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Chemo Preventive Potential of Rutin-Rich Extract Obtained from *Capparis spinosa* Culture against HepG2 Cancer Cell Line Activity

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Abstract

Capparis spinosa, an edible medicinal plant rich in flavonoids, relies on amino acids as key precursors for secondary metabolite biosynthesis in vitro. This study investigated the effects of tryptophan, glutamine, and phenylalanine at varying concentrations on callus growth, flavonoid production, and cytotoxic activity against the hepatocellular carcinoma cell line (HepG2). Callus induction was optimized using 0.5 mg/L 2,4-Dichlorophenoxyacetic acid (2,4-D), achieving a 100% callusing frequency from leaf explants. Elicitation with 100 mg/L tryptophan or 300 mg/L glutamine significantly enhanced callus biomass (4.7 g fresh weight). HPLC analysis revealed elevated rutin levels in callus treated with 100 mg/L glutamine (88.2 µg/g) or 200 mg/L phenylalanine (64.3 µg/g), highlighting their role in flavonoid biosynthesis. The glutamine-elicited extract (100 mg/L) exhibited potent cytotoxicity against HepG2 cells (IC50: 356.65 µg/mL), followed by phenylalanine treatment (IC50: 371.05 µg/mL). Molecular docking studies demonstrated rutin's ability to suppress HepG2 proliferation by targeting promoter regions, downregulating oncogenic signaling pathways, and inhibiting spheroid formation. Mechanistically, rutin induced apoptosis and mitigated oxidative stress, underscoring its antitumor potential. These findings position amino acid elicitation as a viable strategy to enhance rutin yield in *C. spinosa* cultures, offering a sustainable platform for producing anticancer compounds. Further *in silico* and *in vitro* studies are warranted to explore rutin's interactions with specific molecular targets in hepatocellular carcinoma.

Keywords: Caper; Anticancer activity; Tissue culture; Flavonoids; Secondary metabolites; Molecular docking

1. Introduction

The genus *Capparis* belongs to family Capparaceae. Capers cultivate wildly in several regions of the world, including the Atlantic coast of the Canary Islands and Morocco, the Black Sea, and the east side of the Caspian Sea to Iran [1]. The genome of *Capparis spinosa* included 157,728 bp in length and 136 genes, while the plastome included 80 protein-coding genes, 4 rRNA genes, and 31 tRNA genes [2]. Recent genomic studies have identified key genes involved in its secondary metabolite biosynthesis, contributing to its rich profile of bioactive compounds. *Capparis spinosa* had displayed the presence of many bioactive compounds. Various flavonoids have been identified in the caper bush such as rutin (quercetin 3-rutinoside), quercetin 7-rutinoside, quercetin 3-glucoside-7-rhamnoside, kaempferol 3-rutinoside, and astragalin. Capers contain more quercetin than any other plant. These compounds have anti-inflammatory, antioxidant, antimicrobial, immunomodulatory, and antiviral properties [3].

Among these bioactive compounds, rutin (quercetin-3-rutinoside) stands out due to its well-documented medicinal benefits. Rutin is a flavonoid glycoside with strong antioxidant activity, capable of scavenging free radicals and chelating metal ions. Its anticancer potential has been extensively studied. Rutin inhibits human liver cancer cells (HepG2) cell proliferation, invasion, colony formation, and reducing DNA damage while inducing apoptosis at multiple stages, reinforcing its anticancer potential [4]. In addition to increasing the antioxidant enzymes glutathione S-transferase Pi 1 (GSTP1) and NADPH quinone dehydrogenase 1 (NQO1), rutin treatment inhibits cytochrome P450-dependent CYP3A4 and CYP1A1 enzymes [5]. By reducing the production of reactive oxygen species (ROS) and the level of malondialdehyde in HepG2 cells, rutin positively increased antioxidant performance [6,7]. Remarkably, in rats given nitrosodiethylamine and phenobarbital, rutin inhibited liver tumor indicators such as alpha-fetoprotein and carcinoembryonic antigens in an in vivo investigation. Furthermore, according to Chandra and Viswanathswamy [8], rutin increases the decreased level of membrane-bound ATPases. While electrolyte imbalance aids in the evolution of cancer [9], lipid peroxidation activity, which is frequently elevated when in a malignant condition, negatively impacts ATPase activities that impact electrolyte levels [10]. Rutin restores common electrolyte abnormalities such as hyperkalemia, hypercalcemia, hyponatremia, and hypomagnesemia [8].

Rutin has been demonstrated to reduce inflammatory reactions in UVB-irradiated mouse skin, by preventing the rise in phosphorylated the signal transducer and activator of transcription 3 (STAT3) levels [11].To initiate the production of target

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genes, the STAT3 is phosphorylated and moved into the nucleus. The fact that several inhibitory substances negatively affect STAT3-mediated signaling is becoming more widely recognized. Using various natural phytochemicals to therapeutically regulate STAT3 signaling has shown promise [12], but more thorough research is still required.

Biotechnological approaches like callus cultures can use to increase rutin production in caper plant. It yields a huge amount of clones, which can decrease costs [13]. Mainly, buds or shoots are used for in vitro propagation. Modified MS medium supplemented by auxin, cytokinin, and some elicitors is extensively used for the callus induction to enhance secondary metabolite accumulation. Therefore, several studies have been conducted to enhance the synthesis of secondary metabolites in vitro cultures of different plant species, e.g., salicin from *Salix Safsaf* and flavonoid production in *Hydrocotyle bonariensis* tissue cultures [2,14]. By supplementing the culture medium with some amino acids, which are considered a precursor for various phenolic, flavonoids, and alkaloids, researchers can optimize the conditions for plant callus induction and increase the production of valuable secondary metabolites. It can also improve the growth and metabolic activity of the callus cultures and increase the antioxidant activity of the callus cultures [15**,** 16].

This study aimed to investigate the effect of amino acids (phenylalanine, tryptophan, or glutamine) as precursors to enhance the growth and phenolic and flavonoid compounds accumulation in caper callus culture. Additionally, the medicinal properties of rutin, the main compound in *Capparis spinosa,*to study its anticancer activity against HepG2, as well as to conduct *in silico* analysis and molecular docking studies.

2. Materials and Methods

2.1. Plant material

Capers stem cuttings (*Capparis spinosa* L.) were gathered from the southwest region of Horgada in Egypt. Lab experiment was conducted in the tissue culture laboratory at the Horticulture Research Institute, Agricultural Research Centre, Giza, Egypt. Aiming to study the response of caper plants (*Capparis Spinosa* L.) treated with chemical elicitors (phenylalanine, tryptophan, and glutamine) to evaluate their effects on callusing percentage and its fresh weight, rutin content, and chemo preventive activity of the HepG2 cell line.

2.2. *In vitro callus production*

Explants from stem cuttings (internodes) were rinsed for one hour under running water and then immersed for thirty minutes with constant stirring in water containing soap. Under sterile conditions, the explants were placed in 70% alcohol for one minute and then, with constant stirring, in sodium hypochlorite (15%) for ten minutes. The explants were then rinsed three times for five minutes each with sterile distilled water. In sterile conditions, sterilized stem cuttings were divided into tiny explants with a single bud, and they were then cultivated on full strength Murashige and Skoog (MS) media containing kinetin (Kin) at 0.4 mg/L each, in three replicates with 5 explants per replicate [17]. For callus production, after shootlet formation, leaves were cut and cultured on MS media containing 2,4-dichlorophenoxyacetic acid (2,4-D) at 0.5 mg/L. The jars were placed in an incubator under 24 °C and 16 h light growing conditions. Four weeks later, the growing calluses were transferred to the media containing amino acids.

2.3. *Rutin enhancement*

Callus was transferred on three different media containing MS medium with phenylalanine, tryptophan, or glutamine (100, 200, and 300 mg/L for each amino acid) that were brushed from ROTH®. The callus was incubated for 4 weeks under normal conditions; callus fresh weights were taken, and rutin amount was extracted and determined at the end of the incubation period.

2.4. *Chemical analysis*

2.4.1. Determination of total phenols and flavonoids content

Callus of *Capparis spinosa,* which growing on different amino acids treatments, was extracted by methanol at a 1:10 (w/v) ratio for 24–48 hours with intermittent shaking and filtered using Whatman No. 1 filter paper three times to complete extraction. Methanol was evaporated using a rotary evaporator at 40°C to obtain a dry extract.

The content of total phenols was determined in callus methanol extract using a Folin–Ciocalteu (a mix of phosphomolybdate and phosphotungstate) reacts with phenolic compounds in an alkaline medium (7.5% sodium carbonate), The mixture was incubated in the dark for 30 minutes at 25°C. Absorbance was read at 765 nm using a spectrophotometer. Results were compared to a gallic acid standard curve. The method described by Singleton and Rossi [18].

The content of Flavonoids was determined using the aluminum chloride colorimetric method described by Zhishen et al. [19]. The mixture was incubated at room temperature for 30 minutes in the dark. Absorbance was read at 510 nm. Results were compared to a rutin standard curve.

2.4.2. Rutin determination

The determination of rutin in callus methanolic extract (glutamine 100 mg/L, phenylalanine 200 mg/L, and control) was carried out by using high-performance liquid chromatography (HPLC). The separation was accomplished using HPLC Agilent Technologies 1260 Infinity (USA and Canada) with a reversed-phase Zorbax SB-C18 column (4.6 × 250 mm, 3.5 µm) and a DAD detector (210 nm). Separation was achieved using a gradient elution of 0.1% formic acid (A) and acetonitrile (B) at 1.0 mL/min over 25 min, with 5 µL injection volume. Samples were filtered (0.22 µm) prior to analysis, and the method

was validated for linearity, precision, and recovery. The HPLC separation was performed at a controlled column temperature of 25°C (±1°C) to ensure retention time reproducibility and optimal peak resolution for rutin analysis. The procedure was carried out in accordance with Mutuli [20].

2.4.3. Enzymes activity assays

Callus tissue (1 g) was homogenized in 3 mL of ice-cold 10 mM potassium phosphate buffer (pH 7.0) containing 4% (w/v) polyvinylpyrrolidone (PVP) to inhibit phenolic interference. The homogenate was centrifuged at 12,000 × g for 20 min at 4°C, and the supernatant was used as the enzyme extract. The Bradford [21] technique was used to measure the protein content of the extract.

Polyphenol oxidase (PPO) activity: The Coseteng and Lee [22] method can be used to measure the activity of PPO. The polyphenol oxidase enzyme activity was determined by monitoring the oxidation of 50 mM catechol in 100 mM phosphate buffer (pH 6.8) at 420 nm for 1 min at 25°C.

Catalase (CAT) activity: The Kato and Shimizu [23] method was used to test the catalase activity by tracking the decomposition of 15 mM H_2O_2 in 50 mM phosphate buffer (pH 7.0) at 240 nm for 2 min.

Enzyme activities were expressed as units (U) per mg protein, where 1 U equals 1 μmol product formed per minute. All spectrophotometric measurements were performed in triplicate using a UV-Vis spectrophotometer with temperature-controlled cuvette holders.

2.4.4. HepG2 Cytotoxicity Evaluation via MTT

The methanol extract of *Capparis spinosa* callus cultures (treated with 200 mg/L phenylalanine or 100 mg/L glutamine) was tested for cytotoxicity against hepatocellular carcinoma HepG2 cells (obtained from VACSERA, Egypt). Cells were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum, 2 μM L-glutamine, 100 U/mL penicillin-streptomycin, and 250 ng/mL amphotericin B at 37°C in a 5% CO_2 humidified incubator. Upon reaching 75% confluence, cells were trypsinized (1 mL trypsin/EDTA, 5 min, 37°C), seeded into 96-well plates (0.5×10^5 cells/well in serum-free medium), and treated with 20 μL of test extract at varying concentrations for 24 hours. Cell viability was assessed via MTT assay: after incubation, 5 mg/mL MTT (in 0.9% NaCl) was added followed by 4-hour incubation to form formazan crystals, which were dissolved in acidified isopropanol (0.04 N HCl). The half-maximal inhibitory concentration (IC_{50}) was derived from dose-response curves. Triplicate experiments ensured reproducibility, with <100% viability indicating cytotoxicity [24,25].

2.4.5. In silico assessment

This assessment uses advanced computation modelling to predict the potential toxicity of a chemical rapidly. The three-dimensional (3D) structure of the gamma secretase enzyme complex (PDB ID: 7C9I) deposited by Yang et al. [26] was obtained from the protein data bank [27,28]. The resolution of the retrieved protein structures was 3.10 Å. Visualization of the downloaded structure was carried out by PyMol software (The PyMOL Molecular Graphics System, Version 2.0 Schrödinger, LLC). The 3D structure of rutin was downloaded from the PubChem database [29]. The rutin was prepared for docking using the LigPrep module of the Schrödinger package 2020-1 (Schrödinger program, LLC, New York, NY, USA, 2020). LigPrep corrects various structural problems of ligand molecules, such as the addition of hydrogen atoms, a 2D-3D conversion, the correction of bond lengths, and energy minimization. During ligand preparation, the ionization state of ligands was not changed, tautomers were not created, and ligands were allowed to retain their particular chirality. The OPLS3 force field was used for the energy minimization of protein structure.

2.5. *Statistical analysis*

The data were analyzed using one-way ANOVA ($p < 0.05$) under a randomized complete block design (RCBD) to assess the significant effects different treatments. Fisher's LSD post hoc test was then applied to compare group means, with significance set at $p < 0.05$. Assumptions of normality and homogeneity of variance were verified prior to analysis.

3. Results and Discussion

3.1. Callus production from *Capparis spinosa*

Amino acids play a significant role in the growth and development of plant callus, which is a mass of undifferentiated plant cells. Data in Table 1 and Fig. 1 cleared the effect of different concentrations of tested amino acids(phenylalanine, tryptophan, or glutamine) with different concentrations oncallus fresh weight (FW) that were increased to 4.7 g when treated with 100 mg/L tryptophan or 300 mg/L glutamine compared with control (Fig. 1). Al Tahhan [30] induced callus from cotyledon leaves that were cultured on MS medium supplemented with 2.5 mg/L 6-benzylaminopurine, 0.5 mg/L naphthalene acetic acid (NAA) and 0.5 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D). In addition, El-Maghrabi [31] studied the micropropagation of the wild medicinal plant caper *(Capparis spinosa L.).* They used different concentrations of 6- banzyl amino purine (BAP) and kinetin (Kin)added solid MS medium for callus induction.

The reported data was in agreement with studies having been shown that tryptophan supplementation on *Rauvolfia serpentina* increased callus growth and alkaloid-reserpine production [32]. Also, glutamine is an important amino acid for plant growth and development. It has been found to enhance callus induction and proliferation in various plant species. Therefore, phenylalanine can enhance callus growth and the production of secondary metabolites. In callus cultures of *Hyoscyamus muticus*, phenylalanine supplementation led to increased fresh and dry weights of the callus [30].

Table 1: Effect of different concentrations of amino acids on callus fresh weight in*C. spinosa*

Treatments	Callus FW (g)
Control	4.30
Phenylalanine 100 mg/L	3.70
Phenylalanine 200 mg/L	1.12
Phenylalanine 300 mg/L	1.30
Tryptophan 100 mg/L	4.70
Tryptophan 200 mg/L	4.10
Tryptophan 300 mg/L	4.30
Glutamine 100 mg/L	4.20
Glutamine 200 mg/L	4.30
Glutamine 300 mg/L	4.70
LSD 5 %	0.455

Data are displayed as mean values; LSD refers to the least significant difference test at p≤0.05.

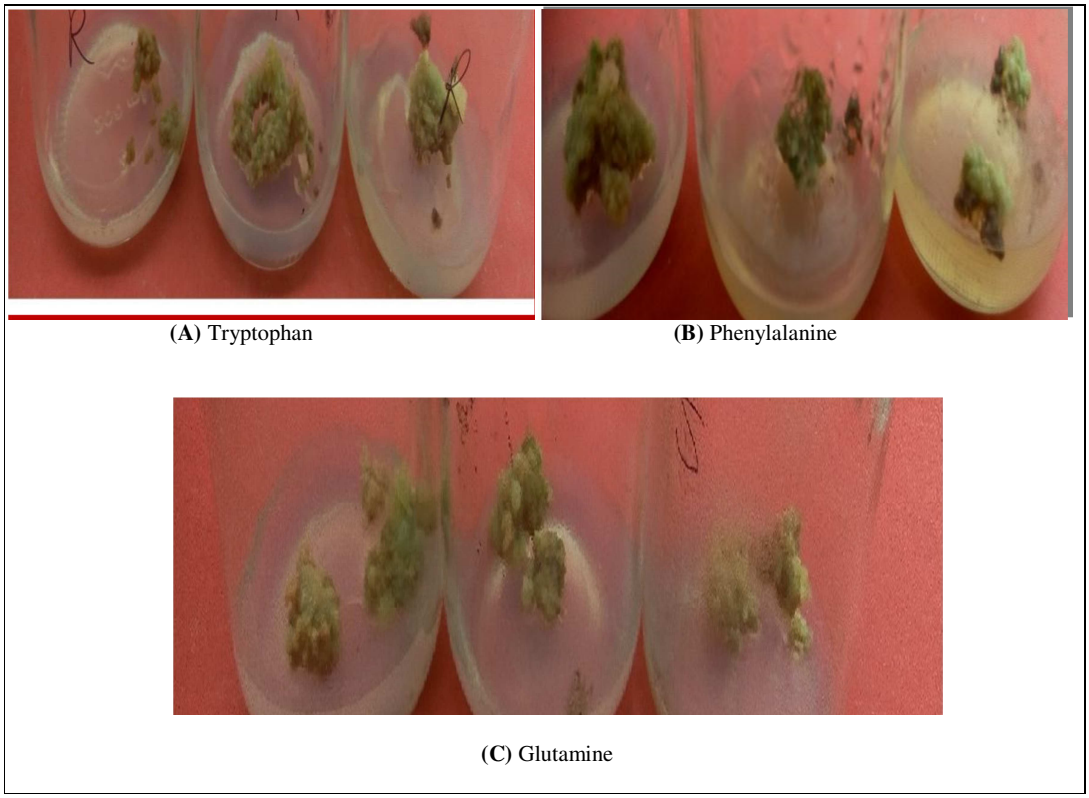

Figure 1: ***Capparis spinosa*** **callus (A) Tryptophan at 100, 200 and 300 mg/L (B) Phenylalanine at 100, 200 and 300 mg/L (C) Glutamine at 100, 200 and 300 mg/L.**

3.2. Total phenols and flavonoids content

Amino acids can promote the accumulation of secondary metabolites such as flavonoids, which are beneficial for the plant's medicinal properties. Flavonoids and phenolic compounds are found in various plant sections. Flavonoids, saponins, tannins, and tocopherol are among the several substances that have been found in *C. spinosa*. Data in Table 2 showed that the flavonoids and phenols were significantly affected by all treatments under study. Also, the highest flavonoids in callus were recorded in phenylalanine 200 mg/L and glutamine 100 mg/L which scored 2.0 mg/100 g FW for each treatment. On the other hand, control, phenylalanine 100 mg/L and tryptophan 300 mg/L treatments had the lowest results (0.1, 0.79, and 0.89 mg/100 g FW, respectively) of flavonoids. In addition, the greatest results of phenols in the callus were recorded in phenylalanine 200 mg/L and glutamine 100 mg/L, which scored 5.96 and 5.92 mg/100 g FW, respectively.

Table 2: Effect of different concentrations of amino acids on total phenols and falvonoids in *C. spinosa* callus

Treatments	Total Phenols (mg/100 g FW)	Flavonoids (mg/100 g FW)
Control	1.07	0.1
Phenylalanine 100 mg/L	1.82	0.79
Phenylalanine 200 mg/L	5.96	2.00
Phenylalanine 300 mg/L	4.51	1.73
Tryptophan 100 mg/L	3.17	1.51
Tryptophan 200 mg/L	2.80	1.00
Tryptophan 300 mg/L	0.85	0.89
Glutamine 100 mg/L	5.92	2.00
Glutamine 200 mg/L	1.84	1.25
Glutamine 300 mg/L	1.84	1.25
LSD 5 %	1.050	0.503

Data are displayed as mean values; LSD refers to the least significant difference test at $p \leq 0.05$.

These flavonoids have an important role in a number of pharmacological effects, including anti-inflammatory, anti-allergic, and antioxidant qualities. The high concentration of alpha-tocopherol in the caper components offers a new way to shield cell membranes from oxidative damage [33,34]. A caper extract in N-butanol induced apoptosis through the mitochondrial route, per a recent study [35]. Additionally, research using both chemical and biological methods has demonstrated the antioxidant activity of the hydro-methanolic extract prepared from *Capparis spinosa* leaves [36].

Moreover, phenylalanine is known to enhance the production of secondary metabolites. For example, in callus cultures of *Rhodiola imbricata*, phenylalanine significantly increased the production of phenylpropanoids like rosarin [37]. Additionally, in *Silybum marianum*, phenylalanine combined with gamma radiation improved the accumulation of phenolic compounds and flavonoids [38]**.** Similarly, tryptophan is a precursor for several important secondary metabolites, such as indole alkaloids and auxins. In callus cultures of *Rauvolfia tetraphylla*, tryptophan supplementation increased the production of reserpine, an important alkaloid used for treating hypertension [39]. Tryptophan also plays a role in the synthesis of signaling molecules like serotonin and melatonin, which are involved in various physiological processes in plants [40]. Therefore, glutamine serves as a nitrogen source and is crucial for plant growth and metabolism. While specific studies on glutamine's effect on callus growth and secondary metabolite production are less common, it is generally known to support overall plant health and development by providing necessary nitrogen for amino acid and protein synthesis [41].

3.3. Rutin content

Rutin is a flavonoid known for its strong antioxidant activity, rutin helps in scavenging free radicals and protecting cells from oxidative damage [42]. The active ingredient rutin produced in *C. spinose* callus in vitro is shown in Table 3 and Fig. 2. Data showed that the administration of callus treated with glutamine at 100 mg/L anadphenylalanine at 200 mg/L strongly accumulated rutin content at 88.2 µg/g and 64.3 µg/g, respectively, compared with callus control, which scored 55.1 µg/g. Administration of phenylalanine and glutamine stimulates the synthesis of secondary products, which increase the rutin accumulation. Abo El-Fadl [43] confirmed that phenylalanine acts as an elicitor, increasing the expression of genes involved in the rutin biosynthesis pathway. Similarly, some studies have shown that phenylalanine can enhance rutin production in gardenia calli cultures; the application of phenylalanine at certain concentrations (e.g., 3 mg/L) resulted in increased rutin content. This is likely due to phenylalanine acting as a precursor in the biosynthesis pathway of rutin [44].

Glutamine has been shown to improve glutamate uptake and inhibit glutamate excitotoxicity, which indirectly suggests its role in cellular metabolism. However, specific studies directly linking glutamine to increased rutin production are scarce [45]. However, specific studies on its effect on rutin production in *Capparis spinosa* callus are limited.

Table 3: Analysis of rutin contents for successive callus containing high amount of flavonoids of *C. spinosain vitro* culture

Treatment	Amount of Rutin (µg/g)
Control of callus	55.1
Phenylalanine 200 mg/L	64.3
Glutamine 100 mg/L	88.2

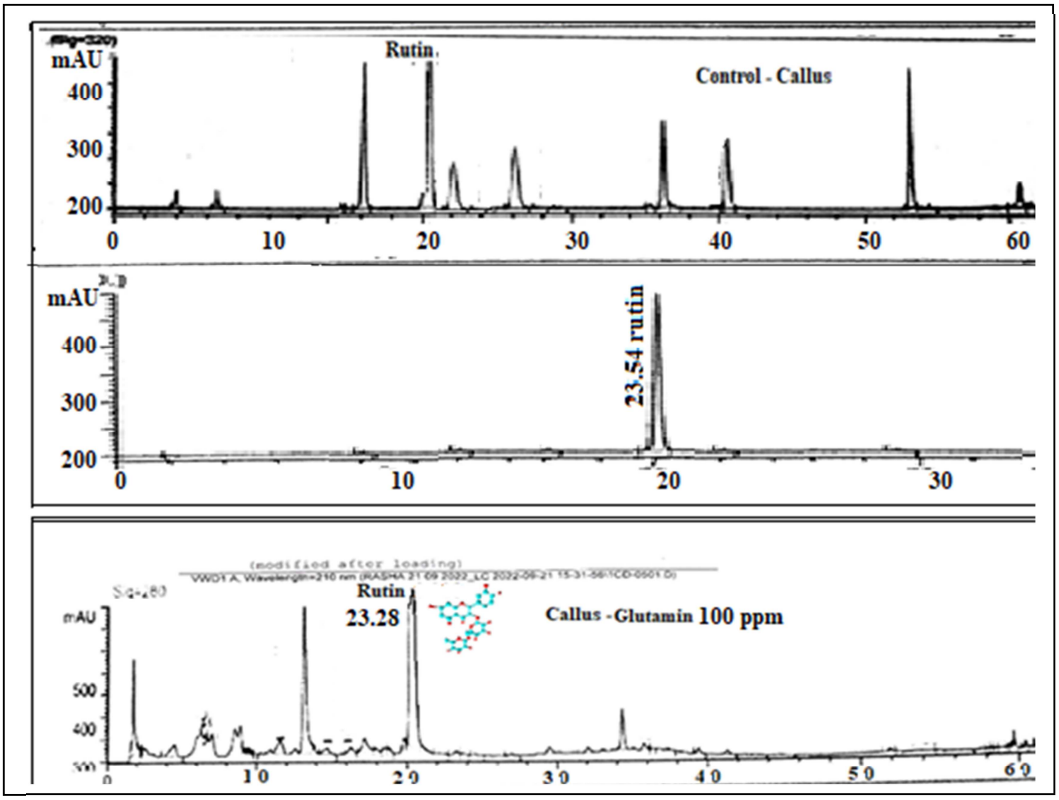

Figure 2: Chromatogram of rutin contents for successive explants containing high amount of flavonoids and antioxidants of *C. spinosein vitro* culture.

3.4. Antioxidant enzymes activity

The treatment of callus cultures with amino acids can have a significant impact on the activity of antioxidant enzymes. The addition of different amino acids to media increased PPO activity to higher levels than control. Fig. 3 showed that adding glutamine at 300 mg/L followed by 100 mg/L to media gave the highest level of activity (U/mg). The same effect was observed for phenylalanine applied at 200 mg/L, which increased the PPO activity to the highest level, followed by phenylalanine at 100 mg/L. Finally, tryptophan increasing activity compared with control by concentrations of 300, 100, and 200 mg/L, respectively. Catalase (CAT) activity is shown in Fig. 4, with the increase in this level with tryptophan at 300 mg/L, which compared with the control. Glutamine scored the lowest values for CAT activity for 200 mg/L. Similarly, phenylalanine scored 1.86 U/mg for phenylalanine at 200 mg/L.

Amino acids such as glutamine, phenylalanine, and tryptophan have been shown to enhance the activity of antioxidant enzymes like catalase (CAT) and peroxidase (POD) in callus cultures. These enzymes play a crucial role in mitigating oxidative stress by neutralizing reactive oxygen species (ROS), leading to improve the antioxidant defense. Catalase activity can also be upregulated during certain stages of plant growth and development to ensure proper cellular function and metabolism [46]. The presence of bioactive compounds like rutin and hesperidin in *Capparis spinosa* contributes to this increase in catalase activity, enhancing the plant's ability to manage oxidative stress [47].

Phenylalanine has been shown to increase PPO activity in callus cultures. As a precursor for phenolic compounds, phenylalanine can stimulate the production of substrates for PPO, leading to higher enzyme activity and increased levels of secondary metabolites like flavonoids and phenolic acids. Glutamine can enhance PPO activity in callus cultures by providing a nitrogen source that supports overall metabolic activity and enzyme function. This can lead to improved oxidative stress management and better growth of the callus [48]. While specific studies on tryptophan's direct effect on PPO activity are limited, its role in plant metabolism and secondary metabolite production suggests it can indirectly influence PPO activity. Tryptophan may enhance overall metabolic processes, contributing to better enzyme function and stress tolerance in callus cultures. The enhanced polyphenol activity might result in the augmented rate of oxidation of phenolic substances resulting in the production of quinones. These quinones play a role in plant defense mechanisms against herbivores and pathogens. The levels of PPO activity can vary depending on the plant part and environmental conditions, which participates in the defense reaction of the host [49]. These enzymes are crucial for maintaining plant health and resilience against various stressors.

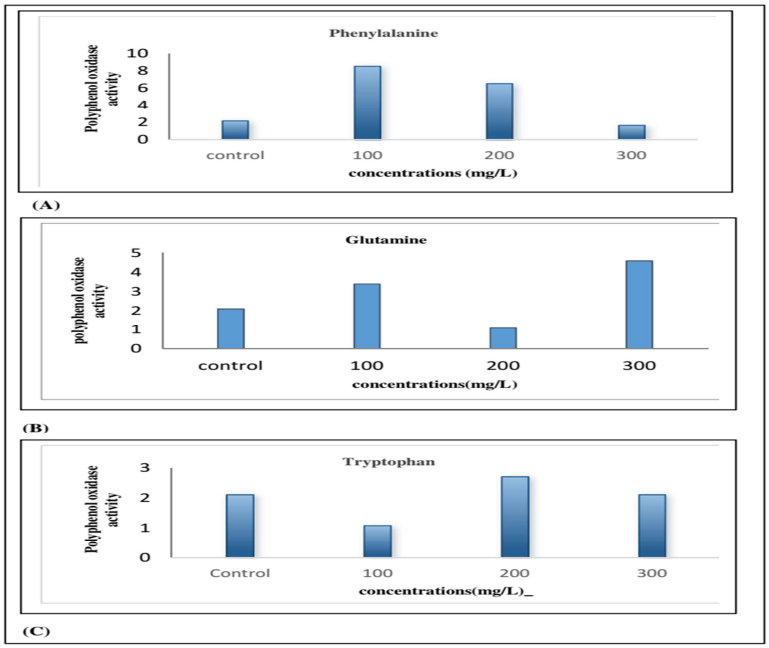

Figure 3: Polyphenol oxidase activity of *Capparis spinosa* callus treated with different amino acids (100,200, and 300 mg/L).

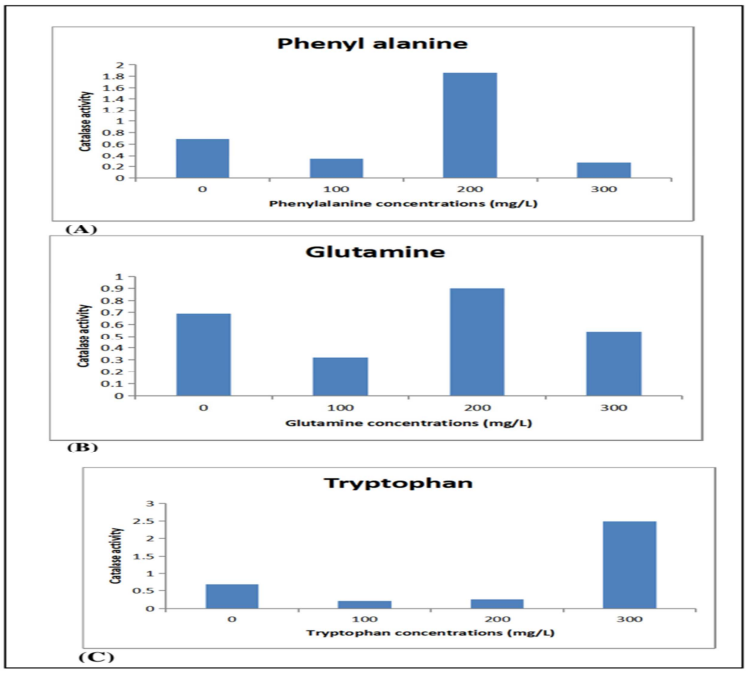

Figure 4: Catalase activity of *C. spinose* callus treated with different amino acids.

3.4.1. HepG2 Cytotoxicity Evaluation via MTT

Data in Table 4 showed that the control callus extract exhibited the lowest anticancer activity, containing 55.1 µg/g rutin and showing a relatively high IC_{50} value (470.83 µg/mL) which reflects the need for a higher concentration to achieve 50% inhibition. Supplementation with phenylalanine at 200 mg/L led to a moderate increase in rutin accumulation (64.3 µg/g) and subsequently improved cytotoxic activity (IC_{50} = 371.05 µg/mL). In contrast, glutamine at 100 mg/L produced the most pronounced effect, resulting in the highest rutin content (88.2 µg/g) and the strongest anticancer activity (IC_{50} = 356.56 µg/mL). When tested on normal Vero cells, the callus extract exhibited very low cytotoxicity (IC_{50} = 805.38 µg/mL), confirming its safety and absence of harmful effects on normal cells.

Table 4: The cytotoxicity of different treatments against human HepG2

Sample	IC_{50}
Control callus extract	470.83
Phenylalanine 200 mg/L Callus extract	371.05
Glutamine 100 mg/L Callus extract	356.56
Vero cells (control)	805.38

The data in Table 5 showed the effect of some extractions obtained from callus treated with phenylalanine 200 mg/L or glutamine 100 mg/L on the toxicity of the HepG2 cells. Also, the extraction of callus obtained without treatments (control) and compared with each cancer cells (HepG2) and normal cell without any treatments. The toxicity of callus treated with glutamine 100 mg/L was more toxic for cancer cells (IC_{50}) followed by phenylalanine 200 mg/L.

The *Capparis spinosa* L. extract most likely has anticancer properties because it contains phenolic and flavonoid components. Additionally, it has been demonstrated that flavonoid compounds have antimutagenic and anticancer effects [50]. Along with preventing proliferation, metastasis in colorectal cell lines and/or angiogenesis, the process by which new blood vessels form to supply nutrients to tumors, thereby limiting tumor growth. Rutin is also known to prevent the growth of cancer cells by causing cell cycle arrest, preventing cancer cells from dividing and proliferating and/or apoptosis, this helps in eliminating malignant cells without harming normal cells [51]. Numerous pharmacological effects, such as antioxidant, cytoprotective, anticarcinogenic, neuroprotective, and cardioprotective properties, have been investigated for rutin, often referred to as rutoside [52]. Because of their hydroxyl groups' capacity to scavenge, phenolic chemicals are crucial plant components.

Table 5: Docking score interaction (Kcal/mol) of natural tested ligand rutin against the target sites of HepG2 cell line cancer cells

Protein receptors	Docking score Kcal/mol	Interaction		
		Hydrogen bond	Pi phospholipid interaction	Van der Waals
EGFR	-8.2	GLU C:762, THR E:790 and ASP B: 800	TYR 801, HIS 805, ARG 841, TRP 881, LYS 875	Gly796, Cys 797, Ser 719, Pro 794, Met 793, Leu 844 Thr 854
CYP3A4	-10.2	AlA 370, ARG 212	Glu 212, His 324, Phe 414, Met 395, Arg 372, Leu 156, Arg 106 and Phe 215	Asn 23, Thr 25, His 41, Ala 44, Ser 45
CYP1A1	-8.1	Glu 226, Asn 245, Tyr 294, Ala 234, Asn 232, Gly 229	His 144, Cys 322, Phe 402	Gly 142, Cys 144, Ile 164

3.5. *In silico* assessment and molecular docking studies

Rutin has been noted to directly bind with the epidermal growth factor receptor (EGFR) as evidenced by a pull-down assay that indicated that EGFR protein was pulled down with rutin–sepharose 4B beads [53]. This finding needs additional verification in different cancer cell lines to know if rutin can effectively inhibit EGFR-induced signaling in HepG2-overexpressing liver cancer**.** The most often used technique for figuring out a pharmaceutical molecule's binding affinity to a particular target is molecular docking experiments [54,55].

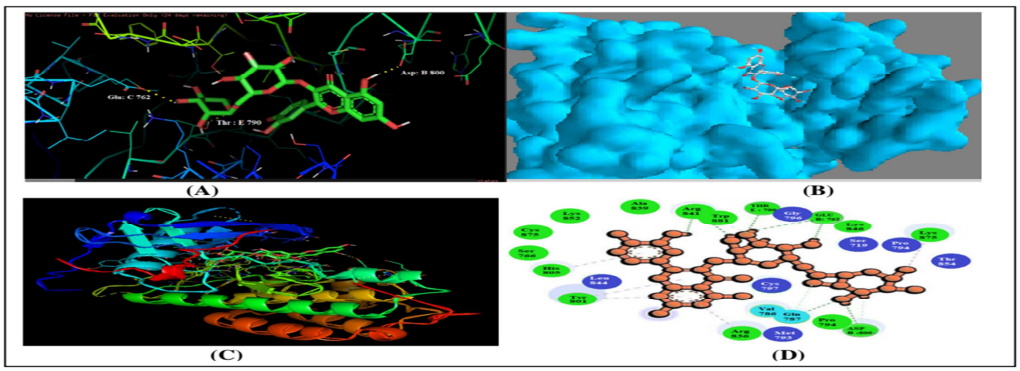

Figure 5: Docking analysis of rutin with the epidermal growth factor site. (**A**) Interaction of rutin with epidermal growth factor site represented with the hydrogen bond surface of the receptor. (**B**) Epidermal Growth Factor site high affinity with rutin. (**C**) Residual interaction of rutin with the Epidermal Growth Factor site. Each color is related to a subunit of protein. (**D**) Epidermal growth factor site represented with the Van der Waals surface of the receptor.

It has been shown that rutin directly binds to the EGF receptor (EGFR) protein, stopping the signaling factors that follow (Fig. 5). Therefore, we used molecular docking to investigate the binding efficacy of rutin against the epidermal growth factor receptor (EGFR). The epidermal growth factor (EGF) signaling pathway stimulates the Ras/Raf proteins and Phosphoinositide 3-Kinase/Protein Kinase B (PI3K/Akt), Mitogen-Activated Protein Kinase (MAPK), Transforming Growth Factor Beta 2 (TGF β2)/Smad2/3 proteins, and Akt/PTEN (Phosphatase and TENsin homolog) signaling pathways [12,56,57].

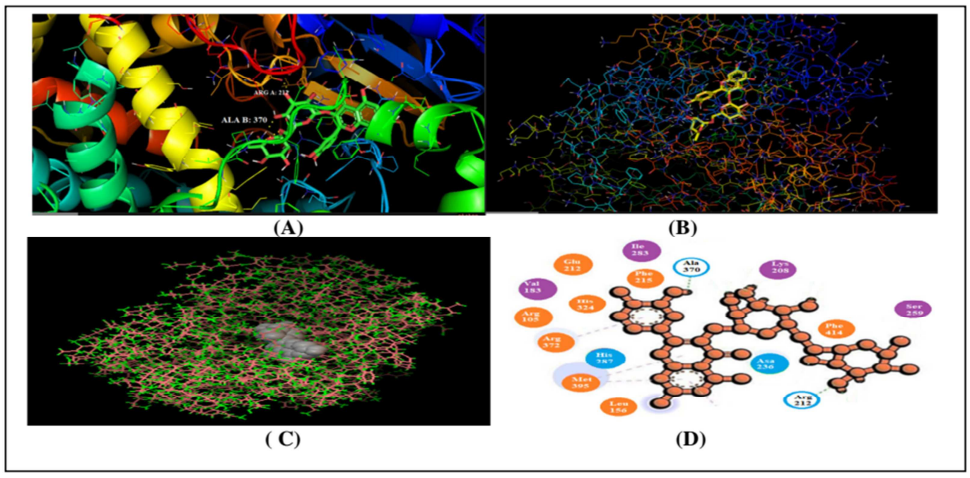

Figure 6: Docking analysis of rutin with the CYP3A4. (**A**) Interaction of rutin with the CYP3A4site represented with the hydrogen bond surface of the receptor. (**B**) CYP3A4site high affinity with rutin (**C**) Residual interaction of rutin with the CYP3A4site. (**D**) CYP3A4site represented with Van der Waals surface of the receptor.

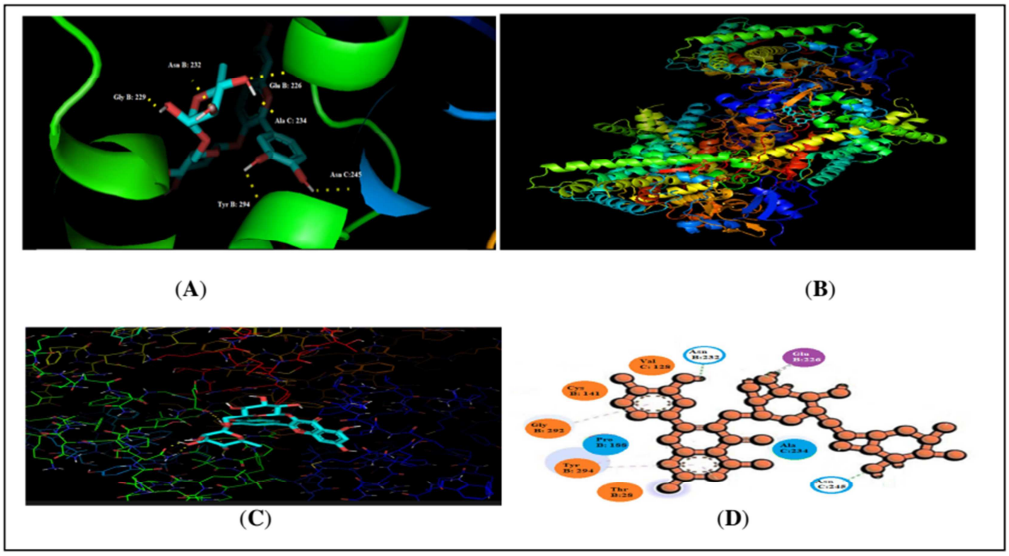

Figure 7: Docking analysis of rutin with the CYP1A1. (**A**) Interaction of rutin with the CYP1A1 site represented with the hydrogen bond surface of the receptor. (B) CYP1A1 site high affinity with rutin (**C**) Residual interaction of rutin with the CYP1A1 site. (**D**) CYP1A1 site represented with Van der Waals surface of the receptor.

As presented in Table 5, rutin had a dock score of -8.2 with the EGFR. Three hydrogen bonds with amino acid residues Glu C:762, Thr E:790 and Asp B: 800 allowed rutin to attach to the three catalytic subunits C; E and B. Furthermore, the EGFR catalytic unit's TYR 801, HIS 805, ARG 841, TRP 881, LYS 875 amino acids showed hydrophobic interaction with rutin. Gly796, Cys 797, Ser 719, Pro 794, Met 793, Leu 844 Thr 854 were frequent amino acid residue establishing Van der Waals binding with EGFR in both complexes. Additionally, grade box dimension used in Docking was 65 x 65 x 65 for all ligand and receptors.

We used the Molecular Mechanics Generalized Born Surface Area (MM-GBSA) approach to determine the binding energy of the rutin-protein receptors complex to gain further insight into their comparative binding efficacy to receptors. The MM-GBSA method is a popular method for calculating the binding energies of protein-ligand complexes. In comparison to Cytochrome P450 3A4 (CYP3A4) binding energy calculation, it was demonstrated that rutin has a greater binding efficiency than the other receptors. The binding energy of the EGFR was -8.2 kJ/mole, while the binding energy of the CYP3A4 was -

10.2 KJ/mole and Cytochrome P450 1A1 (CYP1A1) was -8.1 – 10.2 KJ/mole. The overall binding energy of both complexes was dominated by hydrogen bonds, lipophilic interaction energy, and Van der Waals interaction energy (Figs. 6 and 7). Thus, based on dock score and MM-GBSA binding energy estimation, it can be concluded that rutin binds to the CYP3A4 catalytic subunit more efficiently than EGFR and CYP1A1.

According to Yang [26], the presenilin component of the gamma-secretase enzyme complex serves as a binding site for both substrates and inhibitors. These results suggest that rutin binds to the EGFR, CYP3A3, and CYP1A1 subunits' catalytic/inhibitor binding sites. In cancer, it may be inferred that EGFR, Signal Transducer and Activator of Transcription 3 (STAT3), and Cysteine-dependent ASPartyl-specific proteASE (CASPASE) are key targets for blocking the HepG2 signaling pathway. In the current study, we used computational biology techniques in conjunction with in vitro validation in HepG2 liver cancer cells to examine the rutin's capacity to suppress EGFR, STAT3, and CASPAS-mediated HepG2 signaling. In the molecular docking and molecular dynamics simulation investigation, rutin demonstrated a strand energetically stable interaction with each receptor's catalytic site. Additionally, rutin effectively reduced the activity in the HepG2 promoter and downregulated the mRNA expression of HepG2-responsive genes of liver cancer cells in *in vitro* assays. Additionally, rutin treatment reduced the liver cancer cells' capacity to form spheroids and blocked the HepG2 signaling pathway.

4. Conclusions

This study highlights the potential of plant-derived antitumor compounds, demonstrating that rutin acts as a promising natural inhibitor of HepG2 liver cancer cells, warranting further investigation in clinical applications. The findings provide strong evidence that phenylalanine and glutamine significantly enhance secondary metabolite production in *Capparis spinosa* callus cultures, offering a viable approach for commercial-scale flavonoid enrichment. Moreover, amino acid supplementation serves as an effective strategy for optimizing callus culture conditions, paving the way for advancements in plant tissue culture and biotechnological applications. These insights contribute to the broader field of secondary metabolite biosynthesis, reinforcing the role of biotechnology in enhancing medicinal plant properties for pharmaceutical and industrial use.

5. Conflicts of interest

There are no conflicts to declare.

6. Formatting of funding sources

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Not applicable.

8. References and Bibliography

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