

ANTIBACTERIAL ACTIVITY OF *CAPSICUM* EXTRACT AGAINST SELECTED STRAINS OF BACTERIA AND MICROMYCETES

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ABSTRACT

One of traditional plant that has so many pharmacology effects is chilli fruit (*Capsicum* sp.) that belong to the family *Solanaceae*. Around the world is known five varieties of *Capsicum* which are *C. annuum*, *C. frutescens*, *C. chinense*, *C. baccatum*, and *C. pubescens*. Chilli peppers are known for causing the sensation of heat or burning when consumed. The heat sensation is incited by the type and the amount of a group of capsaicinoids, the alkaloids found only in chilli pepper pods. The HPLC method was used for determination of capsaicin and dihydrocapsaicin content in various dried peppers from genera *C. chinense*. Based on the results of HPLC the hottest pepper has been Bhut Jolokia, followed by Habanero Red Savina, Fatalii Yellow, Habanero Paper Latern, Habanero Maya Red, Habanero Red, and Scotch Bonnet Red. The inhibitory effect of the extract of *Capsicum chinense* pepper type was evaluated, using discs diffusion method, against selected bacteria and micromycetes. Extracts from Bhut Jolokia, Fatalii Yellow, Scotch Bonnet Red and habaneros did not inhibit growth of the any bacteria and micromycetes included in our test.

Keywords: antibacterial activity; capsaicin; chilli peppers; HPLC; micromycetes

INTRODUCTION

For a long time hot pepper fruit has been known all over the world as a delicious spice with a characteristic smell and taste. It is used for preparing spicy sauces and also in Mexican and Asian cuisines. The value of hot pepper consists in its sensorial attributes colour, spiciness and flavour (Perucka and Oleszek, 2000). Chilli pepper and their isolated constituents including capsaicinoids have shown also beneficial therapeutic effects, including antioxidant, anti-inflammatory, anticancer, antimicrobial and anti-immune modulator effects. The capsaicinoids have evolved in chilli peppers as a defence mechanism against mammalian predators; nevertheless, this trait is an important fruit quality attribute and one of the most important reasons chilli peppers are consumed. It is an extraordinarily versatile agent, and its use is ranging, in the fields from pharmaceutical purposes and nutrition (seasoning) to chemical weapons. It has been used as an analgesic against arthritis pain and inflammation (Deal et al., 1991). It has also been reported to show anticancer effect (Morré and Morré, 2003) and to be active against neurogenic inflammation (burning and stinging of hands, mouth and eyes) (Szolcsanyi, 2004). The latter property is the basis for the use of capsaicin in defensive pepper sprays. Capsaicin has also been reported to show protective effects against high cholesterol levels and obesity (Kempaiah et al., 2005). Capsaicin and other members of the capsaicinoids group produce a large

number of physiological and pharmacological effects on the gastrointestinal tract, the cardiovascular and respiratory system as well as the sensory and thermoregulation systems.

Capsaicinoids are derivatives of benzylamin. Differences within their structure depend mainly on their acyl moieties, and three structural elements are involved: first of all the length of the acyl chain (C8-C13), then the way it terminates (linear, iso or anteiso-series), and the presence or absence of unsaturation at the ω -3 (capsaicin type) or ω -4 carbon atom (homocapsaicin type I and II) (Fattorusso and Tagliatela-Scafati, 2008). Capsaicin, a homovanillic acid derivative (8-methyl-N-vanillyl-6-nonenamide), is an active component of the red pepper. The level of the capsaicin in the seasoned pepper is around 0.025%, and in the hot pepper around 0.25% (Holzer, 1991). Capsaicin is represented with 69% in the group of capsaicinoids; dihydrocapsaicinoids with 22%; nordihydrocapsaicinoids with 7%; homocapsaicin and homohydrocapsaicin takes only 1% in the group of capsaicinoids. Capsaicin and dihydrocapsaicin being approximately twice as pungent as nordihydrocapsaicin and homocapsaicin and they are responsible for the hotness of the pepper.

The amount of capsaicinoids in a chilli pepper pod is dependent on the genetic makeup of the plant and the environment where it is grown (Zewdie and Bosland, 2000). The amount of capsaicin in a given variety can vary

depending on the light intensity and temperature at which the plant is grown, the age of the fruit, and the position of the fruit on the plant. Chilli peppers must be harvested at an appropriate degree of development in accordance with the criteria proper to the variety and the area in which they are grown.

Chilli peppers contain also phenolic compounds, flavonoids and carotenoids, besides being a source of vitamin C. Among these, flavonoids are ubiquitous phytochemicals found in plants with a wide group of exploitable activities, including antimicrobial activity, antibiotic synergism and bacterial virulence removal. Once absorbed, they influence several biological functions, including protein synthesis, angiogenesis, cell proliferation and differentiation, thus benefiting a variety of human diseases. The flavonoids found in most peppers are glycosides and aglycones of myricetin, quercetin, luteolin, apigenin and kaempferol (Nascimento et al., 2014). Cinnamic and m-coumaric acids are present in the serrano and pimiento morrón, but not in the habanero. It was concluded that the capsaicinoids composition of the three peppers extract is different, and this may influence their antimicrobial effects. One the capsaicin analogues, vanillin, has shown inhibitory activity towards the growth of yeast (Serruti and Alzamora, 1996) and mould (López-Malo et al., 1998). Also, Kim and Ryeom (1979) reported antibacterial effects of capsaicin from Korean hot pepper on *Bacillus subtilis*, *Bacillus cereus*, and *Sarcina lutea*. The importance of finding natural inhibitors of pathogenic microorganisms has been stressed by López-Malo et al. (1998).

MATERIAL AND METHODOLOGY

Plant material

Four Habanero chilli varieties: Habanero Habanero Red Savina (HRS), Habanero Maya Red (HMR), Habanero Paper Lantern (HPL), Habanero Red (HR), and varieties Fatalii Yellow (FY), Scotch Bonnet Red (SBR) and Bhut Jolokia (BJ) have been used in our experiment. The experiment has been started buying seeds, the sowing, germination, transplanting gradually and care of mature plants, including adaptation of climatic conditions and fertilization. After harvesting the ripe fruits have been stored in a refrigerator at 0 – 4 °C. Samples of chili peppers (6 pieces of each variety were used in the experiment) were dried immediately after harvest in the stage of maturity in a laboratory oven with ventilation. Before the drying chilli peppers were cut to halves or quarters (depending on size) to speed up drying and to prevent undesired changes (moulds). Chili peppers have dried along with the placenta and seeds. Drying was carried out in two stages at 40 ± 5 °C, in a first phase of 24 h, and in the second phase 12 – 24 h depends on water content. After drying, the chili peppers were stored in a sealed glass container in a dry, dark place until analysis (not more than one month).

Extraction procedure and HPLC analysis

HPLC analysis of capsaicin content consisted of sample preparation (removing of placenta and seeds), extraction and liquid chromatography analysis.

Sample extraction

Fresh and dried material, without seeds was cut into pieces. The extraction was carried out with ethanol at a ratio of 1 : 10, sonication lasted 30 min, and 4 h of maceration with extraction efficiency 90%.

HPLC analysis

Column Ascentis Express RP-Amide 2.7 µm, 100 x 2.1 mm, gradient: acetonitrile : 0.2% HCOOH, 0 minutes 30 : 70, and 71 : 29 after 10 min, flow rate 0.5 mL.min⁻¹, injection volume 1 µL, temperature 40 °C and UV detection at 254 nm and 280 nm. Capsaicin content was determined based on a calibration curve and SHU units have been determined by calculation. According to the commonly accepted Scoville organoleptic test (Scoville, 1912), the spicy strength of the investigated samples was calculated by converting the capsaicin content expressed in grams of capsaicin per gram of pepper. This conversion to Scoville heat units was done by multiplying the capsaicin content in pepper dry weight by the coefficient corresponding to the heat value for pure capsaicin, which is 1.6 × 10⁷ and after correction of sample extraction 1.8 × 10⁷.

Antimicrobial activity of extracts from chilli peppers

Bacterial and micromycetes strains and media

For antibacterial activity, reference strains CCM 4223 *Staphylococcus aureus*, CCM 4420 *Salmonella enterica* subsp. *enterica* serovar Enteritidis, ATCC 11338 *E.coli* have been used in this experiment. These bacterial strains were collected from Czech Collection of Microorganisms. Terrain strains *Staphylococcus aureus* isolated from soft cheese bryndza, *Salmonella sp.* and *E.coli* isolates from poultry meat were included also in testing were obtained from the culture collections of Department of Food Hygiene and Technology, and maintained at -80 °C in cryobox. Antifungal activity have been tested against reference strains of micromycetes CCMF 269 *Aspergillus ochraceus*, CCMF 683 *Fusarium gramineum*, CCMF 583 *Penicillium viridicatum* and terrain strains *Aspergillus ochraceus* and *Penicillium purpurogenum* isolated from coffee, and *Fusarium gramineum* isolate from cornflakes obtained from the culture collections of Department of Food Hygiene and Technology, and maintained at -80 °C in cryobox.

For the preparation of the inoculum, bacteria strains were cultured in brain heart infusion (BHI) broth for 24 h at 37 ± 2 °C and standardized for the same absorbency, number 0.5 of the McFarland Nephelometer, which corresponds to the order of 10⁸ CFU.mL⁻¹. For the preparation of the inoculum, micromycetes strains were cultured in Sabouraud dextrose broth for 5 d at 25 ± 2 °C.

Disk diffusion method

The antimicrobial activity of the capsaicin extracts was carried out by disc diffusion method. The capsaicin extracts were prepared in ethanol at a ratio of 1 : 10, sonication lasted 30 min, and 4 h of maceration. Sterile filter paper discs (diameter 6 mm) was impregnated with 20 µL of the extracts, and placed on the agar plate, on which the test microorganisms were uniformly inoculated.

The assay dishes were then left for one hour and subsequently incubated for 24 h at 37 °C for bacteria and for 5 d at 25 °C for micromycetes. The diameter of inhibition was then observed and measured. The diameter of the clear zone shown on plates was measured using callipers and expressed in millimetres as its antimicrobial activity. Each experiment was performed in triplicate.

RESULTS AND DISCUSSION

In our study comparison of various Habanero varieties, Fatalii Yellow, Scotch Bonnet Red and Bhut Jolokia was performed. The results can be compared with following study undertaken to compare the heat levels of Habanero Red Savina and Bhut Jolokia in a replicated field trial; establish whether Bhut Jolokia truly has a higher heat level than Habanero Red Savina.

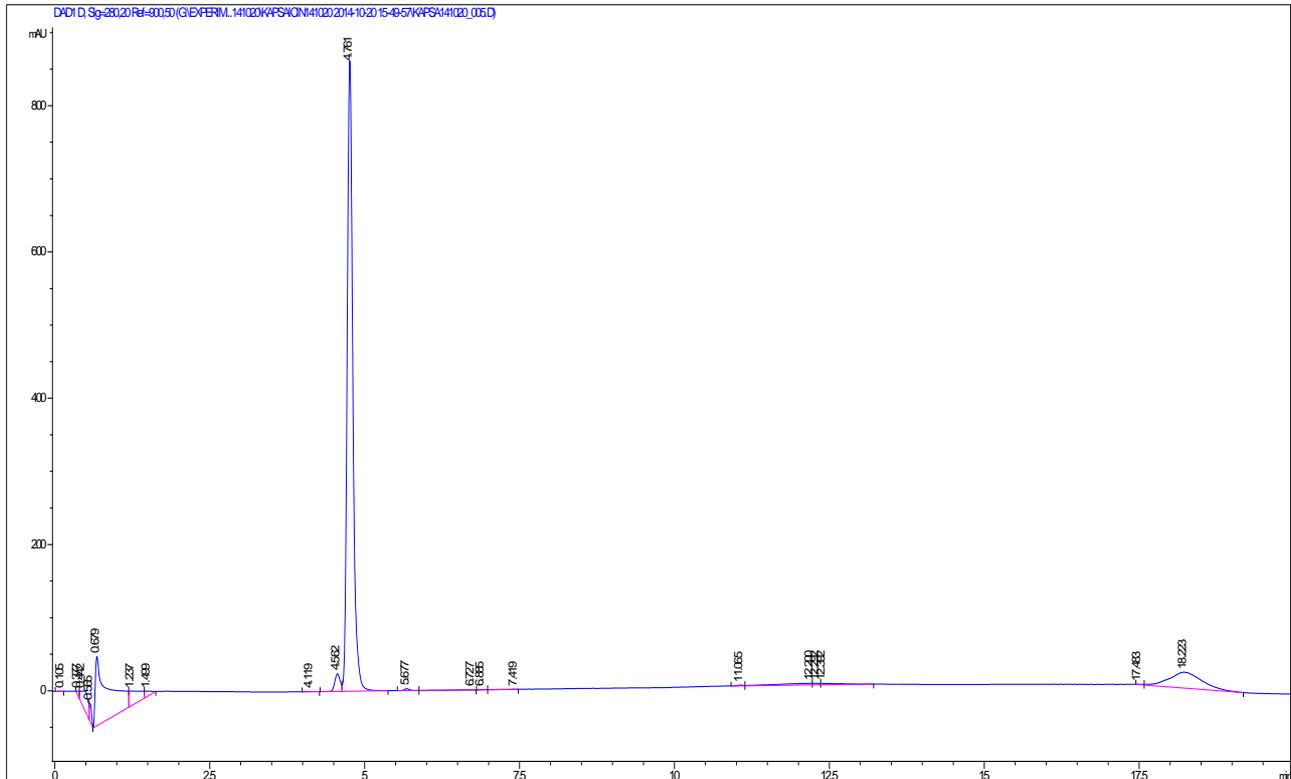


Figure 1 Chromatogram of capsaicin standard determination using HPLC.

Table 1 Capsaicin and dihydrocapsaicin concentrations in extract.

Chilli peppers	Capsaicin ($\mu\text{g.mL}^{-1}$)	Dihydrocapsaicin ($\mu\text{g.mL}^{-1}$)
BJ	2706.3	1738
HRS	886.5	259
FY	663.3	200
HPL	383.5	163
HMR	410.4	124
HR	229.5	110
SBR	126	88

Table 2 Concentration of capsaicin and dihydrocapsaicin in chilli peppers and calculation of SHU after correction of sample extraction.

Chilli peppers	Capsaicin ($\mu\text{g.g}^{-1}$)	Dihydrocapsaicin ($\mu\text{g.g}^{-1}$)	SHU (sum of capsaicin and dihydrocapsaicin) ($18 \times \mu\text{g.g}^{-1}$)
BJ	27145.26	17432.83	802406
HRS	8866.42	2590.41	206223
FY	6595.17	1988.59	154508
HPL	3840.28	1632.29	98506
HMR	4098.58	1238.36	96065
HR	2289.83	1097.52	60972
SBR	1242.97	868.10	37999



Figure 2 Bhut Jolokia.



Figure 3 Bhut Jolokia – dried.



Figure 4 Bhut Jolokia.



Figure 5 Habanero Red Savina.



Figure 6 Habanero Red Savina 1.



Figure 7 Scotch Bonnet Red.

Once the fruit had matured on the plants in the field, a single harvest of 25 random mature fruits from at least 10 plants in each replication was bulked. After harvest, the sample was dried and ground. The extraction of the capsaicinoids and the estimation of capsaicinoid amounts followed the high performance liquid chromatography (HPLC) procedures for the short run method as described by Collins et al. (1995). The HPLC data were converted

from parts per million to SHU by multiplying the parts per million by 16 (Bosland and Baral, 2007). The environment is known to affect the heat level of chilli pepper cultivars (Harvell and Bosland, 1997). Having a replicated field trial with standard control cultivars allows for a better comparison of heat levels among cultivars.

Capsaicin content in dried samples of each chilli peppers was determined on the basis of compliance with the

standard of capsaicin using HPLC. Figure 1 shows a chromatogram of standard of capsaicin determination by HPLC using the optimized conditions described above. The highest peak in the chromatogram represents the standard of capsaicin at a wavelengths of 254 nm and 280 nm. The absorbance for standards of capsaicin have been used in preparation of the calibration curve. The results for the concentration of capsaicin in the analysed samples were calculated using the equation $y = 0,0009x$ (μg).

In Table 1 are ranked dried chilli pepper according to capsaicin and dihydrocapsaicin content in extract expressed as $\mu\text{g}\cdot\text{mL}^{-1}$. Capsaicin content after drying has been increased 4 – 10 fold compared to fresh chilli peppers.

In Table 2 are ranked dried chili pepper according to capsaicin content expressed as SHU units ($18 \times \mu\text{g}\cdot\text{g}^{-1}$) calculated from capsaicin concentration given in Table 2 and after correction of extraction yield (90%).

The results of the analysis for Bhut Jolokia indicated that it possessed an extremely high heat level, 1 001 304 SHUs, whereas Habanero Red Savina recorded a heat level of 248 556 SHUs. Independent tests confirmed this high level of heat for Bhut Jolokia with 927 199 SHUs and 879 953 SHUs from Southwest Bio-Laboratories and Ag-Biotech, respectively (Bosland and Baral, 2007). The results of Bhut Jolokia heat level have been higher compared to our results. The aim of the next study was to determine the content of capsaicin and dihydrocapsaicin in *Capsicum* samples collected from city markets in Riyadh (Saudi Arabia), calculate their pungency in Scoville heat units (SHU) and evaluate the average daily intake of capsaicin for the population of Riyadh. The investigated samples consisted of hot chillies, red chillies, green chillies, green peppers, red peppers and yellow peppers. Extraction of capsaicinoids was done using ethanol as solvent, while high performance liquid chromatography (HPLC) was used for separation, identification and quantitation of the components. The limit of detection (LOD) of the method was 0.09 and 0.10 $\mu\text{g}\cdot\text{g}^{-1}$ for capsaicin and dihydrocapsaicin, respectively, while the limit of quantification (LOQ) was 0.30 and 0.36 $\mu\text{g}\cdot\text{g}^{-1}$ for capsaicin and dihydrocapsaicin, respectively. Hot chillies showed the highest concentration of capsaicin ($4\ 249 \pm 190.3 \mu\text{g}\cdot\text{g}^{-1}$) and the highest pungency level (67 984 SHU) comparable with our results for Bhut Jolokia (Al Othman et al., 2011).

Capsaicinoids are mainly ingested as naturally occurring pungency-producing components of *Capsicum* spices (chili, cayenne pepper, red pepper). Their concentrations typically range from 100 $\mu\text{g}\cdot\text{g}^{-1}$ in chili pepper to 2500 $\mu\text{g}\cdot\text{g}^{-1}$ in red pepper (Parrish, 1996). Pepper varieties from *Capsicum annum*, *C. frutescens* and *C. chinense* were found to contain 220 – 20 000 μg total capsaicinoids. $\cdot\text{g}^{-1}$ of dry weight (Thomas et al., 1998). In another study, cayenne pepper samples had mean capsaicin and dihydrocapsaicin contents of 1320 and 830 $\mu\text{g}\cdot\text{g}^{-1}$ dry weight, respectively (Lopez-Hernandez et al., 1996).

Capsaicinoids are synthesized exclusively in the epidermal cells of the placenta of *Capsicum* fruits and are accumulated in blisters along the epidermis. Their biosynthesis begins approximately 20 days postanthesis, with a number of enzymes being involved in the biosynthetic pathway. The degree of pungency depends on

the *Capsicum* species and cultivars, and the capsaicin and dihydrocapsaicin contents can be affected by different factors such as the developmental stage of the fruit and the environmental growth conditions (Garces-Claver et al., 2006). The biosynthesis of capsaicinoids occurs in the placenta, where the specialised epidermal cells accumulate in vacuoles and excrete on the inner surface of the seed and pericarp; therefore, the capsaicinoids should accumulate preferentially in the placenta rather than in the pericarp. The similar recent studies indicated that capsaicin is mostly located in vesicles or vacuole like sub-cellular organelles of epidermal cells of placenta in the pod. The highest concentrations of capsaicin are found in the ovary and in the lower flesh (tip) and the lowest content of capsaicin can be found in the seeds. The gland on the placenta of the fruit produces capsaicinoids. The seeds are not the source of pungency but they occasionally absorb capsaicin because they are in close proximity to the placenta. No other plant part produces capsaicinoids. The majority, about 89%, of the capsaicin is associated with the placental partition of the fruit and nearly 5 – 6% in the pericarp and the seed. Composition of capsaicin may vary among different varieties of same species and with fruit of a single variety (Arora et al., 2011). A likely explanation for our findings is that the presence of capsaicinoids in the pericarp suggests that capsaicinoids are translocated from the placenta to the pericarp tissue via the cell walls of the epidermal layer of the placenta. Removing of placenta and less likely environmental factors are obviously the reasons why the capsaicin concentration in our chillies is lower.

In our study no antibacterial and antifungal activity of capsaicin and dihydrocapsaicin against selected reference and terrain strains of bacteria and moulds was found. Sterile filter paper discs were impregnated with extracts, and placed on the agar plate, but no inhibition was subsequently observed and measured. Kim et al. (1995) tested the bactericidal activity of carvacrol [2-methyl-5-(1-methylethyl)phenol], geraniol (3,7dimethyl-2,6-octadien-1-ol) and citral (3,7dimethyl-2,6-octadienal) against *S. typhimurium* inoculated on fish cubes and reported that carvacrol at 3% killed the inoculated bacteria, while geraniol killed most of the bacteria and citral killed the least. From this work, it was concluded that the carvacrol showed to be a better antimicrobial. It is noteworthy that this substance is a phenol derivate, such as 3-hydroxycinnamic acid (coumaric acid) which has been reported by Dorantes et al. (2000). Capsaicin and dihydrocapsaicin (responsible for chilli pepper pungency) in the concentrations used in this study did not show an inhibitory effect on the growth of bacteria. It can be seen that *m*-coumaric and cinnamic acids are responsible for the inhibitory action of the four bacteria. It These findings agree with the fact that habanero pepper, which has the highest capsaicin content, was the least effective as a bacterial inhibitor (results not shown). On the other hand, pimiento morrón extract which contains both *m*-coumaric and cinnamic acids but no capsaicins, showed a good inhibitory action on the four bacteria tested. Among the bacteria tested *L. monocytogenes* was the most sensitive to the three chilli extracts, while the most resistant was *S. typhimurium*.

CONCLUSION

From a practical point of view, planting of four varieties of chilli peppers Habanero and Bhut Jolokia, Fatalii Yellow, Scotch Bonnet Red was successfully completed. After harvesting and drying, dried chilli peppers have been analysed by HPLC to determine the content of capsaicin and dihydrocapsaicin. Based on the results, the most pungent chilli pepper is Bhut Jolokia, which has a several times higher content of capsaicin and dihydrocapsaicin (802406 SHU) compared to Habanero Red Savina, Fatalii Yellow, other habaneros and Scotch Bonnet Red. There were found lower values in the content of capsaicin and dihydrocapsaicin, in contrast to the values reported by other studies. The pungency can be influenced with the weather conditions such as heat and it increases with the maturity of fruit. The great impact has also post harvesting processing such as removing of seeds and placenta when capsaicin content is decreased rapidly. Antimicrobial and antifungal activity of capsaicin and dihydrocapsaicin against selected strains of bacteria and moulds have not been proved. Based on the studies of various authors, extracts of pepper from ten different varieties of *Capsicum* contain phenylpropanoids and there were seven different compounds identified at varying concentrations depending on pepper variety (L-phenylalanine, t-cinnamic acid, o-coumaric acid, m-coumaric acid, ferulic acid, caffeic acid and capsaicin) which are intermediates of the capsaicinoids pathway. Non pungent varieties of chilli peppers, with major concentration of cinnamic and caffeic acids and without capsaicin content, presented the highest inhibitory effect. These results confirmed that bacteria are inhibited by some pepper extracts, and also that only some specific phenylpropanoids had a bacteriostatic effect.

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