High value bio-products in "Het Groene Hart"

Use of biomass resources from woody ornamentals in region Boskoop

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Summary

The agricultural area “het Groene Hart” consists of different kind of crop species. This literature study is to find out if and what kind of valuable products can be extracted. The plants which are evaluated are *Buxus sempervirens, Ilex verticillata* and the family of *conifers*.

In ‘Het Groene Hart’ 30.4 hectares of *Buxus sempervirens* are cultivated which delivers 1 ton of biowaste per year. It is found that the leaves and roots of a mature plant contain a fair amount of alkaloids, a valuable compound for pharmaceutical purposes. These chemicals can be used for treatment for HIV, cancer and Alzheimer’s disease. The prices of the several products that contain alkaloid mixtures can vary from a few hundred to some thousand euros per gram.

Ilex verticillata contains three major substances which are thought to have medicinal working. These substances are Pelargonidin-3-xylosyl- glucoside, Pelargonidin-3-glucoside and Ursolic acid. The first two are anthocyanin pigments which are responsible for the red colour of the berries. They have an anti-cancer activity, improvement in vision and neuroprotective effects in humans. The prices for products with high purity in pelargonidin-3-glucoside are roughly around 50 euros per mg.

Ursolic acid that is also present in *I. verticillata* has anti-inflammatory and anti-cancer properties by inducing programmed cell death in tumour cells. It is hard to determine the market value of the other substances as it is influenced by purity. The accumulation of possible bottlenecks led to the conclusion that a biorefinery process might not be economically feasible. The exact amounts of those substances in the discarded biomass need to be determined. The seasonal presence of anthocyanins is also an important factor that needs to be kept in mind, when thinking about the logistics and organization of a biorefinery process.

In conifers, a biologically active substance can be extracted (CC-CP) which contains derivatives of chlorophyll, carotenoids, vitamins E and K, phytosterols, polyprenols and aqualene. Chlorophyll, squalene and phytosterols have anti-carcinogenic function and can prevent DNA damaging, while carotenoids have immunomodulating activity. Vitamin E is a multi-functional compound with especially antioxidant function, vitamin K has many regulations upon blood vessels and polyprenols rouse the immune system. Chlorophyll derivatives are responsible for the highest amount in conifers followed by carotenoids, vitamins and phytosterols.

Besides these chemicals also Taxol® (paclitaxel) is present as an anti-cancer drug, obtained from *Taxus baccata*. Taxol®’s main target is solid tumors. Moreover, resveratrol can be obtained from *Picea abies* barks and has cardio protective effect and it also shows anticancer properties. At last, turpentine can be extracted from various *Pinus* species and it can have several uses as antipyretic, abortifacient, insect repellant, decongestant, etc.

All values of the chemicals are estimated and we see that all plants contain compounds of high value. The market demand and availability of the plants and their compounds can be in specific cases high enough to account for an investment into the establishment of a biobased economy in “het Groene Hart”.

Content

1. Introduction

Today's major problems are caused by the exhaustion of the global oil reserves. Oil is the basis for our whole world wide economy and is therefore used for many purposes, such as petrol, plastics, but also for the pharmaceutical industry, where large complex organic molecules are obtained by mostly chemical conversion processes. The fact that the prices of crude oil increased dramatically over the last few years make it interesting now to look out for an alternative to the crude oil.

In order to reduce the dependency on crude oil the European Union and other countries like Brazil for example, make efforts to find alternative processes, where biomass is thought to replace crude oil and petro-chemicals on long term. This would reduce among other the dependency on politically unstable countries and the fluctuations of the energy prices. If biomass would be used for the production of chemicals and pharmaceuticals these industries will emit fewer amounts of greenhouse gases.

Biomass has a high potential as source for energy, which includes heat, power and fuels, but also as source for important chemical building blocks, food and substances that can be used in pharmaceuticals. The last group is our main point of interest in this report, as we are looking for high value compounds that can be extracted from discarded biomass.

The Green Heart is an agricultural area near Boskoop, between Amsterdam, Utrecht and Rotterdam. Within this agricultural region several plants and crops are cultivated and harvested. The plants that have high amounts of discarded biomass where identified by Graauw in 2013. And in addition they contain promising compounds for medicinal use. These plants are *Buxus sempervirens*, the family of *conifers* and *Ilex verticillata*. On the basis of a report written by Graauw, we were informed about the discarded biomass per plant per year.

In this report we aim to investigate the potential of these plants as a source for high value compounds. The development of a (cost-) effective bio-refinery process begins with the evaluation of the present plants in the region of the "Green Heart". The evaluation will be based on available literature that gives insight into the biochemical composition of all three plants. But also the extraction methods might be influential in the potential as source for high value compounds. Occasionally very expensive substances or methods might be needed to extract compounds from the plants that might make this process unfeasible.

We will mention the possibilities and possible bottlenecks of all plants to serve as a basis for a bio-refinery process.

2. Literature study

The area "het Groene Hart" consists of different kind of crop species. This literature study is to find out if and what kind of valuable products can be extracted. In chapter 2.1 can be found information about the *Buxus sempervirens*. Then in chapter 2.2 can be found of the *Ilex verticillata.* And finally, in chapter 2.3, information about a several conifers can be found.

2.1. *Buxus sempervirens*

The region "het Groene Hart" has 30.4 hectares of *Buxus sempervirens* grown for sale to individuals. Maintenance during the growing season produces 6.7 m^3 per hectare on bio waste. This is approximately 1 ton of bio waste per year.

Products

The main constituents of *Buxus sempervirens* are steroidal alkaloids and amines[1], shown in table 1. Formerly it was used medicinally for purifying the blood and for rheumatism, but it seemed to be far too toxic. Symptoms of poisoning are abdominal pain, vomiting, convulsions and death. Nowadays specific chemicals seemed to have medicinal function if treated with the appropriate dose. By extraction with a weak acid (HOAc 10%) the alkaloids can be extracted[2].

The products out of *Buxus sempervirens* is depending on the age of the plant. In an early age the amount of products is higher, and 8.24% of the dry weight can be classified as alkaloid. While the mature plants contain only 3.8% of alkaloids[3].

Table 1. Yields of alkaloids from *Buxus sempervirens* waste biomass[3].

Alkaloid	Immature		Mature	
	Yield		Yield	
	m/mg	w/mass %	m/mg	w/mass %
Cyclovirobyxine-D	1713,5	6,640	238,0	0,270
Buxaminol-E	139,7	1,130	27,5	0,020
Cyclobuxine-D	88,0	0,340	68,7	0,050
Buxtauine-M	11,3	0,050	566,2	0,480
Cycloprotobuxine-C	2,1	0,040	1020,8	0,860
Buxpiine-K	5,3	0,020	34,5	0,030
Cyclosuffrobuxine-K	5,2	0,020		
Cyclobuxamine-H			2406,5	2,040
Buxenine-G			71,7	0,060
Buxamine-E			47,0	0,040
Cyclobullatine-A			13,1	0,014
Total	**1965,1**	**8,240**	**4494,0**	**3,800**

Application

Veratrine is an acetonic extract of the *Buxus sempervirens*, which contains several alkaloids, exhibit promising anti-cancer activity by triggering both autophagic cell death and apoptosis. Autophagy is the basis metabolic cell degradation of un necessary cells[4]. Apoptosis is the process of programmed cell death, which may occur in multi cellular organisms[5]. These processes activate and enhance the destruction of rapidly growing cells in the body. Suggesting that this plant may contain potential anti-cancer agents for single or combinatory cancer therapy against breast cancer[6].

Alkaloid cyclobuxine-D can be used medical, it can treat the HIV virus[7] and other diseases in which the tumor necrosis factor is involved. The molecular formula of cyclobuxine-D is $C_{25}H_{42}N_2O$ and is shown in figure 1. It can be extracted by crystallization[8].

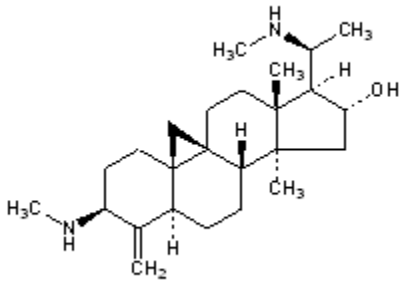

Figure 1, Molecular structure of Cyclobuxine-D

Buxaminol-E is tested on anaesthetised cats; it induced small short-lasting increase in blood pressure followed by marked hypotension. The results suggest that the hypotensive effect of buxaminol-E could be due to activation of muscarinic receptors[9]. The molecular formula of buxaminol-E is $C_{26}H_{44}N_2O$ and is shown in figure 2[10].

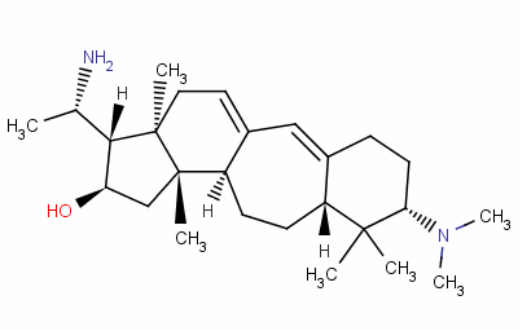

Figure 2, Molecular structure of Buxaminol-E

The alkaloid buxabenzamidienine is later discovered, which is known as an effective treatment for Alzheimer's disease. Alzheimer's disease reduces the acetylcholine level in the brain. This alkaloid increases the acetylcholine level[11]. The molecular formula of buxabenzamidiene is C33H48ON2 and is shown in figure 3.

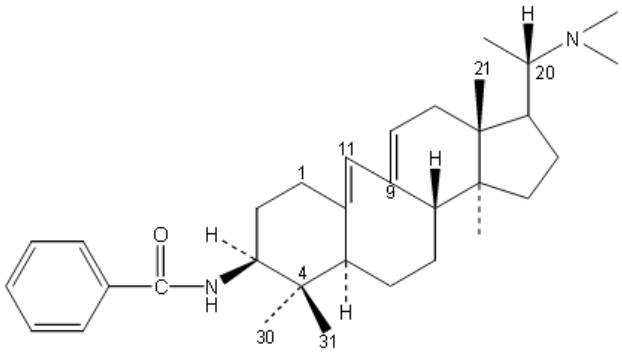

Figure 3, Molecular structure of Buxabenxamidienine

Profit

The components of the *Buxus sempervirens* are very specific and therefore very rare. There is much research into the application of these alkaloids on lab scale. More research has to be done for the application of these alkaloids on the marked. However, the potency of the alkaloids is pharmaceutical and therefore of great potential value. Expected is that the market in the future will start and grow.

2.2. *Ilex verticillata*

Ilex verticillata is a plant species that is available in the region of the "Green Heart" in high amounts. Specific volumes were suggested by Graauw in 2013. The total production of chaff that is generated is calculated to be 2.7 t ha^{-1} a^{-1}. The total area where *I. verticillata* is bred adds up to 22.7 hectares. This means that in total 61.3 ton of chaff is generated per year, according to Graauw.

I. verticillata is a fruit bearing species. The development of those starts in the early summer while maturation is finished in August or September resulting in deep orange or red berries. The fruits stick to the plant till the late winter[12].

It was found that the berries contain three major substances that are thought to have health-promoting biological activities. Pelargonidin-3-xylosyl- glucoside, Pelargonidin-3-glucoside and Ursolic acid are those three identified substances, which will be explained in more detail.

Pelargonidin-3-xylosyl-glucoside (CAS No.: 34425-23-5) and Pelargonidin-3-glucoside (CAS No.: 18466-51-8), shown in figure 4 are anthocyanin pigments, which are responsible for the red color of the berries. The pigments are only present in the skin (epicarp) of the mature fruits and can be extracted in cold MeOH-1 % HCL. A subsequent hydrolysis step is used for cleavage of the present anthocyanin pigments and can be performed by boiling in 4 moll HCL solution for 20 min. Final separation of both perlagonidins can be performed by High-Performance-Liquid-Chromatography[13].

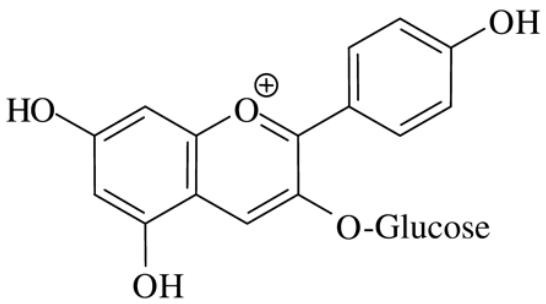

Figure 4. Molecular structure of pelargonidin-3-glucoside

Anthocyanins have attracted much attention due to their high intake rate from daily diets. General research on anthocyanins, but also specific research on perlagonidin-3-glucoside has revealed that their multiple biological activities include anticancer activity, improvement of vision and neuroprotective effects in humans. The anti-obesity activity was up till now only reported in animals, while a possible molecular anti-diabetic mechanism has been investigated in a cell culture system[14].

Specifically for Pelargonidin-3-xylosyl-glucoside a patent was found, that describes the use and application of anthocyanin-polysaccharide complexes that protect pharmaceuticals, cosmetics or food product from harmful external factors like oxygen, UV – VIS light, evaporation and

intermolecular interaction. The use of those complexes reduces loss and isolation of the reactive substances and loss of smell and/or taste in the products[15].

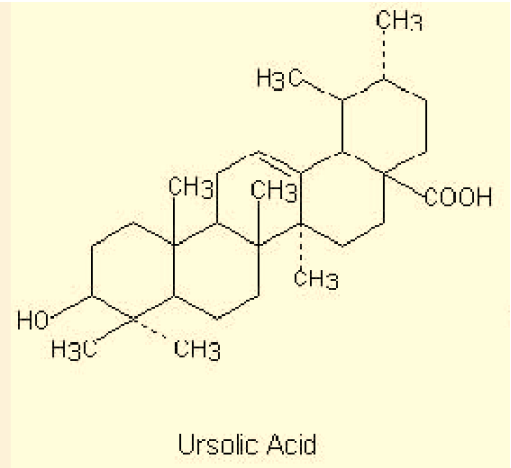

Figure 5 -Molecular structure of ursolic acid ($C_{30}H_{48}O_3$)

Ursolic acid (CAS No.: 77-52-1) (Figure 5) could be detected and extracted in in the berries of *Ilex verticillata*. It is a widespread triterpenoid isolated. In admixture with its isomer (oleanolic acid) it is contained in large quantities in many plants including those which have long been used in folk medicine. Ursolic acid has a wide spectrum of biological activity. An anti-inflammatory and anti-cancer property of ursolic acid could be proven by laboratory experiments. Growths of tumour cell lines were inhibiting including colon, breast, liver, prostate and leukaemia. Furthermore the expression and activity of cyclooxygenase, which sets off pain and inflammation, could be also inhibited. Ursolic acid is able to induce programmed cell death in tumour cells by activation of caspases and modulation of other involved pathways such as proliferation and migration[16].

Isolation of ursolic acid is applied form some fruits, such as from apple peel, but not yet from *Ilex verticillata* berries. We assume that the extraction of usolic acid could be done in a similar pathway as for apple peels. This isolation technique consists out of two main steps: First a liquid/liquid solvent extraction is applied with chloroform, ethyl acetate and/or ethanol. To purify the crude extracts a high speed counter-current chromatograph is used. This is a possible method for isolation ursolic acid, but for industrial scale another purification technique might be more feasible.

Nowadays Ursolic acid is sold and used as an additive for muscle optimizer and to lose weight fast. The market price of the pure product differs extremely from 1-1000000$ per kilogram. The unit price depends on its purity of ursolic acid [17, 18].

Although the demand for those anthocyanins and urolic acid is in all probability present, the isolation of those from chaff is unlikely economically feasible. The concentration of the pigments is at its maximum in august or September. The amount of those pigments is therefore also at its highest in the chaff. Unfortunately no data were available on the percentage of the berries in the total chaff or in the skin. We assume that it is unlikely, that the amount of berries in the chaff is high enough to perform a refinery process. Unless the needed facilities are already present and have free capacities. The prices for Pelargonidin-3-glucoside are roughly around 50 € per mg of product with a purity of 98%[19]. No prices were found for perlagonodin-3-xylosyl-glucoside.

2.3.Conifers

2.3.1. Conifers in general

Coniferous Chlorophyll Carotene Paste (CC-CP) is a natural biologically active substance that can be extracted from the green needles from conifer species. The most important active components of CC-CP are the derivatives of chlorophyll, carotenoids, vitamins E and K, phytosterols, polyprenols, squalene[20].

Chlorophyll

The derivatives of Chlorophyll have a well-known range of biological activity. They are identified as antioxidants and antimicrobials. They incite hematogenesis and response of immunity and assist in ulcers' healing, wounds and burns[21]. They are also known for their antimutagenic, anti-carcinogenic and anti-inflammatory properties[22], and can prevent DNA damages by carcinogens and other toxins [23].

Carotenoids

Carotenoids are known for their antioxidant and immunomodulating activity. *"They affect cellular signaling of the redox system; inhibit oncogenic expression, activity of ornithine decarboxylase, adenylate- and guanylate-cyclase; modulate cytochrome P450 enzymes; inhibit arachidonic acid metabolism; prevent chromosomal instability; retard proliferation and induce cellular differentiation and apoptosis. Beta-carotene and several other carotenoids are metabolized into vitamin A"* [24].

Vitamin E

Vitamin E is a multi-functional compound inside the human body and one of the most significant functions is its antioxidant properties. Also, vitamin E is important for the maintenance of the cellular membrane stability. Moreover, vitamin E takes part in the biosynthesis of protein, cellular division processes and tissue respiration. It also contributes in the reduction of the vascular thrombosis risk, the adjustment of the hormonal balance and immune response, the regulation of cellular signaling and transcription processes and induction of apoptosis [25].

Vitamin K

Vitamin K, by participating in the synthesis of coagulating factors, can regulate blood coagulation, increase the resistance of vessel walls, prevent vascular calcification, regulate resorption of calcium and bone cumulation, and participate in peristalsis[26]. High intake of several forms of vitamin K can reduce the risk of ischemic heart disease[27].

Phytosterols

Phytosterols are plant-origin sterols which have similar structure to cholesterol. They are proven to have antiatherosclerotic, anti-carcinogenic, antioxidant and immuno-stimulating properties[28].

The effect of the anti-carcinogenic action of phytosterols is focused on the structure of the cellular membranes and on regulating cellular signaling; their ability to constrain tumor growth and to encourage apoptosis; to rouse immune response and regulate cholesterol metabolism[29].

Polyprenols

Polyprenols can act as natural bioregulators and can be spotted in minor quantities in a variety of plant tissues[30]. Polyprenols can transport hydrophilic molecular fragments through the cell membranes during synthesis of polysaccharides, glycoproteins and other biopolymers.

One of the richest and most extensively available sources for extraction of polyprenols is live conifer needles[31]. Also, Koichi Ibata and his team investigated the polyprenols in the family of *Pinaceae*. The existence of polyprenols in pine trees had been reported for the needles of *Pinus strobus* , *Pinus sylvestris* and *Picea abies*[32]. Polyprenols are proven to have high biological activity, lack of side effects and exceptionally low toxicity[20].

Polyprenols rouse the immune system, cellular reparation and spermatogenesis, and possess antistress, adaptogenic, antiulcerogenic and wound-healing properties[33]. *"Experiments on mice demonstrated that polyprenols have antiviral activity, in particular, against influenza viruses"* [34].

Squalene

Squalene is an isoprenoid from the polyphenyl compounds group and is an intermediate metabolite in the synthesis of cholesterol. It also possesses antioxidant, immuno-stimulating, hypolipidemic, cholesterol reducing, anti-carcinogenic and anti-inflammatory properties[35].

Experimental and clinical studies showed that squalene can prevent the peroxidation of skin cells, takes part in detoxification from xenobiotics, rouses cellular and nonspecific immune response, and decreases the levels of cholesterol and triglycerides in the blood[35]. *"Epidemiological studies demonstrated that intake of squalene mixed, for example, with olive oil was associated with a reduction in risk of oncological and cardiovascular disease"* [36]. Experimental studies that used an induced carcinogenesis model on rodents showed that squalene constrained carcinogenesis of the large intestine, lungs and skin. Squalene's ability to inhibit activation of oncogene Ras, modulation of oncogenic activation and its antioxidant properties could be the mechanism of its anti-carcinogenic action. Squalene also has antimicrobial properties, in particular, in relation to tuberculosis mycobacteria[37].

So, it is made clear that the biologically active compounds contained in CC-CP have a wide variety of therapeutic-prophylactic properties. The most important properties of CC-CP are shown in Table 2.

Table 2: Therapeutic and prophylactic properties of CC-CP

Property	CC-CP Compounds
Antimicrobial	Chlorophyll, polyprenols, squalene, salts of resin acids, essential oils
Immunostimulating	Chlorophyll, carotenoids, vitamin E, phytosterols, polyprenols, squalene
Antioxidant	Chlorophyll, carotenoids, vitamin E, phytosterols, polyprenols, squalene
Hematogenic	Chlorophyll, carotenoids, vitamin E
Tissue Regenerative	Chlorophyll, vitamin E, polyprenols
Anti-atherosclerotic	Carotenoids, vitamin E, vitamin K, phytosterols, squalene
Anticarcinogenic	Chlorophyll, carotenoids, vitamin E, vitamin K, phytosterols, squalene

Extraction Process

A method that was originally developed by F.T. Solodky is used for processing the green conifer needles in order to extract the CC-CP. In this method, bioactive compounds are extracted from the ground conifer needles by using an organic solvent, the lipids are isolated from the extract and the obtained product is basificated. The resulting product is CC-CP[33, 38]. CC-CP's chemical composition is presented in Table 3.

Table 3: Chemical Composition of CC-CP [38]

Component	Content in CC-CP
Sodium chlorophyllin and other chlorophyll derivatives	400-1600 mg/dl
b-Carotene and other carotenoids	20-120 mg/dl
Vitamin E (a-tocopherol and its acetate)	30-50 mg/dl
Vitamin K group	1.2-2 mg/dl
Phytosterols (mainly b-sitosterol)	1.5-2.9 %
Polyprenols	0.46-1.2 %
Squalene	0.14-0.16 %
Minerals	5-7 %
Sodium salts of fatty, resin, dibasic, oxo- and oxyacids	44-60 %
Waxes	5-8 %
Essential oils	1-1.2 %
Water	up to 100 %

2.3.2 Substances in specific conifer species

Paclitaxel / Taxol®

Taxol® (paclitaxel), shown in figure 6 is a significant anticancer drug and it was first isolated in low concentration from the bark of the western yew, *Taxus brevifolia* [39].

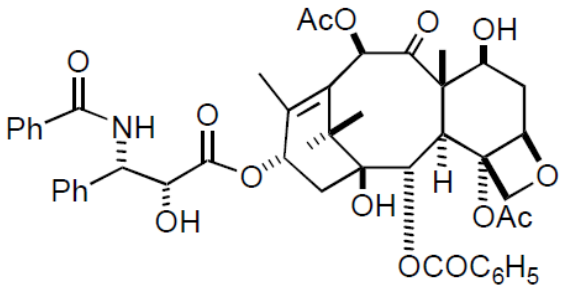

Figure 6:Paclitaxel

History of Taxol®

In 1979 Susan Horwitz with P. Schiff and J. Fant discovered the unique mechanism of action of Taxol®. They found that Taxol® stimulated the get-together of tubulin into stable microtubules and they described the basis for Taxol®'s properties as an antimitotic drug. In December 1992, US Food & Drug Administration (FDA) approved Taxol® for refractory ovarian cancer and for refractory breast cancer in April 1994. Nowadays, Taxol® is being tested on a variety of different cancer situations[39].

Chemistry of Taxol®

Taxol® belongs to the family of taxane diterpenoids or taxoids. There are more than 200 relatives of Taxol® in this family, most of them having the basic (9.3.1.0) pentadecene ring structure, shown in figure 7. More information about the structure of taxol is addressed by Erkan (1998).

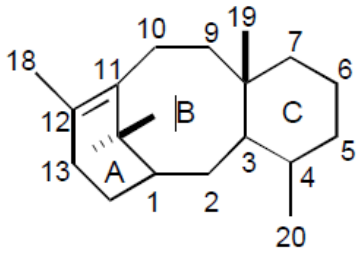

Figure 7: Pentadecene ring structure

Mechanism of Action of Taxol®

"Some limited testing was carried out, however, and the first indication of Taxol® 's excellent activity against solid tumors was when it was shown in 1977 that it had good activity against the B16 melanoma and the MX-1 mammary xenograft in nude mice" [40].

Taxol® has a distinctive mechanism of action in stimulating polymerization of tubulin. Tubulin as a protein of the cells polymerizes reversibly to assemble microtubules, which form the mitotic spindle apparatus. Microtubules are significant parts of the cell division, and they also form the skeleton of the cell.

Taxol®'s main target is solid tumors. *"It is thus possible that the effects of Taxol® on calcium ion fluxes may be involved in cellular signaling mechanisms, or that its primary action might be on tubulin/microtubule isotypes that are different from those involved in mitosis."* [41].

"According to Love, industry experts say Bristol-Myers pays about 40 cents to manufacture a milligram of Taxol, but Bristol-Myers says it charges $4.87 per milligram to drug wholesalers. No wonder the U.S. drug industry was ranked first in Fortune magazine's list of the nation's most profitable industries for each of the last 20 years" [42].

Resveratrol

Resveratrol is a polyphenol phytoalexin, that has various physiological and biochemical actions, such as antiplatelet, estrogenic and anti-inflammatory activities[43].

Besides the cardio protective effect of resveratrol, it also shows anticancer properties, as it has the ability to constrain proliferation of several tumor cells, such as myeloid and lymphoid cancers; cancers of prostate, colon, breast, stomach, thyroid and pancreas; multiple myeloma; neck and head squamous cell carcinoma; melanoma; cervical carcinoma and ovarian carcinoma[44].

Erkki Mannila, Antti Talvitie and Erkki Kolehmainen conducted a research aiming on identifying and isolating resveratrol as a minor stilbene derivative in *Picea abies* barks. They also observed in a preliminary test the improvement over piceatannol in antileukaemic activity[45].

Diterpenoids and Triterpenoids

Reiko Tanaka and his lab team focused on finding tumor-chemopreventive agents in components from materials of conifers (leaves, bark and cones) treated as forestry industry wastes. They previously had found some compounds that showed noteworthy anti-tumour promoting properties in in-vivo 2-stage mouse-skin carcinogenesis test using 7,12-dimethylbenz(a)anthracene (DMBA) and 12-Otetradecanoylphorbol- 13-acetate (TPA): 15,16-bisnor- 13-oxolabda-8(17),11E-dien-19-oic acid, a diterpenoid isolated from the stem bark of *Thuja standishii*, and a chemical derivative of abieslactone, a triterpenoid isolated from the stem bark of Abies species (Pinaceae) [46].

In order to find other terpenoids with anti-tumour properties, they isolated the components from *Picea jezoensis Carr*. Jezoensis (Pinaceae) and *Picea jezoensis* Carr. hondoensis (Mayr) Rehder (Pinaceae). The compounds isolated were serratane-type triterpenoids: 13a,14a-epoxy-3b-methoxyserratan-21b-ol (1), 13a,14a-epoxy-21a-methoxyserratan-21-one (2) and 21a-hydroxy-3b-methoxyserrat-14-en-30-al (3) from P. jezoensis Carr. jezoensis; and 21a-hydroxy-3bmethoxyserrat-14-en-29-al (4), 21a-methoxyserrat- 13-en-3-one (5), 3a-methoxyserrat-14-en-21b-ol (6), and 3b-methoxyserrat-14-en-21b-ol (7) from P. jezoensis Carr. hondoensis along with other analogs, shown in figure 8 [46].

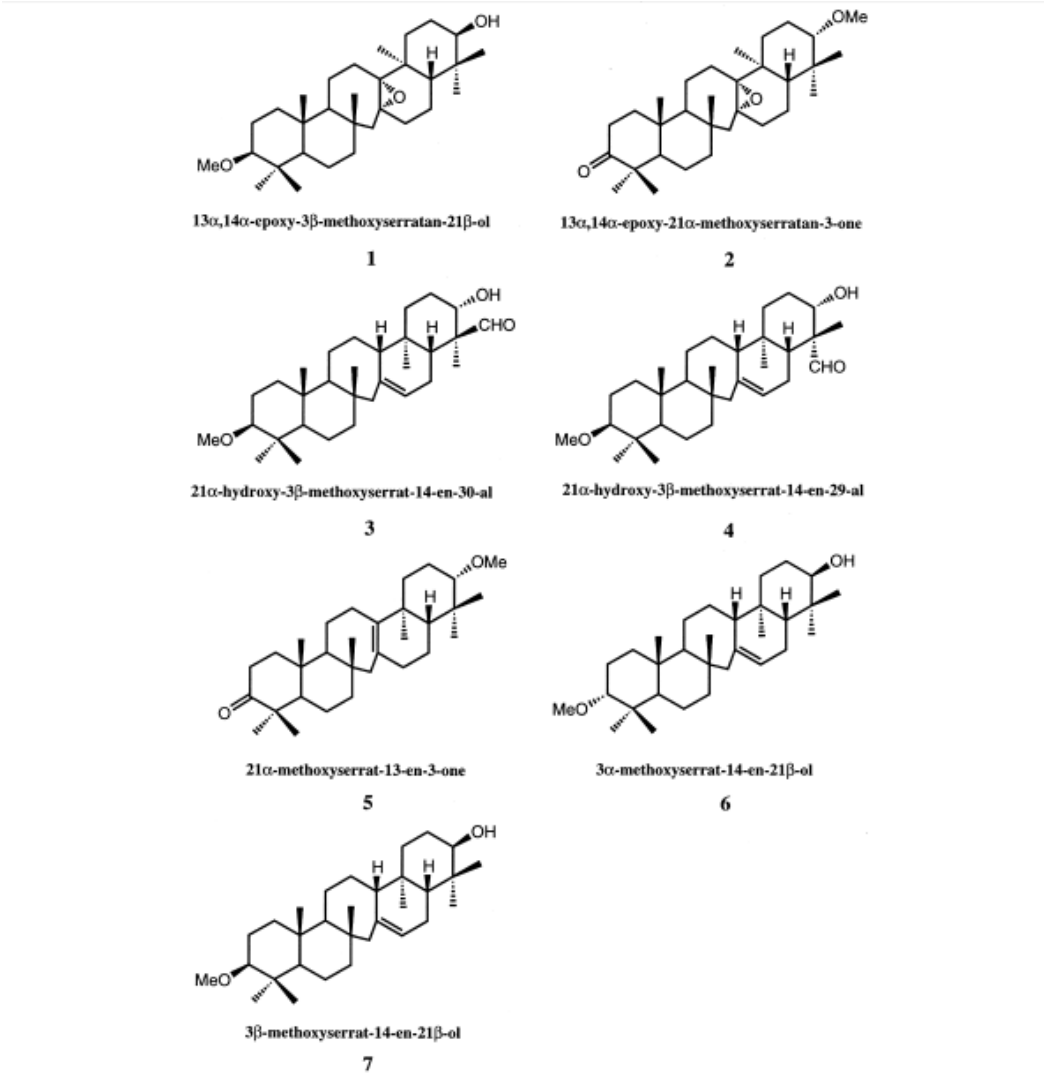

Figure 8: Structures of serratane Triterpenoids from *Picea jezoensis* Carr. jezoensis and *Picea jezoensis* Carr. hondoensis.

Also, a serratane-type triterpene (13α,14α-epoxy-3β-methoxyserratan-21β-ol) showed substantial anti-tumor promoting activity of in-vivo 2-stage mouse-skin carcinogenesis test using 7,12-dimethylbenz(*a*)anthracene (DMBA) as an initiator and TPA as a tumor promoter. Therefore, serratane-type triterpenes can be proven proper lead compounds in the further development of more effective agents with anti-tumor promoting properties for clinical use[47].

*"In our search for naturally occurring next cancer chemopreventive agents, detailed investigation of the stem bark was continued. We currently report the isolation of two unusually migrated serratane triterpenoids, jezananals A (**1**) and B (**2**) from P. jezoensis var. jezoensis, in addition to their stereochemistry and results of in vitro and in vivo anti-tumor promoting activities of compounds **1**, **2**, along with 3β-methoxyserrat-14-en-21β-ol (**3**), 3α-methoxyserrat-14-en-21β-ol (**4**), and 14β,15β-epoxy-3β-methoxyserratan-21β-ol (**5**)"* (Figure 9)[47].

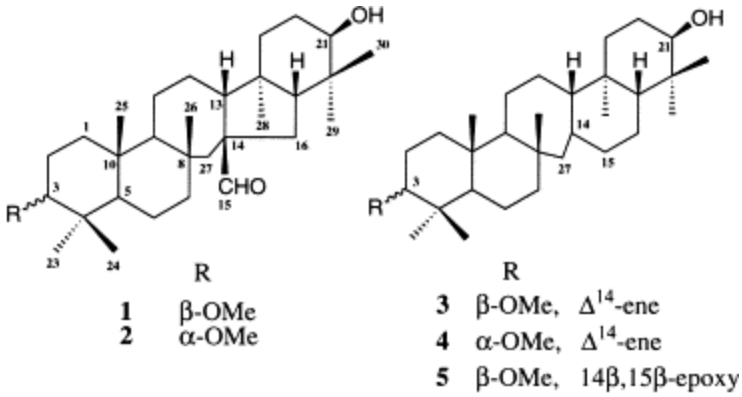

Figure 9: Stereochemistry of serratane triterpenoids

Turpentine

In the past, turpentine oil (terebinthinates) had been used as an herbal treatment against a wide range of illnesses. The value of turpentine oil was highlighted by Hippocrates for its emmenagogic (stimulant of menstrual flow) properties and its ability of preventing nasal discharge. Also, the Greek physician Dioscorides is said to be using turpentine oil for its aphrodisiac, diuretic, decongestant and antiarthritic properties. Moreover, turpentine oil was being used by the Romans against wide range of internal and external diseases, such as depression, lethargy, stroke and pleurisy[48].

On the end of 17th century during the US Civil War, turpentine oil was used by surgeons in treating wounds and amputations, while it was also used as a substitute for quinine in malaria treatment. In the next two centuries, turpentine oil contributed to the treatment of a wide range of infections, such as typhus, croup, dysentery, yellow fever and several parasites. During the late 19th century, turpentine enemas were used for treating abdominal distension, hysteria, and amenorrhea[48].

Turpentine oil (gum turpentine) is consisted of oily, volatile, liquid fraction derivatives from distilling the steam of pine resin. Also, other sources that turpentine oil can be obtained from include the extraction or destructive distillation of wood (wood turpentine) and chemical pulping of pine trees by-products. Even if it is not considered as a petroleum distillate, many reviews include turpentine oil in petroleum distillates because it is an aromatic hydrocarbon that has similar properties, toxic effects, and uses to petroleum distillates. Colophony is the substance that remains after the

evaporation of volatiles in the distillation of crude turpentine. It is a nonvolatile, solid residue (rosin, gum rosin) and it is used as skin sensitizer[48].

Turpentine oil can be used as an antipyretic, abortifacient, anthelminthic agent, insect repellant, rubefacient, decongestant, and a treatment for human myiasis (i.e., infection with dipterous larvae, such as maggots)[48].

3. Findings and economic data

Table 4. Products of different plant species and their applications.

Species	Active substance	Plant part	Applications
Conifers (in general)	❖ Chlorophyll ❖ Carotenoids ❖ Vitamin E ❖ Vitamin K ❖ Phytosterols ❖ Polyprenols ❖ Squalene	❖ Green needles	❖ Anti-microbial ❖ Anti-carcinogenic ❖ Anti-oxidant ❖ Hematogenic ❖ Immunostimulating ❖ Tissue regenerative ❖ Anti-atherosclerotic
Taxus baccata	❖ Paclitaxel / Taxol®	❖ Bark	❖ Anti-carcinogenic ❖ Anti-leukemic
Picea abies	❖ Resveratrol ❖ Triterpenoids (abieslactone)	❖ Bark	❖ Anti-leukemic
Picea jezoensis	❖ Diterpenoids	❖ Stem bark	❖ Anti-tumor
Thuja standishii		❖ Bark	
Pinus sp.	❖ Polyprenols	❖ Needles	❖ Adaptogenic ❖ Anti-stress ❖ Cicatrizant ❖ Anti-ulcerogenic ❖ Anti-viral
	❖ Turpentine	❖ Pine resin	❖ Anti-pyretic ❖ Abortifacient ❖ Anthelminthic agent ❖ Insect repellant ❖ Rubefacient ❖ Decongestant ❖ Treatment for human myiasis
Ilex verticillata	❖ Pelargonidin-3-xylosyl- glucoside	❖ Berries	❖ Anti-cancer ❖ Anti-obesity ❖ Anti-diabetic ❖ Protection of anthocyanin-polysaccharide complexes from UV-VIS light
	❖ Pelargonidin-3-glucoside		
	❖ Ursolic acid		❖ Anti-cancer ❖ Anti-inflammatory
Buxus sempervirens	❖ Buxabenzamidienine	❖ Leaves ❖ Roots	❖ Alzheimer treatment
	❖ Cyclobuxine-D	❖ Roots	❖ HIV-treatment
	❖ Veratrine	❖ Leaves	❖ Blood purification ❖ Anti-rheumatism ❖ Anti-cancer

Table 5. Approximate market prices of some of the most interesting substances (data gathered from Sigma-Aldrich)

	Compound	Price
Conifers	B-carotene (97% purity)	16EUR/g
	Vitamin E acetate	338EUR/g
	Vitamin K [BioXtra, ≥99% purity (sum of isomers, HPLC), mixture of isomers]	43.9EUR/g
	Squalene (98% purity)	280.5EUR/g
	Paclitaxel (from semisynthetic (from *Taxus* sp.), ≥97% (Sigma)	39.8EUR/mg
	Resveratrol ≥99% purity (GC)	1380EUR/g
	7,12-Dimethylbenz[*a*]anthracene (DMBA) ≥95% purity	185EUR/g
	Oil of turpentine (purified)	40.9EUR/L
	Gum rosin/Colophony	41EUR/kg
Ilex verticillata	Ursolic acid (≥90% purity)	533EUR/g
	Callistephin chloride (Pelargonidin 3-O-glucoside chloride)	113.5EUR/mg
Buxus sempervirens	Veratrine	288EUR/g
	Beta-Veratrine	1440EUR/g

4. Possibilities & Bottlenecks

Buxus sempervirens

Acetonic extract of the *Buxus sempervirens* (the total mixture of extracted alkaloids) exhibit promising anti-cancer activity by triggering both autophagic cell death and apoptosis.
Alkaloid cyclobuxine-D can be used medical, it can treat the HIV virus[7] and other diseases in which the tumor necrosis factor is involved.

Buxaminol-E is tested on anaesthetised cats; it induced small short-lasting increase in blood pressure followed by marked hypotension. The results suggest that the hypotensive effect of buxaminol-E could be due to activation of muscarinic receptors.

The alkaloid buxabenzamidienine is later discovered, which is known as an effective treatment for Alzheimer's disease. Alzheimer's disease reduces the acetylcholine level in the brain. This alkaloid increases the acetylcholine level[11].

By harvesting the leaves of young plants may be a higher concentration of alkaloids obtained.

The big bottleneck in the *Buxus sempervirens* applications is that there is not enough research done for practical application.

Ilex Verticillata

The three most interesting compounds for industrial purposes present in *I. verticillata* are urolic acid, perlagonidin-3-glucoside and perlagonidin-3-xylosyl-glucoside. The three compounds, with their health promoting characteristics, may contribute to improve the world wide health-care-system.
The use of the three compounds for pharmaceutical application demands a lot of research. Nowadays, this is still in early research stage and not yet applied for human consumption. But we assume that the potential for those products on the market will increase, once research has revealed their full potential for human usage.

On the other hand we could already show that the prices for highly pure compounds are very promising to make profit. We assume that the capital investment increases in relation with the quality of the product. For large capital investments, a minimum turnover is needed. In case that the total amounts of the extracted compounds are too low, we would not recommend such a high investment. Therefore it is essential to determine the concentration of those compounds in the berries, as well as the percentage (w/w) of the berries in the total biomass waste.

Conifers

The ability of the carotenoids in preventing the development of tumors of various localizations, atherosclerosis and associated cardiovascular diseases and cataracts is confirmed by epidemiological and experimental studies[49].
The protective action of vitamin E in such disorders as atherosclerosis, certain types of cancer, diabetes, chronic inflammation, cataracts and Alzheimer's Disease, is confirmed by epidemiological and experimental studies[25].

Experimental, epidemiological and clinical studies showed that vitamin K can prevent the development of osteoporosis[50].

Epidemiological studies showed that high intake of phytosterols can reduce the risk of cancer of the large intestine, prostate, mammary glands[29], and of the stomach[51] and lungs[52]. *"Some clinical studies showed that phytosterols have the capacity to reduce symptoms of urological pathology, to improve urination as well as quality of life for patients with benign prostatic hyperplasia"* [53].

Polyprenols are proven to have high biological activity, lack of side effects and exceptionally low toxicity[20]. "Experiments on mice demonstrated that polyprenols have antiviral activity, in particular, against influenza viruses" [34].

Experimental and clinical studies showed that squalene can prevent the peroxidation of skin cells, takes part in detoxification from xenobiotics, rouses cellular and nonspecific immune response, and decreases the levels of cholesterol and triglycerides in the blood[35]. *"Epidemiological studies demonstrated that intake of squalene mixed, for example, with olive oil was associated with a reduction in risk of oncological and cardiovascular disease"* [36]. Experimental studies that used an induced carcinogenesis model on rodents showed that squalene constrained carcinogenesis of the large intestine, lungs and skin. Squalene's ability to inhibit activation of oncogene Ras, modulation of oncogenic activation and its antioxidant properties could be the mechanism of its anti-carcinogenic action. Squalene also has antimicrobial properties, in particular, in relation to tuberculosis mycobacteria[37].

Taxol® can be formed from baccatin III, which can be isolated as 10-deacetyl baccatin III in substantial quantities (1g/kg) from the renewable needles of *Taxus baccata* (European Yew). This is almost 10 times more than the amount of Taxol® (0.1g/kg) that can be extracted from the bark of *Taxus brevifolia*. Due to this fact, Taxol® conversion from baccatin III is an attractive but also challenging option[39]. The economic value of Taxol® is high as it is supposed to be the best-selling anticancer drug in history, with the sales reaching almost one billion U.S. dollars in 1997[39] Taxol®'s strong cytotoxicity and decent activity in the P-388 mouse leukemia tests made it worth of research[40]. In summary, it can be said that Taxol®'s unique mechanism of action makes it a unique anticancer drug in the market[39]. In order to obtain large amounts of Taxol® several approaches have been considered, such as total synthesis, plant tissue culture production, use of taxol-producing fungus, extraction and semi-synthesis of 10-deacetylbaccatin III from needles and leaves of *Taxus* species. Only the last two approaches can be proved commercially viable, with the last one being used more widely[39]. "The tetracyclic diterpene moiety of Taxol® , 10-deacetylbaccatin III (10-DAB) shown in figure 10, which is the most demanding portion of Taxol® from the point of view of total synthesis, is readily available from the renewable leaves of *Taxus baccata* (European Yew). 10-DAB can be extracted from the leaves of this tree in high yields of 1g/kg.40" [54]. Thus the conversion of 10-DAB to Taxol® can be considered as an economically beneficial choice for producing Taxol® in large amounts[39].

Reiko Tanaka and his lab team found some terpenoids that showed noteworthy anti-tumor promoting properties in in-vivo 2-stage mouse-skin carcinogenesis test[46].

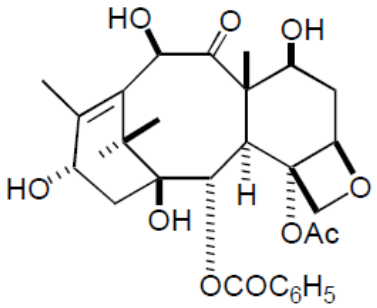

Figure 10: 10-deacetyl-baccatin III

Due to the chemical complexity of Taxol®, its commercial production by total synthesis is not likely to be economically viable[39].

The initial encouraging findings from P-388 mouse leukemia tests was not followed up enthusiastically, however, because of the clear supply problem in getting a multifarious natural product from the bark of a rather uncommon tree, and also because of Taxol®'s low water solubility[40].

5. Conclusion

The aim of this literature research was to determine some possibilities but also bottlenecks for the biorefinery of the biomass that is discarded in the region of the "Green heart". The three major plants that are grown in this region are *Buxus sempervirens, Ilex verticillata* and *Conifers*, that all contain chemical compounds that are having a certain value on the present market. All in all, we found out that most of our important compounds are already on the market in form of nutritional supplements (vitamins, ursolic acid etc.) and pharmaceuticals (Taxol®/paclitaxel, resveratrol, terpenoids etc.). In addition we also found some compounds that might be evolving in future as important substances to support the treatment of certain diseases like e.g. Alzheimer disease, AIDS, cancer. In this category we could identify Cyclobuxine-D, Buxabenzamidienine, and Pelargonidin-3-glucoside etc.

The cultivation of *Buxus sempervirens* results in a biowaste of approximately 1 ton per year. This can be processed with biorefinery in order to extract valuable chemicals from the plant. The main constituents of *B. sempervirens* are steroidal alkaloids and amines. These chemicals can be used for medication of cancer, HIV and Alzheimer's disease, but they are however very specific and therefore very rare. These potential pharmaceuticals will have then a great value when they are extracted and purified. The exact value is unfortunately still unknown.

Ilex verticillata is quite a promising source for biomass with valuable compounds. Nonetheless a lot of challenges need to be solved, before the biorefinery of those products is economically feasible. It is not yet known what the theoretical amount of those products per year is, and if the present market demand is high enough to account for a large scale extraction.

The biowaste produced from conifers is approximately 0.5 ton per year. The most important compounds that can have medicinal uses are CC-CP, Taxol®, resveratrol, several terpenoids and turpentine. Most of them have anti-carcinogenic properties and some of them can have general medical uses like anti-microbial, anti-oxidant, decongestant and anti-pyretic. The prices of these compounds vary and some of them can be proven really profitable for a biobased economy system.

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