Effectiveness of *Vigna unguiculata* seed extracts in preventing colorectal cancer.

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Colorectal cancer (CRC) is one of the most common types of cancer, especially in Western countries, and its incidence rate is increasing every year. In this study, for the first time *Vigna unguiculata* L. Walp. (cowpea) water boiled seed extracts were found to reduce the viability of different colorectal cancer (CRC) cell lines, such as E705, DiFi and SW480 and the proliferation of Caco-2 line too, without affecting CCD841 healthy cell line. Furthermore, the extracts showed the ability to reduce the level of Epidermal Growth Factor Receptor (EGFR) phosphorylation in E705, DiFi and SW480 cell lines and to lower the EC50 of a CRC common drug, cetuximab, on E705 and DiFi lines from 161.7 ng/mL to 0.06 ng/mL and from 49.5 ng/mL to 0.2 ng/mL respectively. The extract was characterized in its protein and metabolite profiles by tandem mass spectrometry and ^{1}H-NMR analyses. A Bowman-Birk protease inhibitor was identified within the protein fraction and was supposed to be the main active component. These findings confirm the importance of a legume-based diet to prevent the outbreak of many CRC and to reduce the amount of drug administered during a therapeutic cycle.

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Introduction

Colorectal cancer (CRC) is one of the most frequent malignancy in the world. The outbreak of the majority of CRC forms is related to environmental factors, such as lifestyle and diet, while only a 5-10% of hereditariety is described[1]. During carcinogenesis and cancer progression, the up-regulation of survival signals is mainly responsible for the abnormal proliferation of CRC cells[2], in which the epidermal growth factor receptor (EGFR) signaling pathway is thought to play a crucial role. EGFR is a transmembrane tyrosine kinase receptor triggering two signaling pathways: the RAS-RAF-MAPK axis involved in cell proliferation and the PI3K-PTEN-AKT pathway involved in cell survival and escaping from apoptosis[3]. In the last decades, many drugs have been developed for the treatment of CRC. Among these, the monoclonal antibodies (MoAbs) cetuximab and panitumumab bind to the extracellular domain of EGFR when it is in the inactive configuration, compete for receptor binding by occluding the ligand-binding region, and thereby block ligand-induced EGFR activation, inducing its internalization and degradation[4]. However, these drugs are expensive and still characterized by some side effects such as severe skin toxicity, occurring in approximately 80% of patients[5], corneal erosion[6], headache, pulmonary damages, general weakness and diarrhea[7]. Moreover, they show efficacy in no more than 30% of patients[8]. Indeed, it has been demonstrated that hyperactivating mutations occurring in downstream effectors (such as KRAS, NRAS and BRAF) represent the main mechanism of primary resistance. However, even in patients without any downstream mutations, the percentage of efficacy of anti-EGFR MoAbs is less than 50%[9-18]. All these concerns underline the need to identify new approaches, such as the application of nutraceuticals able both to elicit and expand the range of chemo-preventive actions, while reducing the amount of administered drugs during a therapeutic cycle[19]. Fruit, vegetables and other edible plant parts are the primary sources for human nutrition and medicine[20]. The increasing knowledge on plant biodiversity and biotechnology has dramatically changed the role of plant food on human health and wellbeing. Nutritional therapy and phytotherapy have emerged as new concepts and healing systems have quickly and widely spread in recent years. Considering that plant foods easily reach the stomach and the gut, these organs represent the most suitable targets to estimate the biological activities of food phytocomplexes[19].

Pulses have received increasing attention in the last decades due to their nutraceutical properties, such as antioxidant, anti-inflammatory, hypoglycemic and other activities[21,22]. Moreover, several studies have shown pulses anticancer properties related to the presence of specific classes of phytochemicals, such as resistant starch fermenting in the gut and being converted into SCFAs, proteins, like amylase and protease inhibitors, globulins and polyphenols[23].

In Europe, the production of pulses is lower than in other continents and mainly limited to peas, chickpeas and faba beans, while in poor and developing countries other species and local cultivars are preferred due to higher accessibility[24]. Among these minor crops, *Vigna unguiculata* L. Walp., also known as cowpea, stands out due to its adaptability to harsh environmental conditions, such as drought and minimum field tillage[25].

Its seeds provide high amount of proteins, peptides, amino acids and other minor metabolites such as folates and minerals (calcium, zinc and iron)[23]; leaves are also sometimes consumed fresh or boiled increasing the uptake of polyphenols and fibers in diet[26,27]. Although the nutritional traits of this species is well documented, relatively little is known about the anticancer properties of this species, mainly due to bioactive peptides and polyphenols but results are controversial and require further investigations[28,29]. Therefore, in this study *V. unguiculata* beans phytoextract was considered to evaluate potential anticancer activities. Specifically, phytochemical analyses were combined with

bioactivity investigations to clarify the role of specific bean components as a possible supplement in EGFR-targeted therapies for CRC.

Experimental

Plant material and phytoextraction

V. unguiculata seeds from three batches were collected (Colfiorito, Italy, batches 17117 and 18039, and Castellani, Italy, batch 011018). Seeds were water boiled for one hour and left resting one more hour in water at room temperature (RT). This treatment aimed at emulating the typical boiling process of conventional food recipes.

Seeds were then incubated overnight at 50°C to dry completely and then they were grinded into a powder. Two grams of dry powder were extracted in 50 mL of milliQ water at RT with a magnetic stirrer for 5 minutes and then centrifuged at 5000 xg for 30 minutes at RT. Supernatants were then recovered and freeze-dried.

Separation of phytoextract components

Molecular weight-based separation

Ultra-2 mL Amicon filters (Merck-Millipore, Germany) with a cut-off of 3 kDa were used to begin separation process of extracts. Freeze-dried extracts were resuspended in water at a concentration of 40 mg/mL. After conditioning tubes with 2 mL of water, extracts were loaded and centrifuged till complete separation at 7197 xg at 4°C. The upper and the lower fractions were collected, freeze-dried and stored at -20°C. Protein content was determined using Coomassie brilliant blue G-250 method (Thermo Scientific Rockford, IL, USA) and using bovine serum albumin (BSA) as standard protein for calibration curve.

DEAE chromatography

DEAE chromatography was performed to further purify bioactive components. The upper fraction was resuspended in 5 mM Tris HCl pH 8 to reach a concentration of 40 mg/mL. 1 mL of DEAE resin (DEAE Sephacel, GE-Healthcare, USA) was centrifuged at 5000 xg for 10 minutes at 4°C to separate the resin from ethanol. Ethanol was removed, then 10 mL of water were added, centrifuged and supernatant was removed. The resin was further resuspended in 10 mL of 5 mM Tris HCl pH 8 for conditioning and centrifuged, then about 400 mg of sample were loaded and incubated on rotating wheel for 60 minutes at 4°C.

The mixture was subsequently loaded onto a column at a flow rate of 1 mL/min and eluted with 10 mL of 5 mM Tris HCl pH 8, 0.5 M NaCl at the same flow rate. The flowthrough and the eluted fractions were recovered and stored. Eluted fractions were dialyzed against PBS buffer (25 mM phosphate buffer pH 7.2, 0.15 M NaCl) with a 13000 Da cut-off membrane overnight at 4°C.

Size exclusion chromatography

A further purification was carried out using the AKTA Purifier Instrument (GE-Healthcare) equipped with a Superose 12 10/300 GL gel filtration column (GE Healthcare, Life Sciences, Little Chalfont, England), pre-equilibrated with PBS buffer. Elution was performed at a flow rate of 0.5 mL/min in the same buffer. A calibration curve was obtained by plotting elution

volume parameters of a set of standard proteins against the logarithm of their molecular weights. Standards employed at 1 mg/mL: Immunoglobulin G (150 kDa), bovine serum albumin (67 kDa), bovine β-lactoglobulin (35 kDa) and bovine cytochrome C (12.7 kDa) (Sigma Aldrich, St. Louis, MO, USA).

Removal of the hydrophobic components

A further step of purification was carried out on the fractions isolated through the size exclusion chromatography by using SPE C-18 Bond Elute cartridges (Agilent Technologies, USA) in order to remove the hydrophobic components. Samples were passed through the cartridge and the unbound fraction was recovered.

Proteomic analysis

HPLC fractions were reduced, derivatized and digested with trypsin (protein: protease ratio 20:1) as described in[30] before MS/MS analysis.

Peptides separation was achieved on a Thermo Easy-nLC 1000, and MS data were acquired on a Thermo Q-Exactive–HF, with a data-dependent top 15 method, the survey full scan MS spectra (300–1650 m/z) were acquired in the Orbitrap with 60000 resolution, AGC target 3e6, IT 20 ms. For HCD spectra resolution was set to 15000, AGC target 1e5, IT 80 ms; normalized collision energy 28 and isolation width of 1.2 m/z.

Raw label-free MS/MS files from Thermo Xcalibur software (version 4.1) were analyzed using Proteome Discoverer software (version 2.2, Thermo Fisher Scientific) and searched with Sequest algorithm against the proteome of NCBI Phaseoleae (release 05th August 2019) with minimum peptide length 6 amino acids, carbamidomethylation as fixed modification, Met oxidation and Arg/Gln deamidation as variable modifications[31].

The mass spectrometry proteomic data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD017846.

^{1}H-NMR metabolic profile

The total cowpea water boiled seed extract was suspended in H_2O:D_2O (9:1) at a final concentration of 10 mg/mL. 3-(Trimethylsilyl)propionic-2,2,3,3-d_4 acid sodium salt (TSP, final concentration 0.5 mM) was used as external reference and alanine doublet at 1.48 ppm as internal reference for chemical shift. The pH of the sample was verified with a microelectrode (Mettler Toledo, Columbus, OH, USA) and adjusted to 7.4 with NaOD and DCl. The acquisition temperature was 25 °C. All spectra were acquired on an Avance III 600 MHz NMR spectrometer (Bruker, Billerica, MA, USA) equipped with a QCI (^{1}H, ^{13}C, ^{15}N/^{31}P and ^{2}H) cryogenic probe. ^{1}H NMR spectra were recorded with *noesygppr1d* pulse sequences (Bruker library) and 256 scans, spectral width 20 ppm, relaxation delay 5 s. They were processed with 0.3 Hz line broadening, automatically phased and baseline corrected. The ^{1}H,^{1}H-TOCSY (Total Correlation SpectroscopY) spectra were acquired with 24 scans and 512 increments, a mixing time of 80 ms and relaxation delay of 2 s. ^{1}H,^{13}C-HSQC (Heteronuclear Single Quantum Coherence) spectra were acquired with 48 scans and 256 increments, relaxation delay 2 s. The NMR data were processed using MestreNova 14.1.0 software (Mestrelab Research, Santiago de Compostela, Spain). Compound identification and assignments were done with the support of 2D NMR experiments and comparison with reported assignments.

Bioactivity assessment

Cell cultures

CCD841 (ATCC® CRL-1790™) human healthy mucosa cell line and CaCo-2 (ATCC® HTB-37™) human colorectal cancer cell line were grown in EMEM medium supplemented with heat-inactivated 10% fetal bovine serum (FBS), 2 mM L-glutamine, 1% non-essential amino acids, 100 U/mL penicillin, 100 µg/mL streptomycin. E705 (kindly provided by Fondazione IRCCS Istituto Nazionale dei Tumori, Milan, Italy) and SW480 (ATCC® CCL-228™) human colorectal cancer cell lines were grown in RPMI 1640 medium supplemented with heat-inactivated 10% FBS, 2 mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin. DiFi human colorectal cancer cell line, kindly provided by Dr. Josep Tabemero (Vall d'Hebron Institute of Oncology, Barcelona, Spain), was grown in Ham's F12 medium supplemented with heat-inactivated 5% FBS, 2 mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin. All cell lines were maintained at 37°C in a humidified 5% CO_2 incubator. ATCC cell lines were validated by short tandem repeat profiles that are generated by simultaneous amplification of multiple short tandem repeat loci and amelogenin (for gender identification). All the reagents for cell cultures were supplied by Lonza (Lonza Group, Basel, Switzerland).

Viability assay

Cell viability was investigated using MTT-based *in vitro* toxicology assay kit (Sigma, St. Louis, MO, USA), according to manufacturer's protocols.

In detail, the different cell lines were seeded in 96-well microtiter plates at a density of 1×10^4 cells/well, cultured in complete medium and treated after 24 hours with increasing concentrations of total extract (0-4000 µg/mL). In order to evaluate the combined effect of Cowpea extract and cetuximab, 24 hours after the seeding the cells were treated with different concentrations of cetuximab (0-100 µg/mL) and at fixed concentrations of Cowpea total extract (200 and 1000 µg/mL).

After 48 hours at 37°C, the medium was replaced with a complete medium without phenol red containing 10 µL of 5 mg/mL MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide). After 4 hours incubation more for CCD841 and 2 hours for CRC cells lines, formazan crystals were solubilized with 10 % Triton X-100, 0.1 N HCl in isopropanol and absorbance was measured at 570 nm using a microplate reader. Cell viabilities were expressed as a percentage against untreated cell lines used as controls. Before each experiment the extract or the related fractions were filtered through a nitrocellulose 0.22 µm filter and the protein concentration was evaluated by using Coomassie brilliant blue G-250 (Thermo Scientific Rockford, IL, USA) and BSA as a standard protein.

Proliferation assay

In order to evaluate the effect of Cowpea total extract on the cellular proliferation of the CRC cell lines, the cells were counted at consecutive time points. The cells were seeded in 35 mm dish at a density of $1\text{-}2\times10^5$, treated with the extract (200 and 2000 µg/mL) 24 hours after seeding and harvested by trypsinization at 24, 48 and 72 hours after treatment. Aliquots of the cell suspension were counted in Burker's chamber. All counts were expressed as total number of cells.

SDS–PAGE and Western blot

To examine the effect of extract on the EGFR phosphorylation, the CRC cell lines and the healthy cell line were seeded at 75×10^4 cells/60 mm dish and treated for 48 hours with the total extract at 200 and 2000 µg/mL. The cells were rinsed with ice-cold PBS and lysed in RIPA buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS), containing protease and phosphatase inhibitors and 1 mM PMSF. Homogenates were obtained by passing 5 times through a blunt 20-gauge needle fitted to a syringe and then centrifuged at 15000 xg for 30 minutes. Supernatants were analyzed for protein content by the BCA protein assay[32]. SDS-PAGE and Western blot were carried out by standard procedures[33]. Twenty or sixty micrograms of proteins were separated on 10% acrylamide/bis-acrylamide SDS-PAGE, transferred into a nitrocellulose membrane (Millipore, Billerica, MA, USA). Membranes were blocked with 5% (w/v) dried milk in PBS for 30 minutes at RT and then probed overnight at 4°C with the appropriate antibodies in 5% (w/v) BSA in PBS. After three 10 minutes washes with PBS containing 0.1% (v/v) Tween 20 (PBS-T), membranes were treated for 1 hour at RT with an HRP-conjugated secondary antibody diluted in 5% (w/v) dried milk in PBS. After three washes in PBS-T, detection was performed using an ECL plus detection system (Millipore, Billerica, MA, USA). Protein levels were quantified by densitometry of immunoblots using Scion Image software (Scion Corp., Frederick, MD, USA). The following primary antibodies (all purchased from Cell Signaling Technology, Danvers, MA, USA) were used: anti EGFR (dilution 1:1000), phospho-EGFR (Tyr1068; dilution 1:1000), p44/42 MAPK (ERK 1/2; dilution 1:1000), phospho-p44/42 MAPK (ERK 1/2) (Thr202/Tyr204; dilution 1:1000), Akt (dilution 1:1000), phospho-Akt (Ser473; dilution 1:1000), GAPDH (dilution 1:10000) and vinculin (dilution 1:10000). IgG HRP-conjugated secondary antibodies (purchased by Cell Signaling Technology, Danvers, MA, USA) were diluted 1:10000.

Statistical analyses

Statistical analyses were performed by using software R, version 3.3.3. Packages used for the analyses and the graphs were lme, lme4, nlme, glmmTMB and ggplot2. The threshold of statistical significance was set at 0.05.

MTT assay

To analyse the impact of the amount of extracts on the viability of the different cell lines a Generalized Linear Mixed Effects Model (GLMM) was used. The response variable (% cells survival) was assumed to be binomial or beta-binomial distributed in case of overdispersion[34]. Fixed effect analysed was the concentration of extract in interaction with the cell line. Since three different batches were tested, they were treated as a random effect.

Proliferation assay

Data from proliferation assays were analysed by a GLMM. Response variable was the number of cells and it was assumed to be negative-binomially distributed. Fixed effect was growth time up to 72 hours after the treatment in interaction with the amount of extracts used for the treatment itself. For each cell lines, 3 replicates were performed and therefore treated as random effect.

Combination experiment between cetuximab and total extract

One Way ANOVA was used to evaluate the effect of the supplementation of two different concentrations of extract on the EC50 of cetuximab. In order to compare the effect of the supplementation of extract at different concentrations (200 µg/mL and 1000 µg/mL) against the control (cells treated only with cetuximab) a Dunnett test was performed.

Densitometric analysis

A Linear Model (LM) was performed to evaluate statistical differences regarding EGFR phosphorylation among the different extract concentrations in the 5 cell lines. The response variable was the level of EGFR phosphorylation while the categorical variable was the interaction between the cell line and the concentration of extract. The same analysis was performed on ERK and AKT phosphorylation levels.

Evaluation of protein-dependence

To verify the existence of a relationship between CCD841 and E705 cell viability and the amount of proteins in the samples, a GLMM was carried out considering a binomial distribution of the response variable and proteins amount in interaction with the cell line as fixed effect. Both total and purified extracts were taken into account, so that the different extract fractions (see paragraph 2.2) were considered the random component within the model.

Results & discussion

Effect of *V. unguiculata* extract on the viability of healthy and colorectal cancer cell lines

Starting from 2 g of dried seeds from 3 different batches the following extract yields were obtained: 102.4 ± 11 mg from the first batch, 203 ± 9 mg from the second and 81.8 ± 3.78 mg from the third. The protein content was equal to 4.168 ± 0.379 mg for the extraction of the first batch, 3.179 ± 0.203 mg for the second batch and 8.142 ± 0.407 mg for the third. Extracts of *V. unguiculata* from the three different batches were tested on healthy mucosa and CRC cell lines with different molecular profiles: Caco-2, E705 and DiFi cell lines, all wild type for EGFR, KRAS, NRAS, and BRAF genes, as well as SW480 cell line, carrying the KRAS G12V mutation and wild-type for the other aforementioned genes. The DiFi cell line is characterized by a strong EGFR gene amplification profile. Fig. 1 reports the results of MTT assay on each cell line at different phytoextract concentrations. Data suggest a dose-dependent effect in E705 ($p < 0.001$), DiFi ($p < 0.001$) and SW480 ($p < 0.001$) cell lines, with a percentage of viability at 2000 µg/mL of 50%, 23% and 45%, respectively. Neither Caco-2 cancer cell line ($p = 0.672$) nor the healthy one CCD841 ($p = 0.301$) were affected by the treatment.

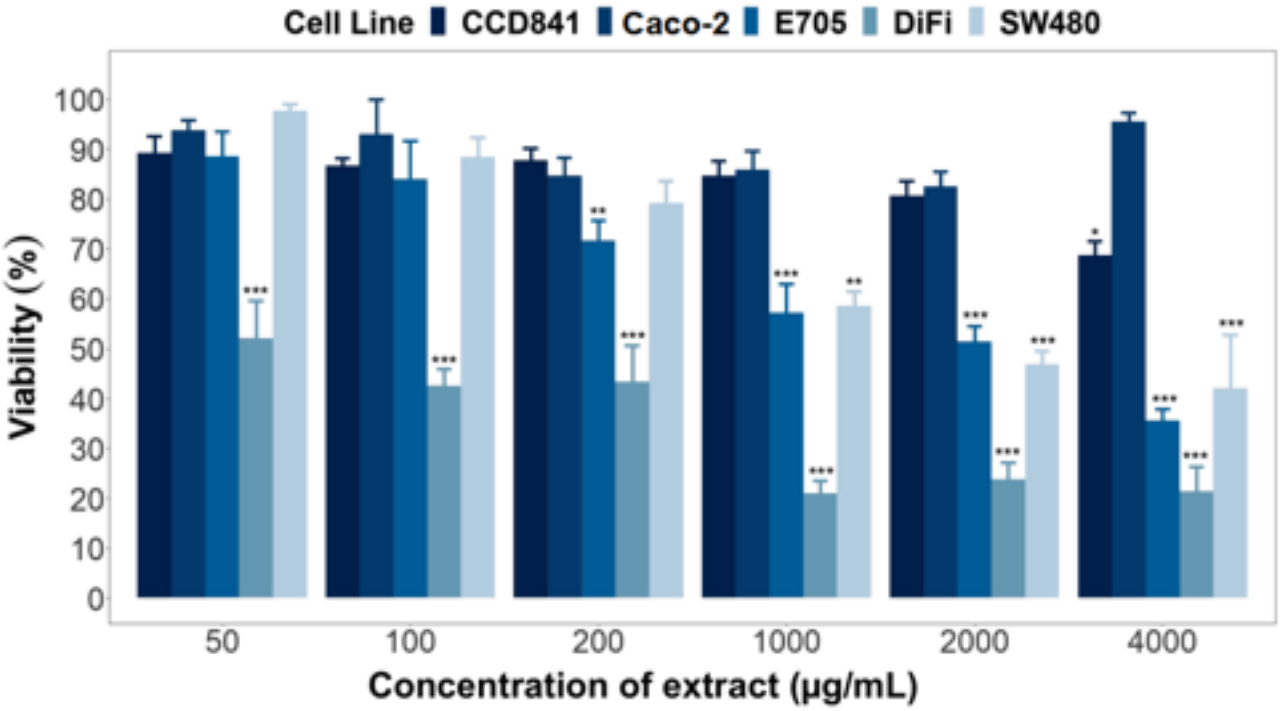


Fig. 1 Effects of *V. unguiculata* extract after 48 hours treatment on the viability of colon cell lines. Data are expressed as the mean percentage of viability of the three batches tested compared to the untreated control (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, with Bonferroni's correction). The experiment was performed with increasing concentrations of phytoextract (from 50 µg/mL to 4000 µg/mL).

Proliferation assays on healthy and CRC cell lines after extract treatment

To evaluate the cytostatic effect of the phytoextract, proliferation assays were performed on healthy and CRC cell lines treated with 200 and 2000 µg/mL extract at different times. Results showed a general cell growth decrease of the CRC lines (Fig. 2). In particular, the effect on Caco-2 was detected only at the highest dose after 60 hours treatment, whereas E705 proliferation showed a significant effect already after 30 hours at both concentrations. Noteworthy, at a concentration of 2000 µg/mL, DiFi cells did not show any growth. Concerning SW480, the reduction in the growth rate at 2000 µg/mL extract was highly significant compared to the untreated control approximately after 60 hours treatment (confidence bands do not overlap). The healthy line CCD841 was found not to be affected by the treatment with the extract at any concentration tested (Fig. 1, Supplementary information). Therefore, our data indicate that components of *V. unguiculata* seed extract may play a role in cancer prevention, especially by slowing down cancer cells proliferation.

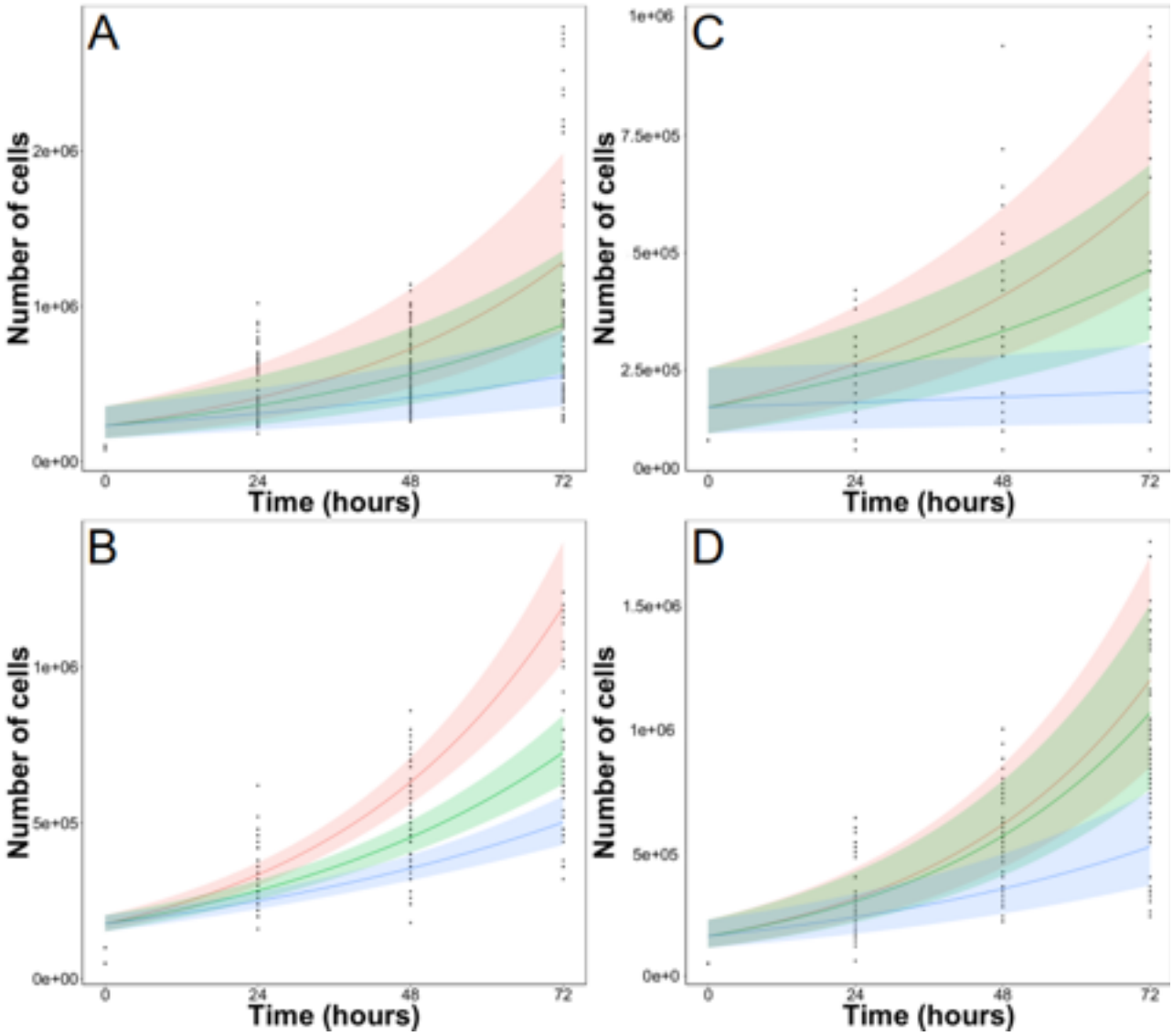


Fig. 2 Cell proliferation. CRC cell lines treated after 24 hours after seeding, harvested and counted at 24, 48 and 72 hours after treatment. A: Caco-2, B: E705; C: DiFi; D: SW480. Coloured lines represent the growth function in time of cells treated with different concentration of extract (red: control, green: 200 µg/mL, blue: 2000 µg/mL). Bands show the 95% confidence intervals. When bands overlap, there is no significant difference among treatments.

Analysis of EGFR phosphorylation and related downstream pathways in response to phytoextract

As EGFR signaling is one of the pathways mainly involved in CRC pathogenesis, the activation of EGFR and of the main downstream effectors (ERK for the MAP kinase pathway, Akt for the PI3k-Akt-mTOR axis) were evaluated through western blot and densitometric analysis after treatment with 200 µg/mL and 2000 µg/mL extract (Fig. 3). Results showed that in E705 cells the level of phospho-EGFR significantly decreased at 200 µg/mL and even more at 2000 µg/mL extract ($p < 0.01$). DiFi and SW480 showed a decrease in phospho-EGFR levels only at 2000 µg/mL ($p < 0.01$). Conversely, no

effects were shown on Caco-2, neither at 200 µg/mL nor at 2000 µg/mL. As expected, no EGFR phosphorylation was found in CCD841. All CRC cell lines displayed high phospho-EGFR levels compared to healthy control[35].

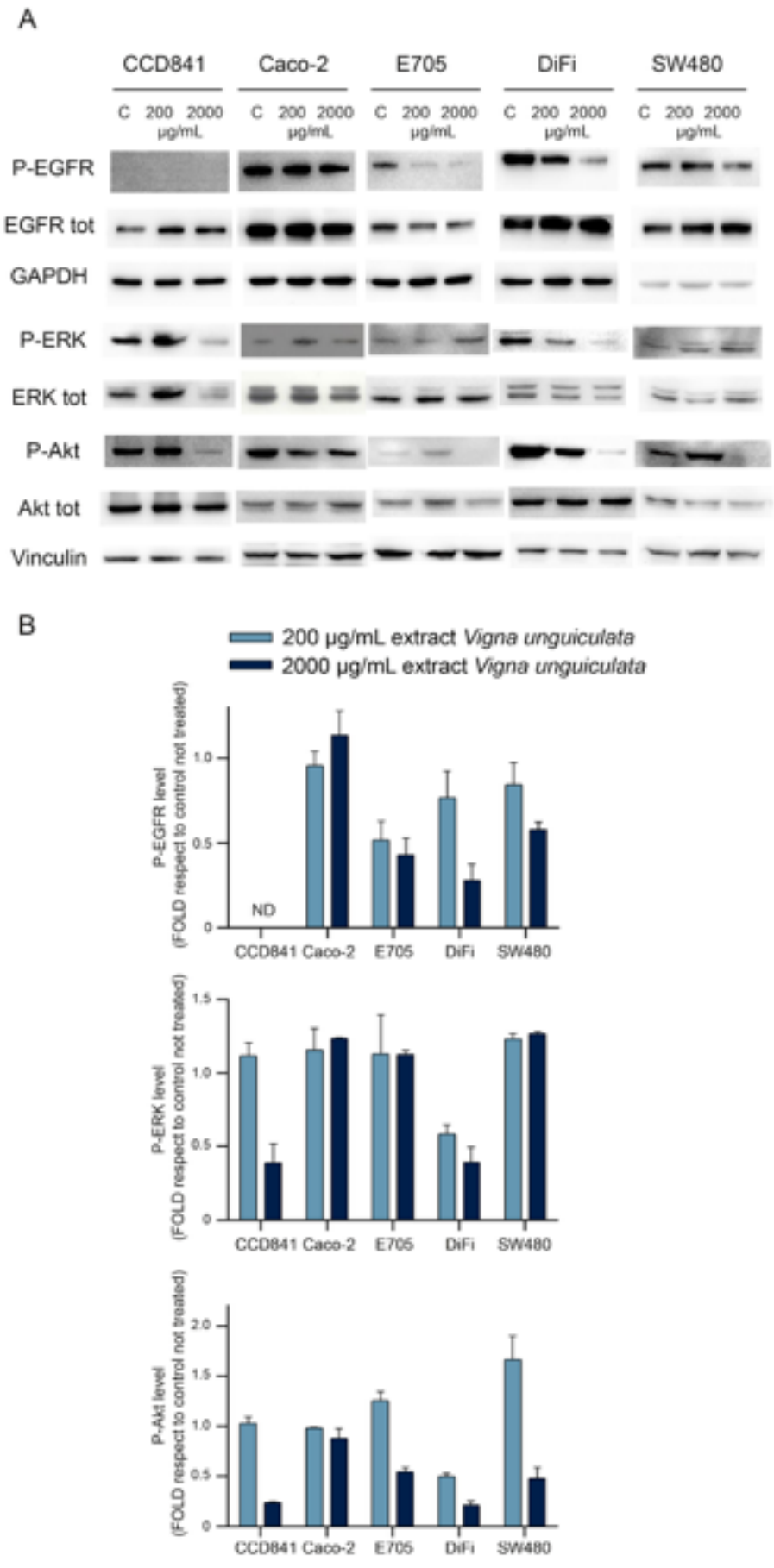
The precise interplay of the two EGFR downstream pathways is still unknown, but most data suggest that the MAP kinase pathway is the most relevant one[8,36,37]. As a matter of fact, the presence of mutations able to hyperactivate this pathway (such as those occurring in KRAS, NRAS and BRAF genes) is the main factor for ruling out EGFR-targeted therapies, that have been developed in the last 10 years[9].

However, concerning the two EGFR downstream pathways, our data reveal that the situation is more complex. Indeed, ERK activation was downregulated only in the DiFi cell line in a dose-dependent manner ($p < 0.001$), while no variations in Caco-2, E705 and SW480 cells were detected. Diversely, a significant decrease in Akt activation was observed in DiFi cell line already at 200 µg/mL extract,while in E705 and SW480 cell lines only at the highest extract dose. In general,the responses of these cell lines were found to be concentration-dependent (E705 $p=0.028$, DiFi $p < 0.001$, SW480 $p=0.009$). A decrease in ERK ($p < 0.001$) and Akt ($p = 0.001$) activation was also observed in the CCD841 normal mucosa cell line, but only at the highest phytoextract concentration and with limited evidences at the viability and proliferation level (Fig. 1 and Fig. 1, Supplementary Information). Overall, the alterations of the activation levels of EGFR and partly of its downstream effectors are in line with the viability and proliferation assays. In E705 and DiFi cell lines, both sensitive to anti-EGFR therapies, the decrease of EGFR phosphorylation and the decrease of ERK and/or Akt activation led us to hypothesize a putative supplementary effect between *Vigna unguiculata* extracts and EGFR-targeted therapies.

The SW480 cell line is characterized by the KRAS G12V mutation and presents a constitutively activated MAP-kinase pathway, so that the significant decrease in EGFR phosphorylation can lead to the reduction of the PI3K-mTOR pathway with a decrease of Akt phosphorylation, leading cells to apoptosis. We cannot exclude that cell proliferation may be mediated by alternative, MAP-kinase independent pathways. The Caco-2 cell line shows a decrease in cell proliferation only at the highest dose but no alterations in the activation of EGFR and its downstream effectors are observed: for this cell line, that is characterized by a complete RAS-BRAF wild-type status, we can hypothesize that the driver is outside the EGFR pathway but acts on it through a putative dimerization of EGFR with other members of the EGFR family[38]. Therefore the phytoextract plays a minor role, in accordance with the smaller decrease observed in cell proliferation. As for the normal mucosa cell line, the decrease of downstream pathways activation at the highest dose does not lead to any alteration in the viability and proliferation levels. It is conceivable that the administration of *Vigna unguiculata* extract treatment may have no effect on normal mucosa cells. This is an extremely important factor to avoid the tedious side effects of the majority of chemotherapies.

Overall, our Western blotting results clearly indicate that not only the MAP kinase axis, but both EGFR downstream pathways are relevant. At clinical level, as mentioned before, the presence of mutations able to hyperactivate this pathway, such as those occurring in KRAS, NRAS and BRAF genes, is the main factor for ruling out EGFR-targeted therapies[9]. On the contrary, little is known about the P3K-Akt-mTOR pathway, if we exclude few, sporadic studies that investigated PIK3CA mutations and PTEN protein deregulations, but without an extensive confirmation by the wide

scientific community[39,40,41]. Having in mind that only up to 30% of RAS-BRAF wild-type cases may profit from the administration of EGFR-targeted therapies, our data suggest a deeper investigation of the P3K-Akt-mTOR axis.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CCD841
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P-EGFR level (FOLD respect to control not treated)
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P-Akt level (FOLD respect to control not treated)
CCD841 Caco-2 E705 DiFi SW480

Fig. 3 Western blotting analysis of EGFR, ERK and Akt phosphorylation in CRC and normal mucosa cell lines. (A) Representative Western blotting performed on protein extracts (20 µg for DiFi cell line and 60 µg for the other cell lines in EGFR analysis and 60 µg for all cell lines in ERK and Akt analysis), using anti-P-EGFR, anti-EGFR, anti-P-ERK, anti-ERK, anti-P-Akt and anti-Akt antibodies. GAPDH and vinculin was used as loading control. (B) Determination of phosphorylation rate by densitometric analysis was performed with Scion Image Software. Data are expressed as the phospho/total ratio and each ratio is normalized on phospho/total ratio of not treated cell line.

Extract ability to supplement cetuximab treatment

The significant reduction in EGFR activation may propose these extracts as potential co-drugs to be administered in combination with EGFR-targeted therapies, that have some adverse side effects and are effective in only up to 30% of treated patients. The ability of the extract to complement the cetuximab EGFR-targeted therapy was evaluated. CRC cell lines were treated with cetuximab (0-100 µg/mL) and 200 µg/mL and 1000 µg/mL extract (Fig. 2, Supplementary information). These concentrations were selected on the basis of extract effect on the two drug-sensitive cell lines, i.e., E705 and DiFi. As far as E705 line is concerned, results show that the EC50 of the drug decreased from 161.7 ± 18.3 ng/mL to 18.4 ± 9.8 ng/mL when E705 were treated with 200 µg/mL and to 0.06 ± 0.01 ng/mL in combination with 1000 µg/mL of extract. Regarding DiFi cell line, a similar pattern to that of E705 was observed. Specifically, the EC50 of the drug decreased from 49.5 ± 1.0 ng/mL to 12.4 ± 5.4 ng/mL in combination with 200 µg/mL of extract and to 0.2 ± 0.1 ng/mL with a treatment of 1000 µg/mL of extract. In both cell lines, differences between treatments and control were statistically relevant (p <0.05). We assume the possible use of lower doses of drug in combination with cowpea extracts in the treatment of patients. A diet regime including pulses' consumption, such as cowpea, could be a turning point in targeted therapies: there are, in facts, evidences showing that consumption of pulses is linked to a lower CRC incidence rate[42-46]. Furthermore, some studies showed that it would be enough to include in the diet the consumption of approximately 100 g of legumes per week to prevent many forms of cancer, including CRC[47].

Chemical characterization of the total extract (^{1}H-NMR)

The metabolic profiling of the total extract was characterized by NMR spectroscopy data exploited for primary and secondary metabolite identification following the approach developed for the analysis of complex plant extracts[48-53]. The identification of metabolites was based on the analysis of mono and bidimensional NMR spectra and is in agreement with data from previous literature[54]. Overall, ^{1}H-NMR profile (Fig. 4) revealed the presence of amino acids (alanine, valine, serine, threonine, methionine, cysteine, arginine, aspartate, glutamate, proline, glycine, tryptophan, tyrosine, phenylalanine), organic acids (acetate, lactate, citrate, succinate, GABA, nicotinic acid), sugars (glucose, sucrose, raffinose among the most abundant), choline and uracil. In addition to previously reported results[54], we also observed trigonelline and a significant amount of broad resonances (indicated by red arrows in Fig. 4), corresponding to proteins, as confirmed also by typical TOCSY correlations in the amide NH region (Fig. 3, Supplementary information).

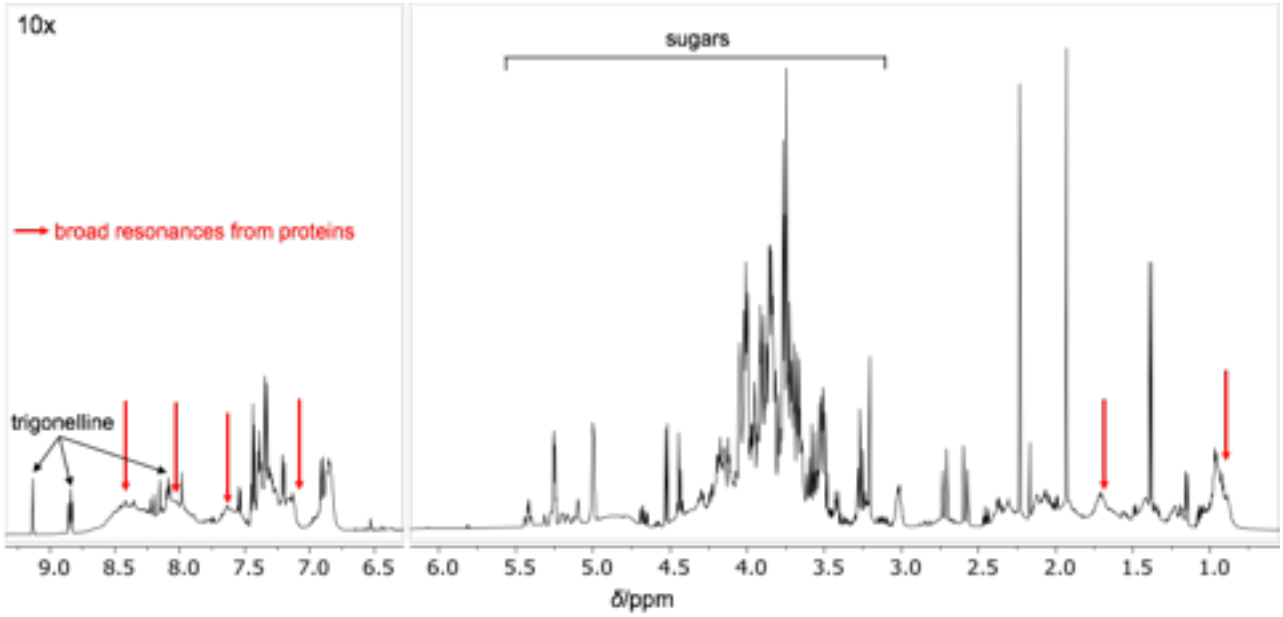

Fig. 4 ^{1}H-NMR profile of a total cowpea water boiled seed extract sample dissolved in H_2O:D_2O 9:1 at a final concentration of 10 mg/mL, 25 °C, 600 MHz.

Fractionations of the bioactive components

To identify the molecule/s responsible for the selective cytotoxicity against CRC cells, the extract was progressively fractionated and tested to follow the bioactive component. The effects of the different fractions are expressed as the viability ratio between E705 cancer cells and CCD841 healthy cell line (Fig. 5A). Only fractions showing a viability ratio below 1 were considered active and further purified. At first, the separation through Centricon suggested that the bioactive component is a compound with a molecular weight higher than 3 kDa. DEAE chromatography and size exclusion chromatography (Fig. 4, Supplementary information) showed that it is a macromolecule with an overall negative charge at physiological pH, with an apparent molecular weight ranging from 8 kDa to 20 kDa. The P1, P2 and P3 fractions obtained by SEC were further loaded onto SPE C-18 Bond Elute cartridge to remove hydrophobic compounds. The active fraction (P1 plus P2) was effective also on the other sensitive CRC lines (Fig. 5B).

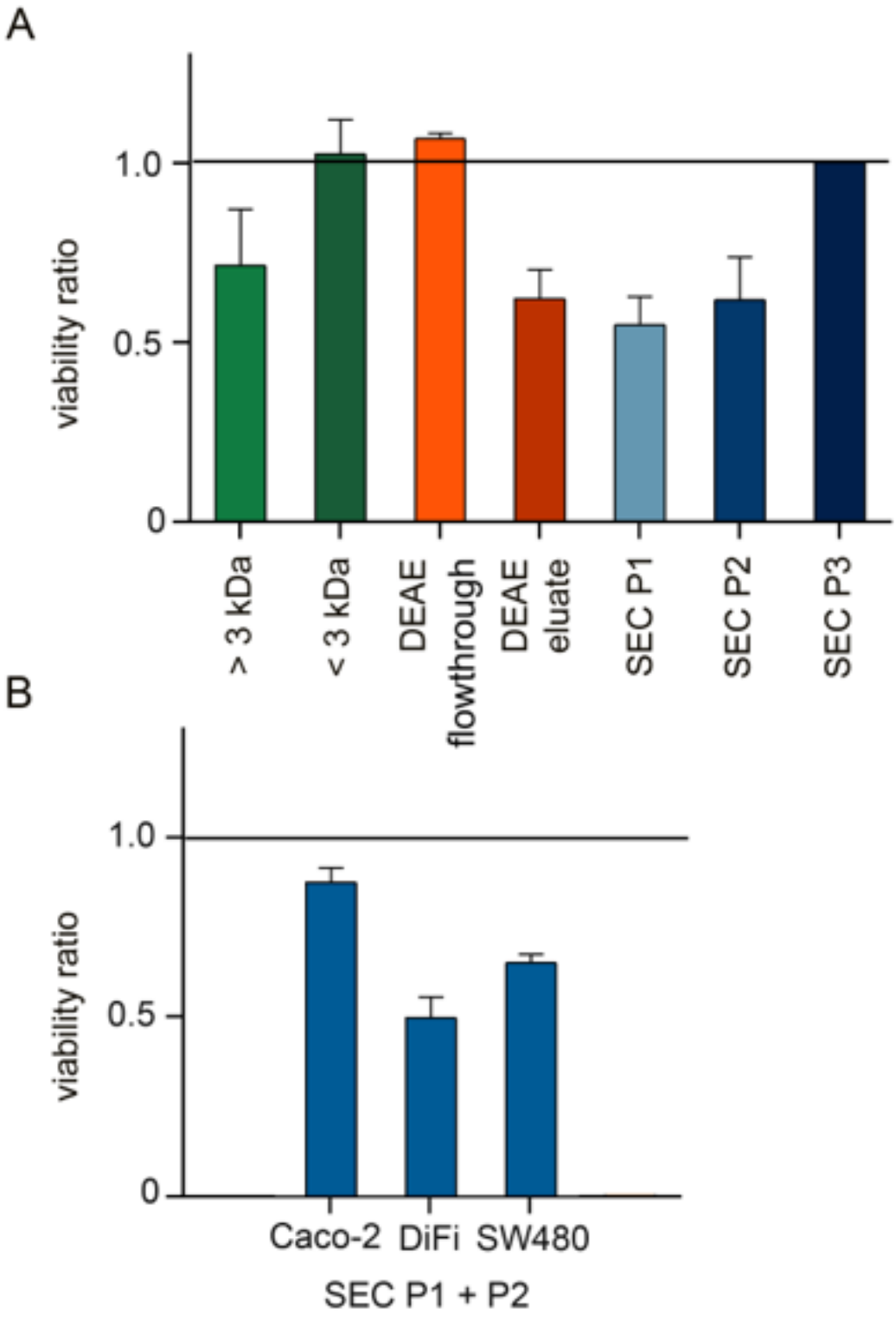

Fig. 5 Viability ratio of E705 compared to CCD841 cells treated with the different fractions obtained by the progressive steps of purification (3 kDa cut-off filter, DEAE chromatography and P1, P2, P3 of the SEC) (A). Viability ratio of Caco-2, DiFi, SW480 compared to CCD841 cells treated with the SEC-purified fraction (P1 plus P2) (B). Values are mean ± SEM.

Protein-dependent selective cytotoxic properties of the extract

The above mentioned purification procedures led to obtain a set of samples with a variety of protein concentrations within the phytocomplex, so that E705 and CCD841 cells were treated with a wide range of samples with different protein concentrations (from 0 to 115 µg/mL). To test the effect of protein concentration on the viability of E705 and CCD841 cells a regression model was set up (see Experimental).

Results highlighted a clear negative effect of protein concentration on the viability of E705 cells with significant effects already at a concentration around 30 µg/mL, while in the healthy cell line the effect was much lower and detectable only at doses higher than 90 µg/mL (Fig. 6). Therefore, proteins appeared to be the effective component in the selective cytotoxic activity of the extract.

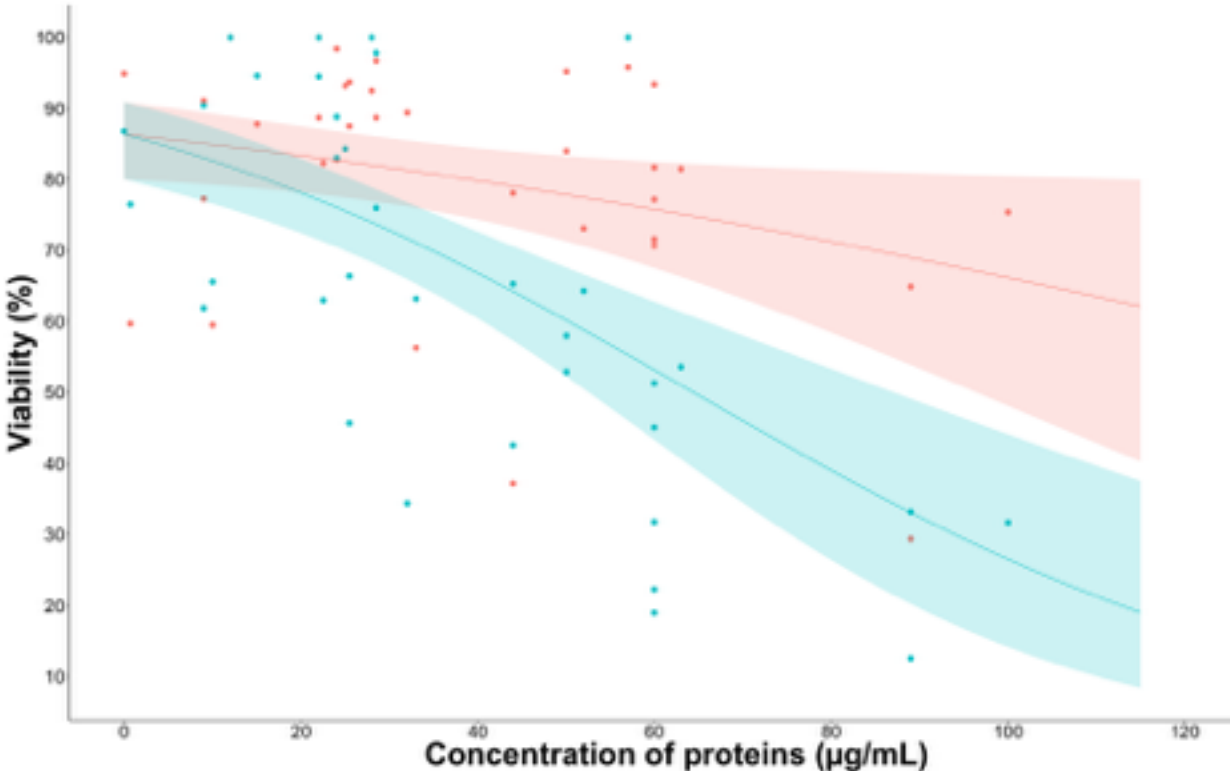

Fig. 6 Model showing the effect of protein amount on CCD841 (red line) and E705 (blue line) cell lines.

Proteomic assessment of bioactive fractions

The protein composition of all the fractions separated by SEC was characterized by LC-MS/MS using a shotgun proteomic approach, a gel free technology that allows to identify all the proteins in a sample by obtaining sequence information without previous purification of each single protein[55]. The differential analysis between the active fraction data (P1 plus P2) and fraction P3, which did not show any biological activity, allowed to identify the proteins exclusively present in P1+P2 fractions, most probably responsible for the bioactivity. The analysis was repeated on two different preparations to verify the reproducibility of the results. The list of these proteins is reported in Table 1.

Table 1. List of the proteins present exclusively in P1 + P2 fractions. The protein composition of all the fractions was determined by a shotgun MS/MS strategy. The Table reports the proteins identified exclusively in P1 and P2 fractions upon differential analysis between P1 plus P2 and P3 mass spectrometry data. AA = number of amino acids, MW = molecular weight.

Accession	Description	Score	Coverage	Unique Peptides	Peptides	AA	MW kDa	Calc. pI
XP_027903254.1	serpin-ZX [*Vigna unguiculata*]	54,39	48,35	2	16	424	46,9	7,64
XP_027916766.1	subtilisin inhibitor 1 [*Vigna unguiculata*]	9,36	43,88	2	2	98	11,1	5,01
XP_027922998.1	Bowman-Birk type seed trypsin and chymotrypsin inhibitor-like [*Vigna unguiculata*]	5,57	18,42	1	1	114	12,4	5,22
XP_027917589.1	heat shock 70 kDa protein [*Vigna unguiculata*]	38,29	7,4	3	3	649	71,1	5,39
NP_001304197.1	heat shock cognate 70 kDa protein 2-like [*Vigna radiata*]	17,40	4,17	2	2	648	71,2	5,25
XP_014497815.1	heat shock 70 kDa protein 17 [*Vigna radiata* var. radiata]	4,35	2,58	1	1	892	99,0	5,60
XP_027923838.1	class I heat shock protein-like [*Vigna unguiculata*]	3,84	11,43	1	1	140	16,3	6,64
XP_027903882.1	glutaredoxin-like [*Vigna unguiculata*]	21,83	21,67	2	2	180	19,6	8,05
XP_014497624.1	probable mediator of RNA polymerase II transcription subunit 37c [*Vigna radiata* var. radiata]	12,52	4,16	2	2	649	71,1	5,29
XP_027920371.1	glycine-rich RNA-binding protein 2, mitochondrial-like [*Vigna unguiculata*]	5,87	9,15	1	1	306	29,9	4,92

Among the different proteins identified, the Bowman-Birk domain trypsin and chymotrypsin inhibitor (BBI) was identified. Many studies are available concerning this family of peptides isolated from pulses (especially lentils and soybean) showing beneficial effects in CRC prevention[47,56,57]. They are small homodimeric peptides of 107 amino acids characterized by high variability among and also within legume species. The most conserved feature is the presence of 4

disulphide bridges within each monomer responsible for their high stability during the digestive processes, so that they are able to resist to pH levels equal to 1.5 without modifications that may affect their bioactivity. This means that the majority of the peptides assumed is able to reach intact the intestine and the colon[47,58].

This study showed that the BBI is maintained after 1-hour boiling of beans before the extraction procedure. Thus, once cooked and eaten they are very likely to reach the colon, where they are able to carry out their chemopreventive function. A recent work studying the effect of purified BBI from *V. unguiculata* on different breast cancer cell lines showed the internalization of the peptide in cells (probably mediated by endocytosis, since no receptors or carrier proteins were found on the cellular membrane to promote its internalization) and its ability to enable the proteasome 20S functionality, activating a series of processes within cells that lead to apoptosis[43]. BBI internalization could explain the cytotoxic effect against CRC non-responsive cell lines to cetuximab.

The main biological function of the Bowman Birk inhibitor peptides in plants is related to defense mechanisms against predation and parasites through the inhibition of the digestion of protein and peptides, preventing insects feeding[59,60]. The biological activity shown by cowpea BBI against many pests is well known, so that many staple species such as rice and tomato have been genetically modified by using this gene to improve their resistance[61,62]. This aspect sheds new light on the possibility of finding suitable cultivation strategies to enhance the nutraceutical value of cowpea beans e.g., cultivation without the use of pesticides that could be hypothesised to elicit the production of these peptides by plants, therefore coupling healthy nutrition to environmental sustainability.

Conclusions

Legumes are fundamental raw food items supporting human diet, not only as a source of macronutrients such as proteins, starch, fibers and micronutrients (amino acids, vitamins, minerals), but also for their bioactive molecules (mainly polyphenols and peptides) able to provide benefits to human health. Our study focused on one minor African species, *V. unguiculata*, not only for its importance for the economy and the sustenance of African population, but also for its adaptability and ease of cultivation. The results showed a chemopreventive action of *V. unguiculata* beans extract against different colorectal cancer cell lines, without affecting the healthy cell line and its ability to reduce cetuximab dose in colon cancer therapy. Based on the proteomic characterization and according to the literature the Bowman-Birk serine-protease inhibitor is supposed to be the main active component. The ability of this peptide to resist against boiling and low pH levels (such in the stomach) increases the feasibility of considering legumes such as the cowpea as fundamental supporters to contrast many different forms of CRC in the dietary context.

Supplementary information

Fig. S1 CCD841 cells proliferation (see Fig. 2 for details).

Fig. S2 Dose-response curves of E705 (**A**) and DiFi (**B**) with drug alone and drug plus two concentrations of extract.

Fig. S3 ^{1}H-^{1}H-TOCSY (**A**) and ^{1}H-^{13}C HSQC (**B**) spectra of a total cowpea water boiled seed extract sample dissolved in H_2O:D_2O 9:1 at a final concentration of 10 mg/mL, 25 °C, 600 MHz.

Fig. S4 FPLC profile of the purified extract.

Electronic supplementary information (ESI) available.

Conflicts of interest

There are no conflicts to declare.

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