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The Antioxidant and Hormonal Effects of *Ginseng Panax* on Lifespan and Stress Resistance in *Caenorhabditis elegans*

Diploma thesis

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Hradec Králové, Heidelberg 2008/2009 **Václav Ježek**

I claim that I worked out my diploma thesis (The antioxidant and hormonal effects of *Panax ginseng* on lifespan and stress resistance in *Caenorhabditis elegans*) individually and with the use of all herein mentioned information sources.

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CONTENT

1. INTRODUCTION

The aim of this study is to find out the basic mechanisms of *Panax ginseng* effects. Two main effects on the model organism *Caenorhabditis elegans* were investigated: antioxidant and hormonal activities. Very important load is to find the basic principels of the relation between cholesterol and *Panax ginseng* and their mutual effect on lifespan and generative ability of *C. elegans*. Antioxidant activity was investigated *in vitro* using two methods: DPPH free radical scavenging activity and superoxide anion radical scavenging activity. The results indicate that extract of *Panax ginseng* (GPE) can not be ranked as a strong antioxidant and this finding was demonstrated in the *in vivo* study of survival assay and by means of quantification of hsp-16.2/GFP expression. Hormonal activity of GPE was investigated by means of the progeny increase and observing of changes in reproduction phases of *C. elegans*. The following experiments were aimed to find out the relation between *Panax ginseng* with main active compounds ginsenosides (steroidal saponins) and cholesterol, their mutual interactions were reflected in an increase or a decrease of GPE toxicity and an impact on the reproduction abilities. The study of the interactions between cholesterol and steroidal saponins, in this case ginsenosides, has the considerable potential to the future. *C. elegans*, as a model organism, has the unique importance in this research owing to its inability to synthesize cholesterol *de novo* and is starting to become a valuable new model for this and other studies related to metabolism of sterols. Its importance bears on its dependence on exogenous sources of sterols, such as ginsenosides. There are many studies that have been engaged in *Panax ginseng*, whereas one part of researchers has confirmed beneficial effect of *Panax ginseng*, the other part has oppugned. This study is looking for the relation between *Panax ginseng* benefitial effect and the content of cholesterol in nourishment. The negative effect of higher concentrations of cholesterol on medical potential of ginsenosides in *C. elegans* can improve its beneficial effect in human medicine.

2. AIMS OF DIPLOMA THESIS

1. to recap the information sources about the antioxidant, hormonal and other activities of *Panax ginseng*
2. to measure the antioxidant activity of *Panax Ginseng* through *in vitro* studies (free radicals and superoxide anion scavenging activity)
3. to investigate the impact of *Panax ginseng* extract on lifespan and the generative abilities of *Caenorhabditis elegans*.
4. to observe the effect of *Panax Ginseng* extract on the oxidative stress resistance in *Caenorhabditis elegans*
5. to search for the basic principles of the mutual relationship between cholesterol and *Panax ginseng* on the model organism *Caenorhabditis elegans*

3. THEORETICAL PART

3.1. *PANAX GINSENG* (C.A. MEYER)

Ginseng refers to species within Panax, a genus of 11 species of slow-growing perennial plants with fleshy roots, in the Araliaceae family. *Panax ginseng* (C.A. Meyer) also known as Korean or Chinese ginseng has been used as a general tonic in traditional oriental medicine to increase vitality, health, and longevity, especially in old person [1]. Ginseng is present in the Pharmacopoeias of China or Europe, and is regarded as a tonic with adaptogenic, stimulant and aphrodisiac properties [2]. Commercially available ginseng is classified into fresh, white, and red ginseng. Red ginseng is made by steaming and drying of the fresh ginseng, suggesting chemical transformations by heat [3]. *Panax red ginseng* C.A. Meyer methanol extract was used in all herein mentioned experiments. There are numerous theories and claims describing the efficacy of ginseng, which can combat stress, enhance both the central and immune systems and contribute towards maintaining optimal oxidative status against certain chronic disease states and aging [4].

On the other hand there is conflicting research about the antioxidant and free radical scavenging or hormonal activity of *Panax ginseng*. Nevertheless there is extensive literature on the beneficial effects of *Panax ginseng* and its constituents. Although the traditional source of ginsenosides from *Panax Ginseng* is the root, both the leaf and berry parts of this plant also contain significant quantities of ginsenosides [5]. There is a wide variation (2-20%) in the ginsenoside content of different species of ginseng and the roots of *Panax Ginseng* contain approximately 3-4 % of saponins [6]. In addition there are many differences in the ginsenoside content in the utilized plant parts, ginseng species and location, cultivation period, harvesting time or methods of preparation [7].

The major active components of ginseng are triterpenoid steroidal saponins referred to collectively as ginsenosides or panaxosides. Ginsenosides is the term developed by Asian researchers, and the term panaxosides was

developed by early Russian researchers. Ginsenosides demonstrate the ability to target a myriad of tissues, producing an array of pharmacological responses. However, many mechanisms of ginsenoside activity still remain unknown. Since ginsenosides and other constituents of *Panax ginseng* produce the effects that are different from one another, and a single ginsenoside initiates multiple actions in the same tissue, the overall pharmacology of *Panax ginseng* is complex [8].

The other active constituents found in most ginseng species include: polysaccharides, peptides, polyacetylenic alcohols, and fatty acids [8]. Significant part of content substances present pectin, B vitamins, various flavonoids, recently investigated panaxans, which have documented hypoglycaemic effects or an acidic polysaccharide, referred to as `Ginsan', with noted immunostimulant activity [9]. Possibly, more detailed research will find many new different constituents and many other ways of their action.

3.2. GINSENOSIDES, THE MAIN ACTIVE COMPONENTS OF *PANAX GINSENG*

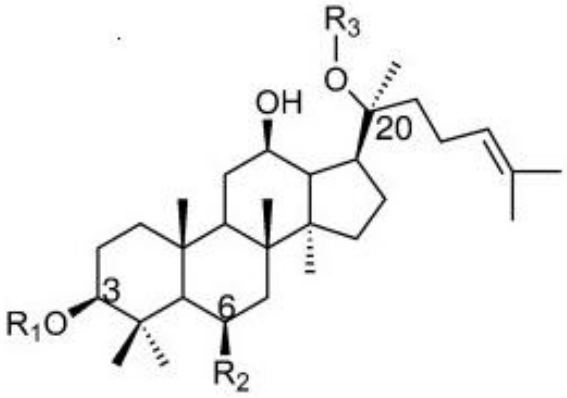
Ginsenosides are primary active components of ginseng, including a group with more than 30 different triterpene saponins, which vary in content and relative proportions among different species of ginseng [10].

They belong to the pharmacological active group of steroidal saponins [11] and have been named ginsenoside saponins, triterpenoid saponins, or dammarane derivatives [12], [13].

The effect of various ginsengs is related in part to the mix of ginsenosides. *Panax ginseng* and other ginsengs contain two main groups: protopanaxadiol (PPD) ginsenosides as Rb1, Rb2, Rc, Rd and protopanaxatriol (PPT) ginsenosides as Rg1, Re, Rf [***Figure 1***].

Figure 1

Structures of main ginsenosides of *Panax ginseng* (C.A.Meyer) [14]



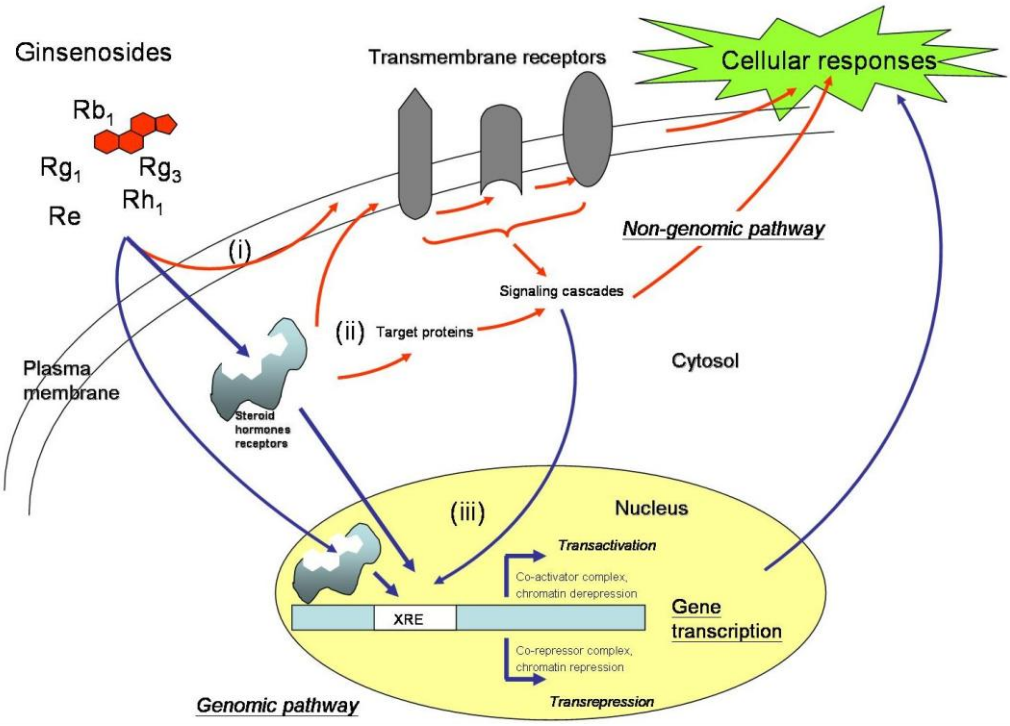
Ginsenoside	R_1	R_2	R_3
Protopanaxatriol			
Rg_1	H	-O-*glc*	*glc*
Rg_2	H	-O-*glc*(2-1)*rha*	H
Re	H	-O-*glc*(2-1)*rha*	*glc*
Rf	H	-O-*glc*(2-1)*glc*	H
Rh_1	H	-O-*glc*	H
F1	H	-OH	*glc*
Protopanaxadiol			
Rb_1	*glc*(2-1)*glc*	H	*glc*(6-1)*glc*
Rb_2	*glc*(2-1)*glc*	H	*glc*(6-1)*ara*(p)
Rg_3	*glc*(2-1)*glc*	H	H
Rh_2	*glc*	H	H
Rc	*glc*(2-1)*glc*	H	*glc*(6-1)*ara*(f)
Rd	*glc*(2-1)*glc*	H	*glc*
Ra	*glc*(2-1)*glc*	H	*glc*(6-1)*glc*
F2	*glc*	H	glc
Compound Y	*H*	H	*glc*(6-1)*ara*(p)
Compound K	*H*	H	*glc*
Compound O	*glc*	H	*glc*(6-1)*ara*(p)
Compound Mc	*glc*	H	*glc*(6-1)*ara*(f)

Abbreviations for carbohydrates are as follows: glc, glucopyranoside; ara(p), arabinopyranoside; ara(f), arafuranoside; rha, rhamnopyranoside.

They are amphiphilic and have the ability to intercalate into the plasma membrane. This leads to changes in membrane fluidity, and thus effects membrane functions, eliciting a cellular response. The ability of ginsenosides to independently target multireceptor systems at the plasma membrane, as well as to activate intracellular steroid receptors, may explain some pharmacological effects. There is evidence to suggest that ginsenosides interact directly with specific membrane proteins. Moreover, like steroid hormones, they are lipid-soluble signalling molecules, which can traverse the plasma membrane and initiate genomic effects. Two factors may contribute to the multiple pharmacological effects of ginseng. The first is the structural isomerism and stereoisomerism exhibited by ginsenosides, which increase their diversity. The second is the ability of ginsenosides to target membrane-anchored receptors and ion channels, as well as nuclear receptors [2], ***Figure 2***.

Figure 2

Schematic overview of ginsenosides- mediated genomic and non-genomic pathways [14]



Ginsenosides exhibit considerable structural variation. They differ from one another by the type of sugar moieties, their number, and their site of attachment. Some sugar moieties present are glucose, maltose, fructose, and saccharose. They are attached to C3, C6, or C20. The binding site of the sugar has been shown to influence biological activity [15].

Ginsenosides also differ in their number and site of attachment of hydroxyl groups. Polar substituents interact with phospholipid head groups in the hydrophilic domain of the membrane. Consequently, the insertional orientation of ginsenosides into the membranes would be influenced by the number and site of polar OH groups. Differences in the number of OH groups were shown to influence pharmacological activity [16]. Another factor that contributes to structural differences between ginsenosides is stereochemistry at C20 and most ginsenosides that have been isolated are naturally present as enantiomeric mixtures [10].

Sugar moieties are cleaved by acid hydrolysis during extraction or by endogenous glycosidases to give the aglycone [6], [17]. There have been results suggest that the natural glycosides ginsenosides are only prodrugs, which can be transformed to active compounds by intestinal micro flora. In this case there is considerable distinction among *in vitro* and *in vivo* tests because orally administrated ginseng must inevitably come into contact with intestinal micro flora in the alimentary tract. Ginsenosides components may be transformed to the pharmacological more effective forms before they are absorbed from the gastrointestinal tract. The transformation of ginsenosides by human intestinal micro flora are therefore great [18], [19].

3.3. THE ANTIOXIDANT AND PROOXIDANT EFFECTS OF *PANAX GINSENG*

The oxidative damage to carbohydrates, proteins, nucleic acids and lipids resulting from contact with free radicals is believed to be the source of early detrimental cellular changes that influence the aetiology of chronic disease and aging. The oxidative status of the individual is balanced by the activity of both

non-enzymatic antioxidant compounds (e.g. tocopherols, β carotene, glutathione) and antioxidant tissue enzymes (e.g. superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px), which together prevent reactive oxygen species formation, or work to mitigate the damage caused to cells by various sources of free radicals [20]. There is abundance of studies that have shown a strong association between ginseng intake and increased levels of the antioxidant activity but in the most cases ginsenosides increase only antioxidant tissue enzyme activity and do not act directly like antioxidant compounds as for example polyphenols. The mechanism of ginsenosides antioxidant activity is not well known as yet [4].

There are the results that ginsenosides have both antioxidant and prooxidant activity dependent on their structure. Various ginsenosides, protopanaxadiol (PPD)-type ginsenosides (Rg3, Rd, Rc, Rh2, Rb1, Rb3), the triterpene dammarane backbone with hydroxyl or glycosides in C-3 and C-20, and protopanaxatriol (PPT)-type ginsenosides (Rg1, Rg2, Rh1, Re, R1), the sugar moieties in C6, and its aglycones (PPD, PPT) were investigated to evaluate the relationship between structure and antioxidant and prooxidant activity using (2,2'-azobis-2-amidinopropane hydrochloride) AAPH-induced haemolysis in human erythrocytes. The dammarane skeleton, either PPD or PPT, played a prooxidant role. For groups of ginsenosides, the PPD mixture decreased haemolysis, while PPT increased haemolysis in a dose-dependent manner, that is, the PPD mixture acted as an antioxidant and PPT as a prooxidant. For individual ginsenosides, among those with no sugar moieties attached to C20 of the triterpene dammarane, the ginsenoside acted as a prooxidant in AAPH-induced haemolysis, that is, Rh2 and Rg2, even in the absence of AAPH, that is Rg3, while sugar moieties at C20 of the triterpene dammarane made the ginsenosides an antioxidants, i.e., Rb1, Rb3, Rc, Rd, Re, and Rg1. A glucose attached to C6 instead of C20 sugar moieties could also make the ginsenosides an antioxidant, i.e., Rh1. Therefore sugar moieties in C20 or glucose in C6 are the key structure making the ginsenosides antioxidants at any central structure of PPD or PPT [21].

On the other hand ginsenosides have more important mechanisms of action, for example only the protective effect on CNS involves the effects on calcium channel blockade, glutamate and gamma aminobutyric acid, antiperoxidation, estrogen-like action, nitric oxide and its synthesis, also the inhibition on cerebral nerve cell apoptosis, etc. [22].

3.4. MAILLARD REACTION AND ITS IMPACT ON THE ANTIOXIDANT PROPERTIES

There are distinguished two basic kinds of common distributed ginseng, white ginseng, is air-dried ginseng, and red ginseng is produced by steaming raw ginseng at 98-100 °C for 2-3 h. Red ginseng is reportedly more pharmacologically active than white ginseng. These improved biological activities of ginseng result from changes in all chemical constituents that occur during steaming treatment. Ginseng saponins, referred to as ginsenosides, are believed to play a pharmacologically important role. Several investigators have reported new red ginseng ginsenosides that are not usually found in white ginseng. It is reported that unique ginsenosides in red ginseng are Rg3, Rg5, Rg6, Rh2, Rh3, Rh4, Rs3 and F4 [23]. In the laboratory was prepared sun ginseng steamed at 120 °C and has been reported to have more potent pharmacological activity [24], [25]. The increase of antioxidant capacity in thermally adjusted ginseng may be also due to the Maillard reaction [26].

The Maillard reaction proceeds between reducing sugars and amino acids or proteins, is thought to be one of the major sources correlated with enhancing activity by heat treatment in various crude drugs. Products of Maillard reaction in ginseng were reported to be increased by steaming [27]. This might be explained by the increased antioxidant activity due to releasing of phenolic compounds during heat processing. Most of antioxidant compounds in food and plants are mainly present as a covalently bound form with insoluble polymers but the steaming treatment might disrupt the cell wall and liberate free antioxidant compounds [28].

Phenolic compounds are commonly found in plants, and they have been reported to have multiple biological effects, including the antioxidant activity. Many studies have revealed that the phenolic contents of plants can be correlated with their antioxidant activities [29]. The phenolic compounds of ginseng, one of the non-saponin components, were defined as maltol, salicylic acid, vanillic acid and p-coumaric acid [*Figure 3*]. They are also known as principal antioxidant components in white and red ginseng but purified ginsenosides did not show significant antioxidant activities [30].

It is insufficient to explain the various pharmacological effects of ginseng with only ginsenosides, especially antioxidant activity related to radical scavenging activity. Almost all ginsenosides had no effect on radical scavenging activity. Therefore, among the various pharmacological effects of ginseng, antioxidant activity, especially radical scavenging activity, can not be explained only with them. In addition, maltol was the major component to increase during processing ginseng at a higher temperature and is closely related to the radical scavenging activity of heat-processed ginseng. These findings indicate that maltol may act as a free radical scavenger and protect against a damage caused by oxidative stress related to these radicals. Maltol is also the most abundant phenolic compound, having a strong free radical scavenging activity. On the other hand, levels of free vanillic and salicylic acids were increased in red ginseng compared to those in white ginseng but the contents of the compounds were not continuously increased by a higher processing temperature. Similarly, sun ginseng had more free phenolic contents than white ginseng, but not more than red ginseng. Sun ginseng was prepared in the laboratory under higher pressure and temperature. Thermal decomposition or the reactions like dehydration in phenolic compounds are thought to occur under high pressure and temperature, so it is thought that some phenolic contents of sun ginseng decreased more than in red ginseng [31]. On the other hand thermally adjustment of ginseng alters the ginsenosides spectrum because sugars bounded in ginsenosides can admit to Maillard reaction and new are formed. There were isolated seven new ginsenosides from sun ginseng, that are Rk1, Rk2, Rk3, Rs4, Rs5, Rs6 and Rs7 [32] and the major

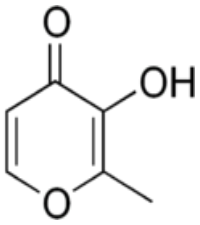
ginsenoside content in sun ginseng is altered too, that are Rg3, Rg5 and Rk1. The amounts of these ginsenosides are absent or present in trace amounts in conventional white and red ginseng [33].

There are two main effects of thermally adjustment, the first effect is the increase of antioxidant activity and the second effect is significant change of ginsenoside spectrum. There was found a high correlation between the contents of total phenolics and antioxidant capacity among the steamed ginseng products which indicates that these products have more phenolic compounds than white ginseng and contribute to the increase of the antioxidant and radical scavenging activities [31].

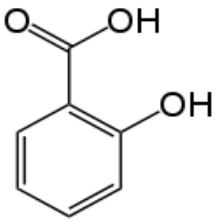
The antioxidant activities of ginseng appeared to have dual actions, such as a direct action of oxygen radical scavenging and an indirect action on the antioxidative cellular enzymes, such as SOD (superoxide dismutase) catalyzing the degradation of superoxide anions to oxygen and hydrogen peroxide and CAT (catalase) converting hydrogen peroxide into water and oxygen. The indirect action on the antioxidative cellular enzymes seems to be prevailing because there are many results that support this finding. The extracts of ginseng have not strong antioxidant or oxygen radical scavenging activity in the direct action but can indirectly increase the activity of some antioxidative cellular enzymes [4].

Figure 3

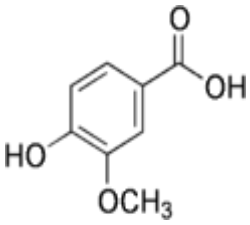
The phenolic compounds, the principal antioxidant components of *Panax ginseng* [31]



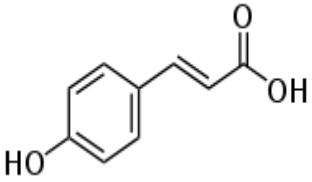
Maltol



Salicylic acid



Vanillic acid



P- Coumaric acid

3.5. *CAENORHABDITIS ELEGANS* AS A MODEL ORGANISM

Certain organisms, such as *C. elegans*, have been used in research laboratories to advance our understanding of life and human diseases. These organisms become "model organisms" because of the advantages in studying them. All model organisms are less costly, fewer ethical constraints are encountered using them and more research data have been generated in the past. Model organisms are those that useful data sets have been already gathered to describe basic biological processes. They are desirable to answer certain questions due to their simplicity of their structure and features. There is a wide range of characteristics common to model organisms, such as rapid development with short life cycles, small adult size, availability, and tractability [34]. In summary a model system is a simpler, idealized system that can be accessible and easily manipulated [35].

C. elegans popularity as a model organism is for its transparency, thus cells of interest can be observed using a dissecting microscope. It is small about (1-1.5 mm), easy to cultivate and has a short life cycle. These advantages allow maintaining large numbers of *C. elegans*. It can be crossed at will. Male and hermaphrodites are the two sexes. Hermaphrodites can self fertilize or mate with males to produce offspring. Thus, cross or self-fertilization can be manipulated as desired. There are numerous tools available to study *C. elegans* and its genome has been completely sequenced [36]. *C. elegans* is also recognized as a powerful model organism for screening potential drug compounds and validating drug efficiency prior to more costly and time consuming *in vivo* studies [37]. *C. elegans* offers a variety of the advantages in the drug development process. For example, the genetics, biochemical pathways, and developmental processes of *C. elegans* have been well characterized and share many basic features with higher organisms, including humans. In addition, *C. elegans* studies are cost effective. The organisms are easy to maintain in the laboratory and can be grown on agar plates or liquid medium with *E. coli* as a food source. Moreover, the reproductive life cycle of *C. elegans* is quite short, taking 3 days from an egg to a fully mature adult. Thus, developmental processes can be studied in a relatively short period of time. Lastly, many *C. elegans* mutant strains are readily available and can be used to explore protein functions and mechanisms of drug action [38].

3.6. *CAENORHABDITIS ELEGANS* AND ITS RELATION TO CHOLESTEROL AND OTHER COMPOUNDS WITH STEROIDAL STRUCTURE

Sterols, in the first line cholesterol, are crucial for the function of the most eukaryotic cells and for the structure of their membranes. In addition to this structural role in the membrane, cholesterol is essential for various signalling processes. Its importance is reflected in the complicated synthesize and the considerable energy demands by its creation. Almost one hundred proteins are employed to synthesize, modify, transport or degrade sterols, consuming many

equivalents of ATP. However, no energy is returned after their catabolism or excretion from the organism [39]. Many biophysical studies have demonstrated that sterols in cellular membranes influence their physicochemical properties, including fluidity, ion permeability [40] or signal transduction [41]. Cholesterol can be synthesized or taken up from food and after transported to specific destinations, incorporated into the membranes, metabolized into active derivatives like hormones before finally being degraded or excreted [39].

Much is known about these processes in mammals, but the nematode *C. elegans* is starting to become a valuable new model for studying of cholesterol metabolism and its functions. Yeast, plants and mammals have complex biosynthetic pathways for sterols that comprise more than thirty enzymes. By contrast, worms cannot synthesize sterols *de novo* [42] and in their natural environment, they depend on exogenous sources, such as plant or fungal remnants, or animal faeces. *C. elegans* express predicted homologues of the enzymes that produce the initial intermediates of the mammalian sterol biosynthetic pathway up to farnesyl pyrophosphate, but can not synthesize squalene or lanosterol, the key metabolites of sterol biosynthesis in yeast, plants and mammals. Despite of the lack of enzymes for *de novo* sterol synthesis, nematodes can modify externally added yeast, plant or mammalian sterols by introducing double bonds, reducing or isomerising them and by dealkylating the side chain [43]. As a result of the inability to synthesize cholesterol *de novo*, only the final products of the plant and mammalian biosynthetic pathways (that is, ergosterol, sitosterol, cholesterol or their close precursors) can fully support a worm growth. On the other hand, metabolic studies and lipid analysis have revealed a nematode-specific modification of sterols: methylation of the ring at C4 [44]. This is remarkable because in mammals, a complex series of almost twenty steps is used for the opposite reaction, demethylation of lanosterol at C4, and no enzymes are known in mammals that can restore these methyl groups [39].

The presence of enzymes for such specific modifications of cholesterol suggests that a pathway might exist to produce a hormone. However, efforts to identify steroid hormones or ecdysones in the nematodes have been

unsuccessful. On the other hand there are several strong arguments suggest that cholesterol is involved in signalling processes. It has been found that an enantiomer of cholesterol can not substitute for cholesterol to support worm growth and reproduction [45]. The biophysical properties of the enantiomer are identical to natural cholesterol, so it should substitute for any structural role of cholesterol in the membranes. So the observation that this enantiomer does not support growth could be explained if its altered conformation prevents specific interaction with an enzyme that mediates steroid hormone production [39].

The distribution of sterols in *C. elegans* is not uniform, and it may affect the membrane structure in a subset of cells. The membranes of these cells must have specific properties that allow cholesterol accumulation, and these membranes may have altered physical properties and raft-like microdomains. The high accumulation of sterols has been observed in the apical surface of their gut, excretory gland cell, pharynx, sensory amphids, nerve ring, sperm and oocytes [46], [47].

There are other mechanisms how to survive in spite of a small amount of cholesterol in the worm membranes, for example the caveolin-1, a protein behaves as a bona fide raft protein [48]. The caveolin-1 suggests the ability to form the rafts in the absence of cholesterol. So, although cholesterol could mediate membrane structure and raft function in a subset of cells, other cell types may assemble rafts through other mechanisms. The phase separation in the membranes can occur in the absence of cholesterol, but adding of cholesterol alters phase boundaries and lipid mobility [49], [50].

All results demonstrate that cholesterol may have both an essentials structural role for a few cells and a hormonal role in signalling, mainly shaping the dauer-formation pathway, the generative abilities or molting. Many studies indicate that *C. elegans* contains a little or no cholesterol in the cell membranes and also indicate its ability to modify dietary steroids [39].

3.7. EFFECT OF GINSENOSIDES ON DEVELOPMENT, GROWTH AND LIFESPAN OF *CAENORHABDITIS ELEGANS*

Very interesting recent study demonstrates that supplement of ginsenosides Rb1 or Rc but not Rg1 to cholesterol deprived medium restored lifespan of *C. elegans* as much as cholesterol and co-supplement of ginsenoside Rc but not Rb1 and Rg1 to cholesterol fed medium not only prolonged lifespan but also extended the maximum days of lifespan. Interestingly, the supplement of ginsenosides Rb1, Rc or Rg1 to cholesterol deprived medium slightly but not significantly extended the lifespan of *C. elegans* [51].

In addition, co-supplement of ginseng total saponins to cholesterol-fed medium produces bigger worms in size and diameter than those grown in cholesterol-fed medium. On the other hand co-supplement of ginsenosides Rb1 or Rc to cholesterol fed medium did not affect bigger worm sizes compared with those grown in cholesterol fed medium. These results show a possibility that the production of bigger worms in a co-administration of total ginseng extract to cholesterol fed medium might be achieved through the total effects of individual ginsenosides rather than those of individual ginsenosides [51].

Interestingly, in the study of the relationship between the structure of ginsenosides and their antioxidative or prooxidative activity, has been determined the order of antioxidative ability in this succession: Rc > Rb1 and Re > Rd > R1 > Rg1 > Rb3 > Rh1 [21]. This is very remarkable that only ginsenosides with the most significant antioxidant activity such as Rc and Rb1 have the beneficial effect on treatment of *C. elegans*. This succession of the antioxidant ability corresponds with the observation that lifespan extending effect of ginsenoside Rg1 on worm growth in cholesterol deprived or fed medium was less effective than those of ginsenoside Rb1 and Rc [51].

In addition to lifespan, it was observed that ginsenoside Rb1 or Rc but not Rg1 was effective to restore the average brood size, development, growth rate, and standard size of the worms in cholesterol deprived medium as ginseng total saponins extract. This can be caused due to the differences of chemical

structures. Rg1 has carbohydrate attached at carbon-6 of steroidal ring and it might play a role in exhibiting less physiologically beneficial effects in cholesterol deprived medium. This fact might be explained that Rg1 can not be effectively utilized by *C. elegans* like desirable food source with steroidal structure. On the other hand these ginsenosides have no significant effect on the worm size in cholesterol fed medium. Whereas total ginseng extract like co-supplement to cholesterol fed medium effects bigger worm size compared with those grown in cholesterol fed medium [51].

3.8. EFFECT OF GINSENG SAPONINS ON CHOLESTEROL METABOLISM

It was reported that ginseng saponins increased transiently lipid synthesis rates in C-acetate administrated rats. The effect of ginseng saponins on lipid synthesis in rats with different diet conditions was also studied. In fasted rats, ginseng saponin did not effect on C-acetate incorporation, but in rats with high carbohydrate, high protein and normal diets, ginseng saponins increased the lipid synthesis. However, in high fat diet groups, ginseng saponins did not increased the lipid synthesis rate [58].

In additional the recent study reported that ginseng saponin lowered a surface tension of water significantly and the CMC (critical micellar concentration) of the pure saponin solution was found been about 2%. When saponin was added to the cholesterol suspension, the formation of micelles seemed to start at the saponin concentration of only 0.1 %, which was one twentieth of the CMC of the saponin alone. These results suggested that under the physiological condition, a small amount of saponins would affect sufficiently for lipid micelle formation for example in intestinal lumen. Therefore lipids might be absorbed easily from small intestine [59].

Very important study reported that the saponins are able to stimulate the absorption and transport of water-insoluble vitamins such as vitamin A and vitamin K [60].

There are many other results suggest that ginseng saponins might be effective on cholesterol absorption and its utilization. These results were obtained from the study aimed at the dependence of cholesterol and ginseng saponins utilization in rat body. In protopanaxatriol saponin fed group, the cholesterol absorption was stimulated significantly. However, protopanaxadiol saponin did not affect on cholesterol absorption. These results suggest that the stimulatory effect of total saponins in cholesterol absorption might be mainly due to the protopanaxatriol saponins. The above results suggest that ginseng saponin fractions facilitate for the absorption and utilization of cholesterol. It was found that the level of blood serum cholesterol was slightly decreased in the test groups; however phospholipid level was increased. It is interesting that the ratios of cholesterol to phospholipid of the blood serum of the test group were lower than that of the control group. It is well known that phospholipid plays a significant role in the transport of lipids including cholesterol; therefore the increase of phospholipid by ginseng saponin administration may facilitate the transport and utilization of cholesterol [61].

4. PRACTICAL PART

4.1. MATERIALS AND METHODS FOR PREPARATION OF EXTRACT FROM *PANAX GINSENG* (GPE)

- **Raw material:**

Red *Panax ginseng* root powder (Sam ginseng, 125.0 g root- powder, Bio-Diät-Berlin GmbH, Germany)

- **Chemicals:**

Methanol (J.T. Baker, Holland)

- **Instruments:**

Rotary evaporator (Rotavator-R, Büchi, Switzerland)

Vacuum concentrator centrifuge (Univapo 150H Concentrator Centrifuge, Fröbel, Germany)

- **Method:**

For the extraction of ginsenosides from red ginseng root powder was used a simple method, using hot methanol. The extract was prepared from 125.0 g of red ginseng powder in 1 litre of hot methanol, the extraction lasted 2 hours. A simple method, using hot methanol, was chosen for its efficiency and good ginsenoside recovery. The sample purification step was simplified to one single filtration step to remove impurities. This method, with a single filtration step, is easy to use, it increases sample output, decreases the chances for loss in ginsenosides and it allows fast, simple, and reliable extraction of ginsenosides from ginseng roots without the need for a highly specialized laboratory [52]. This extract was condensed and the majority of methanol was removed using rotary evaporator and the finally removal of methanol was accomplished in vacuum concentrator centrifuge. The homogenization in a grinding mortar was the closely extract adjustment. The homogenized and purified samples were stored in the freezer at - 20 °C.

4.2. MATERIALS AND METHODS NEEDED TO MAINTAINING OF *CAENORHABDITIS ELEGANS*

- **Materials and chemicals:**

Na_2HPO_4, Merck GmbH (Darmstadt, Germany)

K_2HPO_4, Merck GmbH (Darmstadt, Germany)

NaCl, Merck GmbH (Darmstadt, Germany)

$ZnSO_4.7H_2O$, Merck GmbH (Darmstadt, Germany)

$MgSO_4.7H_2O$, Merck GmbH (Darmstadt, Germany)

$CuSO_4.5H_2O$, Merck GmbH (Darmstadt, Germany)

$CaCl_2$, Merck GmbH (Darmstadt, Germany)

Tri-potassium citrate monohydrate, Merck GmbH (Darmstadt, Germany)

Citric acid monohydrate, Merck GmbH (Darmstadt, Germany)

Bacto™ Agar, Becton, Dickinson and Company (USA)

Bacto™ Tryprone, Becton, Dickinson and Company (USA)

Bacto™ Yeast Extract, Becton, Dickinson and Company (USA)

Cholesterol, Sigma-Aldrich Chemie GmbH (Steinheim, Germany)

$FeSO_4.7H_2O$, Sigma-Aldrich Chemie GmbH (Steinheim, Germany)

EDTA disodium salt, AppliChem GmbH (Darmstadt, Germany)

$MnCl_2.4H_2O$, AppliChem GmbH (Darmstadt, Germany)

NaH_2PO_4 ,Ferak Berlin (West Germany)

KH_2PO_4, Gerbu Biotechnik GmbH (Gaiberg, Germany)

Petri plates, Greiner Bio-One GmbH, (Germany)

- **Methods**
  - **Preparation of bacterial food source [**56**]**

As a food source has been used *E. coli* strain *OP50*. This strain is the uracil auxotroph whose growth is limited on Nematode Growth Medium (NGM) plates. A limited bacterial growth is desirable because it allows easier observation and better mating of the worms. The bacteria were cultivated on LB agar composed of 10.0 g Bacto-tryptone, 5.0 g Bacto-yaest, 5.0 g NaCl, 15.0 g agar and water to 1 litre, by final pH 7.5. The plates containing LB agar

were aseptically inoculated by L Broth solution containing *E. coli OP50*, which was cultivated one day at 37 °C. The L Broth solution is composed of 10.0 g Bacto- Tryptone, 5.0 g Bacto- yeast, 5.0 g NaCl and water to 1 litre by final pH 7.0 using 1 M NaOH. This both media were autoclaved in screw-cap bottles for 20 min. The LB plates were seeded with L Broth solution containing *E. coli OP50* and thereafter cultivated overnight at 37 °C. The *E. coli OP50* streak petri plates and liquid culture were stored at 4 °C.

- **Preparation of NGM Petri plates [**56**]**

C. elegans was maintained in the laboratory on NGM agar which has been aseptically poured into Petri plates. NGM agar was made in several steps. In the first step were 3.0 g NaCl, 17.0 g agar, 2.5 g peptone and water to 1 litre mixed in an Erlenmeyer flask whose mouth was covered with aluminium foil. The incurred mixture was autoclaved for twenty minutes and after this time cooled in 55 °C water bath for 15 minutes. In the second step were added under the sterile conditions 1 ml of 1M $CaCl_2$, 1 ml of cholesterol (5 mg/ml) in ethanol, 1 ml of 1 M $MgSO_4$ and 25 ml 1 M KPO_4 buffer, which was made of 108.3 g KH_2PO_4, 35.6 g K_2HPO_4 and water to 1 litre. All foregoing solutions were autoclaved except solution of cholesterol in ethanol. The final solution was dispensed under the sterile conditions into Petri plates in the last step. The plates were filled 2/3 full of agar and were left at room temperature for 2- 3 days before use to allow a detection of potential contaminants, and to allow excess moisture to evaporate. In this way prepared plates were usable for several weeks.

- **Preparation of liquid culture of *C. elegans* [**56**]**

The first step was the preparation of S-basal medium from 5.85 g NaCl, 1.0 g K_2HPO_4, 6.0 g KH_2PO_4, 1 ml cholesterol (5 mg/ml) in ethanol and water to 1 litre. S-basal medium was autoclaved for twenty minutes and after successful autoclaving assembled with four basic solutions: 1 M potassium citrate pH 6.0, trace metals solution, 1 M $CaCl_2$ and 1 M $MgSO_4$ and all these solution were autoclaved. 1 M potassium citrate was prepared from 20.0 g citric acid monohydrate, 293.5 g tri-potassium citrate monohydrate and water to 1 litre. Trace metals solution was prepared from 1.86 g disodium EDTA, 0.69 g

$FeSO_4.7H_2O$, 0.2 g $MnCl_2.4H_2O$, 0.29 g $ZnSO_4.7H_2O$, 0.025 g $CuSO_4.5H_2O$ and water to 1 litre, this solution was stored in the dark. 1 M $CaCl_2$ was made of 55.5 g $CaCl_2$ solubilsed to 1 litre of water and 1 M $MgSO_4$ made of 120,4 g $MgSO_4$ in 1 litre of water. Under the sterile technique were added 10 ml 1 M potassium citrate pH 6.0, 10 ml trace metals solution, 3 ml 1 M $CaCl_2$ and 3 ml 1 M $MgSO_4$ to 1 litre of S-basal medium. S-medium prepared in this way was usable for several weeks by storing at 4 °C. S-medium used for maintaining of worms was fresh inoculated with the concentrated *E. coli OP50* pellets obtained from LB agar that was incubated overnight. In addition there was prepared S-medium without presence of cholesterol.

4.3. *CAENORHABDITIS ELEGANS* STRAINS, CHEMICALS, METERIALS, STATISTICAL ANALYSIS AND METHODS USED BY ALL EXPERIMENTS

4.3.1. LIFESPAN ASSAY

- **Chemicals:**

5% solution of hypochlorite, Central chemical store in Neuenheimerfeld, Heidelberg University

NaOH, Central chemical store in Neuenheimerfeld, Heidelberg University

- ***Caenorhabditis elegans* strain:**

N2, wild type

All strains were obtained from the Ceanorhabditis Genetic Centre (CGC) situated by NIH National Centre for Research Resources.

- **Materials:**

35-mm-diameter tissue culture dishes, Cellstar, Greiner bio-one GmbH, (Germany)

- **Statistical analyses:**

Lifespan data were subjected to Kaplan-Meier survival analysis using StatView 5 (SAS) software to carry out statistical analysis and to determine

mean lifespan started from the day of egg hatching. The long rank (Mantel-Cox) test was used to compare the animal groups. Animals were examined daily and dead worms, which did not move after touching their heads with a platinum wire, were removed to count. The worms that exploded or bagged were censored at the time of the event and were incorporated into the data set [51]. All figures indicate means and standard error of the mean.

- **Method:**

All worms used in this study were age-synchronized, they were raised from eggs obtained by sodium hypochlorite treatment (bleaching) of hermaphrodites, and this treatment gives only the worm eggs and neutralizes a potential contamination of the stocks. All worms were cultured in a suspension of *E. coli OP50* in S-medium [55] or in S-medium without cholesterol and treated with different concentrations of GPE from the third day after bleaching throughout the complete lifespan. Animals were placed in populations of 25 individuals into 2 ml of S Medium in 35-mm-diameter tissue culture dishes. Worms were transferred to fresh medium daily from 3 to 10 days after hatching to keep them separated from their progeny and every other day thereafter. Animals were examined daily and scored as dead when they no longer responded to light touching their heads with platinum wire.

4.3.2. QUANTIFICATION OF HSP-16.2/GFP EXPRESSION

- ***Caenorhabditis elegans* strain:**

TJ375 (hsp-16.2/GFP)

- **Chemicals:**

Juglone, Sigma-Aldrich Chemie GmbH, (Steinheim, Germany)

NaN_3 , AppliChem GmbH, (Darmstadt, Germany)

- **Instruments:**

The objective, Nikon-eclipse 90i, Nikon Imaging Center, Heidelberg University and the digital camera, Nikon digital sight DS-Qi1Mc.

- **Statistical analysis:**

All images were analyzed for quantifying the GFP fluorescence using NIS-Elements AR 3 software.

- **Method:**

TJ375 worms used in this study were age-synchronized, they were raised from eggs obtained by sodium hypochlorite treatment of hermaphrodites, cultivated in SM with presence or absence of cholesterol containing *E. coli OP50* in concentration 1×10^9 cell/ml and maintained at 20 °C. The worms were treated with GPE in the same concentration of 50 µg/ml on the day after hatching for 48 hours. Then the worms were exposed to oxidative stress by adding of juglon to the medium to achieve the concentration of 20 µM. This exposure to juglon proceeded 24 hours and thereafter was the expression of hsp-16.2/GFP measured directly by observing the fluorescence of the reporter protein GFP. In the TJ375 strain is hsp-16.2/GFP expressed by either heat shock or by oxidative stress, in this case by adding of juglon. The worms from each set of experiments were mounted onto glass slide in solution of S-medium with sodium azide. Fluorescence images were taken at constant exposure times of 4 seconds using the 20× objective Nikon-eclipse 80i with the digital camera Nikon digital sight DS-Qi1Mc. The images included the anterior part from the back of the pharynx, they were outlined, black to white inverted, and the mean pixel density was measured.

4.3.3. SURVIVAL ASSAY

- ***Caenorhabditis elegans* strain:**

N2, wild type

- **Chemicals:**

Juglone, Sigma-Aldrich Chemie GmbH, (Steinheim, Germany)

- **Instruments:**

35-mm-diameter tissue culture dishes, Cellstar, Greiner bio-one GmbH, (Germany)

- **Method:**

Oxidative stress was induced by an acute, lethal concentration of juglone at 80 μM. The treatment of worms with GPE in the concentration 50 μg/ml and in two different media with and without cholesterol started on the day after hatching for 48 hours. Juglone was added to the media and the survivals counted after 24 hours.

4.3.4. EFFECT OF GPE TREATMENT ON GENERATIVE ABILITY OF *CAENORHABDITIS ELEGANS*

- ***Caenorhabditis elegans* strain:**

N2, wild type

- **Chemicals:**

NaN_3 , AppliChem GmbH, (Darmstadt, Germany)

- **Instruments:**

35-mm-diameter tissue culture dishes, Cellstar, Greiner bio-one GmbH, (Germany)

- **Method:**

Hormonal activity was measured by means of the increase of progeny. There were seven monitored groups, which were mutually compared. Each group was represented by three separated Petri plates with two adult worms. All worms used in this study were age-synchronized. This experiment was repeated three times. There were used the control medium with standard contents of cholesterol 5 μg/ml and media with cholesterol and without cholesterol in mixture with two different concentrations of GPE (50 μg/ml and 300 μg/ml). Worms were age-synchronized by bleaching and exposed to the treatment immediately. After two days the experiment started, every day were adult worms transferred to the new plates and old plates with progeny were incubated three days at 20 °C. Three days lasting incubation was necessary for hatching and growth of *C. elegans* to the adult individuals which were scored. It is very important thing that the transfer of the maternal worms must be realized to 24 hours punctually, differently the results are useless. The progeny

were scored during the transfer and by the visual observing. One part of progeny was transferred from the old plates and scored and the other part was counted visualy. For visually counting were used the microscope and special transparent foil that dividend the plates to the standard counted sectors. There was used 100 μL of 10 Mm sodium azide to prohibit the worms from moving among the sectors. Every plate was counted five times, the highest and the lowest quantity of progeny were crossed off and from three remaining values was calculated the average. The quantities of progeny from transferring and visual counting were counted up and the results from three plates were re-counted on one worm. There is very important to notice that not eggs but worms capable to hatch were counted. Accordingly these results did not demonstrate the ability to have more eggs but only the ability to have higher number of viable progeny, which is capable to mature grow up.

4.4. MATERIALS AND METHODS FOR DETERMINATION OF ANTIOXIDANT ACTIVITY

4.4.1. 2, 2- DIPHENYL- PICRYLHYDRAZIL (DPPH) FREE RADICAL SCAVENGING ACTIVITY

- **Chemicals and reagents**

Methanol, J.T. Baker (Holland)

DPPH (2,2'-diphenyl-1-picryl-hydrazyl), Sigma-Aldrich Chemie GmbH (Steinheim, Germany).

- **Instruments**

LKB 4054 UV/Visible Spectrophotometer ultraspec plus made in Biochrom (England).

- **Method:**

DPPH is reduced to hydrazine when it reacts with hydrogen donors. It causes the change of violet color to yellow and the decrease of absorbance. This method was accomplished according to Blois description [57] with some modifications. The basic solution of DPPH in methanol was prepared in

concentration of 0.2 mM and was added in the amount of 500 µL to 500 µL of GPE (Ginseng Panax Extract) at different concentrations in the following range (0.1- 1.7 mg/ml). The absorbance was measured at 517 nm, 30 minutes later. The capability to scavenge the DPPH radicals was calculated by using this equation:

DPPH scavenging activity in %= [(A_0 – A_1) / A_0] × 100

A_0 is the absorbance of the control reaction (500 µL basic DPPH solution and 500 µL methanol) and A_1 is the absorbance in the presence of GPE (500 µL basic DPPH solution and 500 µL methanol solution with different concentrations of GPE).

4.4.2. SUPEROXIDE ANION SCAVENGING ACTIVITY

- **Chemicals and reagents:**

PMS: phenazine metho-sulphate, Sigma-Aldrich Chemie GmbH, (Steinheim, Germany)

NADH: β-Nicotinamide adenine dinucleotide, reduced disodium salt hydrate, Sigma-Aldrich Chemie GmbH, (Steinheim, Germany)

NBT: nitro blue tetrazollium chloride, AppliChem GmbH, (Darmstadt, Germany).

- **Instruments:**

LKB 4054 UV/Visible Spectrophotometer ultraspect plus made in Biochrom, (England).

- **Method:**

Superoxidant scavenging activity was determined by the method of Robak and Gryglewsky [53]. Superoxide radicals are generated in the PMS-NADH (phenazine methosulphate-nicotinamide adenine dinucleotide) system by oxidation of NADH and assayed by the reduction of NBT (nitroblue tetrazolium). The superoxidant radicals were generated in the 0.1 M phosphate buffer pH 7.4 in two basic quantities 900 µL (blank control and blank sample) and 800 µL (control and sample). The molar concentrations of basic

components in the whole mixture were following: NADH (156 μM), NBT (630 μM) and PMS (30 μL). The blank control mixture was prepared from 100 μL NBT and 100 μL PMS and the control mixture was prepared from 100 μL water, 100 μL NADH, 100 μL NBT and the reaction was started by addition of 100 μL PMS. The blank samples were prepared from 100 μL of GPE at different concentrations in the following range (0.2- 3.8 mg/ml), 100 μL NBT and 100 μL PMS. The samples were prepared from 100 μL of GPE at different concentrations, 100 μL NADH, 100 μL NBT and the reaction was started by addition of 100 μL PMS to the mixture. The absorption was measured at 560 nm after 5 minutes of the reaction, which was going at 25°C. The percentage inhibition of superoxid anions was calculated by using the following equation:

$$\textbf{\% superoxide anions inhibition} = [(A_0 - A_1) / A_0] \times 100$$

A_0 is the absorbance of the control reaction and A_1 is the absorbance in presence of GPE.

4.5. STATISTICAL PROCESSING OF RESULTS [66]

Sample Standard Deviation

Sample standard deviation is the square root of the sample variance and is described by the equation:

$$s = \sqrt{\frac{\Sigma(y_i - \bar{y})^2}{n-1}}$$

where n - 1 = degrees of freedom for a sample or number of independent observations, n, in a dataset.

Standard Error

Standard error is the estimate of the variation of a statistic. The estimate of the standard error of the mean (SEM) is described by the following equation:

$$s_y = \frac{s}{\sqrt{n}}$$

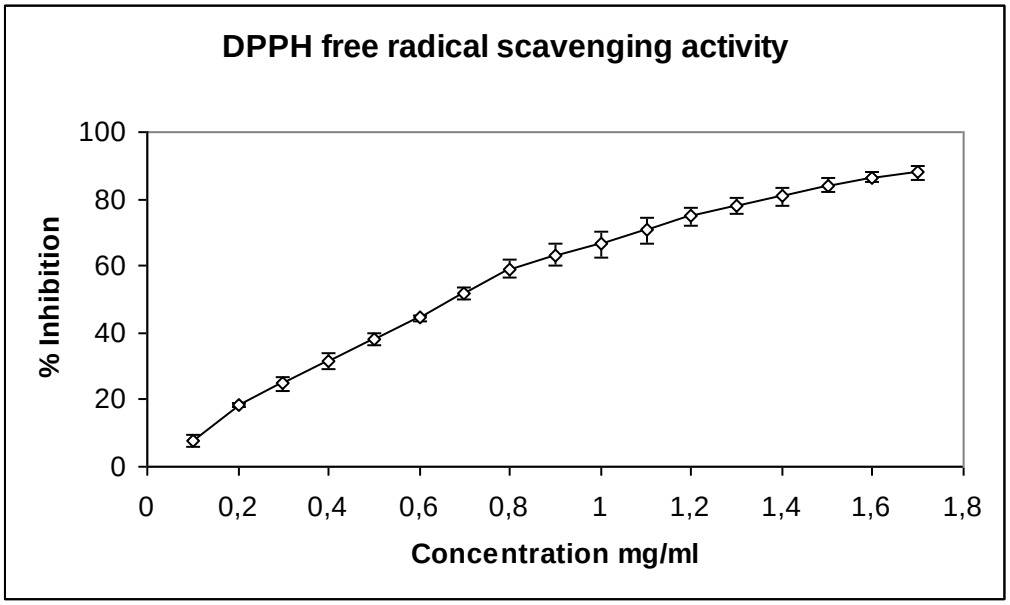
where s is the sample standard deviation and n is the number of samples.

5. RESULTS

Graph A

Free radical scavenging activity of GPE (0.1- 1.7 mg/ml) determined by the DPPH assay. The results were obtained from three independent experiments. IC $_{50}$ was measured from the average graph.

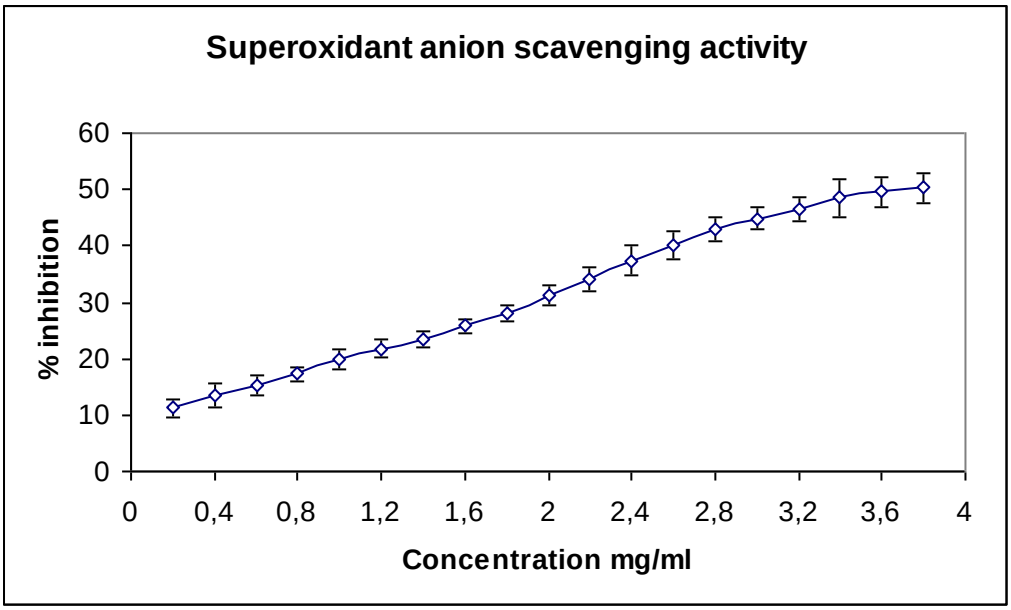
IC $_{50}$ = 0.66 ± 0.09 mg/ml.



Graph A shows DPPH free radical scavenging activity and rates IC_{50} = 0.66 ± 0.09 mg/ml. This concentration is approximetaly 13.3 times higher than the concentration (50 µg/ml) used by treatment with insignificant therapeutic effect.

Graph B

Inhibitory effect of GPE (0.2- 3.8 mg/ml) on superoxide radicals generated in the PMS-NADH system by oxidation of NADH and assayed by the reduction of NBT. The results are expressed as percentage inhibition of the control from three independent experiments. IC $_{50}$ was measured from the average graph. IC $_{50}$ = 3.70 ± 0.27 mg/ml



Graph B shows superoxide anion radical scavenging activity and rates IC_{50} = 3.70 ± 0.27 mg/ml. This concentration is approximately 74 times higher than the concentration (50 µg/ml) used by treatment with insignificant therapeutic effect.

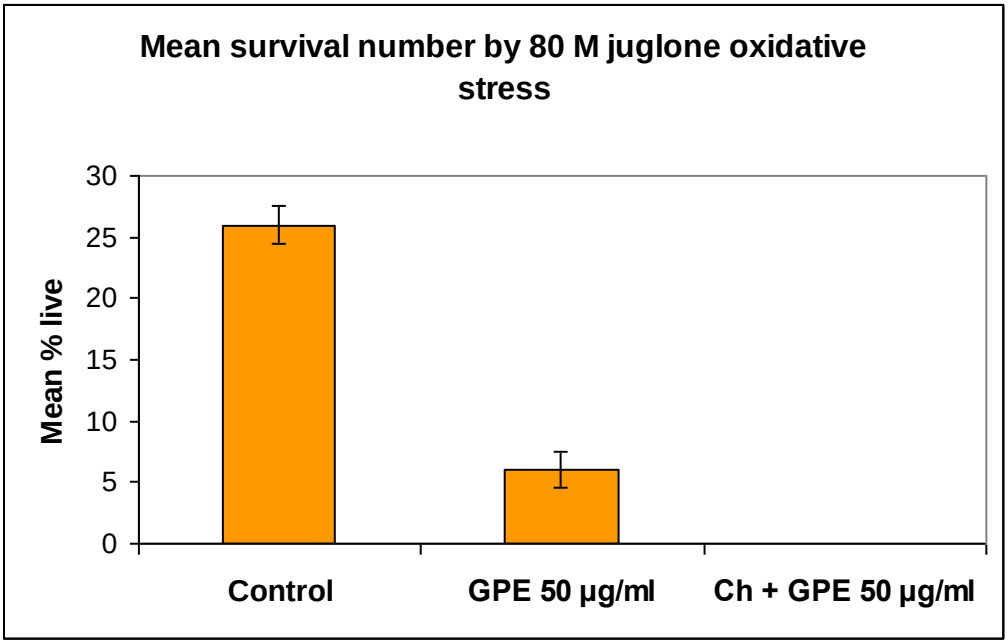
Table B 1

Explanation of the abbreviations used below

Control	**Presence:** cholesterol 5 µg/ml, **Absence:** GPE
Ch + GPE 50 µg/ml	**Presence:** cholesterol 5 µg/ml, GPE 50 µg/ml
Ch + GPE 300 µg/ml	**Presence:** cholesterol 5 µg/ml,GPE 300 µg/ml
GPE 50 µg/ml	**Presence:** GPE 50 µg/ml, **Absence:** cholesterol
GPE 300 µg/ml	**Presence:** GPE 300 µg/ml, **Absence:** cholesterol
Without Ch	**Absence:** GPE and cholesterol

Graph C

This graph shows survival rate after oxidative stress induced by juglon. The effect of GPE treatment (50 µg/ml for 48 h) with and without presence of cholesterol on survival rate of wild type of *C. elegans* under acute, lethal dose of oxidative stress (80 µM juglon for 24 h)



Graph C reflects the survival assay, in this experiment oxidative stress was induced by an acute lethal concentration of juglone by using 80 µM. *C. elegans* was treated with GPE in concentration 50 µg/ml concentration in two different media with and without cholesterol and its treatment started on the day after hatching for 48 hours. Juglone was added to the media and the survivals counted after 24 hours. The results indicate that the combination of cholesterol and GPE in presence of juglone killed all worms in each sample and caused the lethal damages by exposure to oxidative stress. The samples with pure GPE 50 µg/ml do not appear to have protective activity against oxidative stress caused by juglon, since only 6 ± 1.47 % of worms were survived in contrasting with control with survival rate 26 ± 1.58 %. The survival assay shows that pure GPE 50 µg/ml does not protect the worms against oxidative stress induced by juglone and also caused more toxic effect. Since the combination of cholesterol and GPE appears more toxic because all

worms exposed to this mixture did not survive. This fact can be explained by means of two explanations. The first is that all worms which were treated with GPE and cholesterol mixture grew bigger sizes and had more extensive surface in comparison with control. So they could absorb higher quantity of toxic compounds, for instance juglone. The second explanation is that GPE improves accessibility to cholesterol for *C. elegans* and all worms with the higher concentration of cholesterol in organism are more susceptible to oxidative stress. It might explain this observed phenomenon why co-administration of GPE, cholesterol and juglone has more toxic effect.

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Figure 4

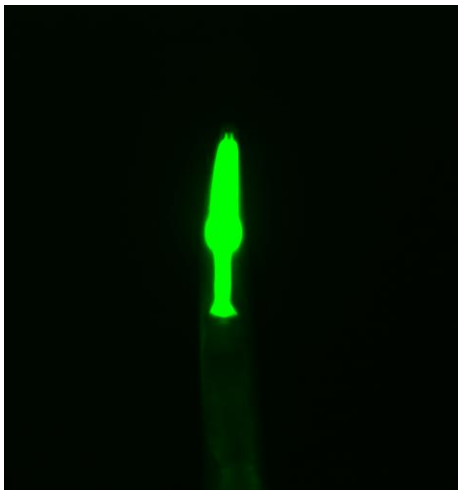


Figure 5

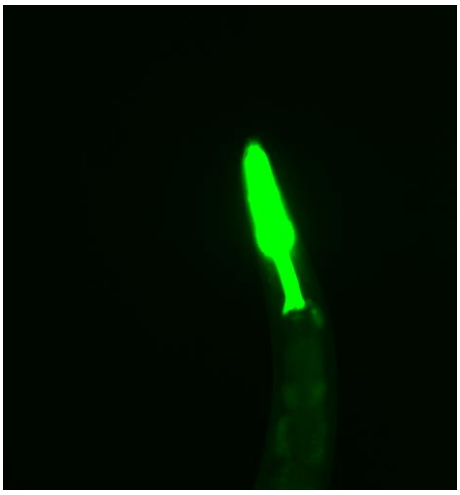


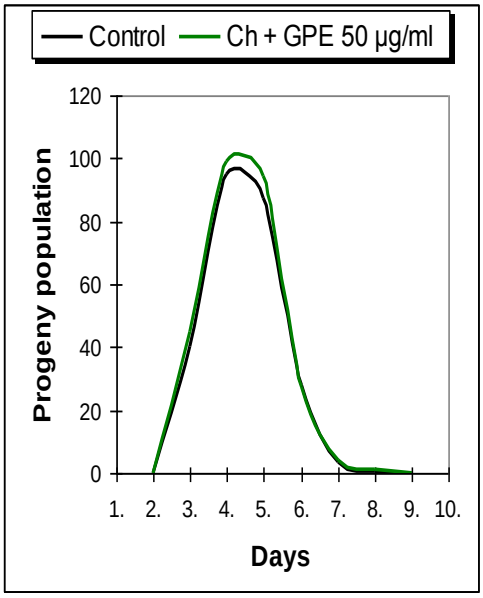
Figure 4- 5

Induction of hsp- 16.2/GFP reporter in response to juglone treatment. TJ375 (hsp- 16.2/GFP) worms were treated with or without GPE 50 µg/ml for 48 h followed by 20 µM juglone for 24 h.

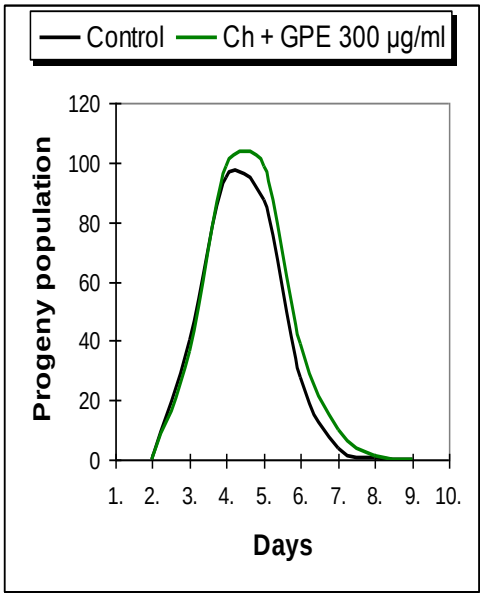
Figure 4 shows the control worm without treatment with GPE in standard medium (cholesterol 5 µg/ml) and ***Figure 5*** shows the worms treated with GPE 50 µg/ml with presence of cholesterol. All worms were exposed to GPE treatment on the day after hatching and after 48 hours were exposed to the oxidative stress by adding of juglone to the medium to achieve concentration of 20 µM. This exposure to juglon proceeded 24 hours and thereafter was the expression of hsp-16.2/GFP measured directly by observing the fluorescence of

the reporter protein GFP. In the TJ375 strain is hsp-16.2/GFP expressed by either heat shock or by oxidative stress, in this case by adding of juglone. ***Figure 4*** and ***Figure 5*** are not distinguished by a significant different of hsp-16.2 expression, the measure of the mean pixel density was unimportant in this case because two independent experiments did not show the antioxidant effect of GPE and a significant visual decrease of hsp-16.2/GFP expression

Graph D 1



Graph D 2



Graph D1- D2

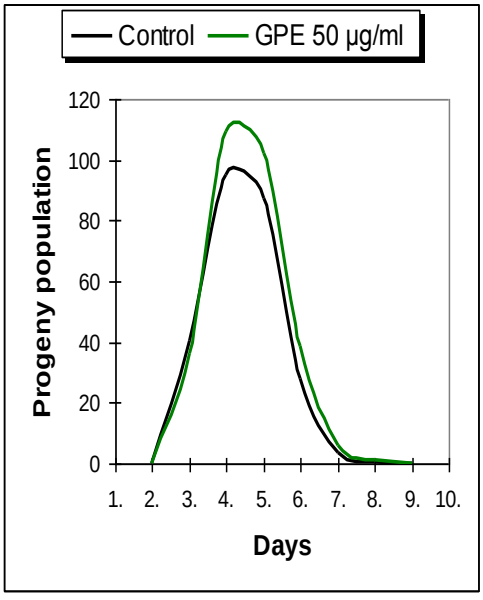
The increase of the number of progeny by GPE (50 and 300 µg/ml) treatment with presence of cholesterol in comparison with the control (cholesterol 5 µg/ml).

Graph D 1 illustrates a possible explanation that the worms utilized both cholesterol (5 µg/ml) and GPE (50 µg/ml) in the concentration, which is not significant toxic in the mixture with cholesterol. This fact has been demonstrated in previous observing of lifespan. The increase of progeny is spaced equally on the curve in comparison with the control curve. The increase of the overall number of progeny is 7.88 ± 6.51 %, mean survival 21.04 ± 0.54

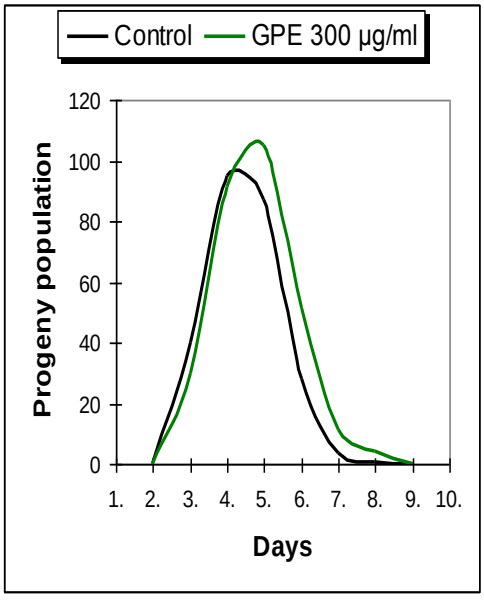
days, mean survival decreased by 2.95 % and maximum lifespan decreased in 2 days in comparison with the control [*Graph F 2,Table B 2*].

Graph D 2 can be explained that the worms utilized both cholesterol 5 µg/ml and GPE 300 µg/ml in the concentration, which is toxic. This fact has been proved in previous observing. GPE can be utilized in this situation preferentially in contrasting with the concentration that is six fold lower (50 µg/ml). The graph shows mild shift to the right in comparison with the control curve. The increase of progeny is more marked in contrasting with previous concentration of GPE (50 µg/ml), the increase is 12.61 ± 4.85 %, mean survival is 15.26 ± 0.31 days, mean survival decreased by 45.94 % and maximum lifespan decreased in 11 days in comparison with the control [*Graph F 5, Table B 3*].

Graph D 3



Graph D 4



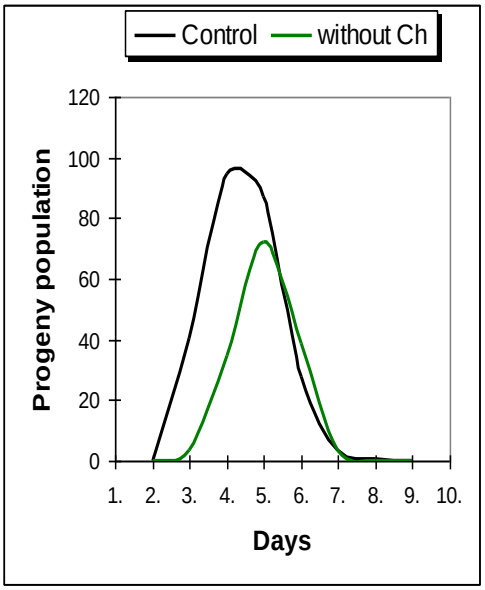
Graph D3- D4

The increase of the number of progeny by GPE (50 and 300 µg/ml) treatment with the absence of cholesterol in comparison with the control (cholesterol 5 µg/ml).

Graph D 3 presents different situation and preferential source of sterols might be derived from GPE in the concentration of 50 μg/ml (there can be present the remanent traces of sterols from bacteria) in contrasting to the previous situation where GPE 50 μg/ml was in the mixture with cholesterol. The graph shows the marked increase of progeny and mild shift to the right in comparison with the control curve. The mild shift to the right indicates the mild extension of the reproduction phase. The increase of the overall number of progeny is 16.73 ± 7.23 %, mean survival 23.35 ± 0.59 days, mean survival increased by 7.80 % and maximum lifespan increased in 1 day in comparison with control [*Graph F 1, Table B 2*].

Graph D 4 shows that the preferential source of cholesterol is derived from GPE in concentration of 300 μg/ml (there can be present the remanent traces of sterols from bacteria) but this concentration is toxic. The graph shows the shift of the whole graph to the right, the mild deceleration of the reproduction phase and the marked extension of the reproduction phase in comparison with the control curve. The increase of the overall number of progeny is 17.17 ± 7.02 %, mean survival 17.66 ± 0.49 days, mean survival decreased by 24.00 % and maximum lifespan decreased in 8 days in comparison with the control [*Graph F 4, Table B 3*].

Graph D 5



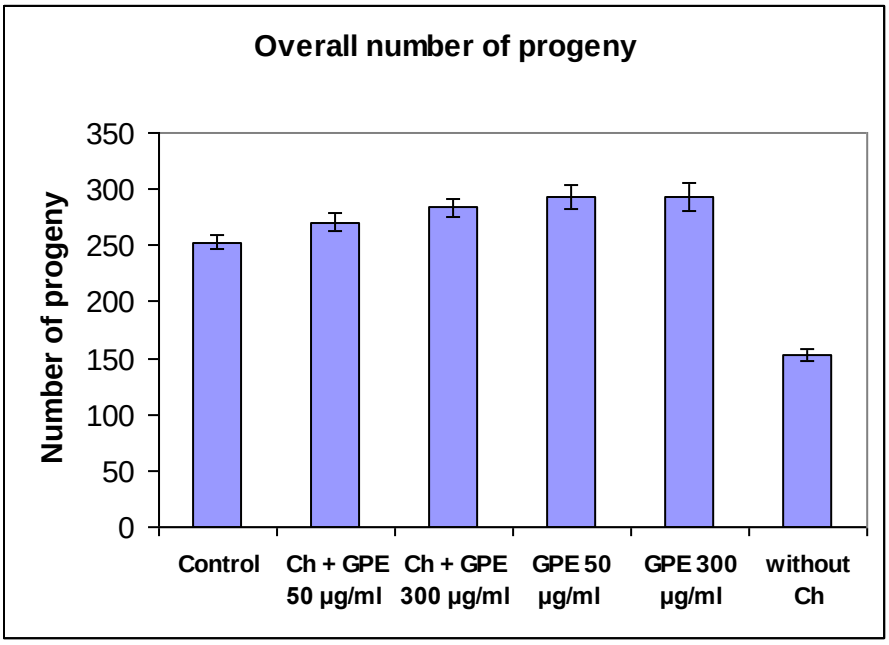
Graph D 5

The decrease of the number of progeny in cholesterol deprived medium in comparison with the control (cholesterol 5 µg/ml). The graph shows the course of the reproduction phase of *C. elegans* and the essential role of sterols in *C. elegans* reproduction.

Graph D 5 points out that without a standard presence of cholesterol or its close derivates, the decrease of progeny is very considerable. This small quantity of progeny is able to hatch from eggs but mostly is unable to reach maturity. Only the small portion of them was able to become fully grown, this fact might be caused with the remanent traces of sterols from bacteria. The graph shows marked deceleration of the reproduction phase. Decrease of the overall number of progeny is 38.73 ± 5.39 %, mean survival 18.19 ± 0.31 days, mean survival decreased by 19.08 % and maximum lifespan decreased in 9 days in comparison with control [*Graph F 7, Table B 4*].

Graph E 1

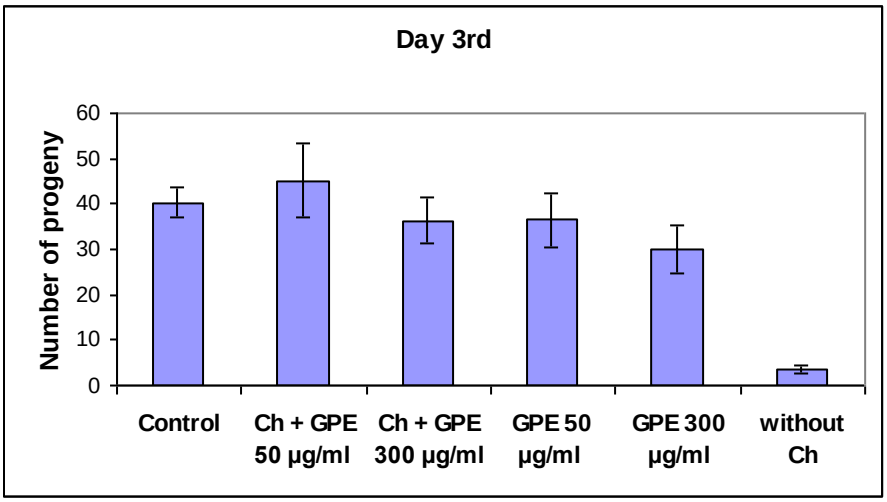
The increase and decrease of the overall number of progeny from all investigated groups in the time range from the third to the eighth day after hatching.



Graph E 1, [*Table A 4*] shows the total number of progeny, we can see the increase of progeny in all samples containing GPE. The final increases are following: Ch + GPE 50 µg/ml is 7.88 ± 6.51 %; Ch + GPE 300 µg/ml is 12.61 ± 4.85 %; GPE 50 µg/ml is 16.73 ± 7.23 % and GPE 300 µg/ml is 17.17 ± 7.02 %. Only medium without presence of cholesterol and GPE shows expected decrease that is -38.73 ± 5.39 %. These results show that the highest number of progeny was achieved by treatment of the worms with GPE 300 µg/ml. This concentration is toxic and GPE might be utilized preferentially in the absence of cholesterol but there can be present the remanent traces of sterols from bacteria. can be present The lowest number of progeny was achieved in cholesterol and GPE deprived medium, this indicates that ginsenosides can be utilized as sterols with better effect on generative ability and that the absence of sterols causes the damage of the generative ability of the worms.

Graph E 2

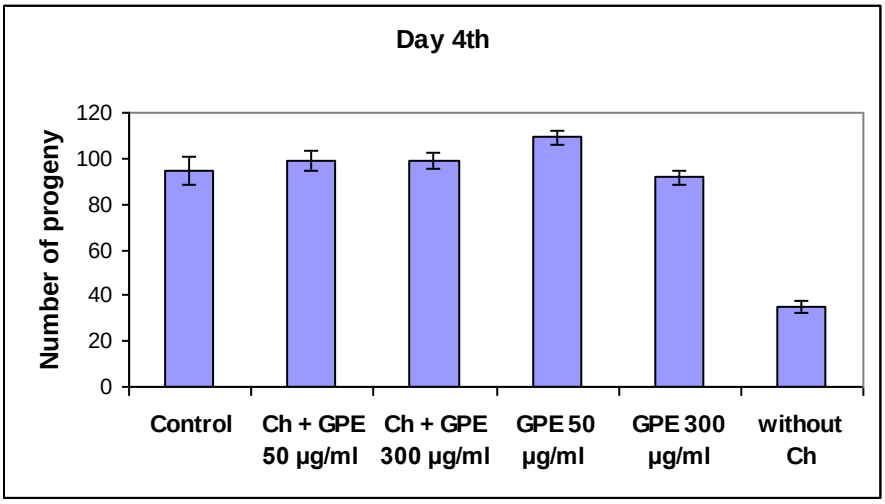
The increase and decrease in the number of *C. elegans* progeny in all investigated media in the third day after hatching.



The third day ***Graph E 2 ,*** [*Table A 1*] we can observe the increase of progeny only in media containing both cholesterol and GPE. The increase is only in medium containing cholesterol and GPE 50 µg/ml and is 11.96 %. The graf shows the decrease of progeny in media without cholesterol but this decrease is not so considerable in media with the presence of GPE. The decrease of progeny in cholesterol and GPE deprived medium is 92.5 %.

Graph E 3

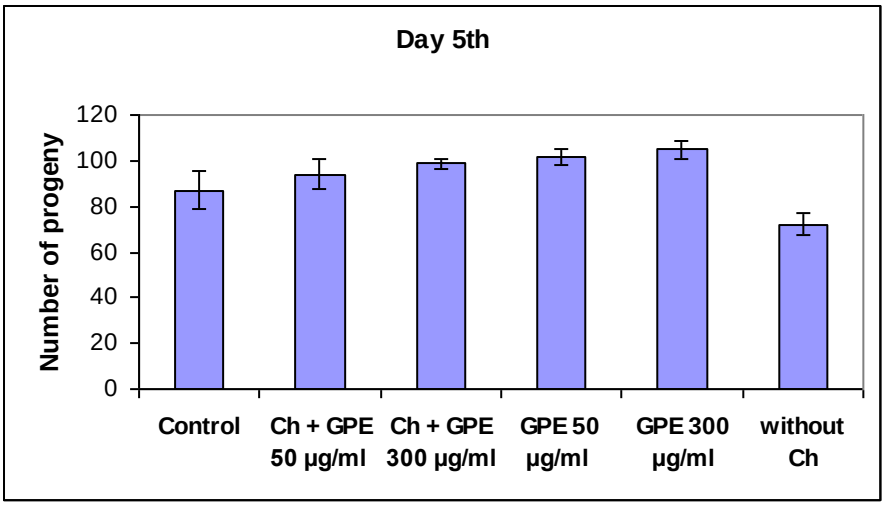
The increase and the decrease in the number of *C. elegans* progeny in all investigated media in the fourth day after hatching.



***Graph* E *3* ,** [*Table A 1*] shows the increase of progeny in all media containing GPE, which is the most significant in medium without cholesterol and with the presence of GPE 50 µg/ml and this increase is 14.99 %. This medium is without cholesterol and with GPE in the concentration that is not toxic. The decrease of progeny continues in medium without the presence of cholesterol and GPE and this decrease is 63.2 %.

Graph E 4

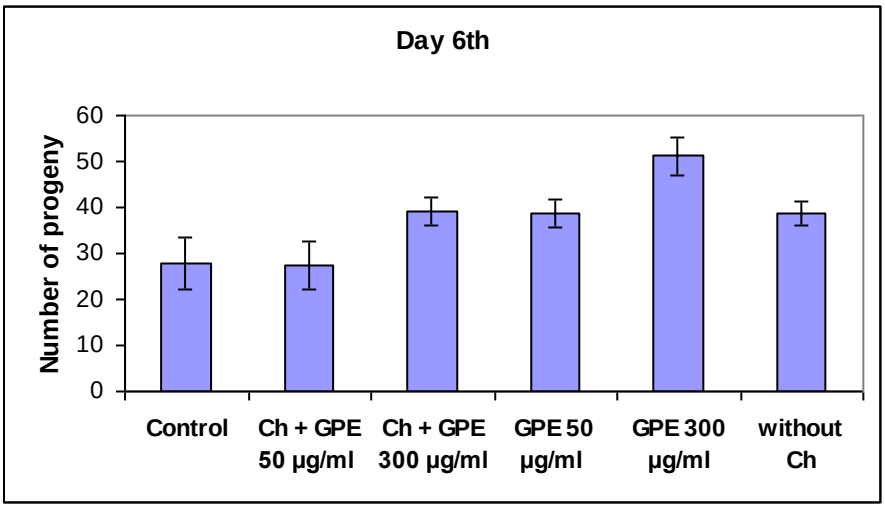
The increase and decrease in the number of *C. elegans* progeny in all investigated media in the fifth day after hatching.



The fifth day ***Graph E 4,*** [*Table A 2*] we can observe the increase of progeny in all media containing GPE again. The increase is the most significant in medium with the absence of cholesterol and presence of GPE 300 µg/ml in the concentration which is toxic and this increase is 20.21 %. The graf shows the decrease of progeny in cholesterol and GPE deprived medium again, this decrease is 17.22 %.

Graph E 5

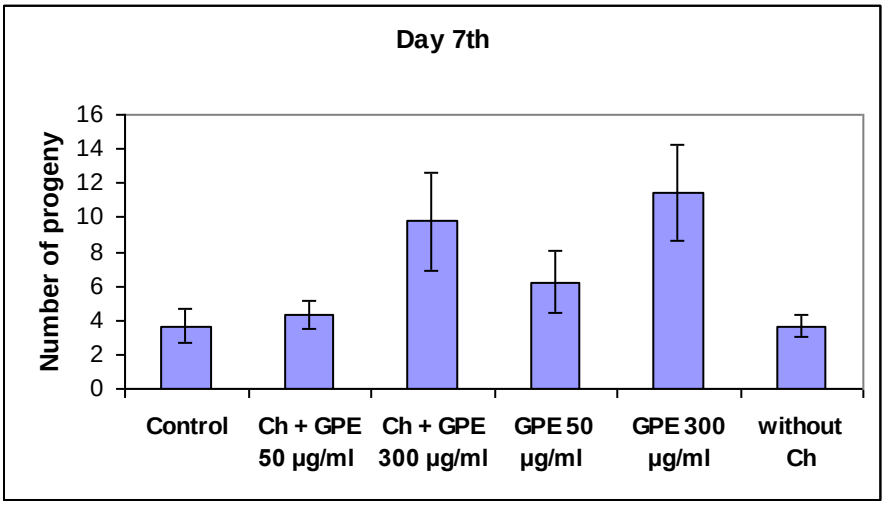
The increase and decrease in the number of *C. elegans* progeny in all investigated media in the sixth day after hatching.



The sixth day ***Graph E 5***, [*Table A 2*] illustrates the increase of progeny practically in all medium with presence of GPE excluding medium containing cholesterol and GPE 50 µg/ml, where is the mean decrease 1.62 %. The increase is the most appreciable in medium with GPE 300 µg/ml and without cholesterol, this increase is 83.98 %. The graph shows the surprising increase of progeny in cholesterol and GPE deprived medium, this increase is 38.80 % in contrasting with the control.

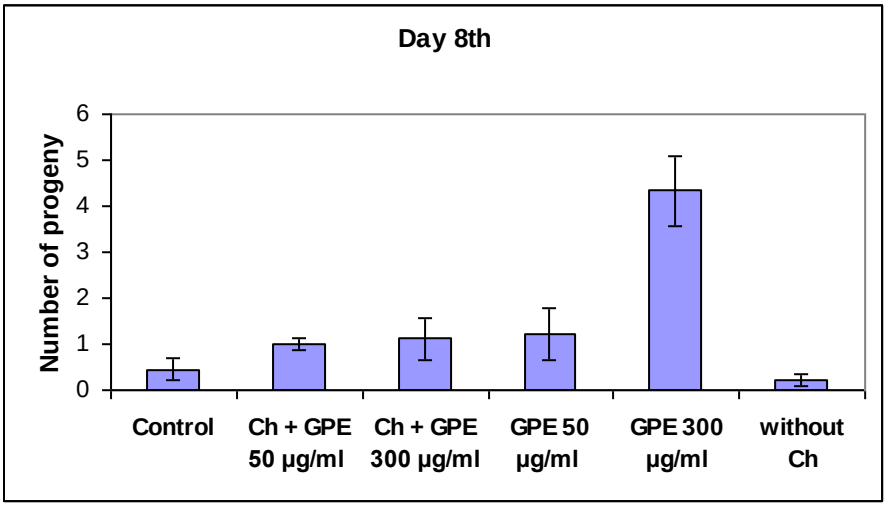
Graph E 6

The increase and decrease in the number of *C. elegans* progeny in all investigated media in the seventh day after hatching.



Graph E 7

The increase and decrease in the number of *C. elegans* progeny in all investigated media in the eighth day after hatching.



The seventh ***Graph E 6***, [*Table A 3*] and the eighth day ***Graph E 7***, [*Table A 3*] show higher quantity of progeny in medium with pure GPE in concentration of 300 µg/ml which is toxic, but the increase is in all media containing GPE. This fact causes the mild shift of the graphs to the right and this is the most appreciable in [*Graph D 4*].

Table A 1

Impact of GPE and cholesterol (5 µg/ml) treatment on the generative ability of *C. elegans*

	Day 3rd: mean number of progeny ± SEM	**Day 4th: mean number of progeny ± SEM**
Control	40.29 ± 3.38	94.89 ± 6.04
Ch + GPE 50 µg/ml	45.11 ± 8.19	99.00 ± 4.34
Ch + GPE 300 µg/ml	36.33 ± 5.03	98.89 ± 3.52
GPE 50 µg/ml	36.44 ± 6.08	109.11 ± 3.03
GPE 300 µg/ml	30.11 ± 5.29	91.56 ± 2.87
Without Ch	3.56 ± 0.73	34.89 ± 2.44

Table A 2

Impact of GPE and cholesterol (5 µg/ml) treatment on the generative ability of *C. elegans*

	Day 5th: mean number of progeny ± SEM	**Day 6th: mean number of progeny ± SEM**
Control	87.11 ± 8.39	27.78 ± 5.52
Ch + GPE 50 µg/ml	94.11 ± 6.32	27.33 ± 5.12
Ch + GPE 300 µg/ml	98.56 ± 2.59	39.00 ± 3.01
GPE 50 µg/ml	101.89 ± 3.49	38.67 ± 2.97
GPE 300 µg/ml	104.89 ± 4.08	51.11 ± 4.21
Without Ch	72.11 ± 4.89	38.56 ± 2.65

Table A 3

Impact of GPE and cholesterol (5 µg/ml) treatment on the generative ability of *C. elagans*

	Day 7th: mean number of progeny ± SEM	**Day 8th: mean number of progeny ± SEM**
Control	3.67 ± 0.99	0.44 ± 0.24
Ch + GPE 50 µg/ml	4.33 ± 0.87	1.00 ± 0.12
Ch + GPE 300 µg/ml	9.78 ± 2.87	1.11 ± 0.45
GPE 50 µg/ml	6.22 ± 1.82	1.22 ± 0.57
GPE 300 µg/ml	11.44 ± 2.80	4.33 ± 0.76
Without Ch	3.67 ± 0.65	0.22 ± 0.15

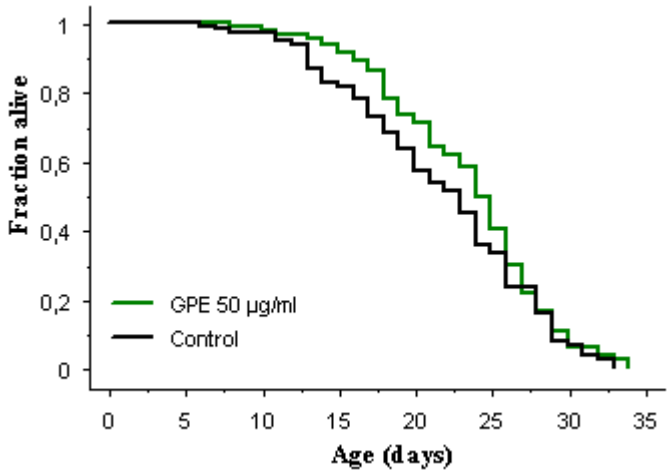
Table A 4

Impact of GPE treatment on the generative ability of *C. elegans* in comparison with control (cholesterol 5 µg/ml)

	Day 3rd - 8th: mean number of progeny ± SEM	**Day 3rd - 8th: mean % increase ± SEM**
Control	252.56 ± 6.24	
Ch + GPE 50 µg/ml	270.89 ± 8.31	7.88 ± 4.51 %
Ch + GPE 300 µg/ml	283.67 ± 8.22	12.61 ± 3.36 %
GPE 50 µg/ml	293.56 ± 10.88	16.73 ± 5.01 %
GPE 300 µg/ml	293.44 ± 12.24	17.17 ± 4.87 %
Without Ch	153.00 ± 5.42	− 38.73 ± 3.74 %

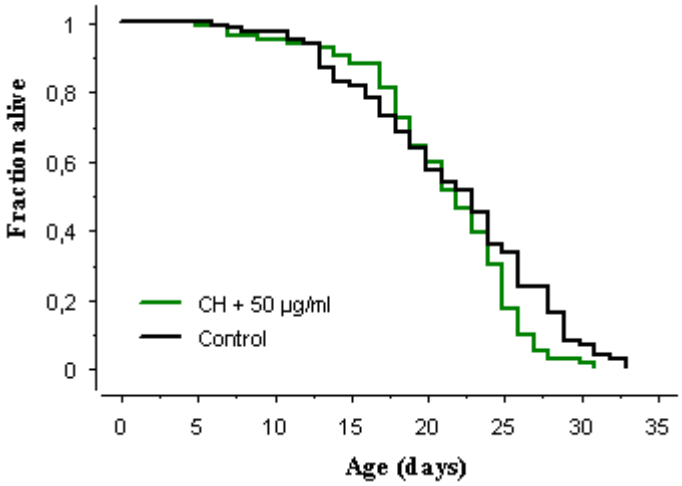
Graph F 1

The comparison of two *C. elegans* lifespans. The first lifespan serves as the control (cholesterol 5 µg/ml) and the second shows the treatment with GPE 50 µg/ml.



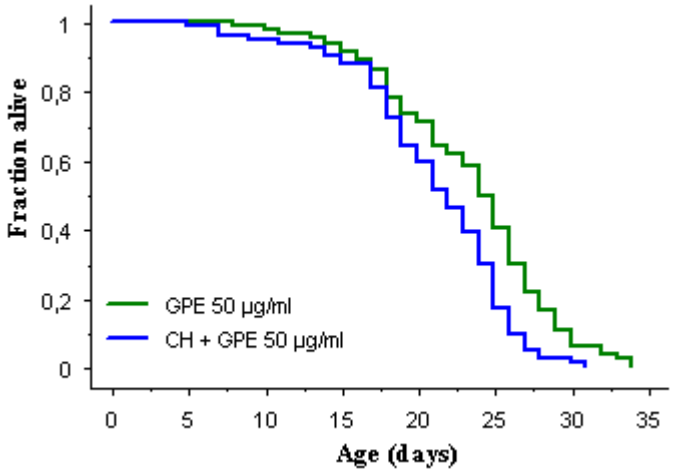
Graph F 2

The comparison of two *C. elegans* lifespans. The first lifespan serves as the control (cholesterol 5 µg/ml) and the second shows the treatment with co-administration of cholesterol and GPE 50 µg/ml.



Graph F 3

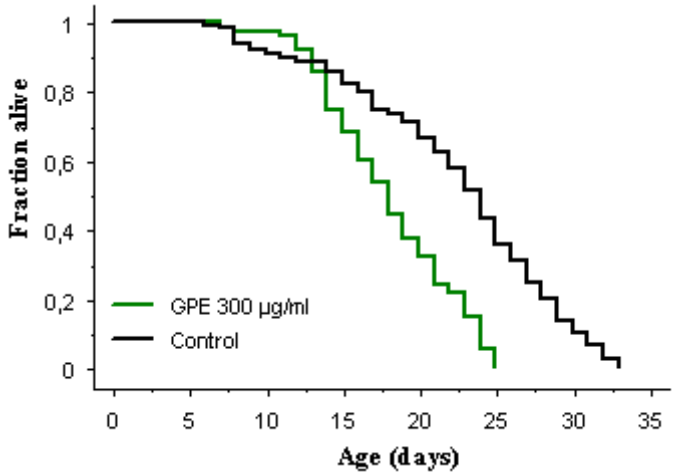
The mutual comparison of two *C. elegans* groups: the first group was exposed to GPE 50 µg/ml and the second to the mixture of cholesterol and GPE 50 µg/ml.



Graph F 3, [*Table B 2*]shows the comparison between medium containing GPE 50 µg/ml with and without the presence of cholesterol. The medium with pure GPE assigns the increase of mean survival by 10.98 % and the extension of maximum lifespan by 3 days and P = 0.0002 in contrasting with medium containing the mixture of GPE 50 µg/ml and cholesterol.

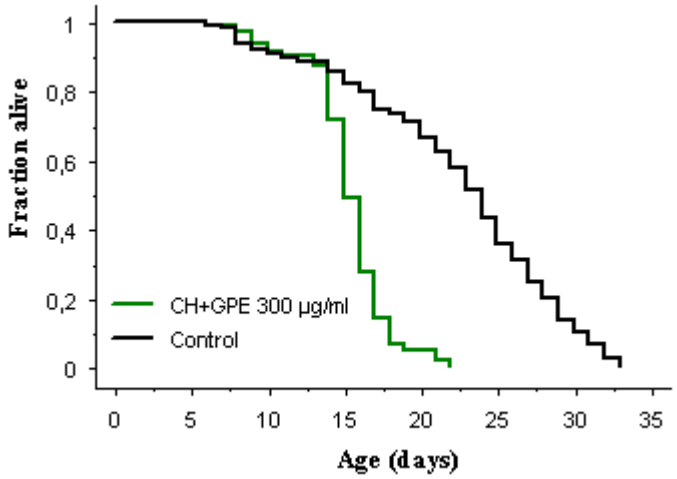
Graph F 4

The comparison of two *C. elegans* lifespans. The first lifespan serves as the control (cholesterol 5 µg/ml) and the second shows the treatment with GPE 300 µg/ml.



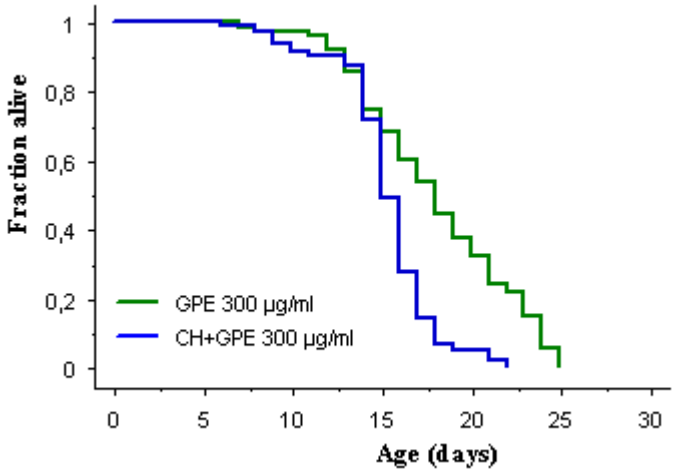
Graph F 5

The comparison of two *C. elegans* lifespans. The first lifespan serves as the control (cholesterol 5 µg/ml) and the second shows the treatment with co-administration of cholesterol and GPE 300 µg/ml.



Graph F 6

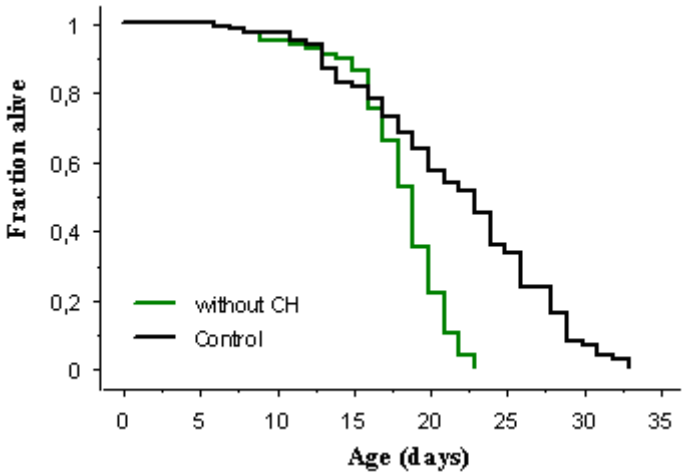
The mutual comparison of two *C. elegans* groups: the first group was exposed to GPE 300 µg/ml and the second to the mixture of cholesterol and GPE 300 µg/ml.



Graph F 6, [*Table B 3*] shows very similar situation by using six fold higher concentration of GPE. This graph shows more significant difference in mean survival. The increase of mean survival is 17.69 %, the extension of maximum lifespan is 3 days and $P < 0.0001$.

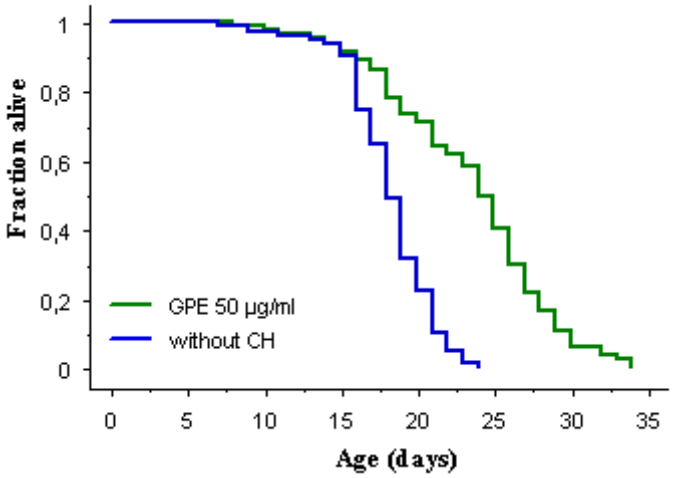
Graph F 7

The comparison of two *C. elegans* lifespans: Control group of *C. elegans* presents cholesterol fed worms and the second group presents cholesterol deprived worms.



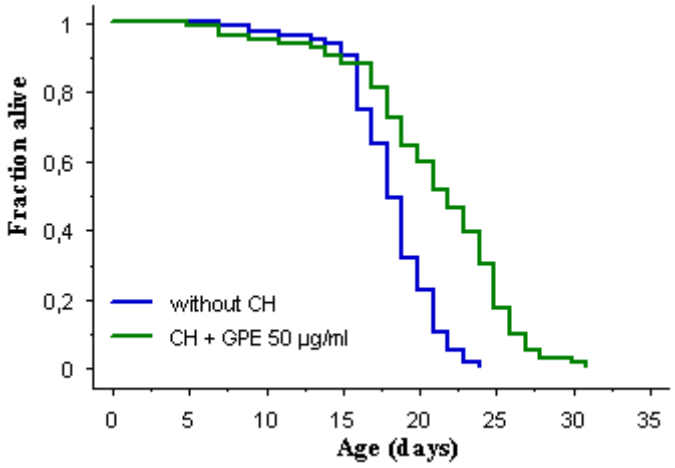
Graph F 8

The comparison of two *C. elegans* lifespans: The first lifespan shows treated worms with GPE 50 µg/ml and the second lifespan shows cholesterol deprived worms.



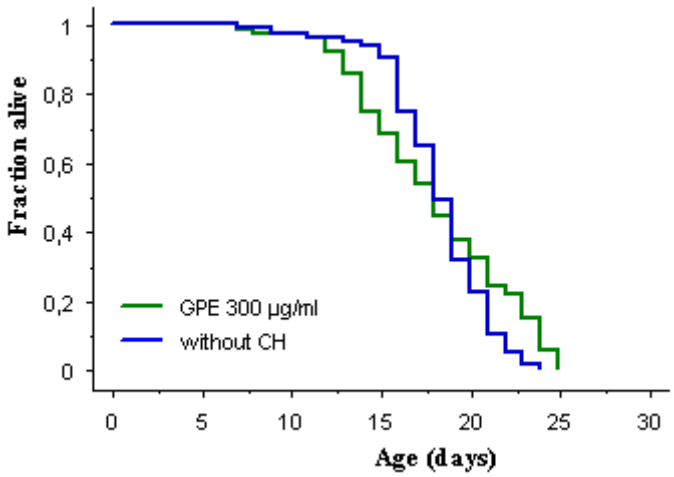
Graph F 9

The comparison of two *C. elegans* lifespans: The first lifespan shows treated worms with mixture of cholesterol and GPE 50 µg/ml and the second lifespan shows cholesterol deprived worms.



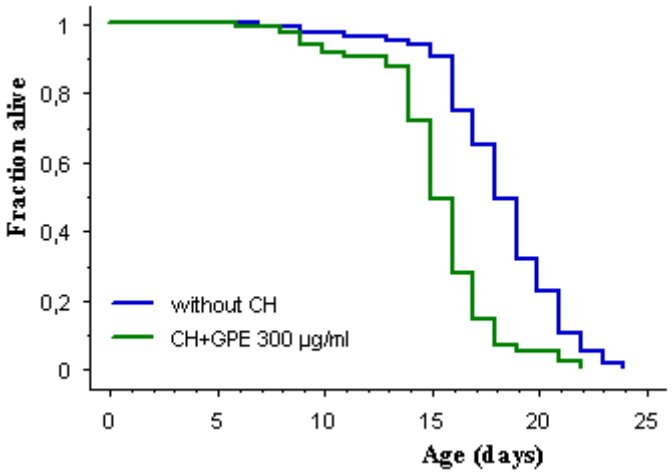
Graph F 10

The comparison of *C. elegans* exposed to the cholesterol deprived medium with *C. elegans* exposed to GPE 300 µg/ml.



Graph F 11

The comparison of *C. elegans* exposed to the cholesterol deprived medium with *C. elegans* exposed to the mixture of cholesterol and GPE 300 µg/ml.



Graph F 7 - **Graph F 11**, [Table B 4] show the relationship between cholesterol deprived medium with all herein mentioned media. The medium containing pure cholesterol, pure GPE 50 µg/ml and their mixture have more beneficial effect on lifespan than the cholesterol deprived medium. Only medium containing both cholesterol and GPE 300 µg/ml shortens mean survival significantly than the cholesterol deprived medium.

Table B 2

Effect of pure GPE 50 µg/ml and impact of its co-administration with cholesterol (5 µg/ml) on lifespan of *C. elegans*.

Treatment (µg/ml)	Mean survival ± SEM	Maximum lifespan	N	P value*
GPE 50	23.35 ± 0.59	34	85	
Control	21.66 ± 0.66	33	90	0.1813
Ch +GPE 50	21.04 ± 0.54	31	88	
Control	21.66 ± 0.66	33	90	0.0282
GPE 50	23.35 ± 0.59	34	85	
Ch +GPE 50	21.04 ± 0.54	31	88	0.0002

**P* value long rank as compared with untreated or in different medium treated worms; mean survival values in days is the mean of population and the standard error. Each group was compared by the nonparametric long rank test and *N* is the number of worms after censoring.

Table B 3

Effect of pure GPE 300 µg/ml and impact of its co-administration with cholesterol (5 µg/ml) on lifespan of *C. elegans*.

Treatment (µg/ml)	Mean survival ± SEM	Maximum lifespan	N	P value*
GPE 300	17.96 ± 0.49	25	76	
Control	22.27 ± 0.74	33	83	< 0.0001
Ch +GPE 300	15.26 ± 0.31	22	69	
Control	22.27 ± 0.74	33	83	< 0.0001
GPE 300	17.96 ± 0.49	25	76	
Ch +GPE 300	15.26 ± 0.31	22	69	< 0.0001

Table B 4

Effect of media containing cholesterol and GPE in contrasting with cholesterol deprived medium

Treatment (µg/ml)	Mean survival ± SEM	Maximum lifespan	N	P value*
Without CH	18.19 ± 0.31	24	90	
GPE 50	23.35 ± 0.59	34	85	< 0.0001
Ch +GPE 50	21.04 ± 0.54	31	88	< 0.0001
GPE 300	17.96 ± 0.49	25	76	0.1703
Ch +GPE 300	15.26 ± 0.31	22	69	< 0.0001
Control	21.66 ± 0.66	33	90	< 0.0001

6. DISCUSSION

This work was initially focused on the antioxidant properties of *Panax ginseng* extract (GPE). *In vitro* studies were accomplished and these studies indicated that GPE has really free radical scavenging activity [*Graph A*] and superoxide anion radical scavenging activity [*Graph B*] however the extract from *Panax ginseng* does not appear to have a strong antioxidant activity. This finding corelates with the data from different study engaged in free radical scavenging activity of red ginseng aqueous extract which demonstrated that this extract is not a strong free radical scavenger [64]. Almost all ginsenosides, the main active compounds of *Panax ginseng*, had no effect on radical scavenging activity. Therefore, the various pharmacological effects of ginseng such as the antioxidant activity, especially radical scavenging activity, can not be explained only with them [31].

IC_{50} of GPE free radical scavenging activity is 0.66 ± 0.09 mg/ml and that is approximately 13.3 times higher concentration than was used for treatment (50 μg/ml) without toxic and with insignificant therapeutic effect. IC_{50} of superoxide anion radical scavenging activity is 3.70 ± 0.27 mg/ml. This concentration is approximately 74 times higher than the concentration used by treatment (50 μg/ml). It is evident that this extract is not pure, composed of one substance with one main mechanism of action but GPE is the mixture containing many active compounds. A lot of them can have the antioxidant activity but simultaneously there are other compounds with potential prooxidant activity or they are able to effect through a lot of different cellular mechanisms which are more significant than the antioxidant activity. This fact might explain why only low concentrations of GPE were applicable by treatment of *C. elegans*.

For example a parallel study engaging in the antioxidant properties of licorice, *in vitro* tests assign very similar results of DPPH free radical scavenging activity and superoxide anion radical scavenging activity but *in vivo* tests on *C. elegans* assign a possible curative effect in the concentrations which are considerably lower than IC_{50}, only 10 μg/ml [62]. This concentration

is only the fragment of measured antioxidant activity from *in vitro* tests likewise in all herein described *in vivo* experiments.

On the other hand there is the study engaged in the antioxidant properties of Epigallocatechin gallate (EGCG) but *C. elegans* is treated approximately with 40 times higher concentration than is IC_{50} of DPPH radical scavenging activity. In this case EGCG is highly pure chemical and this manifold higher concentration than IC_{50} increases lifespan and stress resistance in *C. elegans* significantly [63].

Treatment with GPE 50 μg/ml is not significant beneficial in the absence of cholesterol or significant toxic in the presence of cholesterol. On the one hand GPE appears to have antioxidant *in vitro* activity and causes the increase of progeny but on the other hand its co-administration with cholesterol appears to be more toxic than pure GPE and causes the decrease of mean lifespan.

Co-administration of GPE and cholesterol seems to be unbeneficial to worm`s health because bigger worms has simultaneously bigger surface in the contact with their surroundings. The biger surface might absorb corresponding more marked quantity of the aggressive substances, as juglone, from its environment, that`s why the mixture of cholesterol and GPE could have more significant toxic effect. It was visually observed that the parallel co-administration of GPE and cholesterol caused significant increase of mean size of the worms, the results are not showed but this observation agrees with the observation in different study of Lee J.H. et al. [51]. This fact was braced up by means of the various beginning of GPE treatment. All experiments with progeny were started immediately after hatching and lifespan assay was started three days after hatching. Probably the sooner beginning supports the better utilization of cholesterol in the worm organism. Co-administration of GPE and cholesterol does not improve the generative abilities of *C. elegans* in comparison with the worms treated with pure GPE. This finding is at variance with the results from the study of Lee J.H. et al. [51] in which co-administration of ginseng total saponins and cholesterol leads to the most significant increase of the average brood size and egg laying ability of *C.*

elegans. On the other hand their extract was more purified and for example all phytosterols such as sitosterol or campasterol were removed. Surprisingly many results from their study correspond to herein mentioned findings. For example, co-administration of cholesterol and ginseng extract produces bigger worms and conversely cholesterol deprivation produces smaller worms and impairs the reproduction ability of *C. elegans* in contrasting with cholesterol fed group. On the other hand ginseng extract improves the reproduction ability of *C. elegans* [*Graph E 1*, *Table A 4*] and can completely replace cholesterol as a source of sterols.

All herein mentioned findings indicate that parallel administration of cholesterol and GPE effects negative on mean lifespan. The bigger worm does not mean the healthier and more fertile worm. In addition worms treated parallel with GPE and cholesterol and thereafter exposed to juglone treatment did not survive anyone. Interestingly worms fed with GPE and without cholesterol did not attain good survival results that are only 6 ± 1.47 %. This is considerable decrease in contrasting with the control whose survival rate is 26 ± 1.58 % [*Graph C*]. This finding can be related to the study of Liu Z.Q. et al. [21] engaging in the relationship between structure and the antioxidant or prooxidant activity of ginsenosides using AAPH that induced haemolysis in human erythrocytes. The results are obvious, some ginsenosides acted as a prooxidant in AAPH induced haemolysis. In summary a protective activity of the extracts against oxidative stress is closely connected with their prooxidant and antioxidant rate.

The mutual interactions of cholesterol and GPE (cholesterol and steroidal saponins) must be defined for better understanding of their role in the human medicine. There can be many mechanisms of action, both direct and indirect. Further GPE can effect on hormonal or antioxidant levels, can modify membrane fluidity, improve the utilization of cholesterol or its close derivates etc. For example lipid micelle formation can be included among very important indirect mechanisms of action. In this case the scientific results from the different studies of Joo C.N. et al. [59], [60] or Song J.L. et al. [61] must be presented. These results suggested that under the physiological conditions, a

small amount of the saponins would affect sufficiently for lipid micelle formation and so improve the utilization of cholesterol. Therefore lipids might be absorbed easily for example from small intestine [59]. Very important finding was reported that saponins were able to stimulate the absorption and transport of water-insoluble vitamins such as vitamin A and vitamin K [60]. The other very interesting results were obtained from the study aimed at the dependence of cholesterol and ginseng saponin utilization in rat body. In protopanaxatriol saponin fed group, the cholesterol absorption was significantly stimulated. However, protopanaxadiol saponins did not affect on cholesterol. These results presented in the study of Song J.L. et al. [61] suggest that the stimulatory effect of total saponins in the absorbtion of cholesterol might be mainly due to the protopanaxatriol saponins. The results suggest that ginseng saponin fractions would be facilitate for the absorbtion and utilization of cholesterol [61]. This finding corresponds with the visual observation of the worms treated with the mixture of cholesterol and GPE. The better utilization of cholesterol in presence of GPE might explain bigger sizes of *C. elegans* and this non-specific mechanism of action can be combined and so augmented with specific effect on hormonal system of *C. elegans*.

On the other hand the protopanaxadiol mixture acted as an antioxidant and protopanaxatriol as a prooxidant [21]. These facts show that the effect of GPE is very complicated and one compound may act through many mechanisms and a lot of fundamental principles of action must be found.

The study of mutual interactions between cholesterol and steroidal saponins, in this case ginsenosides, has the considerable potential to the future. *C. elegans*, as model organism, has the unique importance in this research owing to its inability to synthesize cholesterol *de novo* and is starting to become a valuable new model for this study. The importance lies in its dependence on exogenous sources, such as ginsenosides. There are many studies have been engaged in *Panax ginseng*, whereas one part of researchers has confirmed its beneficial effect, the other part has oppugned.

This study is looking for the relation between ginseng beneficial effect and the content of cholesterol in nourishment. The negative effect of higher

concentrations of cholesterol on medical potential of GPE can explain very different results of many studies engaging in ginseng research from different countries. There are considerable differences in ordinary contents of sterols in nourishment and genetic differences in cholesterol metabolism. The impact of *Panax ginseng* on beneficial treatment can be very different in the west with animal fats abundant diet or in the countries with lower content of animal fats in diet.

7. CONCLUSION

The results of this work can be recaped as follows:

1) *Panax ginseng* extract is not a strong free radical scavenger and IC_{50} is 0.66 ± 0.09 mg/ml. In addition this concentration is approximately 13.3 times higher than the concentration used *in vivo* studies (50μg/ml) without considerable toxic effect in mixture with cholesterol and with insignificant effect on extension of lifespan in the absence of cholesterol.
2) *Panax ginseng* extract is not a strong superoxide anion scavenger and its IC_{50} is 3.70 ± 0.27 mg/ml. Further this concentration is approximately 74 times higher than the concentration used *in vivo* studies (50 μg/ml) without considerable toxic effect in mixture with cholesterol and with insignificant effect on extension of lifespan in the absence of cholesterol.
3) *Panax ginseng* extract protects *C. elegans* directly or indirectly against oxidative stress insignificantly. GPE does not suppress the expression of hsp-16.2 measured directly by observing the fluorescence of the reporter protein GFP in the TJ375 strain. In this study was used non toxic concentration of 50 μg/ml and the expression of hsp-16.2 was stimulated by adding of juglone (20 μM/ ml). The survival assay shows also the inability of GPE to protect against oxidative stress induced with lethal dose of juglone. Even GPE 50 μg/ml causes the decrease of survival rate in this experiment in contrasting with the control. On the other hand this experiment demonstrates higher toxicity of lethal dose of juglone in combination with cholesterol and GPE 50 μg/ml than only with the pure GPE 50 μg/ml. The survival rate of the control is 26 ± 1.58 % and by the treatment with pure GPE 50 μg/ml is 6 ± 1.47 %. The mixture of cholesterol with GPE 50 μg/ml is lethal for all worms and the survival rate is 0 %.

4) *Panax ginseng* has an influence on the generative abilities of the worms. We can see the increase of progeny in all samples containing GPE. Progeny increases are following: Ch + GPE 50 μg/ml is 7.88 ± 6.51 %; Ch + GPE 300 μg/ml is 12.61 ± 4.85 %; GPE 50 μg/ml is 16.73 ± 7.23 % and GPE 300 μg/ml is 17.17 ± 7.02 %. The decrease was observed only in the cholesterol deprived medium and this decrease is 38.73 ± 5.39 %. These results show that the highest number of progeny was achieved by treatment of the worms with GPE 300 μg/ml in the absence of cholesterol. This concentration is toxic and GPE might be utilized preferentially in the absence of cholesterol. The lowest number of progeny was achieved in cholesterol and GPE deprived medium. This fact indicates that GPE can be utilized as sterols with better effect on generative ability and that the absence of sterols causes the damage of the generative abilities and shortens mean lifespan of the worms.
5) GPE has more harmful influence on lifespan of *C. elegans* in the combination with cholesterol than the pure GPE without presence of cholesterol. The medium with pure GPE 50 μg/ml assigns the increase of mean survival by 10.98 % and the extension of maximum lifespan by 3 days and $P = 0.0002$ in contrasting with the medium containing the mixture of GPE 50 μg/ml and cholesterol. Very similar situation arises by using six fold higher concentration of GPE (300 μg/ml). There is more significant difference in mean survival. The increase of mean survival is 17.69 %, the extension of maximum lifespan is 3 days and $P < 0.0001$.
6) *C. elegans* mean size depends on presence of sterols in medium, their contents in medium, their type (cholesterol, phytosterols, steroidal saponins: ginsenosides) or on beginning of the treatment. The visual observations show that the smallest worms were in cholesterol deprived medium and the biggest in all mixtures of cholesterol and GPE. Interestingly the most considerable grow up was observed by the

worms fed with GPE immediately after hatching in the comparison with the worms treated three days after hatching.

7) A future research of the relationship between cholesterol and *Panax ginseng* must be concentrated on finding of suitable concentrations of GPE. It seems to be probable that all searched concentrations will be lower than 50 μg/ml. A special strain of *C. elegans* with higher content of cholesterol in its membranes might have great importance in this future research, for example the transgenic strain known as *C. cholegans*. This transgenic strain contains approximately 80 % more cholesterol than the wild-type and so it is closer model to human cell membranes [65].

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9. ABBREVIATIONS

AAPH: 2, 2'-azobis-2-amidinopropane hydrochloride

Ara(f): Arabinofuranoside

Ara(p): Arabinopyranoside

ATP: Adenosine tri-phosphate

CAT: Catalase

CH: Cholesterol

CMC: Critical micellar concentration

CNS: Central nerve system

DPPH: 2, 2-Diphenyl-1-picryl-hydrazyl

Glc: Glucopyranose

GFE: Green fluorescent protein

GPE: Ginseng Panax extract

GSH-Px: Glutathione peroxidase

Hsp: Heat-shock protein

IC_{50}: The half maximal inhibitory concentration

LB: Liquid broth

NADH: β-Nicotinamide adenine dinucleotide

NBT: Nitro blue tetrazollium chloride

NGM: Nematode growth medium

PMS: Phenazine metho-sulphate

PPD: Protopanaxadiol

PPT: Protopanaxatriol

Rha: Rhamnopyranoside

SEM: Standard error of the mean

SM: S-medium

SOD: Superoxide dismutase

10. ABSTRACT

The Thesis: The Antioxidant and Hormonal Effects of *Ginseng Panax* on Lifespan and Stress Resistance in *Caenorhabditis elegans*
Made by: Václav Ježek

The antioxidant and hormonal effect of *Panax ginseng* on lifespan and stress resistance in *C. elegans* were investigated. The best results by treatment of *C. elegans* were achieved in the presence of pure GPE 50 μg/ml without the presence of cholesterol. Surprisingly this medium assigns the increase of mean survival by 10.98 % in contrasting with medium containing the mixture of GPE 50 μg/ml and cholesterol. This negative impact of GPE and cholesterol co-administration on lifespan of *C. elegans* is more obvious by using sixfold higher concentration of GPE (300 μg/ml). In this case the increase of mean survival by treatment with pure GPE is 17.69 %. In addition the highest increase of progeny was observed in medium with pure GPE 300 μg/ml. Surprisingly this concentration was appointed as toxic with negative effect on lifespan of *C. elegans*. It is obvious that GPE does not protect *C. elegans* against oxidative stress significantly because it does not suppress the expression of hsp-16.2/GFP and does not protect against oxidative stress caused by lethal dose of juglone. These findings suggest that the antioxidant activity of GPE does not play prevailing role by treatment of *C. elegans*. All results and information sources indicate that main mechanisms of action are related to an effect on hormonal levels and its close relation with cholesterol metabolism. The results demonstrate that the co-administration of cholesterol and GPE to *C. elegans* leads to the decrease of the curative ability of GPE in contrasting with the administration of pure GPE.

Diplomová práce: Antioxidační a hormonální vliv *Ginseng Panax* na délku života a resistenci na stres u *Caenorhabditis elegans*

Vypracoval: Václav Ježek

V této práci byl zkoumán antioxidační a hormonální vliv léčby *Panaxem ginseng* na délku života a odolnost vůči stresu u *C. elegans*. Nejlepších výsledků při léčbě *C. elegans* bylo dosaženo při koncentraci 50 μg/ml GPE. V tomto případě léčba čistým GPE vykazuje nárůst střední délky života o 10.98 % v porovnání s médiem obsahujícím jak GPE tak cholesterol. Negativní dopad vzájemného podávání GPE a cholesterolu na délku života *C. elegans* se pak ukazuje být výraznějším při šestinásobné koncentraci 300 μg/ml, kde nárůst střední délky života u léčby čistým GPE činí 17.69 %. Nejmarkantnější nárůst potomků u *C. elegans* byl zaznamenán při léčbě čistým GPE 300 μg/ml, což je toxická koncentrace, která významně zkracuje střední délku života u *C. elegans*. Také je zřejmé, že GPE nechrání *C. elegans* významně vůči oxidačnímu stresu, jelikož nepotlačuje expresi hsp-16.2/GFP a nevykazuje ochranu vůči letální dávce juglonu v porovnání s kontrolou. Tato zjištění potvrzují, že antioxidační účinek GPE nehraje významnější roli při léčbě *C. elegans* a všechny výsledky spolu s informačními zdroji směřují k tvrzení, že hlavní mechanismy účinku jsou na hormonální úrovni a v jeho úzkém vztahu s metabolismem cholesterolu. Významné může být také zjištění, že paralelní příjem GPE a cholesterolu vede k poklesu léčebného účinku GPE u *C. elegans* v porovnání s léčbou čistým GPE.