ASSESSMENT OF BIOLOGICAL ACTIVITY OF APIGENIN AND LUTEOLIN ISOLATED FROM *BACOPA MONNIERI*: A STUDY ON STRUCTURE-ACTIVITY RELATIONSHIPS

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Abstract

The aim of the study was to screen bioactive compounds apigenin and luteolin from the ethyl acetate extract of *Bacopa monnieri* (L.), Byone-hmwe, for some biological activities such as antioxidant, antimicrobial, cytotoxic, and antiproliferative activities. The antioxidant activity of apigenin and luteolin compounds was determined by the DPPH assay. These assays found that luteolin (IC_{50} = 1.86 µg/mL) was more potent than apigenin (IC_{50} = 2.36 µg/mL) in antioxidant activity. The luteolin was found to have more potent antimicrobial activity than apigenin against all eight tested microorganisms with inhibition zone diameters (18-25 mm) determined by the agar well diffusion method. The cytotoxicity was examined by brine shrimp lethality bioassay. LC_{50} values of apigenin and luteolin were found to be 296 µg/mL and 200 µg/mL. The *in vitro* antiproliferative activity of apigenin and luteolin was most potent against human cancer cell lines with IC_{50} values of 9.14 µg/mL (MCF-7) and < 6.25 µg/mL Hela (cervical), but no significant activity IC_{50} values > 100 µg/mL(A549) as determined by MTT assay. Both compounds share a similar flavone structure but differ in their hydroxyl substitution patterns, which significantly influence their biological activities.

Keywords: apigenin, luteolin, antioxidant, antimicrobial, and antiproliferative activities

Introduction

Bacopa monnieri, a member of the Scrophulariaceae family, is a small, creeping herb with numerous branches, small oblong leaves, and light purple flowers. In India and the tropics it grows naturally in wet soil, shallow water, and marshes The herb can be found at elevations from sea level to altitudes of 4,400 feet, and is easily cultivated if adequate water is available. Flowers and fruit appear in summer and the entire plant is used medicinally. (Devendra *et al*, 2018). This medicinal plant is locally known as Brahmi. It is known as a memory enhancer, and many preparations of Brahmi are now commercially available in the market. Some important medicinal uses of the plant *B. monnieri* for treatment of different diseases and the traditional formulation have been reported (La and Baraik, 2019). Traditionally, a number of chemical compounds have been isolated from *Bacopa monnieri*. *Bacopa* contains alkaloids (hydrocotyline, brahmine and herpestine), glycosides (asiaticoside and thanakunicide), and flavonoids (apigenin and luteonin) (Behera *et al*, 2016). Flavonoids are a widely distributed group of natural polyphenolic compounds that are found in plants usually in glycosylated form and have been shown to possess a wide range of biological activities, including antioxidant, anti-inflammatory, antibacterial, antiviral, and anticancer properties, making them an attractive target for synthesis and further study. Structurally, flavonoids are functional aromatic compounds constituted by a C_6-C_3-C_6 structure. The reactive α, β unsaturated ketone structure and the presence of hydroxy groups in *o*-hydroxychalcones make their cyclization possible, resulting in different flavonoidic compounds (Leonte *et al*, 2023).The present study aims to evaluate antioxidant, antimicrobial, cytotoxic and antiproliferative activities of apigenin and luteolin in relation to their structural properties.

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Botanical Aspect of *Bacopa monnieri* (L.) Wettst (Byone-hmwe)

Family	Plantaginaceae
Botanical name	*Bacopa monnieri* (L.) Wettst.
Myanmar name	Byone-hmwe
Part used	the aerial parts

Figure1 A Photograph of *Bacopa monnieri*

Materials and Methods

Plant Material

The sample was collected from Twantay Township, Yangon Region in July 2019, and identified as *Bacopa monnieri* (L) Wettest, by the authorized botanist at the Department of Botany, University of Yangon. The sample was dried under the shade for a week, cut into very small pieces, and then ground into a purely fine powder using an electric grinder. The powdered samples were stored in airtight containers.

Extraction and Isolation of Pure Compounds (Apigenin and Luteolin)

Dried powdered aerial parts of *B. monniera* (1000 g) were extracted with 95 % ethanol. After evaporation of the solvent, the resulting crude extract was successively partitioned between organic solvents (Pet-ether, ethyl acetate) and water. The ethyl acetate crude extract (11 g) was chromatographically separated on silica gel 60 (70-230 mesh, Merck) with the pet-ether, ethyl acetate mixture used as an eluent with various solvent ratios from non-polar to polar. Each and every fraction was checked by TLC and a UV detector. The fractions with the same R_f values were combined, and six combined fractions were obtained. Among them, the fraction (V) was rechromatographed by using the same adsorbent and the same eluent as mentioned in the previous column. Pure pale-yellow crystals were obtained and checked on TLC for purity. It gave one spot on TLC (R_f = 0.5) with pet-ether: ethyl acetate (1:1) (v/v). The weight of the isolated compound apigenin was 50 mg and its yield percent was found to be 1.25 % based on the ethyl acetate crude extract. The FT IR and UV-vis spectra were recorded at the University of Yangon and ^{1}H NMR and ^{13}C NMR were measured at the Division of Natural Product Chemistry, Institute of Natural Medicine, and University of Toyama, Japan (Thant *et al*., 2023).

Antioxidant Action by the DPPH Free Radical Scavenging Assay

The antioxidant activity of apigenin and luteolin was evaluated using the stable 1, 1-diphenyl-2-picryl hydrazyl (DPPH) radical, following the methodology established by Jothi *et al.* (2019). To prepare the control solution, 1.5 mL of a 180 µM DPPH solution was combined with 1.5 mL of ethanol in a brown bottle. For the sample solution, 1.5 mL of the same 180 µM DPPH solution (with an absorbance of 0.8941, serving as the control) was mixed with 1.5 mL of the test sample solution at concentrations of 10, 5, 2.5, 1.25, and 0.625 µg/mL The resulting mixture was vortexed thoroughly and allowed to stand for 30 min at room temperature. Absorbance was then measured at 517 nm against a blank using a UV spectrophotometer. Ascorbic acid was utilized as the positive control, and all experiments were performed in triplicate. The mean values obtained for each concentration were used to calculate the percent inhibition of oxidation using the following equation:

$$\% \text{ RSA} = \frac{A_{DPPH-}(A_{smaple-}A_{blank})}{A_{DPPH}} \times 100$$

where, % RSA = % radical scavenging activity

A_{DPPH} = absorbance of DPPH in EtOH solution

A_{Sample} = absorbance of sample and DPPH solution

A_{blank} = absorbance of sample and EtOH solution

The experimental results were performed in triplicate. The data were recorded as mean ± standard deviation.

Screening of Antimicrobial Activity of the Samples by the Agar Well Diffusion Method

The antimicrobial activity of apigenin and luteolin compounds from ethyl acetate extracts from the aerial parts of Byone-hmwe was determined against eight strains of microorganisms namely *Escherichia coli, Bacillus subtilis, Staphylococcus aureus, Pseudomonas fluorescens, Agrobacterium tumefaciens, Bacillus pumilus, Micrococcus luteus, and Candida albicans* by employing the agar well diffusion method (Anibijuwon and Udeze, 2009). These tests were screened at the strains' storage facility, Pathein University.

Preparation of nutrient agar medium

A mixture of meat extract (0.5 g), peptone (0.5 g), sodium chloride (0.25 g) and 1.5 g of agar powder was placed in a sterilized conical flask, 100 mL of sterile distilled water was added to obtain nutrient agar medium. The resulting mixture was heated to dissolve the contents. Then, the pH of this solution was adjusted to 7.2 with 0.1 M sodium hydroxide solution. It was sterilized in an autoclave at 121 ℃ for 15 min.

Determination of antimicrobial activity by the agar well diffusion method

The agar well diffusion method was used to evaluate the antimicrobial activities of the compounds, apigenin and luteolin, against the bacteria and fungi. Different extracts (1 mg each) were dissolved in 1 mL of their respective solvent. After inoculation, plates were dried for 15 min. A hole with a diameter of 8 mm was punched aseptically with a sterile cork borer, and 0.1 mL of extract solution at the desired concentration was introduced into the well. Then, agar plates were incubated under suitable conditions depending upon the microorganisms. The antimicrobial agent diffuses in the agar medium and inhibits the growth of the microbial strain tested. The plates were incubated for 24 h to allow the extract to diffuse through the agar media to form zones of inhibition. The extent of antimicrobial activity was measured by the diameter of the inhibition zone.

Cytotoxicity Effects by the Brine Shrimp Lethality Bioassay

The brine shrimp lethality bioassay, is a method used to assess the cytotoxicity of various substances. In this procedure, brine shrimp (*Artemia salina*) are exposed to different concentrations of test samples mixed with artificial seawater. The setup involves adding 9 mL of artificial seawater and 1 mL of the sample or standard solutions (such as potassium dichromate and caffeine) to each test tube. Following this, 10 live nauplii are introduced into each tube, and

the samples are incubated at room temperature for approximately 24 h. After the incubation period, the number of dead and surviving brine shrimp is counted. This data is then used to calculate the 50 % lethality concentration (IC_{50}), which indicates the concentration of the substance required to kill half of the brine shrimp population. This bioassay is a widely used method for preliminary screening of the toxicity of various compounds. The cytotoxicity of compounds was expressed using Deciga-Campos criteria, to which the toxicity of the two compounds was classified with LC_{50} values: those above 1000 µg/mL are non-toxic, those between 500 and 1000 µg/ml are weakly toxic, those between 100 and 500 µg/mL are medium toxic, and those below 100 µg/mL are toxic (Deciga-Campos *et al*., 2007).

Investigation of Antiproliferative Activity of Apigenin and Luteolin Against Human Cancer Cell Lines

Antiproliferative activity of apigenin and luteolin was investigated by the reduction of 3-(4,5-dimethylthiazol-2-yl)-2.5-diphenyltetrazolium bromide (MTT) to formazan at Division of Natural Product Chemistry, Institute of Natural Medicine, and University of Toyama, Japan. The cell lines used were Hela (human cervix cancer), A549 (lung cancer) and MCF-7 (breast cancer). Minimum essential medium with L-glutamine and phenol red (α-MEM, Wako) were used for cell cultures. All media were supplemented with 10 % fetal bovine serum (FBS, sigma) and 1 % antibiotic antimycotic solution (Sigma). From the above medium solution, 100 mL of this supplemented medium was mixed with 1 mL of non-essential amino acid (NAA) for A-549. The *in vitro* antiproliferative activity of the two compounds was determined by the procedure described by (Kamalakararao *et al.,* 2018). Briefly, each cell line was seeded in 96-well plates (2×10^3 per well) and incubated in the respective medium at 37 °C under 5 % CO_2 and 95 % air for 24 h. After the incubation, the cells were washed with PBS, serial dilutions of the tested samples were added. The sample solution in wells with cells were incubated in an incubator for 72 h. The sample solution with cell and medium was added with 100 µL MTT reagent. And then the wells were incubated in an incubator for 3 h, After the incubation, cells in the medium were aspirated with aspirator. The cell was washed with PBS (5 mL) 2 times. Then, DMSO was added about 100 µL per well and the 96 well plates were placed in the dark for 15 min. And then, the absorbance of each solution was measured at 570 nm by using UV-visible spectrophotometer. The concentrations of the isolated compounds and positive control were in the range (6.25-100) µg/mL. Cell viability was calculated from the mean values of the data from three wells using the equation below and antiproliferative activity was expressed as the IC_{50} (50 % inhibitory concentration) value, 5-fluorouracil (5FU) was used as a positive control.

$$(\%)\ \text{Cell viability} = \frac{A_{(\text{test sample})} - A_{(\text{blank})}}{A_{(\text{control})} - A_{(\text{blank})}} \times 100$$

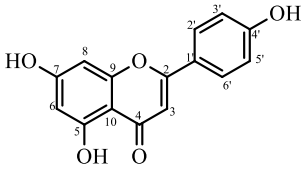
where,

$A_{(\text{test sample})}$ = absorbance of test sample solution
$A_{(\text{control})}$ = absorbance of DMSO solution
$A_{(\text{blank})}$ = absorbance of MTT reagent

Results and Discussion

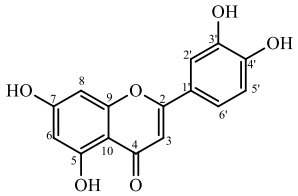
Apigenin: A pale yellow crystal, mp:345 ℃.; FT IR (ν cm^{-1}): 3274 (ν_{O-H}), 1650 ($\nu_{C=O}$), 1604,1555,1495 ($\nu_{C=C}$), 1267 (ν_{C-O-C}), 1015 (δoop(C-H)), UVmax (MeOH) nm: 268, 338; NaOAc nm: 275, 368; NaOAc + H_3BO_3 nm: 269, 344; $AlCl_3$ nm: 276, 340, 381; $AlCl_3$ + HCl nm: 276, 381; NaOH nm: 275, 393.^{1}H NMR (400 MHz, DMSO-d_6) δ_H (ppm): 5.94 (s, 1H, H-3), 12.1(s,1H, Ar-OH), 5.35 (d, J = 1.6 Hz, 1H, H-6), 5.64 (d, J = 2 H, 1H ,H-8), 7.09 (d, J = 8.4

Hz, 2H, H-2', 6'), 6.08 (d, *J* = 8.4 Hz, 2H, H-3', 5').^{13}C NMR (100 MHz, DMSO-d_6) δ_C (ppm): 94.39 (C-8), 99.2 (C-6), 103.2 (C-3), 104 (C-10), 115.9 (C-3' and 5'), 121.5 (C-1'), 128.5 (C-2' and 6'), 157.7 (C-9), 161.5 (C-5), 161.8 (C-4'), 164.1 (C-2), 182.1 (C-4) (Ei Shoubaky *et al.*, 2016, Thant *et al*, 2023).



Apigenin ($C_{15}H_{10}O_5$)

Luteolin: A yellow crystal, m.pt.: 325 ℃.; FT IR (ν cm^{-1}): 3295 (ν_{O-H}),1649 ($\nu_{C=O}$), 1611,1 577, 1432 ($^{\nu}_{C=C}$), 1254, 1117 ($^{\nu}_{C-O-C}$), 861 (δoop(C-H); UVmax (MeOH) nm: 254, 351; NaOAc nm: 267, 364; NaOAc+ H_3BO_3 nm: 261, 374; $AlCl_3$ nm: 271406 ; $AlCl_3$ + HCl nm: 260, 352; NaOH nm: 268, 404.^{1}H NMR (400 MHz, DMSO-d_6) δ_H (ppm): 6.61(s,1H, H-3), 12.92, 10.77, 9.87, 9.349,(s,1H, 5, 7,4', 3'-OH), 7.367 (dd, *J* =2.4, 8 Hz, 1H, H- 6'), 7.335 (d, *J* = 2.4 Hz,1 H, H-2'), 6.836 (d, *J* = 8.8 Hz, 1H, H-5'), 6.382 (d, *J* = 1.6 Hz,1H, H-8), 6.127 (d, *J* =1.6 Hz, 1H, H-6) ^{13}C NMR (100 MHz, DMSO-d_6) δ_C (ppm): 93.8 (C-8), 98.8 (C-6),102.88 (C-3),103.69 (C-10), 145.73 (C-3'), 121.49 (C-1'),116.01(C-5'), 118.99 (C-113.36 (C-2'), 157.27 (C-9), 161.47 (C-5), 149.68 (C-4'), 164.12 (C-7), 163.88 (C-2), 181.C-4) (Puspalata *et al.*, 2019, Thant *et al*, 2023).



Luteolin ($C_{15}H_{10}O_6$)

In vitro Antioxidant Activity

The antioxidant activity of flavones is significantly influenced by the presence and position of hydroxyl groups. For instance, flavones with multiple hydroxyl groups, particularly in the 5, 7, and 4' positions, exhibit stronger radical scavenging abilities. The conjugated double bonds in the flavone structure can stabilize free radicals, contributing to antioxidant properties. The nature of substituents on the flavone ring can also affect antioxidant capacity. Electron-donating groups tend to enhance activity, while electron-withdrawing groups may reduce it (Wang *et al.*, 2018). In the DPPH free radical scavenging assay, 0.625-10 µg/mL of apigenin and luteolin compounds were used. A decrease in absorbance exhibits an increase in radical scavenging activity. The radical scavenging activities of two compounds were expressed in terms of % RSA and IC_{50} (50 % inhibitory concentration). The IC_{50} values of apigenin and luteolin were found to be 2.36 µg/mL and 1.86 µg/mL, respectively. Similarly, the reference ascorbic acid showed IC_{50} value of 1.18 µg/mL. The two compounds were nearly the same as radical scavenging as ascorbic acid. Apigenin has hydroxyl groups at the 3', 4', and 5 positions. These hydroxyl groups contribute to its ability to scavenge free radicals and reduce oxidative stress. The presence of a double bond between C2 and C3 enhances the stability of the flavonoid structure, allowing for effective electron donation to free radicals. Luteolin has hydroxyl groups at the 3', 4', 5, and 7 positions. The additional hydroxyl group at the 7 position compared to apigenin enhances its antioxidant capacity. The presence of multiple hydroxyl groups increases

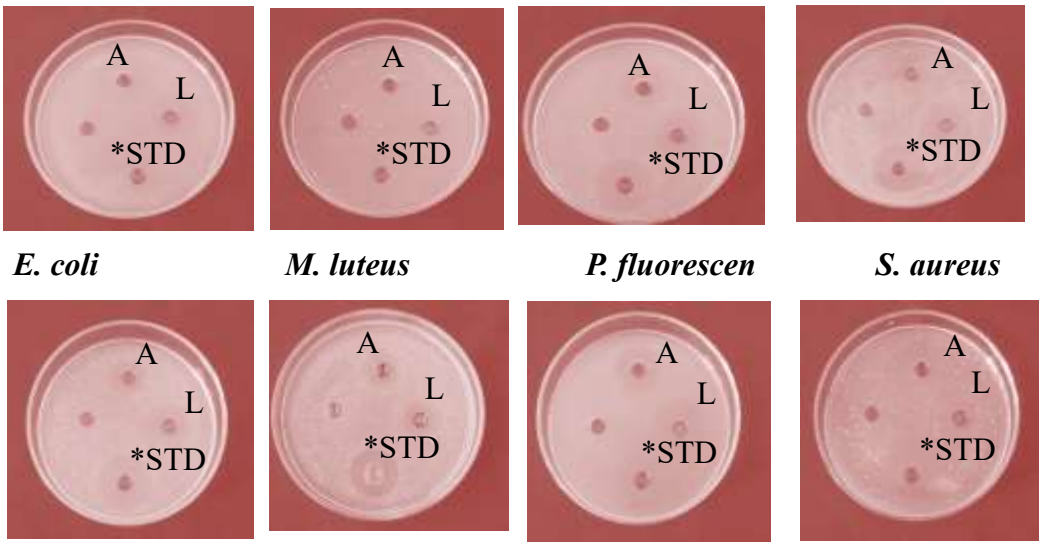
the electron-donating ability, making luteolin a more potent antioxidant than apigenin. The results are shown in Table 1

Table1 Antioxidant Activity of Isolated Compounds from the Aerial Parts of *B.monnieri*

Compounds	% RSA (mean ± SD) in different concentration (µg/mL)					IC_{50} (µg/mL)
	0.625	**1.25**	**2.5**	**5**	**10**	
apigenin	33.91 ± 0.03	41.57 ± 0.13	51.13 ± 0.02	68.90 ± 0.07	79.54 ± 0.03	2.36
luteolin	30.12 ± 0.05	39.02 ± 0.02	62.01 ± 0.18	83.14 ± 0.01	93.77 ± 0.39	1.86
ascorbic acid	26.90 ± 0.37	52.41 ± 0.13	70.55 ± 0.06	88.34 ± 0.06	94.62 ± 0.01	1.18

Antimicrobial Activity

The antimicrobial activity was determined by measuring inhibition zone diameter (Table 2). The activity of apigenin and luteolin were measured by the agar well diffusion method. The greater the zone diameter, the greater the antimicrobial activity of the tested organisms. For bacteria, standard chloramphenicol was used, and nystatin was utilized for fungi. According to the results, luteolin showed the maximum zone of inhibition (25 mm) against *P. fluorescens*, followed by *C. albicans* (21 mm), three gram positive *M. luteus, B. pumilus, and S. aureus (18-21 mm) and* apigenin exhibited *A. tumefaciens* (24 mm) and *B. pumilus* (23 mm), *C. albicans* and *S. aureus* (21 mm), and P. *fluorescens* (20 mm). Luteolin showed greater antimicrobial activity than apigenin. The phenolic compound's hydroxyl group may be able to inhibit the target microorganisms. The presence of aromatic rings in flavones is crucial for their interaction with microbial cell membranes, which can lead to increased antimicrobial activity. Apigenin exhibits antimicrobial activity against various bacteria and fungi. Its structure allows it to interact with microbial cell membranes, disrupting their integrity. The specific positioning of hydroxyl groups contributes to its ability to penetrate microbial cells and exert its effects. Luteolin generally shows stronger antimicrobial activity than apigenin, likely due to its additional hydroxyl group and overall structural stability.



A = Apigenin L = Luteolin

Figure 2 Photographs showing screening of antimicrobial activity of apigenin and luteolin from *B. monnieri*

Table 2 Inhibition Zone Diameters of Apigenin and Luteolin on Some Tested Microorganisms

	Inhibition Zone Diameter (mm)		
Type of Microorganisms	**Apigenin**	**Luteolin**	***STD**
Agrobacterium tumefaciens	24	20	17
Bacillus pumilus	23	20	15
Bacillus subtilis	18	18	22
Candida albicans	21	21	25
Escherichia coli	18	20	24
Micrococcus luteus	18	19	20
Pseudomonas fluorescens	20	25	22
Staphylococcus aureus	21	19	24

Diameter of agar well = 8 mm

9 mm-14 mm = Low activity *STD for bacterial = chloraphenicol

15 mm- 20 mm = Medium activity for fungus = Nystatin

21 mm above = High activity

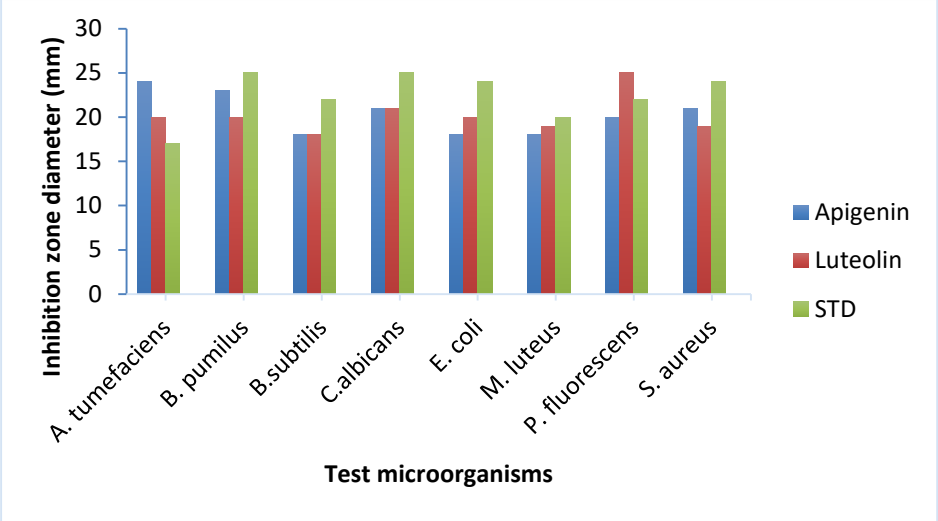


Figure 3 A bar graph of antimicrobial activity of apigenin and luteolin

Cytotoxicity Effects of Apigenin and Luteolin

The brine shrimp assay assess toxicity by counting the number of surviving nauplii after 24 h of exposure to the test substances. The cytotoxicity of apigenin and luteolin was evaluated using the brine shrimp lethality bioassay. The cytotoxic effects were quantified as LC_{50} values (50 % Lethality Concentration). Potassium dichromate ($K_2Cr_2O_7$) and caffeine were selected as standards; $K_2Cr_2O_7$ is recognized as a well-established toxic agent in this assay, while caffeine represents a natural product. The LC_{50} values of apigenin and luteolin were, respectively, 296.00 µg/mL and 200.00 µg/mL and for the standard, 56.44 µg/mL $K_2Cr_2O_7$ and > 1000 µg/mL caffeine. The resulting LC_{50} values were compared with the Deciga-Campos criteria. The two compounds showed medium toxicity due to LC_{50} values between 500 and 1000 µg/mL. The structural integrity of the flavone, such as the presence of a C2-C3 double bond, is often essential for its cytotoxic activity, as it influences the compound's capacity to interact with cellular targets.

Table 3 Brime Shrimp Cytotoxicity of the Isolated Pure Compounds and Standards

Test Sample	% Mortality of Brine Shrimp at Different Concentrations of Sample (µg/mL)					LC_{50} (µg/mL)
	50	100	200	400	800	
apigenin	23.30	30.00	48.83	56.67	63.33	296.10
luteolin	26.67	33.33	50.00	66.67	76.67	200.00
*potassium dichromate	36.67	53.34	70.00	86.67	100.00	56.44
*caffeine	0	0	6.67	16.67	23.34	>1000

*Used as Cytotoxic standards

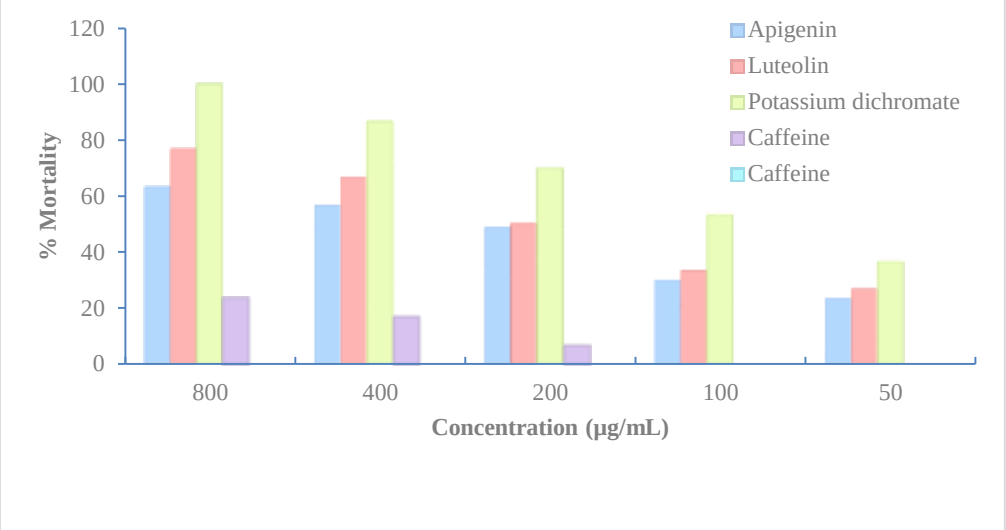


Figure 4 A bar graph of cytotoxicity of the two compounds and standards

Antiproliferative Activity of Apigenin and Luteolin Against Three Human Cancer Cell lines

Antiproliferative activity is the activity relating to a substance used to prevent or retard the spread of cells, especially malignant cells, into surrounding tissues. The MTT assay was used to assess the antiproliferative activity of apigenin and luteolin on three cancer cell lines, A549 (lung cancer cell line), Hela (cervix cancer cell line), and MCF-7 (breast cancer cell line). The anticancer effect was expressed as IC_{50} values (50 % inhibitory concentration). The lower the IC_{50} values, the higher the antiproliferative activity. According to these results, apigenin and luteolin demonstrated the most potent antiproliferative activity, particularly against two cancer cell lines, with IC_{50} values of 9.14 µg/mL for MCF-7 and < 6.25 µg/mL for Hela, when compared to the standard 5 FU, however**,** showed no significant activity IC_{50} values >100 µg/mL for A549 (Table 4). Apigenin has been shown to induce apoptosis and inhibit cell proliferation in various cancer cell lines. The positioning of hydroxyl groups plays a crucial role in its ability to modulate signalling pathways related to cancer cell growth. Luteolin has been shown to induce apoptosis in cancer cells more effectively, likely due to its additional hydroxyl group and overall structural characteristics that enhance its interaction with cellular targets.

Table 4 Antiproliferative Activity of the Isolated Compounds and the Standard

Test Sample	IC_{50} (µg/mL) of Various Samples against Tested Cell Lines		
	A549	**MCF-7**	**Hela**
apigenin	>100	9.14	< 6.25
luteolin	>100	9.14	< 6.25
5-fluorouracil	4.29	77.12	7.42

A549 = lung cancer, MCF-7 = breast cancer, Hela = cervix cancer

Conclusion

In this research work, the following could be deduced: the biological activities of flavones are closely related to their structural characteristics. The antioxidant activity of luteolin showed higher free radical scavenging activity than apigenin. The antimicrobial activity of apigenin showed larger inhibition zone diameter than luteolin. Apigenin and luteolin were found to possess significant antiproliferative activity against human cancer cell lines such as MCF-7 breast cancer cell line (IC_{50} = < 6.25 µg/mL) and Hela cervical cancer cell line (IC_{50} = < 6.25 µg/mL) except A549 lung cancer cell line. Both apigenin and luteolin exhibit significant antioxidant, antimicrobial, and other activities, but their effectiveness is influenced by their structural differences. Luteolin, with its additional hydroxyl group and specific substitution patterns, generally demonstrates stronger biological activities compared to apigenin. The structural activity relationship (SAR) of apigenin and luteolin, two well-known flavonoids, can be analyzed in terms of their antioxidant, antimicrobial, and antiproliferative activities. Both compounds share a similar flavone structure but differ in their hydroxyl substitution patterns, which significantly influence their biological activities, contributing to the development of new therapeutic agents.

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