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BOERHAVIA DIFFUSA: MEDICINAL POTENTIAL, ANTIOXIDANT AND ANTICANCER EFFECTS, WITH M-CSF AND GM-CSF GENE EXPRESSION ANALYSIS IN HCT116 CELL LINES.

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Abstract
Humans historically relied on nature for essential needs, including medicine. Medicinal plants, especially in developing nations, play a vital role in healthcare. *Boerhavia diffusa*, known as Punarnava, is an herb with diverse phytoconstituents, offering various health benefits. This study aimed to evaluate the phytoconstituent value, specifically total flavonoid content and antioxidant activity of *Boerhavia diffusa*. Additionally, the research explored its anticancer potential, focusing on the HCT116 cell line. *Boerhavia diffusa* leaves were extracted using hexane, chloroform, methanol, and water. Phytochemical analysis was conducted, and the total flavonoid content was estimated. Antioxidant activity was assessed using DPPH, FRAP, and H2O2 assays. Anticancer activity was examined on HCT116 cell lines, and potent extracts underwent column purification. The active fractions were further purified using HPLC. The isolated compound (Kaempferol) was tested for anticancer activity, and its IC50 was determined. Gene expression analysis was performed to evaluate the regulatory effects of Kaempferol on M-CSF and GM-CSF genes. Methanol and water extracts showed higher flavonoid content and antioxidant activity compared to hexane and chloroform extracts. Kaempferol, isolated from water extract, exhibited significant anticancer activity against HCT116 cell lines with an IC50 of 56.144 µg/ml. Gene expression analysis indicated Kaempferol's potential regulatory role in M-CSF and GM-CSF genes. *Boerhavia diffusa* extracts, particularly methanol and water extracts, demonstrated potent antioxidant and anticancer properties. Kaempferol, identified as a key bioactive compound, showed significant cytotoxicity against HCT116 cells and potential regulatory effects on immune-related genes. These findings support the medicinal potential of *Boerhavia diffusa* and warrant further exploration in healthcare applications.

Keyword: Boerhavia diffusa, Flavonoids, Antioxidant activity, Anticancer potential, HCT116 cell lines

Introduction
Throughout history, humans have relied on nature to provide them with their basic needs, including food, housing, medicine, clothes, flavors, fertilizers, and transportation. This is especially true in developing nations, where herbal medicine has a long history of use. Medicinal plants continue to play a significant role in the healthcare system for vast segments of the world's population. Both

industrialized and developing countries are increasingly recognizing and developing this plant based medical products for health care and economic benefits. It has been observed that nature serves as the ultimate healer, aiding in the recovery from illnesses and promoting a state of well-being to sustain a healthy life. The significance of plants in our healthcare system cannot be overstated. With their profound pharmacological relevance, medicinal plants of natural origin have captured the attention of numerous researchers. These natural resources hold immense importance as valuable reservoirs of medicines, particularly when it comes to developing novel and pharmacologically effective molecules (Morales *et al*., 2017; Sharma *et al*., 2017).

Plant extracts contain a diverse array of free radical scavenger molecules, such as phenolics, anthocyanins, carotenoids, and vitamins. Among these, standard phenolics, including phenolic acids and flavonoids, are particularly recognized for their potent antioxidant activity. They effectively absorb and neutralize free radicals, contributing to their beneficial effects. The secondary bio-active metabolites found in plants showcase robust biological properties, presenting a wide range of effects, including anti-inflammatory, anti-cancer, anti-bacterial, and antiviral (Benabderrahim *et al*., 2019; Gunathilake *et al*., 2018). For example, phenolic compounds, such as flavonoids, demonstrate an anti-cancer effect, may reduce bone resorption, and exhibit cardio-protective properties, among other beneficial effects. (Hazafa *et al*., 2020; Buyel, 2018).

In the current research study, we extracted bioactive compounds from leaves and investigated their potential pharmacological effects. In this study, we focused on *Boerhavia diffusa*. Our aim was to evaluate its phytoconstituent value, specifically the total flavonoid content and antioxidant activity. Moreover, we successfully demonstrated the anticancer activity of berry leaves in HCT116 cell lines.

Boerhavia Diffusa

Boerhavia diffusa, also known as Punarnava, belongs to the family Nyctaginaceae (Nayak *et al*., 2016). It is a perennial and annual herb having pale rose-colored flowers (Struwig *et al*., 2013). The plant has been reported to contain a variety of components, including alkaloids, lipids, flavonoids, glycoproteins, triterpenoids, proteins, steroids, carbohydrates, and lignin. It further contains specific compounds such as β-sitosterol, α-2-sitosterol, palmitic acid, ester of β-sitosterol, tetracosanoic, hexacosanoic, stearic, arachidic acid, ursolic acid, hentriacontane, β-ecdysone, and triacontanol (Ali *et al*., 2022). Punarnava has been reported to provide hepatoprotective, anti-convulsant, anti-hypertensive, antiproliferative, immunomodulatory, anti-diabetic, antioxidant, anti-inflammatory, and anti-microbial effects (Prathapan *et al*., 2017).

Materials and Methods

Materials

Powder of leaves *Boerhavia diffusa* were purchased from an e-commerce site. Chemicals of analytical-grade reagents were used. Mercuric chloride, methanol, ethanol, sodium carbonate, 2,2-diphenyl-1-picrylhydrazyl (DPPH), ascorbic acid, MTT reagent, and dimethyl sulphoxide (DMSO). The HCT116 cell line used in the study was sourced from the National Centre for Cell Science (NCCS) in Pune.

Methods

Soxhlet extractions of leaves

Powdered (*Boerhavia diffusa*) samples were soxhlet extracted using different solvents following the protocol of Chauhan *et al*., 2021. Briefly, a 5 g sample was extracted with 500 mL of Hexane, Chloroform, Methanol and Water for 24 cycles at 4 cycles per hour. After that, the extracts were evaporated, weighed, and diluted in 20 mL of their respective solvent.

Phytochemical analysis of extracts

Phytochemical analysis was conducted to identify specific types of chemical compounds in a plant extract. Alkaloids were assessed using Mayer's, Dragendorff's, and Wagner's reagents, each resulting

in distinctive precipitates—cream-colored, orange, and brown, respectively (Mir et al., 2016). Flavonoids were identified by the development of a pink or tomato-red color following the addition of magnesium ribbon and concentrated hydrochloric acid (Mahesh *et al*., 2013). Tannins were confirmed by the manifestation of a blue hue upon treatment with ferric chloride ($FeCl_3$) (Kewlani *et al*., 2023). The presence of cardiac glycosides was determined through the Keller-Killiani test, wherein a greenish-blue color emerged (Mir *et al*., 2016). Steroids were detected via the Liebermann-Burchard test, revealing a characteristic blue-green ring. Saponins were assessed using the Frothing test, with stable foam exceeding 1.5 cm indicating their presence. Carbohydrates were examined using Molisch's reagent, leading to the observation of a brown ring at the liquid interface. Glucose was specifically identified using Benedict's test, resulting in a distinctive red brick color upon treatment with Benedict solution (Shibu et al., 2017).

Total Flavonoid content

The estimation of flavonoid content was conducted using the aluminum chloride colorimetric method (Pontis et al., 2014). Briefly, 0.5 ml of a solution containing 1.2% aluminum chloride and 1M potassium acetate was added to 0.5 ml of the extract. The reaction mixture was then diluted to a total volume of 3 ml using methanol and left to incubate at room temperature for 30 minutes. After incubation, the absorbance of the solution was measured at a wavelength of 415 nm. A blank sample was prepared by combining all reagents except for the extracts.

Standard solutions of quercetin were prepared within the concentration range of 0.01 mg/ml to 0.150 mg/ml. The Total Flavonoid Content (TFC) was calculated using the equation derived from the standard curve of quercetin and was expressed as milligrams of quercetin equivalent (QE) per 100 grams of dried leave sample.

Antioxidant assay of crude extract

2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay

Based on the scavenging of the stable 2, 2-diphenyl-1-picrylhydrazyl (DPPH) free radical, the crude extract's DPPH free radical scavenging activity was assessed. The samples were diluted to a concentration of 1 mg/ml. By adding 1.0 ml of 0.1 mM DPPH in methanol and 0.1 ml of extract, the reaction mixture was prepared. The mixture was incubated at room temperature in the dark for 30 minutes. At 517 nm, the amount of DPPH inhibition was determined by observing the decline in absorbance (Sharma *et al*., 2011). The positive control was ascorbic acid. Radical scavenging activity was measured as the sample's free radical inhibition percentage and calculated using the following equation:

$$\%Inhibition = \left(\frac{Ao - At}{Ao}\right) * 100$$

Were,

A_0= Absorbance of control (blank without sample)

A_t= Absorbance in presence of the sample.

Each test was run three times, and a graph with mean values was plotted.

Ferric Reducing Antioxidant Power Assay (FRAP) assay

The FRAP (Ferric Reducing Antioxidant Power) test, following the methodology outlined by Benzie et al. in 1996, commenced with the dilution of samples to a concentration of 1 mg/ml. An aliquot of 100µL from the appropriately diluted extract was then added to 3 mL of the standard reaction solution. Absorbance measurements were taken at 593 nm immediately and six minutes later at room temperature, with the entire process repeated three times for reliability. A standard curve was constructed using $FeSO_4$ concentrations ranging from 200 to 1000 µM. The resulting FRAP values for both standards and samples were quantified in units of µM Fe [II] per gram of dry weight.

Hydrogen Peroxide (H_2O_2) assay

A portion of the 40 mM H_2O_2 solution (0.6 ml) and the diluted crude extract (0.1 ml) was added to 2.4 ml of 0.1 M phosphate buffer of pH 7.4 was added to the mixture, shaken rapidly, and incubated for 10 minutes at room temperature. The reaction mixture's absorbance was then measured at 230 nm. Ascorbic acid served as the positive control (Kumar *et al*., 2020).

The H_2O_2 scavenging activity was calculated as follows:

$$\%Inhibition = \left(\frac{A1 - A2}{A1}\right) * 100$$

Where,

A1= Absorbance of the ascorbic acid

A2= Absorbance of the sample.

Column purification of crude extracts

Crude extracts were screened for their anticancer activity against HCT116 cell lines. Extract with potent anticancer activity was subjected to column purification following the protocol of (Zhang et al., 2014). Briefly, the crude extract was dried and packed into a column with silica 60–120 mesh using hexane as the solvent. Carefully applying the crude extract on the silica layer without disturbing it, the purification process utilized a stepwise increase in solvent polarity with varying ratios of chloroform, and methanol (Chloroform (20 ml); Chloroform: Methanol (16:4); Chloroform: Methanol (10:10); Chloroform: Methanol (4:16) and Methanol (20 ml) with a flow rate of 5 ml per min. The eluted fractions were screened for anticancer activity, and subsequently, specific fractions were further purified using High Performance Liquid Chromatography.

Anti-Cancer Activity

To determine the anticancer activity of crude and fractions, all of the samples were dried and diluted to a concentration of 1 mg/ml for further evaluation. HCT116 cell lines were incubated with the highest concentration of the crude and fractions. The percentage cytotoxicity was evaluated and fractions showing potential anticancer activity were further processed by HPLC purification.

HPLC purification of column fraction

The potent fraction with anticancer properties was analyzed using HPLC with a method adapted from Khuluk et al., 2021. Standard solutions of flavonoids were prepared in HPLC-grade methanol, and after filtration, fractions underwent sonication. An Agilent 1200 series HPLC system with a C18 column was used, featuring a programmed gradient of methanol and 0.2% formic acid over 60 minutes. The UV/Vis detector aided in identifying and quantifying eluted compounds.

Anticancer activity and half Maximal Inhibitory Concentration (IC50) of the HPLC purified compounds

Following HPLC purification, the obtained compounds were evaluated for their potential anticancer activity against HCT116 cell lines. The purified compounds were subsequently dried and diluted to a concentration of 1 mg/ml for testing. The IC50 values of HPLC purified compounds having anticancer activity were determined following the protocol of Mbarek et al., 2007). Cell lines, cultivated for 24 hours, were treated with properly diluted purified compounds. After a 24-hour incubation at 37°C with 5% CO_2, supernatant was collected, and MTT reagent was added. After a two-hour incubation at 37°C, DMSO was added to solubilize formazan crystals, followed by agitation for 15 minutes. Absorbance was measured at 490 nm. Negative controls include media and DMSO in the absence of the tested compound. The % cell viability was calculated using the formula

$$\%\ Cell\ viability = \left(\frac{A1}{Ao}\right) * 100$$

Where:
Ao is the absorbance of the control
A1 is the absorbance of the extracts.

Gene expression analysis
To isolate RNA from HCT116 cells, cultured at a concentration of approximately $2.6*10^4$ cells/ml, the cells were treated with purified compounds at IC50 concentrations in a six-well microtiter plate. Control wells with untreated cells were also included. After a 72-hour incubation, cells were trypsinized, and the pellet was obtained by centrifugation. RNA isolation followed the protocol by Rio et al., 2010, involving Trizol, chloroform, and isopropanol. The RNA pellet was suspended in 70 µl of nuclease-free water and stored at -20°C. RNA quality and quantity were assessed using gel electrophoresis and a UV-Vis spectrophotometer (measured at 260 nm and 280 nm), respectively.
cDNA first strands were synthesized using the Thermo Scientific RevertAid First Strand cDNA Synthesis Kit #K1621. RNA samples (5 µg) were added to oligo (dt)18 primer and nuclease-free water, incubated at 65°C, and then chilled. Enzyme mix was added, and the reaction proceeded at 70°C. The cDNA products were stored at -20°C.
Real-time PCR with ABI PRISM 7000 SDS used a 50 µl reaction mixture, including Sybr green master mix, cDNA, and primers. Quality control measures included no template controls. Gene expression analysis employed the comparative CT method, normalizing target gene CT values to GAPDH and comparing to a calibrator sample. Results were quantified using the $2^{-\Delta\Delta CT}$ formula, indicating the ratio of relative gene expression in compound-treated cells to the initial control replicate.

Result and Discussion
Methanolic extract of Punarnava yielded 54%, water extract 44%, hexane extract 38%, and chloroform extract 28%. This highlights the effectiveness of methanol as a solvent for *Boerhavia diffusa* extraction, providing insights for optimal extraction procedures.

Phytochemical analysis of extracts
The phytochemical analysis reveals that *Boerhavia diffusa* water extract (BDW) is rich in flavonoids, glycosides, and saponins (Table 1), suggesting its potential for addressing conditions related to oxidative stress due to the antioxidant and anti-inflammatory properties of flavonoids. This information is valuable for selecting extracts with specific bioactive components for targeted medical or pharmaceutical applications.

Table 1: Phytochemical analysis of different extracts of *Boerhavia diffusa.* BDH: *Boerhavia diffusa* hexane extract, BDC: *Boerhavia diffusa* chloroform extract, BDM: *Boerhavia diffusa* extract, BDW: *Boerhavia diffusa* water extract, +: mildly present, ++: moderately present, +++: abundant, -: absent

Sample	Alkaloid			Flavonoid	Phenol	Glycosides	Tannins	Carbohydrate			Saponins	steroids
	Mayer's test	Dragendorff's test	Wagner test					Molisch	Fehling's	Benedicts		
BDH	-	-	-	-	+	-	-	+	+	+	+	-
BDC	+	+	-	+	++	-	-	++	+	++	+	-
BDM	+	+	+	++	+++	++	++	+	+	+	-	-
BDW	++	++	+	+++	++	++	++	-	-	-	-	-

Total Flavonoid content

The hexane extract displays a quercetin equivalents (QE) concentration of 0.031 mg/ml and a total flavonoid content (TFC) of 0.756 mg/g. The chloroform extract shows values of 0.037 mg/ml for QE and 0.884 mg/g for TFC. The methanol extract exhibits the highest flavonoid content with a QE concentration of 0.067 mg/ml and a TFC of 1.604 mg/g. Discrepancies in TFC among extracts may stem from variations in extraction methods. Methanol extraction proves most efficient in yielding flavonoids. Flavonoids, recognized for their antioxidant properties, are crucial in safeguarding plants against UV light. This information underscores the impact of extraction methods on flavonoid content and emphasizes the potential antioxidant benefits associated with *Boerhavia diffusa* methanol extracts.

Antioxidant assay of crude extract

Antioxidant activity of *Boerhavia diffusa* extracts is represented in Table 2. The methanol (BDM) and water (BDW) extracts demonstrate higher antioxidant potential compared to hexane (BDH) and chloroform (BDC) extracts. BDM and BDW exhibit strong DPPH scavenging (81.223% and 83.038%, respectively), high FRAP values (4866.667 and 3333.333 µM Fe (II)/g dry wt.), and substantial H_2O_2 scavenging (62.61% and 63.51%). These results suggest the superior antioxidant properties of methanol and water extracts, indicating their potential for health-related applications.

Table 2: Antioxidant activity of different extracts of *Boerhavia diffusa.* BDH: *Boerhavia diffusa* hexane extract, BDC: *Boerhavia diffusa* chloroform extract, BDM: *Boerhavia diffusa* methanol extract, BDW: *Boerhavia diffusa* water extract. Data are mean ± std. dev. and n=3.

Sample	DPPH (% scavenging activity)	FRAP value (µM Fe (II)/g dry wt.)	H_2O_2 % scavenging
BDH	23.376±1.7	733.333±115.470	17.12
BDC	42.114±0.738	1600	30.18
BDM	81.223±0.441	4866.667±230.940	62.61
BDW	83.038±0.215	3333.333±115.470	63.51

Purification and isolation of single compound

The crude extracts were initially screened to evaluate their potential anticancer properties. Among them, only the water extract demonstrated significant anticancer effects against the HCT116 cell line, prompting its selection for further purification. The purification process involved column chromatography, resulting in around 20 fractions for each sample. Anticancer activity screening was then conducted on these fractions at their highest concentrations. Fractions showing positive anticancer activity underwent additional purification steps, which included evaporation, weighing to quantify the remaining material, and dissolution in HPLC-grade methanol. Fractions F19 and F20 exhibited substantial anticancer activity against HCT116 cell lines. To isolate individual compounds, these fractions underwent further purification using High-Performance Liquid Chromatography (HPLC). Kaempferol was purified with retention time of 9.968 min (Figure 1) and a concentration of 4.57 µg/ml.

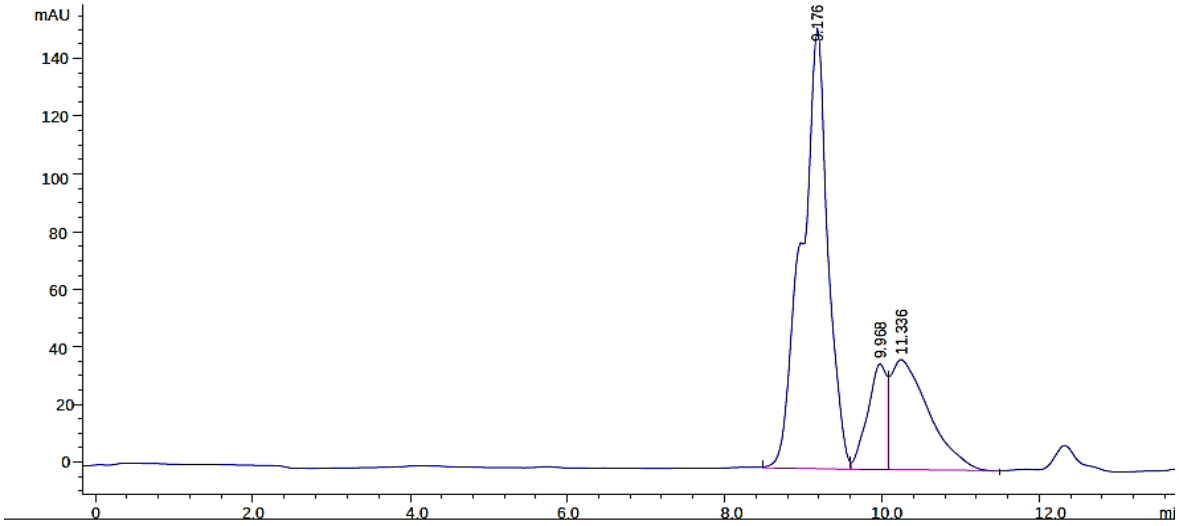

Figure 1: HPLC chromatogram of fraction 19 showing retention time of 9.968 for kaempferol with other unknown compound peaks.

Anticancer Activity and IC50 estimation for the purified compounds

Following the purification process, all isolated compounds, including Kaempferol obtained from Fraction F19, were subjected to screening against HCT116 cell lines at their maximum concentrations. Kaempferol, a compound derived from Fraction F19, exhibited notable anticancer activity. Subsequent to this observation, an estimation of the IC50 (half-maximal inhibitory concentration) for Kaempferol was conducted. The results indicate a concentration-dependent impact on cytotoxicity, with pronounced peaks in anticancer activity observed at concentrations of 62.5 µg/ml, 75 µg/ml, 87.5 µg/ml, 100 µg/ml, and 112.5 µg/ml (Figure 2). These concentrations resulted in a significant reduction in cell viability, underscoring the potency of Kaempferol against HCT116 cell lines. The IC50 value, representing the concentration at which the compound inhibits cell growth by 50%, was determined to be 56.144 µg/ml. Kaempferol was reported to induce apoptosis Kaempferol, at a concentration of 50 µM, triggers programmed cell death (apoptosis) in colorectal cancer cells (SW480, HCT116, HCT-15). This effect is linked to increased reactive oxygen species (ROS), higher levels of the tumor suppressor protein p53, and activation of the p38/MAPK pathway, indicating its potential as an anti-cancer agent in colorectal cancer (Lu et al., 2018). This finding further supports the potential therapeutic significance of Kaempferol in combating the proliferation of HCT116 cancer cells.

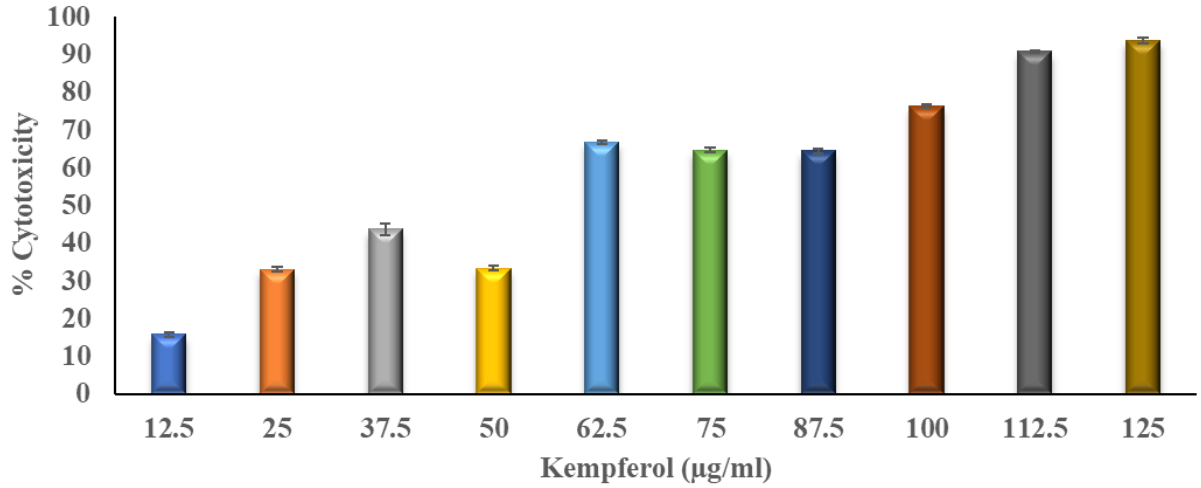

Figure 2: % Cytotoxicity at different concentrations of kaempferol against HCT116 cell lines. Data are represented as mean ±std dev.

Gene expression analysis

Between 1.9 and 1.96, the extracted RNA has a high degree of integrity and purity and is in good condition. The relative gene expression levels of M-CSF and GM-CSF are evaluated using the 2^{-ddCt}. The gene exhibits a significant 4.39-fold elevation in M-CSF with kaempferol administration, indicating a higher level of expression. This suggests that kaempferol may have a positive regulatory effect on the expression of M-CSF. On the other hand, the GM-MCF gene shows a downregulation of 0.72-fold relative to the control under the same conditions (Figure 3), suggesting a lower expression level. Both results imply that the gene responds differently to M- and GM-CSF conditions, which may indicate that the gene is involved in the cellular processes that both cytokines affect. These findings highlight the potential role of kaempferol in regulating the expression of M-CSF and GM-MCF genes. Further research is needed to understand the specific mechanisms underlying these differential responses and their implications for cellular processes affected by M- and GM-CSF. kaempferol and quercetin have been reported to induce GM-CSF production in prostate cancer PC-3 cells, potentially activating an immune response. The mechanism involves vesicular trafficking, microtubule dynamics, and calcium ion regulation. The conditioned medium enhances dendritic cell chemotaxis via GM-CSF, suggesting an immunotherapeutic role for these flavonoids in prostate cancer (Bandyopadhyay et al., 2008)

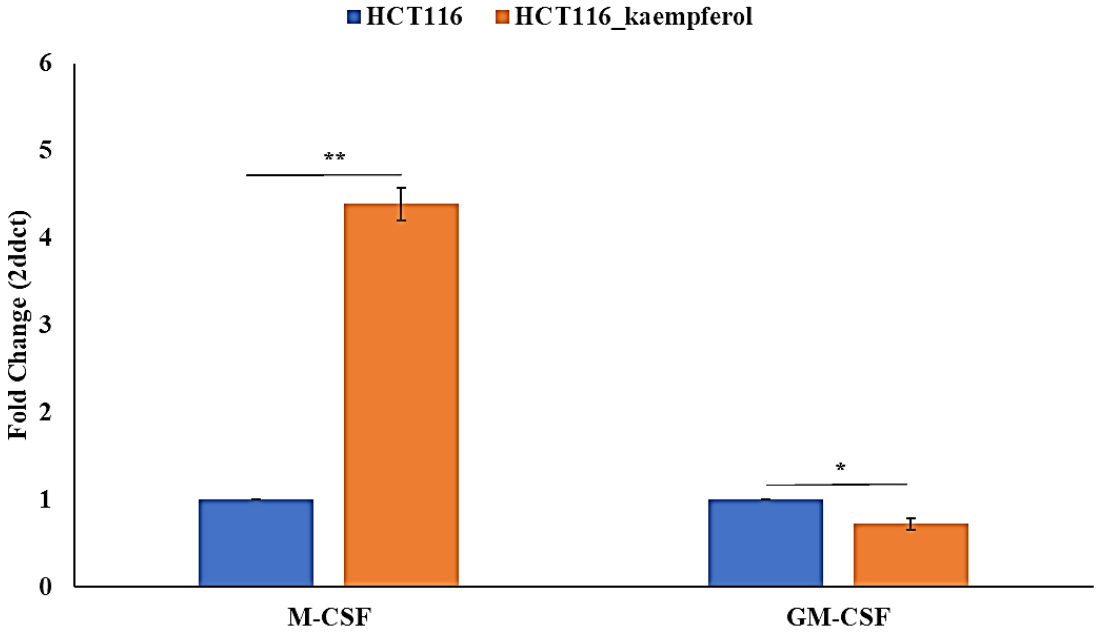

Figure 3: Relative gene expression of M-CSF and GM-CSF. All data are represented as mean ± std dev., where n=3, Significance is denoted by asterisks, with "" indicating high significance (p<0.005), and * indicating (p<0.05).**

Conclusion

The study underscores the efficacy of methanol in *Boerhavia diffusa* extraction and identifies rich phytochemical compositions, especially in water extracts. These extracts exhibit potent antioxidant properties and significant anticancer effects, with Kaempferol showing notable efficacy. Gene expression analysis suggests Kaempferol's potential regulatory role in M-CSF and GM-CSF genes. Overall, the findings emphasize the health-related potential of *Boerhavia diffusa* extracts, particularly those obtained with methanol and water, paving the way for further exploration in medicinal applications.

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