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Genotoxicity testing of *Geranium macrorrhizum* extracts

Master Thesis

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Table of Contents

Introduction....3
1. Literature review....3
  1.1 Purpose of genotoxicity testing....3
    1.1.1 Genotoxicity testing of plants' constituents....4
  1.2. Phytochemicals present in plants and their properties....5
  1.3 Taxonomy and properties of *Geranium macrorrhizum*....19
2. Materials and methods....25
  2.1 Cytokinesis block micronucleus (CBMN) assay....25
  2.2 Comet Assay....28
3.Results and discussion....33
Conclusions....42
Summary....43
References....44

Introduction

Plants have played very important life throughout human history. Ancient time humans were consuming solely plants until they learnt that they can actually cook the animals they have hunted and removed the potential harm. Lots of people have lost their life because of toxicity caused by plants since people simply did not have an idea which plants are toxic for consumption and which are not. Humanity learnt which plants or toxic and which are not by trial-error method. Today, methods regarding to toxicity are well developed compared to early days, however there are still unknowns present regarding to plants and their toxicity. *Geranium macrorrhizum* is a plant which its toxic effects of root and leaves extracts are not very well studied. However, there are several studies regarding to *G. macrorrhizum* essential oil due to the compound which are abundant inside of the essential oil, germacrone, because of its abundancy in other plants. In this research CBMN assay and Comet assay will be performed to determine genotoxicity of *G. macrorrhizum* extracts.

1. Literature review

1.1 Purpose of genotoxicity testing

Genotoxicity testing is a part of safety measures applied to the different variety of substances which humans use. Genotoxicity tests are applied on products like cosmetics, agricultural products, food additives, pharmaceuticals to name a few. Genotoxicity tests are done in two types: They can be either *in vitro* or *in vivo*. Commonly, *in vitro* tests are used but it is not possible to eliminate *in vivo* tests, which are done on animals, completely. In last few decades, people are trying to optimize *in vitro* tests to reduce the necessity of *in vivo* tests, caused by the situation referred as false positives. Development of new tools like creating genotoxicity and carcinogenicity databases and creating a list of reference chemicals are supporting this movement (Corvi et al., 2017).

In vitro and *in vivo* test batteries consists of 4 tests each and they are listed below:

In vitro test battery:

- Bacterial reverse mutation assay
- Mammalian chromosomal aberration assay
- Mammalian cell gene mutation test

- Mammalian cell micronucleus test

In vivo test battery:

- Mammalian erythrocyte micronucleus test
- Bone marrow chromosomal aberration
- Transgenic rodent somatic and germ cell mutation assay
- Mammalian alkaline comet assay

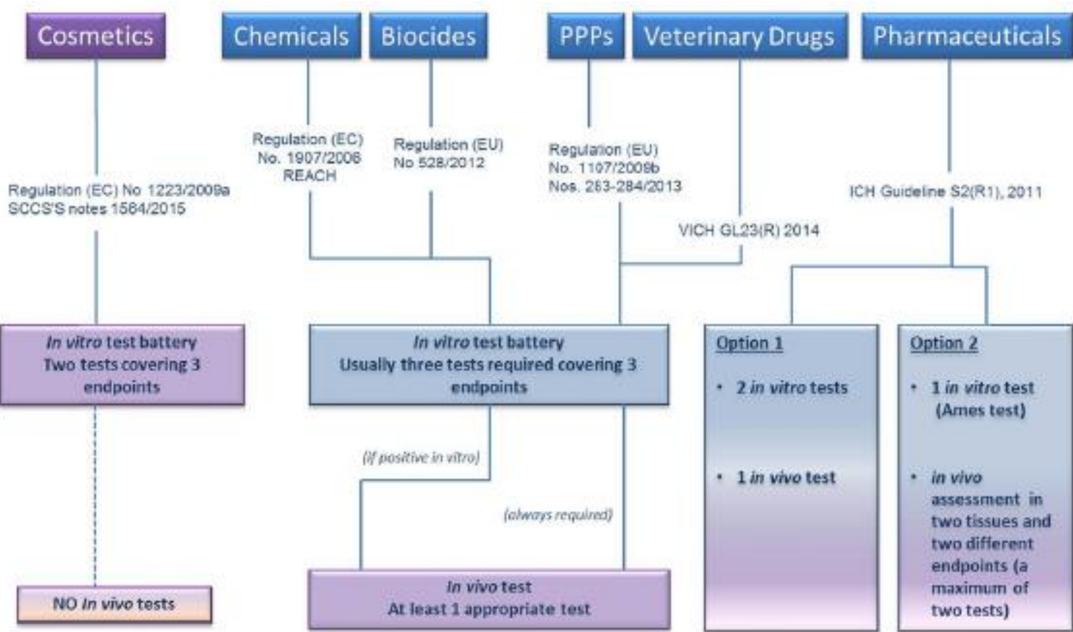


Fig. 1. Summary scheme of testing requirements and corresponding guidelines for genotoxicity testing across European Union. (retrieved from Corvi et al, 2017)

1.1.1 Genotoxicity testing of plants' constituents

In a systematic review performed back in 2016 (Sponchiado et al., 2016), which looked through articles published between 2000 and 2015 from Medline, Science Direct and Web of Science databases, 28.4 % of the conducted studies out of 458 resulted as positive genotoxicity. Initial search resulted as 2289 record, however only 458 of them were able to pass inclusion criteria researchers have set. It was reported that 24 different toxicity assays were used in that 458 study. The most common three test performed by researchers were

micronuclei test (171), ames test (170) and comet assay (109). Plant genotoxicity will be further discussed under phytochemicals section.

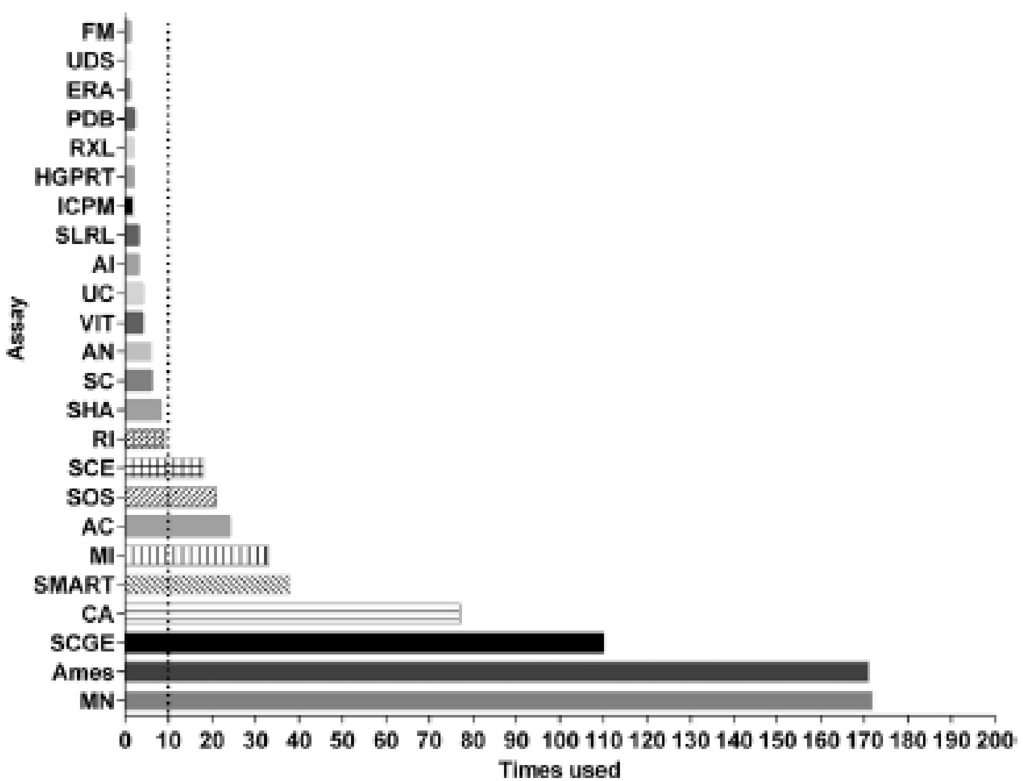


Fig. 2. Graph of most commonly used methods for determining genotoxicity in medicinal plants from a quantitative study. (retrieved from Sponchiado et al, 2016)

1.2. Phytochemicals present in plants and their properties

Phytochemicals can be briefly described the chemicals which are found in plants. There are a lot of different subclasses of phytochemicals but the main classes of phytochemicals are polyphenols, carotenoids, alkaloids, glucosinolates, polyacetylenes, polysaccharides, lectins, terpenes, capsaicinoids, betalains, allium compounds and chlorophyll (Campos-Vega&Oomah 2013). Most of phytochemicals provide plants defense against enemies of plant, mainly against herbivores and insects. There are some extreme examples of this defense, one of them was reported that common yew (*Taxus baccata*) caused the death of 35 cattle out of 43 because the phytochemical named taxine it had inside. The toxicity may not always kill the

enemy but it can cause other problems. It was reported that corn lily (*Veratrum californicum)* consumption of female sheep resulted as cyclopsia in lambs, which is a very rare birth defect Providing defense is not the only benefit of phytochemicals for plants, they also help regulation of pollination, fertilization and growth of the plant. (Molyneux et al., 2007). Phytochemicals were also used by humans to kill their enemies during ancient times. It was found that extracts of a plant known as deadly nightshade (*Atropa belladonna*) was applied on arrows to make them poisonous. There are some non-proved rumors that Roman emperor Augustus was killed by his wife by toxic phytochemicals in this plant (Wink 1998). It is estimated that one fifth of the known plants are used in pharmaceutical industry for treatment of harmful diseases. These phytochemicals are extracted, isolated and purified by several methods. Extraction can be microwave assisted, ultrasonic assisted or solvents can be used. Thin layer chromatography and column chromatography are the most commonly used methods to extract phytochemicals because of their convenience (Altemimi et al., 2017).

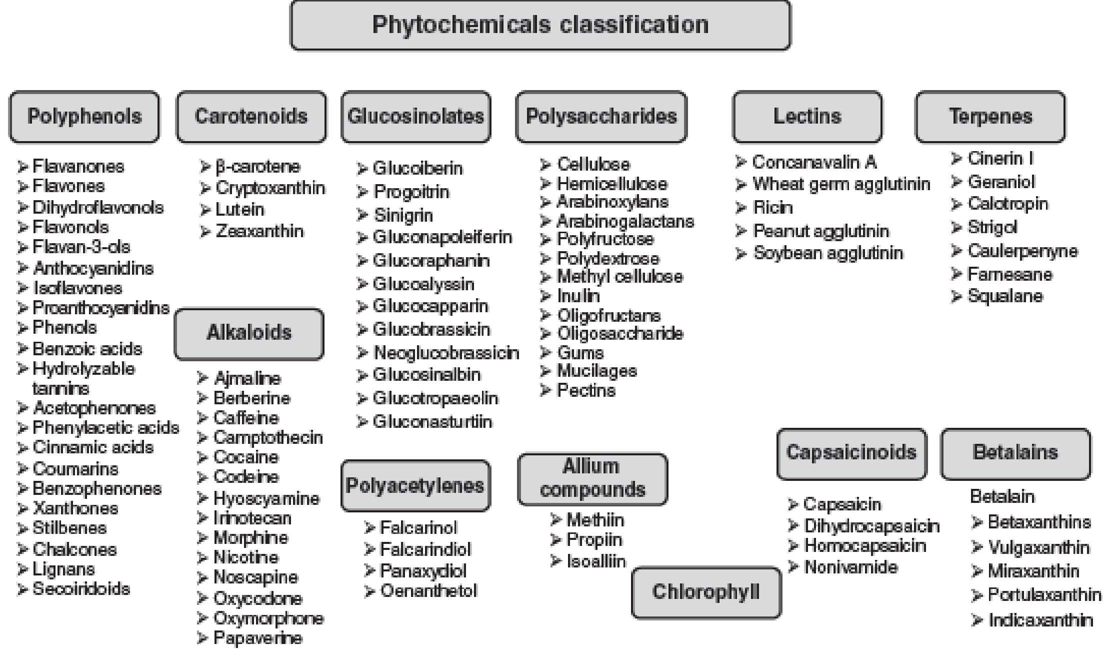


Fig. 3. Classification of phytochemicals. (retrieved from Campos-Vega&Oomah, 2013)

Terpenes. Terpene name derives from turpentine, which is known as balsam produced by several species of *Pinaceae*. Terpenes are classified according to their isoprenoid units in their structure. Largest groups in this classification are: monoterpenes (2), sesquirterpenes (3), diterpenes (4), sesterterpenes (5), triterpenes (6) and tetraterpenes (8). The numbers between

parentheses showing the number of isoprenoid units. (Campos-Vega&Oomah 2013). Monoterpenes are divided into three subgroups as acyclic, bicyclic and monocyclic. Monoterpene containing essential oils shows antibacterial activity and they are used in cosmetics (Perveen 2018). Monoterpenes also act as anti-tumor agents. It was reported that geraniol, a monoterpene alcohol causes 30% mortality in human colon cancer cell line. Geraniol shows effect on human epithelial colorectal adenocarcinoma by causing cell cycle arrest with IC_{50} value of 250 µM. Monoterpene carvone is very effective against CEM (human acute lymphoblastic cell leukemia cell line) K-562 (human chronic myelogenous leukemia cell line), MCF -7 (breast carcinoma cell line) and P-815 (mouse lymphoblast like mastocytoma cell line) cell lines with IC_{50} values of 0.11 µM, 0.17 µM, 0.63 µM and 0.16 µM respectively. Another monoterpene thymoquinone is effective against A431 (HT-29 (colon carcinoma cell line) and KB-V1 (cervix carcinoma cell line) cell lines by inducing cell cycle arrest and apoptosis with IC_{50} values of 10 µM, 46.8 µM and 25.8 µM respectively (Sobral et al., 2014).

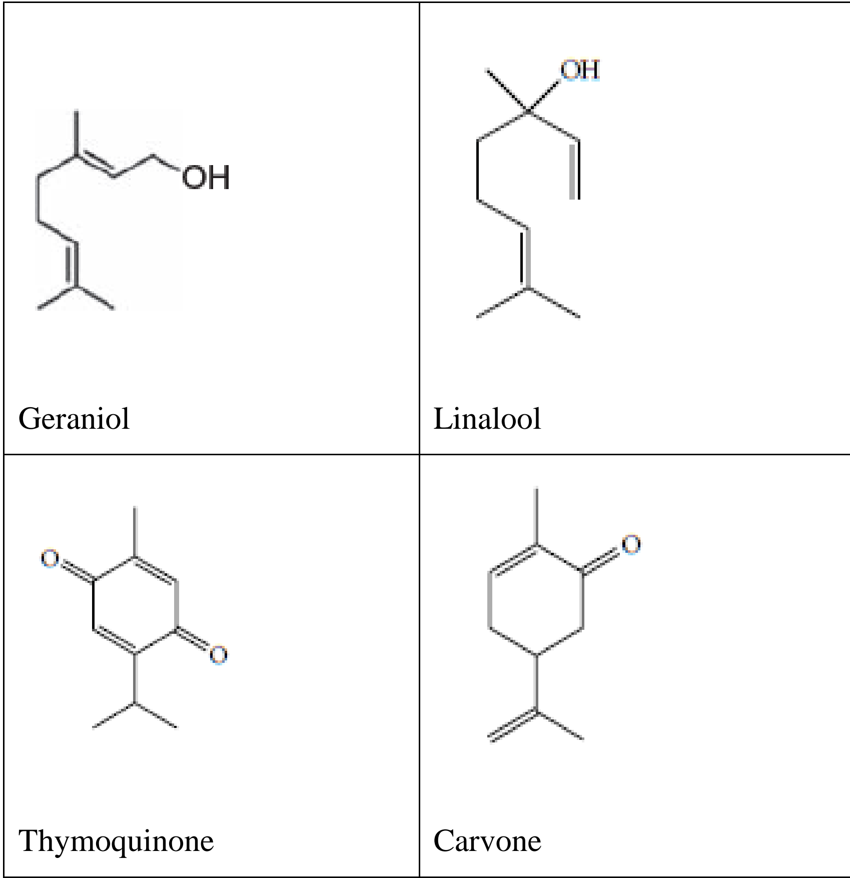


Fig. 4. Chemical structures of some terpenes

Sesquiterpenes are found in bicyclic, cyclic, lactone ring and tricyclic forms. Most of latex produced by plants contains sesquerterpines and this latex can be potential anti-microbial and anti-insecticidal. Two sesquiterpenes isolated from *Onopordum illycrum,* namely onopordopricin and vernomelitensin were reported to be reducing activity of nuclear factor kappa B (NF-κB) with IC_{50} value of 8.6 μM and 3.6 μM (José Serrano Vega et al., 2018). Diterpenes like elegolone, forskolin, granayotoxin and marrubenol present cardiovascular activity (Perveen 2018) Triterpenes pardinol B, pardinol E and pardinol F, and pardinol H which are produced by *Tricholoma pardinum* showed cytotoxic effect against several human cancer cell lines (HL-60, SMMC -7721, A-549, MCF-7 and SW450) with IC_{50} value of fewer than 20 μM (Zhang et al., 2018).

Polyphenols are phytochemicals which are abundant in human diets. Polyphenols are classified according to number of aromatic rings and groups which bind to these rings. Mainly there are two groups of polyphenols: Flavonoids and non-flavonoids. Flavonoids are classified under six main classes: anthocyanins, chalcones, flavanones, flavones, flavonols and isoflavones. Onions, kales, berries and capers contain high amount of flavonols. Main source of flavanones are citric fruits. Flavones are abundant in parsley, thyme and oregano. Anthocyanidins (which is the form of anthocyanins present in foods) are found in red vegetables mostly: red onion, radish, red cherries and plums to name a few. Non-flavonoids are classified according to their carbon atom number. Main classes of non-flavonoids are: Acetophenones, benzophenones, chalcones, coumarins, hydroxycinnamic acids, lignans, secoiridoids, simple phenols and stilbenes (Andrés-Lacueva et al., 2010). Flavonoids are inhibitors to aldose reductase, cyclo-oxygenase (COX), lipoxygenase and xantine oxidase (XO) enzymes. Cyclo-oxygenase enzyme has two isoforms, COX-1 and COX-2. COX-2 enzyme is responsible for inflammation and pain and *in silico* studies between flavonoids and COX-2 shows some flavonoids have anti-inflammatory property by virtue of a specific bond that might result as inhibition of COX-2. Flavonoids are involved in mechanisms related to nuclear factor kappa B (NF-κB) inhibition. *In vitro* studies reported that flavonoids may inhibit low density lipoprotein (LDL) oxidation. Flavonoid macluraxanthone inhibited the activity of acetylcholinesterase (AChE) and butyrylcholinasterase (BChE) with IC_{50} values of 8.47 μM and 29.8 μM (Pance et al., 2016). Brussachalcone A and B inhibit SARS-CoV $3CL^{pro}$ (3-chymotripsin-like protease) with IC_{50} values of 88.1 μM and 57.8 μM, SARS-CoV

PL^{pro} (papain like protease) with IC_{50} values of 9.2 µM and 11.6 µM and MERS-CoV $3CL^{pro}$ protease with IC_{50} values of 36.2 µM and 27.9 µM *in vitro* (Park et al., 2017).

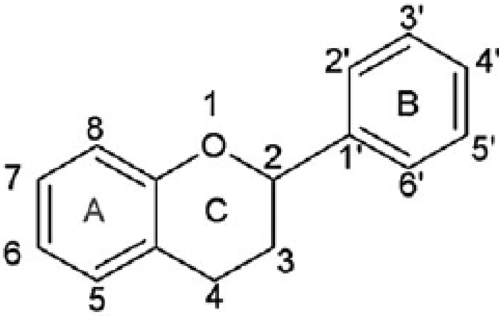


Fig. 5. Basic chemical skeleton of flavonoids

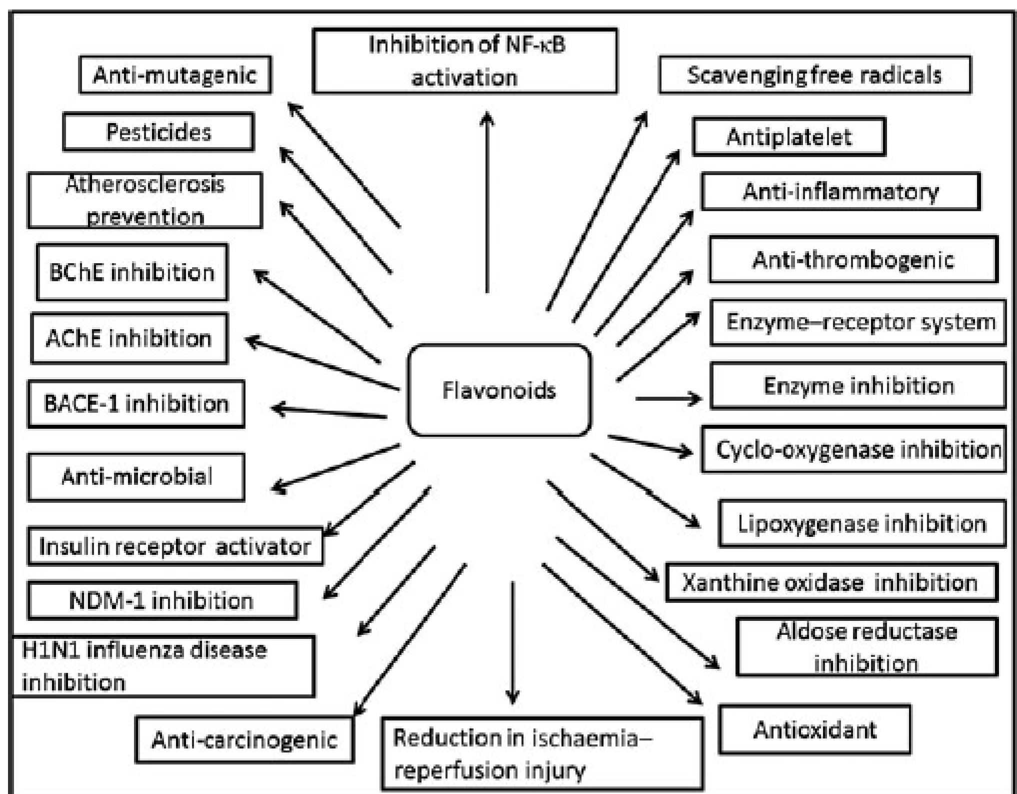


Fig. 6. Summary of flavonoid bioactivity (retrieved from Pance et al, 2016)

Carotenoids are pigments which are fat soluble. There are two main groups of carotenoids: carotenes and xantophylls, which are differentiated by presence or absence of oxygen in their structure. Carotenoids are known by their ability to absorb light, especially blue light. Light absorbing property of carotenoids helps plant to perform photosynthesis and improve vision in human. Four carotenoids (α- carotene, β- carotene, β-cryptoxanthin and γ-carotene) are known to act as vitamin A and important in human diets. Most famous one among them is β-carotene and is widely present in carrots (Campos Vega&Oomah 2013) Carotenoids are well known as their anti-oxidant property and they belong to group of lipophilic antioxidants. Studies have shown that carotenoids are very effective scavengers of peroxyl radicals and singlet oxygen ($^1O_2$) Carotenoids use either chemical or physical quenching to deactivate singlet oxygen Provitamin A and non-provitamin A were found to inhibit transformed fibroblast growth. β-carotene and lycopene were discovered to block cell growth on several human cancer cell lines. Some studies have shown a relation between increased β- carotene uptake and reduced risk of coronary heart disease (Diplock et al., 1998).

Fig. 7. Chemical structure of β-carotene

Glucosinolates are phytochemicals which arise from glucose and amino acid. They are abundant in plants belonging to *Brassicaceae* family. Glucosinolates are classified into three main groups according to their amino acid precursor: aliphatic glucosinates, aromatic glucosinates and indole glucosinates. Glucosinolates hold an important role in biotransformation of xenobiotics. They induce both phase I and phase II reactions, provide protection against oxidative damage caused and responsible for detoxification of carcinogens (Ishida et al., 2014). Isothiocyanates, one of the degradation products of glucosinolates have several properties. They affect carcinogenesis by inhibiting angiogenesis and metastasis. Isothiocyanates indirectly enhance glutathione levels and by this, they increase antioxidant capacity of cell indirectly. They take part in mitochondrial release in cytochrome c and this helps the regulation of apoptosis. Indirect targeting of cyclins by isothiocyanates allows them

to regulate cell cycle. Isothiocyanates also take part in NF-κB regulation (Traka& Mithen 2009)

Fig. 8. Chemical structure of glucosinolate

Lectins are defined as carbohydrates that bind to proteins, with the exception of enzymes and antibodies. The name of lectin derives for Latin verb *legere*, meaning ‘to select’ The first sort of lectin definition dates back into late 19th century. Lectins were also called phytohemaglutinins because back in 1898, similar abilities of lectins compared to agglutinins found in animal serum were discovered; they both cause cell clumping. When their specific agglutination activity towards carbohydrates was described, they were separated from agglutinins and hemagglutinins. Plant lectins are classified in to seven groups according to their structure: type 2 ribosomal inactivating proteins (type 2 RIP), monocote mannose binding lectins (MMBL), legume lectins, jacalin related lectins, *Cucurbitaceae* phloem lectins, chitin binding lectins composed of hevein domains and amaranthins. There are several lectin types that are not suitable to classified in to any of the seven families because the lack of structural resemble and sequence information to trace back the origin of them. Lectins have variety of roles in plants. Legume lectins are used as storage by plant however they act as defense mechanism once plant come across an enemy or eaten up by a plant consuming organism. Chitin binding lectins provide defense against insects and bacteria. Type 2 RIP lectins provide defense against herbivores and seed consuming rodents because of toxicity of ricin and abrin, in addition to their antiviral activity *in vitro* against some plant viruses. They can also act as storage proteins, like legume lectins. Mannose binding act as defense against insects and also can be used as storage proteins. Jacalin-like lectins are defensive against seed consuming predators They can also act like storage proteins, like the previously mentioned lectins. Amarathins acts against seed predators. *Cucurbitaceae* phloem lectins are found in

phloems as their name suggests and this type of lectins blocks infection of phloem by interacting with PP1 (an abundant phloem protein) (van Damme et al., 1998).

Alkaloids are groups of chemicals which consist too many chemicals and therefore, there are several classifications of alkaloids. When alkaloids are classified according just purely by their chemical structure, they can be classified as: acridones, acromatics, bisindoles, carbolines, ephedras*, ergots, imidazoles, indoles, indolizines, manzamines, oxindoles, phenylethylamides*, phenlylisoquinolines, piperidines*, purines*, pyridines, pyrolidines, pyrrolizidines, pyroloindones, quinolines, quinozolines, quinozolines, sesquerterpines*, simple tetrahydroisoquinolines, steroids, tropanes and terpenoids. However some of these classes are not entirely alkaloids. From biosynthetic point of view, alkaloids are classified under three categories: True alkaloids, proto alkaloids and pseudo alkaloids. The unmarked groups above shows true alkaloids and the groups marked by an asterisk denote groups which includes pseudoalkaloids (Aniszewski 2015) Some alkaloids have therapeutic effect on several neurological disorders like Alzheimer's disease, Huntington disease, Parkinson's disease and epilepsy. Berberine, salsoline and galantamine are involved in Alzheimer's disease by inhibiting monoamine oxidase and acetylcholinesterase (AChE). Morphine binds to μ-opioid receptor in central nervous system and this results as increased level of γ-aminobutyric acid (GABA) Phenserine and isorhynchophylline reduces alpha synuclein (α-Syn), a protein that is highly expressed when a person is suffering from Parkinson's disease. Piperine plays role in neurotransmitter regulation and elevates serotonin and GABA levels. Arecoline prohibits demyelination of white matter and shows therapeutic efficacy in schizophrenia by targeting oligodendrocytes directly. Caffeine is well known with its ability to act as antagonist to adenosine and this property of caffeine resulted as decreased neurotoxicity in trial models of Alzheimer's disease (Hussain et al., 2018).

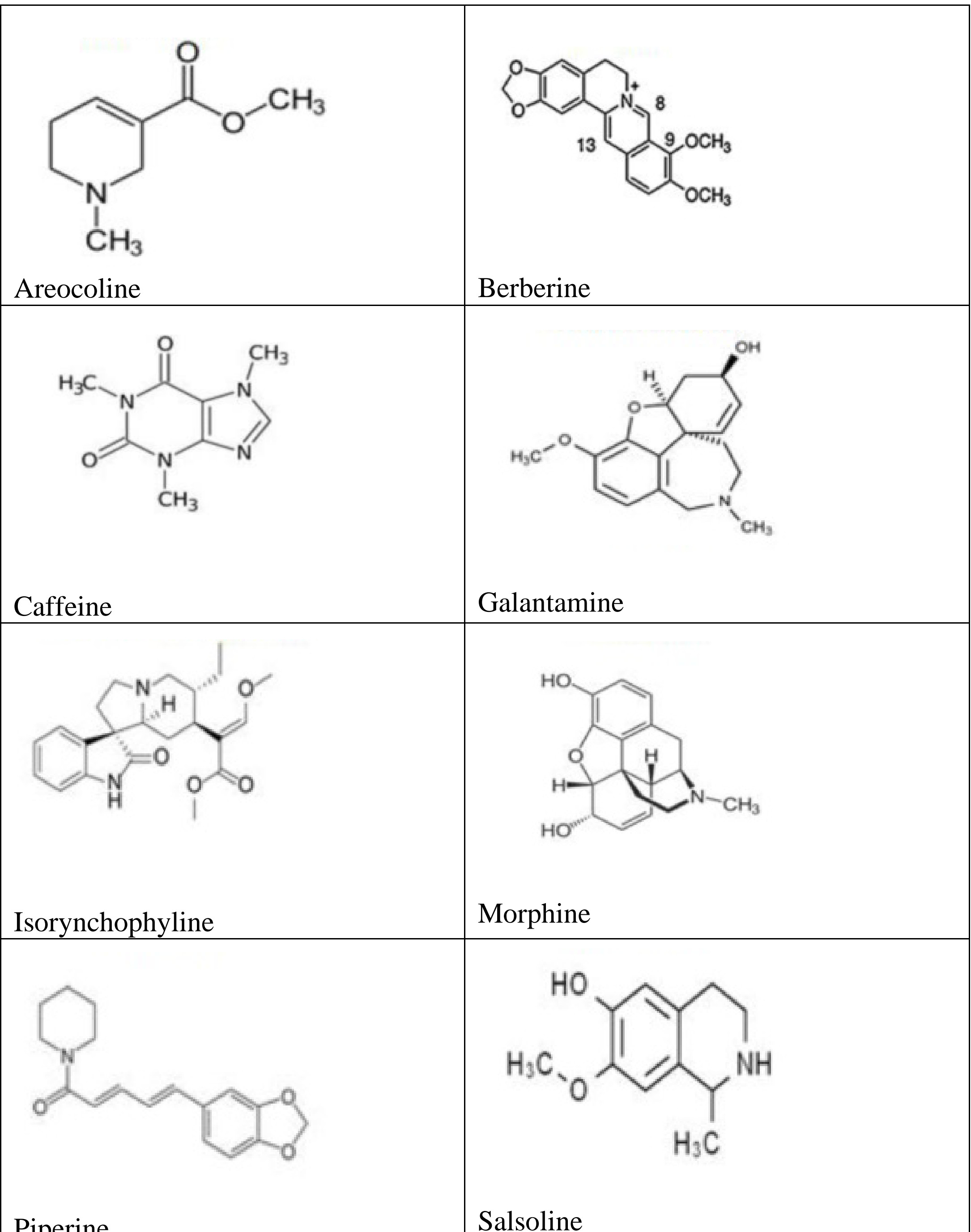


Fig. 9. Chemical structures of some alkaloids

Polyacetylenes are chemicals which have two and more conjugated C≡C bond. Polyacetylenes occur in many different structures, showing that they may have several precursors. More than 2000 of polyacetylenes are known and majority of them are synthesized in higher plants. However, the comparison made among these structures shows most of them are derived from unsaturated fatty acids. They are found in several commonly used plants in human diet: Carrot, tomato, lettuce, parsley, aubergine, artichoke to name a few. Plants belonging to *Asteraceae* family are well-known producers of polyacetylenes, with more than 50% of known polyacetylenes are synthesized by these plants. Polyacetylenes are not desired in human diets because of being hazardous. Some groups of polyacetylenes are known to be neurotoxic in high concentrations. Cicutoxin and oenanthotoxin, two polyacetlyenes found in hemlocks, are known to act on central nervous system. These polyacetylenes cause spams and respiratory paralysis and eventually death of organism. A lot of people and livestock died because of this plant and polyacetylenes inside, the most famous one being Greek philosopher Socrates. In addition to this, polyacetylenes are potential skin sensitizers, causing skin irritations, allergic reactions and even dermatitis. Later on it was found out a polyacetylene named falcanirol is causing this problem however the same chemical shown being acting as antifungal by inhibiting spore germination. Falcanirol is also considered to be used in treatment of neurological disorder virtue of having ability to promote neuritogenesis of paraneurons. Falcanirol and falcanirol type polyacetylenes may also act serotonin receptors and this might indicate that they may have a role in serotonin related pharmacological effects. Falconirol and related polyacetylenes were found out the reason behind the anti-inflammatory property of purple carrots. Despite the mechanism is not known yet, falconirol and related type polyacetylenes are shown potential anticarcinogenic and exhibit cytotoxic effect by suppressing tumor cell proliferation. (Christensen 2010)

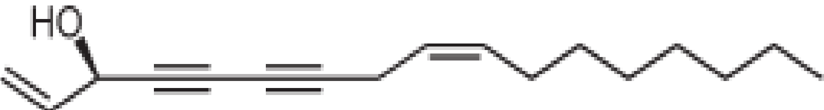


Fig. 10. Chemical structure of falconirol

Allium compounds are the compounds found in plants of *Allium* genus, as their name suggests. The most known species of this genus is garlic (*Allium sativum* L.) and the word

allium means garlic in Latin. The benefits of allium compounds were known since ancient times. Ancient civilizations were using garlic for a variety of reasons: Healing wounds, intestinal disorders, respiratory infections, skin diseases, bacterial infections and even treatment of epidemical diseases of past like cholera, thymus and tuberculosis. Two main classes of compounds inside *Allium* plants were found to be decreasing the risk of prostate and gastric cancers, by epidemiological studies. These classes are apolar sulphur compounds and polar saponins. Sulphur compounds are the main reason behind pungent smell of *Allium* plants and these compounds rapidly react and forms other compounds when the plant is crushed or cooked. Allicin is the first kind of these sulphur compounds discovered. It is the major biologically active compound of fresh garlic. Allicin shows antimicrobial activity by inhibiting pathogenic protozoa, gram positive and gram-negative bacteria, Antimicrobial activity of allicin is thought to be due to inhibition of sulphur dependent enzymes. This compound is also known to act as bactericidal against some well-known pathogenic bacteria like *Escherichia coli, Helicobacter pylori, Staphylococcus aureus, Vibrio cholerae* and *Salmonella typhimurium.* Allicin exhibits anticarcinogenic activity by several ways: Inhibiting procacinogen activation by interacting with cytochrome p450, changing the carcinogen metabolism, inducing apoptosis and blocking cell proliferation and showing anticlastogenic effect to name a few. Additionally, allicin reacts with sulphydryl groups of tubulin and blocks the polymerization of tubulin. Saponins have anti-inflammatory, antispasmodic and antifungal activity. They also show cytotoxic activity on several cancer cell lines such as adenocarcinoma, leukemia, fibrosarcoma, glioblastoma, melanoma, colorectal carcinoma, reticulum cell carcinoma and cervical carcinoma (Lanzotti et al., 2014).

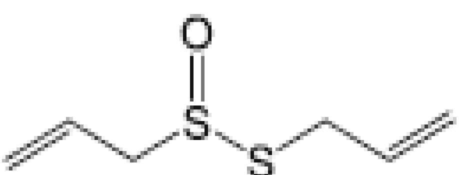


Fig. 11. Chemical composition of allicin

Chlorophyll is a pigment found in chloroplasts of green plants and photosynthetic bacteria. It has an important role for plants because it takes part in photosynthesis. Chlorophyll is the pigment which is most commonly found at plants, being present five times more than

carotenoids. Chlorophyll absorbs blue and red light well but absorb green light poorly. That is the reason why chlorophyll gives green color. Chlorophyll shows very similar structure to heme group of hemoglobin. There are many groups of chlorophylls present and chlorophyll a is the most abundant one in plants (Stefanos &Addison 2014) Dietary chlorophyll derivatives consist two groups: Lipophilic derivatives and water soluble derivatives. Some of lipophilic derivatives are chlorophyll a and b, metal-free pheopytins Zn-pheopytins, metal-free pyropheohitins, Zn-pyropheohitins. Some of water soluble derivatives are pheoporbides, chlorophyllides and sodium copper chlorophyll (SCC), which is a commercial derivative. Results from various studies show SCC is effective both directly and indirectly against mutagens like DMBA, aflatoxin B1, chromium VI oxide, benzo[a]pyrene. Metal free derivatives present suppressive effect against *Salmonella typhimurium* and *Escherichia coli*. Studies found a possible link between high chlorophyll containing diets can reduce risk of colon cancer at humans. Studies specified on cell culture based assays of chlorophyll derivatives shown that they modify pathways of xenobiotic detoxification, induce apoptosis, and act as antioxidants in vivo and trap mutagens ex vivo, despite poor bioavailability (Ferruzi & Blacksee 2007)

Fig. 12. Chemical structure of chlorophyll

Betalains are another type of pigments which are produced by 17 families belonging to *Charyophalles* order. They are not only specific to *Charyophalles* however, some higher fungi species are found out to contain betalains. All types of betalains derive from one core molecule, betalamic acid. They are water soluble. Betalains are divided into two main categories: Violet betacyanins and yellow betaxanthins. Betacyanins are produce by

interaction of betalamic acid and *cyclo*-DOPA or its glucosyl relatives whereas interaction between betalamic acid and amino acids or its derivatives results as betaxanthins. Betalains are present in variety of foods but the most known betalain containing plant is red beet. (Slimen et al., 2017) They exhibit *in vitro* radical scavenging activity towards radicals such as lipoperoxyl, DPPH and $ABTS^{+}$. Many studies show betalains demonstrate better radical scavenging activity compared to trolox, a vitamin E analog, and ascorbic acid. Betalains inhibits low density lipoprotein (LDL) oxidation and membrane lipid peroxidation. In addition to this, they block DNA damage induced by hydrogen peroxide. It was reported betalains could induce quinone reductase, which is a phase II enzyme, and provide hepaprotective effect. Betalains may have activity towards controlling the problems causing hyperglycemia related cardiac fibrosis. At a diabetic model study done on rats by feeding them by a high-fructose diet, betalains helped restoring of redox, in addition to decreasing levels of collagen cross-links and advanced glycation end products. There are several patented betalain-rich supplements and they are presented as beneficial for several illnesses. One of them which is derived from red beets shows the ability of reducing blood sugar and glucose absorption. The most vital property of betalains is their anticancer activity. Betalains show cytotoxic activity towards human lymphoma, human carcinoma, melanoma, colon adenocarcinoma liver hepatoma cells and breast cancer cell line. Apart from role in human diet, betalains are used in coloring several food supplies such as gelatin deserts, gravy mixes, marshmallow candies, powdered drink mixes and soft drinks (Khan 2016).

O
H
HOOC
N
H
COOH

Fig. 13 Chemical structure of betalamic acid

Capsaicinoids are alkaloids which belong to various species related to *Capsicum* genus. Main compound of capsaicinoids is capsaicin, as the name suggest but there are other capsaicinoids like dihidrocapsaicin, homocapsaicin, homodihidrocapsaicin, nordihydrocapsaicin as well Their chemical structure consist of vanillylamine head and fatty acid tail. Capsaicin is known as the reason of pungent taste behind pepper and around 60% of capsaicinoid content of hot peppers are capsaicin molecule (Antonious 2018) Capsaicin is odorless, hydrophobic and highly volatile. Plants use capsaicin for defensive purposes against herbivores. Capsaicin can be used in human diet because of its anti-obesity effect. Studies shown capsaicin increases lipid oxidation and energy consumption. Topical application of capsaicin resulted as less fat accumulation in adipose tissue in mice (Sharma et al., 2013). It was reported that capsaicinoids causes apoptosis to several human cancer cell lines *in vitro*. Capsaicinoids enhance the effect of cisplatin at human stomach cancer and is considered as a potential anti-cancer substance to cancers such as breast cancer, colon cancer and prostate cancer. When capsaicinoids are taken in low dose, they increase mucosal blood flow and help gastric mucosa repair. Additionally, capsaicin is used as analgesic in analgesic creams. However, exposure of high dose to capsaicinoids may result as peptic ulcer and contributes to gastrointestinal problems. All of this activity of capsaicin and capsaicinoids led to discovery of TRPV1 (Transient receptor potential vanilloid subfamily member 1) or more commonly known as capsaicin receptor, which are present in various parts in human body (Rollyson et al., 2014).

H_3CO HO N H R

Fig. 14. General chemical structure of capsaicinoids.

Dietary fibers are described as plant polysaccharides which human digestive system cannot digest. They are also known as non-starch polysaccharides and have several groups depending on their characteristics. Total dietary fibers are classified according to their solubility in water. Insoluble dietary fibers are mostly the compounds which forms cell wall of a plant. Cellulose, lignin and cutin are some examples of insoluble dietary fibers. Pectins, beta-glucans,

arabinoxylans, galactomannans are some examples of soluble dietary fibers. Major source of dietary fibers are fruits and vegetables; however, the dietary fiber content may change due to growth and storage of plants. Dietary fibers are associated with prevention of cardiovascular diseases, diabetes, obesity and intestinal health. Additionally, dietary fibers can act as antioxidants (Saura-Calixto et al., 2010).

1.3 Taxonomy and properties of *Geranium macrorrhizum*

Geranium macrorrhizum, also known by names like cranesbill and big root cranesbill is a perennial, semi evergreen plant which belongs to *Geraniaceae* family (which includes around 750 species) and *Geranium* genus. *Geranium* genus is well known member of *Geraniaceae* family with *Pelargonium* genus. Some *Pelargonium* species are known by the name *Geranium* and this leads to confusion between these two genus. The name of the plant derives from the Greek word geranos, which means crane. 423 species are accepted inside *Geranium* genus and divided into 3 subgroups: *Erodiodeca*, *Geranium, Robertium* (Bautista Ávila et al., 2013).

Subgenus	Section	Number of Species
Erodioidea	*Erodioidea*	3
	Aculeolata	1
	Subacaulia	15
	Brasiliensia	3
Geranium	*Geranium*	339
	Dissecta	4
	Tuberosa	19
	Neurophyllodes	6
	Paramensia	2
	Azorelloida	1
	Polyantha	7
Robertium	*Trilopha*	5
	Divaricata	2
	Batrachioidea	4
	Ungiculata	5
	Lucida	1
	Ruberta	4
	Anemonifolia	2

Table 1. Classification of *Geranium* genus. Retrieved from Bautista Ávila et al, (2013)

Geranium macrorrhizum generally grow up in Balkan countries and mostly in mountains and hills (Radulovic et al., 2010). It grows up to 20-30 cm tall and 80-120 cm wide and shows tolerance to drought (Gardenia.net) The name of the plant in different languages spoken in Balkans could be translated as 'health' (Chalchat et al., 2002; Radulovic et al., 2010; Radulovic et al., 2012). In past, people were using the extract of geranium to heal wounds and stop excessive bleeding. Extracts of *G. macrorrhizum* have been used to treat illnesses related to kidneys and gallbladder at both humans and animals. It was reported these extracts were used to treat liver diseases (Radulovic et al., 2012). The root and leaf extracts derived from *G. macrorrhizum* have radical scavenging and antioxidative abilities. In addition to the abilities listed above, these extracts were also found to have cardiotonic and sedative properties (Miliauskas et al., 2004).

G. macrorrhizum does not have only these abilities, it has other benefits which have an impact to human life indirectly. It was reported that *G. macrorrhizum* extracts are shown to be

effective on several pests like ticks, moths, flies in their different stages of development, acting as pesticide (Tabanca et al., 2013). *G. macrorrhizum* leaves and roots water extracts (100% concentration) showed 83% and 74% efficacy against *M.persicae* and ethanol extracts (1%) of leaves and water extracts showed 97.89% and 95.25% efficacy (Velcheva et al., 2001).

G. macrorrhizum contains several different compounds in its composition and there are several reports about composition of *G. macrorrhizum* essential oil. Most of the reports shows germacrone, a sesquerpetine, is the most abundant compound inside the essential oil (Chalchat et al., 2002; Sharopov et al., 2017). In a study conducted at 2010, compounds of aerial parts oil (a yellowish semi-solid oil) and rhizome oil (a green-yellow oil) of *G. macrorrhizum* were investigated. Terpenoids were found to be most abundant phytochemical found in both parts, with 88.2% and 90.7% respecitvely.78.3% of 88.2% in rhizome oil and 85.7% of 90.7% of aerial parts oil terpenoids were sesquiternepoids. Germacrone was found to be abundant in *Geranium macrorrhizum* aerial parts oil, whereas δ -guaiene was found out to be major compound in rhizome oil. The same study compared the minimal bactericidal concentrations of essential oil parts of *G. macrorrhizum*. for selected bacterial (*Bacillus subtilis*, *Clostridium sporogenes*, *Escherichia coli*, *Klebsiella pneumoniae* and *Staphylococcus aureus*) and fungal (*Aspergillus fumigatus*, *Aspergillus restrictus*, *Candida albicans* and *Penicillium chrysogenum*) strains. Aerial parts oil had less minimum bacterial concentration required compared to rhizome oil for bacterial strains except *K. Pneumoniae*, *B. subtilis*. Rhizome oil required less minimum bactericidal concentration for fungal strains *A. fumigatus* and *P. Chrysogenum* (Radulovic et al., 2010). At a further study, two compounds derived from epoxidation of germacrone (1,10 Epoxygermacrone and 4,5 Epoxygermacrone) were found out to be much more efficient against *B. subtilis* compared to germacrone but due to their very low presence in essential oil, their effect is not significant (Radulovic et al., 2014). Gallic acid, ellagic acid, 4-galloyl quinic acid and quercetin with its three glycosides (quercetin-3-β-glucopyranoside, quercetin-3-β-galactopyranoside and quercetin- 4'- β -glucopyranoside) were the major compounds extracted from *Geranium macrorrhizum* extracts in various fractions (Miliauskas et al., 2004). As their antioxidant properties of these compounds, suggested that *G. macrorrhizum* may be used in food preserving (Radulovic et al., 2012). It is important to note that the wild type geranium and ornamental geranium may have different essential oil composition. A study done on ornamental VB and wild type GH populations of *G. macrorrhizum* resulted as different essential oil composition from the

information mentioned above. In VB population linalool (26.45 %) was the major component in essential oil and β-Elemenone (30.53%) was the major component in GH population (Navarro-Rocha et al., 2018). The composition of essential oil from plants may vary because of their different habitats and different genetics across the species, as it was the case with the example mentioned above.

Fig. 15: *Geranium macrorrhizum.* The plant blooms magenta/purple flowers around late spring to early summer (retrieved from www.gardenia.net/plant/geranium-macrorrhizum-czakor-cranesbill)

Germacrone is a type of sesquiterpene found in high amounts not only *G. macrorrhizum* but some other plants, like *Curcuma zeodoria*. Chemical formula of germacrone is $C_{15}H_{22}O$. Germacrone shows anti-inflammatory, antitumor, antifungal and hepaprotector activities by the result of several studies conducted on this chemical (Wu et al., 2017). It was reported that germacrone can block replication of some types of influenza virus *in vitro* by reducing RNA transcription and protein expression. *In vivo* studies conducted on mice showed that germacrone treatment of mice resulted as lower mortality rate compared to untreated mice. Tissues taken from lung of mice shows reduced amount of virus titers when germacrone dose

is increased. Therefore, germacrone is believed to be a potential agent that can be used in influenza treatment at future (Liao et al., 2013). Germacrone can be a potential substance in glioma treatment. In a study conducted at 2014, researchers found out germacrone blocks proliferation of glioma cells by causing apoptosis and taking part in regulation of the proteins related in cell cycle arrest (Liu et al., 2014). In addition of these properties, germacrone acts as acaricidal to *Tetranychaus urticae*, insecticidal to *Culex quinquefasciatus*, ixodicidal to *Hyalomma lusitanicum* and antifeedant to *Spodoptera littoralis, Myzus persicae* and *Rhopalosiphum padi* (Galisteo Pretel et al., 2019).

Fig. 16. Chemical composition of germacrone

Quercetin is a flavonol which belongs to flavonoids and found in plants and vegetables. Chemical formula of quercetin is $C_{15}H_{10}O_7$. It is commonly found as quercetin glucuronide, quercetin glycoside, quercetin sulfate and methylated quercetins forms. It has very poor solubility in water and also poor bioavailability. Poor bioavailability may be result of low absorption or rapid elimination of quercetin in gastrointestinal tract. Quercetin shows immunosuppressive effect on mouse dendritic cell function and stabilizes mast cells It has anti-inflammatory effect on pulmonary epithelial cell line, N9 microglial cells and rat liver epithelial cell *in vitro. In vivo* studies have shown quercetin decreased the clinical signs of arthritis in chronic rat adjuvant induced arthritis and accelerated recovery of motor function of mice which suffered from acute traumal spinal cord injury. Quercetin increased cytokine secretion and exhibited protection against irradiation included inflammation at a study conducted on mice (Li et al., 2016). In a study dated back to 2012 (Nguyen et al., 2012), quercetin inhibits SARS-CoV $3CL^{pro}$ (3-chymotripsin-like protease) with 82% rate and IC_{50} value of 73 µM *in vitro*. In another study done in 2017 (Park et al., 2017), quercetin inhibit SARS-CoV $3CL^{pro}$ protease with IC_{50} value of 52.7 µM, SARS-CoV PL^{pro} (papain like

protease) with IC_{50} value of 8.6 μM and MERS-CoV $3CL^{pro}$ protease with IC_{50} value of 34.8 μM *in vitro.*

HO OH OH OH OH O O

Fig. 17 Chemical composition of quercetin

Two previous studies have analyzed biological activity of butanol-ethanol and water extracts of *G. macrorrhizum.* At a study done in 2004 (Miliauskas et al., 2004), radical scavenging activity of seven compounds inside *G. macrorrhizum* leaves extract was observed. Quercetin derivatives quercetin-3-β-glucopyranoside and quercetin-3-β-galactopyranoside showed higher radical scavenging value compared to other compounds tested. Quercetin showed third best radical sacvenging ability followed by ellagic acid, gallic acid, quercetin- 4'- β - glucopyranoside and 4-Galloyl quinic acid. Only quercetin- 4'- β -glucopyranoside and 4-Galloyl quinic acid showed lower radical scavenging ability than reference antioxidant,which was rosmarinic acid. Water-butanol (WB) fraction have showed best H_2O_2 scavenging ability followed by ethanol-water (EW) fraction. Ethanol-butanol (EB) have showed better superoxide scavenging ability.

At a study focused on singlet oxygen scavenging, cytotoxic and genotoxic properties of *G. macrorrhizum*, it was reported (Venstukonis et al., 2010) that quercetin may be responsible for protective effects of *Geranium macrorrhizum* against $^1O_2$, rather than gallic acid. According to that study quercetin showed an IC_{50} value of 170 μM whereas gallic acid had an IC_{50} value higher than 170 μM. Genotoxicity of *G. macrorrhizum* extracts was identified by chromosomal aberrations and sister chromatid exchanges. Ethanol-butanol (EB) and water-water (WW) fraction showed very similar sister chromatid exchange in a dose dependant manner. Chromose aberrations however,yielded different result. EB fraction show more number of aberrations compared to WW fraction and there were not any chromosome breaks

or chromosome exchanges detected at WW fraction. 150 µg/ml concentration gave highest number of aberration in EB fraction,whereas 200 µg/ml concentration gave highest number of aberration in WW fraction.

2. Materials and methods

Cytokinesis block micronucleus assay (CBMN assay) and Comet assay (also known as SCGE) methods were used to perform this research. Three donors were used during these experiments. Donor 1 is male,22 years old and smokes. Donor 2 is male,24 years old and does not smoke. Donor 3 is female,26 years old and does not smoke. The *G. macrorrhizum* extracts used in this experiment were provided from Kaunas University of Technology, Faculty of Chemical Technology.

2.1 Cytokinesis block micronucleus (CBMN) assay

CBMN assay is a commonly used method in genotoxicity testing and human biomonitoring. There are papers published for the optimal usage of CBMN, in addition to an OECD guideline. This guideline suggests usage of cytochalasin B 44 hours after phytohaemaggluttin stimulation to achieve maximum sensitivity and efficiency. The most common protocol used to perform CBMN assay is protocol designed by Fenech et al. (2002) for HUMN (Human MicroNucleus) project. The protocol has criteria for different cell categorizations. The criteria for a cell to be considered as binucleated are listed below (Fenech et al., 2003):

- The cell should have two nuclei.
- The membrane of the candidate binucleated cell should be intact and distinct from the other cells.
- The two nuclei shall not overlap each other but they may touch each other if they are easily distinguishable.
- The two nuclei are expected to be around same size, shape and staining intensity
- The two nuclei should have intact membranes.

The criteria for a cell to be considered as binucleated are listed below (Fenech et al., 2003):

- Micronuclei are non-refractile
- Micronuclei are round or oval shaped and

- Micronuclei are not connected to nucleus. They may touch to nucleus but they shall not overlap.
- Micronuclei have same staining intensity as nucleus. However, micronuclei may have more staining intensity rarely.
- Micronuclei are around 1/256 to 1/9 th of the nucleus.

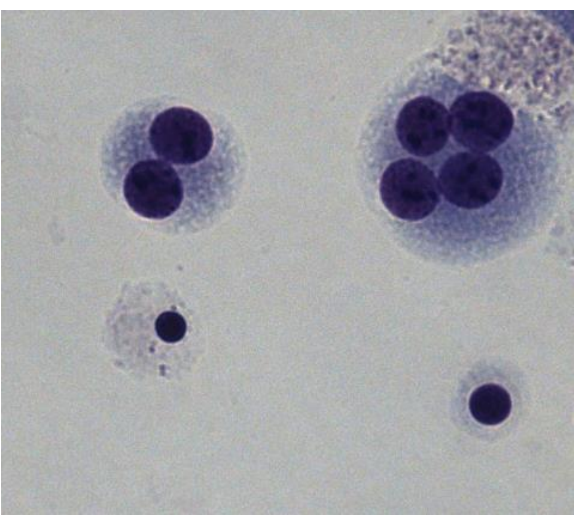
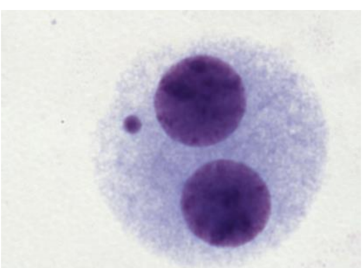


Fig. 18. View of a CBMN sample under microscope. Two mononucleated cells, one binucleated cell and one tetranucleated cell can be seen.



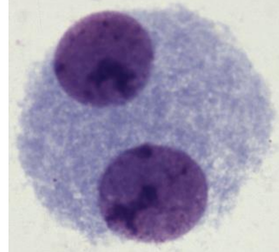


Fig. 19. The binucleated cell with micronuclei on the left and a normal binucleated cell on the right

The list of steps applied during the CBMN procedure:

1. Peripheral blood is taken from the donor just before the experiment begins
2. Lymphocyte cultures are set up by stimulating the cultures with PHA (7.8 μg/ml), RPMI 1640 medium supplemented with 12% heat inactivated newborn calf serum and 40 μg/ml gentamycin. Cells are left in incubator.

3. Cultures are treated with desired test compounds 24 hours after culture initiation. Test solutions are freshly prepared, just before treatment.

Sample/Doses	Concentration
C	-
SC	7µL/ml ethanol
PC	0,02µg/ml Doxorubicin
50	50µg/ml GM extract
100	100µg/ml GM extract
150	150µg/ml GM extract
200	200µg/ml GM extract
250	250µg/ml GM extract

Table 2. Concentration of materials used in samples at CBMN Assay.

4. Cytochalasin B at a concentration of 6 µg/ml is added 44 hours after PHA stimulation. CytB is added to arrest the cells in cytokinesis stage and allow binuclear cell formation.
5. Cells are harvested 28 hours later and centrifuged at 400X for 8 minutes.
6. Cells are treated with a cold (4°C hypotonic KCl solution (0.56%) and centrifuged at 400X for 8 minutes again right after solution treatment.
7. Cells are fixed for 20 minutes with methanol: glacial acetic acid (5:1) mixed with equal amount of 0.9% NaCl.
8. After the first fixation, cells are fixed twice with methanol: glacial acetic acid mixture (5:1) with 20 minutes interval.
9. When slides are dried, they are stained with Giemsa dye (5% in Soerensen's buffer) for 3-7 minutes.
10. Slides are analyzed using Lucia software. Slides are put into the column of the robot. The robot transfers the slide inside the column under microscope via an arm and starts to scan the slide for binucleated cells. Required time for analysis per slide differs but it takes around half an hour per slide to be analyzed by the robot. After the analysis has been completed, the software will show how many binucleated cells inside the sample,

how many of them have a micronuclei or even multiple micronuclei and the captured pictures of the all cells. From than onwards, the experimenter should look up through the captured pictures because the robot may have taken some cells as binucleated but these cells are not satisfying the criteria to be considered as binucleated. For example, the robot can score a tetranucleated cell as binucleated because of the overlapping nuclei. Another example of this misscoring is, the abrupt difference between two nuclei, the cell having very less or none cytoplasm. There may be cells which actually have micronuclei but left out by the robot. The opposite situation can happen, as the robot scores some binucleated cells as micronuclei but that micronuclei turns out to be a piece of debris or a drop of a dye.

2.2 Comet Assay

Comet assay can be briefly described as a single cell electrophoresis assay to evaluate DNA damage. This assay is used in genotoxicity testing, human biomonitoring and cancer risk assessment. There are two types of comet assay: Alkaline comet assay and neutral comet assay. Alkaline comet assay is performed under denaturing condition and used to analyzed single stranded DNA breaks. In contrast to this, neutral comet assay is performed under non-denaturing condition and used to analyzed double stranded DNA breaks. This is proven to be not right and most likely to be caused by faulty comparisons between Comet assay and other methods which measures DNA breaks (Collins et al., 2008). First sort of single cell DNA damage detection was done back in 1978 by Rydberg and Johanson. They stained cells with acridine orange and measured green:red ratio with a photometer. Later at 1984, Östling and Johanson developed the method to allow direct visualization of damage inside individual cells. Their methods consisted suspension of cells in thin agarose on a slide followed by lysis by detergents and salts, electrophoresis under neutral conditions and finally cell staining with a fluorescent dye. However, this method had a problem, lysis conditions were not effective enough to remove all proteins. Singh et al. (1988) adapted the method to allow both double stranded and single stranded DNA breaks (Cotelle&Férard 1999) There are no absolutely defined comet assay protocols but the Singh version is the most commonly used one, as this was the case for this experiment as well. Results of the comet assay can be scored by different types (Collins et al., 2008):

1.A graticule or photomicrograph can be used to determine the length of comet tail however this is a laborious method.

2.Comets can be inspected and classified according to their tail intensity,0 being no tail at all and value between 1 and 4 representing the DNA damage. The score of 100 comets are summed up this value will be the overall score in arbitrary units.

3. Image analysis with an appropriate software downloaded in to a computer and supported by a camera.

4. Automated systems which detects comets by its own, without any human assist.

Performing a viability check to the desired cells before comet assay is an useful procedure and generally applied. Viability check can be done by many ways. One way to check viability is to use trypan blue. This method relies on integrity of the cell membrane. Alive cells have integrated cell membranes, therefore does not allow trypan blue inside and remain white/colorless. In contrast, dead cells do not have the cell membrane integrity as their alive counterparts and trypan blue enters dead cells without any issues, making them look blue. Performing trypan blue method is relatively easy and simple: 10 μl of cell suspension is mixed with 10 μl of 0.4% trypan blue. This mixture is transferred on to slide, covered with a coverslip and analyzed under inverted microscope at 40x magnification. For each dose, at least 100 cells should be analyzed and relative percentage of dead/alive cells is calculated. The viability range of cells is expected to be around 70%-80% of the all cells.

Another method of assessing cell viability is determination by CasyTT analyzer. This analysis is also dependent to cell membrane integrity. Cells are exposed to electric current during this process: alive cells have integrated cell membrane and the membrane shows resistance during the calculations. Dead cells membranes cannot show the resistance to electric current and allows the current pass through easily. 10 μl of CasyTonner is mixed with 10 μl of cell suspension and put into the machine. Only a single dose of experiment can be analyzed each time due to structure of the analyzer.

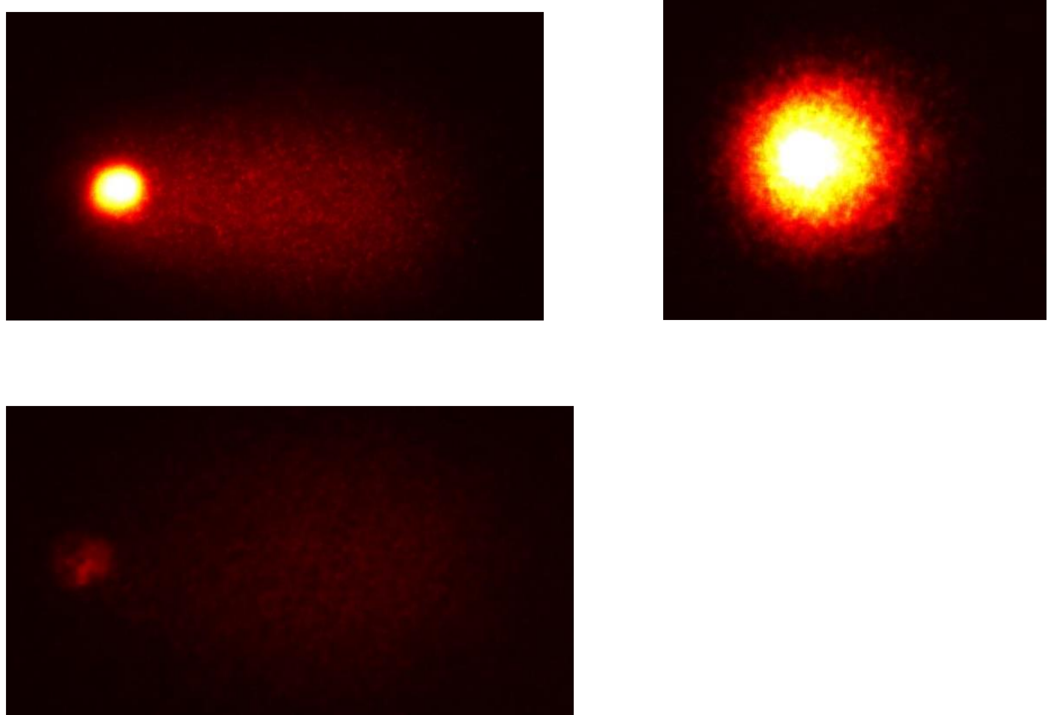


Fig. 20: Cells under comet assay software. Upper left figure shows comet with a long tail, upper right shows comet with a short tail and bottom figure shows the ghosts. The cells which the head is apart from tail are called ghosts.

The list of steps applied during Comet Assay procedure:

1. Peripheral blood is taken from the donor just before the experiment begins.
2. Lymphocytes are isolated by Lymphoprep density centrifugation according to instruction of manufacturer.
    - Lymphoprep is mixed thoroughly and added into tube.
    - Blood is diluted with equal amount of culture medium.
    - Blood is layered on top of the Lymphoprep. While this layering, blood should not be mixed with Lymphoprep.
    - Tube is centrifuged at 800X for 20 minutes at room temperature
    - Upper plasma layer is removed by a Pasteur pipette and discarded.
    - Mononuclear cell layer is removed and retained at plasma: Lymphoprep interface without disturbing erythrocyte/granulocyte pellet.
    - Retained mononuclear cells are washed with medium once.

3. Cells are resuspended in RPMI 1640 medium following isolation. Cells are adjusted about $2X10^5$ lymphocytes/ml and treated with desired test compound for 1 hour at 37°C.
4. Test solutions are freshly prepared, just before treatment. Hydrogen peroxide (20µM concentration) is used as positive control and ethanol (diluted with RPMI as 1:1 ,0.3% v.v final concentration) is used as solvent control. For each sample, test material and controls are tested concurrently.

   (5 µg/µl concentrated extract → 5mg of extract + 1ml solvent

   1 µg/µl concentrated extract → 100 µl from 5µg/ml concentrated extract + 400 µl solvent

Sample/Doses	Concentration
C	-
SC	0.3% v.v
PC	0,15µg/ml (20 µM) H_2O_2
50	50 µg/ml from 1 µg/µl concentrated extract
100	100 µg/ml from 1 µg/µl concentrated extract
150	30 µg/ml from 5 µg/µl concentrated extract
200	40 µg/ml from 5 µg/µl concentrated extract
250	50 µg/ml from 5 µg/µl concentrated extract
300	60 µg/ml from 5 µg/µl concentrated extract

Table 3. Concentration of materials used in samples at Comet Assay .

5. 40µl of freshly prepared cell suspension is mixed with 40µl of 1% low melting point agarose. The obtained mixture is spread onto slides which are precoated with 1%

normal melting point agarose and covered with a coverslip. Two slides are prepared for each experimental point.

6. The agarose is allowed to solidify for 10 minutes at 4°C. Coverslips are removed and another layer of agarose (80µl of 0.5% low melting point agarose this time) is added. Slides are covered with coverslip and agarose is left to solidify for 1 minutes at 4°C again.
7. Slides are immersed in freshly prepared lysis solution (2.5M NaCl, 100 mM Na_2EDTA, 10 Mm Tris, with 1% Triton X-100 and 10% DMSO added just before use, pH 10) after coverslips are taken off and put in a dark place for 1.5 hours at 4°C
8. Slides are removed from lysis solution and placed into electrophoresis tank, which is filled with 540 ml of electrophoresis buffer (1mM Na_2EDTA and 300 mM NaOH, pH 13)
9. Slides stay in electrophoresis tank for 20 minutes without running the electrophoresis to allow DNA unwinding and expression of alkali-labile damage. 20 minutes later, electrophoresis is carried out at 21 V and 300 mA for 30 minutes. This will allow damaged DNA/fragments to move towards the anode.
10. When electrophoresis is finished, the slides are neutralized with 200 ml Tris buffer (0.4 M Tris, pH 7.5) for 30 minutes. Later, slides are stained with 80µl ethidium bromide (20µl/ml) for 5 minutes. Neutralization and staining should be performed under dimmed light in order to prevent additional DNA damage.
11. Slides are analyzed using Lucia Comet Assay software. Slides are placed on to microscope and software is set with appropriate settings. The lamp is opened when slide is going to be analyzed, in order to prevent extra damage can be cause by light. This is the reason why analyzing under minimal light is recommended. When a comet is found, the name of the slide should be registered so that the next comets can be submitted to the created subfolder. Submitting comet is not a complicated process, when a comet is found, double left click to Mouse while being at the center of the comet will register the comet to folder. Right clicking while being at the center of the comet will show information about the comet. The information shown are head DNA percentage, tail DNA percentage and radius. 100 comets can be submitted to subfolder created as the name of slide, after 100 comets, software will prompt a message that slide is finished. Software will produce several graphs regarding the properties of comets registered. The data will be sent to Excel via software and this Excel file will

be used for the preparation of results. The property of comets which should be taken into consideration to determine genotoxic property of the extract is DNA damage.

3.Results and discussion

Research plan was to perform at least two micronuclei assay (two experiment per extract, four in total) and five comet assay analysis, as per discussion with thesis supervisor. One experiment of micronuclei assay and three comet assay experiments were scheduled to be performed until the coronavirus pandemic halted the progress.

The results obtained from the CBMN assay and Comey assay are given below.

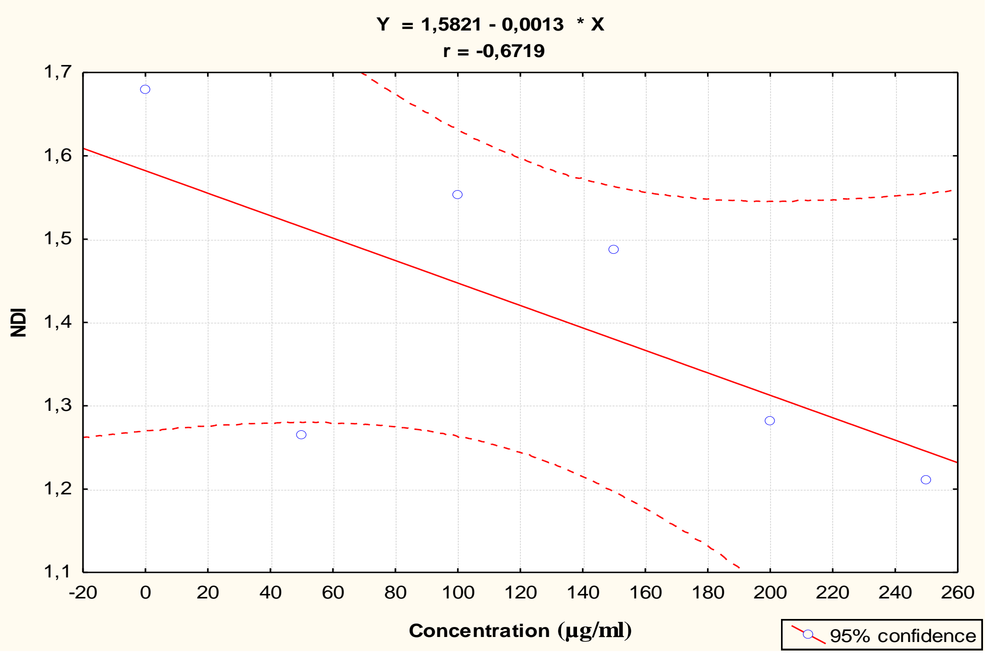


Fig. 21. Nuclear Division Index of human peripheral blood cultures treated with *Geranium macrorrhizum* root extracts (Donor 1)

In this graph, the doses between 100 µg/ml and 250 µg/ml resulted in a decreasing trend,which were expected. However 50 µg/ml dose have NDI only higher than 250 µg/ml, a case which was not expected. Exact NDI values are 0 µg/ml (SC) → 1,628, 50 µg/ml → 1.266, 100 µg/ml → 1.554, 150 µg/ml → 1.448, 200 µg/ml → 1.282, 250 µg/ml → 1.212 This experiment had $p>0.05$

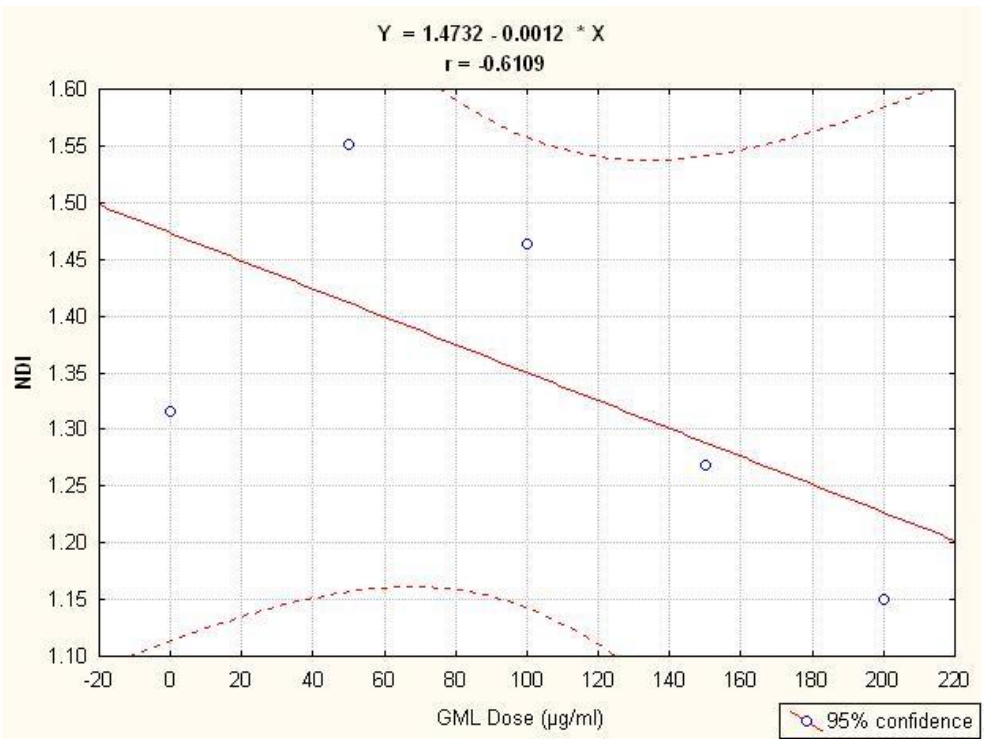


Fig. 22. Nuclear Division Index of human peripheral blood cultures treated with *Geranium macrorrhizum* leaves extracts (Donor 2)

In this graph, increase of dose resulted as decreasing NDI which were expected However, at the highest dose planned in CBMN experiments (250 µg/ml), there were not enough cells so this dose was excluded from the results. Exact NDI values are 0 µg/ml (SC) → 1.316, 50 µg/ml → 1.552, 100 µg/ml → 1.464, 150 µg/ml → 1.268, 200 µg/ml → 1.15 This experiment had $p>0.05$

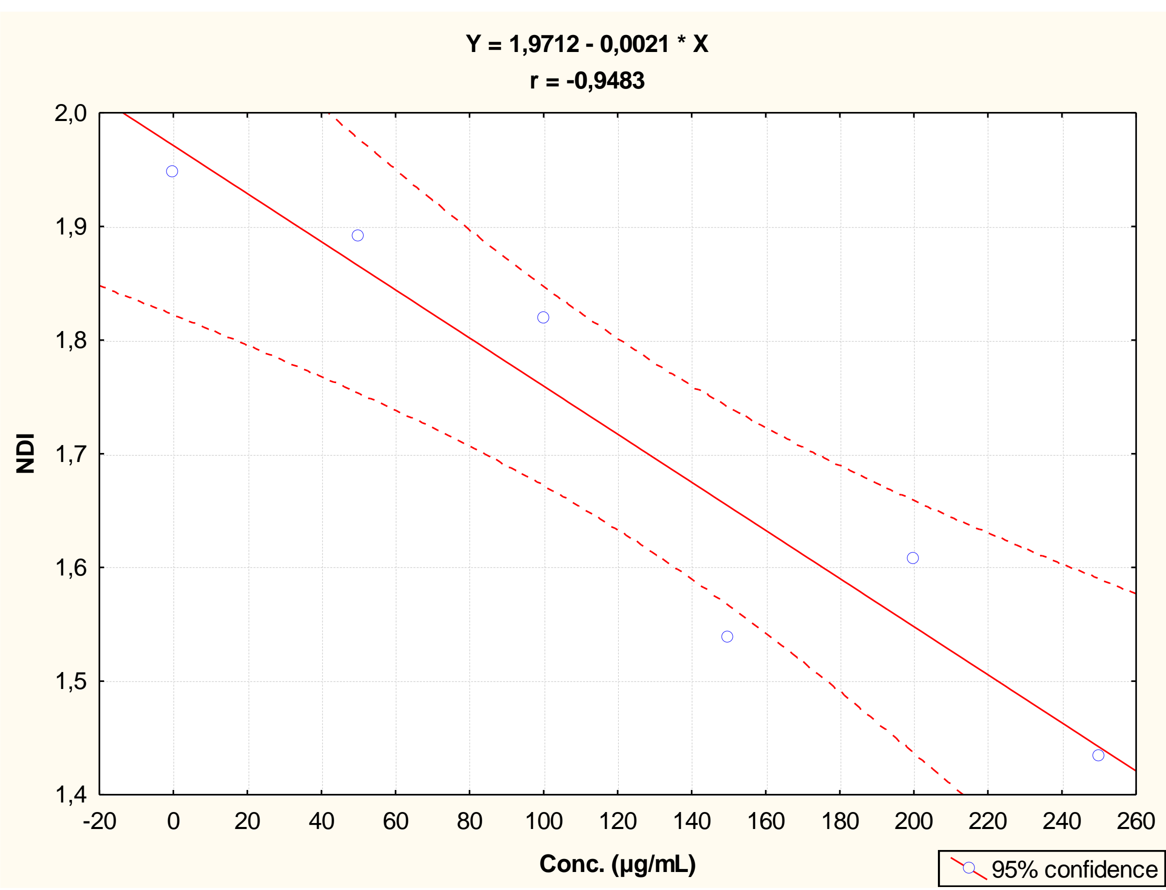


Fig. 23. Nuclear Division Index of human peripheral blood cultures treated with *Geranium macrorrhizum* root extracts (Donor 2)

In this graph, increase of dose resulted as decreasing NDI which were expected with the exception of 200 µg/ml concentrated extract having higher NDI compared to 150 µg/ml concentrated extract. Exact NDI values are 0 µg/ml (SC) → 1.948, 50 µg/ml → 1.892, 100 µg/ml → 1.82, 150 µg/ml → 1.538, 200 µg/ml →1.608, 250 µg/ml → 1,484 This experiment had $p<0.05$

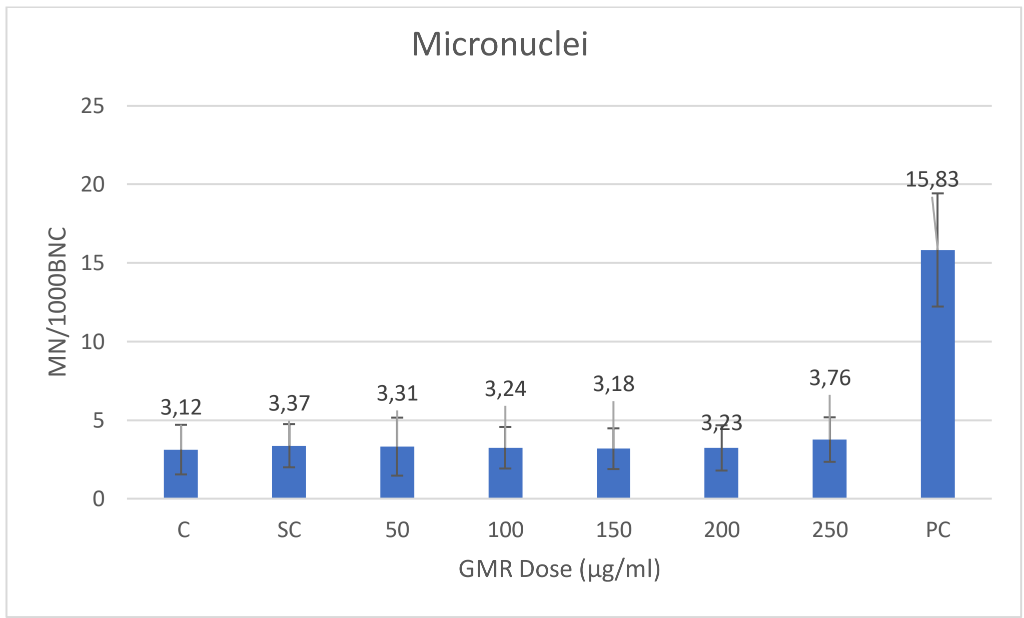


Fig. 24: Rate of micronuclei in human peripheral blood lymphocytes treated with *Geranium macrorrhizum* root extracts (Donor 1)

In this graph, rate of micronuclei stayed almost same level regardless of increased dose so this may show *G. macrorrhizum* root extracts does not effect rate of micronuclei at all. Positive control was expected to have around 5 times more micronuclei ratio compared control sample and this was the case for this experiment.

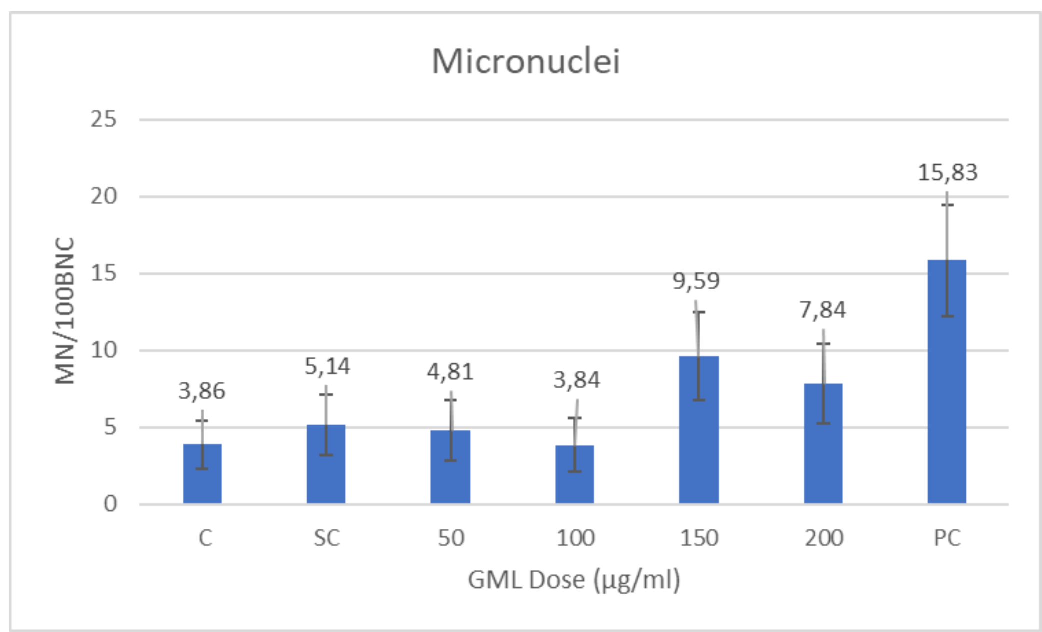


Fig. 25: Rate of micronuclei in human peripheral blood lymphocytes treated with *Geranium macrorrhizum* leaves extracts (Donor 2)

In this graph, there is a nearly 2.5 times increase in micronuclei ratio when dose is increased from 100 µg/ml to 150 µg/ml. 250 µg/ml), there were not enough cells so this dose was excluded from the results. Positive control was expected to have around 5-6 times more micronuclei ratio compared control sample and this was the case for this experiment.

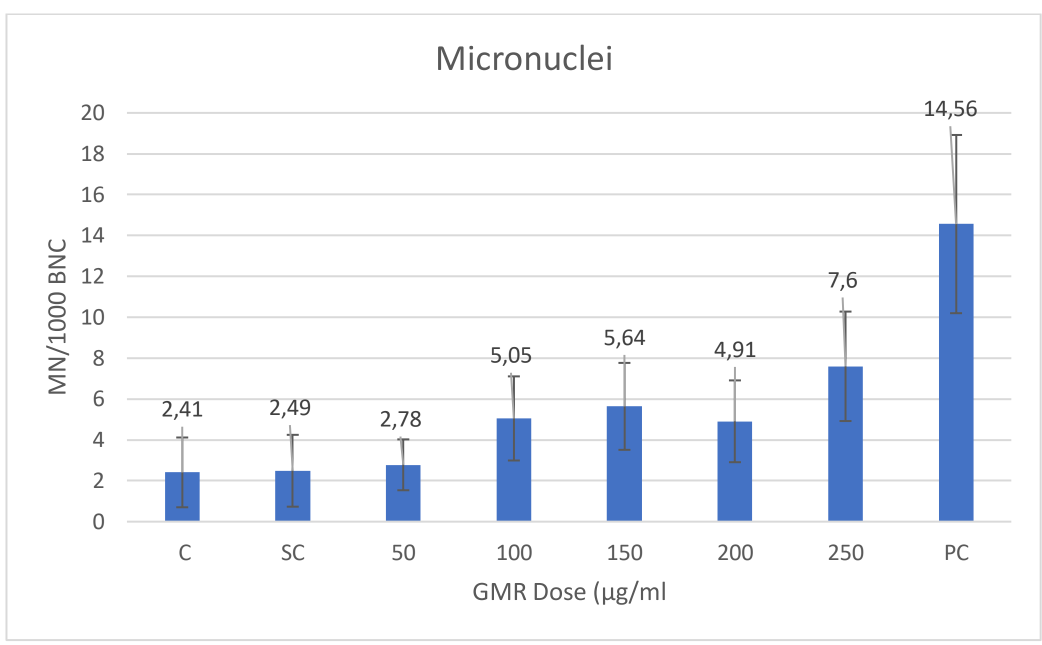


Fig. 26. Rate of micronuclei in human peripheral blood lymphocytes treated with *Geranium macrorrhizum* root extracts (Donor 2)

In this graph, there is a nearly 2. times increase in micronuclei ratio when dose is increased from 50 µg/ml to 100 µg/ml. Positive control was expected to have around 5-6 times more micronuclei ratio compared control sample and this was the case for this experiment.

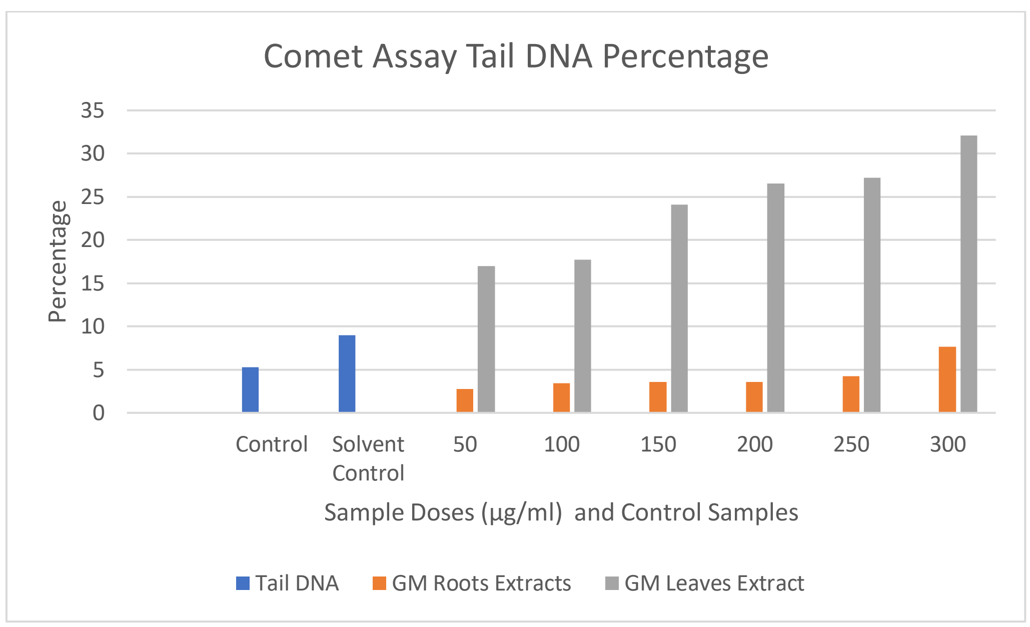


Fig. 27. Comet Tail DNA Percentage in human lymphocytes treated with *Geranium macrorrhizum* extracts (Donor 3)

In this graph, all doses clearly indicates that *Geranium macrorrhizum* leaves extracts caused more damage compared to *Geranium macrorrhizum* root extracts. Roots extracts cause at same levels of damage (except 300 µg/ml), however leaves extracts showed a constant increase in damage caused when the dose is increased.

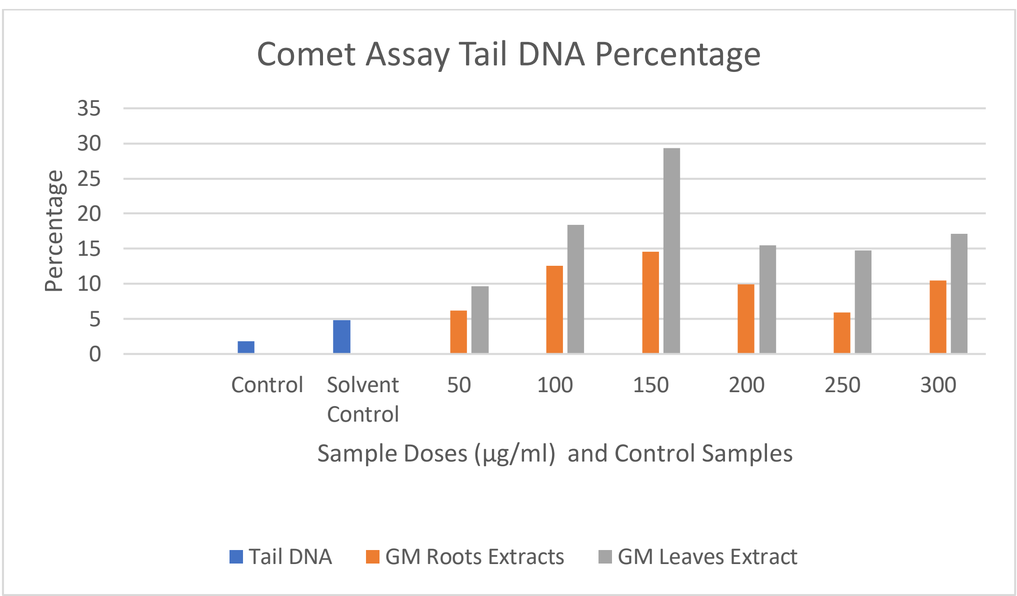


Fig. 28 Comet Tail DNA Percentage in human lymphocytes treated with *Geranium macrorrhizum* extracts (Donor 2)

In this graph, all doses clearly indicates that *Geranium macrorrhizum* leaves extracts caused more damage compared to *Geranium macrorrhizum* root extracts. In both roots extracts and leaves extracts 150 µg/ml dose seems to be most damage causing,followed by 100 µg/ml. Therefore it can be said that the levels of damage caused is not related to dose for this experiment,since the expectation were 300 µg/ml dose to cause most damage and 50 µg/ml to cause least damage.

From the results presented above, it can be clearly seen that samples treated with leaves extract have lower NDI compared to samples treated with root extracts. Lower NDI means cells are divided less when exposed to extract. In addition to this, leave extracts caused more damage to samples compared to root extracts. This can be understood by higher tail DNA amount at the samples treated with leaves extracts. When these two statements are combined,it can be said that leaves extract may shows more genotoxicity compared to root extracts. The highest dose (250 µg/ml) of leaves extract did not yield enough amount of binucleated cells despite the careful observation from both robot and experimenter. The second set comet assay did not provide 100 cells as required. The results from Donor 1 root extacts and Donor 2 leaves extracts had p value higher than 0.05.

The main limitation that halted the progress of the research was coronavirus pandemic. Another limitation was the lack of articles studying on genotoxicity of *Geranium macrorrhizum* root and leaves extracts. Only two articles were found to study on *Geranium macrorrhizum* root and leaves extracts (Miliauskas et al., 2004; Venstukonis et al., 2010). Their genotoxicity testing results were quite similiar to our results - *Geranium macrorrhizum* extracts did not induced chromosomal damage but increased frequency of sister-chromatid exchanges that are related to primary DNA damage, like the Comet assay.

There were studies regarding to other Geranium species and some of them were discussed below. A study conducted on *Geranium wallichianum* raw rhizome extracts have shown that it has antibacterial activity (Ismail et al., 2012).

At a study model investigating the effects of *Geranium schiedeanum* in a thioacetamide induced livery on rats, *Geranium schiedeanum* was found as non toxic as LD_{50} value was greater than 5000 mg/kg. Additionally, *Geranium schiedeanum* supplement decresed alanine aminotransferase (ALT) and aspartate aminotransfase (AST), enzymes that their activity is used as necrosis marker, levels in blood (Gayosso-De-Lucio et al., 2014).

In a study performed on some selected compounds obtained from *Geranium psilostemon*, gallic acid showed most cytotoxic acitvity against HeLa cell line with IC_{50} value of 15 µg/ml and showed second best activity against L1210 and V79 cell line with IC_{50} value of 8.1 µg/ml and 15 µg/ml,after 1,3,6-tri-O-galloyl-β-glucopyranose. Regarding comet assay, tail length, tail intensity and tail moment parameters were used. Gallic acid was found to have longest tail length with a value of 51.04, highest tail intensity with a value of 36.79 and highest tail moment with a value of 10.53 compared to other compounds tested. 1,3,6-tri-O-galloyl-β-glucopyranose gave the shortest tail length with a value of 36.06, lowest tail intensity with a value of 14.86 and lowest tail moment with a value of 3.86 (Sabuncuoğlu et al., 2017).

Conclusions

From the results obtained above, the conclusions below can be reached:

- *Geranium macrorrhizum* leaves extracts show more toxicity compared to *Geranium macrorrhizum* root extracts.
- Both leaves and root extracts did not significantly increased number of micronuclei in human lymphocytes.
- *Geranium macrorrhizum* leaves extracts exhibit more genotoxic activity in Comet assay as compared to *Geranium macrorrhizum* root extracts.
- Overall, *Geranium macrorrhizum* root extracts may be considered as non-genotoxic *in vitro*, while leaves extracts may be considered as slightly genotoxic *in vitro*.

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Genotoxicity testing of *Geranium macrorrhizum* extracts

Master Thesis

SUMMARY

Plants have played very important life throughout human history. Since ancient times, humans use plants for various reasons, not only to feed themselves. However, at these times, the possible harm a plant can cause were not well known due to the fact that science was not developed enough. Lots of people have lost their life because of toxicity caused by plants. Humanity learnt which plants they can eat and which they cannot eat firstly. Later on, humans discovered they can use some plants for their wounds and illnesses as treatment. Today, methods regarding to toxicity are well developed compared to early days, however there are still unknowns present regarding to plants and their toxicity. The aim of this research to observe if *Geranium macrorrhizum* root and leaf extracts are suitable to be used in human diets as per their cytotoxic and genotoxic properties.

Keywords: *Geranium macrorrhizum*, genotoxicity, comet assay, CBMN

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