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Chemical Characterization of *Rosmarinus officinalis L.*Hydrodistillation of By-Products, Evaluating Their Antioxidant, and Anticancer Activities.

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Abstract

Essential oils derived from Mediterranean plants are widely used; nevertheless, the hydrodistillation waste produced with these oils are relatively unexplored and underutilized. With only partial data available in the literature, we analysed the chemical composition of by-products from hydrodistilled rosemary leaves. The solid residue of rosemary by-product (RSB) was extracted using 80% ethanol. The extract was tested for antioxidant activity and demonstrated a high effect. The overall phenolic content was 188 mg GAE/g, whereas the flavonoid content was 12 mg QE/g. caffeic acid (31.2%), naringenin (27.8%), apigenin (7.39%), hesperetin (5.06%), and coumaric acid (3.94%) were the most common components of RSB. the inhibitory concentration that inhibits 50% of the cancer cell population (IC_{50}) for the cytotoxic action of RSB extract against HCT 116 (human colon) and PC3 (human prostate) cancer cell lines at varied concentrations (31.25-1000 µg/mL) was 31.4 µg/mL for HCT 116 and 33.4 µg/mL for PC3.We conclude that the solid residue of rosemary by-product leaves exhibits significant potential in the development of phyto-medicines with anticancer properties. Drugs derived from rosemary solid by-product (RSB) could serve as an alternative medicinal source due to their anticancer activity. Furthermore, this study suggests that the ethanol extract of this by-product possesses the greatest potential for anticancer activity against (HCT 116) Human colon and human Prostate (PC3) cancer cell lines.

Keywords, Rosemary Hydrodistillation, Bioactive Compounds, Antioxidant, and AnticancerActivities

1. Introduction

Rosemary (*rosmarinus officinalis* L.) is a medicinal and aromatic herb native their. Mediterranean region that can be used as an oil, spice, or extract. Owing to their abundant bioactive compounds, essential oils boast a broad spectrum of uses, and their global trade is experiencing an average annual growth of 10% **[1]**. They are utilized across a wide range of industries, including flavor, aromatherapy, cosmeceuticals, cosmetics, aroma chemicals, nutraceuticals, and pharmaceuticals, collectively command an annual market value surpassing A thousand billion dollars [2]. The yield of oil produced varies depending on the extraction procedure and the type of plant material employed, often yielding a few milliliters per hundred grams of dried plant [3-11]. The increasing demand for oils has prompted the development of various new strategies to improve crop production and composition. Prior to hydrodistillation, plant materials are routinely treated with microwaves, ultrasound, ohmic heating, or enzymes to break down cell walls, enhancing solvent access to cellular contents and enabling bioactive component liberation [3, 4, 8, 11-16]. However, regardless of the production process, essential oils produce a huge number of byproducts [17]. Hydrodistillation's potentially valuable byproducts are classified into three groups: hydrolat, water residue, and solid residue. Hydrolates frequently contain dissolved elements of essential oils, which preserve a significant number of volatile compounds [18-22], whereas water and solid remains contain many polyphenols [8-23-28]. Although they include useful components, these fractions are typically neglected or considered waste. Hydrolats are aromatic aqueous solutions made up of water-soluble volatile chemicals produced from essential oils. Hydrolats have much fewer volatile components than essential oils. However, it has been established that hydrolats may include higher concentrations of some primary, primarily oxygen-containing molecules [29-32]. The proportions of various component groups vary between hydrolats and essential oils. Hydrolats, for example, contain a high concentration of oxygenated monoterpenes while having a low quantity of sesquiterpene hydrocarbons and hydrophobic monoterpenes [33,34]. Hydrolats are the most commonly used residues of essential oil production and are employed in food manufacturing, cosmetic manufacturing, fragrance manufacturing, and aromatherapy [20-22, 35–36].

Plant remains from hydrodistillation and water extract leftover. Filtering this mixture yields two independent sources of polyphenols: the leftover material, or the robust residue [23, 24, 26-28], and the water extract produced through hydrodistillation [25, 27, 37]. Plant materials are high in antioxidants and contain large amounts of water-soluble chemicals that remain dissolved in water after hydrodistillation [25, 27]. It is critical to recognize that water residues stored in open air may get polluted with bacteria and fungi, which can be harmful to the environment if discharged [2]

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The solid leftovers from hydrodistillation can be used as secondary raw materials to extract various bioactive. Traditional procedures such as solvent extraction in a shaker and Soxhlet apparatus, as well as contemporary approaches such as ultrasound baths, can produce considerable levels of phenols and flavonoids in the final extracts [8, 23, 24, 26, 27]. When comparing several extraction methods for processing solid hydrodistillation residues, ultrasound is the most successful. It operates at lower temperatures and takes less time, making it better suited for protecting polyphenols against heat degradation [38–40].

In this study, we assessed the composition of the solid residue following rosemary hydrodistillation methods. We also assess the antioxidant, total flavonoid, total phenolic, and anticancer properties of the rosemary by-product extract's solid residue.

In this study, we have estimated the composition of the solid residue after the rosemary hydrodistillation procedures. Additionally, we determine the antioxidant, total flavonoid, total phenolic, and anticancer activity of solid residue of rosemary by-product extract.

2. Experimental

2.1. Materials and Chemicals

Rosemary has been obtained from a local market (Zagazig, Egypt). All solvents used throughout the present work were obtained from different companies. 1, 1-Diphenyl-2, picrylhydrazyl (DPPH), β-carotene, quercetin, gallic acid and Tert-butyl hydroquinone (TBHQ), were purchased from Sigma (St. Louis, MO, USA).

HCT 116 cell (human colon cancer cell line), and PC3 cells (prostate carcinoma cell) were obtained from VACSERA Tissue Culture Unit (Giza, Egypt).

2.2. Preparation of rosemary extracts (RSB).Rosemary was dried in a vacuum oven (Thermo Fisher Scintific Inc., Japan) at 45˚C for 72 h and grounded to a fine powder in a mill (Retsch, Model ZM 1000, Haan, Germany).

Approximately 100 g of dried plant material underwent steam distillation for about 4 hours in a pilot-scale apparatus to extract essential oil. The residual solid rosemary by-product (RSB) post-distillation was initially sun-dried for Forty-eight hours to achieve a moisture content below 10%, then milled in a laboratory grinder to a size smaller than 0.5 mm. The RSB was preserved at 4°C for subsequent analysis.

The solid residue of rosemary by-product (RSB) was extracted using the method described in reference [40]. A quantity of 100 g of RSB was extracted with 1000 ml of 80% ethanol at room temperature for Twenty-four h. The extract was then centrifuged at 5500 rpm for ten minutes. The remaining residue was re-extracted twice under identical conditions and filtered through filter paper. The 80% ethanolic extract was subsequently concentrated under reduced pressure and lyophilized to yield a powder. The final yield was 5.62 g, which was stored at -20°C for further study.

2.3. *Phytochemical analysis of* ***(RSB)***

2.3.1. Determination of total phenolic compounds

The concentrations of TPC in (RSB) were estimated by a UV spectrophotometer (Jenway6705-UV/VIS), based on a colorimetric oxidation/reduction reaction **[42].** The used oxidizing reagent was Folin–Ciocalteu reagent [**43**]. TPC [mg gallic acid equivalent (GAE)/g extract] was computed depending on the calibration curve as follow:

$$y = 0.024x + 0.095$$

$$R^2 = 0.9937$$

where x is the concentration (mg GAE g−1 extract), y is the absorbance, and R2 is the correlation coefficient.

2.3.2. Determination of total flavonoids.

Quantification of the yellow color produced by the interaction of total flavonoid (TF) contents with aluminum chloride (AlCl3) was measured as described by [44] with some modification. A 0.5 mL of RSB solution (10 mg in 10 mL solvent) was mixed with a 3 mL aliquot of 10 g/L AlCl3 ethanolic solution and after one h, the absorbance was measured at 420 nm. TF contents expressed as quercetin equivalent (QE) was measured depending on the calibration curve using the following equation:

$$y = 0.0148x - 0.0135$$

$$R^2 = 0.9996$$

where R2 is the correlation coefficient, y is the concentration (µg QE), and x is the absorbance.

2.3.3 LC/Ms-Ms for RSB

The RSB analysis was carried out utilizing liquid LC-ESI-MS/MS with an Exion LC AC system for separation and a SCIEX Triple Quad 5500+ MS/MS system equipped with electrospray ionization (ESI) for detection.

The separation was done using a ZORBAX SB-C18 Column (4.6×100 mm, 1.8 µm). The mobile phases included two eluents: A, 0.1% formic acid in water, and B, acetonitrile (LC grade). The mobile phase protocol was as follows: 2% B from 0-1 min, 2-60% B from 1-21 min, 60% B from 21-25 min, and 2% B from 25.01 to 28 min. The flow rate was set at 0.8 ml/min, with an injection volume of 3 µl. For MRM analysis of selected polyphenols, both positive and negative ionization modes were used in the same run, with the following parameters set: curtain gas, 25 psi; Ion Spray voltage, 4500 and -4500 for positive

and negative modes, respectively; source temperature, 400°C; ion source gas 1 & 2, 55 psi; declustering potential, 50; collision energy, 25; and collision energy spread, 10.

2.4. Antioxidant activity Determination of RSB.

2.4.1 DPPH radical-scavenging activity

The electron donation ability of the obtained extracts was measured according to Hatano et al. **[45**] by bleaching of the purple-colored solution of DPPH. One hundred µL of each extract (10 mg extract/10 mL solvent) was added to 3.9 mL of 0.1 mm DPPH dissolved in methanol. After 120 min, the absorbance at 517 nm was measured against control [**46**]. Percentage of antioxidant activity of free-radical DPPH was determined as follows:

$$\textbf{Antioxidant activity (Inhibition) percentage} = [(A_{control}-A_{\mathbf{RSB}})/A_{control}]\times 100.$$

A control is the absorbance of the control reaction, while A_{RSB} is the absorbance in the presence of RSB. TBHQ and gallic acid were utilized as positive controls.

2.4.2. β-Carotene/linoleic acid bleaching

Extracts and synthetic antioxidants were investigated for their ability to prevent β-carotene bleaching [47].

A control with no extract was also tested. The antioxidant activity was calculated using the following procedure:

$$\textbf{Antioxidant activity (\%)} = [1 - (Abs^{0}_{RSB} - Abs^{120}_{RSB})/ (Abs^{0}_{control} - Abs^{120}_{control})] \times 100$$

Abs0 RSB represents the initial absorbance of RSB at 0 time, Abs^{120} RSB designates the absorbance of RSB after 120 minutes, Abs^{0}control represents the initial absorbance of the control at 0 time, and Abs^{120}control represents the absorbance of the control after 120 minutes.

2.5. Antitumor Activity determination of RSB

Sample cytotoxicity on cells is determined using the MTT technique.

The effect of RSB concentrations ranging from 31.25-1000 µg/mL on human cell line viability was tested in vitro using MTT-assay. Normal cells (Vero cells) and cancer cells (HCT 116 and PC3) were obtained from the VACSERA Tissue Culture Unit in Giza, Egypt. A 96-well tissue culture plate was filled with 1 X 10^5 cells/ml (100 µl/well) and cultured at 37°C for 24 hours to generate a full monolayer. The growing media was then withdrawn from the wells. The confluent cell monolayer was washed twice with washing solution. Two-fold dilutions of the test sample were produced in RPMI medium with 2% serum. 0.1 ml of each dilution was applied to separate wells, with three wells set aside as controls with only maintenance medium. The plate was incubated at 37°C for evaluation. The cells were tested for toxicity signs such as partial or complete monolayer loss, cell rounding, shrinkage, or granulation. An MTT solution (5mg/ml in PBS) supplied by BIO BASIC CANADA INC was made. Each well received 20 µl of this solution. The plate was shaken at 150 rpm for 5 minutes to completely mix the MTT and medium. It was then incubated at 37°C with 5% CO2 for 1-5 h to allow the MTT to metabolize. The media was discarded, and the plate was dried with paper towels as needed. Formazan, a metabolic product of MTT, was resuspended in 200 µl DMSO. The plate was then shaken at 150 rpm for 5 minutes to completely dissolve the formazan. The optical density was measured at 560nm after eliminating the background at 620nm. The optical density of cells is directly proportional to their amount. The cell viability and cytotoxicity percentages were estimated using the following formulas:

$$\textbf{Cell viability (\%)} = (Abs_{RSB} / Abs\ control) \times 100$$

The following formula was used to determine the tested substance's cytotoxic activity (%):

$$\textbf{Cytotoxic activity (\%)} = 100\ \% - \textbf{cell viability (\%)}$$

The RSB concentration producing 50% growth inhibition is termed IC_{50}.

2.6. Statistical analysis

Experiments were repeated three times and findings were provided as mean ± standard error. The ANOVA variance analysis was performed using the general linear models (GLM) approach in the Statistical Analysis System software (SAS version 9.1, SAS Institute, 2003). A p-value of < 0.05 indicated statistical significance.

3. Results and discussion

3.1. The Total Active Components in RSB

Phenolic compounds, extensively researched and reported by [48] and [49], possess at least one aromatic ring with hydroxyl groups known as reducing agents. These natural antioxidants, including phenolics and flavonoids, exhibit a broad range of pharmacological effects such as anti-allergic, antibacterial, anti-inflammatory, neuroprotective, and anticancer properties, and also shield plants from pathogenic microbial attacks.

The medicinal properties of Plants exist because of phytochemicals. These phytochemicals are secondary metabolites that are produced in sufficient amount under stressed conditions, allowing the plant to protect itself from detrimental

environmental effects. Consuming phytochemicals through diet may offer health advantages, such as protection against chronic degenerative conditions including cardiovascular, neurodegenerative diseases, and cancer.

The study found a phenolic content of 188 mg GAE g−1 RSB and a flavonoid content of 12 mg QE g−1 RSB. Phenolic chemicals are known to inhibit the cyclooxygenase and lipoxygenase pathways [50][51][52]. Flavonoids have been demonstrated to inhibit cell proliferation by inhibiting the Ornithine decarboxylase enzyme, a rate-limiting enzyme in polyamine biosynthesis linked to DNA synthesis and cell proliferation in a variety of tissues [53][54][55]. Flavonoids can also impede microbe development by depolarizing their membranes and preventing DNA, RNA, and protein synthesis [56]. The flavonoids and phenols found in this plant may talk more about the therapeutic effects of rosemary byproducts.

LC/MS-MS for RSB

Analysis of rosemary by-product extract revealed the presence of 16 compound. As shown in **Table 1** and **Figure 1** the primary components in RSB were (caffeic acid 31.2%, naringenin 27.8%, apigenin 7.39%, hesperetin 5.06%, and coumaric acid 3.94%).

Caffeic acid has already been found in rosemary [57] and [58]. The greater quantities of caffeic acid found in rosemary (300-1500 µg) are consistent with our studies [59]. However, Kivilompolo, Oburka, & Hyotylainen (2007) discovered that rosemary contains less than 50 µg of caffeine [60]; [61]. Differences in phenolic acid levels in rosemary in the literature can be attributed to genotypic and environmental differences within species, plant section selection, sample collection time, and technique of determination. According to [62], rosemary has the highest content of syringic acid (3.46 µg/g DW), however Hossain et al. also discovered it in rosemary [63].

Except for cumin, which contains 4.18 µ/g, other culinary herbs and spices exhibited comparable amounts of chlorogenic acid. The literature [63] offers findings that are congruent with ours.

Quercetin has already been discovered in rosemary [63]. Our analysis found quercetin levels of 11.88 µg/g DW, similar to another study that found 0.20-10 µg/g DW in rosemary [63].

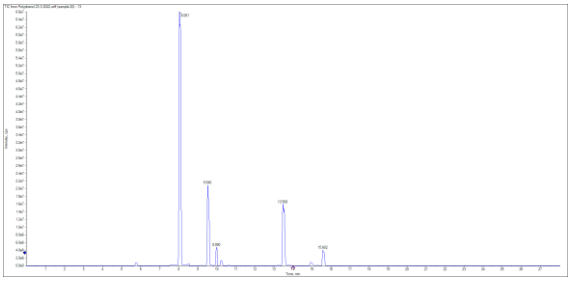

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Figure 1. Chromatogram of LC/MS-MS analysis of RSB ethanol extract

Table 1, phenolic and flavonoid compounds in RSB using (LC/MS-MS).

Compound	Rosemary Ethanol			
	RT	µg/g	µg/g	DW%
Chlorogenic acid	7.36	37.86	5.3	1.072521
Daidzein	N/A	N/A	N/A	0
Gallic acid	3.93	13.75	1.925	0.389518
Caffeic acid	8.08	101.96	154.2	31.217
Rutin	9.73	0.87	0.121	0.024646
Coumaric acid	9.5	139.29	19.5	3.945892
Vanillin	9.57	14.85	2.07	0.42068
Naringenin	14.98	982.34	137.52	27.82833
Querectin	13.62	81.34	11.38	2.304249
Ellagic acid	9.95	13.67	1.913	0.387252
3.4- Dihydroxybenzoic acid	5.77	72.60	10.164	2.056657
Hesperetin	15.61	178.90	25.046	5.067989
Methyl gallate	7.47	0.15	0.021	0.004249
Kaempferol	15.34	2.70	0.378	0.076487
Ferulic acid	10.26	126.30	17.682	3.577904
Syringic acid	8.41	39.35	5.50	1.114731
Apigenin	15.05	261.15	36.561	7.398017
Catechin	N/A	N/A	N/A	0
Luteolin	13.52	463.43	64.88	13.12833

3.2. The antioxidant activity of RSB

The antioxidant activity of the extract was assessed using two different assays: DPPH and β-Carotene/linoleic acid bleaching. This approach provides a comprehensive view of the extract's electron or free radical scavenging capabilities. These methods, which are based on distinct principles, are widely utilized to evaluate the antioxidant potential of complex samples [64].

The RSB extract is a potent antioxidant, as demonstrated by DPPH scavenging activities depicted in Figure 2, in comparison to the standards TBHQ and gallic acid. All analyzed samples exhibited an increase in antioxidant activity over time, indicating a concentration-dependent pattern of free radical scavenging ability.

Also as shown in **Figure 3**, RSB prevented bleaching of β-carotene by scavenging linoleate-derived radicals. Scavenging linoleate-derived radicals resulted in a higher concentration of β-carotene (85.13a) than TBHQ (31.3) or gallic acid (18.1).

Phenolic chemicals and flavonoids have been linked to antioxidative activity in biological systems, serving as scavengers of singlet oxygen and free radicals [65]. Antioxidant activity is strongly related with the presence of phenolic compounds [66]. In biological systems, free radicals are known as reactive oxygen species (ROS), which are the most physiologically relevant free radicals. ROS produced by cells include hydroxyl radicals, hydrogen peroxides, and superoxide anions [67].

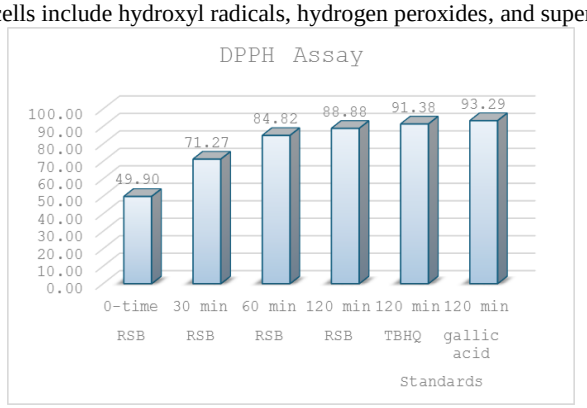

Figure 2:- Antioxidant activity of RSB against DPPH• as compared with TBHQ and gallic acid

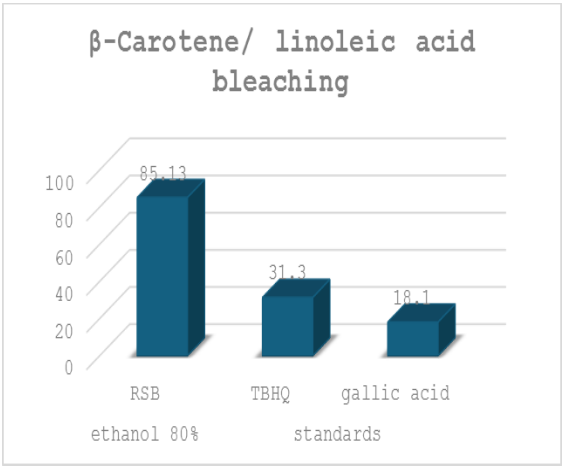

Fig 3: Inhibition of RSB in β-carotene-linoleic acid emulsion as compared with TBHQ and gallic acid.

3.3. Cytotoxicity effect of ethanolic 80% solid residue of rosemary by-product extracts on (Hct 116) Human colon and human Prostate (PC3) cancer.

The MTT assay was used to assess the cytotoxic activity of RSB extract against **(HCT 116) Human colon and human** Prostate **(PC3) cancer** cell lines with different concentrations (31.25-1000 µg/mL) as reported in tables (2 and 3). The antiproliferative activity of the plant extract on cancer cell lines was expressed in IC_{50} value. The IC_{50} is the inhibitory concentration required to suppress the cancer cell population by 50%.

The extracts caused an inhibition variation in the cell growth according to the kind of extract and type of cell line, the phenolic chemicals found in medicinal plants are bioactive and have a significant function in cancer prevention. They have a complementary and overlapping mode of action, including antioxidant activity, free radical scavenging, and modulation of carcinogen metabolism, all of which alter important cellular and molecular mechanisms related to carcinogenesis, a multistep process involving tumor cell transformation, survival, proliferation, angiogenesis, and metastases, as discovered by [**68**].

The anticancer properties of rosemary and its primary derivatives are linked to a number of actions, including antiangiogenic properties, antioxidant effects, epigenetic activities, changes in hormone signaling regulation of immune and anti-inflammatory responses, modifications to specific metabolic pathways, and increased expression of tumor suppressor

genes. Rosemary is best recognized for its antioxidant properties, which make it an effective cancer treatment. It neutralizes free radicals and protects DNA, proteins, and lipids from oxidative damage [**69**]. However, under some conditions, rosemary derivatives have been shown to have harmful effects by generating reactive oxygen species (ROS)

Rosemary and its derivatives are renowned for their epigenetic effects. Histone deacetylases (HDACs), enzymes that regulate gene expression by targeting histone acetyl groups, have abnormal expression patterns linked to tumor formation [**70]**. HDAC2 is known to be overexpressed in tumor cells, inhibiting p53 expression and programmed cell death. Rosemary extract may accelerate mitochondrial-dependent cell death by increasing the proapoptotic protein Bax and decreasing the antiapoptotic protein Bcl-2. [**71, 72**]

The prospect of rosemary interacting with hormone receptors is the most exciting aspect of the rosemary-prostate cancer relationship. The androgen receptor (AR) is a well-known prostate cancer target. However, antiandrogens' effects are typically transient. In almost half of the patients, Molecular alterations within the AR lead to antiandrogen resistance. [**73]**

Table 2 : Percent cell viability of ethanolic 80% solid residue of rosemary by-product extracts of HCT 116 cell line.

ID	Con c. µg /ml	Viability %	Toxicity %	IC_{50} µg /ml
HCT 116	--------	100	0	
RSB	1000	4.968383017	95.03161698	31.41
	500	4.968383017	95.03161698	
	250	5.420054201	94.5799458	
	125	5.691056911	94.30894309	
	62.5	23.75790425	76.24209575	
	31.25	57.00090334	42.99909666	

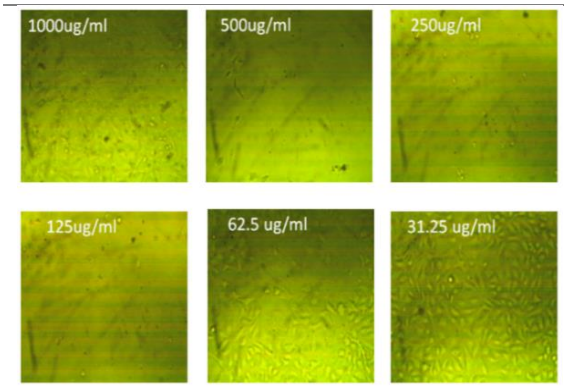

Fig 4: Effect of ethanolic 80% solid residue of rosemary 80% solid by-product extracts on human colon cancer cell line (PC3).

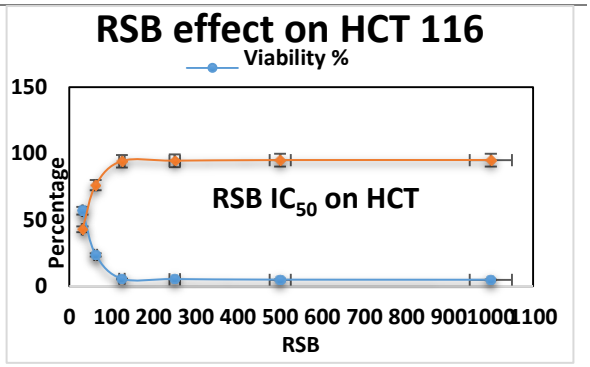

Fig 5: Percent cell viability and toxicity of ethanolic residue of rosemary by-product extracts of HCT 116

Table 3, Percent cell viability of ethanolic 80% solid residue of rosemary by-product extracts of PC 3 cell line.

ID	Con c. µg /ml	Viability %	Toxicity %	IC_{50} µg /ml
PC3	----------	100	0	
RSB	1000	4.675324675	95.32467532	33.14
	500	4.848484848	95.15151515	
	250	5.021645022	94.97835498	
	125	5.108225108	94.89177489	
	62.5	23.03030303	76.96969697	
	31.25	58.61471861	41.38528139	

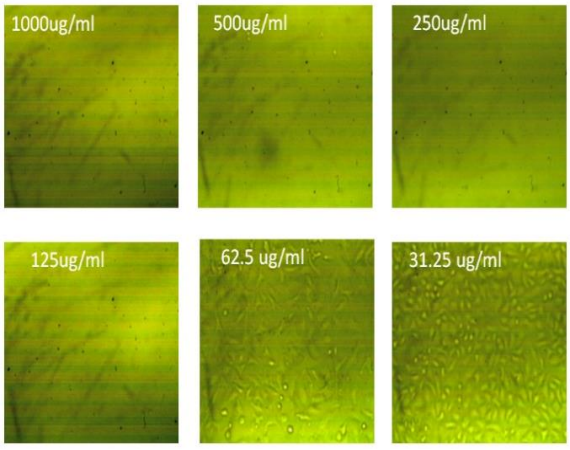

Fig 6: Effect of ethanolic 80% solid residue of rosemary by-product extracts on human prostate cancer cell line(PC 3).

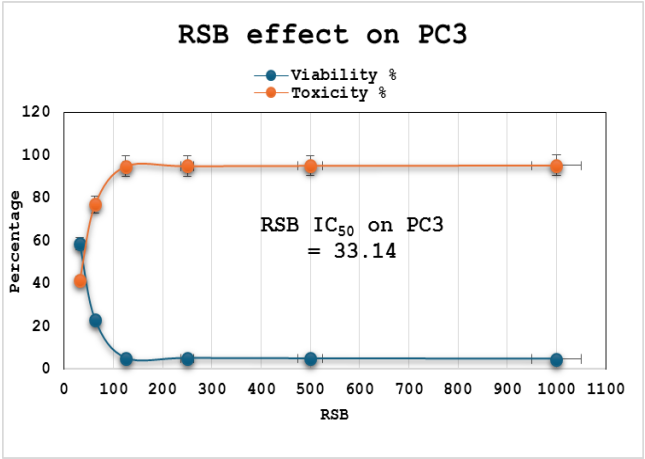

Fig 7: Percent cell viability and toxicity of ethanolic 80% solid residue of rosemary by-product extracts of PC3 cell line.

4. Conclusion

Chemical composition constituents of solid residue of rosemary by-product (RSB) indicated the presence of phenolic components as caffeic acid, naringenin, apigenin, hesperetin, and coumaric acid. RSB has antioxidant, and anticancer.
The solid residue of rosemary by-product leaves shows great promise in the creation of phytomedicines with anticancer characteristics. Because of their anticancer properties, drugs generated from rosemary solid by-product (RSB) may serve as an alternative therapeutic source. Furthermore, this investigation indicates that the ethanol extract of this by-product has the strongest anticancer activity against (HCT 116) human colon and human prostate (PC3) cancer cell lines.

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