Egypt. J. Chem. **Vol. 68**, No. 11 pp. 193 - 200 (2025)

Egyptian Journal of Chemistry

http://ejchem.journals.ekb.eg/

Profiling Bioactive Compounds and Assessing In Vitro Anticancer Activity of Wild *Ruta graveolens* (Rue) Subfractions

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Abstract

The ethnopharmacological importance of Rue lies in its traditional use for treating various diseases. Ruta graveolens (Rue) is widely used for a range of medicinal purposes. The aim of this study was to examine the effects of Rue subfractions against breast and liver cancer. Additionally, the polyphenolic profile of Rue alcoholic extract was analyzed using HPLC, and the in vitro antioxidant activity of different solvent extracts of Rue was evaluated. HPLC analysis identified twenty-four phenolic acids, with pyrogallol as the main component, followed by vanillic acid, along with twenty flavonoids, with rutin being the predominant flavonoid. Furthermore, Rue fractions exhibited anticancer activity, particularly the EtOAc subfraction, which demonstrated superior anticancer effects in both HepG2 and MDA-MB cell lines. Based on these findings, it can be concluded that the EtOAc subfraction of Rue, which contains a high amount of rutin, may serve as a safe and natural anticancer agent. Overall, this study highlights the potential of Rue subfractions as valuable and promising natural sources for developing new therapeutic compounds in the future.

Keywords: *Ruta graveolens*, polyphenolic profiling; HPLC; anticancer; Rutin

1. Introduction

Globally, cancer is the leading cause of mortality among various deadly illnesses. By 2040, it is projected that over 30 million new cancer cases will emerge, with the highest burden occurring in low-income countries [1]. The number of cancer cases is rising worldwide, in both industrialized and developing nations, due to several factors, including an aging population, sedentary lifestyles, poor diets, and other risk factors.

Currently, cancer is treated using radiation, chemotherapy, surgery, or a combination of these methods. While these treatments are therapeutically beneficial, they often cause severe suffering, particularly surgery and radiotherapy. Additionally, some cancer cells have developed resistance to multiple anticancer drugs, many of which also have significant adverse effects [2].

As a result, there is an urgent need for clinically effective medications that selectively target cancer cells while sparing healthy ones. Consequently, numerous efforts have been made to identify new anticancer bioactive compounds from various sources, including plant metabolites [3-10].

To supplement and enhance current medicines, it is imperative to develop novel, cost-effective anticancer drugs with minimal adverse effects. Bioactive compounds are of particular interest as lead substances for drug development due to their considerable chemical diversity and biochemical specificity. In fact, bioactive compounds such as polyphenols and related chemicals serve as active components in numerous medicinal plants (MP) [11-14].

Many of these compounds have been evaluated for their potential therapeutic benefits in various diseases, including Alzheimer's and cancer. Medicinal plants, recognized as some of the most important natural sources of antioxidants, have been extensively studied for their antioxidant and antimicrobial activities. These plants contain high concentrations of polyphenols—bioactive substances known for their metal-chelating, radical-scavenging, hydrogen-donating, and reducing properties [15-17].

Phenols are a broad group of natural plant products, all of which exhibit a broad spectrum of biological activity that makes them attractive as drugs. Given the risk of synthetic compounds proving to be toxic or harmful once placed on the market [18], bioactive substances from natural sources are already in demand. Such bioactive substances often exhibit antioxidant activity and resist the development of chronic diseases such as cancer. At the same time, the disadvantage of providing medicinal metabolites derived from plant sources is that the abundance of such biologically active substances is often very low [19].

The medicinal value of plants of the Rutaceae family is well established [20-21]. Because of its antibacterial, antifungal, anti-leishmanial, and anti-plasmodia qualities, this natural product has been the subject of extensive research, which shows its potential use in the treatment of a variety of illnesses, including cancer, Alzheimer's, depression, and other conditions [22].

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Receive Date: 13 January 2025, Revise Date: 09 March 2025, Accept Date: 23 March 2025

DOI: 10.21608/ejchem.2025.352867.11162

The genus Ruta is one of the plants from the Rutaceae family that has been investigated. Rue is the most prevalent species [23]. An extensive range of pharmacological activity, including antioxidant, insect repellent, larvicidal, antiandrogenic, antidepressant, antihyperglycemic, antihyperlipidemic, anti-inflammatory, and anticancer properties, have been described for Rue's biological properties [23-24]. Rue contains more than 100 bioactive substances from various classes of natural products such as alkaloids, essential oils, and polyphenolic substances [25-26]. Many of these bioactive substances are therapeutically active [27-29].

However, the bioactive constituents of Rue have not been accurately characterized, limiting its widespread use in modern medicine. Therefore, further research is needed to better understand its chemical composition and biological activity. The present study aimed to explore Rue from Egyptian flora to identify new natural bioactive compounds. Accordingly, the in vitro biological evaluation of Rue subfractions for their anticancer properties was conducted. Additionally, the polyphenolics composition of these subfractions was determined using HPLC.

2. Results and Discussion

Polyphenol profile

The polyphenol content in plants has been shown to depend on biological factors, as well as soil and environmental conditions. Furthermore, the solubility of phenolic compounds is influenced by the type of solvent used, the degree of polymerization of the polyphenolic compounds, and their interactions.

Natural phenolic bioactive substances including phenolic acids, flavonoids, tannins, coumarins, and others have been reported to have major biological activities [33-34]. Polyphenolic profile of alcoholic extract was analyzed using HPLC. HPLC Analysis of the alcoholic extracts of Rue showed numerous UV-detectable compounds. In this work, the alcoholic extract of rue was found to contain bioactive substances as shown in Table 1.

Forty-four phenolic compounds were identified using HPLC analysis in rue alcoholic extract (Table 1, 2), including 24 phenolic acids and 20 flavonoids. The major peaks in the rue alcoholic extract were assignable to Luteo,6-arabinose-8-glucose>Pyrogallo>Rutin>Apig.6- glucose -8- rhamnose>e-Vanillic. These compounds were previously identified for rue extract [35], and found that the aerial part extract of rue contained significant amounts of alkaloids, coumarins, flavonoids, and tannins.

Analysis of the phenolic composition revealed that kaempferol was found exclusively in the stems and leaves of cultivated Rue, whereas naphthoresorcinol and luteolin were present only in the stems and leaves of wild Rue. The variation in phenolic bioactive compounds between Rue origins can be attributed to differences in climate and location. Additionally, variations were observed between different parts of the Rue plant; for example, ferulic acid is found in stems and leaves but absent in flowers; Salicylic acid is found in the stems and flowers but not in the leaves. The variation may be related to the morphological differentiation that occurs during the phenological cycle [36].

Table 1: HPLC identification of phenolic compounds in rue EtOH extract

Phenolic µg/g			
Gallic	7.68	*p*-coumaric	7.66
Pyrogallol	658.12	Ferulic	3.83
4-amino- *benzoic*	11.60	Iso-Ferulic	2.12
protocatchuic	111.60	Resveratrol	0.8
Cateachin	40.68	Ellagic	20.15
Chlorogenic	39.14	e- Vanillic	379.94
Catechol	42.92	Alpha-Coumaric	4.16
Epicatechin	32.04	Benzoic	217.16
Caffeine	3.83	3,4,5-methoxy-cinnamic	2.61
P-OH- benzoic	40.90	Coumarin	8.33
Caffeic	15.16	Salicylic	115.38
Vanillic	31.41	Cinnamic	10.71

Table 2: HPLC identification of flavonoids in rue EtOH extract

Flavonoids µg/g			
Luteo,6-arabinose-8-glucose	685.07	Apig.7-o-neohespiroside	18.67
Luteo,6- glucose -8-arabinose	142.05	Kamps-3,7-dirhamoside	29.99
Apig.6-arabinose-8-gala*ctose*	11.36	Apigenin-7-glucose	8.27
Apig.6-rhamnose-8-glucose	170.13	Quercetin	4.97
Apig.6- glucose -8- rhamnose	404.55	Quercitin	39.11
Luteo-7-glucose	74.47	Hesperetin	38.67
Luteolin	84.53	Kampferol	187.98
Naringin	6.61	Rhamnetin	12.89
Rutin	629.03	Apigenin	2.94
Rosmarinic	3.76	Acacetin	76.56

Cytotoxicity of rue subfractions

The MTT assay was performed in the present work. The concentration required for 50% inhibition (IC_{50}) for the extract activity was calculated using different concentrations of fractionated ethanol extract of Rue by different solvents (Table 3).In this work, subfractionated ethanol extract of Rue exhibited antiproliferative effects on HepG2 and MDA-MB cells, and this antiproliferative effect was also observed previously [37]. The EtOAc subfraction of rue exhibited the highest anticancer activity in both cell lines (5.83µg/ml in Hep-G2, 60.8 µg/ml in MDA-MB), followed by the petroleum ether subfraction (14 µg/ml in Hep-G2). On the other hand, petroleum showed the lowest anticancer activity (243.6 µg/ml in MDA-MB) followed by EtOH fraction (102 µg/ml in MDA-MB). The anticancer activity of rue subfractions followed the order: EtOAc>petroleum>EtOH>$CHCl_3$in the Hep-G2 cell line. While in MDA-MB cell lines followed the order EtOAc > $CHCL_3$>EtOH petroleum subfraction.

Table 3: Antitumor Activity of fractionated ethanolic *Rute* extract against HepG2 and MDA-MB Cells

	MDA-MB				Hep-G2			
Inhibition %	EtOH	Pet-ether	$CHCl_3$	EtOAc	EtOH	Pet-ether	$CHCl_3$	EtOAc
12.5	5±0.01	4±0.02	18±0.25	29±0.35	41±1.86	39±1.74	18±1.2	48±1.42
25	19±0.95	18±0.76	29±0.17	34±0.92	44±2.51	61±2.14	37±2.4	58±1.98
50	20±1.02	20±0.93	47±0.13	44±0.85	73±1.46	65±4.51	39±1.6	62±3.15
100	45±0.06	23±0.09	61±0.24	68±1.96	77±3.25	67±2.6	65±3.5	72±3.1
IC_{50} (µg/ml)	102±0.19	243.6±4.25	70.22±2.12	60.8±1.45	26.7±2.47	14±0.85	68±2.3	5.83±0.05

The antitumor activity of flavonoids is still a point of discussion. ROS can damage DNA and division of cells with unrepaired damage that leads to mutation. Also, it has been stated that flavonoids as antioxidants such as apigenin and luteolin are stated to be potent inhibitors of cell proliferation [38]. A large clinical study suggested the presence of an inverse association between flavonoids intake and the subsequent incidence of lung cancer [39].

The observed reduction in nitric oxide generation is believed to be caused by the presence of several flavonoids in rue, as well as other natural antioxidants such phenolic bioactive compounds like coumarins and their derivatives [40]. To elucidate such impacts, more research is required.

Homeopathy and traditional medicine around the world have long used rue extracts for therapeutic purposes. Flavonoids, coumarin derivatives, furoquinolines, essential oils, undecanone, and other active ingredients are all present in rue [41]. One of Rue's flavonoid compounds, rutin, has anticancer properties [42]. Numerous in vitro investigations have assessed the possible anticancer agent properties of rue [43-45]. Rue extract has been shown to inhibit tumor growth and exert a cytotoxic effect on various cancer cell lines [45]. When consumed alone [43-44] or in combination with calcareous phosphate [41], Rue demonstrates a cytotoxic effect in vitro. Additionally, it has been found to inhibit tumor growth in mice [46]. A reduction in tumor size was observed when Ehrlich Ascites Carcinoma cells were treated with Rue methanol extract. Rue also induces cell death in brain cancer cells, and its mode of action may be linked to alterations in telomere dynamics, leading to mitotic catastrophe and apoptosis [47].

Remarkably, Rue also prevented the growth of liver tumors caused by N0-nitroso-diethylamine [47]. In gamma-irradiated rats, extracts of Rue showed evidence of genotoxic and clastogenic potential [48]. Increased apoptosis and decreased

clonogenicity and dose-dependent cell survival were noted in cancer cells treated with Rue extract for prostate, breast, and colon cancer. Twelve treatments with Rue alkaloids, cytotoxic and apoptotic effects on lung and breast cancer cells were also noted [42]. Rutin (Figure 1), a flavonoid with nitric oxide scavenging properties, is the major active bioactive compound found in rue [49]. The literature now in publication states that the rue plant has a rutin content of around 2% [50].

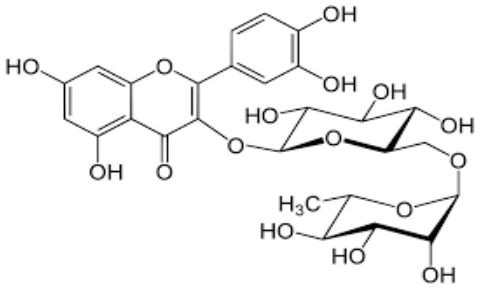

Figure 1: Rutin structure

Rutin is a glycoside that contains the flavonoid quercetin, which the gastrointestinal tract can hydrolyze to produce quercetin [51]. The medicinal properties of rutin, a naturally occurring flavonoid, have been thoroughly investigated [52-53]. Leukemia, breast, liver, ovarian, endometrial, gastric, and colon cancers have all been demonstrated to be significantly inhibited by rutin [54-56]. By causing apoptosis, inhibiting angiogenesis, and lowering oncogene expression, rutin has been demonstrated in numerous studies to be able to regulate the progression of cancer (Figure 2, Table 4) [57-59]. It has been proposed that rutin contributes significantly to Rue's medicinal benefits.

Table 4: Rutin Anticancer mechanisms of on different cell lines

Type	Mechanisms	Reference
Hepatic carcinoma (HTC hepatic cells)	Cytotoxic	[60]
Colorectal (CRC)	Cell cycle arrest apoptosis	[61]
Leukemia (HL-60)	Reducetumor size	[62]
Neuroblastoma (LAN-5)	Decrease BCL2 expression BCL2/BAX ratio along with reduction in levels of MYCN mRNA level and the secretion of TNF-α	[63]
Colon cancer (SW480)	Less harmful effects on body weight and relative organ weight in mice Increasemean survival time	[64]
Melanotic melanoma (B16 melanoma)	Inhibit melanin formation Augmentation of tumor mass	[65]
Pulmonary metastasis (B16F10)	Reduce metastatic nodules	[66]

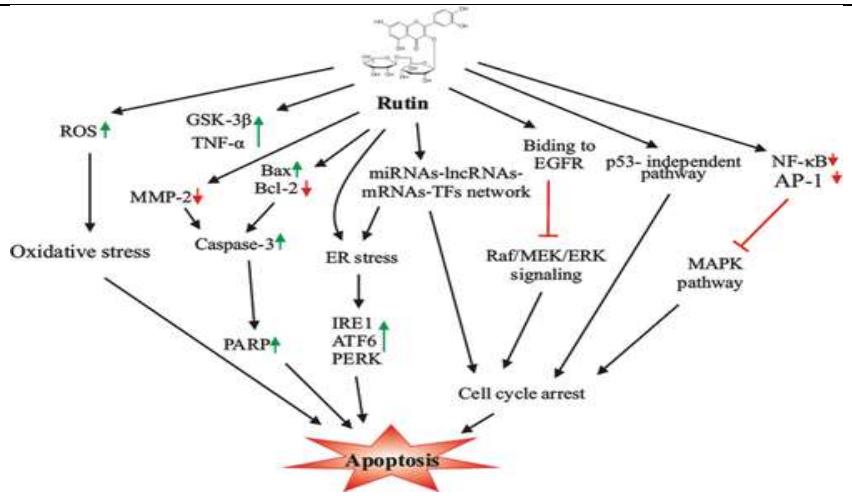

Figure 2: Anticancer molecular mechanisms of rutin

At the end of discussing the role of flavonoids found in rue, we can conclude that flavonoids are complex because of the heterogeneity of the different molecular structures and the scarcity of data on bioavailability. Furthermore, the insufficient methods to measure the oxidative damage in vivo and the measurement of endpoints remain difficult. However, in conclusion the in vivo studies that already performed give a hopeful picture for the future.

3. Experimental

Plant material

Wild Rue was collected from El-Kanater El-Khairyah, Qalyubia Governorate, Egypt, in June 2022. A voucher specimen of the sample is preserved in the herbarium of the National Research Center. The plant was dried in the shade until it reached a moisture content of 10% and was then stored at room temperature for further study.

Extraction and fractionation

The areal parts of Rue (1 kg) were extracted with 70% ethanol (EtOH). The extract was evaporated under reduced pressure to get EtOH fraction, which was suspended in water and partitioned successfully with petroleum ether (Pet-ether), chloroform ($CHCl_3$), and ethyl acetate (EtOAc) [30].

Polyphenolic profiling using HPLC

The alcoholic extract of Rue was analyzed using HPLC according to Kim et al. [31]. The HPLC system was a HP 1100 chromatograph (Agilent Technologies, Palo Alto, CA, USA) equipped with an auto-sampler, quaternary pump, and a diode array detector. The measurements were integrated by ChemStation chromatographic software interfaced with a personal computer. The analytical column was ZORBAX Eclipse XDB C18 column (15 cm x 4.6 mm I.D., 5 µm, USA).

MTT assay

HepG2 and MDA-MB cells were cultured in Dulbecco's modified Eagle medium containing 100 µg/ml streptomycin (Nacalai), 100 U/ml penicillin, and 10% heat-inactivated fetal bovine serum (Biosera). HepG2 cells were seeded in 96-well plates for 24 h and incubated in the presence of test samples for another 72 h. Then 10 µl of 5 mg/ml MTT was added. After incubation for 4 h, cells were extracted with DMSO and measured for absorbance at 535 nm [32].

4. Conclusions

Based on our study and supporting literature, we report that the successful subfractionation of ethanol extract from Ruta graveolens (Rue) exhibited significant antiproliferative activity. Our findings identified polyphenolic compounds as the key bioactive molecules responsible for inhibiting cancer cell growth, suggesting that these compounds contribute to Rue's anticancer potential. This highlights Rue as a promising natural source of bioactive molecules that could serve as alternatives to synthetic anticancer drugs. However, the precise mechanism of action by which these flavonoids exert their optimal therapeutic effects remains to be fully understood. To further validate these findings, we recommend evaluating the anticancer potential of the diamond subfraction in an animal model, paving the way for future advancements in natural cancer therapy.

5. Conflicts of interest

"There are no conflicts to declare".

6. Formatting of funding sources

Not applicable

7. Acknowledgments

We would like to express our great thanks and appreciation to the National Research Centre, Dokki, Egypt for providing facilities for this work.

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