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ΜΕΤΑΠΤΥΧΙΑΚΗ ΔΙΑΤΡΙΒΗ

THE IMMUNOMODULATING ACTIVITY OF *CAPSICUM L.* VARIANTS: EMPHASIS ON PROLIFERATION, CYTOKINE PRODUCTION AND CLASS II MHC ANTIGEN EXPRESSION

Ο ΡΟΛΟΣ ΣΤΕΛΕΧΩΝ ΤΟΥ *CAPSICUM L.* ΣΤΗΝ ΤΡΟΠΟΠΟΙΗΣΗ ΤΗΣ ΑΝΟΣΟΛΟΓΙΚΗΣ ΑΠΟΚΡΙΣΗΣ: ΕΜΦΑΣΗ ΣΤΟΝ ΠΟΛΛΑΠΛΑΣΙΑΣΜΟ, ΠΑΡΑΓΩΓΗ ΚΥΤΟΚΙΝΩΝ ΚΑΙ ΕΚΦΡΑΣΗ ΤΩΝ ΤΑΞΗΣ ΙΙ ΑΝΤΙΓΟΝΩΝ ΙΣΤΟΣΥΜΒΑΤΟΤΗΤΑΣ

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To my lovely aunt, Movsesyan Galina Martirosovna,
and to the memory of my uncle, Movsesyan Kamo Armenakovich,
who will always stay in my heart.

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Abstract

Discovery and identification of novel safe drugs, without severe side effects, is an important goal of research in cancer chemotherapy. Cell growth regulation is a key objective in anticancer research. On the other hand, the enhancement of host immune system through the regulation of cytokines in the cytokine network as defense mechanisms, as well as expression of MHC class II molecules have been recognized as possible pathways of inhibiting tumor growth without harming the host. Chili plays multiple roles in pharmacological and biological functions as a well-known folk medicine and also a spice. Preliminary observations had detected an anti-proliferative effect of chili extracts which placed this preparation in the anti-cancer therapeutic products. In order to evaluate the functional activity of dietary red sweet and hot peppers, this study was conducted to assess cell proliferating and immunomodulating activity of aqueous extracts from sweet and hot chili peppers *in vitro* on murine spleen cells (BALB/c) with particular focus on the proliferative state of spleen primary cells and specific cell populations (macrophages, T- and B lymphocytes), cytokine production, expression of MHC class II molecules and apoptotic status of cells upon the application of extracts. In addition, significant effort was given on the identification of the active molecules in the extracts which would be responsible for the above effects. It was found that extracts from sweet pepper reduced cell growth, while hot extracts increased cell proliferation. Interestingly, hot extract stimulated the growth of B lymphocytes and suppressed proliferation of macrophages while sweet extract, on the contrary, stimulated macrophages. Both extracts suppressed the proliferation of T lymphocytes. A significant increase in IFN-gamma, IL-2 and TNF-alpha production was observed upon exposure of spleen cells to all extracts. Both sweet and hot extracts stimulated biosynthesis of MHC class II molecules. Neither apoptosis nor necrosis was detected in spleen cells upon administration of either extracts. Subsequent fractionation by DEAE-Sephacel ionic chromatography revealed certain fractions with significant proliferative and anti-proliferative activities, both highly active in cytokine production. High amounts of active components, such as polyamines, proteins, carbohydrates and

carotenoids were found in the extracts, while the fractions derived from the extracts contained lower amounts of these compounds. Based on these results, the aqueous extracts from sweet and hot peppers could be proposed as a potential source of food material for a novel anticancer activity. However, further studies are necessary to assess wide scale potential of extracts. The identification of the active member(s) in these extracts will provide new insight in the up- or down-regulation of immune response by natural products.

ΠΕΡΙΛΗΨΗ

Η ανακάλυψη και ταυτοποίηση νέων ασφαλών και χωρίς παρενέργειες φαρμάκων αποτελεί ένα σημαντικό στόχο στην έρευνα της αντι-καρκινικής χημειοθεραπείας. Η ρύθμιση της κυτταρικής ανάπτυξης είναι μια θεμέλια επιδίωξη της αντι-καρκινικής έρευνας. Από την άλλη μεριά, η ενίσχυση του ανοσοποιητικού συστήματος του ξενιστή μέσω της ρύθμισης των κυτοκινών στα πλαίσια της κυτοκινικής πλεκτάνης ως μηχανισμού άμυνας, όπως και η έκφραση των τάξης ΙΙ MHC μορίων, αποτελούν πιθανά μονοπάτια για την καταστολή της ανάπτυξης όγκων χωρίς να βλάπτουν τον ξενιστή. Το πιπέρι, εκτός του ότι χρησιμοποιείται ως καρύκευμα, έχει πολλαπλούς φαρμακολογικούς και βιολογικούς ρόλους. Προκαταρτικές παρατηρήσεις αποκάλυψαν ότι εκχυλίσματα από πιπέρι έχουν αντι-πολλαπλασιαστικό ρόλο, γεγονός που τοποθέτησε αυτό το σκεύασμα στα προϊόντα αντι-καρκινικής θεραπείας. Για να εκτιμηθεί η λειτουργική ενεργότητα των διαιτητικών μη-καυτερών και καυτερών πιπεριών, η παρούσα μελέτη σχεδιάστηκε ώστε να εξετάσει την πολλαπλασιαστική και ανοσοτροποποιητική ενεργότητα των υδατικών εκχυλισμάτων μη-καυτερών και καυτερών πιπεριών σε κύτταρα σπλήνας ποντικού (BALB/c) *in vitro*, με ιδιαίτερη έμφαση στην πολλαπλασιαστική δράση τους στα πρωτογενή κύτταρα σπλήνας και ειδικών κυτταρικών υποπληθυσμών (μακροφάγα, Τ- και Β λεμφοκύτταρα), την παραγωγή κυτοκινών, την έκφραση τάξης ΙΙ MHC μορίων και το αποπτωτικό προφίλ των κυττάρων μετά από την επίδραση των εκχυλισμάτων. Επιπλέον σημαντική προσπάθεια δόθηκε στην ταυτοποίηση των ενεργών μορίων που ήταν παρόντα στα εκχυλίσματα και θα μπορούσαν δυνητικά να είναι υπεύθυνα για τα παραπάνω αποτελέσματα. Βρέθηκε ότι τα εκχυλίσματα των μη-καυτερών πιπεριών μείωσαν την κυτταρική ανάπτυξη, ενώ τα εκχυλίσματα των καυτερών πιπεριών αύξησαν τον κυτταρικό πολλαπλασιασμό. Ενδιαφέρον παρουσίασε το γεγονός ότι τα εκχυλίσματα των καυτερών πιπεριών αύξησαν τον κυτταρικό πολλαπλασιασμό των Β λεμφοκυττάρων και κατέστειλαν τον πολλαπλασιασμό των μακροφάγων, ενώ αντίθετα τα μη-καυτερά εκχυλίσματα ενεργοποίησαν τα μακροφάγα, ενώ και οι δυο τύποι εκχυλισμάτων κατέστειλαν τον πολλαπλασιαμό των Τ λεμφοκυττάρων. Η έκθεση των

κυττάρων σπλήνας σε όλους τους τύπους των εκχυλισμάτων οδήγησε σε σημαντική αύξηση στην παραγωγή των IFN-γ, IL-2 και TNF-α και αύξηση την έκφρασης τάξης II MHC μορίων. Κανένα από τα εκχυλίσματα δεν οδήγησε σε απόπτωση ή νέκρωση των κυττάρων σπλήνας. Περαιτέρω κλασμάτωση των εκχυλισμάτων με χρωματογραφία ιοντο-ανταλλαγής DEAE-Sephacel αποκάλυψε ορισμένα κλάσματα με σημαντική πολλαπλασιαστική και αντι-πολλαπλασιαστική ικανότητα και υψηλή ενεργότητα στην παραγωγή κυτοκινών. Μεγάλες ποσότητες στοιχείων όπως οι πολυαμίνες, οι πρωτεΐνες, οι υδατάνθρακες και τα καροτενοειδή βρέθηκαν στα εκχυλίσματα, ενώ τα κλάσματα που προήλθαν από τα εκχυλίσματα, περιείχαν πολύ μικρότερες ποσότητες από αυτά τα βιομόρια. Βασιζόμενοι σε αυτά τα αποτελέσματα, τα υδατικά εκχυλίσματα από το καυτερό και μη-καυτερό πιπέρι θα μπορούσαν να προταθούν ως μια εν δυνάμει πηγή τροφικού υλικού με νέες αντι-καρκινικές δράσεις. Παρόλα αυτά, περαιτέρω μελέτες είναι απαραίτητες για να εκτιμηθεί η ευρείας κλίμακας δυναμικότητα των εκχυλισμάτων. Η ταυτοποίηση των βιομορίων σε αυτά τα εκχυλίσματα θα προσφέρει νέες προοπτικές στην ρύθμιση της ανοσολογικής απόκρισης από φυσικά προϊόντα.

I. Introduction

For centuries people have used plants for healing. Plant products – as parts of foods or botanical potions and powders – have been used with varying success to cure and prevent diseases throughout history.

Despite the current preoccupation with synthetic chemistry as a vehicle to discover and manufacture drugs, the contribution of plants to disease treatment and prevention is still enormous. Botanical drugs are fully accepted and widely prescribed in China, Japan, India and other Asian and African countries. In addition, some countries in Europe, such as Germany, allow physicians prescribe botanical drugs. Currently, no botanical drugs are sold in the USA, but a few are under clinical development.

So why do we need botanical drugs? The new chemical entities paradigm of the twentieth century attempts to treat complex diseases with a 'single golden molecular bullet'. The first flaw in this paradigm appeared relatively recently when problems of resistance to antimicrobial and anticancer drugs became apparent. The multifactorial nature of many complex diseases, such as diabetes, heart disease and cancer is also an important consideration. Most of these diseases cannot be ascribed to a single genetic or environmental change but arise from a combination of genetic, environmental or behavioral factors (Raskin, 2002).

Therefore finding a source of novel chemotherapeutics continues to be a focus of effort. Identification of naturally occurring substances capable of inhibiting, retarding, or reversing the multi-stage processes of complex ailments is under considerable attention. Diets rich in vegetables are known to reduce risks of many diseases including cancer, implicating edible plants as potential sources of protective agents.

Scientists have suggested that it appears extremely unlikely that any single substance is responsible for all of the associations seen between plant foods and cancer prevention because of the great variety of dietary flavonoids and many types of potential mechanisms reported (Yi *et al.,* 2006). It has been suggested that the combination of phytochemicals in fruits and vegetables is crucial for their potential anticancer activities.

In this context the consumption of red chili peppers, apart from their use as a food additive in various cuisines, is of great importance for therapeutic purposes to treat various peripheral painful conditions such as rheumatoid arthritis and diabetic neuropathy. This species revealed also various antigenotoxic, antimutagenic, and anticarcinogenic effects, suggesting to be an important dietary product with a potential chemopreventive activity (Johnson, 2007).

However, there are only a few studies focusing on the immonomodulating potential of red peppers. No data were found on the effect of red peppers on the biosynthesis of MHC class II molecules, which can have crucial role in cancer immunotherapy. Also no reports on the effects on chili peppers on proliferation of spleen primary cells and different cell populations have been published.

The objective of the present study is to examine the *in vitro* effects of sweet and hot chili aqueous extracts on murine spleen cells (BALB/c) with particular interest in:

- proliferative activity of spleen primary cells and different cell population (macrophages, B- and T-lymphocyets)
- cytokine production
- expression of MHC class II molecules
- apoptotic status
- identification of the active molecules in the extracts which could be responsible for these effects

II. Literature Review

2. 1. Genus *Capsicum*

2. 1. 1. Botanical Traits

Kingdom:	*Plantae*
Division:	*Magnoliophyta*
Class:	*Magnoliopsida*
Subclass:	*Asteridae*
Order:	*Solanales*
Family:	*Solanaceae*
Genus:	*Capsicum L.*

The Latin name Capsicum, was given by the French botanist de Tournefort for unclear reasons. A popular theory holds that the name Capsicum was derived from the Greek kapto, meaning "to bite", which appropriately describes the main characteristic of the fruit. Others argue that the name Capsicum is derived from capsa, the Latin word for box, referring to the fact that the pepper pod is hollow, divided into compartments containing the seeds (Szallasi and Blumberg, 1999 and ref. therein).

The chili pepper is the fruit of species of the plant *Capsicum* spp from the nightshade family, *Solanaceae*. The name, which is spelled differently in many regions (chili, chile or chilli), comes from Nahuatl via the Spanish word *chile*. The term chili in most of the world refers exclusively to the smaller, hot types of *Capsicum*. The mild larger types are called *bell pepper* in the USA, simply *pepper* in Britain and Ireland, *capsicum* in Australasia and *paprika* in many European countries.

Common Names: Cayenne, Cayenne Pepper, Chili Pepper, Chabai Achong, Aji Dulce, Filfil, Hungarian Pepper, Kirmizi Biber, La Chiao, Mexican Chili, Paprika, Peppers, Piment Doux, Pimiento, Red Pepper, Sweet Pepper, Capsicum frutescens, lombok riewit, piment, lombok besar, cabe besar.

2. 1. 2. Species and Cultivars

The most common species of chili peppers are:

- *Capsicum annuum*, which includes many common varieties such as bell peppers, paprika, jalapeños, and the chiltepin;
- *Capsicum frutescence*, which includes the tabasco peppers;
- *Capsicum chinese*, which includes the hottest peppers such as the naga, habanero and Scotch bonnet;
- *Capsicum pubescens*, which includes the South American rocoto peppers;
- *Capsicum baccatum*, , which includes the South American aji peppers;

Peppers are commonly broken down into three groupings; bell peppers, sweet peppers, and hot peppers (Ahn *et al.*, 2006). Most popular pepper varieties fall into one of these categories or as a cross between them (See Appendix 1).

2. 1. 3. Origin

Chili peppers, probably, native of the Tropics, but the original habitat is obscure: China; Dominican Republic; Europe; Haiti; Gabon; Hawaii; India; Iraq; Malagasy; Malaya; USA; Mexico; Peru; Ecuador; Mongolia; Panama; Philippines; Samoa; Spain; Tonga; Trinidad.

2. 1. 4. History

Chili peppers have been a part of the human diet in America since at least 7500 BC and perhaps earlier. There is archaeological evidence at sites located in southwestern Ecuador that chili peppers were already well domesticated more than 6000 years ago (Perry *et al*., 2007) and is one of the first cultivated crops in America.

Capsicum was described by the Greek Therophrasteus (370-286 BC). The Roman poet Martialis (around the 1st century) described "Pipervee crudum" (raw pepper) to be long and containing seeds. The description of the plants does not fit pepper, which does also not grow well in European climates. In an archaeological dig in Lund (Sweden), archaeologists claimed to have found a *Capsicum frutescens* in a layer dating to the 13th century (Hjelmqvist, 1995).

Christopher Columbus was one of the first Europeans to encounter them (in the Caribbean), and called them "peppers" because of their similarity in taste (though not in appearance) with the Old World peppers of the *Piper* genus.

Chilis were cultivated around the globe after Columbus' time. Diego Álvarez Chanca, a physician on Columbus' second voyage to the West Indies in 1493, brought the first chili peppers to Spain, and first wrote about their medicinal effects in 1494. Hot pepper was cultivated in monastery gardens in Moravia as early as 1566 (Szallasi and Blumberg, 1999 and ref. therein).

From Mexico, at the time the Spanish colony that controlled commerce with Asia, chili peppers spread rapidly into the Philippines and then to India, China, Korea and Japan with the aid of European sailors. The new spice was quickly incorporated into the local cuisines.

An alternate sequence for chili pepper's spread has the Portuguese picking up the pepper from Spain, and hence to India. The evidence provided is that the chili pepper figures heavily in the cuisine of the Goan region of India, which was the site of a Portuguese colony. The journey of chili peppers from India was through Central Asia to Europe (Collingham, 2006).

2. 1. 5. Uses

The dried fruit is a powerful local stimulant with no narcotic effect; it is most useful in atony of the intestines and stomach. It has proved efficacious in dilating blood vessels and thus relieving chronic congestion of people addicted to drink.

Used externally, it is a strong rubefacient stimulating the circulation, aiding the removal of waste products and increasing the flow of nutrients to the tissues. It is applied as a cataplasm or linament. Internally, they are used for stomach ache. *Capsicum frutescens* are used in native practice in typhus, intermittent fevers, dropsy, gout, dyspepsia, and cholera. It is used in sore throat, scarlatina, hoarseness, dyspepsia, yellow fever, and occasionally in diarrhea (Ching *et al.,* 2001).

Capsaicin has been used for many treatments including rheumatism, in folk medicine. Chili also relieves painful muscle spasms in the shoulder, arm, and spine areas, bursitis, the pain of shingles, and the pain of diabetic neuropathy. It has also been powdered and placed inside socks as a traditional remedy for those prone to cold feet. Now, there is also substantial human experience with capsaicin in the form of non-prescription (in the USA) or prescription (in the EU) topical analgesics.

The fruit is also antihemorrhoidal, antiseptic, carminative, diaphoretic, digestive, sialagogue and stomachic. It lowers cholesterol and works against arthritis and rheumatism. These pungent fruited peppers are important in the tropics as gastrointestinal detoxicants and food preservatives. Chili applied to the skin desensitizes nerve endings and so has been used as a local anaesthetic (http://www.kakawachocolates.com/index.php?main_page=page_4).

Paprika (ground dried pod of mild capsicum, *Capsicum annuum* L.) may be used safely for the coloring of foods, generally, in amounts that are consistent with good manufacturing practice (Johnson, 2007).

Fruit - raw or cooked. Very hot and normally used as a flavoring. The fruit can be dried and ground into a powder for use as flavouring. Seed - dried, ground and used as a pepper. Leaves - cooked as a potherb.

With regard to the varied, nonculinary uses of hot pepper, there is apparently little new under the sun. For example, Incas burned dried chili peppers to combat the invading Spaniards by temporarily blinding them. Four centuries later, the first U.S. patent was issued for the use of capsaicin for martial (tear gas) purposes (Szallasi and Blumberg, 1999 and ref.

therein). Capsicum-derived ingredients are used in 19 cosmetic products at concentrations as high as 5% (Johnson, 2007).

2. 1. 6. Applications of Chili Tincture

- Cardiovascular support; cleans the blood and stimulates the entire system. Helps clear blockages from arteries, veins and lymphatic system. Also stops bleeding and dissolves blood clots; improvement of circulation and lowering of blood pressure;
- Improves brain function; one of the most well-known effects is that of increasing blood circulation to the head- and brain area. Effective against cluster and migraine headaches.
- Can stop bleeding extremely fast, immediately flush the wound with the tincture (1 - 5 full droppers) after this pack the wound with Chili powder.
- Improves circulation, and reduces or stop bleeding from stomach ulcers. When taken internally, it stimulates circulation. Used against indigestion and heartburn.
- Breaks up congestion and speeds healing of colds and flu. Used as a diaphoretic (sweat inducing-herb).
- Helps lower cholesterol and prevent blood clots: thins the blood and heal the heart after a heart attack.
- Alleviate sore throats and tonsillitis; treats psoriasis and muscle strain.
- Provides far more vitamin C than citrus fruits, and is one of the highest sources available for this vitamin (http://www.tropilab.com/cayennepeppertincture.html).

2. 1. 7. Known Hazards

Pungent-fruited peppers may cause painful irritation when used in excess, or after accidental contact with the eyes. Although no reports have been seen for this species, many plants in this family produce toxins in their leaves. The sap of the plant can cause the skin to blister. (http://www.pfaf.org/database/search_name.php?ALLNAMES=capsicum)

2. 2. Active Components in Chili

2. 2. 1. Vanilloid Compounds

The active principle that causes the heat in chili peppers is a crystalline alkaloid generically called *capsaicin*. It is produced by glands at the junction of the placenta and the pod wall. The capsaicin spreads unevenly throughout the inside of the pod and is concentrated mostly in the placental tissue.

Capsaicin is an incredibly powerful and stable alkaloid seemingly unaffected by cold or heat, which retains its original potency despite time, cooking, or freezing. It has no flavor, color, or odor. Capsaicin is a nonpolar molecule, slightly soluble in water, but very soluble in alcohols, fats, and oils. Capsaicin is one of the most pungent compounds known, detectable to the palate in dilutions of one to seventeen million.

2. 2. 1. 1. Capsaicinoids

The word capsaicin actually describes a complex of related components named capsaicinoids by the Japanese chemists S. Kosuge and Y. Inagaki in 1964. Capsaicinoids are composed of a vanillylamide moiety and an acyl chain containing 8–13 carbon atoms. Depending on their acyl structure, three classes of compounds are grouped (Figure 2.1): capsaicins possessing a methyl branched acyl residue with a carbon–carbon double bond (A); dihydrocapsaicins analogous to the previous class, but being saturated compounds (B); and *N*vanillyl-*n*-acylamides composed of saturated, unbranched alkyl chains (C). The compounds belonging to the capsaicin and dihydrocapsaicin class structurally vary, depending on the position of the double bond and/or the position of the methyl group. Beside the most abundant compounds capsaicin, dihydrocapsaicin, and nordihydrocapsaicin, several minor capsaicinoids have been described. The natural pattern and contents of individual capsaicinoids in pepper fruits vary with species and variety, growing conditions and time of harvest (Schweiggert *et al.*, 2006 and ref. therein).

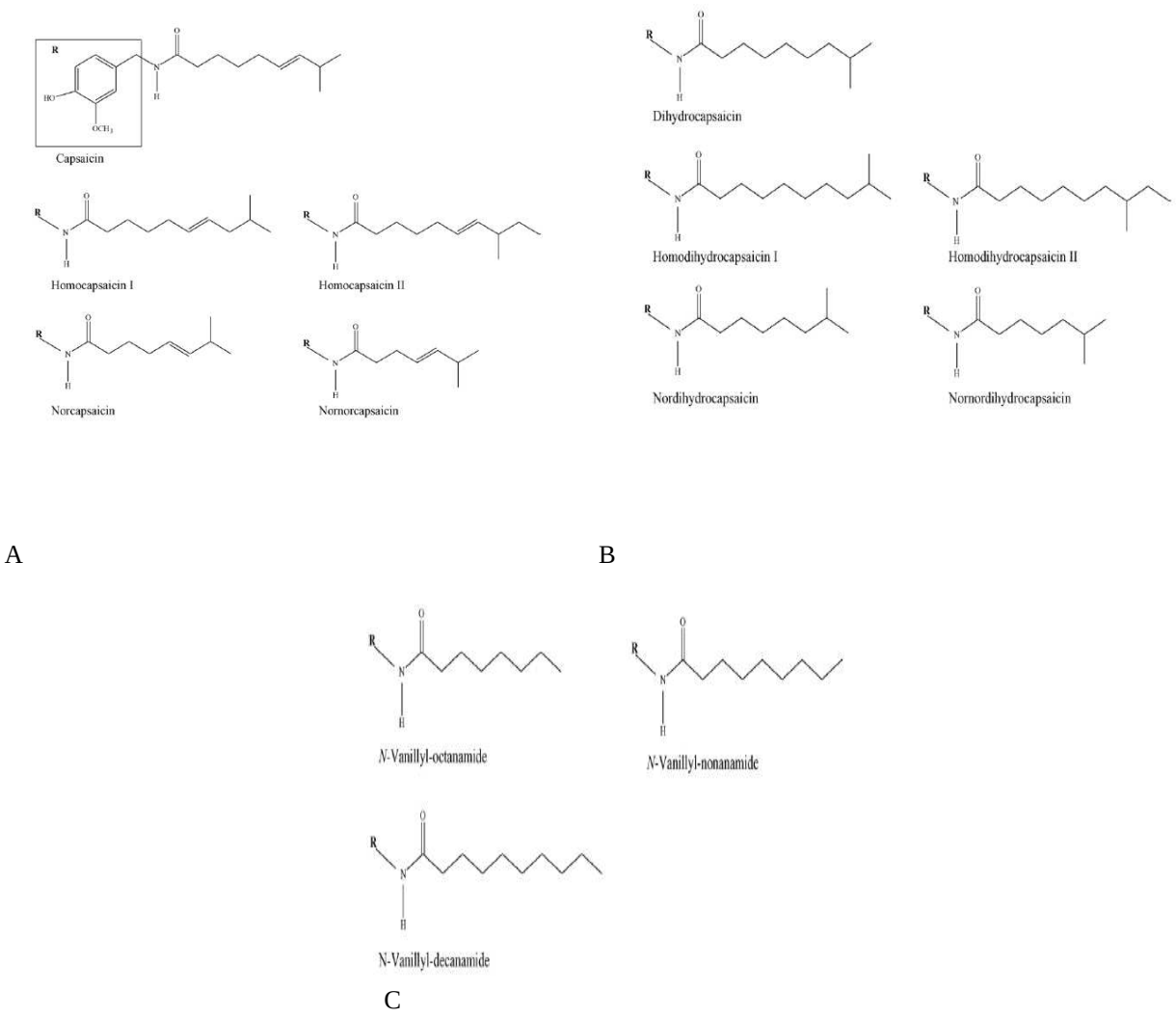
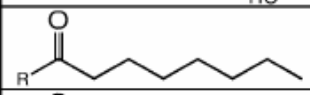
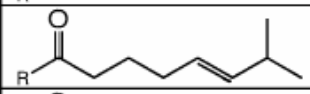
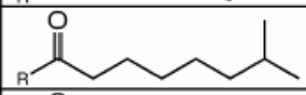
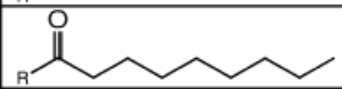
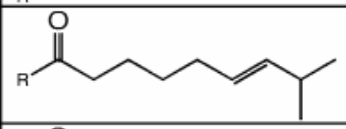
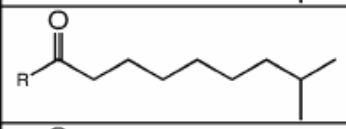
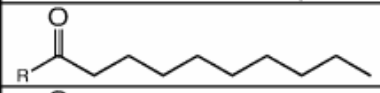
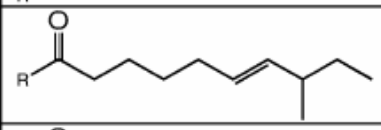
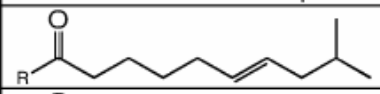
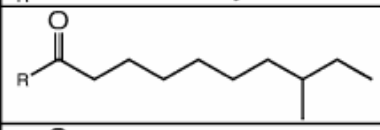
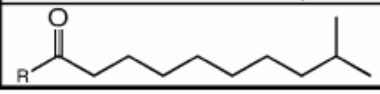


Figure 2.1. Structures of selected homologues of the 'capsaicin group': *A-capsaicins possessing a methyl branched acyl residue with a carbon–carbon double bond; B-dihydrocapsaicins analogous to the previous class, but being saturated compounds; and C-Nvanillyl-n-acylamides composed of saturated, unbranched alkyl chains (Schweiggert et al., 2006).*

The common naturally occurring capsaicinoids, are listed in Table 2.1. The most abundant in chilis, the major capsaicinoids, are 6-ene-8-methylcapsaicin, 8-methyldihydrocapsaicin, and 7-methylnordihydrocapsaicin (Thompson *et al.*, 2005).

Table 2.1. Naturally-occurring capsaicinoids *(Thompson et al., 2005).*

Number	Compound Name	Structure
1	N-vanillyl octanamide	
2	5-ene-7-methyl norcapsaicin	
3	7-methyl nordihydrocapsaicin	
4	N-vanillyl nonanamide	
5	6-ene-8-methyl capsaicin	
6	8-methyl dihydrocapsaicin	
7	N-vanillyl decanamide	
8	6-ene-8-methyl homocapsaicin	
9	6-ene-9-methyl homocapsaicin	
10	8-methyl homodihydrocapsaicin (tentative identification)	
11	9-methyl homodihydrocapsaicin	

The *trans*-geometric isomer of capsaicin, or *trans*-8-methyl-*N*-vanillyl-6-nonenamide (Figure 2.2), is the most abundant pungent molecule in chili peppers. Although there are two geometric isomers of capsaicin, only *trans*-capsaicin occurs naturally (Cordell and Araujo, 1993). The capsaicin content of chili peppers ranges from 0.1 to 1% w/w (Govindarajan and Sathyanarayana, 1991).

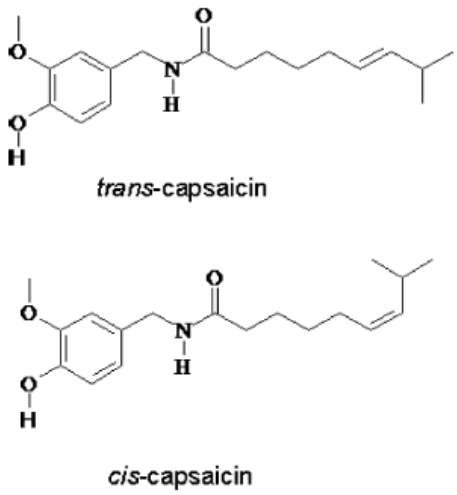


***Figure 2.2. Isomers of capsaicin molecule**.*

The actual percent of capsaicin and other capsaicinoids will vary depending on the peppers used and method of extraction. In fact, the United States Pharmacopoeia defines capsaicin as a product which contains >55% capsaicin and the combination of capsaicin and dihydrocapsaicin to be >75%; total capsaicinoid content may be as little as 90% (United States Pharmacopoeia, 2005).

A vanilloid analogue, derivative of vanillylamine-vanillic acid was determined in *Capsicum annum* L. extract by Qin Zhou group. According to the literature, vanillylamine is formed biosynthetically from phenylalanine *via* cinnamic acid, *p*-coumaric acid, caffeic acid, protocatechuic aldehyde and vanillin, and it may be readily converted into vanillic acid as a degradation product in plant extracts (Zhou Q. *et al.*, 2004 and references therein).
Phenylpropanoids from extracts of ten different varieties of *Capsicum annuum* L. were reported also by C. Acero-Ortega *et al.*, 2005. 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. Earlier, Dorantes *et al. 2000,* studying chili extracts from habanero, serrano and pimiento morrón varieties, reported content of following capsaicinoids (Table 2.2):

Table 2.2. Content of some capsaicinoids in the habanero, serrano and pimiento morrón extracts (mg/ml extract) *(Dorantes et al., 2000):*

Capsaicinoid	Habanero	Serrano	Morrón
o-coumaric acid	0.089±0.01	0.90±0.01	0.18±0.01
m-coumaric acid	–	0.31±0.01	0.21±0.01
Trans-cinnamic acid	–	0.47±0.01	0.21±0.01
Capsaicin	5.88±0.03	0.63±0.01	–
Dihydrocapsaicin	0.86±0.01	0.059±0.001	–

[a] Data represent an average of three replicates (±S.D.).

It was previously reported that the content of capsaicins in habanero, serrano and pimiento morrón 1.95, 0.40 and 0.12 g per 100 g of chilies (dry weight), respectively (Dorantes *et al*., 2000 and ref. therein).

2. 2. 2. Other Compounds

Additionally, pepper extracts are expected to contain chemical entities other than vanilloid compounds. Major glycolipids in monogalactosyl diacylglycerol (MGDG), digalactosyl diacylglycerol (DGDG) and sulfoquinovosyl diacylglycerol (SQDG) were extracted from dried sweet pepper *Capsicum annuum var. angulosum* (Kuriyama *et al*., 2005).

High phenolic content in *Capsicum annuum var. aviculare* (Tepin) were reported by Oboh and Rocha. In 100 g of fresh weigh they registered 218.2 mg of free and 42.5mg of bound poliphenols, respectively (Oboh and Rocha, 2007).

Ripe fruits of chili *Capsicum annuum* L. are good source of carotenoid pigments. Capsanthin, mutatoanthin, lutein, zeaxantin, β-cryptoxanthin and β-carotene were obtained from the paprika extract of different varieties of pepper (*Capsicum annuum*) by Mouly *et al*., 1999. Years later, studying antioxidant content of various fruits and vegetables ascorbate, β-carotene, lutein, lycopene in paprika extract were reported by Bunkova *et al*., 2005.

The capsanthin and capsorubin fatty acid esters are the main components of paprika pigments; they represent 50% of the pigments (Mouly *et al.*, 1999 and ref. therein). The red

carotenoids of chili are mainly 3-hydroxy-k-end group components: capsanthin, capsorubin and capsanthin 3,6-epoxide (Fig. 2.3) (Maoka *et al.*, 2001).

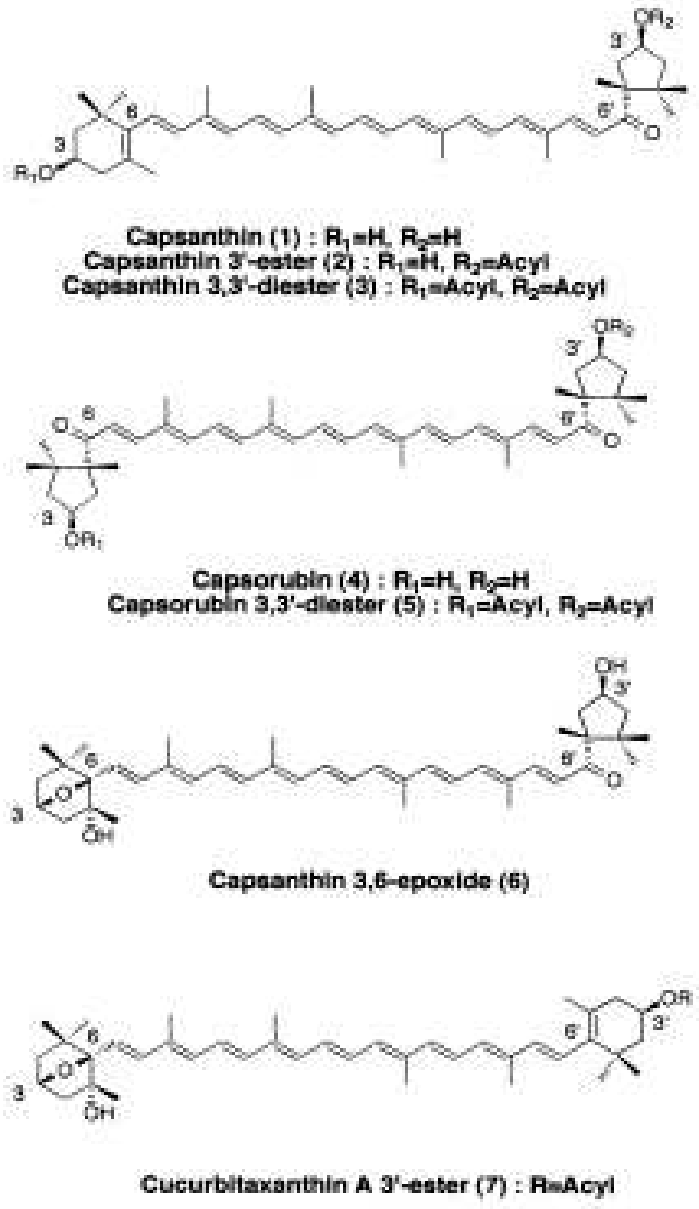


Figure 2.3. Capsanthin (1) and related carotenoids (2–7) isolated from the fruits of red paprika Capsicum annuum L. *(Maoka et al., 2001).*

Red chilis are very rich in vitamin C, vitamin A and α-tocopherol. In addition, peppers are a good source of most B vitamins, and vitamin B6 in particular. They are very high in potassium and high in magnesium and iron (Ahn *et al.*, 2006).

2. 2. 3. Polyamines

The main polyamines are putrescine (Put), spermidine (Spd) and spermine (Spm). They are synthesized from ornithine and are interconvertible. Their chemical structure is as follows: putrescine, $^{+}$H3N-(CH2)4-NH3^{+}; spermidine (Spd), $^{+}$H3N-(CH2)3-NH-(CH2)4-NH3^{+}; spermine (Spm), $^{+}$H3N-(CH2)3-NH-(CH2)4-NH-(CH2)3-NH3^{+}. An exogenous supply of polyamines is provided by dietary intake and by intestinal absorption from the products of bacterial metabolism. Polyamine uptake occurs almost entirely in the gut, and afterward the various forms are metabolized in different tissues under the strict regulation of ornithine decarboxylase. Polyamines establish hydrogen bridges with hydroxylic solvents such as water and alcohols, and for this reason, they are water soluble (Kalac and Krausov, 2005).

Polyamines can be synthesized from ornithine by a reaction catalyzed by the enzyme ODC, which produces putrescine. Spermidine derives from putrescine after the addition of a propylamine group derived from the decarboxylated S-adenosylmethionine (SAM) by the action of spermidine synthase. Spermidine is similarly converted into spermine by the enzyme spermine synthase that adds a second propylamine group from SAM to the spermidine (Figure 2.4).

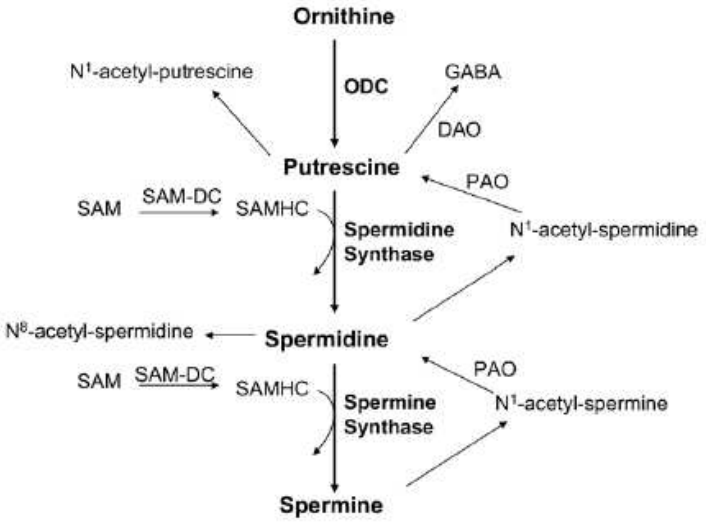


Figure 2.4. Synthesis and interconversion of polyamines. *DAO, diamine oxydase; GABA, γ-aminobutyric acid; ODC, ornithine-decarboxylase; PAO, polyamine-oxydase; SAM, S-adenosylmethionine; SAM-DC, S-adenosylmethionine decarboxylase; SAM-HC, S-adenosylmethionine homocysteamine (Kalac and Krausov, 2005).*

The normal adult diet provides a daily supply of polyamine estimated at several micromoles. The polyamine content of foods varies widely, ranging from a few nanomoles to micromoles per gram; furthermore, the proportions of the different polyamines vary with food type. Polyamines are found not only in foods of vegetable origin (fruits and vegetables), but also in those of animal origin (milk, eggs, meat, and fish) (Larqué *et al.*, 2007).

Kalac and colleagues analyzed polyamine content in selected foods. Very low Put contents were observed in processed meats, pork liver and kidney, while the highest mean contents exceeded 55 mg kg^{-1} in stewed green pea, grapefruit and fresh pepper. Higher Spm than Spd contents were typical for foods of animal origin, while the opposite was observed in plant products. Mean Spd contents, exceeding 20 mg kg^{-1}, were found in dry soybean, stewed green pea, yellow pea puree and roasted chicken breast (Kalac, *et al.,* 2005). A high Put concentration was found in green pepper (706.6 ± 37.6 nmol g^{-1} FW) (Zapata *et al.*, 2004).

In plants the content of polyamines is associated with stress. Tang *et al.,* (2007) have found that enhanced stress tolerance in transgenic pine expressing the *Capsicum annuum* pathogen and freezing tolerance-related protein 1 (CaPF1) gene is associated with the polyamine biosynthesis and this pepper transcription factor may be used to engineer pine species for multiple stress tolerance (Tang *et al*., 2007).

Polyamine levels change under stress conditions, in some cases increasing as a consequence of the stress. Decrease of Put in the variety of plants exposed to high salinity was reported by Zapata, (2004). However, salinity caused a significant increase in Spd concentrations in lettuce, pepper, broccoli, beetroot and tomato. Significant increases in Spm levels (from 1.9 to 9.4-fold) were found in lettuce, melon, pepper, broccoli and tomato seedlings under saline treatment. General trends in decreased Put levels accompanying with increasing Spm and/or Spd reflects the shift of Put pool to Spd and Spm synthesis (Zapata *et al.* 2004).

Interestingly, Put treatment (0.1 mmol/L) influenced enhancement of growth and capsaicin production in the cell suspension cultures of *C. frutescens* (Sudha, 2003).

Polyamines are fully protonated at physiologic pH and are bound by strong interactions with polyanionic macromolecules such as DNA and RNA, with only around 7–10% of the total

cell content remaining as free polyamines. Polyamines are also bound to membrane structures such as phospholipids, especially in erythrocytes. Polyamines play an important role in regulating cell growth and proliferation, the stabilization of negative charges of DNA, RNA transcription, protein synthesis, apoptosis, and the regulation of the immune response (Larqué *et al.*, 2007).

2. 3. Daily Intake and LD50 Values of Capsaicin

Hexane, chloroform, and ethyl acetate extracts of *Capsicum frutescens* fruit at 200 mg/kg resulted in death of all mice (Johnson, 2007). Capsaicin dissolved in dimethyl sulfoxide (DMSO) has been studied for determination of LD50 values by several administration routes in different species. The order of sensitivity (as per LD50), from least to most, by the intraperitoneal route was reported to be: hamster (>120mg/kg), rabbit (>50mg/kg), rat (9.5mg/kg), mouse (6.5–7.65mg/kg), and guinea pig (1.1mg/kg). Human exposure to dietary capsaicinoids in the USA and in European countries is about 1.5mg/day, which translates into, at most, 0.025mg/kg/day. In Mexico and in the Asian countries like Korea, Thailand, and India, the intake of capsaicinoids can be as high as 150 mg/day, i.e., 2.5mg/kg/day. A report from the European Commission's Scientific Committee on Food (2002) concluded that the available evidence does not allow establishment of a safe maximum exposure level for capsaicinoids in food. The daily intake of the chili pepper consumers was estimated to be 4 mg capsaicinoids/kg body weight and a safety factor of 20 was applied. In addition, general limits of 5 ppm for foods and beverages, 10 ppm for hot foods and beverages, 20 ppm for hot ketchup and 50 ppm for tabasco, harissa, hot pimento oils and similar preparations expressed as total capsaicinoids were suggested.
http://europa.eu.int/comm/food/fs/sc/scf/out120_en.pdf

2. 4. Effect of Chili Extracts and Capsaicin

The therapeutic potentials of spices, including chili, and their side effects are known for a long time in folk medicine. However, formal studies of the toxicological potential of capsaicin in vivo began in 1935, when a capsaicin extract was reported to produce a fall in blood pressure accompanied by variable effect on the respiration, an increase in salivary secretion, and a relatively small increase in gastric secretion in dogs. Pure trans-capsaicin when delivered directly into the systemic circulation induces transient increases in heart rate and blood pressure and does not cause alterations in cardiac repolarization. Capsaicin was found to be rapidly eliminated in dogs. Based on in vitro metabolism studies with capsaicin using human and dog liver microsomes it is believed that capsaicin will more likely follow the same elimination pattern in humans as observed in dogs. Studies for possible target organ toxicities didn't revealed any deaths, article-related organ weight changes, and macroscopic or microscopic observations, except vocalization by the dogs receiving 0.3mg/kg/day capsaicin, minimally higher alanine aminotransferase (ALT) for male and female dogs with statistically significant difference for females only. The latter may indicate the liver as a possible target organ when capsaicin is delivered at high doses directly into the systemic circulation (Chanda, 2005 and ref. therein).

2. 4. 1. Antimicrobial Effect

The antimicrobial properties of chili pepper have been widely exploited. The inhibitory action of the pepper extracts was evaluated against *Listeria monocytogenes* Murray, Webb and Swann Scott A by the agar diffusion test. The results showed that three of the 10 extracts had an antibacterial effect. Guajillo San Luis pepper presented the highest inhibitory effect, and contained the major concentration of cinnamic and caffeic acids. Analyses of individual phenylpropanoid to the bacteriostatic effect showed no significant differences in the *t*-cinnamic, *o*-coumaric, *m*-coumaric, ferulic acids and capsaicin among

the samples. A possible mechanism of action of compounds is the inhibition of glucose uptake and ATP production in the cells of *L. monocytogenes* (Acero-Ortega *et al.*, 2005).

The inhibitory effect of three chilli (*Capsicum annum*) extracts against *Listeria monocytogenes*, *Staphylococcus aureus*, *Salmonella typhimurium* and *Bacillus cereus* was investigated by Dorantes and collegues. The identified capsaicinoids and their derivatives were evaluated as microbial inhibitors. It was concluded that cinnamic and *m*-coumaric acids contributed to the inhibition of the four bacteria via the chilli extracts. The pimiento morro´n extract showed the greatest effectiveness on the inhibition of the four bacteria, giving an inhibition zone of 12, 11, 7 and 5 mm. The serrano pepper gave inhibition zones of 9, 7, 6.5, and 1.5 mm; and the habanero 8, 2, 5 and 1.5 mm, accordingly (Dorantes *et al.*, 2000). Vanillin, an analogue of capsaicin, has also been shown for inhibitory activity towards the growth of yeast and mould (Dorantes *et al.*, 2000 and ref. therein).

Inactivation of *Salmonella typhimurium* and dose-related antibacterial and bacteriostatic activity of the bell pepper (*Capsicum annuum*) extract on *Pseudomonas fluorescens* in raw meat were found by Careaga *et al*., 2003.

However, Sagdic and coauthors have found no inhibitory effect of red pepper on growth of 14 lactobacilli from intestine (*Lactobacillus plantarum* C27, *L. plantarum* C32, *L. plantarum* C39, *L. plantarum* P33, *L. plantarum* P51, *L. plantarum* P53, *L. fermentum* C47, *L. fermentum* P37, *L. fermentum* P38, *L. fermentum* P46, *L. fermentum* P54, *L. coryniformisubsp. torquens* C33, *L. animalis* C26, *L. acidophilus* P49 and *Lactobacillus* sp. C41) and pathogenic bacteria that cause food-born illness (*Staphylococcus aureus* ATCC 28213, *S. aureus* ATCC 2392, *S. aureus* 28h, *S. aureus* 29h, *S. aureus* 10b, *S. aureus* 10c, *Escherichia coli* ATCC 25922 and *Yersinia enterocolitica* ATCC 1501) (Sagdic *et al*., 2003). It was noted that some spices (mustard, pepper and coriander) stimulate the growth of some lactic acid bacteria used as starter cultures.

Capsaicin has in vitro activity against *H. pylori*. This may provide a new and alternative treatment approach in eradication of clarithromycin- and metroniclazole-resistant strains. In the experiments with sixteen clinic isolates (including eight metroniclazole resistant strains) and references strain (NCTC 11637) of *H. pylori* with various concentrations of the capsaicin solution, bactericidal effect was observed even at the lowest prepared concentration of capsaicin (25 mu g ml^{-1}). The best effect was seen at

concentration of 50 mu g ml^{-1} within 4 h of incubation. The profound effect of capsaicin on *H. pylori* suggests that small doses of capsaicin can help in treatment of gastric and duodenum ulcer (Zeyrek and Oguz 2005). Capsaicin also exhibits anti-fungal activity (Renault *et al.*, 2003).

2. 4. 2. Antioxidant Effect

The antioxidant activity of chili peppers has also been shown in variety of studies. Antioxidant compounds and their antioxidant activity in 4 different colored (green, yellow, orange, and red) sweet bell peppers (*Capsicum annuum* L.) were investigated by Sun T. *et al*., 2007. The total phenolics content of green, yellow, orange, and red papers were 2.4, 3.3, 3.4, and 4.2 mu mol catechin equivalent/g fresh weight, respectively. The red pepper had significantly higher total phenolics content than the green pepper. Among the 4 different colored peppers, red pepper contained a higher level of beta-carotene (5.4 mu g/g), capsanthin (8.0 mu g/g), quercetin (34.0 mu g/g), and luteolin (11.0 mu g/g). The yellow pepper had the lowest beta-carotene content (0.2 mu/g/g), while the green one had undetectable capsanthin and the lowest content of luteolin (2.0 mu g/g). All 4 colored peppers exhibited significant abilities in preventing the oxidation of cholesterol or docosahexaenoic acid (DHA) (C22:6) during heating.

Total reducing capacity (TRC) and contributions of hydrophilic reducing capacity (HRC) and lipophilic reducing capacity (LRC) to the TRC as well as three antioxidant compounds were measured: ascorbic acid, beta-carotene and lycopene were measured in fresh and processed peppers *(Capsicum annuum)* and analyzed by Greco *et al*. Fresh pepper had the highest TRC, the highest HRC and the greatest content of ascorbic acid. HRC and ascorbic acid content decreased with processing, whilst LRC was generally increased. Ascorbic acid was the major component of HRC in all samples (ranging from 72% in peperonata to 88% in fresh pepper), confirming the high content of this vitamin in peppers. Lycopene was detected only in peperonata. Many liposoluble compounds present in the lipophilic extract were not identified (only 6-20% of LRC was beta-carotene) (Greco *et al.*, 2007).

Ferric reducing activity of capsicum is 2.46 mM for 100 g of the food prepared as a water/methanol extract (Collins, 2005 and ref. therein).

Capsaicin can act as an antioxidant, as revealed by attenuation of oxidative damage or lipid peroxidation in various organs of experimental animals. Capsaicinol, a nonpungent analog recently isolated from chili pepper, exhibited more potent antioxidant activity than does capsaicin (Surh, 2002 (a) and ref. therein).

Polyphenols exhibit a wide range of biological effects because of their antioxidant properties. Extract from *Capsicum annuum* var. *aviculare* (Tepin) had a significantly high Fe (II) chelating ability, OH radical scavenging ability as well as significant inhibitory effect on basal and the various pro-oxidant (25 mu M Fe(II), 7 mu M sodium nitroprusside and 1 mM quinolinic acid)-induced lipid peroxidation in rat's brain and liver in a dose-dependent manner. These effects were probably due to high amounts of free polyphenols, which can also explain the inhibition in the MDA (malondialdehyde) production in the brain and liver homogenates (Oboh and Rocha, 2007).

Kwon *et al.* have found that several pepper extracts had high alpha-glucosidase inhibitory, activity, which was not correlated to total phenolic content and free radical scavenging-linked antioxidant activity (Kwon *et al.*, 2007).

2. 4. 3. Anti-mutagenic Effect

As a result of widespread human exposure, capsaicin has been the subject of several genotoxicity assays and limited tumorigenicity assays. Early reports of the genotoxicity and tumorigenicity of capsaicin in vitro and in vivo are inconsistent and contradictory.

Antimutagenic effect of selected plant extracts (carrot, paprika, tomato, spinach, onion, kiwi and green tea) was analysed by two independent tests of genotoxicity: i) the Ames test with *Salmonella typhimurium* TA98 and ii) cytogenetic analysis of peripheral blood lymphocytes (CAPL) without and/or with metabolic activation. Samples were allowed to be positive antimutagens based on their ability to inhibit the mutagenic effects of standard mutagens. Extracts from paprika, carrot and spinach as well as solutions of standard all trans-β-carotene and some standard flavonoids showed antimutagenic (40–60%

of inhibition) or weakly antimutagenic (20–40% of inhibition) effects (Bunkova *et al*., 2005).

Both positive and negative effects have been found in classical genetic toxicology assays, including the Ames, mouse lymphoma cell mutation, mouse in vivo bone marrow micronucleus and chromosomal aberration in human peripheral blood lymphocytes (HPBL) with capsaicin. In the Ames assay, pure *trans*-capsaicin was not mutagenic to *Salmonella typhimurium* or *Escherichia coli* when dissolved in dimethylsulfoxide and was weakly mutagenic in mouse lymphoma L5178Y cells tested at concentrations extending into the toxic range. *Trans*-capsaicin did not induce micronuclei in bone marrow cells when tested to the maximum tolerated dose of 800 mg/kg per day in male and 200 mg/kg per day in female CD-1 mice using a 0 h plus 24 h oral dosing and 48 h sampling regimen. Finally, *trans*-capsaicin did not induce structural or numerical chromosomal aberrations when evaluated for its ability to induce clastogenicity in blood lymphocytes (Chanda *et al*., 2004).

Capsaicin was assessed in bacterial gene mutation, *in vitro* chromosome aberration, and rodent bone marrow micronucleus tests by Proudlock group. The results confirm the absence of genotoxic activity of high-purity capsaicin in the bacterial mutation and chromosome aberration tests. In addition, no evidence of cytotoxicity or genotoxicity was seen in the rat bone marrow micronucleus test, where systemic exposure to pure capsaicin was achieved using the subcutaneous route and a rising dose toleration protocol. It was concluded that pure capsaicin is not active in the standard battery of genotoxicity assays recommended by the International Conference on Harmonisation for evaluation of new medicines. Earlier reported *in vitro* genotoxic activity was attributed to the mutagenic impurities in commercial grades of the material (Proudlock *et al.*, 2004).

Capsaicin displayed inhibitory effects on metabolism and covalent DNA binding of aflatoxin B. Extracts of hot red pepper also exerted protection against aflatoxin B -induced bacterial mutagenesis (Kim *et al*., 1991). Capsaicin induced the formation of micronuclei and sister chromatid exchange in a dose-dependent manner in the cytokinesis-blocked lymphocytes (Marques *et al*., 2002).

Four different Thai traditional chili peppers, namely bird pepper (*Capsicum frutescens*), red chili spur peppers (*Capsicum annuum*), green bell peppers and sweet pepper (*C. annuum*) were investigated for their antimutagenic properties against urethane by using

the somatic mutation and recombination of wing hair of *Drosophila melanogaster* as an indicator. Each chili was prepared in three formulations commonly used for chili food processing; raw paste (chili ground in water), pickled in vinegar or stir-fried in palm oil. The various processing methods for chilies differentially extracted the antimutagenic chili components. It was found that the specific chili as well as the method of processing influenced the observed antimutagenic properties against urethane. The authors suggested that each chili contains a unique complex mixture of many antimutagens. Co-treatment and pre-treatment experiments showed that both direct and indirect protective mechanisms are involved in an 'activation' process to give antimutagenesis effects (Laohavechvanich *et al.*, 2006).

2. 4. 4. Anti-inflammatory Effect

Capsaicin is considered to exhibit anti-inflammatory properties. Oral feeding of the spice principles capsaicin (5 mg/kg body weight) to rats on specific dietary lipids had beneficial effects on arthritis. The most significant effect of spice principle feeding was in delaying the progression of arthritis. However, the spice principles had very little effect on the overall incidence of arthritis in experimental animals when the chronic stage of the disease was manifested (Joe and Lokesh, 1997).

It was demonstrated that capsaicin inhibited the production of pro-inflammatory mediators such as prostaglandin E2 (PGE2), Cyclo-oxygenase-2 (COX-2) and nitric oxide by nuclear transcription factor kappa B (NF-κB) inactivation in murine peritoneal macrophages. Interestingly, the inhibitory action of capsaicin on the release of pro-inflammatory molecules was not mediated by a vanilloid receptor-1 (VR-1), which is a specific receptor for capsaicin, indicating the involvement of an alternative mechanism (Kim *et al*., 2003).

Topical application of capsaicin has been reported to alleviate the painful osteoarthritis of the hands (Bina and Lokesh, 1997). The authors have shown also that dietary curcumin and capsaicin lower the generation of proinflammatory mediators such as reactive oxygen species and nitric oxide released by macrophages. The spice species can

delay the progression of the disease and also reduce the extent of inflammation in arthritic animals. The anti-inflammatory effects of spice principles were comparable with that of known anti-inflammatory drugs such as aspirin, indometbacin and piroxicam.

Capsaicin, when treated to macrophages, repressed calcium-ionophore stimulated pro-inflammatory responses, such as generation of superoxide anion, phospholipase A activity and membrane lipid peroxidation. Although topical application of capsaicin can initially induce ear edema in mice and neurogenic inflammation in human skin, reapplications of the compound suppressed the subsequent inflammatory response. A single oral dose of capsaicin 5 mg/kg body weight lowered the carrageenan-induced inflammation in rats by 29% (Surh *et al.*, 1998).

A comparison of the extent of carrageenan-induced paw inflammation showed that both dietary curcumin and capsaicin moderately lowered inflammation, while the spice principles in combination were more effective. Dietary curcumin and capsaicin significantly decreased the activity of 5'-lipoxygenase activity in the polymorphonuclear lymphocytes in carrageenan-injected rats, the decrease being even higher in the case of combination of these two spice principles (Manjunatha and Srinivasan, 2006).

The anti-inflammatory action of capsaicin may be mediated by peroxisome proliferators-activated receptors (PPAR) activation in LPS-stimulated RAW 264.7 cells, which regulates the production of the pro-inflammatory cytokine TNF-α. It was reported that capsaicin suppresses the production of TNF-α by acting as an agonist for PPAR in LPS-stimulated murine macrophages RAW 264.7. Capsaicin may be a naturally occurring ligand for PPAR, which can be useful therapy against inflammation (Park *et al.*, 2004).

While many studies have demonstrated the anti-inflammatory activity of capsaicin, some other investigators observed opposite properties of this compound. Thus, capsaicin, through selective excitation of C-fibers or suppression of substance P, causes neurogenic vasodilation and plasma extravasation. Prostaglandin biosynthesis by bull seminal vesicle homogenates has also been stimulated in the presence of capsaicin. However, although capsaicin can cause neurogenic inflammation *per se* under certain physiologic conditions, it also has analgesic and anti-inflammatory activities and is used currently in topical creams and gels (e.g., Axsain and Zostrix) to mitigate neurogenic pain (Surh, 2002).

2. 4. 5. Immune System Effects

Mitogenic responses of B and T lymphocytes from the spleens of newborn, albino NMRI mice single dosed with capsaicin injection in 50 mg/kg in stock solution were examined after 4 to 6 weeks. The mitogens added to spleen cell cultures were: lipopolysaccharide (LPS), pokeweed mitogen (PWM), dextran sulfate, concanavalin A (ConA), and phytohemagglutinin (PHA). It was found that the growth of mice injected with capsaicin was significantly lower (83% of normal) at day 28 and 35 post injection compared to control mice. In all, however, the authors concluded that neonatal capsaicin treatment did not affect the overall immunocompetence of animals tested, because essentially normal responses to B and T lymphocyte mitogens were obtained (Johnson, 2007).

The regional lymph node response to an antigen stimulus was evaluated using the plaque-forming cell (PFC) assay after injection of 50 mg/kg capsaicin. Capsaicin caused a > 80% reduction in the number of cells secreting antigen-specific antibody. This effect was reversed by infusion of substance P after antigen stimulation. The authors suggested that the reduced response of capsaicin-treated rats to an antigenic stimulus was due to an effect of capsaicin on the substance P-containing primary afferent nerves, instead of a toxic effect of capsaicin on the immune system (Johnson, 2007).

The effect of capsaicin on lymphocyte proliferation using groups of five immunized with ovalbumin specific pathogen-free rats was investigated in presence of suboptimal, optimal, or supraoptimal concentrations of ConA. For neonatal and adult rats, the lymphocyte proliferation rate in the presence of suboptimal concentrations of ConA was significantly lower in spleen mononuclear cells (SMCs) from rats dosed with capsaicin than in cells from untreated rats. Proliferative responses of SMC to supraoptimal concentrations of ConA (10 μg/ml) were not decreased significantly in animals dosed with capsaicin. The authors concluded that, compared to normal rats, rats with capsaicin-induced neurologic dysfunction have an altered immunologic response (Nilsson and Ahlstedt, 1988).

The injection of capsaicin resulted in a significant decrease in the intestinal immune response, i.e., significant decrease in intestinal antibodies to cholera toxin in C57/black mice immunized with cholera toxin (Jarrah *et al*., 1995).

The modulation of immune responses by dietary capsaicin was evaluated. Groups of pathogen-free female BALB/c mice were fed diets containing 5, 20, 50, or 100 ppm capsaicin for 3 weeks. Control mice received diet only. No significant differences in growth rate or weight gain between test and control groups were reported. Additionally, relative weights of the liver and spleen were not found to be significantly different when all test groups were compared. In mice fed 20 ppm capsaicin, the number of splenocytes and antibody-producing cells was increased. Mice fed 20 ppm capsaicin also had enhanced T-cell mitogen-induced lymphocyte proliferation. Maximum stimulation of antibody and TNF-alpha production was also associated with this dietary concentration of capsaicin (20 ppm). Increased serum immunoglobulin (IgG and IgM) concentrations were reported for groups fed 20 and 50 ppm capsacin in the diet. The authors suggested that dietary capsaicin may differentially enhance immune status as well as select immune functions (Yu *et al.*, 1998). Capsaicin treatment resulted in marked inhibition of natural killer cell activity and antibody-dependent cytotoxicity (Santoni *et al.,* 1995).

A neurotoxic dose of capsaicin produced phasic changes in functional activity of sensory neurons and venous blood neutrophils of Wistar rats. Early after capsaicin injection, the sensitivity of neutrophils to bacterial stimuli decreased against the background of neuropeptide release from the sensory terminals (Zhukova and Makarova 2002).

2. 4. 6. Effect on Diabetes

One very promising avenue of research lies in the prevention and control of hyperglycemia and diabetes; according to a study the amount of insulin required to lower blood sugar after a meal is reduced if the meal contains chili pepper (Ahuja *et al*, 2006).

Canadian researchers believe that chilies might play a vital role in treating Type I diabetes. They have proved the nervous system helps trigger diabetes. It was reported that $TRPV1^+$ pancreatic sensory neurons control islet inflammation and insulin resistance. Eliminating these neurons in diabetes-prone NOD mice prevents insulitis and diabetes, despite systemic persistence of pathogenic T cell pools. Insulin resistance and β cell stress of prediabetic NOD mice are prevented when $TRPV1^+$ neurons are eliminated. Delivering the

neuropeptide substance P by intra-arterial injection into the NOD pancreas reverses abnormal insulin resistance, insulitis, and diabetes for weeks. This finding upsets conventional wisdom that Type 1 diabetes, the most serious form of the illness that typically first appears in childhood, was solely caused by auto-immune responses. It was also concluded that there are far more similarities than previously thought between Type 1 and Type 2 diabetes. (Razavi *et al*., 2006).

Another study showed that calcitonin gene-related peptide (CGRP)-expressing fibers in the pancreatic islets also express TRPV1. These effects were accompanied by enhanced insulin secretion and a virtually complete loss of CGRP- and TRPV1-coexpressing islet-innervating fibers. These data indicate that CGRP-containing fibers in the islets are capsaicin sensitive, and that elimination of these fibers contributes to the prevention of the deterioration of glucose homeostasis through increased insulin secretion in Zucker diabetic fatty (ZDF) rats, which are used to study various aspects of human type 2 diabetes. Based on these data it was proposed that the activity of islet-innervating capsaicin-sensitive fibers may have a role in the development of reduced insulin secretion in human type 2 diabetes mellitus (Gram *et al*., 2007).

2. 4. 7. Other Effects

Using a randomized cross-over design, the effects of 4-week regular consumption of chili, as a flavouring agent, on some indicators of metabolic and vascular function, included serum lipids and lipoproteins, insulin, C-peptide, plasma glucose, basal metabolic rate, blood pressure, augmentation index (an indicator of arterial stiffness) and subendocardial-viability ratio (a measure of effective myocardial perfusion pressure time) was examined. It was concluded that four weeks of regular chili consumption has no obvious beneficial or harmful effects on metabolic parameters but may reduce resting heart rate and increase effective myocardial perfusion pressure time in men (Ahuja *et al.,* 2007).

There is no scientific evidence that a spicy meal based on red hot chili pepper may worsen hemorrhoidal symptoms and, therefore, there is no reason to prevent these patients from occasionally enjoying a spicy dish if they so wish (Altomare *et al.*, 2006).

Capsaicin has the ability to inhibit platelet aggregation possibly through blockade of phospholipase A2, a key enzyme responsible for formation of arachidonic acid from the membrane lipid (Srinivasan, 2005 and ref. therein).

Capsaicin, along with other phytochemicals, such as curcumin, [6]-gingerol, and resveratrol, has inhibitory effects on P-glycoprotein which mediates resistance to various classes of chemotherapeutic agents including vinblastine, vincristine, daunorubicin, doxorubicn, colchicine, paclitaxel, and etoposide, by actively extruding the drugs from the cells to lower the intracellular concentrations. Therefore it has potencies to cause food–drug interactions (Nabekura *et al*., 2005).

Capsaicin pretreatment also protects against the free radical-induced pulmonary damage in rats exposed to such gaseous chemical irritants as sulfur dioxide and nitrogen dioxide. Ultraviolet-induced lipid peroxidation in liposomal membrane was similarly attenuated by capsaicin (Surh *et al.*, 1998).

Three spices, chili, black pepper, and turmeric, were tested for the effect of their aqueous extracts on the sensitivity of three bacteria, Escherichia coli, Bacillus megaterium, and Bacillus pumilus spores, to gamma-radiation. It was found that the extracts of the three spices offered protection to these organisms against inactivation by gamma-radiation. These spice extracts were also tested for their protection of naked plasmid DNA. Radiation-induced degradation of plasmid pUC18 DNA was reduced in the presence of the spice extracts. The maximum protection was offered by the chili extract followed by that of black pepper and turmeric (Sharma *et al.*, 2000).

Effect of capsaicin on photosynthetic activity was reported by Spyridaki and coauthors. It was found that crystal structure of capsaicin is bound to the quinone (QB) site of a bacterial photosynthetic reaction center and inhibit photosynthetic activity with an IC50 of ~10 µM by replacing QB from its binding site at the interface between the aqueous medium and the membrane (Spyridaki *et al.*, 2000).

2. 5. Capsaicin (Vanilloid) Receptor

The role of capsaicin as a modulator of the sensory nervous system has been widely studied. Capsaicin is a highly selective agonist for the transient receptor potential vanilloid receptor 1 VR1, also known as TRPV1 (Table 2.3). VR1 forms a ligand-gated nonselective cation channel in the plasma membrane that mediates some of the pleiotropic effects exerted by capsaicin and its analogues.

Table 2.3. Biochemical Pharmacology of Vanilloid Receptors *(Szallasi and Blumberg, 1999).*

Receptor family Signal transduction	TRP family of store-operated calcium channels non-selective cation channel
Size	95kDa (cloned VR1); 300 kDa (radiation inactivation size)
Typical natural agonists	RTX, capsaicin, isovelleral, scutigeral
Other activators	low pH, noxious heat
Antagonistat	capsazepine (competitive); ruthenium red (functional)

Vanilloid-sensitive neurons are heterogeneous morphologically (expressed in C-and Aδ-fibers), neurochemically, and functionally and they encompass several subclasses of dorsal root ganglion (DRG) neurons. Vanilloid-sensitive neurons transmit noxious information (usually perceived as itching or pain) to the CNS (afferent function), whereas peripherial terminals of these neurons are sites of release for a variety of proinflammatory neuropeptides (efferent function). These neuropeptides are believed to play an important role in initiating the cascade of neurogenic inflammation (Szallasi and Blumberg, 1999).

VR1, which can also be stimulated with heat and physical abrasion, permits cations to pass through the cell membrane and into the cell when activated (Welch *et al.,* 2000). By binding to the VR1 receptor, the capsaicin molecule produces the same effect that excessive heat or abrasive damage would cause, explaining why the spiciness of capsaicin is described as a burning sensation. According to an emerging concept, hydrogen ions, heat, and capsaicin (or putative endogenous capsaicinoids) may act synergistically to activate VR1 (Tominaga *et al.,* 1998). Thus, a combination of otherwise harmless heat and tissue acidosis may become very painful.

Apparently, it is not capsaicin but heat that has the capability of opening the channel pore of VR1, whereas capsaicin and protons only serve to lower the heat threshold of the receptor. Consequently, even room temperature is able to gate VR1 in the presence of mildly acidic conditions and/or capsaicin (Tominaga *et al*., 1998).

The binding of capsaicin molecule to VR1 receptor is illustrated in the Fugure 2.5.

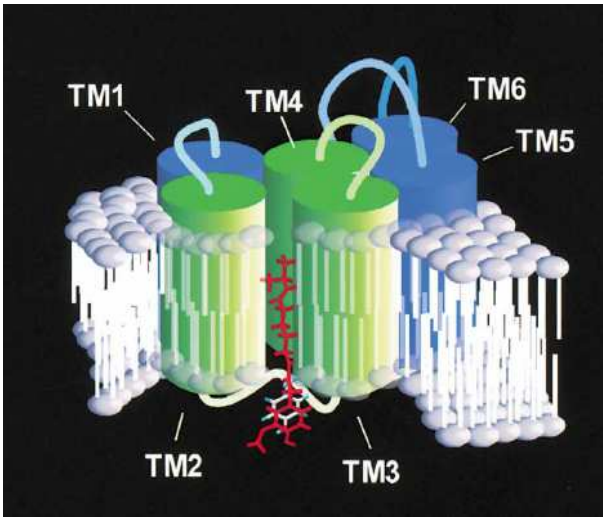


Figure 2.5. Structural model of capsaicin bound to the vanilloid receptor. The vanillyl moiety of capsaicin (red) is shown to interact with an aromatic residue located in the cytosolic region linking TM2 and TM3. Within the plane of the membrane, the ligand interacts with the TM2-3 region at the channel periphery (green). Structural rearrangements induced by ligand binding may be transduced through TM4 (green) into the channel core (Jordt and Julius, 2002).

Responses to capsaicin may lead to desensitization, depending on the dose of capsaicin and the duration of exposure. Receptor desensitization and long-term loss of sensitivity occurs through a variety of related mechanisms, including conformational changes in VRs and, ultimately, closing of channel pores, by depletion of neuropeptide transmitter substances and by downregulated loss of binding sites in peripheral nerves and the central nervous system. In humans, intradermal administration of high doses of capsaicin produces degeneration with subsequent regrowth of local nerve endings. Pharmacological and physiological studies have demonstrated that capsaicin, produces its sensory effects by activating a Ca^{2+}-permeable ion channel on sensory neurons. Studies on structure-activity relationships of agonists and the development of the antagonist capsazepine have shown that the activation of this ion channel is mediated by a classical receptor-based mechanism (Johnson, 2007). Capsaicin responses in sensory neurons exhibit robust potentiation by cAMP-dependent protein kinase (PKA), which reduces VR1 desensitization and directly phosphorylates VR1 (Bhave *et al*., 2002).

Proinflammatory neuropeptides release from sensitive nerve endings in response to vanilloinds initiate the cascade of neurogenic inflammation. Among these neuropeptides, substance P (SP) and calcitonin gene-related peptide (CGRP) are the best studied. SP preferentially interacts at NK-1 receptors (NK-1R). Stimulation of NK-1Rs in endothelial cells leads to plasma extravasation (edema formation) by opening gaps at postcapillary venules. SP can stimulate myoepithelial cells in submucosal glands to produce mucus and activate alvelolar macrophages. Finally, SP was shown to stimulate human neutrophils and T lymphocytes, although these actions are probably not mediated by tachykinin receptors (Szallasi and Blumberg, 1999). Topical capsaicin, or red pepper extract, which is FDA approved, can be enormously helpful in treating diabetic neuropathy by releasing substance P (an amino acid peptide associated with pain) http://www.pathmed.com/p/136,438.html. Another report shows depletion of substance P from local sensory nerve terminals by topical application of capsaicin, and hence suppressed the substance P-mediated flare response in human skin (Surh *et al.*, 1998). Topical exposure of capsaicin, sometimes in combination with an analgesic or local anesthetic to reducing burning, has been used to give long-lived relief from pain in a wide range of debilitating medical conditions, including posttherapeutic neuralgia following shingles, arthritis, polyneuropathy (Proudlock *et al.*, 2004 and ref. therein).

Since CGRP increases production of insulin-like growth factor-I (IGF-I) in fetal osteoblasts *in vitro*, it is possible that sensory neuron activation by capsaicin increases production of IGF-I. Harada and Okajima investigated the effect of topical application of capsaicin and related compounds on dermal IGF-I level and facial skin elasticity in humans. It was found that application of 0.01% capsaicin significantly increased dermal IGF-I levels from 30 to 180 min in mice. Topical application of 0.01% capsaicin to faces of 17 healthy female volunteers for seven days significantly increased cheek skin elasticity ($p<0.01$). These observations suggest that topical application of capsaicin and related compounds might be useful in the treatment of detrimental morphological changes of the skin in patients with growth hormone deficiency and those in the elderly by increasing dermal IGF-I levels (Harada and Okajima, 2007).

To investigate the role of C-fibers on hernatopoiesis, wistar rats were neonatally injected with either capsaicin or its vehicle, and used at adult ages (8-10 weeks). In

capsaicin-pretreated rats, the levels of substance P (SP) in bone marrow fluid were markedly reduced in comparison with the vehicle group (13.1 +/- 4.5 pg/ml versus 47.3 +/- 5.5 pg/ml, $p < 0.05$). In bone marrow, the number of total leukocytes was 28% higher in capsaicin-pretreated group, and this accompanied by a higher number of neutrophils, particularly of the immature forms. The mononuclear cell and eosinophils counts did not differ significantly among vehicle and capsaicin groups (Franco-Penteado *et al*., 2006).

The dendritic cell, a key cell type of the vertebrate immune system, expresses VR1. Engagement of VR1 on immature dendritic cells such as by treatment with capsaicin leads to maturation of dendric cells as measured by up-regulation of antigen-presenting and costimulatory molecules. These data demonstrate a powerful influence of a neuroactive ligand on a central aspect of immune function and a commonality of mechanistic pathways between neural and immune functions (Basu and Srivastava, 2005).

Capsaicin was found to activate mast cells directly in a VR-mediated fashion (Szallasi and Blumberg, 1999). The finding that vanilloids activate sensory neurons and mast cells at similar concentrations forms another bridge between vanilloid-sensitive sensory nerves and neuroimmune regulation.

Proinflammatory cytokines like tumor necrosis factor-a (TNF-α) and interleukin-1β can enhance the capsaicin sensitivity of rat DRG neurons in culture. Following pretreatment with 10 ng/ml TNF-α, a greater than 2-fold increase in the peak amplitude of the inward current evoked by 100 nM capsaicin was described. Of relevance is the finding that intradermal injection of TNF-α or interleukin-1β lowers the response threshold to noxious stimulation (Szallasi and Blumberg, 1999).

Based on the highly selective agonistic property of capsaicin toward TRPV1 receptors, drug products containing pure synthetic *trans*-capsaicin are under evaluation as topical and injectable analgesic therapies (Bley, 2004).

2. 6. Anti-cancer Properties of *Capsicum* Peppers

2. 6. 1. Molecular Basis of Chemoprevention

Recent advances in our understanding at the cellular and molecular levels of carcinogenesis have led to the development of a new promising strategy for cancer prevention, that is, chemoprevention. Chemoprevention is defined as the use of specific chemical substances natural or synthetic or their mixtures to suppress, retard, or reverse the process of carcinogenesis. It is one of the novel approaches of controlling cancer alternative to therapy that has some limitations and drawbacks in the treatment of patients.

Carcinogenesis is characterized by a complex process that involves a series of individual steps. From the stand point of the experimental carcinogenesis in rodents, tumor development has been generally considered to consist of three distinct steps—initiation, promotion, and progression;

- Initiation is an irreversible event that begins when the cells in normal tissues are exposed to a carcinogen and their genomic DNA undergoes damage that remains unrepaired or misrepaired.
- Promotion is expansion of the damaged cells to form an actively proliferating multi-cellular premalignant tumor cell population.
- Progression is the irreversible process which produces a new clone of tumor cells with increased proliferative capacity, invasiveness, and metastatic potential.

Conventional classification of chemopreventive agents is based on the underlying mechanisms by which they exert protective effects in a specific stage of multistep carcinogenesis. According to this system, chemopreventers are subdivided into two major categories, i.e., blocking agents and suppressing agents. Blocking agents are typically those compounds that can inhibit initiation either by inhibiting the formation of carcinogens from precursor molecules or reactive metabolites from the parent carcinogens, or by preventing the ultimate electrophilic and carcinogenic species from interacting with critical cellular target molecules, such as DNA, RNA, and proteins. Suppressing agents are considered to inhibit malignant expression of initiated cells, in either the promotion or the progression stage.

Rational and successful implementation of chemopreventive strategies rely on the precise understanding of underlying molecular mechanisms. Inhibition of initiation is unlikely to be a practical approach to chemoprevention, since there are diverse types of initiators present in our environment and it is not feasible to find a chemopreventive agent that can nullify the initiating activities of all the carcinogens to which we are exposed. Therefore, recent chemopreventive strategies are more concerned with identifying substances with antiproliferative or antiprogressive activities that can suppress the transformation of initiated or precancerous cells to malignant ones, rather than search for anti-initiators (Surh, 1999).

2. 6. 2. Chili Pepper in Cancer Research

The role of capsaicin in carcinogenic processes is quite controversial. Although some investigators suspect that capsaicin is a carcinogen, co-carcinogen, or tumor promoter, others have reported that it has chemopreventive and chemotherapeutic effects.

It has been suspected that excessive ingestion of hot peppers is a high risk factor for gastric and hepatic cancers. An epidemiologic study conducted in Mexico City (Lopez-Carillo *et al*., 1994) demonstrated that, chili pepper consumers were at greater risk for stomach cancer than non-consumers. Epidemiological study among ethnic-cultural groups in the United States consuming high pepper containing cookeries, performed by Archer and Jones 2002, proved evidence that capsaicin is a human carcinogen. Other epidemiological and mortality studies have reported excess cancer of the pharynx, esophagus, larynx, liver, pancreas, stomach, and 'all cancer' and lowered rates of lung, colon, rectum, and breast cancer among high pepper consumers or among Mexican-Americans (Archer and Jones, 2002). However, an early Italian case-control study revealed that chili consumption was protective against stomach cancer (Buiatti *et al*., 1989). Furthermore, the overall frequency of gastric cancer in Mexico, where peppers are more heavily consumed than any other countries except possibly Korea, is relatively low (Surh *et al*., 1998). The same conclusion was made by scientists at the University of Nottingham who found that capsaicin can kill cells by directly targeting their energy source, indicating that people could control or prevent

the onset of cancer by eating a diet rich in capsaicin (Athanasiou *et al.,* 2006). In addition, the rate of stomach cancer in the United States has been declining despite increased consumption of chili peppers in the past two decades. Therefore, it seems unlikely that pepper consumption is linked to high risk for stomach cancer in humans. It should be noted that the etiology of human gastric carcinogenesis is complicated by multifactorial risk factors, such as salty, smoked, or pickled foods, cigarette smoking and insufficient daily intake of fruits and vegetables as well as *Helicobacter pyroli* infection (Surh *et al*., 1998).

Capsaicin exerted protective effects against ethanol-induced gastric mucosal injury in rats. Ethanol-induced haemorrhagic erosion, lipid peroxidation and myeloperoxidase activity in rats were ameliorated by intragastric capsaicin treatment, which was associated with suppression of COX-2 (Park *et al.,* 2004).

Capsanthin and related carotenoids showed potent in vitro anti-tumor-promoting activity with inhibitory effects on Epstein–Barr virus early antigen (EBV-EA) activation induced by the tumor promoter 12-O-tetradecanoylphorbol-13-acetate (TPA). Among them, capsanthin diester and capsorbin diester showed strong inhibitory effects. Furthermore, capsanthin, capsanthin 30-ester and capsanthin 3,30-diester, exhibited potent anti-tumor-promoting activity in an in vivo mouse skin two-stage carcinogenesis assay using 7, 12-dimethylbenz[a]anthracene as an initiator and TPA as a promoter (Maoka *et al*., 2001).

Capsaicin brought about morphological changes characteristic of apoptosis and/or necrosis in H460 human lung cancer cells (Athanasiou *et al.,* 2006).

The antiangiogenic activity of capsaicin using *in vitro* and *in vivo* assay systems was shown by Min *et al.,* 2004. *In vitro,* capsaicin inhibited vascular endothelial growth factor (VEGF) -induced proliferation, DNA synthesis, chemotactic motility, and capillary-like tube formation of primary cultured human endothelial cells. Capsaicin inhibited both VEGF-induced vessel sprouting in rat aortic ring assay and VEGF-induced vessel formation in the mouse Matrigel plug assay. Moreover, capsaicin was able to suppress tumor-induced angiogenesis in chick chorioallantoic membrane assay. Capsaicin caused G1, arrest in endothelial cells. This effect correlated with the down-regulation of the expression of cyclin D1 that led to inhibition of cyclin-dependent kinase 4-mediated phosphorylation of retinoblastoma protein. Signaling experiments show that capsaicin inhibits VEGF-induced p38 mitogen-activated protein kinase, p125(FAK), and AKT activation, but its molecular

target is distinct from the VEGF receptor KDR/Flk-1. Taken together, these results demonstrate that capsaicin is a novel inhibitor of angiogenesis and suggest that it may be valuable to develop pharmaceutical drugs for treatment of angiogenesis-dependent human diseases such as tumors (Min *et al*., 2004).

These extracts and fractions of *Capsicum annuum* L. *var. angulosum* Mill were investigated for their cytotoxicity, anti-human immunodeficiency virus (HIV), anti-Helicobacter pylori (*H. pylori*), urease inhibition and multidrug resistance (MDR) reversal activity. Some fractions of hexane and acetone extracts showed higher cytotoxic activity against three human oral tumor cell lines (squamous cell carcinoma HSC-2, HSC-3, submandibular gland carcinoma HSG) than against three normal human oral cells (gingival fibroblast HGF, pulp cell HPC, periodontal ligament fibroblast HPLF), suggesting a tumor-specific cytotoxic activity. No fractions displayed anti-HIV activity, but some hydrophobic fractions showed higher anti-*H. pylori* activity; urease inhibition activity and MDR reversal activity. The higher MDR activity of these fractions against MDR gene-transfected L5178 mouse lymphoma T cells may possibly be due to their higher content of carotene or polyphenol. (Shirataki *et al*., 2005).

In vitro effects of capsaicin on human T-cell leukemia virus type 1 (HTLV-1)-associated adult T-cell leukemia (ATL) were examined. Capsaicin treatment inhibited the growth of ATL cells both in dose- and time-dependent manner. The inhibitory effect was mainly due to the induction of cell cycle arrest and apoptosis. Capsaicin treatment also induced the degradation of Tax and up-regulation of IkBa resulting in the decrease of nuclear factor (NF)-κB/p65 DNA binding activity. In addition, the Bcl-2 level was found to be decreased (Zhang *et al*., 2003).

However, there are data proving chilli consumption and cancer. Analysis of pregnancy diet of mothers of 315 cases under age six at diagnosis of medulloblastoma/primitive neuroectodermal tumor (PNET), a common brain tumor in children, and 315 controls showed that chili peppers (Odds Ratio for highest compared to lowest category = 1.8, 95% confidence interval: 3.0) associated with medulloblastoma/PNET (Bunin *et al.,* 2005). Tumors incidence in both the intestine and colon were noted in 27 from 30 male Sprague-Dawley albino rats (weights = 120 to 150 g) after red chilli powder diet (8 mg/day/100 g body weight in water) for 15 weeks. At

microscopic examination, areas of dysplasia, but no areas of overt malignancy, were observed (Johnson, 2007 and ref. therein). Association between red chili pepper consumption and gallbladder cancer, liver and pancreas cancer has been also reported (Oikawa *et al.,* 2006 and ref. therein). Capsaicin is found to cause strand scission in calf thymus and plasmid DNA in the presence of Cu (II). This breakage is mediated by reactive oxygen species arising from capsaicin, especially the hydroxyl radical (Singh *et al.,* 2001).

Besides possible deleterious constituents with suspected carcinogenicity, hot chili or red peppers contain substances that may counteract the effects of carcinogens. Peppers are indeed good sources of vitamin C and b-carotene. Moreover, red pepper and its major pungent principle capsaicin have been shown to be gastroprotective (Surh *et al*., 1998).

2. 6. 2. 1. Effect on Human Cytochrome P450 (CYP)

The human cytochrome P450 (CYP) superfamily, containing 57 genes, contributes to the metabolism of a variety of xenobiotics including therapeutic drugs, carcinogens, steroids and eicosanoids. Many herbal constituents have been found to inhibit various CYPs. The nature of inhibition may be competitive, non-competitive, or mechanism-based. The latter is characterized by NADPH-, time- and concentration-dependent enzyme inactivation, occurring when some herbal constituents are converted by CYPs to reactive metabolites that are capable of irreversibly binding to CYPs. Mechanism-based inhibitors require at least one cycle of the CYP catalytic process to form reactive metabolites.

Capsaicin inhibits constitutive enzymes CYP 3A1, 2A2, 2B1, 2B2, 2C6 and 2C11. It has found to inhibit also cytochrome P450 2E1 an isoform that is involved in metabolic activation as well as detoxification of diverse low molecular weight carcinogens (Surh *et al.*, 1998).

Capsaicin undergoes bioactivation by CYP2E1 to reactive species. The major bioactivation pathways include: a) epoxidation of the vanillyl ring moiety to produce an arene oxide; b) one-electron oxidation of the ring hydroxyl group to a phenoxy radical; and c) O-demethylation at the aromatic ring and subsequent oxidation of the resulting catechol metabolite to semiquinone and quinine derivatives (Figure 2.6). The resultant reactive

species are capable of binding covalently to the active site of CYP2E1 as well as DNA. The interaction with target cell DNA would trigger mutagenicity and malignant transformation. However, metabolism of capsaicinoids by CYPs may also represent a detoxification process (in contrast to bioactivation), resulting in a reduction in cytotoxicity (Zhou S. *et al.*, 2004).

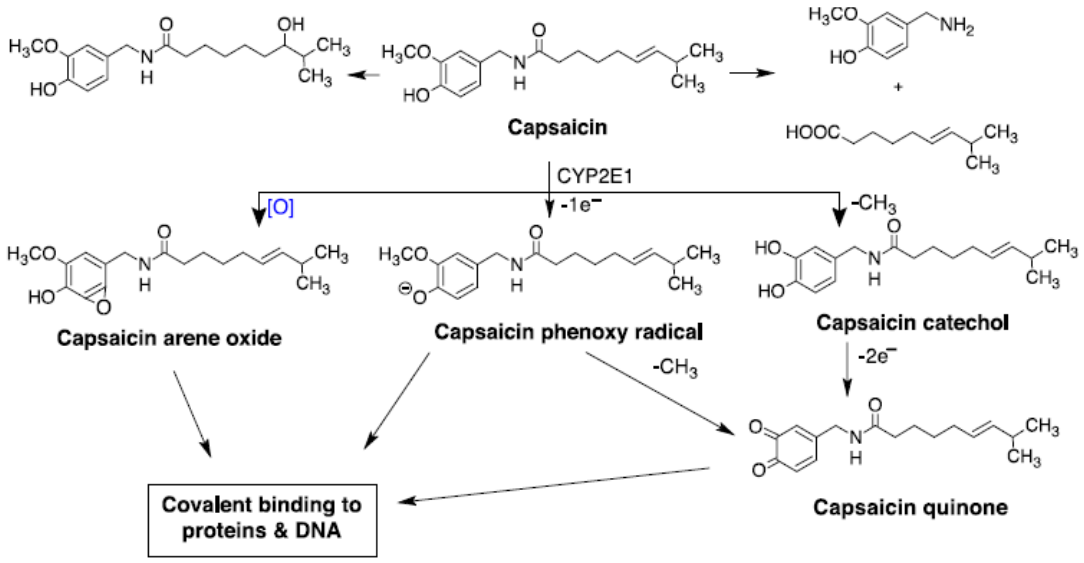


Figure 2.6. The major bioactivation pathways of capsaicin *(Zhou S. et al., 2004).*

Using liver microsomes from hamsters given a single oral dose of capsaicin 2 mg or 10 mg/kg body weight as an enzyme source and methoxyresorufin as a typical substrate of cytochrome P450 1A2, Teel *et al*. has demonstrated a small but statistically significant inhibition of CYP 1A2 activity after capsaicin pretreatment. The suppression of CYP 1A2 by capsaicin correlated with its antimutagenic activity against selected heterocyclic amines (Teel *et al.*, 1997).

Capsaicin modulates microsomal cytochrome P450-dependent monooxygenase activities, thereby affecting metabolism of carcinogens and other xenobiotics (Surh *et al.*, 1998). Capsaicin also suppresses the activity of rat epidermal aryl hydrocarbon hydroxylase that is linked to the cytochrome P450 1A isoform responsible for the metabolism of benzo[*a*]pyrene and other polyaromatic hydrocarbons. Metabolism and subsequent covalent DNA binding of benzo[*a*]pyrene in human and murine keratinocytes were attenuated by capsaicin (Modly *et al.*, 1986).

2. 6. 3. Molecular Mechanisms of Anti-tumor Activity of Capsaicin

One of the important cellular mediators of cellular processes in response to diversified extracellular stimuli including oxidants, UV, mitogens, cytokines, viruses, tumor necrosis factor-a (TNF-α), interleukin-1 (IL-1), etc. is a nuclear transcription factor NF-κB. Upon activation by external signals, NF-κB is migrated to the nucleus where it binds to a specific segment of DNA, thereby triggering expression of a variety of rapid-response genes involved in some important physiological reactions, such as inflammation, cell adhesion, viral replication, immune response, and proliferation. Reactive oxygen species (ROS) has been suggested as a second messenger in a signal transduction pathway for activation of NF-κB. Capsaicin abrogates the activation of NF-κB by TPA, as well as by TNF-α, which may provide a mechanistic basis for the possible anti-tumor promoting activity of capsaicin (Surh, 1999 and ref therein). Capsaicin also has been reported to inhibit constitutive activation of NF-κB mediated by ROS endogenously generated via the NAD(P)H: quinine oxidoreductase system in malignant melanoma cells. The inhibition of NF-κB activation by capsaicin is thought to be mediated through multiple mechanisms, one of which involves the MAP kinase cascades (Surh, 2002 (b)).

Another eukaryotic transcription factor that is responsive to ROS and is hence, regulated by cellular redox status is activator protein 1 (AP-1). The regulation of AP-1 in response to external stimuli is considered to be mediated by members of mitogen- activated protein kinase (MAPK) family, among which c-*jun*-N-terminal kinase (JNK; also known as stress-activated protein kinase abbreviated as SAPK) and extracellular signal-regulated kinase (ERK) isoforms are best characterized. There is some evidence that JNK and ERK exert opposite effects on apoptosis. Capsaicin induced apoptosis in cultured Jurkat cells through generation of ROS. When added to these cells at a concentration of 300 mM, capsaicin rapidly activated JNK: the activity was detectable as early as 5 min after the treatment, while ERK activity was not affected under the same experimental conditions. Despite the activation of JNK by capsaicin, there was no induction of AP-1 activity. On the contrary, capsaicin blocked the TPA-induced AP-1 activation. The latter finding, together with the previous observation by Singh *et al*. on suppression of TPA-induced NF-kB

activation by capsaicin, may provide important molecular basis for anti-tumor promoting potential of this vanilloid (Han *et al.*, 2001).

2. 7. Apoptosis

Cell death can occur by two quite different mechanisms: apoptosis and necrosis. *Necrosis*, also called pathological death, occurs after cells have been exposed to extreme physiological conditions or is evoked by agents like complement or lytic viruses. *In vivo* necrotic cell death is often associated with extensive tissue damage resulting in an intense inflammatory response (Van Furth *et al.*, 1988). The main characteristics of necrosis are swelling of organelles and of the cells, resulting in cell lysis due to loss of membrane integrity, postlytic DNA fragmentation and random digestion of DNA (Wylie, 1980). *Apoptosis*, or programmed cell death, is the most common from of eukaryotic cell death. It is a natural form of cell death controlled by constitutively expressed machinery that induces condensation of nucleoplasm and cytoplasm, blebbing of cytoplasmic membranes, and fragmentation of the cell into apoptotic bodies that are rapidly recognized and eliminated by adjacent cells. According to the current understanding, morphological and biochemical alterations in nuclear and chromatin structures of cells that undergo apoptosis are controlled mainly, if not completely, by the mitochondria (Qiao *et al.*, 2005). In other words apoptosis is a biological suicide mechanism preserving homeostasis and is essential in many physiological processes, such as embryogenesis, maturation of the immune system, or development of the nervous system.

Considerable attention has been focused on dietary and/or pharmacological manipulation of apoptosis as a novel and promising strategy for cancer chemoprevention, as well as therapy. The maintenance of homeostasis in normal mammalian tissues reflects a critical balance between cell proliferation and cell death via apoptosis. In contrast, apoptosis may be inhibited or perturbed in tumors in which the rate of cell proliferation exceeds that of cell loss. If misregulation of apoptosis results in a failure of tissue size regulation, which eventually leads to the malignant transformation, apoptotic cell death could be induced to augment interventions designed to suppress or reverse the development of cancer.

2. 7. 1. Induction of Apoptosis by Capsaicin

Capsaicin preferentially repressed the growth of some transformed cells of human origin, including HeLa, ovarian carcinoma, mammary adenocarcinoma, and human promyelocytic leukemia HL-60 cells in culture (Morre *et al.*, 1995). The inhibition of cell survival was accompanied by induction of apoptosis as revealed by characteristic morphological changes and fragmented appearance of nuclear DNA. Similar findings with human and mouse melanoma cell lines in terms of induction of apoptosis or growth inhibition was reported a year later. Interestingly, direct injection of capsaicin into the B16 mouse melanoma transplanted in C57BL/6 mice significantly suppressed the growth of tumors (Morre *et al.*, 1996).

2. 7. 1. 1. Molecular Mechanisms of Capsaicin-induced Apoptosis

Although the antiproliferative activity of capsaicin has been ascribed to its ability to induce apoptosis, relatively little is known about the molecular basis for the programmed cell death induced by this edible phytochemical. It was reported that capsaicin-induced apoptosis in cultured cells derived from human cutaneous squamous cell carcinoma (SCC) occurs through inhibition of mitochondrial respiration having mitochondrial redox system as a primary target for capsaicin in SCC-derived cells (Hail *et al.*, 2002). Similar findings of mitochondria depolarization were recently reported. Capsaicin evoked concentration-dependent increases and decreases, respectively, in mitochondrial hydrogen peroxide production which supports the hypothesis that (E)-capsaicin is mitochondrial inhibitor, able to activate apoptosis and/or necrosis via non-receptor mediated mechanisms, and also support the use of TRPV1 ligands as anti-cancer agents (Athanasiou *et al.*, 2007).

Other investigators have demonstrated that capsaicin-induced apoptosis in some transformed cells and in activated T cells is associated with the suppression of plasma membrane NADH-oxidoreductase (PMOR), an enzyme that transfers electrons from cytoplasmic NADH via coenzyme Q (ubiquinone) to external electron acceptors such as oxygen. PMOR is thought to be involved in the control of cell growth and proliferation by

maintaining the proper NAD+/NADH ratio required for cell viability. However, PMOR activity in normal tissues and in nontransformed cells is responsive to growth factors and hormones, whereas PMOR activity in tumor tissues and transformed cells is not (Morre *et al.*, 1995, Morre *et al.*, 1996).

Coenzyme Q is a lipophilic and mobile electron carrier of the plasma membrane electron transport system that is essential for cell growth and for the cellular response to redox changes. Preincubation of human lymphoblastoid cells with coenzyme Q prevents capsaicin-induced apoptosis, suggesting that capsaicin, a quinone analogue, induces apoptosis by competing with coenzyme Q in the plasma membrane redox system. Capsaicin can also inhibit the NADH: coenzyme Q oxidoreductase (i.e., complex I) activity of the mitochondrial electron transport system (Surh, 2002 (b) and ref. therein). It has been suggested that capsaicin, may interfere with the coenzyme Q binding site, which may redirect the normal electron flow in the complex and generate excess reactive oxygen species (ROS) and a pro-oxidant environment in the plasma membrane. The pro-oxidative can result in oxidation of thiol groups in mitochondrial permeability transition pores, leading to dissipation of transmembrane potential, which is a prerequisite for the induction of apoptosis. Therefore, overproduction of extramitochondrial ROS due to the inhibition of PMOR could contribute to the apoptosis that is induced by capsaicin and related vanilloids (Surh, 2002 (b) and ref. therein).

Milder endogenous redox stress that results from ROS spontaneously generated by an NAD(P)H:quinone oxidoreductase activity has been shown to play a functional role in the constitutive activation of the transcription factor, nuclear factor-κB (NF-κB) in several malignant human melanoma cell lines. It was found that capsaicin inhibits NAD(P)H:quinone oxidoreductase, reduced superoxide production and proliferation of M1619 melanoma cells, suggesting that the redox coupling between NAD(P)H:quinine oxidoreductase and coenzyme Q is a more important source of growth-signaling ROS in transformed cells than it is in normally regulated nonmalignant cells (Surh, 2002 (b) and ref. therein). In another study, capsaicin is shown to induce apoptotic death of cultured human gastric cancer cells, which appeared to be mediated via overexpression of the *p53* tumor suppressor gene and/or c-*myc* proto-oncogene (Kim *et al.*, 1997).

Induction of apoptosis of both androgen receptor (AR)-positive (LNCaP) and -negative (PC-3, DU-145) prostate cancer cell lines associated with an increase of p53, p21, and Bax was reported by Mori *et al*., (2006). Capsaicin inhibited NF-κB activation by preventing its nuclear migration due to inhibiting proteasome activity which suppressed the degradation of IκBα, preventing the activation of NF-κB (Mori *et al*., 2006).

Capsaicin-induced apoptosis has been suggested to be regulated by Bcl-2 and the protein phosphatase, calcineurin (Surh *et al.*, 1998). The concentration-dependent inhibitory effect of capsaicin on SK-Hep-1 hepatocellular carcinoma cells growth, found by Jung *et al.*, 2001, was mainly due to the induction of apoptosis as evidenced by DNA fragmentation and nuclear condensation. Furthermore, capsaicin prominently reduced the ratio of anti-apoptotic Bcl-2 to pro-apoptotic Bax and consequently increased caspase-3 activity. These results demonstrate that capsaicin efficiently induced apoptosis in SK-Hep-1 cells through a caspase-3-dependent mechanism, which may contribute to its chemopreventive function (Jung *et al*., 2001).

In a recent study, similar effects were found by Jun *et al*., 2007. They investigated the apoptotic effect of capsaicin through a mitochondria-mediated pathway from upstream effectors, such as Bax and Bcl-2 to downstream substrate, poly (ADP-ribose) polymerase in highly metastatic B16-F10 murine melanoma cells. It was found that capsaicin inhibited growth of B16-F10 cells in a concentration-dependent manner. Proapoptotic effect of capsaicin was evidenced by nuclear condensation, internucleosomal DNA fragmentation, in situ terminal nick-end labeling of fragmented DNA (TUNEL), and an increased sub G1 fraction. Treatment of B16-F10 cells with capsaicin caused release of mitochondrial cytochrome c, activation of caspase-3, and cleavage of poly (ADP-ribose) polymerase in a dose-dependent manner. Furthermore, Bcl-2 expression in the B16-F10 cells was slightly down-regulated by capsaicin treatment. In contrast, there were no alterations in the levels of Bax in capsaicin-treated cells (Jun *et al*., 2007).

There are reports of capsaicin evoked apoptosis, which may be blocked by omission of extracellular calcium and by TRPV1 antagonists, indicating the requirement for a functional vanilloid receptor (Athanasiou *et al.*, 2007 and ref. therein).

2. 8. Cytokines

Cytokines are small secreted proteins which mediate and regulate immunity, inflammation, and hematopoiesis. They must be produced *de novo* in response to an immune stimulus. They generally (although not always) act over short distances and short time spans and at very low concentration. They act by binding to specific membrane receptors, which then signal the cell via second messengers, often tyrosine kinases, to alter its behavior (gene expression). Responses to cytokines include increasing or decreasing expression of membrane proteins (including cytokine receptors), proliferation, and secretion of effector molecules.

The largest group of cytokines stimulates immune cell proliferation and differentiation. This group includes Interleukin 1 (IL-1), which activates T cells; IL-2, which stimulates proliferation of antigen-activated T and B cells; IL-4, IL-5, and IL-6, which stimulate proliferation and differentiation of B cells; Interferon gamma (IFN-gamma), which activates macrophages; and IL-3, IL-7 and Granulocyte Monocyte Colony-Stimulating Factor (GM-CSF), which stimulate hematopoiesis. Tumor necrosis factor-alpha (TNF-alpha) is a proinflammatory cytokine greatly involved in the pathophysiological changes associated with acute and chronic inflammatory conditions, including septic shock, autoimmune diseases, wasting, rheumatoid arthritis, inflammatory bowel disease and the respiratory distress syndrome.

2. 8. 1. Cytokines in Immunotherapy

Stimulation of the immune system to reject and destroy tumors is called cancer immunotherapy. The use of cytokines has a long history in immunotherapy, with interferon-gamma being the first cytokine used in tumor immunotherapy in 1957. Cytokines can regulate the immune response and are secreted by immune effector cells as well as a large variety of other cells, including tumor cells. Several cytokines are capable of mediating tumor regression in some malignancies. Interleukin-2 and TNF-alpha are two of the most extensively studied cytokines for tumor immunotherapy purposes (Gan *et al.*, 2003).

The secretion of TNF-alpha, IL-1, -6 and -10 and prostaglandin E2 (PGE2) in the presence of capsaicin was analysed in the experiment of Saade and co-authors. Treatment of primary cultures of adult rat DRG neurons at day 4, with either ET (5 microg in 1 ml) or with capsaicin (0.1, 1 or 10 microg in 1 ml) for 30 min showed that capsaicin produced a significant increase of the levels of IL-6, IL-10 and PGE2, reaching more than 10 folds their initial concentrations. This increase was comparable to albeit slightly less than that observed with ET challenge. The optimal effects were obtained with the concentration of 1microg of capsaicin. It was concluded that capsaicin challenge to DRG culture upregulates the level of pro and anti-inflammatory mediators (Saade *et al.,* 2004).

The chilli extract treatment of leukocytes induces IFN-gamma production, decreases antibody production while increases cell proliferation. Chili extract didn't significantly affect the responsiveness of B-lymphocytes to a mitogenic stimulus or the cellular immune response. The extract induces differentiation and promotes growth of a fibroblast resembling cell population (Athanassakis, unpublished).

Substance P, as it was said before, plays a major role in the regulation of the interaction between immune and nervous systems. SP administration stimulates Con A-induced proliferation of spleen and peripheral blood lymphocytes from normal and neonatally capsaicin treated rats, which correlated with enhanced IL-2 production and expression of activation antigens such as IL-2 receptor or chain (CD25) and RT1B MHC class II molecule. Moreover, SP markedly increased the percentage of CD5(+) and CD4(+) T lymphocytes in the peripheral blood of capsaicin-treated rats. Concomitant administration of SP with the non-peptide Neurokinin-l receptor (NK1R) antagonist SR140333 completely inhibited the SP-mediated augmentation of Con A-induced PBL proliferation and IL-2 production as well as of CD4(+) CD25(+) and CD4(+) RT1B(+) T cell numbers in normal and capsaicin-treated rats. SR 140333 also blocked the increased percentage of peripheral blood CD4 (+) T cells induced by SP in capsaicin-treated rats (Santoni *et al.,* 1999).

2. 9. Major Histocompatibility Complex (MHC)

The Major Histocompatibility Complex (MHC) is a set of molecules displayed on cell surfaces that are responsible for lymphocyte recognition and "antigen presentation". The MHC molecules control the immune response through recognition of "self" and "non-self" and, consequently, serve as targets in transplantation rejection. The Class I and Class II MHC molecules belong to a group of molecules known as the Immunoglobulin Supergene Family, which includes immunoglobulins, T-cell receptors, CD4, CD8, and others.

The major histocompatibility complex is encoded by several genes located on human chromosome 6 (Figure 2.7). Class I molecules are encoded by the BCA region while class II molecules are encoded by the D region. A region between these two on chromosome 6 encodes class III molecules, including some complement components http://www.cehs.siu.edu/fix/medmicro/mhc.htm.

Figure 2.7. Human HLA complex on the chromosome 6.

2. 9. 1. MHC Class II Molecules

Class II molecules are composed of two transmembrane polypeptide chains, both encoded by the D region. These polypeptides (alpha and beta) are about 230 and 240 amino acids long, respectively, and are glycosylated, giving molecular weights of about 33 kDa and 28 kDa. These polypeptides fold into two separate domains; alpha-1 and alpha-2 for the alpha polypeptide, and beta-1 and beta-2 for the beta polypeptide. Between the alpha-1 and beta-1 domains lies a region capable of binding (via non-covalent interactions) a small peptide of about 10 amino acids. This small peptide is "presented" to a T-cell and defines the antigen "epitope" that the T-cell recognizes http://www.cehs.siu.edu/fix/medmicro/mhc.htm.

MHC class II molecules presenting MHC class II restricted antigens play an important role in the activation of CD4+ T cells, which are the central orchestrating cells of an effective and long-lasting immune response. MHC class II molecules are expressed on the surface of antigen-presenting cells (APCs) such as B cells, macrophages, and dendritic cells (Hong *et al*., 2007).

2. 9. 1. 1. Role of MHC Class II Molecules and CD4+ T Cells in Tumour Immunology

As a consequence of genetic instability of tumours, neo-antigens will be generated. Several classes of tumour-associated antigens (TAA) have been recognized. TAA can be peptides that are selectively expressed by tumours as a result of genetic mutations or epigenetic/post-translational modification. TAA can also be proteins that are normally expressed on germ cells and thus novel to the immune system (e.g. MAGE-1). Finally, TAA include over-expressed normal antigens (e.g. Her2/neu) as well. Classically, tumour-associated antigens derived from apoptotic or necrotic cells are taken up by professional antigen presenting cells and presented by MHC class II molecules to CD4+ T cells. These CD4+ T cells ultimately activate CD8+ cytotoxic T cells (CTL) that recognize tumour cells via MHC class I molecules and are prepared to lyse them directly. Tumour cells expressing MHC class I and II molecules together with co-stimulatory molecules can evoke an immune response.

On the other hand, in the absence of these conditions or in the case of those tumours producing immunosuppressive cytokines and enzymes (IL10, TGFb and IDO), immune escape and tolerance will result.

In developing efficient anti-tumour responses, most attention was initially paid to the generation of a CD8+ CTL response, since these CTLs can lyse tumour cells directly. Two other reasons contributed to this preoccupation. First, most tumours, except for hematopoietic tumours and melanoma, express only MHC class I molecules. However, recently hundreds of class II peptides were isolated directly from primary dissected solid tumors, especially from renal cell carcinomas, and from colorectal carcinomas and transitional cell carcinomas, prostate tumor microenvironment (Dengjel *et al.*, 2006, Nanda

et al., 2006). Second, MHC class II antigens turned out to be much more difficult to discover (MHC class II molecule binding groove is more promiscuous in binding peptides, the peptides are composed of more amino acids (up to 31) and the binding groove is more open-ended compared to MHC class I molecules). Today, increasing evidence points out the important role of presenting MHC class II restricted antigens to CD4+T helper cells in the generation of an effective immune response. First, T-helper 1 (Th1) cytokines like IFN-γ and TNF-α are necessary for activating CD8+ CTLs. So, CD4+ T cells orchestrate the immune response by secreting different cytokines to Th1 or to Th2 direction or to innate immune system activation. All of these could contribute to efficient anti-tumour responses (Chamuleau *et al.,* 2006).

2. 9. 1. 2. Role of MHC Class II Molecules in Cancer Immunotherapy

The potential efficacy of immunotherapy for the treatment of cancer has led to the development of novel experimental strategies. Many of these innovative approaches focus on the activation of tumor-specific T lymphocytes because the cell-mediated arm of the immune system can be effective in destroying tumor cells and in providing long-term protection (memory) against the recurrence and/or outgrowth of primary and/or metastatic tumor cells (Chamuleau *et al.,* 2006).

The primary goal of immunotherapy should be to overcome tolerance and to re-educate the immune system, when the tumour burden is reduced to the status of minimal residual disease (after surgery and/or radiotherapy and/or chemotherapy). This can be achieved either by immunizing patients actively (with highly immunogenic tumour cell based or dendritic cell based vaccines) or passively (with immune cells recognizing tumour cells).

Considering the role of MHC class II molecules in actively immunizing vaccines one could think of at least two strategies. The first would be to turn tumour cells themselves into MHC class II positive and TAA presenting cells that activate T cells directly. A second possibility would be to extend the MHC class II presentation pathway in dendritic cells by forcing them to present endogenous TAA (Dolan *et al.,* 2006). Therefore, the successful

targets for cancer immunotherapy do express MHC class II molecules, which leaves no obvious reason to ignore MHC class II molecules as a mediator in anticancer immune therapy (Marsman, 2005).

2. 10. Goals of the present study

The aim of the present work was to study the *in vitro* effects of sweet and hot chili aqueous extracts on the proliferation of murine spleen cells (BALB/c) and different cell population (macrophages, B- and T-lymphocyets), as well their immunomodulating capacities and activity in regulation of immune response via alteration of cytokine production, expression of MHC class II molecules and apoptosis development in primary spleen primary cells. Furthermore, attempt was made to identify the active members of sweet and hot aqueous extracts which could be responsible for the profound effects.

III. Materials and Methods

3. 1. Chili Pepper Extracts

Three different extractions methods were applied in preparations of chili pepper extracts. Cold water extraction, hot water and saline extraction were performed by incubating 15 g of each powdered sweet pepper (Πάπρικα-κόκκινο πιπέρι γλυκό, Carrefour, Μαρινόπουλος A.E., Ελλάδα) and powdered hot pepper (Πάπρικα-κόκκινο πιπέρι καυτερό, Carrefour, Μαρινόπουλος A.E., Ελλάδα) in 45 ml of cold double-distilled (dd) H_2O, hot water (65 ºC) and 0.9% NaCl, respectively, for 48 hours at room temperature. The slurry was centrifuged at 3200 rpm for 15 min. The supernatant was passed through a Whatman 3mm paper. Afterwards, the extract was passed through 0.2µm bacterial filter (Filtropur S 0.2, SARSTEDT) and kept at 4 ºC.

3. 2. Experimental Animals

BALB/c mice were housed in the Animal Care Facility of the University of Crete, Department of Biology in rooms with controlled daylight of 12 h, controlled humidity and temperature range of 22–24°C. Commercial pellet diet and water were made available to animals *ad libitum.*

3. 3. Primary Cell Culture

Male BALB/c mice were sacrificed by cervical dislocation, placed on a dissection tray and soaked with 70% ethanol. Using sterile scissors, the skin of the rodent, placed ventral side up, was initially cut for separation from the abdominal wall and then, using the second set of instruments the abdominal wall was cut exposing the viscera. Spleen was carefully trimmed free from the pancreas and connecting tissue, placed into a Petri dish with HBSS-Hank's Balances salt Solution culture medium (Gibco). Single cell suspension was obtained using a metal sieve or sterile needle, washed and transferred into Dulbecco's

Modified Eagle's Medium (DMEM), supplemented with 10% Fetal Bovine Serum FBS (Gibco). Finally, cells were set in culture at the concentration of 1×10^6 cell/ml at 37 °C, 5% CO_2.

3. 4. Cell Viability Assay

Cell viability was assayed using the Trypan Blue exclusion method. This method is based on the principle that living cells exclude the dye, whereas dead cells will take up the blue dye. The blue stain is easily visible and cells can be counted using a light microscope.

The effect of extracts on cell growth was evaluated by direct cell counting using a haemocytometer. Spleen cells (5×10^5) were cultured in a 96-well plates with decreasing concentrations of chili extracts (50, 25, 10, 5, 2.5, 1, 0.5 and 0 %, v/v). After 6, 24 and 48h, the cells were harvested and cell viability was evaluated by Trypan Blue dye (Gibco) exclusion.

3. 5. Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA, is a biochemical technique to determine if a particular protein is present in a sample and if so, how much. It is based on the recognition of the antigen by specific antibody (first antibody). The second antibody that reacts to antigen-antibody complexes is covalently bound to an enzyme which in the presence of a chromogenic or fluorogenic substrate produces a signal detectable by an ELISA reader.

The supernatants from the spleen cell cultures incubated with the experimental extracts were diluted 1:1 with coating buffer, immobilized in duplicates on the 96-well flat bottom plates (SARSTEDT, USA) and incubated at 4 °C overnight. Wells were washed 3× in 100 µl 0,05% Tween 20 and then blocked with 200 µl of PBS-BSA 2% to prevent the nonspecific antibody binding. The plates were incubated for 1.5 hours at room temperature and washed 3× with 100 µl of PBS/Tween. 100 µl of primary antibodies (anti-mouse monoclonal antibodies against IFN- γ, IL-2, IL-3, IL-10, TNF-alpha and GM-CSF (Endogen)) in 1/1000 dilution in 0.1% PBS-BSA, were added in each well and incubated for 1.5 hours at room temperature. Extensive washing of the plates was followed by addition of 100 µl of

secondary antibody (1/5000 dilution in 0.1% PBS-BSA of anti-mouse IgG Peroxidase Conjugate (Sigma) and incubated for 1.5 hours in dark. 100 µl/well equal amounts of peroxide substrate (TMB) and peroxide solution (PIERCE, PerBIO) were applied in dark for colour development. The reaction was stopped in 15 min with 50 µl of stop solution. Optical density (OD) was measured at 450 nm using ELISA Microplate Photometer (Digiscan, ASYS Hitech GmbH, Engendorf, Austria). Each experiment was repeated at least four times. The results are expressed as percent of OD values increase over background (±S.E.M.).

3. 6. Cell Proliferation Assay

The Proliferation Assay allows determining the number of cells that are growing within a culture and gives the possibility to compare the effect of various agents on the proliferative activity of the cells. Cell proliferation was assessed by ^{3}H-Thymidine (^{3}HTdR) incorporation experiments. The principle of the method is based on the fact that during each cell division the cells will incorporate ^{3}H-Thymidine into their DNA. The more cell divisions (or the higher the proliferation rate) the more radioactivity will be incorporated into the DNA.

An amount of 5×10^{5} cell per well was cultured in V-bottom 96-well plates (Sarstedt, USA). Fresh medium (DMEM) with experimental extracts in various concentrations was added to the growing cells and incubated at 37°C, 5 % CO_2. Cells in the absence of extracts served as controls. 10 µl of ^{3}HTdR solution (Amersham) were added in each well 18 hours prior to cell harvest. Cells were harvested onto fiberglass filters (Flow Labs) using a cell harvester. The filter membrane was dried and radioactivity was quantified using a Scintillation β-counter BECKMAN LS 1701. The amount of radioactivity correlates with the number of proliferating cells in the well. Each test group was performed in triplicate and each experiment was repeated four times.

3.7. Immunomagnetic (Biomagnetic) Separation of Murine Primary Cells to Macrophages, B and T lymphocytes

This cell separation technique is achieved by an antibody-antigen reaction. Coated with a specific ligand (antibody), the magnetic beads can bind to the desired target molecule (antigen) and be removed from the suspension via magnet.

Spleens from BALB/c mice were isolated into a Petri dish with HBSS medium and pushed against the sieve with the rubber end of a syringe plunger to put cells into single cell suspensions. Then cells were carefully collected and transferred into a clean tube, centrifuged and resuspended in DMEM. The resulting suspension was incubated in a Petri dish for 24 hours in a 37°C, 5% CO_2.

Isolation of T and B lymphocytes: After the 24h-incubation, the cells were removed from the plate by gentle collection of the supernatant. The plate was carefully washed few times with culture medium so as not to disturb the attached cells. All washes were pooled with the initial supernatant and constituted the "non-adherent" fraction. The collected supernatant was centrifuged at 1200 rpm for 6 min, room temperature to gently pellet the cells. The supernatant was aspirated and discarded. The banded cells were resuspended in PBS/BSA 0.1% and anti-Ly 5 antibody solution (CALTAG, USA) in 1/100 dilution and incubated for 20-30 min at room temperature. After washing twice with 500 µl PBS and centrifugation at 1200 rpm for 6 minutes the cells were resuspended in 1 ml of PBS. 15 µl of Dynabeads Goat anti-Mouse IgG (Dynal Biotech ASA, Oslo, Norway) were added to the cells and the suspension was incubated 20-30 min at room temperature. Afterwards, the cells were transferred into 5 ml tubes and the final volume of 2 ml was reached using PBS addition. The tube was placed in the Dynal MPC (Dynal Biotech ASA, Oslo, Norway) to collect rosetted cells. The depleted supernatant, containing the negatively isolated cells, was transferred to a new tube. The bead-bound cells were B cells and the cells of supernatant contained untouched mouse T cells.

Isolation of macrophages: The adherent cells were removed from the Petri dish with vigorous pipetting and scraper and washed repeatedly with culture medium until no cells were detected under the microscope. The collected cells were considered as macrophages.

3. 8. Indirect Immunofluorescence

Immunofluorescence is a technique allowing the visualization of a specific protein or antigen in cells or tissue sections by binding a specific antibody chemically conjugated with a fluorescent dye such as fluorescein isothiocyanate (FITC).

There are two major types of immunofluorescence staining methods: 1) direct immunofluorescence staining in which the primary antibody is labeled with fluorescence dye, and 2) indirect immunofluorescence staining in which a secondary antibody labeled with the fluorochrome is used to recognize a primary antibody. Immunofluorescence staining can be performed on cells fixed on slides and tissue sections. Immunofluorescence stained samples are examined under a fluorescence microscope or confocal microscope.

B lymphocytes (5×10^5/well) were seeded in a V-bottom 96-wells plate, (SARSTEDT, USA) overnight at 37°C, 5 % CO_2 and the cells detached from the magnetic beads were transferred into new wells and incubated with experimental agents for 24h at the same conditions. Macrophages (1×10^6/well) were seeded on the slide-spacer in a 24-well plate (SARSTEDT, USA) and let to adhere overnight at 37°C, 5 % CO_2. Experimental agents were added for 24 hours. Cells in the absence of any agent served as controls. After 24 hours the cells were first reacted with 2% PBS-BSA, then with the 1/100 dilution of test antibody in 0.1 % PBS-BSA (anti-mouse I-A^d (Becton Dickinson Immunocytometry Systems, USA), HB3 anti-mouse monoclonal antibody to MHC class II molecules (Clone IBL-5/22) (Immunology lab, UOC, Greece), anti-mouse L_3T_4 (Cat No1440; Becton Dickinson Immunocemistry System, USA/Belgium); anti-mouse Lyt-2 (Cat No 1350; Becton Dickinson Immunocemistry System, USA/Belgium); Anti-Ly-5 (B220) Rat monoclonal antibody against mouse Ly-5 marker (CALTAG, USA); anti-mouse CD11b (Serotec, UK) and, finally, with FITC-conjugated anti-mouse IgG secondary antibody (Sigma) in 1/200 dilution in 0.1 % of PBS-BSA). All incubations were performed at 4°C for 45 min; the incubation with secondary antibody was performed in dark. Extensive washing with PBS 1× followed each step of the procedure. The cells were then fixed with 25% glycerol, transferred onto slides and covered with a coverslip. The slide-spacers with adhered macrophages were transferred to the microscope slides and covered with coverslips in excess of 25% glycerol. Fluorescence intensity was evaluated using a fluorescent

microscope Nikon ECLIPSE E800 fluorescent microscope by counting 20–30 cell fields in each group. Cells with weak or no staining were scored as negative. Positive cells were considered those with bright to very bright staining.

3. 9. Fluorescence Activated Cell Sorting (FACS)

FACS is a technique of individual cell sorting based on their fluorescent markers. It is used to score fluorescent cells and perform cell cycle studies.

B lympocytes (5×10^5/well) were seeded in V-bottom 96-wells plate, (SARSTEDT, USA) overnight at 37°C, 5 % CO_2 and the cells detached from the magnetic beads were transferred into the new wells and incubated with experimental agents for 24h at the same conditions.

Macrophages (1×10^6/well) were seeded in 6-well plate overnight at 37°C, 5 % CO_2. Experimental agents were added for 24 hours. Cells in the absence of any agent served as controls. The cells were detached from the plate surface using scraper, fixed with) Paraformaldehyde (PFA) 4% for 10 min on ice, transferred into V-bottom 96-well plate and first reacted with 0.02% PBS-NaN_3, then with the 1/100 dilution of test antibody in BSA-PBS-NaN_3 (anti-mouse I-A^d (Becton Dickinson Immunocytometry Systems, USA); HB3 anti-mouse monoclonal antibody to MHC class II molecules (Clone IBL-5/22) (Immunology lab, UOC, Greece); anti-Ly-5 (B220) Rat monoclonal antibody against mouse Ly-5 marker (CALTAG, USA); anti-mouse CD11b (Serotec, UK) and, finally, with FITC-conjugated anti-mouse IgG secondary antibody (Sigma) in 1/200 dilution in 0.1 % of PBS-BSA).

All incubations were performed at 4°C for 45 min; the incubation with secondary antibody was performed in dark. Extensive washing with PBS-NaN_3 0.02% followed each step of the procedure. Cells in 500 μl of PBS-NaN_3 0.02% were counted by flow cytometer FACS Calibur Flow Cytometer, Becton Dickinson and analyzed by WinDMI 2.9 software. As positive controls, Ly-5 and CD11b antibodies were used for the detection of B lymphocytes and macrophages, respectively.

3. 10. Cellular DNA Fragmentation Assay

The Cellular DNA Fragmentation assay measures apoptosis, necrosis, or cell mediated cytotoxicity by quantitating the fragmentation and/or release of BrdU-labeled DNA. 5'-Bromo-2'-deoxy-uridine (BrdU), a synthetic nucleoside which is an analogue of thymidine, is used as metabolic labeling agent by the nuclear DNA of target cells. The assay is a sandwich enzyme-linked immunosorbent assay (ELISA) where BrdU-labeled DNA can be detected easily and quantified using a monoclonal antibody against BrdU.

To characterize the type of cell death, kinetic assays were set up and the appearance of DNA fragments in the culture supernatant as well as in the cytoplasm was detected using Cellular DNA fragmentation ELISA-kit Cat. No. 11 585 045 (Boehringer-Mannheim). The procedure was performed according to the manufacturer's instructions. Briefly, cells were prelabeled in tissue culture flask with BrdU for 12–18 h at 37°C, 5% CO_2. BrdU-labeled cells (1×10^6 cells/ml) were transferred into the 96-well round bottom microplate and incubated for 2, 4, 6, 8, 12, 24, 36, 48 and 72 hours in the presence of chili extracts (0.5%) at 37°C, 5% CO_2. At the end of the incubation, cells were centrifuged 6 min at 1200 rpm and 100 µl of supernatant was isolated for the ELISA procedure. To obtain the DNA fragments for the cytoplasm, 200 µl of incubation solution was added to the cells and incubated for 30 min at room temperature. After the centrifugation at 1200 rpm 100 µl of the cell lysate were collected for the ELISA procedure. The positive control (denaturated genomic DNA) was obtained by NaOH treatment which consisted of a 30 min incubation of BrdU-labeled cells with 125 µl 0.25M NaOH and subsequent addition of 125 µl 0.25M HCl and 250 µl 0.2M K_2HPO_4/KH_2PO_4 pH 7, following centrifugation at 1200 rpm.

For the ELISA assay the anti-DNA antibody was adsorbed onto the wells of a 96-well flat bottom plate overnight at 4°C and blocked with 200 µl incubation solution for 30 min at room temperature. Afterwards, the plate was washed with washing solution and incubated with experimental supernatants for 90 min at room temperature. The immunocomplexed BrdU-labeled DNA-fragments were fixed and denaturated by microwave irradiation for 5 min on medium power (500W). This procedure is necessary for the accessibility of the BrdU antigen. Anti-BrdU antibody peroxidase conjugate (100 µl) was added and incubated for 90

min in dark at room temperature. After extensive washing, the quantitation of the bound anti-BrdU-POD in the immunocomplex was performed using 100 µl of peroxidase substrate (TMB). The reaction was stopped by the stop solution and the absorbance at 450 nm was measured using an ELISA plate reader.

3. 11. Protein Quantitation Assay

Total protein concentration was estimated by the method of Bradford (1976). This assay is based on the observation that the absorbance maximum for an acid solution of Coomassie brilliant blue G-250 shifts from 465 nm to 595 nm when binding to protein occurs.

Standard samples of 0.1-1.4 µg/ml of BSA were prepared as dilutions of BSA stock solution. To each 5 µl of standard and experimental samples, 250µl of Bradford reagent (BIO-RAD, cat500-0006) were added and mixed well for 30 seconds. The absorbance was measured at 595 nm in Synergy HT Fluorimeter, Bio-TEK. Protein concentrations were calculated from a standard curve by plotting the absorbance with the concentration.

3. 12. SDS-Polyacylamide Gel Electrophoresis of Proteins

Polyacrylamide gel electrophoresis (SDS-PAGE) separates molecules in complexes according to size and charge. During electrophoresis there is an interaction of samples, gel matrix buffers and electric current resulting in separate bands of individual molecules.
The Mini-Protein 3 Electrophoresis Cell V15-17 (GIBCO) gel apparatus was assembled according to the manufacturer's instructions. The 10 or 12 % resolving gel was prepared and loaded up to 2/3 of the glass plate sandwich. Ethanol was loaded above the surface of the resolving gel to assess efficient solidification. The 4% stacking gel was prepared and then loaded over the resolving gel up to the top of the glass plates. A Teflon comb was immediately inserted into the stacking gel. Care was taken as not to trap any air bubbles.

After the stacking gel had polymerized, the comb was removed carefully and the wells that had formed were washed with ddH_2O to remove any unpolymerized acrylamide solution. The glass plates were removed from the casting stand and fit to the inner unit of the gel apparatus. 1×Tris-glycine buffer (pH 8.3) was used to fill the two chambers of the gel apparatus. Protein samples were prepared with 2×SAB (Loading Buffer), heated in water bath for 5-6 min then centrifuged in the microcentrifuge at 13,000 rpm for 1 min to precipitate the cell debris. The samples were loaded on the gel by repeated loading (Sheen and Ali-Khan, 2005). Molecular markers of known proteins sizes were loaded with the samples. The gel was then run at a constant current at (30 mA) until the dye reached the end of the glass plates.

The gel was stained in Coomassie brilliant blue R-250 for 30 min and destained in distaining solution for 2-3 h at room temperature. Alternative silver staining was performed to detect smaller amounts of protein. The silver staining was also applied to the Coomassie-destained gels. The gel was kept in the 50% of methanol overnight. Then it was placed into a solution of $K_2Cr_2O_7$ for 10 min, followed by a $AgNO_3$ solution for 30 min in dark. Extensive 3-5 times washes with dd H_2O (10 min each), were performed after each step. The gel was developed with the solution of Na_2CO_3 for 5-30 min in dark till the bands appeared. The reaction was stopped with 10% acetic acid.

3. 13. Protein/Peptide Identification/Characterization

Three protein bands were cut under sterile conditions, placed in an eppendorffs and stored at -20°C. The identification analysis was performed by the Protein Analysis Group at the Functional Genomics Center Zurich, according to the following procedure:
Gel bands were cut in small pieces and washed 2× with 100 µl 100 mM NH_4HCO_3/50% acetonitrile, washed 1× with 50 µl acetonitrile. All three discarded supernatants were supplied with 15 µl trypsin (0.01 ng/µl in 10 mM Tris/2 mM $CaCl_2$, pH 8.2) and 15 µl buffer (10 mM Tris/2 mM $CaCl_2$, pH 8.2) and incubated at 37° C overnight. Supernatants were removed and gel pieces extracted using 2× with 100 µl 0.1% TFA/50% acetonitrile. All three supernatants were combined and dried. Samples were dissolved in 15 µl 0.1% formic

acid. The samples were desalted by using a Ziptip C18 and mixed 1:1 with matrix solution (5mg/ml HCCA in 0.1% TFA / 50% ACN). The MALDI/MS/MS measurements were performed automatically. Database searches were performed by using the Mascot search programs. The sequence similarities of proteins were found by Basic Local Alignment Search Tool (BLAST) using www.expasy.org proteomics server.

3.14. DEAE Sephacel Gravity Column Ionic Exchange Chromatography

Ion exchange chromatography is a column chromatography that uses a charged stationary phase. Gravity column chromatography is based on the differences in rates of movement through the solid medium under the influence of gravity. Different exit times from the bottom of the column differentiate various elements of the original sample.

The fractionation of chili extracts was performed in sterile conditions. DEAE Sephadex resine (Sigma) was autoclaved for 20 min with ddH_2O, 100 mM Tris buffer (pH 7.5) and elution solutions 10, 50, 100, 250, 500, 750 and 1000 mM NaCl. The appropriate amount of DEAE Sephadex (2 ml of resin for 1 ml of sample) was placed in a 15 ml falcon tube. Ethanol was removed by washing resin 5 times with ice-cold dd H_2O by repeated inversion, and following centrifugation at 1,000 - 2,000 rpm and 4°C. Afterwards, it was washed twice with Tris buffer; each time the resin was equilibrated for 5 min with gentle inversion prior to spinning down. Final equilibration with the starting buffer (the buffer in which the sample is diluted, i.e. dd water) for a period of 5 min was performed. The pH of the equilibrated mix was adjusted to7.56. The sample was added on the spanned resin and equilibrated by mixing on the rotary shaker (in cold room) at a speed of 3 for 40 min. After loading the gel onto the Poly-Prep Chromatography Columns (Bio-Rad) the flow through was collected and the column was washed with 5 column volumes of starting buffer. The fractions were eluted with 3 column volumes of the appropriate salt solutions and kept at 4°C. The column was washed with 3 column volumes of 0.1M NaOH and immediately

followed extensive water wash to remove strongly bound substances and avoid loss of DEAE-groups.

3. 15. High Performance Liquid Chromatography (HPLC)

HPLC is a type of column chromatography and used to separate components of a mixture by using a variety of chemical interactions between the substance being analyzed and the chromatography column. The principal of this method is on a force of analyte through a column of the stationary phase or adsorbent by pumping a liquid (mobile phase) at high pressure through the column. The different time at which a specific analyte elutes from the columns is called the retention time and is considered a reasonably unique identifying characteristic of a given analyte. Separation and quantization of benzoylated polyamines of benzoylated free (S) fraction of chili extracts and fractions was done by HPLC as described by Kotzabasis *et al.,* 1993.

Polyamine extraction: Perchloric acid (PCA) 5% (v/v) [PCA; 10:1 (v/w)] (Merck) was added to 200 µl of extract and kept at 4ºC for 30 min for precipitation. After centrifugation at 11000-12000 rpm 4ºC for 20 min the supernatant was separated into a glass tube.

Benzoylation of Polyamines: For benzoyation of polyamines 1 ml of 2N NaOH, 10 µl of Cadaverin 5mM (Sigma) and 10 µl of benzoylchloride (Sigma) were added to 200 µl of the polyamines aliquots and vortexed for 30s. After a 20 min incubation at 25ºC, 2 ml of saturated NaCl was added to the samples and vortexed carefully to stop the reaction. The benzoyl-polyamines were extracted in 3 ml of diethyl ether (Merck). The samples were centrifuged at 6000 g for 5 min (Econospim; Sorvall-Instrument, Dupont) and the ether phase was collected to the new glass tube and evaporated over a water bath (68ºC) to dryness. The benzoyl-polyamines were redissolved in 100 µl of 63% (v/v) methanol and 20 µl of this extract were transferred into the HPLC vials.

HPLC analysis of polyamines: Analysis of benzoyl-polyamines was performed with a Hewlett-Packerd 1100 HPLC equipped with DPU multichannel integrator, a diod array system (Hewlett-Packard), and a 85B PC. A C-18 narrow-bore column (2.1 ×200 mm, 5-µm particle size; Hypersyl; were injected automatically into a fixed volume of 20 µl and

chromatographed at 25ºC. The elution flow rate was 200µl/min. The quantity of polyamines is expressed in nmol g^{-1} dry weight (DW) ±S.E.M.

3. 16. Anthrone-sulfuric Acid Colorimetric Assay

A modification of the anthrone-sulfuric acid colorimetric assay for glucose-based carbohydrates was performed according to Laurentin and Edward, 2003. The principle of the method is that the heat and a strong acidic environment produce both hydrolysis of glycosidic bonds and dehydration of monomers to produce furfuraldehyde derivatives. These compounds react with anthrone and produce colored products.

40 µl water (blank), standard (0.05, 0.1, 0.15, 0.2, 0.25, 0.3, and 0.4 g/l glucose), or sample was added to individual wells of the microtitration plate. This plate was covered with cling film, vortex-mixed gently, and incubated at 4 °C for 15 min. Then, 0.1 ml of 0.2% solution of anthrone reagent (Sigma) in concentrated sulfuric acid was added to each well using a 12-channel micropipette; the plate was sealed with acetate tape, vortex-mixed gently but thoroughly, and incubated at 92 °C in a non-shaking water bath. After 3 min, the plate was transferred to a non-shaking water bath at room temperature for 5 min to stop the reaction and then placed into an oven at 45 °C for 15 min to dry off. The 3-min incubation is critical as this allows optimal development of color without deformation of the plate due to the high temperature in the water bath. In this regard, plates should not be left in the water bath for more than 5 min. Absorbance at 630 nm was read in a Synergy HT Fluorimeter plate reader (Bio-TEK) and total carbohydrate concentration, as glucose or starch (0.9× glucose concentration) equivalents, was worked out.

3. 17. Thin Layer Chromatography (TLC)

Thin Layer Chromatography (TLC) has been traditionally regarded as a simple, rapid and inexpensive method for the separation, tentative identification and visual semiquantification of a wide variety of substances.

Pigments extraction: Pigments were extracted from 500 mg pepper powder with 100% methanol in a water bath for 2-3 min at 70°C. The slurry was centrifuged twice at 1500 rpm and the upper phase was used for TLC.

TLC analysis of pigments: The experimental samples were triply applied to the chromatographic sheets, immersed in the flat-bottomed glass plate into 100% acetone to achieve a straight strip. Afterwards, the sheet was placed vertically in the chamber with solvent (petrol ether/propanol-2/water (100:10:0.25) for 15-30 min. The care was taken to maintain the gas phase formed from a mixture of air and solvent vapors in the chamber. Then, the sheets were dried with hot air flow. Each band was scratched into the separate glass tube, dissolved in 500 µl of acetone 100% and centrifuged at 1200 rpm. The resulted supernatants were kept at -20°C and analyzed for the absorption spectra in visible light at 380-750 nm using Perklin Elmer UV/VIS Spectrometer Lambda 20.

3. 18. Microscopy

Photographs were taken in the absence or presence of chili extracts, using a Inverted Microscope (Lietz Diavert, USA) and digital camera Kodak EasyShare C 310.

3. 19. Statistical Analysis

All of the experiments were performed at least three times. Values were expressed as mean ± S.E.M. Statistical comparisons were made by ANOVA and *P*-values <0.05 were considered significant.

IV. Results

4. 1. Effect of Sweet and Hot Pepper Extracts on Spleen Cell Viability

Sweet and hot pepper extracts were obtained following three different extraction protocols as described in the Materials and Methods section and initially tested on spleen cell viability experiments. Spleen cells from BALB/c mice were cultured for 6, 24 or 48 h with 0, 0.5, 1, 2.5, 5, 10, 25 and 50% v/v of the extracts and cell viability was assessed by trypan blue exclusion (Figure 4.1).

The results showed a significant decrease of cell viability in a dose- and time-dependent manner after incubation with the capsicum extracts. In general, hot pepper extracts were more toxic than sweet pepper extracts. The results showed that the type of extraction affected the extract's properties. Cold water extract of sweet pepper (SI) had more toxic effect as compared to the hot water extracts (SII). While there was some cell viability in cases of cells cultured with 10% SII extracts after 48h of incubation, SI extracts showed a high toxicity even after 24h. The threshold for cell survival with the saline extract (SIII) was at 5 % v/v concentration. In cases of hot pepper extracts, hot water extraction appeared to be more toxic (HII) as compared to the cold water extraction. After 24 and 48h of incubation with HII, viable cells could only be detected at 10% and 1% v/v extract concentrations, respectively. Hot pepper extracted with saline (HIII) had the most toxic properties since the cell survival threshold was at 1% v/v concentration of extract.

The highest concentrations of all sweet and hot extracts tested (50% v/v) were toxic to cell cultures within 6h. Saline extracted sweet and hot peppers (SIII and HIII) in 25 and 10% concentrations were toxic for cell cultures. The same concentrations of HI extract showed their toxic properties after 24h and 48h, while cells incubated with SI survived only at 10% for first 6 hours. Using hot water extracts, total cell death was registered at 24h with 25% sweet (SII) and hot (HII) extract applications. Incubation for 48h with 10% extract was fatal in case of hot pepper extract (HII). Reduction of extract concentration to 2.5% had positive trend on cell survival in all extracts except from hot water and saline extracted hot

peppers (HII and HIII). The concentration of extracts in 0.5% v/v does not seem to alter cell viability in any case. Therefore, this concentration was chosen for further experimentation.

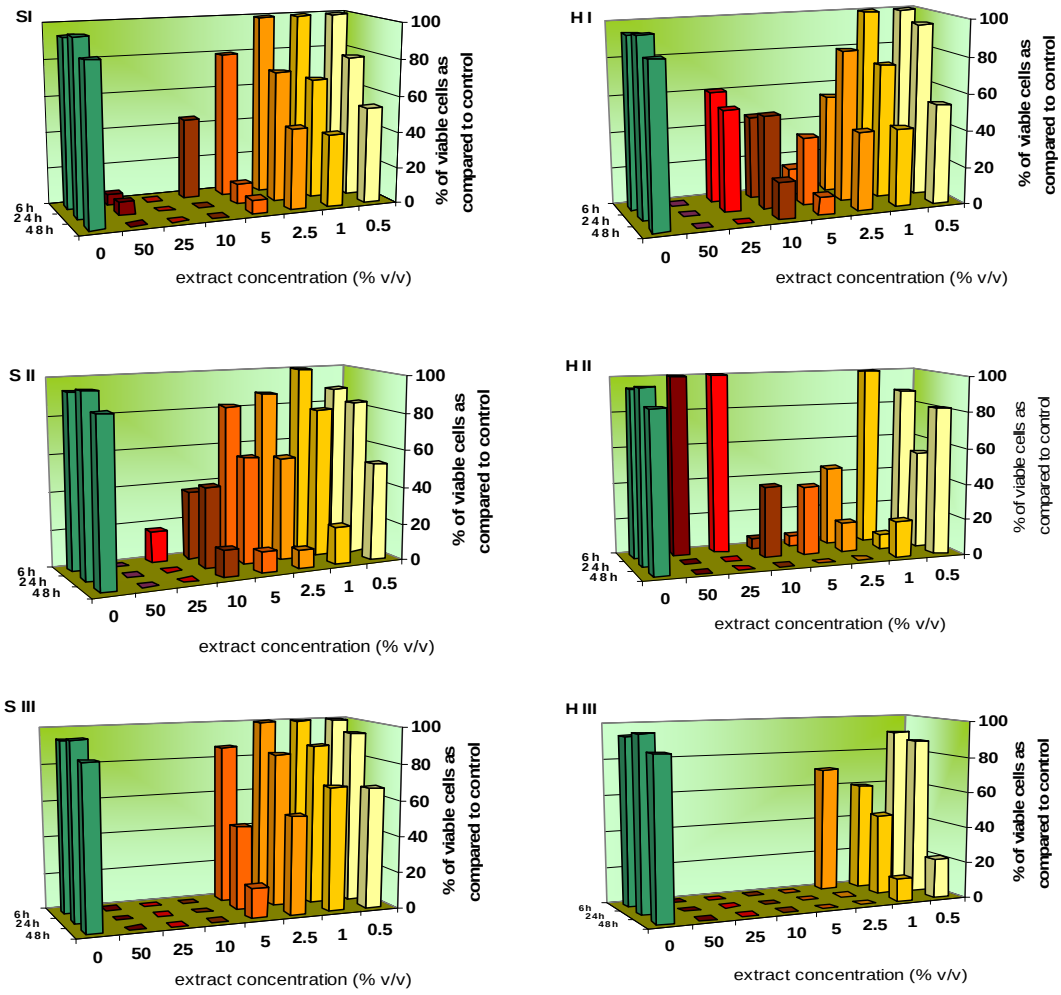
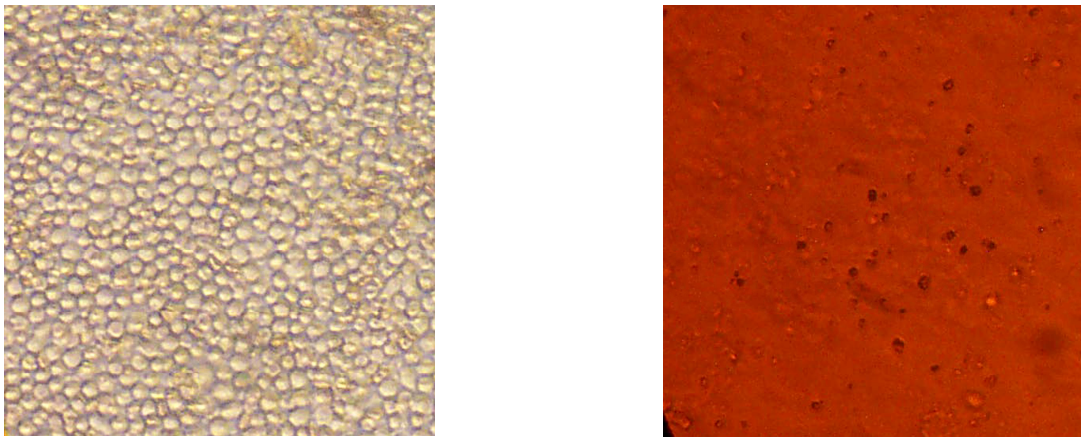


Figure 4.1. Effect of sweet and hot extracts on spleen cells viability. *Spleen cells were incubated with decreasing concentrations (50, 25, 10, 5, 2.5, 1, 0.5 v/v) or without (control) capsicum extracts for 6, 24 and 48h, respectively. Total number of viable cells was evaluated by Trypan blue dye exclusion. The count rates were expressed as percentage of corresponding control; (S-extract from sweet pepper; H-extract from hot pepper; I, II and III- cold water, hot water and saline extractions, respectively).*

At toxic concentrations no cells were detected or they appeared smashed (Figure 4.2). This toxic effect was not due to the acid pH of all extracts since the correction of pH to neutral didn't reverse the extracts' toxicity.



A B

Figure 4.2. Primary cells from BALB/c spleens cultured without (A) and with capsicum extracts (B). *Cell cultures (1×10^6 cells/ml) were incubated at standard conditions of 37°C and 5% CO_2 with 0 and 50 % v/v concentrations of extracts for 4 days in final volume of 1 ml in 24-well plates.*

4. 2. Effect of *Capsicum* Extracts on Cytokine Production

To detect the immunomodulating activity, all experimental extracts were checked for their ability to stimulate cytokine production from spleen cells. Thus spleen cells were cultured for 24h at a 1×10^6 cells/ml concentration with 0.5% v/v capsicum extracts as defined above and culture supernatants were tested for the presence of IFN-gamma, IL-2 and TNF-alpha production by ELISA techniques.

The results showed that spleen cells increased the production of IFN-gamma, IL-2 and TNF-alpha upon exposure to all extracts (Figure 4.3). Sweet and hot pepper extracts significantly increased IFN-gamma production ($p<0.01$). According to the results, the production of IFN-gamma was higher in response to the sweet pepper extracts as compared to the hot extracts. Non significant variation of IFN-gamma production was registered between the different types of sweet (SI, SII, SIII) or hot (HI, HII, HIII) extracts.

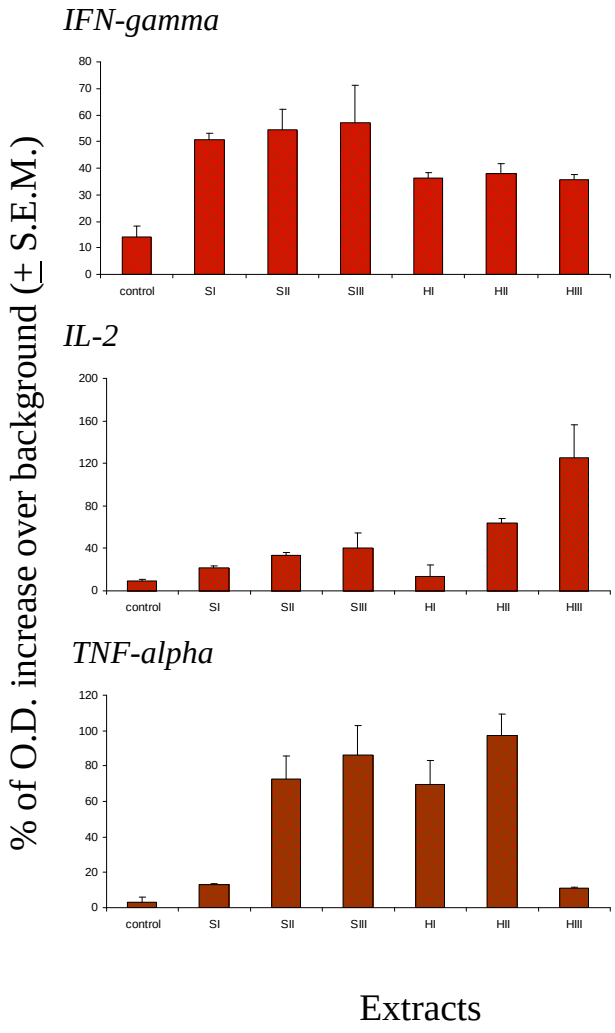


Figure 4.3. Effect of sweet and hot extracts on the production of IFN-gamma, IL-2 and TNF-alpha*. Spleen cells were cultured for 24h at a 1×10^6 cells/ml concentration with 0.5% v/v capsicum extracts in 24-well plate. IFN-gamma, IL-2 and TNF-alpha production in the culture supernatants were tested by ELISA techniques. The results were expressed as % of optical density increase over background. Data represent the mean ± S.E.M. values from three assays in duplicate wells.*

The production of interleukin-2 was significantly ($p<0.003$) affected by hot water and saline extracts of hot pepper, where almost 7 and 13 fold increase of IL-2 production was obtained by the HII and HIII applications, respectively. Non-significant ($p<0.6$) increase in IL-2 secretion was recorded only upon application of cold water extracted hot pepper (HI). The application of SII, SIII, HI and HII to the cell cultures significantly ($p<0.01$) stimulated TNF-alpha production. As for IL-2, TNF-α production followed the order of induction SI<SII<SIII, indicating thus a higher immunomodulating activity of sweet pepper extracted with saline, as compared with hot and cold water extractions. In the case of hot pepper, the hot water extraction seemed to be more effective as compared to cold water extraction, whereas saline extraction (HIII), which induced high levels of IL-2 production, showed the lowest stimulation of TNF-alpha.

4. 4. Effect of *Capsicum* Extracts on Primary Cells Proliferation

To examine the effect of extracts on cell growth, cell proliferation assays using ^{3}H-thymidine (^{3}HTdR) incorporation experiments were performed. Spleen cells (5×10^{5}) were cultured for 24h with 0.5% v/v extracts from sweet and hot chili peppers. The cultures were pulsed with 1µCi of ^{3}HTdR 18h prior to cell harvest.

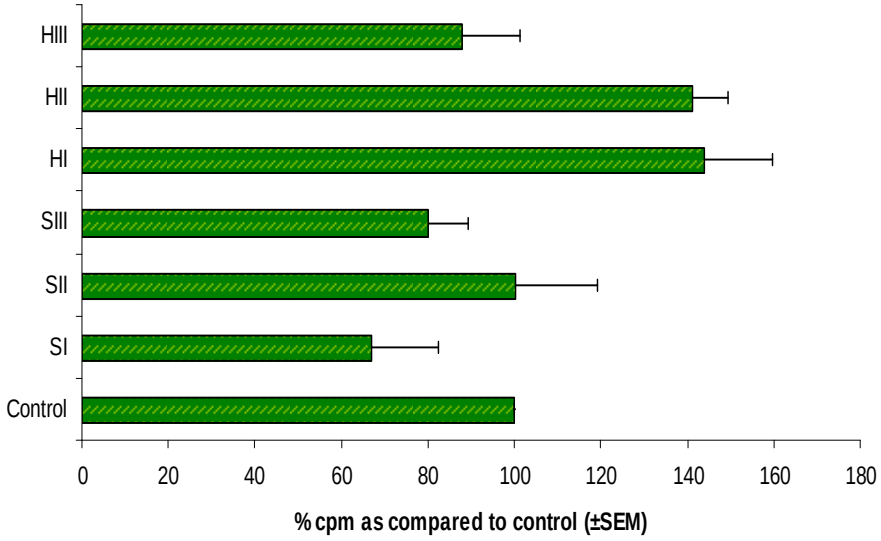


Figure 4.4. Effect of capsicum extracts on primary cells proliferation. *5×10^{5} cells/well were treated without (control) or with capsicum extracts at the indicated dose in 0.5% v/v for 24 h. ^{3}H-Thymidine incorporation assays were performed and the incorporation rates were expressed as percentage of corresponding control, which was considered as 100%.. Data represent mean ± S.E.M. values from four assays in triplicate wells.*

The results showed that SI and SIII inhibited primary cells growth by 40 and 20% respectively, as compared to extract-free cultures. Stimulation of cell growth was observed using the HI and HII extracts, which increased by 40% ^{3}HTdR uptake as compared to non-treated controls. No major differences were detected in cell growth by SII and HIII extracts as compared to controls (Figure 4.4).

Comparing the extraction methods of sweet and hot peppers, there was clear evidence that cold water-extracted sweet and hot peppers showed significant modulatory capacities and therefore it was decided to continue the experiments with these particular extracts.

4. 5. Effect of *Capsicum* Extracts on Proliferation of Different Cells Populations

To establish whether the anti-proliferative effect of extracts was affecting specific cell populations, macrophages, T and B lymphocytes were isolated by negative selection from spleen cells using magnetic bead-isolation techniques. The secondary effect of chili extracts on the proliferative activity of the different cell populations was analyzed by applying the supernatants from the cultures with sweet, hot extracts and untreated control cells to the isolated spleen cell populations.

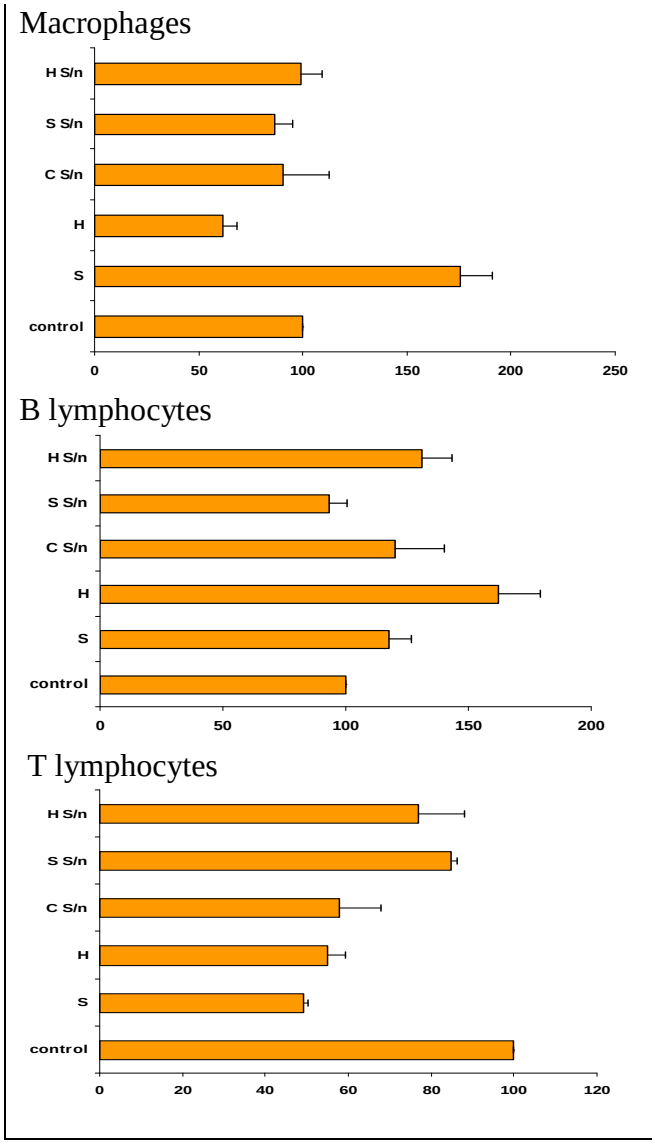


Figure 4.5. Effect of capsicum extracts on proliferation of different cells populations. *Macrophages, T and B lymphocytes isolated by negative selection of spleen cells using magnetic beads were incubated in 5×10^5 cells/well concentration without (control) or with capsicum extracts at the indicated dose in 0.5% v/v for 24 h. The secondary effect of chili extracts on the proliferative activity of the above cell populations was analyzed by application of 50% v/v supernatants from cells incubated with sweet (S S/n), hot extracts (H S/n) and untreated control cells (C S/n). The ^{3}HTdR incorporation rates were expressed as percent of cpms compared to non-treated controls, which was considered as 100%. Data represent mean ± S.E.M. values from three assays in triplicate wells.*

Chili extracts affected the cell proliferation rate, suggesting that they are also active on different isolated cell types at similar concentrations. The results showed that the hot pepper extracts significantly stimulated ($p<0.006$) B lymphocyte-growth and suppressed the proliferation of macrophages ($p<0.001$), while sweet pepper extract stimulated ($p<0.04$) both macrophages and B lymphocytes (Figure 4.5). The proliferation of T lymphocytes was suppressed by both sweet and hot extracts ($p<0.0001$).

No significant differences were found in the proliferation of macrophages exposed to supernatants, while sweet extract stimulated and hot extracts suppressed the growth of macrophages ($p<0.002$). Although supernatants from control cells (C S/n) and cells incubated with sweet extracts (S S/n) had no significant influence on the proliferative state of B lymphocytes, supernatant from hot-extract-supplied cells (H S/n), as well as extracts themselves, increased proliferation of B cells ($p<0.02$). It was noted also, that the proliferation trend of B lymphocytes exposed to of supernatants was similar to the direct exposure to S and H extracts. The percentage of T cells was significantly lower in all treatments as compared to control ($P <0.004$), except from the one using hot extract supernatants (H S/n).

4. 6. Effect of *Capsicum* Extracts on the Expression of MHC Class II Molecules

To evaluate a possible activation pathway in the extract-treated cells, the levels of MHC II molecules on B lymphocytes and macrophages indirect immunofluorescence and FACS experiments were performed, using a specific antibody to MHC II molecules-HB-3 ($H\text{-}2A^d$) as a primary antibody and FITC-conjugated goat anti-mouse IgG as a secondary antibody.

4. 6. 1. Effect of *Capsicum* Extracts on the Expression of MHC Class II Molecules on B Lymphocytes

Application of the sweet and hot extracts resulted in increase of the MHC class II molecules expression on B lymphocytes (Figure 4.6). While 15.3% fluorescent cells were detected in the untreated control, this percentage increased to 18.2 and 27.5% upon incubation with sweet and hot extracts, respectively. Considerable increase in the number of MHC class II molecules was detected also in the cells incubated with the supernatants, indicating a secondary effect of extracts on the expression of MHC class II molecules. The percent of fluorescent cells in the presence of cell supernatants incubated with sweet, hot extracts and untreated control cells was 21.5%, 34.9% and 33.6% respectively.

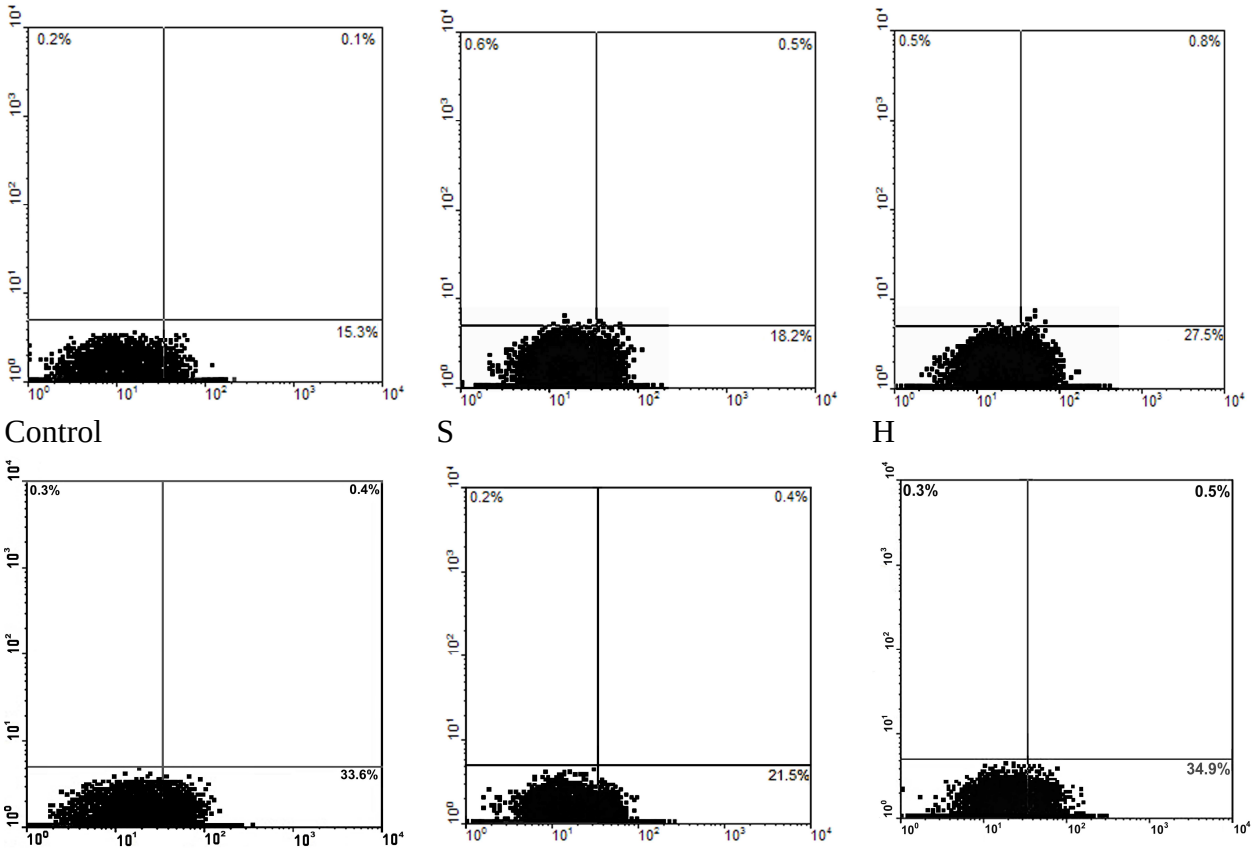


Figure 4.6. Expression of MHC class II molecules by B lymphocytes. *Immunomagnitacally isolated B lymphocytes were cultured in 0.5% v/v sweet (S) and hot (H) extracts, and in 50% v/v supernatants from cells incubated with sweet (S S/n), hot extracts (H S/n) and untreated control cells (C S/n) for 24 hours. Cells were labeled with FITC-conjugated anti-mouse IgG secondary antibody, counted in FACS Calibur Flow Cytometer analyzed by WinDMI 2.9 software.*

It should be noted that due to positive immunoselection of B cells a possible non-specific binding of secondary antibody to the magnetic beads could result in increased background. Furthermore, the probability of autofluorescence should not be ignored. Keeping these possibilities in mind, the increase of MHC class II molecules over background is presented in the figure 4.7.

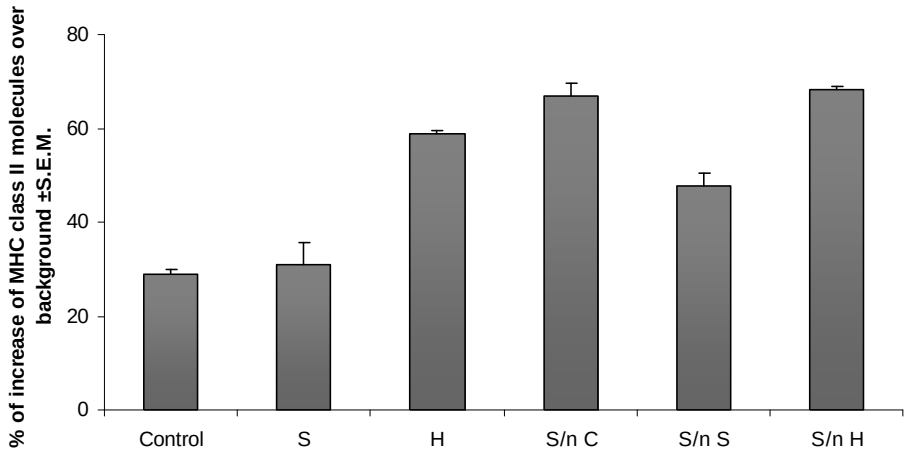


Figure 4.7. Increase of expression of MHC class II molecules on B lymphocytes over background. *Immunomagnitacally isolated B lymphocytes were cultured in 0.5% v/v sweet (S) and hot (H) extracts, and in 50% v/v supernatants from cells incubated with sweet (S S/n), hot extracts (H S/n) and untreated control cells (C S/n) for 24 hours. FITC-labeled cells were counted in FACS Calibur Flow Cytometer and analyzed by WinDMI 2.9 software.*

According to these results, significant increase ($p<8.02E-05$) in MHC class II molecule expression was recorded on the surface of B cells incubated in the extract from hot pepper. However, no differences were found in sweet-extract-incubated cells. Interesting results were recorded in the cases of application of supernatants from cells incubated with sweet, hot extracts and untreated control cells. All treatments increased ($p<0.001$) MHC class II molecules on the surface of B cells. The effect of the supernatant from cells cultured with hot extract didn't differ from the one from untreated control cells. Despite the increased levels of class II molecules in the presence of the supernatants from cells incubated with sweet extract, this treatment was of less efficient compared to the others.

4. 6. 2. Effect of *Capsicum* Extracts on the Expression of MHC Class II Molecules on Macrophages

Increase of MHC class II molecule expression was also observed in the case of macrophages cultured in the presence of sweet and hot extracts (Figure 4.8). The percent of fluorescent cells appeared to be 14.4% and 13.5% upon application of sweet and hot extracts respectively, as compared to 10.7% of untreated controls.

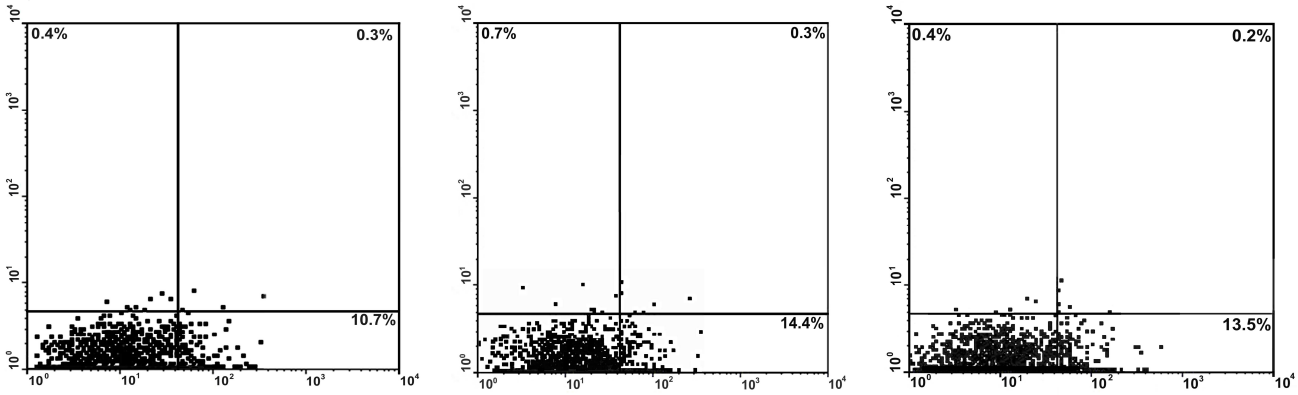


Figure 4.8. Expression of MHC class II molecules by macrophages. *Macrophages were cultured in 0.5% v/v sweet (S) and hot (H) extracts for 24 hours. Cells were labeled with FITC-conjugated anti-mouse IgG secondary antibody, counted in FACS Calibur Flow Cytometer and analyzed using the WinDMI 2.9 software.*

The increase of MHC class II molecules over background revealed significant differences in the cells cultured with the experimental extracts as compared to the extract-free medium ($p<0.008$). The proportional distribution of MHC class II molecules between sweet and hot extracts was of the same range and appeared to be 1.7 times higher than controls (Figure 4.9).

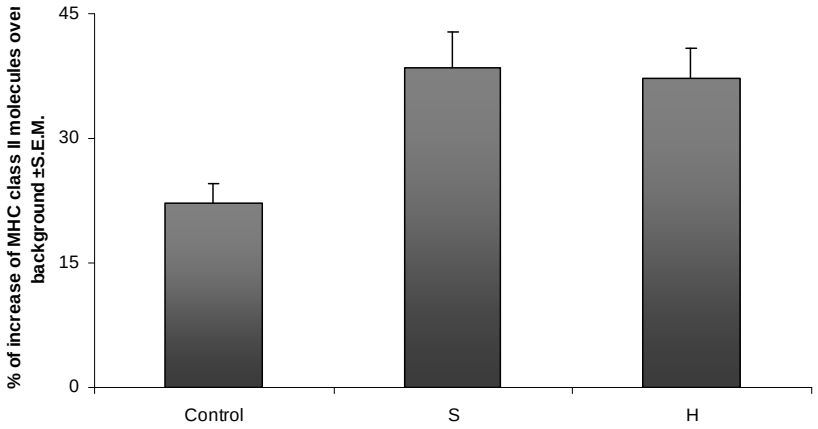


Figure 4.9. Increase of expression of MHC class II molecules on macrophages over background. *Macrophages, isolated from the spleen primary cell suspension were cultured in 0.5% v/v sweet (S) and hot (H) extracts for 24 hours. FITC-labeled cells were counted in FACS Calibur Flow Cytometer and analyzed by WinDMI 2.9 software.*

4. 7. Type of Cell Death Induced by *Capsicum* Extracts in Primary Cells

To characterize the type of cell death occurring in spleen cells upon the application of experimental extracts, a kinetic assay was set up and the appearance of DNA fragments in the culture supernatant (in case of necrosis) as well as in the cytoplasm (in case of apoptosis) was detected using Cellular DNA Fragmentation ELISA kit.

According to the results (Figure 4.10), within the first 2 hours of incubation with the experimental extracts, DNA fragments were detected only in the cytoplasm of the spleen primary cells cultured with the extract from sweet pepper ($p<0.007$). The appearance of DNA fragments in the cell lysates indicates that spleen primary cells incubated with sweet extract underwent apoptosis. However, further tests in 4, 6 and 8 hours didn't reveal significant changes in apoptotic state of the spleen cells upon application of the same extract. The induction of apoptosis observed at the beginning of the experiment could be explained by the impurity profiles present in the sweet extract, which didn't have prolonged and/or strong effect.

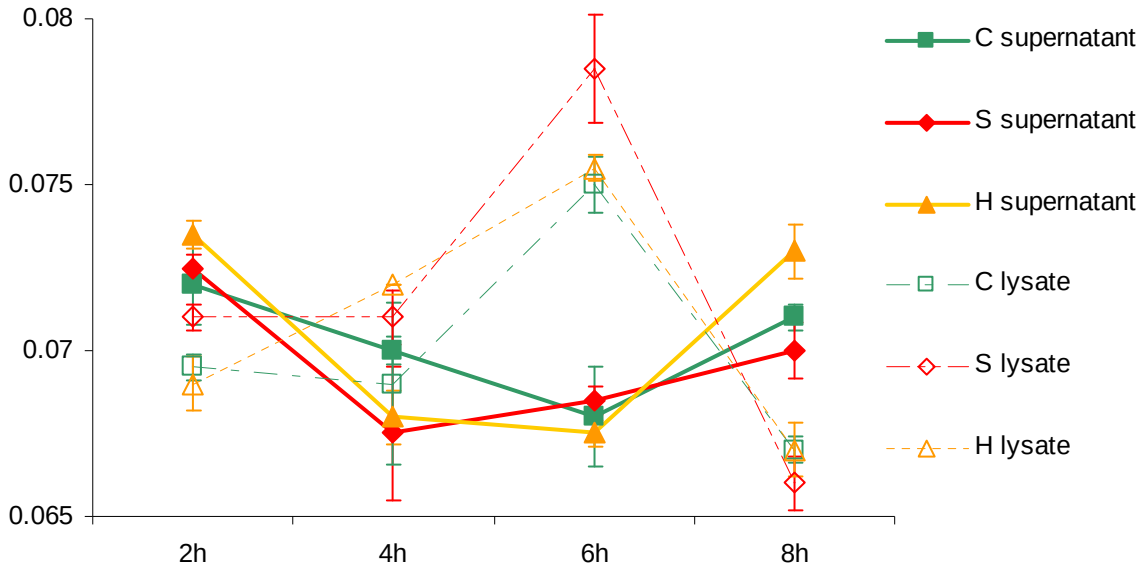


Figure 4.10. Kinetics of cell death induced by the capsicum sweet and hot pepper extracts in spleen primary cells ±S.E.M. *10^6 BrdU labeled cells/well were incubated either in the presence of 0.5% v/v sweet (♦, ◊) or hot (▲, Δ) extract or in the absence of any extract (■, □), for 2, 4, 6 and 8 h at 37°C and 5% CO_2. After the time indicated, 100 µl/well supernatant (■, ♦, ▲) and 100 µl/well lysate (◊, Δ, □) were removed and tested by ELISA.*

Further incubation up to 4 hours didn't have any considerable effect on the cells in the presence of the both extracts. Upon 6 hours of exposure to the sweet and hot pepper extracts, DNA fragments appeared first in the cell lysate, however, no significant differences were found between the treatments and control cells. No BrdU-labeled DNA fragments were detected in the supernatant during the first 6 hours after exposure to the extracts except in the case of sweet extract discussed above, indicating that DNA fragmentation occurred prior to plasma membrane lysis. This increase of cytoplasmatic DNA followed by the raise of DNA fractions in the cultures supernatants indicates a phenomenon that takes place during cell necrosis. It should be noted that at this stage, the effects of hot extract was more remarkable ($p<0.03$). The shift from apoptotic to necrotic cells detected in this experiment can be explained by the possibilities that after apoptosis cells underwent secondary necrosis. Further incubation of cells for 12, 24, 36 and 48 hours didn't show any differences in cell death kinetics (the results are not shown).

4. 8. Protein Profile of Sweet and Hot *Capsicum* Extracts

In order to find out the proteins that could be responsible for the functional activity of the experimental extracts, both sweet and hot extracts were analyzed by acrylamide gel electrophoresis. The SDS-PAGE of both extracts showed qualitative and quantitative differences in protein profiles (Figure 4.11). The major bands of both extracts (arrows) were cut in sterile conditions and processed for protein/peptide identification/characterization analysis to Functional Genomics Center Zurich.

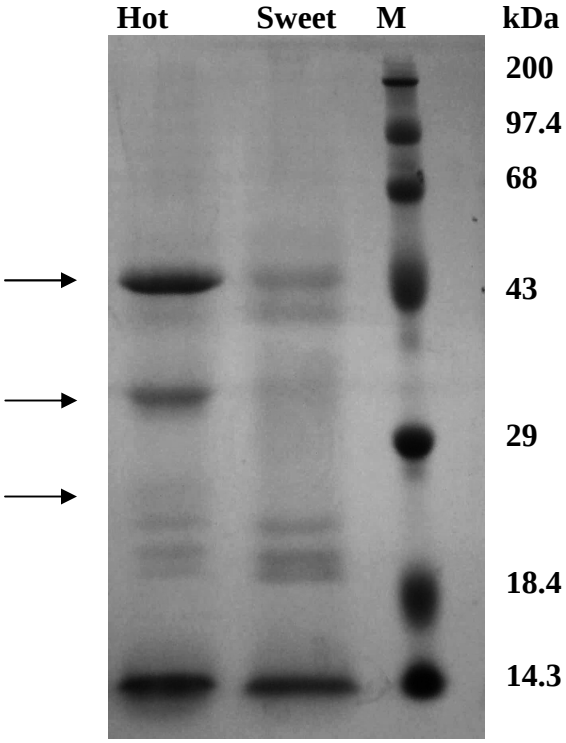


Figure 4.11. Protein profile of sweet and hot capsicum extracts. *Total amount of 150 µl chili extracts in ¼ dilutions was loaded on 4 and 12% SDS polyacrilamide gel by repeated loading of samples (50 µl) into the wells. Prestained Protein Marker (Gibco) was loaded in amount of 10 µl. Proteins were stained with Coomassie Blue*.

4. 8. 1. Protein Identification Analysis

The MALDI/MS/MS measurements were performed and database searches were performed by using the Mascot search programs. According to the results 1st band contained 25 proteins with scores greater than 79, which were considered significant ($p<0.05$) (Figure 4.12).

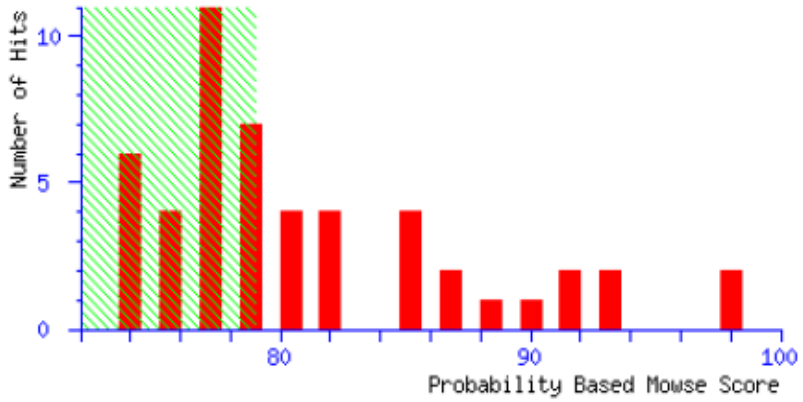


Figure 4.12. Proteins of the 1st band. *Ions score is -10*Log(P), where P is the probability that the observed match is a random event. Protein scores greater than 79 are significant (p<0.05). Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.*

The first homology matches according to the protein scale were the following:

Q628H0 -Mass: 83031 Score: 98 Expect: 0.00068 Queries matched: 6 Cluster: Hypothetical protein CBG00387; n=1; from *Caenorhabditis briggsae*

Q0K287- Mass: 61991 Score: 98 Expect: 0.00076 Queries matched: 4 Cluster: Putative peptidase, M20A subfamily; n=1; *Ralstonia eutropha* H16 Rep: Putative peptidase

Q1AZG5- Mass: 39557 Score: 93 Expect: 0.0023 Queries matched: 2
Cluster: Nitrilase; n=1; *Rubrobacter xylanophilus* DSM 9941

Q1N5D6 -Mass: 51982 Score: 93 Expect: 0.0023 Queries matched: 5
Cluster: Adenylate cyclase; n=1; *Oceanobacter sp*. RED65

The Protein Summary Report is attached in the Appendix 2.

The second band contained 10 matches with protein scales greater than 79 ($p<0.05$) (Figure 4.13).

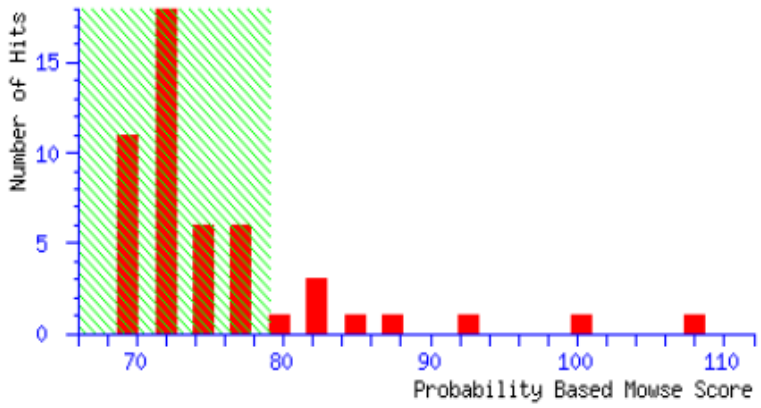


Figure 4.13. Proteins of the 2nd band. *Ions score is -10*Log(P), where P is the probability that the observed match is a random event. Protein scores greater than 79 are significant (p<0.05). Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.*

Q4DJF4 -Mass: 182153 Score: 108 Expect: 6.8e-05 Queries matched: 18
Cluster: Kinesin, putative; n=1; *Trypanosoma cruzi*

Q9C5J3 -Mass: 108219 Score: 101 Expect: 0.00034 Queries matched: 18
Cluster: Hypothetical protein At5g25060; n=1; *Arabidopsis thaliana*

Q2INP5 -Mass: 110397 Score: 92 Expect: 0.003 Queries matched: 12
Cluster: Surface antigen [D15] precursor; n=1; *Anaeromyxobacter dehalogenans* 2CP-C

Q628H0 -Mass: 83031 Score: 88 Expect: 0.00068 Queries matched: 6 Cluster: Hypothetical protein CBG00387; n=1; from *Caenorhabditis briggsae*

Homology searches by BLAST within different plants revealed high score matches between proteins in the 1st and 2nd bands with many proteins of *Ostreococcus lucimarinus* CCE9901, indicating the probability of contamination of hot pepper powder with this single-celled alga.

The 3rd band contained 1 match with protein scales greater than 79 ($p<0.05$) (Figure 4.14).

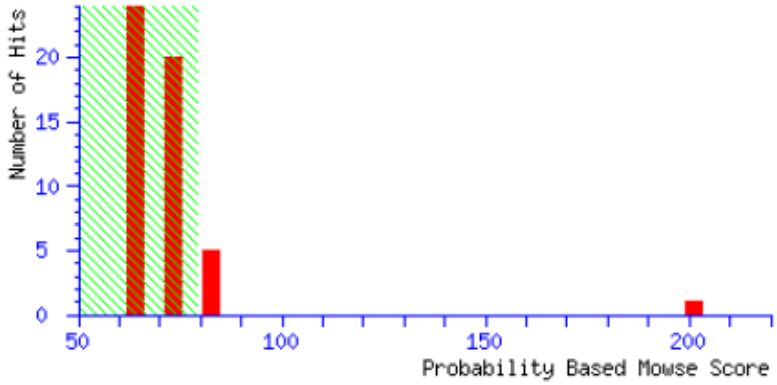


Figure 4.14. Proteins of the 3rd band. *Ions score is -10*Log(P), where P is the probability that the observed match is a random event. Protein scores greater than 79 are significant (p<0.05). Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits*

Q6L3H2-Mass: 59862 Score: 201 Expect: 3.4e-14 Queries matched: 3 Cluster: Putative vicilin, identical; n=1; *Solanum demissum*

4. 9. Effect of Fractions from Sweet and Hot Peppers on Cell Proliferation

For the identification of ingredients of experimental extracts, sweet and hot pepper extracts were further fractionated. Ionic exchange chromatography using DEAE-Sephacel anion-exchange gravity flow column resulted in seven fractions sequentially eluted with 0.01 (1), 0.05 (2), 0.1 (3), 0.25 (4), 0.5 (5), 0.75 (6) and 1 M NaCl (7). Fractions were tested for their functional activity on murine spleen cells in the experiments analyzing their effects on cell proliferation and cytokine production similar to ones with crude extracts. Concentrations of fractions applied to the cells were of correspondence to the extracts concentrations applied in starting experiments.

As it is shown in the Figure 4.15, certain fractions with proliferative and anti-proliferative activities were revealed.

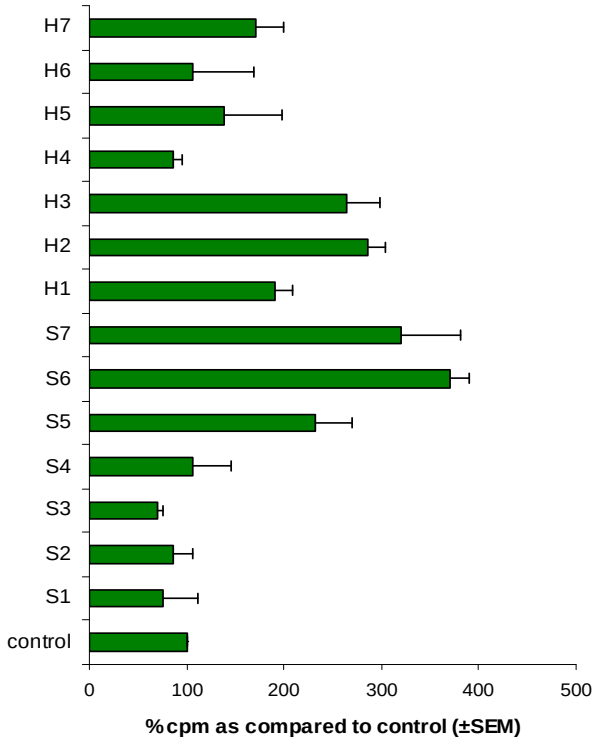


Figure 4.15. Effect of fractions from capsicum extracts on primary cells proliferation: *5×10^5 cells/well were treated without (control) or with capsicum extracts at the indicated dose in 3% v/v for 24 h. The cultures were pulsed with 1µCi of 3HTdR 18h prior cell harvesting. The ^{3}H-Thymidine incorporation rates are expressed as percentage of corresponding control which is considered as 100%. Data represent mean ± S.E.M. values from four assays in triplicate wells.*

Within the fractions of sweet pepper extracts, highly significant proliferative activity ($p<0.0008$) was recorded in the spleen cells cultured with S5-S7 fractions. The maximal proliferation of primary cells cultured with S6 fraction exceeded 3.7 times the one of control cells ($p< 5.54019E-08$). The first fractions of sweet extracts (S1-S4) didn't considerably affect cell growth, except S3 fraction, which showed significant suppressive effect ($p<4.7E-05$) inhibiting cell growth by 30%. Interestingly, opposite to the sweet extract, the first fractions of hot pepper extracts (H1-H3) had stimulatory effect on cell growth ($p<0.0001$). In this case, the highest proliferative activity was manifested by the H2 fraction. The cells incubated with H2, showed a 2.8-times higher proliferation rate than non-treated cells. H5-H7 fractions did not have noticeable effect on cell growth. No significant decrease in the cell proliferation rate was recorded by any of the hot pepper fractions. Among the hot fractions, the highest growth-inhibitory activity cell-growth-suppression was recorded using the H4 fraction and corresponded to 86% as compared to the control.

Two fractions from both sweet and hot pepper extracts were chosen as cell proliferation stimulators (S6 and H2) and cell proliferation inhibitors (S3 and H4) for the assessment of cytokine production.

4. 10. Effect of Selected Fractions from Sweet and Hot Peppers on Cytokine Production

Selected fractions were tested for cytokine production (Figure 4.16). According to the results, none of the applied fractions significantly altered the production of IFN-gamma. Nevertheless, it was noted that primary spleen cells cultured with the S3 fraction, in contrast to other fractions, showed increased release of IFN-gamma. Despite the fact that all applied fractions had tendency to increase the GM-CSF amount, significant increase of GM-CSF production ($p<0.03$) was recorded in the spleen cells incubated with the H4 fraction. Biosynthesis of TNF-alpha was also affected in the presence of sweet and hot fractions. However, the significant changes were recoded in the cases of S6 ($p<0.03$) and H4 ($p<0.005$), where 2.8 and 3.2 fold increase in TNF-alpha production was recorded in the culture supernatants respectively.

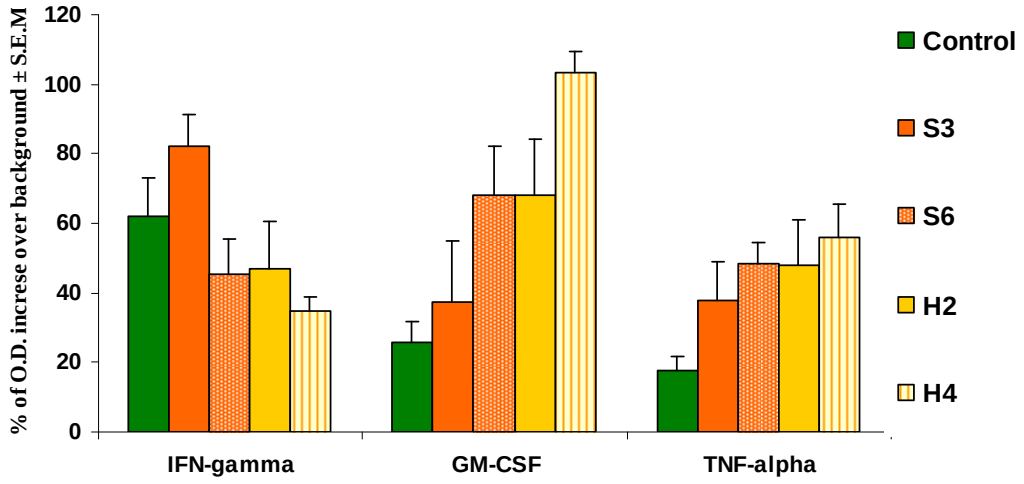


Figure 4.16. Effect of selected fractions from sweet and hot extracts on the production of IFN-gamma, GM-CSF and TNF-alpha*: Spleen cells (1×10^6 cells/ml) were cultured with 3% v/v of selected fractions (S3, S6, H2 and H4). Culture supernatant was collected after 24h of incubation and the profile of cytokine production was examined by ELISA. Anti-mouse monoclonal antibodies to IFN-gamma, GM-CSF and TNF-alpha were used as first antibodies; Anti-mouse IgG Peroxidase Conjugate was used as secondary antibody. Results represented as % of optical density increase over background. Data represents mean ± S.E.M. values from three assays in duplicate wells.*

4. 11. Protein Profiles of Selected Fractions from Sweet and Hot *Capsicum* Extracts

SDS electrophoresis of the selected fractions didn't reveal any band when the fractions were applied in the initial concentration. Fraction samples were further concentrated 50 times using protein concentrating tubes and applied to the SDS gel.

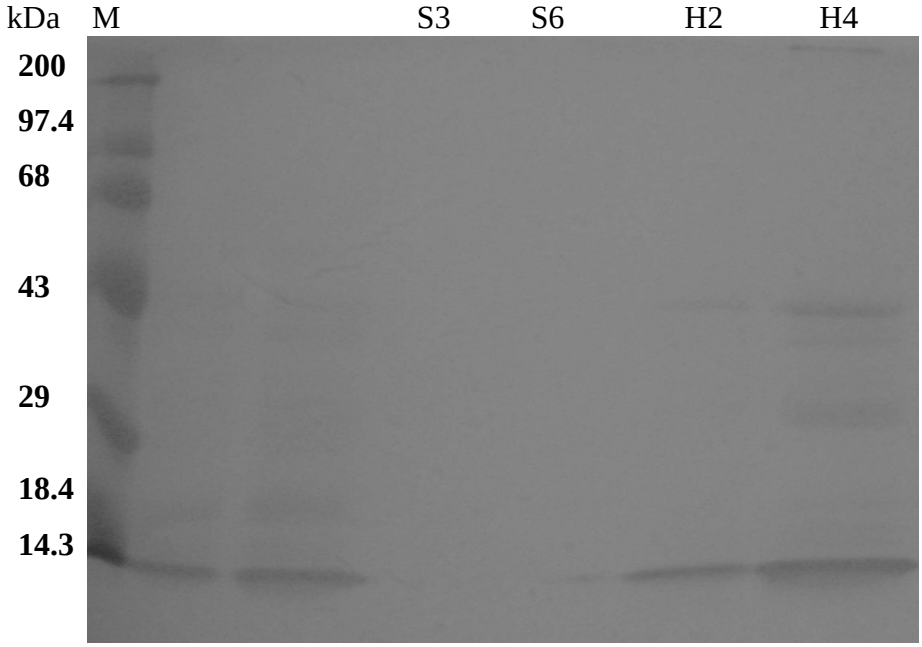


Figure 4.17. Protein profile of selected fractions from sweet and hot capsicum extracts. *Total amount of 50 µl chili extracts was loaded on 4 and 10% SDS polyacrilamide gel. Prestained Protein Marker (Gibco) was loaded in amount of 10 µl. Proteins were stained with Coomassie Blue*.

The results presented in Figure 4.17 show that no protein band was detected in the sweet fractions. The profile of the hot fractions was similar to the one of crude hot extract (Figure 4.17). Considerable high amount of proteins was at the H4 fraction. According to the results it can be concluded that the activity of the sweet fractions was not mediated by the active proteins. In case of hot extracts it is difficult to estimate the role of protein(s), however the protein profiles in proliferative (H2) and anti-proliferative (H4) fractions are different.

4. 12. Polyamines in Sweet and Hot pepper Extracts and Selected Fractions

In order to find out whether non-protein components in the extracts and their fractions were responsible for the functional alterations of spleen primary cells, extracts from sweet and hot peppers as well as active fractions selected from the previous experiments were analyzed for polyamine availability using high performance liquid chromatography (HPLC). As it is shown in Table 4.1, which represents the mean values of two independent experiments, the extracts from sweet and hot peppers differed in their polyamine contents. The used powders derived from mature peppers at the red stage when the fruit is a bright red color. In spite of the fact that both extracts showed the same proportional distribution of polyamines: Put>Spd>Spm, the total content of each compound varied widely among the two extracts. Amount of putrecin (Put) in the hot extract 35 times exceeded one of the sweet extract. Interestingly, the concentrations of spermidin (Spd) and spermin, (Spm) in the sweet pepper extract were higher than the ones in the hot extract. Moreover, these polyamines in the hot extract were detected only during the first experiment.

Put, Spd and Spm were not detectible in neither fractions of sweet extracts (S3 and S6), nor in the H4 fraction of hot pepper. The second fraction of hot extract (H2) was the only analyzed fraction that contained polyamines. The contents of Put and Spd in H2 were 2.32 and 0.18 nmols/mg, respectively; however the amount of Spm was not detectable.

Table 4.1. Content of polyamines in sweet and hot extracts and selected fractions. *Benzoylated polyamines in the ether fraction were analyzed by HPLC with a Hewlett-Packard 1100. The quantities of polyamines are represented in nmols/mg of dry weight. Means ± S.E.M. of two independent experiments with two replicates are shown. Notes: nd-not detectible.*

	Put		Spd		Spm	
Hot pepper extracts	102.82	± 24.09	0.49	0	0.11	0
H2 fraction	2.32	± 2.06	0.18	0	nd	nd
H4 fraction	nd	nd	nd	nd	nd	nd
Sweet pepper extract	2.95	± 0.60	2.17	± 0.39	1.45	± 0.36
S3 fraction	nd	nd	nd	nd	nd	nd
S4 fraction	nd	nd	nd	nd	nd	nd

4. 13. Content of Carbohydrates in Sweet and Hot Extracts and Selected Fractions

The total amount of carbohydrates in sweet and hot red pepper extracts and in the selected fractions was analyzed by the anthrone-sulfuric acid colorimetric assay. According to the results, carbohydrates were detected in the all tested samples (Table 4.2). Considerable high carbohydrates contents were recoded in both sweet and hot red pepper extracts, however, the ratio of total carbohydrates in the extract of sweet pepper: hot pepper is 1.2 indicating excess of this compounds in the sweet pepper extract.

Different amounts and distributions of carbohydrates were found in the active fractions of the sweet and hot extracts. The lowest amount of total carbohydrates was recoded in the S6 fraction of the sweet extract (0.07g/l), which exhibited proliferative properties on the murine spleen cells. Interestingly, the hot pepper (H4), which was showing the inhibitoriest activity on spleen cell growth, had the highest carbohydrate value, exceeding almost by 3.5 times the anti-proliferative activity of the sweet pepper fraction S3 and was the richest in total carbohydrate contents. At the same time, equal amounts of 0.9 g/l of total carbohydrates were detected in S3 and H2 fractions, which were shown to suppress and stimulate cell growth, respectively.

Table 4.2. Content of carbohydrates in sweet and hot extracts and selected fractions. *The total amount of carbohydrates was measured by anthrone-sulfuric acid colorimetric assay for glucose-based carbohydrates. The quantities of carbohydrates are represented in g/l. Means ±S.E.M. of two independent experiments with three replicates are shown.*

	Total amount of carbohydrates (g/l)	
Sweet pepper extract	68.65	± 0.68
S3 fraction	0.09	± 0.02
S6 fraction	0.07	± 0.01
Hot pepper extract	56.47	± 0.32
H2 fraction	0.09	± 0.02
H4 fraction	0.31	± 0.09

4. 14. Pigments in Sweet and Hot Peppers

In view of the impossibility to extract water-dissolved pigments from aqueous extracts of sweet and hot peppers into organic solvents, crude sweet and hot pepper powders were used for pigment separation analysis.

Thin-layer chromatography (TLC) with petrol ether/propanol/water (100:10:0.25; v:v:v) resulted in 12 bands and varied from light yellow to terracotta colors, indicating the presence of carotenoids (Figure 4.18 and 4.19).

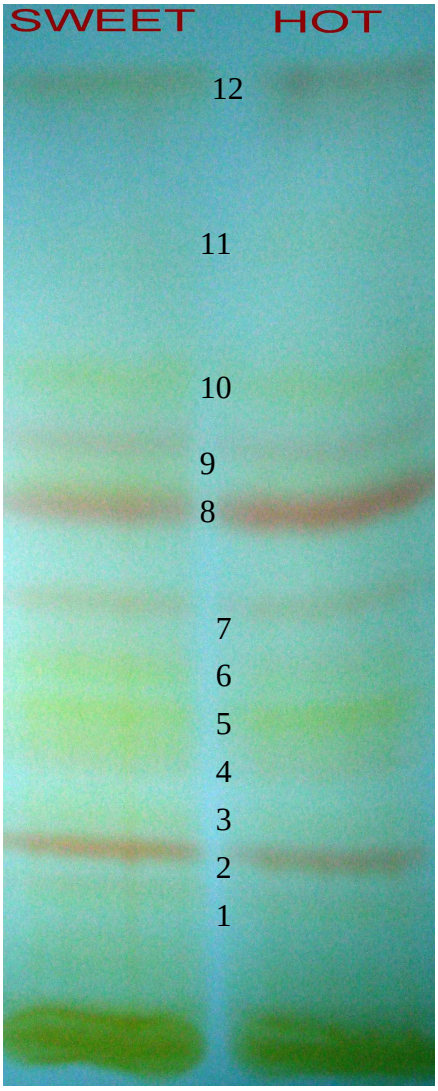
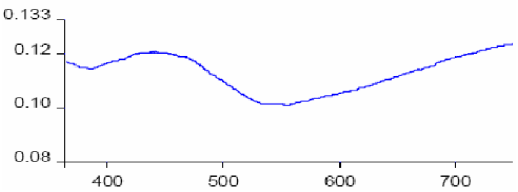


Figure 4.18. Thin Layer Chromatography (TLC) of sweet and hot peppers pigments. *Pigment from crude sweet and hot pepper powder extracted in petrol ether/propanol-2/water (100:10:0.25; v:v:v) were separated by TLC. Absorbance spectra of the bands in visible light (380-750nm) were recorded in UV/VIS Spectrometer Lambda 20 (Perklin Elmer) at room temperature.*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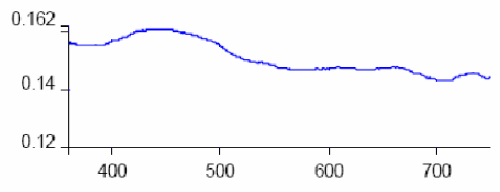
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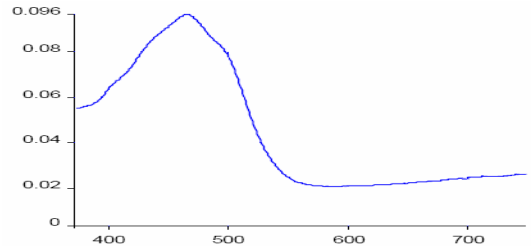
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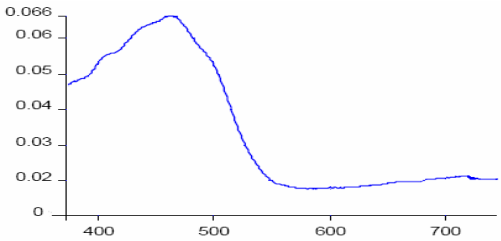
2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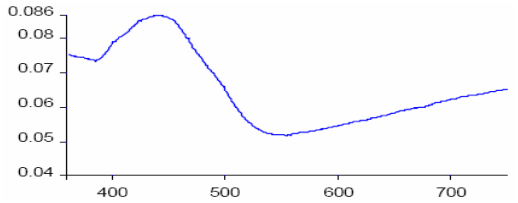
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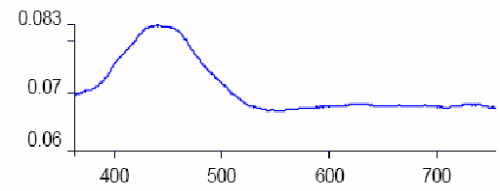
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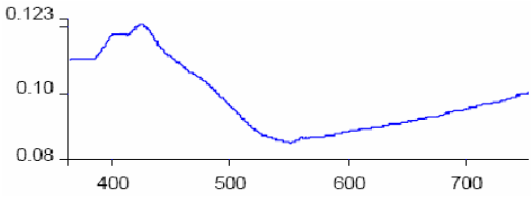
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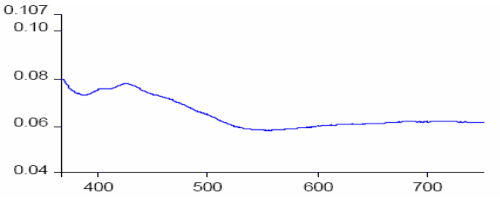
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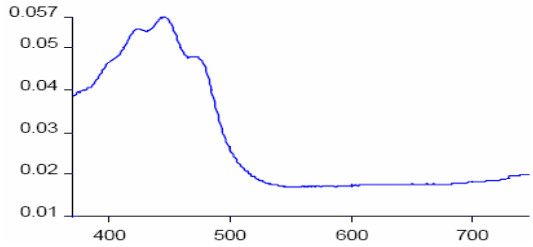
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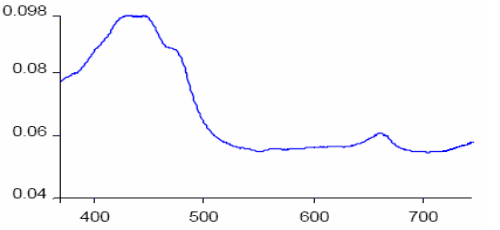
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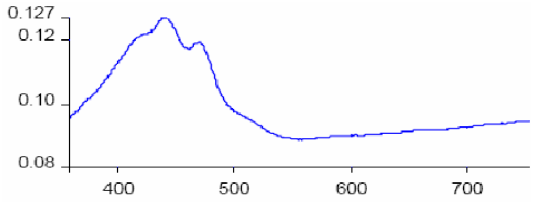
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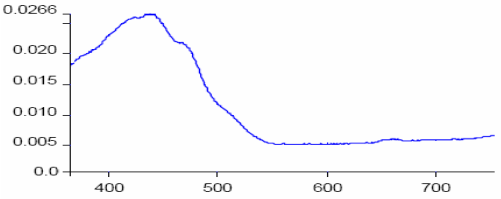
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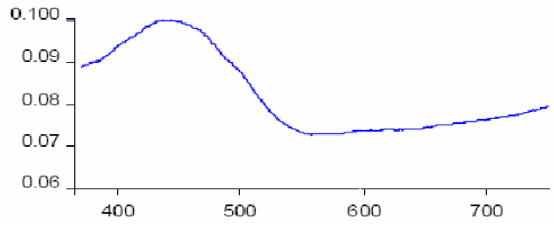
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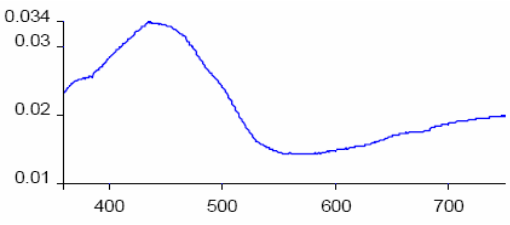
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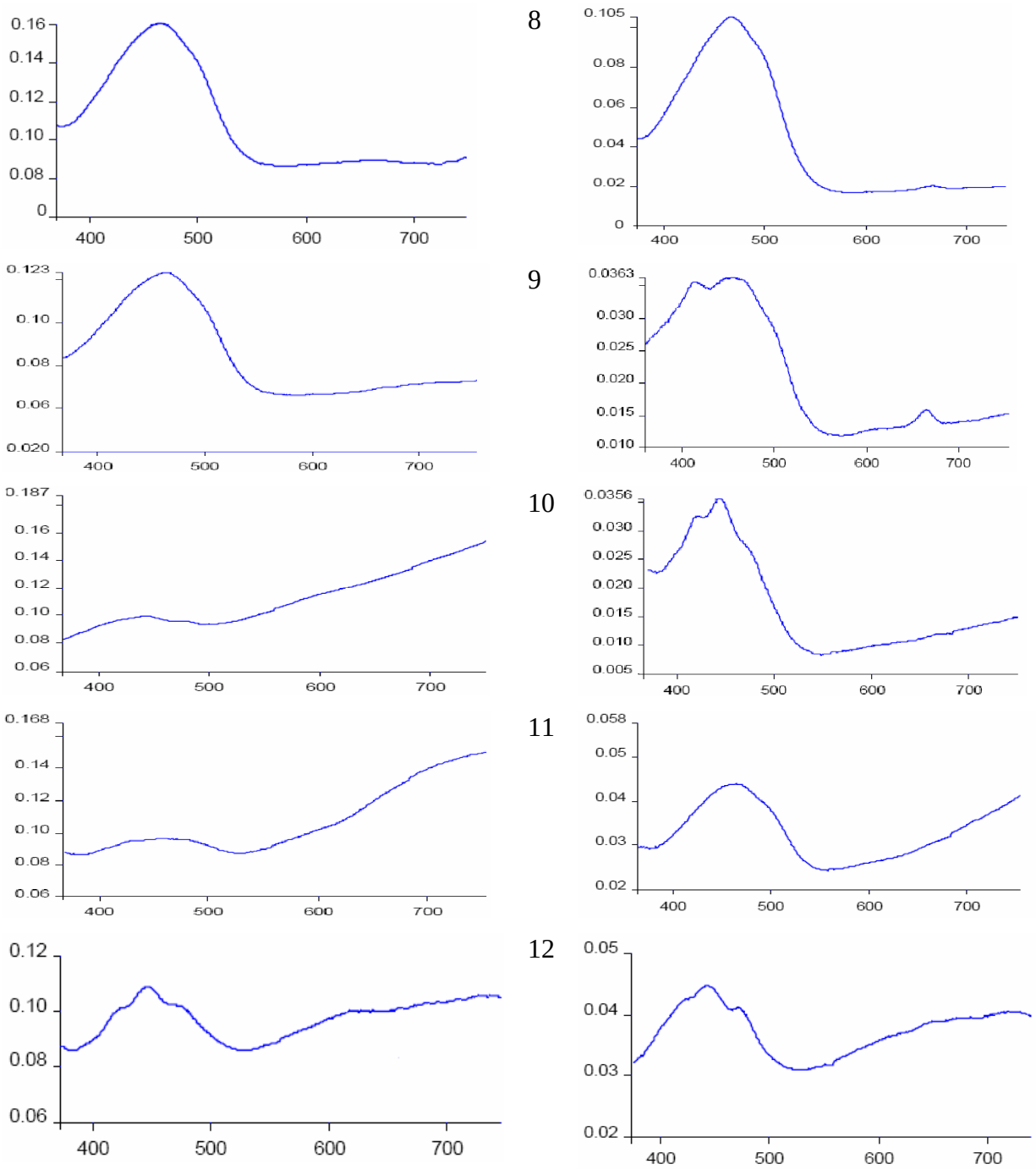

Figure 4.19. Analysis of absorption spectra of the selected bands from the TLC plate. *Pigment from crude sweet and hot pepper powder extracted in petrol ether/propanol-2/water (100:10:0.25; v:v:v) were separated by TLC. Absorbance spectra of the bands in visible light (380-750nm) were recorded in UV/VIS Spectrometer Lambda 20 (Perklin Elmer) at room temperature.*

The resulting bands were further measured for absorption spectra in visible light (380-750 nm) and are presented in the Figure 4.18. Almost all spectra showed small shoulders indicating insignificant contamination levels, probably caused by different pigments. Absorbance spectrum of all bands was in 400-500nm, which characterize carotenoids. However the possible availability of chlorophylls with absorption maximums at 430 and 453 nm (chlorophyll α and b, respectively) should not be ignored. The small pick in the 5^{th} and 9^{th} bands of hot pepper in the absorption area of chlorophylls can propose that these particular bands had traces of chlorophyll α, with absorbance maximum at 662 nm. Comparing the obtained absorbance maxima of the bands with known data from the literature, as well as their chromatographic behavior, it may be proposed that the pigment of the last band (12) may correspond to β carotene with a peak of absorption maximum at 440 nm. The nature of other pigments remains open to further investigation.

The relative quantitative estimation of the pigments in the sweet and hot peppers is represented in the Table 4.3. According to the results, the amount of pigments in the sweet pepper was higher than hot pepper.

Table 4.3. Relative quantitative values of the pigments in sweet and hot peppers. *Absorbance spectra of the bands from sweet and hot pepper powder in visible light (380-750nm) were recorded in UV/VIS Spectrometer Lambda 20 (Perklin Elmer) at room temperature. Data represent the values of absorption maximums over background.*

Bands	Sweet Pepper	Hot Pepper
1	0.033	0.022
2	0.076	0.048
3	0.036	0.018
4	0.04	0.023
5	0.039	0.04
6	0.037	0.0216
7	0.025	0.019
8	0.075	0.087
9	0.061	0.0243
10	0.006	0.0296
11	0.013	0.02
12	0.025	0.013

V. Discussion

Discovery and identification of novel safe drugs, without severe side effects, is an important goal of research in cancer chemotherapy. Cell growth regulation is a key objective in anticancer research. On the other hand, the enhancement of host immune system through the regulation of cytokines in the cytokine network as defense mechanisms, as well as expression of MHC class II molecules have been recognized as important pathways for inhibiting tumor growth without harming the host.

Chili plays multiple roles in pharmacological and biological functions as a well-known folk medicine and also as a spice. Preliminary observations had detected an anti-proliferative effect of chili extracts. Chili peppers also are well known for their antioxidant and anti-inflammatory properties which along with the anti-proliferative activity and regulation of apoptosis contribute to cancer chemoprevention or chemoprotection. Literature data indicate that effects of capsaicin, at some degree, rely on the doses, routes of administration and types of tissues affected.

In order to evaluate the functional activity of dietary red sweet and hot peppers, the present study was conducted to assess cell proliferating and immunomodulating activity of aqueous extracts from sweet and hot chili peppers on murine spleen cells (BALB/c) with particular focus on the proliferative state of primary spleen cells and specific cell populations (macrophages, T- and B lymphocytes), cytokine production, expression of MHC class II molecules and apoptotic status of cells upon the application of extracts. In addition, significant effort was given on the identification of the active molecules in the extracts which would be responsible for the above effects.

5.1. Proliferating and Anti-proliferating Activity of *Capsicum* Extracts

Cell growth regulation is a key objective in anticancer research. Therefore the assessment of extracts' effect on spleen cell growth was of great importance. Many studies have shown the growth-inhibitory effect of capsaicin on several cancer cells; however no study has been done for the growth-inhibitory potential of the chili extracts on primary spleen cells. In

viable cell count assays, capsaicum extracts induced dose and time depended suppression of spleen cells growth. Interestingly, the different extracts have been found to retain proliferative and antiproliferative activities assessed by ^{3}H-thymidine incorporation assay, which can be explained by the different compositions of the extracts as well as their extraction type.

The potential efficacy of immunotherapy for cancer treatment has led to the development of novel experimental strategies. Many of these innovative approaches focus on the activation of tumor-specific T lymphocytes, because this cell mediated arm of the immune system can be effective in destroying tumor cells and in providing long-term protection (memory) against the recurrence and/or outgrowth of primary and/or metastatic tumor cells. In the light of this proposal, interesting findings on the effect of extracts on the different cell populations were recorded. Both sweet and hot extracts inhibited the proliferation of T cells. Nevertheless this suppression cannot be considered fatal for the organization of immune response, since nothing is known about the activity of these specific T cells. Sweet extract increased the number of macrophages and B cells, while the proliferative activity of hot extracts was profound on B lymphocytes.

Several mechanisms could be responsible for the pro- or antiproliferative activities of the extracts. DNA synthesis was assessed by the ability of cells to incorporate ^{3}H-thymidine into their DNA in primary cell culture after chili extracts treatment, therefore it can be concluded that growth inhibition might be associated with inhibition of DNA synthesis.

The inhibitory effect could be also due to inhibition of DNA polymerase. The effects of the fraction containing major natural glycolipids from sweet pepper *Capsicum annuum var. angulosum* such as monogalactosyl diacylglycerol (MGDG), digalactosyl diacylglycerol (DGDG) and sulfoquinovosyl diacylglycerol (SQDG) on calf replicative polymerase *a* and rat DNA repair-related polymerase *h* was studied by Kuriyama *et al.*, 2005. The application of SQDG fraction showed that sweet pepper had the inhibitory activity toward polymerase *a*, suggesting that SQDG was able to penetrate the cancer cells and reached the nucleus inhibiting polymerase *a* activity. On the other hand, the glycolipid fraction had no effect on polymerase *h.* It was proposed that the glycolipid fraction from plants might be containing not only SQDG but also other compounds which interfered with the polymerase *h* inhibitory activity by SQDG. However, the water extract containing the

watersoluble fraction and the ethanol extract containing the fatsoluble fraction did not influence the cell proliferation of human cancer cells.

Beside this, the growth-inhibitory potential of extracts could be due to the induction of cell cycle arrest at the G1 phase of the cell cycle upon treatment. G1 cell cycle arrest has been found to be a common feature of many anticancer drugs. Recent studies support another hypothesis, according to which Tax may induce cell cycle progression through protein–protein interaction. Tax, a HTLV-1 transcriptional transactivator protein, can interact with various cellular proteins, such as nuclear factor (NF)-κB, cAMP response element binding (CREB) protein and serum response factor (SRF), activating thus the transcription of proto-oncogenes (c-*fos*, c-*jun*, *fra-1* and c-*myc*), cytokines (IL-2, IL-6, TGF-β and GM-CSF) and cytokine receptor (IL-2R). Tax can also repress the transcription of cellular genes, such as DNA polymerase β and *bax*. So Tax is considered to play a crucial role in several pathways on the transformation of T cells by HTLV-1. Cell cycle arrest in G1 phase by capsicum extracts treatment may be associated with the down-regulation of Tax expression. This theory was proposed by Zhang *et al*., 2003 on the human T-cell leukemia virus type 1 (HTLV-1)-associated adult T-cell leukemia (ATL) cells.
However, it wouldn't be wise to exclude the other possible mechanisms of G1 cell cycle arrest, like the induction of CDK inhibitors.

Although there are a number of publications focusing on the antiproliferative potential of capsaicin or spicy pepper extracts, the test substances used in these studies vary according to source, purity and impurity profile. It was reported that *trans*-capsaicin appears to have very limited genotoxic potential and displays a different profile than impure capsaicin or chili extracts in genotoxicity assays (Chanda *et al.*, 2005).

The results of a study by Fischer *et al*. (2001) indicated that capsaicin (in 50% ethanol/50% water) blocked receptor-stimulated Ca^{2+} entry in Jurkat T cells (human leukemic T-cell line). Capacitative calcium entry (CCE) through Ca^{2+} channels is an absolute requirement for normal activation of T lymphocytes. Capsaicin also rapidly blocked voltage-gated K^+ current in a nonvoltage-dependent manner.

The relationship between blockage of inward Ca^{2+} current by capsaicin and functional immunosuppressive activity-TCR activation was evaluated using Jurkat cells.

Both TCR-dependent and TCR-independent production of IL-2 (an immune modulator) was suppressed by capsaicin in a dose-dependent manner. The dose-dependence of capsaicin-induced reduction of IL-2 was comparable to its blockage of inward Ca^{2+} current, indicating the functional relevance of capsaicin-induced blockage of lymphocyte capacitative calcium entry. Based on the results of this study, the authors stated that capsaicin and its analogs may have potential use as immunomodulatory drugs (Fisher *et al.,* 2001).

Chili extracts were tested for its ability to affect B cell and T cell mitogenic responses. According to the results, chili extracts showed a dose depended suppression on cell proliferation and didn't affect the ability of cells to respond neither to the B-cell mitogenic stimulus of LPS nor to the T cell mitogenic stimulus of ConA (Avetisyan, 2005).

5. 1. 1. Induction of Apoptosis by *Capsicum* Extracts

According to the results of the present study, the differences in proliferative and antiproliferative profiles of spleen primary cells incubated with sweet and hot peppers did not seem to cause apoptosis or necrosis. Similar results were found by Zhang *et al.,* 2003. They reported that capsaicin induced neither apoptosis nor necrosis in normal peripheral blood resting T cells which were treated with up to 100 µM for 72 h. Another study also reported that capsaicin did not induce any growth inhibition in non-cancerous, spontaneously immortalized human breast epithelial cell line MCF10A, which suggests a capsaicin inhibitory effect only on undifferentiated cells (Soule *et al.,* 1990).

Nevertheless, short-term apoptotic state within the first hours of incubation was recorded upon application of the sweet extract. This effect is probably caused by impurity profiles of the extract.

Induction of capsaicin-induced apoptosis in the cancer cells widely reported in the literature is proposed to be due to different mechanisms. Many findings suggest that vanilloids can target both mitochondrial and plasma membrane electron transport systems, thereby generating ROS that can mediate apoptosis. However, it can be argued that such inappropriate ROS generation may also have a deleterious effect on nonmalignant cells as well, and if this is the case, the vanilloids would be metabolic poisons rather than valuable

candidates for use in preventive therapy for skin cancer or other cutaneous disorders (Surh, 2002).

Other investigators have demonstrated that capsaicin-induced apoptosis in some transformed cells and in activated T cells is associated with the suppression of plasma membrane NADH-oxidoreductase (PMOR), an enzyme that transfers electrons from cytoplasmic NADH via coenzyme Q (ubiquinone) to external electron acceptors such as oxygen. PMOR is thought to be involved in the control of cell growth and proliferation by maintaining the proper NAD+/NADH ratio required for cell viability. However, PMOR activity in normal tissues and in nontransformed cells is responsive to growth factors and hormones, whereas PMOR activity in tumor tissues and transformed cells is not (Morre *et al.,* 1995, Morre *et al.,* 1996). Nevertheless it is not yet clear whether NAD(P)H oxidoreductase is crucial for cell proliferation. The availability of several different genetically tractable animal models, in which the gene encoding NAD(P)H oxidoreductase is mutated or deleted, will help us better understand the functional role of this enzyme and possibly facilitate the discovery of drugs that selectively kill tumor cells by targeting this enzyme (Surh, 2002).

It is well known that activation of caspases trigger the apoptotic process in various cells. One of the characterized mechanisms for pro-caspase-3 activation involves translocation of the respiratory chain protein, cytochrome c, from the mitochondria to the cytoplasm. Bcl-2, an anti-apoptotic oncoprotein, has been shown to act on mitochondria and prevent the release of cytochrome c and thus caspase activation while Bax accelerates these processes. Capsaicin induced activation of caspase-3 in Hepatocellular carcinoma SK-Hep-1 cells at the same concentration which caused prominent decrease of Bcl-2 and increase of Bax. It is suggested that decreased level of Bcl-2 may accelerate caspase-3 activation during capsaicin-induced apoptosis of SK-Hep-1 cells (Jung *et al.,* 2001).

Capsaicin is found to be a potent inhibitor of NF-κB activation. NF-κB is a ubiquitous transcription factor that binds to a specific DNA sequence as a dimeric complex composed of various combinations of members of the Rel/NF-κB family, homodimers or heterodimers of RelA (p65), p50, c-Rel, p52 and RelB. In resting lymphocyte, NF-κB dimers are sequestered in the cytoplasm in an inactive form by association with an inhibitory IκB subunit, mainly Iκ-Bα. Following cellular activation, multiple kinases lead to

phosphorylation of Iκ-Bα and proteosome-mediated degradation, resulting in the release of an active NF-κB complex that translocates to the nucleus. In the nucleus, NF-κB binds its response elements and activates various genes involved in the inflammation, immune response and cellular growth control. The activation of NF-κB is also essential for the inhibition of apoptosis (Zhang *et al*., 2003).

Activation of NF-κB plays an important role in prevention of apoptosis due to elevated expression levels of several NF-κB-inducible cytokines, such as IL-6, IL-10, IL-15, and IFN-gamma. Decrease in the NF-κB activity, at least in part, may be responsible for induction of apoptosis in ATL cells observed by Zhang *et al.*, 2003. In addition to the decreased NF-κB activity, downregulation of Bcl-2 may be also responsible for the capsaicin-induced apoptosis.

5. 2. Immunomodulating Activity of *Capsicum* Extracts

5. 2. 1. Effect of *Capsicum* Extracts on Cytokine Production

The enhancement or potentiation of host defense mechanisms has been recognized as a possible means of inhibiting tumor growth without harming the host. The production of cytokines is a key event in the initiation and regulation of an immune response. Therefore, the regulation of cytokine production is important in induction of immune reactions. Many compounds are now used routinely to modulate cytokine production, and therefore the immune response, in a wide range of diseases, such as cancer.

Cytokines are mediators of all aspects of immunoregulation, so the capacity to induce or enhance cytokine production could be a major mechanism by which chili extracts exert immunomodulatory effects. IFN-gamma is secreted by T lymphocytes and natural killer cells only. IFN-gamma has antiviral, immunoregulatory, and anti-tumour properties. It alters transcription in up to 30 genes producing a variety of physiological and cellular responses. Interleukin-2 stimulates the proliferation of cytotoxic T lymphocytes, helper T lymphocytes, natural killer cells, lymphokine-activated killer cells and macrophages, all of which can participate in immunological antitumor mechanisms. Tumor necrosis factor-

alpha (TNF-alpha) is a peptide, which plays a pivotal role in host defense, secreted from activated macrophages and T cells. It may act on monocytes and macrophages in an autocrine manner to enhance various functions, such as cytotoxicity to tumor cells, and to induce the expression of a number of other immunoregulatory and inflammatory mediators (Gan *et al*, 2003).

However, because of the short half-lives of cytokines such as IFN-gamma, IL-2 and TNF-alpha in serum, systemic administration of high doses of IL-2 and TNF-alpha is needed, resulting in severe side effects, such as vascular leak syndrome, edema, anemia, fever and hypotension. One strategy to reduce the incidence and/or severity of cytokine therapy is induction therapy. In the present study it was shown that administration of the sweet and hot capsicum extracts upregulated the production of IFN-gamma, IL-2 and TNF-alpha by the spleen primary cells. The results suggest that chili extracts may induce immune responses and possess potential therapeutic efficacy in cancer.

Unfortunately, there are only few data about the effect of chilli extracts on cytokine production. According to Athanassakis, 50% v/v of chilli extract didn't have any significant effect on IL-2, IL-4, IL-10 and TNF-alpha production by human primary leukocytes from peripheral blood samples of healthy donors, while there was an increase of IFN-gamma by 54% compared to untreated control (Athanassakis I., unpublished). On the other hand, capsaicin, the active compound of chilli extract, significantly (10 folds) increases the levels of IL-6, IL-10 and PGE2 in the primary culture of adult rat DRG neurons (Saade *et al.*, 2004).

Capsaicin significantly inhibited the production of TNF-alpha by macrophages in a dose-dependent manner. It was shown that capsaicin acts as a ligand for PPARc, which regulates the production of the pro-inflammatory cytokine TNF-alpha, indicating that the anti-inflammatory action of capsaicin may be mediated by PPARc activation in LPS-stimulated RAW 264.7 cells (Park *et al.*, 2004).

TNF-alpha is reported to impair pre-adipocyte differentiation and induce lipolysis and apoptosis. Capsicum extract treatment of 3T3-L1 adipocytes decreased TNF-alpha mRNA levels, but had no apparent affect on apoptosis (Ahn *et al.*, 2006).

5. 2. 2. Effect of *Capsicum* Extracts on the Expression of MHC Class II Molecules

The present study provides evidence for the first time that sweet and hot pepper extracts can upregulate the expression of MHC class II molecules on the B lymphocytes and macrophages. Therefore, it can be proposed that the extracts play certain role in the regulation of immune response.

One of the main roles in the regulation of the immune response belongs to the MHC class II molecules. MHC class II molecules activate CD4+ T cells, which are the central orchestrating cells of an effective and long-lasting immune response (Hong *et al*., 2007). These CD4+ T cells ultimately activate CD8+ cytotoxic T cells (CTLs) that recognize tumour cells via MHC class I molecules and are prepared to lyze them directly. Tumour cells expressing MHC class I and II molecules together with co-stimulatory molecules can evoke an immune response. Therefore, new strategies to induce MHC class II can point out the important role in the generation of an effective immune response.

The effect of the capsicum extracts on the biosynthesis of the MHC class II molecules had not been yet reported. Increase in MHC class II molecules expression by B lymphocytes and macrophages upon administration of the extract from hot and sweet peppers can be a promising approach in term of cancer immunotherapy, introducing dietary spices as a potent immunovaccine.

The activation of the MHC class II molecules is very likely connected to the production of cytokines. This hypothesis supports the fact that MHC class II were in excess upon the application of the supernatants from the cells incubated with the experimental extracts as compared to the supernatants from non-treated control cells. It is known that cytokines like IFN-gamma and TNF-alpha, produced by T-helper 1 (Th1) cells are necessary for activating CD8+ CTLs. So, CD4+ T cells orchestrate the immune response by secreting different cytokines to Th1 or to Th2 direction or to innate immune system activation. At the same time, the activation of biosynthesis of MHC class II molecules could of an immunoprotective approach. As it was shown, the activation of production of cytokines by spleen primary cells and expression of MHC class II by B lymphocytes and macrophages via application of the sweet and hot extracts could contribute to efficient anti-tumour responses.

5. 3. Composition/Impurities of Sweet and Hot Extracts

In the literature there is a large amount of available data on capsaicin and also a limited data on botanical ingredients derived from *Capsicum* species.

5. 3. 1. Carbohydrates in the Sweet and Hot Extracts

During the past three decades, many polysaccharides and polysaccharide-protein complexes have been isolated from mushrooms, fungi, yeasts, algae, lichens and plants. The biological activities of these polysaccharides have attracted more attention recently in biochemical and medical fields because of their immunomodulatory and antitumor effects (Ooi and Liu, 2000). The search for novel polysaccharides with antitumor properties stems from the basic shortcomings of cancer chemotherapy and radiotherapy. In particular, the great majority of chemical compounds, which have been shown to be cytotoxic to cancer cells, are also toxic to normal cells (Kim *et al*., 1996).

The present study showed that aqueous extracts from sweet and hot pepper contain carbohydrates, however further study is necessary to identify the active carbohydrates that could be responsible for the functional activity of the extracts.

In a recent study by Gan *et al.*, 2003 it was demonstrated that *Lycium barbarum* polysaccharide-protein complex (LBP3p), the third fraction of LBP, could significantly suppress the growth of S180 solid tumor in vivo and restore the immune status of S180-bearing rats, as witnessed by macrophage phagocytosis, the formation of antibody by spleen cells, spleen lymphocyte proliferation and the activity of cytotoxic T lymphocytes (CTL).

Many mushroom polysaccharides and polysaccharide-protein complexes have been considered as antitumor and immunomodulating agents. Some researchers showed that the antitumor action of mushroom polysaccharide complexes, such as PSK, a protein-bound polysaccharide extracted from the mycelia of Coriolus versicolor and the polysaccharice-protein complex (PSPC) isolated from the culture filtrate of Tricholoma lobayense was due to the potentiation of the host immune system through the regulation of cytokines in the cytokine network. It has been reported that PSK induces the expressions of genes for IL-1a,

IL-1h, IL-6, IL-8, TNF-a, etc. in human peripheral blood mononuclear cells in vitro (Liu *et al.*, 1996).

5. 3. 2. Polyamines in the Sweet and Hot Pepper Extracts

Put, Spm and Spd universally occurring in plant organs are involved in a wide array of processes, ranging from triggering organogenesis to protecting against stress. Putrescine contents are commonly the highest among polyamines, which were also observed in the present experiments. Some of the tested foods have a considerably high mean putrescine level (above 40 mg kg^{-1}), namely oranges, orange juice, mandarins, grapefruit juice and the processed foods sauerkraut, ketchup, frozen green peas and fermented soy products (Kalac and Krausov, 2005). Unfortunately data on polyamine contents in peppers are limited and dieticians have thus limited plausible information.

It is known that putrescine content increases by bacterial activity during inappropriate storage and processing. Since protein analysis showed high contamination level with microorganisms in the hot pepper, the high amount of putescine in the hot pepper extract could be explained by this evidence.

The possible therapeutic use of dietary polyamines is of great interest. However, there is still a lack of information in this field, in contrast with the large number of reports and reviews concerning the metabolism of polyamines.

The importance of polyamines in cellular growth and proliferation is well established in humans. In hyperplasic diseases ODC activity and polyamine levels are very high and can be used as biomarkers (Bachrach, 2004). In addition, the high rate of polyamine synthesis in the fetus and the considerable and significant variations of these amines during the first days of life in preterm and term babies have been associated to their role in cell growth and development in these subjects (Larqué *et al.*, 2007 and ref therein). The synthesis of polyamines, especially spermidine, gradually increases in the mammary gland during pregnancy and lactation (Larqué *et al.,* 2007 and ref therein). In blood, high concentrations of polyamines have been reported during stages of maximum development, whereas they decrease in adults.

The effects of polyamines on cell proliferation have been related to their role in stabilizing the negative charges of DNA and of the chromatin structure, the regulation of several transcriptional factors, and protein synthesis. However, excessive accumulation of polyamines induces apoptosis, perhaps because of the increased oxidative stress associated with the accumulation of hydrogen peroxide during the catabolism of polyamines by PAO. In the intestinal epithelium, polyamine depletion by DFMO reduces apoptosis and PAO activity, whereas an exogenous putrescine supply or induction of the intracellular putrescine pool restores apoptosis (Moinard, 2005).

Polyamines are involved in the differentiation of immune cells and the regulation of the inflammatory response. An insufficient polyamine intake could influence the development of food hypersensitivity. In suckling rats, oral administration of spermine or spermidine induced early postnatal gut maturation and changes in immunoglobulin A levels. It was reported also that spermine supplementation of neonatal mice increased the percentage of intraepithelial lymphocytes expressing antigens such as TCRab, CD4, CD5, and CD54, as occurs in natural maturation (Larqué *et al.*, 2007 and ref. therein).

During local inflammation reactions involving damaged or killed cells, spermine supplementation induces cell migration and growth. In such situations, polyamines exert a negative effect on macrophage activation with complex interactions between nitric oxide metabolism and polyamines. Moreover, it has been described that polyamines may exert a suppressor effect on the pulmonary immunologic and intestinal immunoallergic responses (Moinard *et al*., 2005).

High levels of polyamines are observed in rapidly dividing cells and tissues such as tumour cells. However, tumour cells have the ability to uptake extracellular polyamines, both dietary and produced by gastrointestinal bacteria. Reduction of the main exogenous sources including food and microflora-derived polyamines, has been shown to be a promising therapeutic strategy. Stimulation of the antitumoural immune response is an additional effect of polyamine deprivation (Kalac and Krausov, 2005). However, it has not yet been experimentally proved that altering of the dietary polyamine intake can help cancer patients.

Spermine increases proliferation and differentiation of lymphocytes isolated from childrens' tonsils. In an experiment with transgenic rats, insufficient hepatic pools of spermine and

spermidine failed to initiate the regenerative process following partial hepatectomy. An increased intake of dietary polyamines seems to have a favourable potential for post-operation patients or during wound healing (Larqué *et al.*, 2007 and ref therein).

5. 3. 3. Pigments in the Sweet and Hot Pepper Extracts

The experimental extracts were rich in pigments, with relative prevalence of those in sweet extract. Initial identification showed the presence of different carotenoids and β-carotene in particular. The protective effect of β-carotene on DNA damage has been reported. β-carotene reduced the frequency of chromosome aberrations induced by three different alkylating agents in bone marrow cells of Chinese hamsters (Bunkova *et al.,* 2005).

Capsanthin and related carotenoids showed potent in vitro anti-tumor-promoting activity with inhibitory effects on Epstein–Barr virus early antigen (EBV-EA) activation induced by the tumor promoter 12-O-tetradecanoylphorbol-13-acetate (TPA). Among them, capsanthin diester and capsorbin diester showed strong inhibitory effects. Furthermore, capsanthin, capsanthin 30-ester and capsanthin 3,30-diester, exhibited potent anti-tumor-promoting activity in an in vivo mouse skin two-stage carcinogenesis assay using 7, 12-dimethylbenz[a]anthracene as an initiator and TPA as a promoter (Maoka *et al*., 2001).
Previously, on the basis of reactivity with DNA it was demonstrated that several antioxidants, such as melatonin, propyl gallate, catechins, beta-carotene, alpha-tocopherol, etc. or those metabolites caused oxidative DNA damage to cellular and isolated DNA (Singh *et al*., 2001).

5. 3. 4. Proteins in the Sweet and Hot Pepper Extracts

The different activity profiles of sweet and hot extracts reported here suggest that the purity and source of extracts should be an important consideration for functional evaluations. Protein identification analysis reviled contamination evidence in the hot pepper extracts. The identified microorganism *Ostreococcus lucimarinus* CCE9901 belongs to the

Prasinophyceae, an early-diverging class within the green plant lineage, and is reported as a globally abundant single-celled alga.

In the literature there are reports about contamination of the capsicum extracts. Pesticide impurities and heavy metals may form part of the composition of these plant-derived ingredients. Mycotoxin contamination is also a common problem concerning food safety as it causes a variety of toxic effects in human and animals. In recent years, several European countries have found aflatoxin B1 (AFB1) and ochratoxin A (OA) levels exceeding legal limit in chili samples imported from developing countries (Saha *et al*., 2006). The isolation of fungi from and the production of aflatoxins by species of *Aspergillus* in samples of red pepper of the genus *Capsicum* have been reported (Johnson, 2007 and ref. therein).

Concerning quality estimations, the European Scientific Committee for food has fixed the legal limit for AFB1 at 5 μg kg-1 for spices. Though there is currently no legal limit for OA, the European Commission has been discussing a limit of 10 μg kg−1 in spices. The total PCB/pesticide contamination should be limited to not more than 40 ppm, with not more than 10 ppm for any specific residue, and agreed on the following limitations for other impurities: arsenic (3 mg/kg maximum), heavy metals (0.002% maximum), and lead (5 mg/kg maximum) (Saha *et al*., 2006).

Conclusion

The current research shows that aqueous extracts from sweet and hot *Capsicum* extracts have activity on the proliferation of spleen primary cells as well as on the proliferation of specific cell populations, such as macrophages, T and B lymphocytes. Sweet and hot extracts showed different profiles in their proliferative potential on the cells. The immunomodulating capacity of the extracts was reflected by the alteration of the cytokine production and expression of MHC class II molecules by spleen cells. The extracts did not induce either apoptosis or necrosis to spleen cells. The extracts contained high amount of carbohydrates, polyamines, mainly Put and proteins, while the fractions derived from the extracts contained only minimal amounts of these compounds.

Based on these results, the aqueous extracts from sweet and hot peppers could be proposed as a potential source of food material for a novel anticancer activity. It can be concluded that *Capsicum* extracts might be used as a chemotherapeutic or chemopreventive agent, but further studies will be necessary to assess their wide scale potential. The identification of the active molecules in these extracts will provide new insight in the up- or down-regulation of immune response by natural products.

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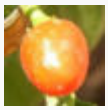
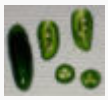
APPENDIX 1

Capsicum annuum

Capsicum annuum is a popular species native to South America. It is cultivated world-wide. Despite being a single species, the capsicum annum has many forms, with a variety of names, even in the same language. In American English it is commonly known as the chili pepper, although not all varieties would be recognized by most speakers under this name. In British English, they are all called *peppers*, whereas in Australian English there is no commonly-used name encompassing all its forms, with the name *capsicum* being commonly used for bell peppers exclusively; however the name *chilli* is often used to encompass the hotter varieties. Its forms are varied, from large to small, sweet to sour, very hot to bland.

The plant is a herbaceous annual, with a densely branched stem. The plant reaches 0.5–1.5 m. Single white flowers bear the fruit which is green when unripe, changing principally to red, some varieties may ripen to brown or purple. While the species can tolerate most climates, they are especially productive in warm and dry climates.

	Name	Hotness (SR)*	Length	Description
	Anaheim	500 - 2,500	15 cm	Smooth, narrow fruit first cultivated in California. Often used for chile relleno. When mature, takes on a red color and is referred to as a Colorado.
	Bell	0	15 cm	Cultivar group of large rectangular fruit without noticeable heat. Green when unripe; the ripe fruit can be red, yellow, orange, white, purple, blue, or brown depending on the specific cultivar.
	Cascabel	3,000	2.5 cm	Small, round fruit that is usually dried and has a distinct nutty flavor. The name is Spanish for "rattle" or "jingle bell," and derives from the rattling noise made by the seeds inside the dried pod.
	Cayenne	30,000 - 50,000	12.5 cm	Long, thin fruit that was transported by the Portuguese to China and India, where it is used widely. Often dried and ground into powder.
	Cherry	3,500	2.5 cm	Named for the fruit it resembles, this cultivar's fruit is small, red, and round. It is typically used fresh, or pickled and jarred.

	Chilaca	1,000 - 2,000	15 cm	Popular in Mexican cuisine. Almost always encountered dried; in this state, it is referred to as a Pasilla. The Pasilla has a dark brown color and a smoky flavor.
	Chiltepin	50,000 - 100,000	0.5 cm	Small, hot fruit that is often eaten by birds. The plant is thought to be the oldest member of the Capsicum genus. Evidence indicates that this has been consumed by humans as far back as 7,500 B.C.
	de Arbol	15,000 - 30,000	8 cm	Slender fruited cultivar grown primarily in Mexico. Name is Spanish for "tree-like."
	Jalapeño	2,500 - 10,000	9 cm	Very popular, especially in the United States. Often pickled or canned. A smoke-dried jalapeño is referred to as a chipotle.
	Pepperoncini	0 - 500	8 cm	Sweet-tasting and mild, is used extensively in Italian and Greek cuisine. Very frequently pickled.
	Poblano	1,000 - 2,000	13 cm	Large, heart-shaped, dark green fruit that is extremely popular in Mexico. Often used to make chile relleno. When dried, referred to as an Ancho.
	Serrano	8,000 - 22,000	5 cm	Thin, tapered fruit that is green when unripe but turns red when mature. Due to its thin skin, it does not need to be peeled before use.
	Thai	75,000 - 150,000	4 cm	Thin fruit with a pointed tip. Often used in the cuisines of Southeast Asia, especially (as the name implies) Thailand.

Capsicum frutescens

	Name	Hotness (SR)	Length	Description
	Tabasco	30,000-50,000	4 cm	Native to Mexico, this fruit is now grown in large amounts in Louisiana by McIlhenny Company for the sauce of the same name.

Capsicum chinense

Capsicum chinense or "Chinese capsicum" is a misnomer since all *capsica* originate in the New World. Nikolaus Joseph von Jacquin (1727-1817), an Austrian botanist, erroneously named the species in 1776, because he believed that they originated in China.

	Name	Hotness (SR)	Length	Description
	Dorset Naga	876,000-1,000,000	4 cm	Bred from a Naga Jolokia in Dorset, England. It is believed to be one of the hottest, if not the hottest, capcicum cultivar.
	Habanero	100,000 - 300,000	5 cm	Often (mistakenly) referred to the hottest, the habanero is nonetheless hotter than most commonly available cultivars. The habanero has a subtle fruity flavor and a floral aroma.
	Naga Jolokia	855,000 - 1,000,000	6 cm	Cultivar that originated in northeast India and is among the hottest. There is some debate as to which species the Naga Jolokia belongs, as it contains genes from both *C. chinense* and *C. frutescens*.

	Scotch bonnet	150,000 - 325,000	5 cm	Named because of its resemblance to a tam o'shanter, this fruit is closely related to the habanero and is similarly hot. Due to its heat and distinct flavor, it is often used in Caribbean cuisine.

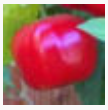
Capsicum baccatum

These have a distinctive, fruity flavor, and are commonly ground into colorful powders for use in cooking, each identified by its color.

	Name	Hotness (SR)	Length	Description
	Aji	30,000 - 50,000	7.5 cm	An aromatic fruit that is most popular in Peru. Often consumed raw in salsas and salads.

Capsicum pubescens

Capsicum pubescens is among the oldest of domesticated peppers, and was grown up to 5000 years ago. It is probably related to undomesticated plants that still grow in South America (cardenasii, eximium, and others).

	Name	Hotness (SR)	Length	Description
	Rocoto	50,000 - 200,000	2.5 cm	Egg-shaped fruit with black seeds. Popular in Latin America, particularly in salsas and for stuffing.

* The hotness of the fruit in this list is indicated in the Scoville scale (SR), which ranges from no heat at zero to pure capsaicin at 16,000,000. A Scoville unit is the factor by which the capsaicin-containing substance must be diluted to render the resulting solution imperceptible to a tester (for example, a teaspoon of a 5,000 Scoville unit hot sauce would have to be diluted with 4,999 teaspoons of a sugar water solution to negate its potential to cause a sensation on the palate).

http://www.g6csy.net/chile/database.html

APPENDIX 2

ΠΑΝΕΠΙΣΤΗΜΙΟ ΚΡΗΤΗΣ
ΤΜΗΜΑ ΒΙΟΛΟΓΙΑΣ

ΠΡΟΓΡΑΜΜΑ ΜΕΤΑΠΤΥΧΙΑΚΩΝ ΣΠΟΥΔΩΝ
«ΜΟΡΙΑΚΗ ΒΙΟΛΟΓΙΑ ΚΑΙ ΒΙΟΤΕΧΝΟΛΟΓΙΑ ΦΥΤΩΝ»

ΜΕΤΑΠΤΥΧΙΑΚΗ ΔΙΑΤΡΙΒΗ

THE IMMUNOMODULATING ACTIVITY OF *CAPSICUM L.* VARIANTS: EMPHASIS ON PROLIFERATION, CYTOKINE PRODUCTION AND CLASS II MHC ANTIGEN EXPRESSION

Ο ΡΟΛΟΣ ΣΤΕΛΕΧΩΝ ΤΟΥ *CAPSICUM L.* ΣΤΗΝ ΤΡΟΠΟΠΟΙΗΣΗ ΤΗΣ ΑΝΟΣΟΛΟΓΙΚΗΣ ΑΠΟΚΡΙΣΗΣ: ΕΜΦΑΣΗ ΣΤΟΝ ΠΟΛΛΑΠΛΑΣΙΑΣΜΟ, ΠΑΡΑΓΩΓΗ ΚΥΤΟΚΙΝΩΝ ΚΑΙ ΕΚΦΡΑΣΗ ΤΩΝ ΤΑΞΗΣ ΙΙ ΑΝΤΙΓΟΝΩΝ ΙΣΤΟΣΥΜΒΑΤΟΤΗΤΑΣ

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