained from a Fe^{2+} reference solution, measured concurrently under similar circumstances, to assess the antioxidant capacity of the samples in the CUPRAC test for cupric ion reduction. A CUPRAC test was conducted to quantify the decrease of cupric ions. A premixed reaction mixture consisting of 1 mL of 10 mM $CuCl_2$, 1 mL of 7.5 mM neocuproine in ethanol, and 1 mL of 1 M ammonium acetate buffer (pH 7.0) was formulated. Subsequently, 0.5 mL of the sample solution was included into this combination. For the control, 0.5 mL of the sample solution was included into a reaction mixture devoid of $CuCl_2$, resulting in a total volume of 3 mL. The sample and the control were incubated at ambient temperature for 30 minutes. Absorbance was recorded at 450 nm, and the CUPRAC activity was determined as the difference between the sample's absorbance and that of the blank. The outcomes were quantified in milligrammes of Trolox equivalents per gramme (mg TE/g).

Metal Chelating Activity

To assess the metal chelating activity, a 2 mM $FeCl_2$ solution was diluted to 0.1 mL using 5 mL of the rooibos extract. Subsequently, 0.2 mL of a 5 mM ferrozine solution was added, and the mixture was shaken for 10 minutes. The absorbance was recorded at 562 nm. The inhibition percentage of Ferrozine-Fe^{2+} complex formation was determined using the formula:

I = (Abs control - Abs sample)/Abs control × 100.

EDTA was used as the positive control.

Total Antioxidant Capacity (TAC)

The TAC of the rooibos extract and ascorbic acid (positive control) was assessed utilising the DPPH radical scavenging and ORAC assays. The DPPH test consisted of combining 1 mL of 0.1 mM DPPH solution with 1 mL of each sample, incubating in darkness for 30 minutes, and thereafter measuring the absorbance at 517 nm. To perform the ORAC test, add 25 µL of sample to 150 µL of fluorescein solution in a 96-well microplate. The mixture was incubated at 37°C, and the reaction was started by

adding 25 µL of AAPH solution. Fluorescence was quantified every minute for 60 minutes, with values represented in mg TE/g 30. All experiments were performed in triplicate.

Statistical Analysis

All tests were performed in triplicate to guarantee the repeatability and reliability of the results. Data are expressed as mean ± standard deviation (SD) to indicate variability within each set of triplicates. Statistical comparisons between the experimental groups were conducted using an independent t-test, after confirming normality and equal variances with the Shapiro-Wilk test and Levene's test, respectively. For comparisons involving multiple groups, one-way ANOVA was used, followed by post-hoc analysis with Bonferroni correction to adjust for multiple comparisons Statistical significance was established at P-values < 0.001. Effect sizes were computed to assess the magnitude of the observed changes.

Table 1: Comparative analysis of aqueous rooibos extract inhibition and viability effects on cancer cell lines

Aqueous rooibos extract	PC-3%	HCT-116%	HepG2%
Inhibition	67.24	80.58	86.02
Viability	32.76	19.42	13.98
SD (±)	3.12	1.84	1.74

Results

Inhibitory Effect of Aqueous Rooibos Extract on Three Cancer Cell Lines

The experimental evaluation of aqueous rooibos extract revealed dose-dependent inhibition of the three cancer cell lines. Notably, the effect of the extract increased as the concentration increased from 200 µg/ml to 1,000 µg/ml (Table S1, Supplementary Material). Treating cells with 1,000 µg/ml aqueous rooibos extract produced a significant inhibitory effect, with inhibition rates of 67.24% and 80.58% for the PC-3 and HCT-116 cell lines, respectively. The highest inhibitory effect was observed on the $HepG_2$ cell line, with an inhibition rate of 86.02%. This highlights the potential effect of rooibos extract for targeting hepatocellular carcinoma.

The viability of PC-3 cells was 32.76% ± 3.12%. A lower viability of 19.42% ± 1.84% was noted for the HCT-116 cell line, correlating with the higher inhibition rate. The lowest cell viability was observed for the $HepG_2$ cell line at 13.98% ± 1.74%, consistent with the fact that this cell line had the highest inhibition rate among the tested cell lines (Table 1).

Table 2: IC_{50} values of aqueous rooibos extract and cisplatin in cancer cell lines using the MTT assay

Compound	PC-3	HCT-116	HepG2
Aqueous rooibos extract (1,000 µg/ml)	46.41.01 ± 2.07*	2,431.51 ± 98.71*	1,399.41 ± 62.73*
Cisplatin (1,000 µg/ml)	6.64 ± 0.28	2.53 ± 0.21	3.67 ± 0.25

These data were measured using the MTT test during a 24-h period. *p < 0.001.

IC_{50} of Aqueous Rooibos Extract and Cisplatin in Cancer Cell Lines

In the three cancer cell lines, the IC_{50} values of aqueous rooibos extract and cisplatin were measured at 1,000 µg/ml rooibos and 1,000 µg/ml cisplatin. Table 2 shows the IC_{50} values of aqueous rooibos extract and cisplatin in different cancer cell lines (PC-3, HCT-116, and $HepG_2$) as determined by the MTT assay.

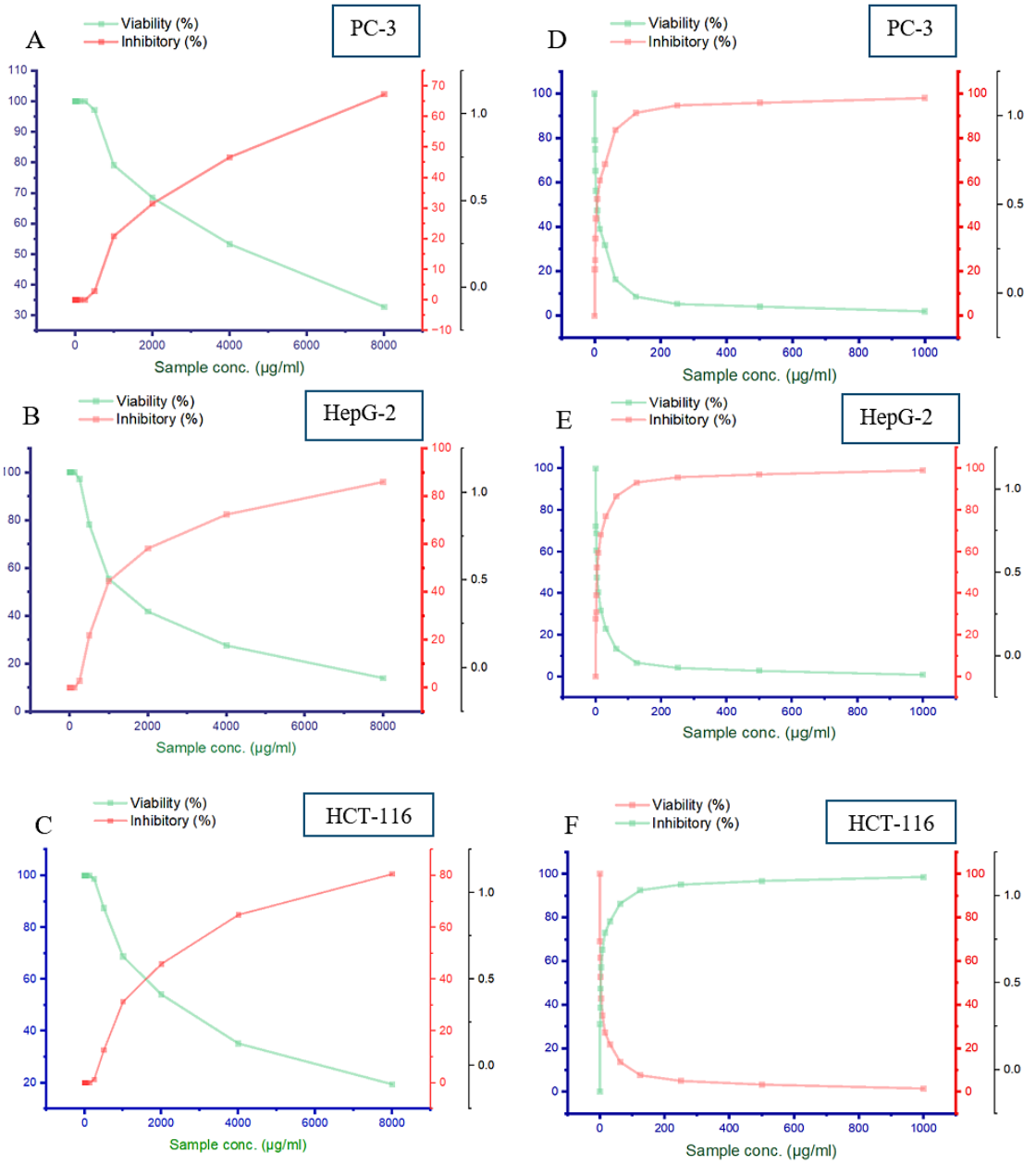


Fig 2: The relationship between rooibos extract and cell viability and inhibition (%) over 24 h in PC-3, HCT116, and HepG2 cells. Graphs A, B, and C show viability (%), illustrating decreasing cell growth in response to rooibos extract treatment. Inhibition (%) shows an increasing trend with rooibos treatment. Graphs D, E, and F show cell growth and viability in the presence of cisplatin

Cisplatin, a well-established chemotherapeutic agent, exhibited consistently lower IC_{50} values across all cell lines, indicating a stronger inhibitory effect on cancer cell proliferation. For PC-3 cells, the IC_{50} value for cisplatin was measured at 6.64 ± 0.28 µg/ml, demonstrating high effectiveness. Interestingly, the IC_{50} value for the inhibitory effect of cisplatin on HCT-116 colorectal cancer cells was even lower at 2.53 ± 0.21 µg/ml, confirming its high efficacy in inhibiting these cells. Additionally,

the IC_{50} for $HepG_2$ hepatocellular carcinoma cells was recorded at 3.67 ± 0.25 µg/ml, showcasing substantial effectiveness against this cancer type. In contrast, treating PC-3 cells with aqueous rooibos extract resulted in an IC50 value of 46.41 ± 2.07 µg/ml ($p < 0.001$), indicating a notable but lesser potency compared to cisplatin. For HCT-116 cells, the IC_{50} of rooibos extract was significantly higher at 2,431.51 ± 98.71 µg/ml ($p < 0.001$), suggesting a much lower sensitivity to the extract. The $HepG_2$ cell line was also inhibited by rooibos extract, with an IC_{50} value of 1,399.41 ± 62.73 µg/ml ($p < 0.001$), (Figure 2, Table 2).

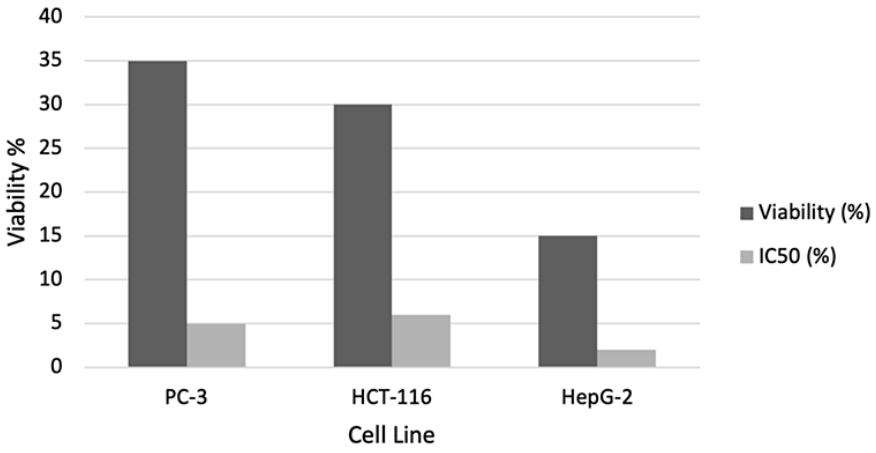


Fig 3: Cell viability percentage and IC_{50} across three cell lines: PC-3, HepG-2, and HCT-116. The results were obtained using an MTT assay. HepG-2 had the lowest viability at 15%, followed by HCT-116 at 30% and PC-3 at 35%. High viability percentages indicate a lower cytotoxic effect of rooibos on the cell line. The IC_{50} values were relatively low across the cell lines, with PC-3 at 5%, HepG-2 at 2%, and HCT-116 at 6%. Lower IC_{50} values suggest a higher potency of rooibos in inhibiting cell viability

Comparing the IC_{50} values revealed that although aqueous rooibos extract displays varying levels of cytotoxicity across cancer cell lines, it generally presents higher IC_{50} values than cisplatin. $HepG_2$ cells were the most sensitive to rooibos extract among the cell lines studied. These findings contribute valuable insights into the potential application of aqueous rooibos extract as an alternative or complementary drug in cancer treatment, particularly for hepatocellular carcinoma (Figure 3).

Thirteen components were isolated and identified in aqueous rooibos extract, and molecular docking studies targeting the 1M17 domain of the EGFR gene were conducted to determine which of these components exhibits the most potent inhibitory effect against the hepatocellular cancer EGFR enzyme (Supplementary Table S2, Figure S2, and S3).

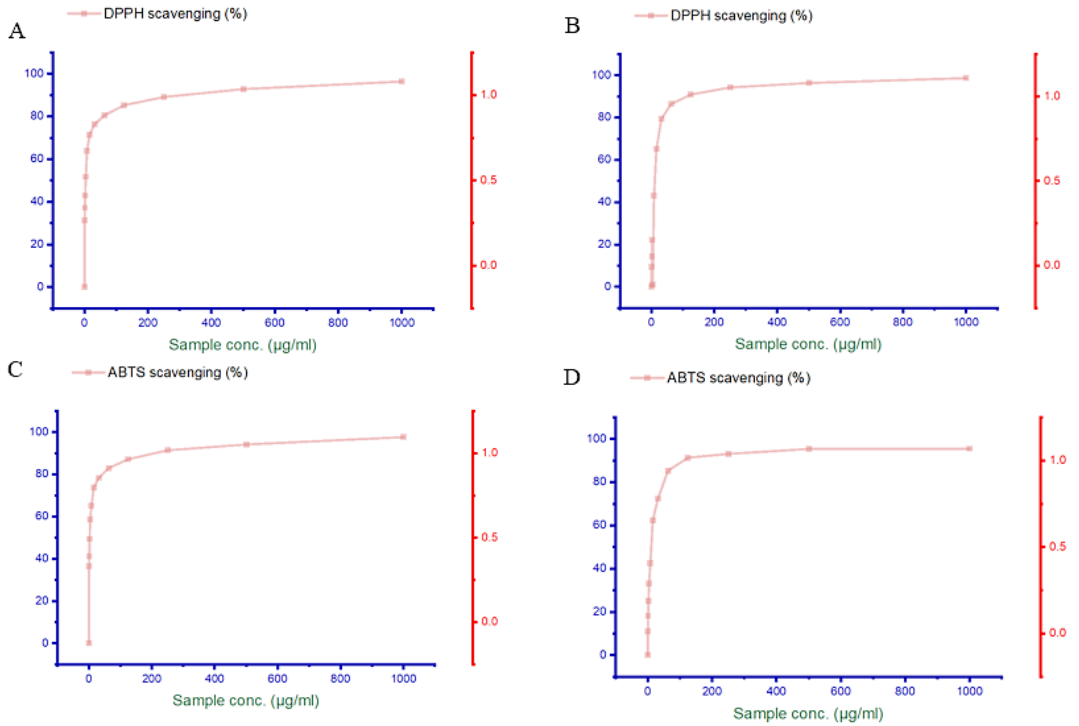
Various assays were employed to assess antioxidant activity and properties. The DPPH assay showed a low IC_{50} value for rooibos extract (3.62 ± 0.10 µg/ml), with a significant difference compared to ascorbic acid ($p < 0.001$); ascorbic acid exhibited a higher scavenging activity (IC_{50} = 6.58 ± 0.03 µg/ml). Similarly, rooibos extract demonstrated a low IC_{50} value (2.13 ± 0.13 µg/ml), confirming its strong effect on scavenging ABTS radicals. The difference between rooibos extract and ascorbic acid was significant ($p < 0.001$), with ascorbic acid having a higher effect (IC50 = 6.94 ± 0.03 µg/ml). The FRAP assay demonstrated that rooibos extract had a moderate capacity for reducing ferric ions (IC_{50} = 6.70 ± 0.18 µg/ml) compared to ascorbic acid (IC_{50} = 10.25 ± 0.23 µg/ml; $p < 0.001$). When evaluating the CUPRAC values, rooibos extract showed a lower ability to reduce cupric ions (IC_{50} = 14.77 ± 0.04 µg/ml) compared to EDTA (IC_{50} = 23.84

± 0.80 µg/ml; p < 0.001), indicating that rooibos extract has less antioxidant potential than EDTA. The H_2O_2 assay confirmed that rooibos extract can neutralize hydrogen peroxide (IC_{50} = 4.97 ± 0.01 µg/ml) to a greater extent than EDTA (IC_{50} = 14.77 ± 0.04 µg/ml; p < 0.001). However, the metal chelating activity was not significantly different, with IC_{50} values of 20.89 ± 0.09 µg/ml for rooibos extract and 21.16 ± 1.67 µg/ml for EDTA. In summary, significant differences were observed in the ABTS, DPPH, FRAP, CUPRAC, and H_2O_2 assays, demonstrating the strong antioxidant potential of rooibos extract compared to ascorbic acid and EDTA. Meanwhile, the metal chelating activity showed no significant difference compared to EDTA, highlighting the varying antioxidant capacity of rooibos extract across different assays (Table 3, Figure 4).

Table 3: Antioxidant activity of rooibos extract compared to EDTA and ascorbic acid.

Compounds (1,000 µg/ml)	DPPH (µg/ml)	ABTS (µg/ml)	H_2O_2 (µg/ml)	FRAP (µg/ml)	CUPRAC (µg/ml)	Metal chelating activity (µg/ml)
Aqueous rooibos extract	3.61 ± 0.29*	2.13 ± 0.13*	4.97 ± 0.65*	6.65 ± 0.29*	6.94 ± 0.67*	6.58 ± 0.63
EDTA positive control	-	-	-	-	23.28 ± 4.73	21.16 ± 2.08
Ascorbic acid standard	10.21 ± 0.77	10.66 ± 0.89	14.77 ± 0.69	20.89 ± 1.25	-	-

* Statistically significant difference at p < 0.001.



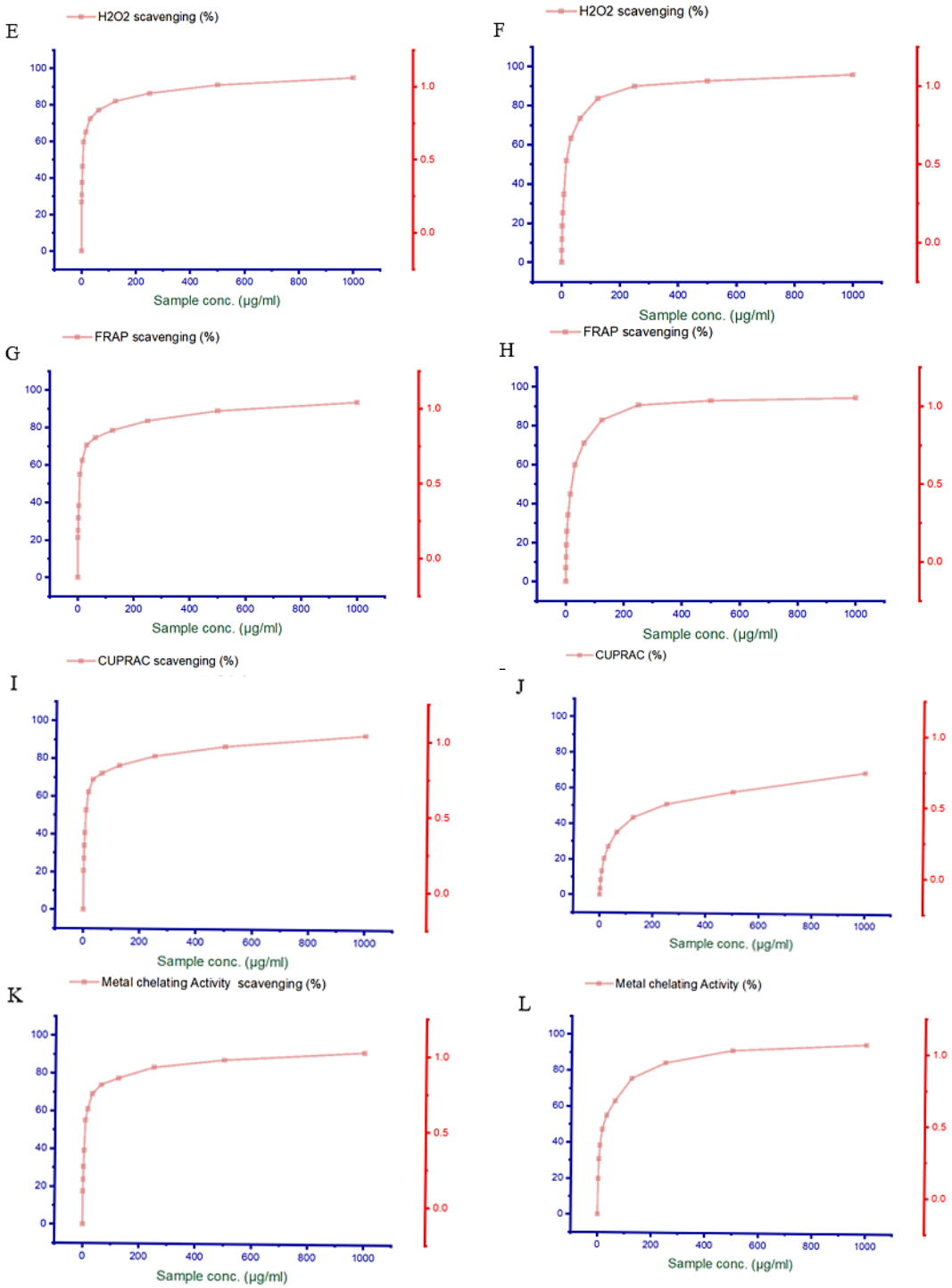


Fig. 4: Antioxidant activity of aqueous rooibos extract compared to control. A) DPPH assay (rooibos), B) DPPH assay (ascorbic acid), C) ABTS assay (rooibos), D) ABTS assay (ascorbic acid), E) H2O2 assay (rooibos), F) H2O2 assay with ascorbic acid, G) FRAP assay (rooibos), H) FRAP assay (ascorbic acid), I) CUPRAC assay (rooibos), J) CUPRAC assay (EDTA), K) Metal chelating activity assay (rooibos), L) Metal chelating activity assay (EDTA).

The TAC was measured to determine the ability of rooibos extract to prevent oxidative damage. The aqueous extract had a TAC of 68.91 ± 4.07 mg GAE/g, whereas ascorbic acid had a slightly higher TAC at 74.19 ± 3.97 mg GAE/g. However, statistical analysis indicated that the difference was not significant, suggesting that the antioxidant capacity of rooibos extract is nearly equivalent to that of the ascorbic acid standard (Table 4).

Table 4: TAC of aqueous rooibos extract and ascorbic acid

Sample	TAC ± SD (mg GAE/g)
Rooibos	68.91 ± 4.07
Ascorbic acid (positive control)	74.19 ± 3.97

Discussion

Liver, colorectal, and prostate cancer are major global health issues. Liver cancer occurrence and mortality rates have risen significantly since the 1980s, particularly in sub-Saharan Africa and Southeast Asia.[33] Colorectal cancer, the third most frequent cancer globally, mostly affects older people and is associated with lifestyle factors such as diet, lack of physical exercise, and smoking.[34] Rooibos is recognized for its antioxidant capabilities as it contains substances such as aspalathin and nothofagin, which potentially reduce cancer development. However, further investigation is required to understand its effectiveness in preventing and treating cancer.[34-36]

This study investigated the potential anticancer properties of aqueous rooibos extract. The effect of a 1,000 µg/ml concentration on cancer cell line viability was examined using an MTT assay after a 24-h treatment period. Additionally, the antioxidant capacity of rooibos extract was assessed with various assays. The extract's effectiveness was compared to a standard (ascorbic acid) and a positive control (EDTA) by measuring their IC_{50} values. Finally, the TAC of the extract was measured to determine its overall ability to combat oxidative stress.

Our findings indicate that aqueous rooibos extract substantially limits the growth of the PC-3, HCT-116, and $HepG_2$ cell lines. Additionally, it has strong antiproliferative effects, especially on hepatocellular carcinoma cells (Figure 2,3). Previous research showed that 100 µg/ml of pharmaceutical-grade green rooibos extract (GRT) inhibited prostate cancer cell growth.[23] While cisplatin demonstrated strong efficacy across all cell lines with consistently low IC_{50} values, rooibos extract displayed a more selective effect. Rooibos extract exhibited variable cytotoxicity across the cell lines. $HepG_2$ cells displayed the highest sensitivity to the extract, with the most significant IC_{50} value (Table 1). This suggests that rooibos extract has a potential targeted effect on liver cancer cells. However, the effect on PC-3 and HCT-116 cells was less pronounced. The precise mechanisms by which rooibos extract exerts its cytotoxic effect remain unclear; research has shown that antioxidants reduce oxidative stress by neutralizing reactive oxygen species, potentially reducing the risk of cellular mutations and cancer cell proliferation.[37]

Comparing our findings (Table 2) to previously reported IC_{50} values of GRT on prostate cancer cells (98.5 and 226.6 µg/mL), cytotoxicity appears to vary significantly depending on both the extract type and the cancer cell line. This variation could be linked to differences in Akt signaling pathway activity across cancer cell lines. GRT suppresses proliferation by inhibiting Akt signaling and potentially downregulating c-Myc, disrupting the cancer cell's ability to grow and survive.[18] Moreover, Afriplex GRT, a rooibos-based product, is protective against CCL4-induced liver damage in rats.[38] Additional research is necessary to explore this potential mechanism in the context of rooibos extract and its components.

This study demonstrates that aqueous rooibos has potent antioxidant properties (Figure 4). The antioxidant efficacy of rooibos extract was measured using a variety of assays, aligning with broader research on the influence of the extraction method on antioxidant activity. Boiled water was used as the extraction solvent; the extract effectively neutralized ABTS and DPPH radicals. This aligns with previous research indicating that water can efficiently extract antioxidants, hence influencing their measurement.[39]

A strong correlation has been observed between polyphenol content and *In Vitro* antioxidant capacity, particularly in relation to flavonoids and phenolic acids.[40] However, another study showed that these factors may be less significant for green rooibos;

both cold and hot brews exhibited similar total polyphenol content and antioxidant capacity.[41]

Furthermore, rooibos exhibited a moderate capacity to reduce ferric ions, although it was less effective than ascorbic acid (Table 3). The CUPRAC test confirmed the antioxidant potential of rooibos extract. Additionally, the H_2O_2 scavenging assay demonstrated the extract's efficiency in neutralizing hydrogen peroxide. Furthermore, the extract inhibited EDTA in the metal chelating assay, which is crucial for preventing free radical formation. Since there are no catechins in rooibos, this may contribute to the reduced antioxidant activity observed in the DPPH and FRAP assays. However, rooibos contains a variety of other bioactive compounds with antioxidant properties. These include significant amounts of aspalathin, isoorientin, orientin, and rutin, as well as smaller quantities of iso-vitexin, vitexin, isoquercitrin, hyperoside, quercetin, luteolin, and chrysoeriol. The presence of these compounds suggests that although catechins are absent, the antioxidant potential of rooibos is supported by these diverse phenolic constituents.[42,43] Moreover, previous studies showed significant positive effects of rooibos kombucha in lowering oxidative stress in fibroblasts exposed to oxidation caused by H_2O_2 treatment, both before and following kombucha treatments.[44]

The TAC of aqueous rooibos extract closely matched that of ascorbic acid (Table 4). However, the TAC varies considerably between unfermented and fermented rooibos, with unfermented rooibos tea displaying a higher antioxidant content. This finding is crucial for both consumers and researchers interested in the nutritional and therapeutic benefits of rooibos tea, as it highlights the importance of the fermentation process in influencing the tea's antioxidant properties.[8]

Conclusion

This study demonstrates the significant anticancer potential of aqueous rooibos extract, particularly against hepatocellular carcinoma ($HepG_2$) cells, which exhibited the highest sensitivity to the extract with an IC_{50} value of 1,399.41 ± 62.73 µg/ml. The extract's cytotoxic effects were confirmed by dose-dependent inhibition across various cancer cell lines, including PC-3 and HCT-116, where IC_{50} values were notably higher. Additionally, the antioxidant capacity of rooibos extract, with a TAC of 68.91 ± 4.07 mg GAE/g, highlights its ability to combat oxidative stress, although it was slightly lower than that of ascorbic acid (74.19 ± 3.97 mg GAE/g). Overall, these findings underscore the potential of aqueous rooibos extract as an alternative or complementary therapeutic agent in cancer treatment, warranting further exploration into its mechanisms of action and clinical applications.

Limitations

Despite our promising results, this study has several limitations. First, *In Vitro* studies may not fully replicate rooibos extract's complex interactions and bioavailability in living organisms. Additionally, the rooibos extract concentration may vary due to differences in the plant source, harvest time, and extraction methods, which could affect the reproducibility and generalizability of the findings. Furthermore, *in vivo* studies are required to confirm the antioxidant and anticancer effects observed *In Vitro* and evaluate the potential therapeutic applications of rooibos extract in living systems. Additionally, the selectivity index, which evaluates the relative safety and efficacy of the aqueous rooibos extract and cisplatin by comparing their IC_{50} values in cancerous versus normal cell lines, was not determined. This information is critical for assessing the therapeutic potential and safety profile of these compounds. Future research should include *in vivo* trials to establish rooibos extract's anticancer and antioxidant activities and explore its metabolism and bioavailability. Standardising plant supply, harvest time, and extraction methods improves repeatability and generalisability. Studies should examine its synergistic effects with conventional chemotherapeutics, molecular mechanisms underpinning its bioactivity, and clinical trials to confirm its therapeutic potential in humans.

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Conflict of Interest
The author declares no conflict of interest.

Data Availability Statement
The manuscript incorporates all datasets produced or examined throughout this research study, and the raw data presented in this study are available on request from the corresponding author.

Ethics Statement
This study did not involve any experiment on humans and animals. Therefore, approval from any ethics body was neither required nor sought.

Informed Consent Statement
This study did not involve human participants, and therefore, informed consent was not required.

Clinical Trial Registration
This research does not involve any clinical trials.

Author Contributions
Manal Malibary: The sole author was responsible for the conceptualization, methodology, data collection, analysis, writing, and final approval of the manuscript.

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