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Phytochemical composition, antioxidant and antiproliferative activities of *Rosmarinus officinalis* leaves

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Phytochemicals in *Rosmarinus officinalis* leaves, their total phenolic content, antioxidant potential and antiproliferative activity against human prostate (DU145), colon (CT26) and cervical (HeLa 229) cancer cells were investigated. Extraction was done separately using hexane, dichloromethane, ethyl acetate and methanol. A total of 32 compounds were identified, eight of which were reported for the first time. The highest phenolic content was 476.80 ± 0.69 µg/ml for the methanolic extract which also had the highest antioxidant activity with a minimum inhibitory concentration of 5.39 ± 0.09 mg/ml. Extracts exhibited the highest toxicity against prostate cancer cells and the least against cervical cancer cells.

Introduction

Cancer is one of the leading causes of mortality worldwide [1]. It is characterized by irregular proliferation of malignant cells in a series of stages with different biochemical, molecular and cellular events [2]. Cancer is caused by both internal factors (such as mutations, hormones and immune conditions) and external factors like chemicals, radiation and infectious microorganisms [3, 4].

Treatment of cancer is costly, and this has been exacerbated by the resistance of tumor cells to the available antineoplastic drugs. Due to their lack of specificity, the conventional cancer therapies present severe side effects and in most developing countries are inaccessible to cancer patients [5]. Thus, traditional medicine is gaining more attention in chemoprotective management of cancer [1]. Over 3,000 plant species have been reported to have anticancer properties [6]. An example is *Camptotheca acuminate* from which the anticancer drug Camptothecin has been developed [6].

Although a number of plants have been claimed to have antitumor properties, they have not been fully investigated for the development of novel anticancer drugs [7]. *Rosmarinus officinalis* L. (Rosemary) is one of the plants used in the traditional management of cancer in Uganda. However, its safety to humans as well as identification and isolation of the main phenolic compounds as the presupposed source of anticancer activity has not been fully documented. In the current study, we report on the phytochemicals in the leaves of *R. officinalis*, their total phenolic content, antioxidant potential and antiproliferative activity against human prostate (DU145), colon (CT26) and cervical (HeLa 229) cancer cells.

Experimental part

Ethical approval

This study was approved by Centre for Traditional Medicine and Drug Research, Kenya Medical Research Institute Scientific and Ethics Review Unit, Kenya (Approval No. KEMRI/RES/7/3/1).

Sampling and sample preparation

Leaves of *R. officinalis* were collected from cultivated plants in Wakiso district of Uganda ($0^0$23'36" N, $33^0$0'9" E) with permission from Uganda Natural Chemotherapeutics Research Institute, Kampala, Uganda where they were identified by Kyoshabire Medius (a taxonomist). A voucher sample (No. 50907) was deposited at Makerere University Herbarium, Kampala, Uganda on 7th August 2019.

Laboratory samples were air-dried in mesh bags and ground into a fine powder using a laboratory mill. Weighed 150 ± 0.1 g of the powder were separately extracted with 775 ml of *n*-hexane, dichloromethane (DCM), ethyl acetate and methanol in 1000 ml conical flasks for 96 hours at room temperature. The crude extracts were filtered using a cheese cloth, Whatman No. 1 filter paper and concentrated to dryness on a rotary evaporator (Rotavapor BUCHIR-100, Switzerland) [8]. The extracts were transferred into sample bottles which were placed in a desiccator of anhydrous sodium sulphate. The yields of the extracts were calculated (**Equation 1**) and they were transferred into tightly stoppered bottles which were kept at 4 ℃.

$$\text{Percentage yield} = \left(\frac{A}{A0}\right) \times 100 \qquad (1)$$

Where A is the amount of crude extract obtained after drying and A_0 is the weight of the leaves used for extraction.

Fractionation was done for the methanol extract because it had the highest yield. The dried crude methanol extract was divided into two parts; one portion (1.5 g) was kept in the crude form and the other portion (8 g) was subjected to column chromatography fractionation.

Antiproliferative activity of the extracts

The Vero, prostate (DU145), colorectal (CT26) and cervical (HeLa 229) cancer cells were

separately thawed in a water bath at 37 ^{0}C. Growth media (20 ml) was added to 1 ml of each of the cell lines in T-75 culture flasks and incubated at 5% carbon dioxide and 37 ^{0}C to revive the cells. Culturing was done for three days until when the cells obtained at least 80% confluence. The excess media was poured off, leaving the cells attached to the surface of the flask and the flask was washed 3 times with phosphate buffer saline (PBS). Excess PBS was poured off and then 500 µL of Trypsin-EDTA was added into the flask having cells attached to the surface. This was spread evenly on the inner surface of the flask by tilting the flask back and forth and then incubating for 3 minutes. Trypsin was added to detach the cells off the surface of the flask. Growth media (10 ml) was added immediately to stop the action of Trypsin. Growth media was purged gently to allow breaking of clumps between cells.

In vitro antiproliferative assay was done for both crude extracts and the solid phase extracted methanolic isolates using MTT assay [9]. Briefly, the cancer cells were washed 3 times with 5 ml of PBS after attainment of 100% confluence and harvested by trypsinization. The number of viable cells was determined by Trypan blue exclusion test. Approximately 2 × 10^4 cells/ml suspension of both Vero and cancer cells were seeded in 96-well plates and incubated for 24 hours.

Measured 15 µl of the extracts and the commercial drug doxorubicin at seven different concentrations (1000, 333.33, 111.11, 37.03, 12.34, 4.11 and 1.37 µg/ml) were added from rows H to B and the plates incubated for 48 hours. Row A acted as the negative control (extracts or the drug were not added to it).

After incubation, 10 µl of MTT dye solution was added to each of the wells in the plates and incubated for 4 hours. The media was then poured off from the wells of the plates leaving cells alone attached to the surface. Measured 50 µl of DMSO was added to solubilize the formazan crystal formed by viable cells. Absorbance was then read on a scanning multi-well spectrophotometer at 562 nm [10].

Absorbance values higher than the control cells indicated an increase in the rate of cell proliferation and vice versa [11]. The percentage viability was evaluated by determining absorbance with the corresponding chemical concentrations. Linear regression analysis at 95% confidence limits and R^2 were used to define dose-response curves of percentage viability of cells against concentration. Percentage of cell viability was calculated using **Equation 2** and **Equation 3**.

$$\% \text{ cell viability} = 100 - \% \text{ cytotoxicity} \quad (2)$$

$$\% \text{ cytotoxicity} = \frac{A-B}{A} \times 100 \quad (3)$$

Where A is the optical density of control and B is optical density of test drug.

Data was analyzed to obtain the minimum inhibitory concentration (IC_{50}) and median cytotoxic concentration (CC_{50}) of the extracts on cancer and Vero cells, respectively [12]. The selectivity index (SI) was calculated as the ratio of CC_{50} to IC_{50} [7, 13].

Determination of total phenolic content and antioxidant activity

The TPC of the extracts were determined using Folin-Ciocalteu reagent as described by previous authors [14, 15]. Briefly, 0.5 ml of the extract dissolved in 1 mg/L of methanol in falcon tubes. Gallic acid solutions of 0, 20, 40, 80 and 100 µg/ml were also added into the tubes in methanol : water (50 : 50 v/v) were mixed with 0.5 ml of Folin-Ciocalteu reagent diluted 10-fold in distilled water in falcon tubes and allowed to stand at room temperature for 5 minutes. Exactly 1.5 ml of sodium carbonate (20 g in 100 ml of distilled water) solution was then added, followed by 8.5 ml of distilled water. After 90 minutes, the absorbance was measured using UV-1900 UV Vis Spectrophotometer (Shimadzu Corporation, Japan) at 755 nm using Gallic acid as the standard solution [16].

Antioxidant activity was assessed using DPPH radical scavenging assay as described by Awah and Verla [14]. Briefly, 8.5 ml of methanol was added to 0.1 g of the extracts. From these, 200 µg/ml was made by transferring 0.167 ml of sample stock solutions in different falcon tubes and the volume made up to 10 ml. The solutions were then mixed with 1 ml of 0.1 mM DPPH in methanol. The mixture was shaken vigorously and allowed to stand at room temperature in the dark for 25 minutes. Blank solutions were prepared with 1 ml of methanol while the negative control was 1 ml of 0.1mM DPPH solution in 2 ml of methanol. Thereafter, the absorbance of the assay mixtures was measured at 517 nm using a UV visible spectrophotometer to measure the decolourization to yellow diphenylpicrylhydrazine. DPPH radical inhibition was calculated using **Equation 4**.

$$\% \text{ inhibition} = \left(\frac{A_S - A_O}{A_o}\right) \times 100 \qquad (4)$$

Where A_o = the average absorbance of blank (untreated cells) and A_S = absorbance of the sample (treated cells).

Characterization of compounds in R. officinalis methanolic leaf extract

The functional groups in the extract fractions were analyzed by Fourier Transform Infrared Spectroscopy (FTIR) using a Shimadzu FTIR spectrometer (Nicolet NEXUS 470, Thermo Scientific, USA). Aliquots (0.1 g) of the fractions were dissolved in 10 ml of methanol. Exactly 0.6 ml of the sample solution was poured on Attenuated Total Reflection (ATR) crystal and the spectra were read at 4500 to 400 cm^{-1}. The frequencies of the different components were recorded. The resolution was 4 cm^{-1} for 20 scans on each sample [17, 18]. The analysis was repeated twice for spectra confirmation.

Solid phase extraction and clean up was done for the methanol and ethyl acetate fractions. The end-capped C18 cartridge of sorbent mass, 500 mg; particle size, 50 µm; pore diameter, 48Å; surface area, 526 m^2/g was conditioned with 5 ml of 10% methanol in acidified water. Measured 20 ml of each fraction solution was loaded into a C-18 (Supelco, Sigma-Aldrich Germany) column and allowed to flow under gravity. The co-extracted substances were eluted from the sorbent with 100 ml of aqueous acetic acid (2% v/v). The column was dried using a pressure pump in the vacuum manifold for 5 minutes and total retained phenols were eluted with 1.2 ml of 0.1% formic acid acidified methanol [19]. Purified extracts were filtered through a 0.1 µm filter prior to liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS) analyses [20].

LC-MS/MS was used to identify compounds in the clean-up fractions. The auto-sampler LC system (Finnigan, Thermo Electron Corporation, USA) was coupled to an MS detector (Agilent Technologies, 6420 Triple Quad, USA). Sample solutions of 5 µL were injected into C-18 reverse phase column (Poroshell 120 EC-C18 3 × 50 mm, 2.7 µm, USA) at 40 °C. Data acquisition software was for 6400 Series Triple Quadrupole (Version B.08.00, Qualitative analysis software Version B.07.00 Service Pack 1). Solvent A was made of a mixture of 0.1% formic acid in water and 0.1% ammonium formate in water. It was made by adding 1 ml of formic acid to 1000 ml of water and then a solution of 1.0 g of ammonium formate dissolved in 1000 ml of deionized water and the two solutions were mixed to form solvent A. Solvent B was made of 0.1% formic acid in methanol which was made by adding 0.6 ml formic acid to 600 ml of methanol.

The elution was conducted at a column flow rate of 0.5 ml/min, the pressure of 350 bars, a column temperature of 40 ^{0}C at gradient elution for 35 minutes [21]. From 0 to 0.5 minutes, elution was 95% solvent A and 5% solvent B, 0.5-12 minutes was 58% A and 42% B, at 12-15 minutes was 40% A and 60% B, 15-20 minutes was 5% A and 95% B, 20-25 minutes was 5% A and 95% B, 25-25.5 minutes was 90% A and 10% B and then 25-35 min was 95% A and 5% B. The eluent was monitored at Electron spray ionization connected to an ion trap MS (ESI-MS) under negative ion mode at full scan mode of 55-500 m/z [22]. Identification of the compounds was based on retention time in reversed-phase LC and MS spectral features [21].

Statistical analysis

Experiments were done in triplicate and data presented as means ± standard deviations. ANOVA was used to establish any significant differences between extracts and controls. Correlations between antioxidant activity and antiproliferative activity were established using Pearson's correlation coefficient. Analyses were

performed at $P < 0.05$ using Minitab statistical software (Release 17, Minitab Inc., USA).

Results and discussion

Percentage yield

The yield of the different extracts, obtained as the percentages of initial mass of the sample macerated is shown in **Table 1**.

Table 1. Organic extract yield of *R. officinalis* leaves

Solvent	Yield (g)	Percentage yield
Methanol	81.210	54.14
Ethyl acetate	65.115	43.41
Dichloromethane	58.005	38.67
Hexane	32.025	21.35

Methanol gave the highest yield (54.14%) while *n*-hexane gave the least yield (21.35%). This is could be due to the differences in polarity as methanol being the most polar gave the highest yield. It could be because it extracted many compounds from the leaves. Differences in solvent polarities used for extraction is known to play a key role in increasing the solubility of phytochemical compounds [23, 24]. Further, differences in the structure of phytochemical compounds also determine their solubility in solvents of different polarities [25]. Indeed, the four solvents used had different polarities arranged as hexane < DCM < ethyl acetate < methanol. Therefore, the results of the current study confirmed the effect of varying solvent polarities on the yield of plant extracts and confirmed the richness of *R. officinalis* leaves in polar phytochemicals. The results obtained are consistent with those of Widyawati et al. [26] who assessed the effects of solvent polarity on the phytochemical yields from *Pluchea indicia* leaf extracts.

Antiproliferative activity of R. officinalis leaf extracts and fractions

The anticancer activity was determined for both crude solvent extracts and fractionated methanol extract. The minimum inhibitory concentrations (µg/ml) required to give 50% of cell death (IC_{50}) by the crude extracts and positive control (doxorubicin) on the prostate, colorectal and cervical cancer cells are shown in **Table 2**. Doxorubicin showed the highest activity on all cancer cell lines compared to the plant extracts ($P < 0.05$). This was evidenced by its very low IC_{50} values (4.36 ± 0.22, 6.39 ± 0.47 and 3.64 ± 0.33 µg/ml for prostate, colorectal and cervical cancer cell lines) compared to the plant extracts.

Table 2. IC_{50} values (µg/ml) of *R. officinalis* leaf extracts against prostate, colorectal and cervical cancer cells

Extract	DU145	CT26	HeLa 229
Methanol	147.38 ± 0.53	301.99 ± 0.53	432.47 ± 0.41
Ethyl acetate	182.48 ± 0.50	460.08 ± 0.14	522.80 ± 1.06
DCM	1459.10 ± 0.86	928.57 ± 0.49	931.63 ± 1.19
Hexane	Not active	1104.04 ± 0.06	1001.10 ± 0.41
Doxorubicin	4.36 ± 0.22	6.39 ± 0.47	3.64 ± 0.33

For results of antiproliferative activity, $IC_{50} < 10$ µg/ml is considered potentially very toxic; IC_{50}

between 10 and 100 µg/ml is potentially toxic; IC_{50} between 100 and 1000 µg/ml is potentially harmful and $IC_{50} > 1000$ µg/ml is potentially non-toxic [27]. As shown in **Table 2**, the methanol extracts were highly toxic on all the cancer cell lines studied compared to other extracts. This is because it showed the least IC_{50} values which means, only a small concentration of the extract is required to reduce the number of cancer cells by 50%. Ethyl acetate extract was the second most active, followed by DCM extracts and then finally hexane extracts. This order was also recorded for the TPCs as well as the antioxidant activity of the extracts. This shows that the phenols responsible for the antioxidant activity as well as cytotoxicity of these cancer cells are polar. Correlations between antioxidant activity of the crude extract and antiproliferative activity were established using Pearson's correlation coefficient. It was found that the antioxidant activity is positively correlated with the antiproliferative activity of the crude extracts against cervical and colorectal cancer cell lines. However, there was a negative correlation for prostate cancer cell lines. The correlation was not statistically significant in all cancer cell lines ($P > 0.05$).

Previous studies reported that *R. officinalis* extracts (6.25-50 µg/ml) inhibited viability of DU145 and PC3 prostate cancer cells with IC_{50} of about 8.82 µg/ml [28]. The extracts were also effective against colon cancer cell lines: HT-29, HCT116, W480, and HGUE-C-1 for doses between 1.5 to 100 µg/ml with IC_{50} between 16.2 and 25 µg/ml [29-33]. For For HeLa (cervical adenocarcinoma), inhibition was at 1.56-400 µg/ml with IC_{50} between 10.02 and 23.31 µg/ml [34, 35].

The mechanism of anticancer activity of *R. officinalis* extracts is not clear though. Many studies attributed its antiproliferative activity to enhanced apoptosis and cell death [36]. Increased poly Adenosine diphosphate ribose polymerase (PARP) cleavage, an indicator of enhanced apoptosis was reported for colon, pancreas, breast and lung cancer cell lines [37]. Rosemary extract also increased nitric oxide production and tumor necrosis factor production in pancreatic and liver cancer cells [38, 39], indicating enhanced cell death capabilities and nitric oxide-induced apoptosis. For ovarian cancer cells, enhanced apoptosis was associated with increased gene expression of mitochondrial-regulated apoptosis proteins cytochrome c [40]. These proteins are in the electron transport chain, and along with heat shock protein 70 (hsp70) involved in protein folding protects the cell from heat stress and toxic chemicals. Other mechanisms of apoptosis by Rosemary extracts include enhanced protein expression of pro-apoptotic Bax and cleaved-caspase 3 [32, 41], increased expression of binding immunoglobulin protein (BiP) and enhancer-binding protein homologous proteins (CHOP) which induce endoplasmic reticular stress [33, 41], and the unfolded protein response in prostate and colon

cancer cells [31, 33, 41, 42]. Rosemary extracts have also been reported to exert antioxidant effects in colon, breast and leukemia cell lines, protecting cells from oxidative DNA damage [37].

The results of antiproliferative activity of the fractions from methanolic extract of *R. officinalis* leaves are shown in **Table 3**. Ethyl acetate fraction showed the highest anticancer activity with IC_{50} of 6.39 ± 0.26, 261.31 ± 0.27 and 119.34 ± 0.38 µg/ml for DU145, CT26 and HeLa 229 cancer cells, respectively. On the other hand, hexane fraction had no activity against CT26 and HeLa 229 cells and had an IC_{50} of 1019.26 ± 0.28 µg/ml for DU145 cells.

Table 3. IC_{50} values (µg/ml) of the fractions of methanolic extract of *R. officinalis* on the cancer cells

Fraction	DU145	CT26	HeLa 229
Methanolic	28.28 ± 0.49	272.32 ± 0.56	385.43 ± 0.52
Ethyl acetate	8.54 ± 0.47	196.02 ± 0.03	181.47 ± 0.50
DCM	812.49 ± 0.50	773.41 ± 0.35	569.30 ± 0.58
Hexane	Not active	972.26 ± 0.44	902.69 ± 0.60
Methanol SPE	488.90 ± 1.01	521.29 ± 0.50	578.74 ± 0.65
Ethyl acetate SPE	429.30 ± 0.26	512.02 ± 0.04	550.75 ± 0.53
Doxorubicin	4.36 ± 0.22	6.39 ± 0.47	3.64 ± 0.33

SPE: solid phase extract.

The CC_{50} of the extracts and the fractions were determined (**Table 4**).

Table 4. CC_{50} values of the tested *R. officinalis* leaf extracts and fractions on Vero cells

Extract/Fraction	CC_{50} (µg/ml)
Methanol extract	468.55 ± 0.51
Ethyl acetate extract	599.27 ± 0.24
DCM extract	1253.00 ± 0.62
Hexane extract	Not applicable
Ethyl acetate fraction	401.09 ± 0.08
Methanol fraction	378.38 ± 0.55
DCM fraction	1644.64 ± 0.58
Hexane fraction	Not applicable
Methanol SPE	1897.12 ± 0.11
Ethyl acetate SPE	1841.27 ± 0.47
Doxorubicin	6.36 ± 0.45

The methanolic fraction of *R. officinalis* showed the least CC_{50} value of 378.38 µg/ml which is potentially harmful while the methanolic solid phase extract showed the highest CC_{50} value of 1897.12 ± 0.11 µg/ml which is potentially non-toxic. The results obtained showed that all the extracts under investigation were less toxic to normal Vero cells, compared to the positive control (doxorubicin) with CC_{50} = 6.36 ± 0.45 µg/ml which is potentially very toxic.

The fractions were comparatively more cytotoxic than the corresponding crude extracts while the solid phase extracts were less cytotoxic when compared to the crude extracts. This could be attributed to greater activity of the polyphenols than in crude extracts where they had interferences [43]. It was observed that the isolates obtained through solid phase extraction showed lower toxicity than the crude extracts and fractions. This could be due to synergistic effects in the crude extracts [30]. Among the fractions, ethyl acetate fraction showed better activity on

the cells than the methanol fraction. This could be due to the fact that ethyl acetate solvent was passed through the column before methanol and it had extracted most of the active compounds from the plant extracts. This was still carried on to the solid phase extracted isolates where it was observed that ethyl acetate isolates showed higher activity ($p < 0.05$).

To further understand the cytotoxicity of the extracts when used for cancer therapy, the selectivity indices were calculated (**Table 5**). The selectivity index (SI) is the ability of an extract to inhibit the growth of cancer cells more than it does to the normal cells. An extract with the SI > 3 is considered to be highly selective and has the potential to be used in the management cancer [13]. Selectivity is the most important feature of an effective anticancer drug and a clear understanding of how much selectivity a new drug should have to be clinically effective is essential [13].

Table 5. Selectivity indices of *R. officinalis* leaf extracts and fractions

Extract/fraction	DU145	CT26	HeLa 229
Methanolic extract	3.18	1.55	1.08
Ethyl acetate extract	3.28	1.30	1.15
DCM extract	0.86	1.08	1.07
Hexane extract	NA	NA	NA
Methanolic fraction	14.18	1.47	1.04
Ethyl acetate fraction	44.31	1.93	2.09
DCM fraction	2.02	2.13	2.89
Hexane fraction	NA	NA	NA
Methanolic SPE	3.88	3.64	3.28
Ethyl acetate SPE	4.29	3.60	3.34
Doxorubicin	1.459	0.995	1.747

SPE: Solid phase extract, NA: Not applicable

Solid phase extracted clean ups had the highest selectivity indices since they showed selectivity on all cells, followed by the ethyl acetate and methanolic fractions and then the crude extracts then the positive control (doxorubicin). The results showed that doxorubicin was not selective on Vero cells as its selectivity indices were quite lower than 3 [13]. Selective cytotoxicity is a pivotal requirement for anticancer drugs.

Total phenolic content and antioxidant activity of the extracts

The TPC of the extracts were determined using the Folin-Ciocalteau method. Folin-Ciocalteau reagent consists of a mixture of sodium molybdate, sodium tungstate and other reagents which when added to plant extracts react with phenolic compounds to produce a solution of a blue complex which absorbs at 760 nm. The assay relies on the transfer of electrons in alkaline medium from phenolic compounds to phosphomolybdic/phosphotungstic acid complexes [44, 45]. A calibration curve (**Figure 1**) was prepared for the quantitative analysis and the linearity for gallic acid standard was established from the range of 1 µg/ml to 100 µg/ml which was fitted on the line $y = 0.0025x$.

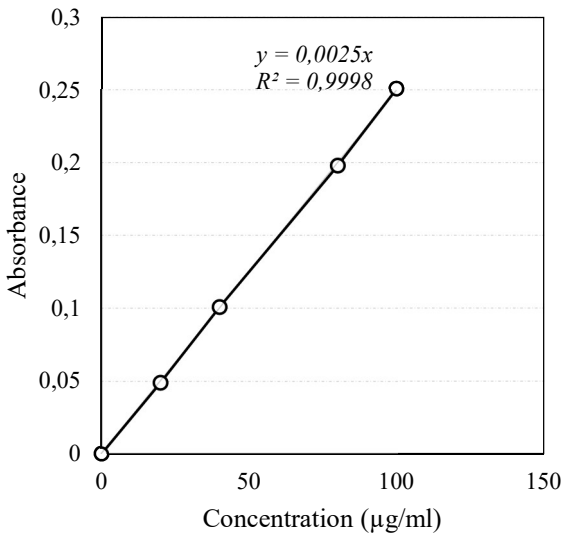


Figure 1. Calibration curve for TPC using Gallic acid standard.

The methanol crude extract gave the highest TPC of 476.8 ± 0.69 µg/ml (**Table 6**). Methanol is a polar protic solvent [23, 46] and thus, it extracted more polyphenols which are inherently polar and their solubility is through hydrogen bond formation [47]. Further, ANOVA test showed that there were significant differences ($p < 0.05$) among the mean TPC of the different solvent extracts. From the results of the antioxidant activity assay (**Figure 2**), the IC_{50} of methanolic extract (5.39 ± 0.09 mg/ml) was the lowest as compared to 0.06 ± 0.01 mg/ml for ascorbic acid (control). This is because most phenolic compounds responsible for antioxidant activity have polar functional groups which are easily dissolved in polar protic solvents like methanol [26]. The antioxidant activity of plant phenolic compounds is attributed to their redox properties which allow them to act as reducing agents, hydrogen donators, singlet oxygen quenchers and metal chelators [48]. The DPPH test measures the hydrogen atom or electron donating capacity of extracts to the stable radical DPPH formed in solution [49].

Table 6. Total phenolic content of *R. officinalis* leaf extracts

Extraction solvent	Total phenolic content (µg/ml GAE)
Methanol	476.8 ± 0.69
Ethyl acetate	74.80 ± 0.80
Dichloromethane	37.47 ± 0.92
n-hexane	21.33 ± 0.83

GAE: Gallic acid equivalent

In a study which used *R. officinalis* from different regions of Algeria, Fellah et al. [50] reported TPC ranging from 58.26 ± 0.31 to 114.10 ± 0.15 mg GAE/g dry weight.

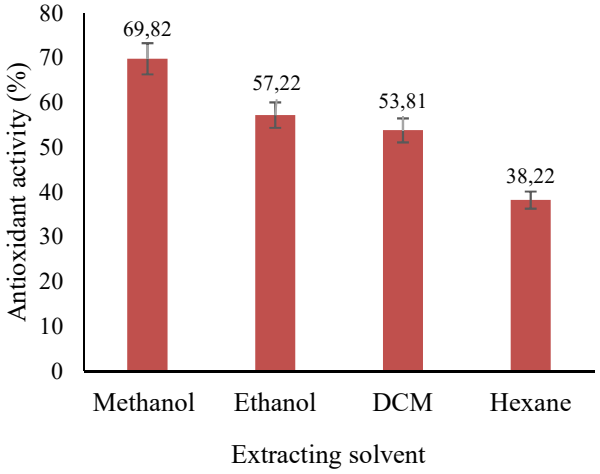


Figure 2. Antioxidant activity of *R. officinalis* leaf extracts

Similarly, antioxidant activity with IC_{50} values of 8.6 ± 0.5 to 19.4 ± 1.5 µg/ml was reported for *R. officinalis* leaf extracts by Garbarino et al. [51]. Further, Bourhia et al. [52] reported TPC of 146.63, 92.39, 83.27 and 74.15 µg GAE/mg for *R. officinalis* harvested from El Jadida, Taounate,

Beni Mellal and Marrakesh regions of Algeria, respectively. The plants exhibited antioxidant activity with IC_{50} values of 0.302, 0.258, 0.236 and 0.176 mg/ml, respectively.

The antioxidant properties of rosemary have been attributed to its richness in isoprenoid quinones, which act as chain terminators of free radicals and as chelators of reactive oxygen species [53, 54]. Further, compounds such as rosmarinic acid and hesperidin found in rosemary extracts in this study have been cited in the literature as important free radical scavengers [55, 56].

Characterization of compounds in R. officinalis methanolic leaf extracts

In the FT-IR spectrum (**Figure 3**), the intense absorption at 3400 cm^{-1} was due to stretching of phenolic groups present in the extracts. The band at 2900 cm^{-1} was due to stretching of hydroxyl groups like alcohols and water while the absorption at 2800 cm^{-1} could have been due to a C-H group stretching of sp^3 hybridized (R_3C-H) portion. Absorption at 1700 cm^{-1} is due to stretching of C=O group. The bend at 1550 cm^{-1} is due to C=C bonds, typical of aromatic compounds (containing a benzene ring). Absorption at 1400 cm^{-1} was due to asymmetric in-plane bending of $–CH_3$ while at 1350 cm^{-1}, the absorption was due to symmetric in-plane bending of $–CH_3$. The stretch at 1250 cm^{-1} is due to nitro groups ($-NO_2$). The absorption at 1100 cm^{-1} was due to C-O stretching vibration. The weak bands at 1000 cm^{-1} and 900 cm^{-1} could be due to C-H bending and terminal $C=CH_2$ groups respectively. These assignments are based on previous studies on phenolic compounds in plants [17, 57, 58]. These confirmed the presence of phenolic compounds in the extract. The various functional groups observed in the extracts reflected the biochemical profile of the leaf extract which could be responsible for the various medicinal properties of this plant leaf, including antiproliferative activity. Phytochemicals such as phenolics, carotenoids, terpenoids and alkaloids from plants have been reported to be key actors in cancer therapy [1, 6, 59].

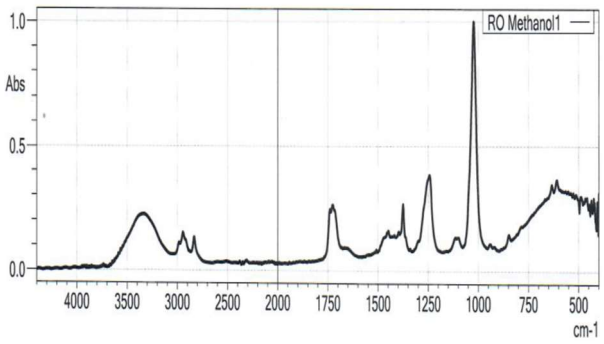


Figure 3. FTIR spectrum of the methanolic fraction of *R. officinalis* leaf extract.

LC-MS/MS qualitative analysis afforded the identification of 32 compounds in *R. officinalis* methanolic leaf extract (**Table 8**). These compounds included polyphenols (such as gallic acid, rosmanol, rosmarinic acid), flavonoids, terpenoids and alkaloids. The standards used for quality control in the study (gallic acid and rutin) showed similar LC chromatograms and MS spectra with their corresponding compounds in

the samples. Of the 32 compounds identified, were eight compounds reported for the first time in this plant. These are procyanidin, hydroxyplorentin, cephalin, isoquercetin, latifoliamide, diadzin, hyperin and emetine (**Figure 4**). Mena et al. [60] reported the presence of (poly)phenolic compounds in *R. Officinalis* leaves. Using ultra-high-performance liquid chromatography-electrospray ionization-mass spectrometry afforded the identification and quantification of 57 compounds, 14 of which were reported for the first time.

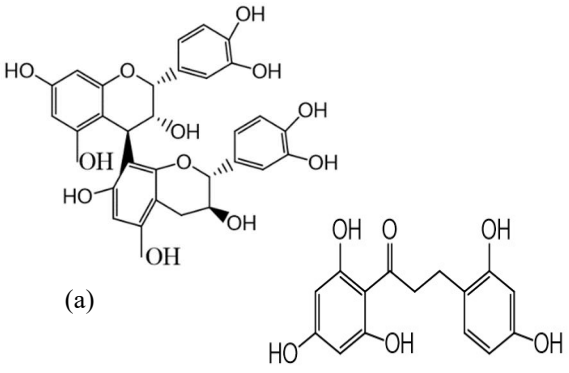
Table 8. Compounds identified in *R. officinalis* leaf extract

Peak	Rt (s)	m/z	MF	Fragments (CE)	Compound
1	2.060	191.1	$C_7H_{12}O_6$	127.0 (24), 93.0 (32)	Quinic acid
2	2.967	163.1	$C_7H_8O_2$	117.1 (40)	Anustoline
3	3.082	179	$C_9H_8O_4$	135 (10), 134 (20)	Caffeic acid
4	3.128	235.2	$C_{27}H_{30}O_{16}$	86.1 (16), 58.2 (36)	Rutin
5	8.306	220.1	$C_{30}H_{26}O_{13}$	56.1 (52)	Procyanidin
6	8.466	304.2	$C_{15}H_{15}O_6$	182.1(16), 82(48)	Hydroxyphlorentin
7	8.480	261.2	$C_{15}H_{14}O_6$	176.1(0), 55.2(28)	Catechin
8	9.790	359.1	$C_{18}H_{16}O_8$	123.0 (20), 161.0 (100)	Rosmarinic acid
9	13.81	313.1	$C_{17}H_{14}O_6$	283.0 (32), 298.1 (24)	Cirsimaritin
10	13.92	345.2	$C_{20}H_{26}O_5$	283.2 (100), 301.2 (49)	Rosmanol
11	15.14	283.1	$C_{16}H_{12}O_5$	268.0 (100.0)	Genkwanin
12	15.57	150.1	$C_{40}H_{80}NO_8P$	65.1(48), 65.1(44)	Cephalin
13	16.04	487.3	$C_{30}H_{48}O_5$	-	Asiatic acid
14	17.405	290.1	$C_{15}H_{10}O_7$	168(16), 77(60)	Quercetin
15	18.558	208.1	$C_{21}H_{20}O_{12}$	163.1 (8), 105.1 (24)	Isoquercetin
16	18.669	163.1	$C_{21}H_{24}N_2O_3$	130.1 (28)	Latifoliamide
17	18.746	208.1	$C_{21}H_{20}O_{12}$	163.1 (8), 105.1 (24)	Isoquercetin
18	18.826	244.2	$C_{21}H_{20}O_9$	91.1 (36), 86.2 (8)	Diadzin
19	21.91	471.3	$C_{30}H_{48}O_4$	-	Benthamic acid
20	22.35	471.3	$C_{30}H_{48}O_4$	-	Augustic acid
21	25.425	195.1	$C_{14}H_6O_8$	83 (40)	Ellargic acid
22	29.116	147	$C_9H_6O_2$	103.1 (20), 91.1 (20)	Courmarin
23	29.133	169	$C_7H_6O_5$	125 (10), 79 (20)	Gallic acid
24	29.148	318.2	$C_{21}H_{24}O_{10}$	196 (16), 82.1 (32)	Phlorizin
25	29.161	272.2	$C_{21}H_{20}O_{12}$	215.1 (20), 171.1 (40)	Hyperin
26	29.175	234.1	$C_{27}H_{32}O_{14}$	84.1 (20)	Naringin
27	29.179	220.1	$C_{28}H_{34}O_{15}$	84.1 (16)	Hesperidin
28	29.234	153	$C_7H_6O_4$	109 (10), 108 (20)	Gentisic acid
29	29.384	177.1	$C_{16}H_{18}O_9$	98.1 (24), 80.1 (28)	Chlorogenic acid
30	29.405	136.1	$C_{29}H_{40}N_2O_4$	119 (4), 91 (16)	Emetine
31	29.719	209.2	$C_{17}H_{23}NO_3$	124.1 (24), 93.1 (32)	Atropine

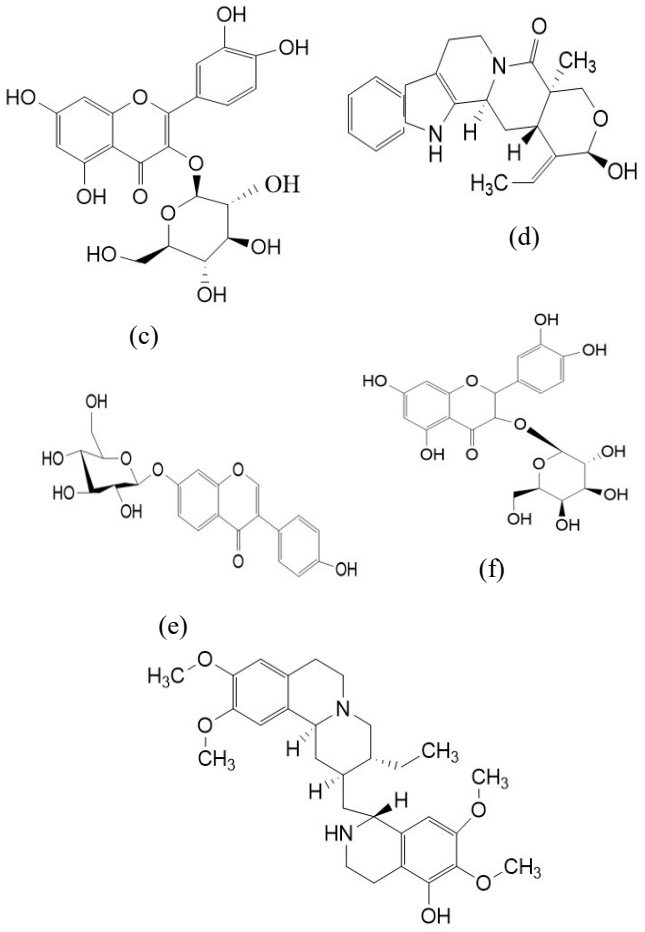
Peak	Rt (s)	m/z	MF	Fragments (CE)	Compound
32	30.25	455.4	$C_{30}H_{48}O_3$	-	Ursolic acid

Rt: Retention time, MF: Molecular formula, m/z-Mass to charge ratio, CE- Collision energy

The rosemary extract contained 24 flavonoids (mainly flavones), 5 phenolic acids, 24 diterpenoids (carnosic acid, carnosol, and rosmanol derivatives), 1 triterpenoid (betulinic acid) and 3 lignans (medioresinol derivatives). Carnosic acid was reported as the dominant phenolic compound in the extracts [60]. The compounds identified were Medioresinol, *p*-Coumaric acid, Luteolin-rutinoside, Luteolin-hexoside, Isorhamnetin-3-*O*-hexoside, 4-hydroxybenzoic acid, Apigenin-7-*O*-glucoside, Homoplantaginin (Hispidulin 7-glucoside) among others which have been previously identified in this plant [38, 61-64]. Five phenolic acids (a hydroxybenzoic acid, two hydroxycinnamic acids and two rosmarinic acid derivatives) were identified, substantiating previous observations in this species [61, 64].



(a) (b)



(c) (d) (e) (f) (g)

Figure 4. Structures of some molecules identified in *R. officinalis* leaves for the first time (a) procyanidin, hydroxyplorentin, (c) isoquercetin, (d) latifoliamide, (e) diadzin, (f) hyperin, and (g) emetine.

Some of the compounds identified such as ursolic, rosmarinic and gallic acids were previously reported to have anticancer activity [36, 65]. Thus, the results of this study supports the traditional use of this plant in cancer therapy in Uganda.

Conclusions

The results of this study showed that *R. officinalis* extracts has phenolic compounds with antiproliferative activity against human prostate (DU145), colorectal (CT26) and cervical (HeLa 229) cancer cells. Selectivity of *R. officinalis* leaves in antiproliferative activity followed the order: solid phase extracted clean ups > ethyl acetate and methanolic fractions > crude extracts. Further studies should evaluate the anticancer activity of the extracts on other cancer cell lines because some of the polyphenols could be inactive on the cell lines investigated in this study yet active on the other cell lines that have not been studied. Studies on the anticancer potential of some of the identified unstudied compounds should be taken. The chemical composition and antiproliferative activity of *R. officinalis* roots should be done.

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