ORIGINAL ARTICLE

Effect of Chemical Modification involving Phenolic Hydroxyl Group on the Biological Activity of Natural Coumarins

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ABSTRACT

Two coumarins have been isolated from the seeds of two apples' cultivars named Red Delicious and Granny Smith. The influence of their phenolic hydroxyl groups on the biological activities has been investigated. This investigation involved the chemical transformation of these functional groups into less hydrophilic moieties and the detection of the impact of this transformation on the biological activities. The investigated biological activities included the antioxidant, antiproliferative, antibacterial, and antifungal effects. The antioxidant potential was tested by figuring the capacity of these derivatives to trap hydroxyl and DPPH radicals. The antiproliferative potential was tested by MTT photometric assay against eight cancer lines named HeLa, SK-OV-3, AR42J, MCF-7, AB12, KYSE-30, LC540, and AMN3. The antibacterial potential was visualized via a well-defined disc diffusion assay versus six common pathogenic bacterial strains, which are: *Escherichia coli*, *Salmonella typhi*, *Klebsiella pneumonia*, *Haemophilusinfluenzae*, *Shigelladysenteriae*, and *Pseudomonas aeruginosa*. By applying the same general assay, the antifungal potential was highlighted against three pathogenic fungi named *Candida albicans*, *Aspergillusflavus*, and *Aspergillusniger*. From the outcomes of the tested biological activities, we concluded that the phenolic hydroxyl groups exert a beneficial effect on the antioxidant and antiproliferative activities of the natural derivatives, and a detrimentalrole on their antimicrobial activity.

Keywords: Coumarins, Antioxidant, Antiproliferative, Antibacterial, Antifungal, Hydroxyl group, Chemical modification.

INTRODUCTION

From ancient times to date, nature has beenthe main source forbiochemical agents which possess multifarious biologically biased activitiesowing to the variation in their chemical characteristics and targeted biomolecules[1]. Exploring the chemical structures of the isolated natural products and investigating their valuable pharmacological activities may accelerate the progress of the drug innovationprocess[2,3].

Products inspired by nature and based in their chemical structures on coumarin backbone have magnetized a senior attention, part of which is directed toward the exploration of their biomedical activities[4]. Models of these actions include the antibacterial[5], antifungal[6], antioxidant[7], anticancer[8], anticholinesterase[9], and anticonvulsant[10] effects. Coumarin-based derivatives can be isolated from many natural sources including the plant realm, in which these derivatives havebeen discovered in various plant parts[11].

Structural modification of bioactive agents may afford many auxiliary applications[12]. Examples include scouting the biotargets, modes of action, and binding interactions of novel agents, defeatingthe multi-drug resistanceof agents with antimicrobial or antitumor activity, repurposingcurrently marketed drugs, modulating their metabolic fates, and pharmacokinetic parameters, simplifying the complex structures of agents with high-molecular weights to afford simpler and easier to synthesize molecules having similar bioactivity[13].

For natural products,the eventual target of structural modification is to optimize their drug-like properties. This modification usually manifests by removing, adding, or replacing functional groups to evaluate their impact on the biomedical and biophysicochemical properties[14].The phenolic hydroxyl group included in the chemical structures of many natural pharmacophores may handle a charming influence on the aforementioned properties[15].

The current study aimsto investigate the impact of the phenolic hydroxyl groups foundin the chemical structures of two natural coumarins on their biological activity.These two natural productshave been previously isolated from the seeds of two apples' cultivars: Red Delicious and Granny Smith. Theinvestigation is carried out by chemically modifying these functional groups to less hydrophilic moieties. The estimated biological activities include antioxidant, antiproliferative, antibacterial, and antifungal effects.

MATERIAL AND METHODS

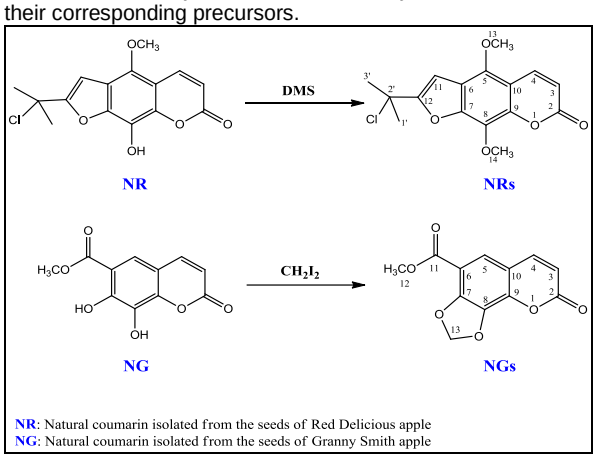
Human cancer cell lines, reagents, and chemicals appointed for this study wereobtained from documented international suppliers named Sigma-Aldrich, CHEM-LAB, Scharlau,Bio-World, Haihang,and others. Thin-layer chromatography (TLC) was engaged by eluting the spots seeded on silica gel GF_{254} (type 60)plates with a mixture of CH_2Cl_2:EtOH (3:1). The melting points (m.p.) of the semisyntheticcoumarins were recorded on an electrochemical CIA 9300 equipment viaan open-capillary style. The existenceof specific functional groupswas inspected by analyzing the FTIR spectra of the products acquired from Bruker-Alpha ATR spectroscopy.UVD-2950 (LABOMED) apparatus was employed to detect the

maximum absorptions (λ_{max})of the natural and semisyntheticcoumarins at the ranges of ultraviolet and visible wavelengths. The chemical structures of the semisyntheticcoumarins were established by studying their ^{13}C-NMR (75 MHz) and^{1}H-NMR (300 MHz) spectra recorded by Bruker 300 MHz AVANCE III HD NMR Spectroscopy.

Synthesis of the semisynthetic coumarins

The facile synthesis of the semisynthetic coumarins (**NRs** and **NGs**) from their corresponding natural precursors (**NR** and **NG**) is illustrated in Scheme 1.

Scheme 1: The synthesis of the semisynthetic coumarins from their corresponding precursors.



*Synthesis of 2-(2-chloropropan-2-yl)-4,9-dimethoxy-7H-furo[3,2-g]chromen-7-one (**NRs**)*

Amixture of **NR** (0.554 g, 1.8 mmol) and dry potassium carbonate (0.5 g, 3.6 mmol) wasblended for 30 min in a solvent-free medium utilizing mortar and pestle. The resulted mixturewastempered by heating at 70°C for 1 hr and diluted minutely with dry ethyl acetate. Amixture of dimethyl sulfate (0.2 ml, 2 mmol, DMS) in 10 ml dry ethyl acetate was prepared and stepwise added to the first mixture. The reaction mixture wasrefluxed for 3 hr under dry conditions and then filtered.The acquired filtrate waswashed with H_2O, andthe separated organic layer condensed under vacuum. The residue waspoured into a mixture of powdered ice and H_2O. Upon filtration, the solid was washed with cold H_2O and recrystallized from an ether: EtOH (2:1) mixture(16).

NRs: Yellow powder; m.p.=189-192°C; λ_{max} (EtOH)=318 nm;R_f=0.72; % yield=71.89 (0.414 g); FTIR (v, stretching, cm^{-1}): 3094, 3054 (=C-H), 2892 (C-H, alkyl), 1733 (C=O, ester), 1632, 1590 (C=C), 1554 (C=C, aromatic), 1250, 1050 (C-O-C, alkyl-aryl ether), 734 (C-Cl); ^{1}H-NMR (DMSO-d_6, 300 MHz): **δ**= 8.08 (1H, d, *J*= 9 Hz, H-4), 6.70 (1H, s, H-11), 6.22 (1H, d, *J*= 9 Hz, H-3), 4.35 (6H, s, H-13, H-14), 2.01 (6H, s, H-1', H-3') ppm; ^{13}C-NMR (DMSO-d_6, 75 MHz): **δ**= 160.8 (C,C2), 159.4 (C, C-12), 146.7 (C, C-5), 143.8 (CH, C-4), 139.6 (C, C-7), 137.6 (C, C-9), 132.6 (C, C-8), 115.5 (CH, C-3), 114.4 (C, C-10), 112.9 (C, C-6), 103.3 (CH, C-11), 64.2 (CH_3, C-13), 63.9 (CH_3, C-14), 62.6 (C, C-2'), 30.9 (CH_3, C-1', C-3') ppm.

*Synthesis of methyl 8-oxo-8H-[1,3]dioxolo[4,5-h]chromene-4-carboxylate (**NGs**)*

A suspension of **NG** (0.944 g, 4 mmol) in 75 ml dry ethyl acetate wasadded to aconical flask enveloped with an aluminum sheet and settled in a salt-ice bath. When the temperature of the suspension fell to 0°C, aprecooled solution of CH_2I_2 (0.16 ml, 2 mmol) in dry ethyl acetate (6 ml) was dropwise added. The reaction mixture was stirred for 12 hr at 90°C, concentrated to dryness,treated with H_2O (50 ml), and extracted by $CHCl_3$ (3×25 ml). The collected hydrophobic layer was dried on $CaCl_2$, vaporized under vacuum, and the product recrystallized from CH_2Cl_2(17).

NGs: White powder; m.p.=177-179°C; λ_{max} (EtOH)=279 nm; R_f =0.68; % yield=48.02 (0.476g); FTIR (v, stretching, cm^{-1}): 3061 (=C-H), 2904 (C-H, alkyl), 1726, 1703 (C=O, ester), 1670 (C=C), 1588 (C=C, aromatic), 1249, 1034 (C-O-C, aryl-alkyl ether); ^{1}H-NMR (DMSO-d_6, 300 MHz): **δ**= 7.76 (1H, d, J= 9 Hz, H-4), δ 7.53 ppm (1H, s, H-5), δ 6.22 ppm (1H, d, *J*= 9 Hz, H-3), 5.95 (2H, s, H-13), 4.20 (3H, s, H-12)ppm; ^{13}C-NMR (DMSO-d_6, 75 MHz): **δ**= 170.2 (C, C-11), 160.9 (C, C-2), 155.2 (C, C-7), 145.4 (C, C-9), 143.7 (CH, C-4), 137.5 (C, C-8), 123.3 (CH, C-5), 115.4 (CH, C-3), 113.1 (C, C-10), 110.1 ppm (C, C-6), 93.5 (CH_3, C-13), 53.5 (CH_3, C-12) ppm.

Biological evaluation

Antioxidant potential: The potential of natural coumarins (**NR**, **NG**) and their matchingsemisynthetic derivatives (**NRs**, **NGs**) to trap the free radicals of DPPH (2,2-diphenyl-1-picrylhydrazyl) and hydroxyl wasquantified in correlation to ascorbic acid (**AA**) as a standard antioxidant. For each tested derivative, six concentrations (200, 100, 50, 25, 12.5, 6.25 µM) were contrived from a reference methanolic (1mM) solution in the double-dilution manner.With each assay, the percentage of trappingpotentialof the derivative expressed as TP% was measured by applying the following mathematic rule: TP% = (A_a – A_d/ A_a) × 100. The$A_a$and A_d represent the absorbances of **AA** and the derivative, respectively. From a diagram that exhibited the correlation between the log concentration of the investigated derivative and TP%, the $TP_{50}$was calculated for three independent tryouts employing non-linear regression.

To test the potential of the derivative for trapping the DPPH radicals, the methanolicsolutions of the sample (1.5 ml) and DPPH (0.5 ml, 0.1 mM) werecombined. The mixture was laminated with an aluminum sheet to preserve from light, and brooded for 30 min at 25°C. Subsequently, the TP% was followed spectrophotometrically at 517 nm utilizinga standard composed of DPPH (0.5 ml, 0.1 mM) and absolute methanol (1.5 ml)[18].

In the hydroxyl radicals trapping assay, the investigated solutionwas prepared by mixing the incoming solutions sequentially: the sample (1.5 ml), potassium phosphate buffer pH 7.8 (2.4 ml, 200 mM), $FeCl_3$ (60 µl, 1 mM), o-phenanthrolinemonohydrate (90 µl, 1 mM), and hydrogen peroxide (150 µl, 170 mM). Following an incubational period of 5 min at 25°C, the investigated mixture wasexamined spectrophotometrically at 560 nm againsttheblankcomposed of the mixed solutions minus the sample[19].

Primary antiproliferative potential: The cells of the selected tumor line were sowed at a density of $4×10^4$cellper hole in a 96-hole sheet. The holes,incorporated with a compatible medium to the applied cell line,were treated

individually in the next 24 hr with mounting concentrations (6.25-200 µM) of the investigated derivatives. The antiproliferative potential of these derivativeswas assessed after 72 hr utilizing the tetrazolium dye, MTT. Cell viability assaywas conducted by withdrawing the medium, applying thetetrazolium dye(28 µl, 3.27 mM), and thenbrooding the treated cells for 1.5 hr at 37°C. The antiproliferative percent symbolized as A_p% of each derivative was calculated via the formula: $A_p\% = (H_u - H_t)/H_u \times 100$. The$H_u$and H_t represent the absorbances of the untreated and treated holes, respectively.The IC_{50} values of the investigated derivatives were determined for three separate experiments by plotting the A_p% versus log concentration and calculated by non-linear regression(20).

Antimicrobial potential: In the antibacterial assay, the selected strain was incubated at 37°C in5 ml nutrient broth for 16 hr. The final inoculum of 1.5×10^8 CFU/ml was acquired by adjusting the turbidity of the incubated mixture to 0.5 McFarland standard utilizing normal saline.Discs(0.2 cmin diameter) prepared from WhatmanGrade 3 filter papers were moistenedwith the DMSO solution (10 µl, 20 mg/ml) of the investigated derivative. The incubated mixture (100µl) and molten agar (20 ml) werecombined under aseptic conditions and flowed into cell-culture plates. The prepared discs were seeded on the surface of solidified agar by using aseptic forceps. Upon incubation for one day at 37°C, the inhibition sector (I) of the individual derivativewas detected in millimeters via Mitutoyo digital vernier caliper series 500. The activity index (A_I) of the investigated derivative was calculated by applying the mathematical law: $\mathbf{A_I = I_D / I_R}$[21]. The symbols $\mathbf{I_D}$ and$\mathbf{I_R}$represent the inhibition sectors achieved by the investigated derivative and reference, respectively.

In the antifungal assay, a similar technique was followed with only twoadjustments;incubating for two days at 30°C, and using Potato dextrose agar as a culturing medium[22].

RESULTS AND DISCUSSION

Chemical modification: The isolation and structural characterization of the natural coumarins (**NR**, **NG**) have been describedpreviously[23,24]. To evaluate the impact of the phenolic hydroxyl groups of these coumarins, two semisynthetic coumarins (**NRs**, **NGs**) were synthesized in such a way to eliminate the ability of these functional groups to act as a hydrogen-bond donor. This structural modification may consequently influence the physicochemical properties, including the hydrophilicity[25].

For **NR**, the nucleophilicity of its phenolic hydroxyl group was improved via the deprotonation achieved by potassium carbonate. The resulted phenoxide attacks the alkylating agent, DMS, affording the formation of the semisynthetic derivative **NRs**. As a result, the influence of the phenolic hydroxyl group was covered by etherification[16]. Concerning **NG**, its catecholic hydroxyl groups were shielded by their incorporation into 1,3-dioxolane ring under the effect of CH_2I_2[17].

Biological evaluation

Antioxidant effect: The trapping capacity of the natural and semisynthetic derivatives was testedversus DPPH and hydroxyl radicals. Many research papers reported the effects of various substituents on the antiradical efficiency of many natural and synthetic coumarins[26,27,28]. This efficiency has been correlated to the number of phenolic hydroxyl groups linked to the aromatic component of the coumarin backbone[26] and to the capability of the substituent ortho to the hydroxyl group to grant electrons[27]. This correlation is matched with the outcomes reported in Table 1 and Figure 1.In comparison with natural coumarins, the antiradical activity of their parallel semisynthetic derivativesis significantly declined. This may indicate the important role of phenolic hydroxyl group (s) in the antiradical activity of the natural coumarins.

Primary antiproliferative effect: The investigated derivatives were screened for their primary antiproliferative activity utilizing MTT dye, and six different concentrations. This investigation also incorporated 5-fluorouracil (5-FU) as a standard antiproliferative drug, and DMSO as a solvent. The cancer cell lines involved in this preliminary test included HeLa (Epitheloid cervix carcinoma, 93021013), SK-OV-3 (Caucasian ovary adenocarcinoma, 91091004), AR42J (Rat exocrine pancreatic tumor, 93100618), MCF-7 (Caucasian breast adenocarcinoma, 86012803), AB12 (Mouse malignant mesothelioma,10092306), KYSE-30 (Human Asian esophageal squamous cell carcinoma, 94072011), LC540 (Rat Fischer Leydig cell testicular tumor, 89031604), and AMN3 (murine mammary adenocarcinoma).

The outcomes manifested in Table 2 and Figure 2 report three mainimports. Firstly, the investigated derivatives showhigher IC_{50} values in comparison with that of 5-fluorouracil. Secondly, the antiproliferative activity of the natural derivatives versus the test cell lines is superior to that of their matching semisynthetic products. Finally, the decline observed in the antiproliferative activity of the semisynthetic derivatives is parallel to the lowering in their antioxidant activity. In the literature, many studies have assigned the antitumor activity of diverse natural and synthetic coumarins with their antioxidant activity[29,30,31].

Antimicrobial effect: The natural and semisynthetic derivatives were scanned for their antimicrobial activity utilizing a well-defined agar disc dissemination method[17]. This method involved the employment of DMSO as a negative control and a standard antimicrobial agent as a positive control, which was either ciprofloxacin (10 µg/disc, **CP**) for the antibacterial activity or nystatin (100 units/disc, **NY**) for the antifungal activity.

The test pathogens involved six standard bacterial and three standard fungal sorts. The experimental bacteria were *Escherichia coli* (ATCC 25922, **Ec**), *Salmonella typhi*(ATCC 6539, **St**), *Klebsiella pneumonia* (ATCC 700603, **Kp**), *Haemophilusinfluenzae*(ATCC 49247, **Hi**), *Shigelladysenteriae*(ATCC 13313, **Sd**) and *Pseudomonas aeruginosa*(ATCC 27853, **Pa**). The fungal sorts encompassed *Candida albicans* (ATCC 10231, **Ca**), *Aspergillusflavus*(ATCC 9643, **Af**), and *Aspergillusniger*(ATCC 16888, **An**).

The data recorded in Tables 3-6 and their graphical representations displayed in Figures 3-6 reveal fourmain points. The first is that the antimicrobial activity of the investigated derivatives was lower than that of the standard. The second issue is thatthe semisynthetic derivatives showed a towering antimicrobial effect in comparison with their corresponding natural products. The

third one is that the semisynthetic derivative **NRs**hada more inhibitory effect on the growth of the tested bacteria than those of the **NGs** and natural derivatives. The last issue is that the semisynthetic derivative**NGs**hada more inhibitory effect on the growth of the tested fungi than those of the **NRs** and the natural derivatives.

The towering antimicrobial activity of the semisynthetic derivatives may be assigned to the replacement of the hydroxyl group found in their corresponding natural derivativeswithless hydrophilic moiety. This replacement may increase the total lipophilicity of the semisynthetic derivatives resulting in the enhancement of theirpermeation into the microorganisms[32,33]. Besides, it is believed that the presence of two aryl-alkyl ether groups in the ortho or para position to each other could enhance the antimicrobial activity of various naturaland semisynthetic coumarins[34].

Table 1: Results of the antioxidant activity of natural and semisynthetic coumarins.

Derivative symbol	Scavenger activity versus DPPH free radicals TP_{50} (µM) ± SD (*n*=3)	Scavenger activity versus hydroxyl free radicals TP_{50} (µM) ± SD (*n*=3)
AA	46.29 ± 0.67	50.33 ± 0.91
NR	64.18 ± 0.90	68.48 ± 0.95
NG	48.20 ± 0.86	52.84 ± 0.76
NRs	89.31 ± 1.05	101.06 ± 0.90
NGs	114.05 ± 0.81	107.14 ± 0.72

Table 2: Results of the primary antiproliferative activity of the investigated derivatives.

Cancer cell line	Derivative symbol				
	5-FU	NR	NG	NRs	NGs
HeLa	13.11 ± 0.80	20.18± 1.00	25.11 ± 0.90	57.63± 1.10	55.54 ± 1.05
SK-OV-3	22.16± 1.05	29.58± 0.90	31.58± 1.00	62.91± 0.95	73.36± 0.95
AR42J	19.86± 0.95	28.09± 1.10	30.32± 1.15	44.67± 0.80	62.48± 0.95
MCF-7	12.46 ± 1.10	22.81± 1.10	24.17 ± 0.85	47.82± 1.20	54.56 ± 0.90
AB12	18.93± 1.25	28.90± 1.35	28.69± 0.80	61.94± 1.05	59.18± 1.00
KYSE-30	29.38± 1.05	40.12± 1.05	33.88± 0.95	60.87± 1.45	67.55± 1.15
LC540	23.67± 0.85	52.47± 1.10	47.17± 1.05	83.04± 1.20	76.48± 1.05
AMN3	24.64 ± 1.20	37.63 ± 1.10	42.11 ± 1.15	49.37 ± 1.00	59.32 ± 1.05

The outcomes are represented as IC_{50} ± SD. The IC_{50} value wascomputed in µM, while the standard deviation (SD) was calculatedfor three separate experiments.

Table 3: Results of the antibacterial activity of the natural and semisynthetic derivatives.

Bacterium	CP	NR	NG	NRs	NGs
Ec	32.63 ± 0.90	10.54± 1.15	12.98± 1.05	22.16± 1.30	19.16± 1.25
St	26.12 ± 1.05	9.84± 0.95	10.02± 1.15	19.50± 1.00	14.05± 1.20
Kp	31.47 ± 1.00	12.47± 1.05	11.59± 0.95	20.81± 0.95	20.57± 1.00
Hi	27.46 ± 1.25	10.46± 1.00	12.11± 1.05	20.67± 1.00	18.82 ± 1.15
Sd	24.56 ± 1.00	8.22± 1.00	13.28± 1.35	21.04± 1.20	21.24 ± 1.05
Pa	35.32 ± 1.05	6.22± 0.95	11.67± 1.15	18.24± 1.05	23.59 ± 0.95

The outcomes represent the means of the inhibition sectors expressed in mm ± SD, whichwas detected for three separate experiments.

Table 4: The outcome assumed from examining the antifungal activity of the natural and semisynthetic derivatives.

Fungus	NY	NR	NG	NRs	NGs
Ca	19.08± 0.90	7.18± 1.15	4.44± 1.05	11.45± 1.10	14.05 ± 0.85
Af	13.67±1.05	6.89±1.00	5.37± 0.85	9.11± 1.25	11.36 ± 1.05
An	12.22± 0.95	6.93± 0.90	4.28± 0.85	8.14± 1.20	9.22 ± 1.10

The outcomes represent the means of the inhibition sectors expressed in mm ± SD, whichhas detected for three separate experiments.

Table 5: The values of $A_i$forthe natural and semisynthetic derivatives versus the experimental bacteria.

Bacterium	NR	NG	NRs	NGs
Ec	0.32	0.40	0.70	0.59
St	0.38	0.38	0.75	0.54
Kp	0.40	0.37	0.66	0.65
Hi	0.38	0.44	0.75	0.69
Sd	0.33	0.54	0.86	0.87
Pa	0.18	0.33	0.52	0.67

Table 6: The values of $A_i$forthe natural and semisynthetic derivatives versus the experimental fungi.

Fungus	NR	NG	NRs	NGs
Ca	0.38	0.23	0.60	0.73
Af	0.50	0.39	0.67	0.83
An	0.57	0.35	0.66	0.75

Figure 1: Graphical representation of the results of antioxidant activity of the investigated derivatives and positive control.

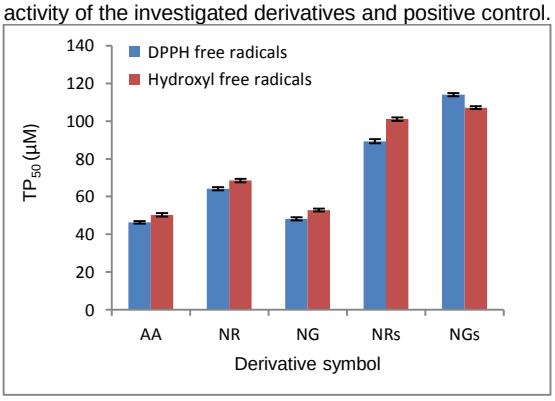


Figure 2: Graphical representation of the data collected from assaying the antiproliferative activity of the investigated derivatives and positive control.

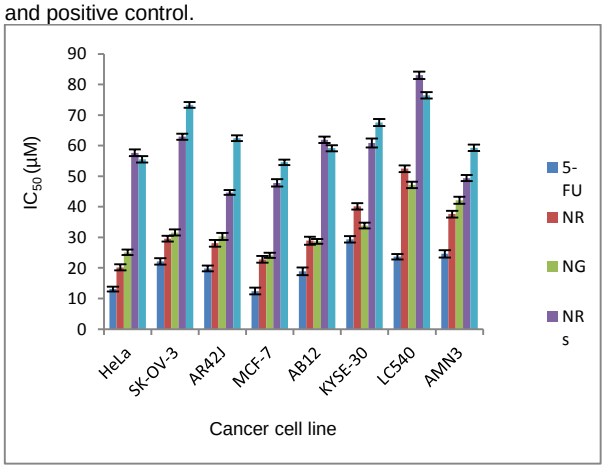


Figure 3: Graphical representation of the data collected from examining the antibacterial activity of the investigated derivatives and positive control.

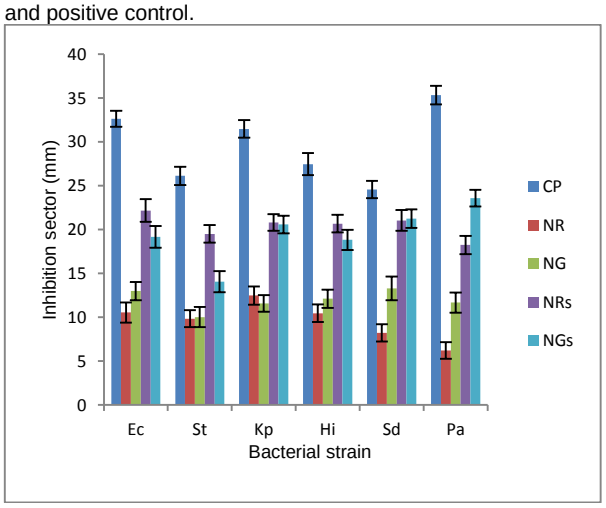


Figure 4: Graphical representation of the data collected from examining the antifungal activity of the investigated derivatives and positive control.

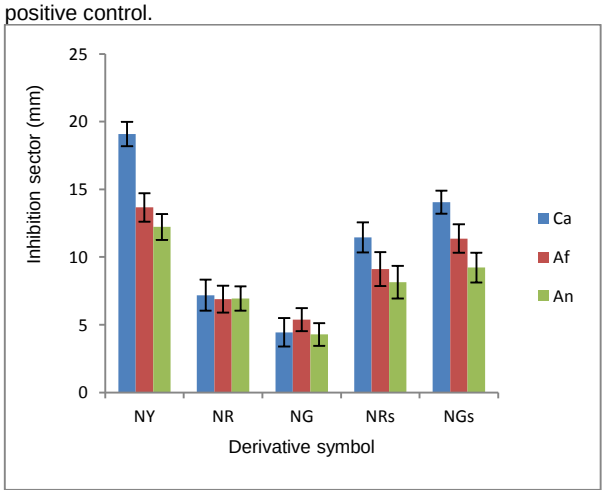


Figure 5: Graphical representation of the A_I values for the investigated derivatives as antibacterial agents.

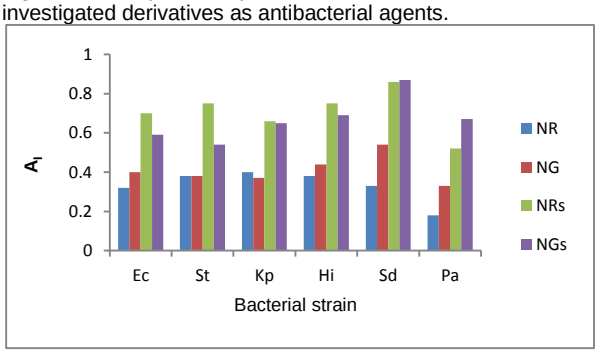
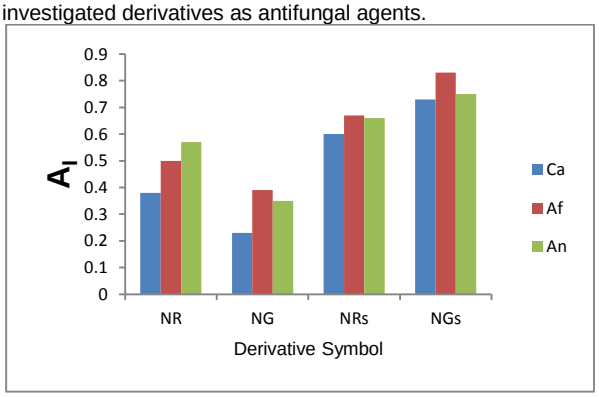


Figure 6: Graphical representation of the A_I values for the investigated derivatives as antifungal agents.



CONCLUSION

This work reported the chemical modification of two natural coumarins to evaluate the role of their hydroxyl groups in the biological activity. It can be concluded from the results of this study that the phenolic hydroxyl groups are important for the antioxidant and the antitumor activities, while they may contribute to lower antimicrobial activity.

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Conflict of interest: There are no conflicts of interest.

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