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Contents

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Review Article

Raspberry pomace - composition, properties and application

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ABSTRACT

Raspberry pomace can be valorised due to its nutritionally favourable effect on human health. It is an important source of polyphenols, ellagic acid, ellagitannins, tocopherols, unsaturated fatty acids, and dietary fibre. Thus, raspberry pomace can be considered as a potential raw material to receive products rich in polyphenols or dietary fibre, which can provide healthy properties to food when used as an additive. This review presents the chemical composition and antioxidant properties of raspberry pomace. The possibilities of its usage in industry are also briefly reviewed.

Keywords: Raspberry; Pomace; Fruit; Antioxidant activities; Bioactive substances; Waste disposal.

1. INTRODUCTION

Nowadays, because of the rising interest in functional food, especially bioactive compounds, food producers are looking for new sources and carriers of those substances. Due to their health properties, consumers search for products that allow them to maintain a proper physical and mental fitness and also well-being [1]. Raspberry pomace is the residue that remains after the extraction of juice from raspberry. Dried raspberry pomace, a fruit industry by-product, is considered as a potential food ingredient. Its pomace contains plenty of valuable components such as carbohydrates, proteins, fats, fibre, flavours, pectins, vitamins, similar to the composition of whole raspberries [2]. Moreover, raspberry pomace is rich in a large group of various phenolics especially ellagitannins, proanthocyanidins, anthocyanins, flavonols, and phenolic acids (especially, ellagic acid) which are also predominant in berries [3]. It has been reported [4] that these compounds have beneficial properties for human like antioxidant and antimicrobial activities and a wide range of physiological properties, such as anti-allergenic, anti-atherogenic, anti-inflammatory, antimicrobial, antioxidant, antithrombotic, cardioprotective and vasodilatory effects. The antioxidant activity of phenolics is provided by the hydroxyl groups and phenolic hydrogen for donation [3].

The aim of this review is to collect recent data on chemical composition and antioxidant properties of raspberry pomace and to present a great potential of usage of raspberry pomace in various fields of industry.

2. CHEMICAL COMPOSITION

Raspberry pomace, a fruit waste, received during pressing raspberries during juice production

consists, mainly of seeds and pulps. On average, raspberry pomace is characterized by a high content of total dietary fibre 59.5%, acid detergent fibre 46%, cellulose about 27%, crude fat about 11%, crude protein 10%, lignin 11.7%, cutin 6%, acid detergent ash 2.2% (Table 1) [5].

Table 1. Approximate composition of raspberry pomace (dry matter basis).

Parameters	**(%)**
Crude fat	11.1
Crude protein	10.0
Total dietary fiber (TDF)	59.5
Acid detergent fiber	46.0
Lignin	11.7
Cutin	6.0
Acid detergent ash	2.2
Cellulose	26.9

On the other hand, results obtained by Laroze et al. (2010) show that raspberry residue composition consists mainly of crude fibre (59.76%) and nitrogen free extract (31.02%). The high content of crude fibre suggests that raspberry pomace is a source of antioxidants. Crude fibre contains polyphenols which are associated with non starch polysaccharides such as pectin, cellulose, β-glucans, hemicellulose, gums, and lignin. Moreover, raspberry residue shows low protein, ash and oil content (1.87%; 5.97%; 1.38%, respectively). Due to the fact that raspberry residue ash contains a low percentage of minerals and heavy metals, from which it is known that it can act as pro-oxidants like iron, a positive impact on the antioxidant capacity of raspberry waste is likely [6].

Furthermore, raspberry pomace contains small amounts of vitamins (E, C), but only vitamin C is presented at a significant level and responsible for anti-inflammatory activity [7, 8]. In addition, a lot of volatile compounds are found in raspberry pomace (Fig. 1) such as alcohols, esters, acids, ketones and carbonyls [9]. Additionally, raspberry pomace is an important source of unsaturated fatty acids and tocopherols.

Besides, the main sugars in raspberry pomace are glucose, fructose and sucrose. Raspberry pomace is also a source of sodium, potassium, calcium, phosphorus and magnesium [7].

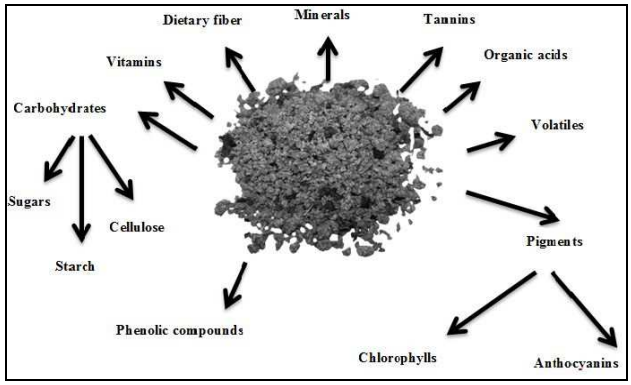

Figure 1. Simplified schematic representation of the remarkable components of raspberry pomace.

3. OCCURRENCE OF BIOACTIVE COMPONENTS IN RASPBERRY POMACE

Bioactive compounds (often called antioxidants) are defined as chemical substances which in small quantities have an ability to prevent or reduce the oxidation of easily oxidisable molecules [10].

Antioxidant activity is closely associated with antioxidants which have antimicrobial activities against human pathogens [11]. More specific, the function of these compounds is to slow down or to stop damaging cellular DNA, lipids, and proteins caused by reactive oxygen species (ROS) [12].

Raspberry pomace, in particular, is a rich source of antioxidants. The biological activity of those compounds is mainly exercised by dietary fibre, tocopherols, unsaturated fatty acids, carotenoids, vitamin C and polyphenols such as tannins (especially ellagitannins), anthocyanins, flavanols, flavonols and phenolic acids [13, 14].

3.1. Dietary fibre

The chemical composition of raspberry pomace makes that it belongs to a valuable group of fruit by-products [15]. By-products from raspberry processing contain prominent amounts of bioactive components including dietary fibre which is highly

desirable for dietary purposes [16]. The content of total dietary fibre (TDF) in raspberry pomace is very high, about 60% [5]. Its composition demonstrates the high content of lignin (63.16%), which means a presence of phenolics. There are also other components, but in less amounts, namely pectin (15.38%), hemicellulose (14.89%) and cellulose (5.36%) [6].

Due to the nutritional benefits of dietary fibre, producers are keen on using by-products as food ingredients. For instance, enriched cookies with 50% of a non-crumbled raspberry pomace resulted in the desired high content of dietary fibre. It has been noticed that differences in a flavour in such kind of cookies depend on the quantity and form of pomace used [17].

The addition of raspberry pomace to shortcrust cookies caused an increase of their fruity smell and taste, as well as an increased sour taste while the sweet taste was less perceptible (Fig. 2). It was confirmed that the more raspberry pomace is added, the stronger the fruity smell, fruity taste and sour taste. An increased crumbliness of cookies was reported after adding a 50% of whole seed raspberry pomace [1].

3.2. Fatty acids and tocopherols

Raspberry seed oil from raspberry by-product has a unique fatty acid profile [18, 19]. Dimić et al. reported that the oil content of raspberry pomace was about 14% on dry basis. It had a dark yellowish-orange colour due to lower chlorophyll content (about 200 mg/kg). The total content of carotenoids was around 40 mg/kg [20].

Oil from raspberry seeds possesses an important nutritional profile. It is a rich source of fatty acids, vitamin A, vitamin E and α-, γ-, σ-tocopherols. Raspberry seed oil is abundant in unsaturated fatty acids such as linoleic, α-linolenic, and oleic acid (96% of the total fatty acids). The extraction of raspberry seed oil with chloroform resulted in a fatty acid composition as follows: C16:0, 2.7%; C18:0, 0.2%; C18:1, 18.7%; C18:2, 55.5%; and C18:3, 32.6%. Oomah et al. investigated that raspberry seed oil contains neutral lipids, free fatty acids, and phospholipids with 93.8, 3.5, 2.7%, respectively. Additionally, raspberry seed oil is a superior source of tocopherols, mainly γ-tocopherol (137-272 mg/100 g). The ratio of the tocopherol isomers (α, γ, σ) in raspberry seed oil was 20:75:5. The high γ-tocopherol content can indicate the prevention of degenerative diseases [9].

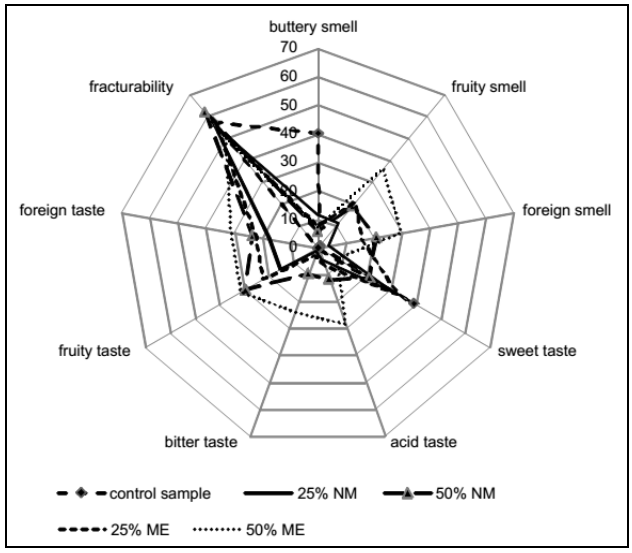

Figure 2. The influence of adding crumbled (ME) and non-crumbled (NM) raspberry pomace on the sensory qualities of shortcrust cookies (based on [1]).

3.3. Carotenoids

Carotenoids belong to the class of natural pigments, occurring in plant materials including fruits and vegetables. They are responsible for the yellow to red colour of those plants. Some of them demonstrate provitamin A activity. Carotenoids are polyenoicterpenoids having conjugated trans double bonds, including carotenes (*β*-carotene, lycopene). These compounds are polyene hydrocarbons, and xanthophylls (lutein, zeaxanthin, capsanthin, canthaxanthin, astaxanthin, and violaxanthin) which means that they have oxygen in the form of hydroxyl-, oxo-, and epoxy groups [16]. Carotenoids have a great potential to human health. It was proved that they occur as biological antioxidants, protectors of cells and tissues against free radicals and inhibitors of the proliferation of the cells [16, 21].

3.4. Vitamin C

Vitamin C, also known as ascorbic acid and dehydroascorbic acid, is widely used as a food additive for humans and other animal species. The deficiency of vitamin C causes the disease called scurvy in human organisms. Dehydroascorbic acid is the minor part of vitamin C content and the oxidised form of ascorbic acid. Ascorbic acid possesses antioxidant activity and prevents oxidative stress-related diseases. Thus, it can be considered as a scavenger of reactive oxygen species. However, humans are not able to synthesise ascorbic acid because of the lack of enzyme L-gluconolactone oxidase. Therefore, plants appear to be able to synthesise ascorbic acid from D-glucose or D-galactose [22].

3.5. Phenolic compounds

Numerous studies show that raspberry pomace is a superior source of phenolics. The results of the quantitative analysis of antioxidant components are shown in Table 2.

The study conducted by Vulić et al. (2011) indicates that raspberry pomace extracts contain a high amount of total flavonoids: 591.65 mg per 100 g of fresh pomace. Besides, the total anthocyanin content appeared to be 65.21 mg per 100 g of fresh pomace [23]. Regarding another study, the total phenolic content of raspberry was higher (234 ± 5.1 mg gallic acid per 100 g of fresh fruit) [24]. It has also been reported that the total anthocyanin content of raspberry pomace extract is 68.0 mg per 100 g fresh fruit [23].

The antioxidant composition commonly occurring in raspberry pomace is presented in Table 3.

3.5.1. Flavonoids

Flavonoids represent the largest group of plant phenolics, accounting for over half of the eight thousand naturally occurring phenolic compounds. Flavonoids consist of classes like: anthocyanins, flavones, flavanols, flavanones, flavans, isoflavones and flavonols. Furthermore, flavonoid compounds are classified in bioflavonoids, chalcones, flavonolignans, prenylflavonoids, glycoflavons, aurones [25]. In raspberry pomace was noticed the dominant presence of flavonol glycosides, namely quercetin and kaempferol glycosides [12].

Table 2. Total anthocyanins, flavonoids and polyphenolics in berry pomace extracts [based on Vulić et al. 2011].

Berrypomace extracts	Antioxidant compounds content (mg/100 g fresh pomace)		
	Total anthocyanins	Total flavonoids	Total polyphenolics
Raspberry	65.21	591.65	637.77
Blackberry	149.12	245.48	804.50
Strawberry	19.48	296.11	488.12
Bilberry	1279.49	1047.39	1116.24

Table 3. Antioxidant compounds identified in raspberry pomace.

Antioxidative compounds	Major compounds	References
Anthocyanins	cyanidin-3-sophoroside*, cyanidin-3-glucoside, cyanidin-3-glucorutinoside, cyanidin-3-rutinoside, pelargonidin-3-sophoroside, pelargonidin-3-glucoside	47
Flavonols (flavonol glycosides)	quercetin glycosides, kaempferol glycosides	12
Flavanols	catechin, epicatechin	10
Polymeric tannins	ellagitannins, proanthocyanidins	7, 48
Phenolic acids	hydroxycinnamic acid, chlorogenic acid	3

*Anthocyanin dominant

The flavonoids are formed in the condensation reaction of a phenylpropanoid (C6-C3) compound with malonyl coenzyme A. Flavonoids have the basic skeleton of diphenylpropanes (C6-C3-C6) [3]. The broad range of functions of flavonoids gives wide prospects for applications, not only in prevention but also in therapy of many diseases, for instance: cancers, atherosclerosis, cardiovascular disease, diabetes, and so on [25]. Flavonoids as ubiquitous compounds in plants constitute an important element in the human diet. It

is estimated that on average one person eats in a day about 1 g flavonoid compounds [26].

3.5.2. Anthocyanins

Anthocyanins, which are classified as pigment compounds in the tissues of berries, constitute one of the major groups of polyphenols in berry pomaces [23, 27]. The basic structures of anthocyanins are the anthocyanidins (or aglycons). When anthocyanidins are bound to sugar molecules, anthocyanins are obtained. The most common sugar substitutes on the anthocyanidins are glucose, fructose, galactose, rhamnose, xylose, and arabinose [22, 28].

Anthocyanins are usually presented in coloured flavylium cation form, which depends on the pH [3]. Therefore, at pH 1, the flavylium cation (red colour) is the predominant species and contributes to the purple and red colours of raspberries.

Anthocyanins belong to compounds which are easy to oxidise, thus they are usually the best antioxidants. Several studies have suggested that the anthocyanin content and their corresponding antioxidant activity, contribute to the fruits protective effect against degenerative and chronic diseases [28]. It has also been reported that they characterize anticarcinogenic activity. It has been proven that the antioxidant activity of berries is directly proportional (linear correlation) to the anthocyanins content [29]. The results received by Soto Rodriguez Gil demonstrated that the main anthocyanins found in black raspberry pomace extract were cyanidins (95% of the anthocyanins), namely cyanidin-3-rutinoside (68.8%), cyanidin-3-sambubioside-5-rhamnoside (18.2%), cyanidin-3-glucoside (7.1%) and pelargonidin-3-glucoside (6%). In addition, in the study by Soto Rodriguez Gil anthocyanin content was 3800 mg per kg of black raspberry pomace [30].

3.5.3. Polymeric tannins

Proanthocyanidins, regarded as condensed tannins, are dimers, oligomers, and polymers of catechins which are bound together by C-C links. Catechins are monomer form of flavan-3-ols and proanthocyanidins are the polymer form of those compounds [22, 31, 32]. Proanthocyanidins have, similar as flavan-3-ols, the C6-C3-C6 flavonoid skeleton and give a characteristic bitter taste to many berries. Flavan-3-ols commonly occurring include: (+)-catechin, (-)-epicatechin, gallocatechin, and epigallocatechin. Procyanidins and prodel-phinidins are made of epicatechin units and epigallocatechins, respectively [33]. There were found prominent amounts of proanthocyanidins in berries [3]. In raspberry residue proanthocyanidins are formed of procyanidins and propelargonidins [13].

Ellagitannins with gallotannins form the group of hydrolyzable tannins. Ellagitannins are presented especially in the family *Rosaceae*, genus *Rubus*, namely raspberries, cloudberries, and blackberries [34]. These berries as well as its pomace produce ellagitannins based on stable glucose conformation [11]. The ellagitannin monomers often form dimers, trimers and even higher oligomers via phenolic oxidative coupling reactions [34, 35].

The major ellagitannins which have been identified in raspberries (*Rubus idaeus* L.) and raspberry pomace are the dimeric sanguiin H-6 and the trimeric lambertianin C (Fig. 3) and comprising 81% of the total ellagitannins in raspberries. Also, raspberries contain ellagitannins such as monomeric casuarictin, potentillin, pedunculagin, sanguiin H-10, dimeric nobotanin A, and tetrameric lambertianin D [3, 11, 34].

Moreover, ellagitannins are complex derivatives of ellagic acid. They contain one or more hexahydroxydiphenic acid (HHDP) moieties esterified usually to glucose. Hydrolysis of ellagitannins with acids or bases yields that HHDP is lactonized to ellagic acid (Fig. 4) [36].

It has been reported that free ellagic acid was detected in berries. The highest level was found in cloudberries and wild red raspberries, whereas ellagic acid glycosides were detected only in raspberries, of which wild raspberries contained the highest level [36].

3.5.4. Phenolic acids

Phenolic acids in raspberry pomace are represented mainly by cinnamic acids and benzoic acid derivatives. Hydroxybenzoic acids, occurring

in raspberry pomace, consist of salicylic acid, p-hydroxybenzoic acid, gallic acid, and ellagic acid. The last one, ellagic acid, is predominant in raspberry pomace and is presented in the free form or esterified to glucose. Hydroxycinnamic acids, which are widely distributed in berry pomaces, include p-coumaric, caffeic, sinapic, and ferulic acids. Hydroxycinnamic acids are commonly found as derivatives of caffeic acid. Chlorogenic acid, which is an ester of caffeic and quinic acid (5-O-caffeoylquinic acid), belongs to the one of the main hydroxycinnamates found in plants [22, 37].

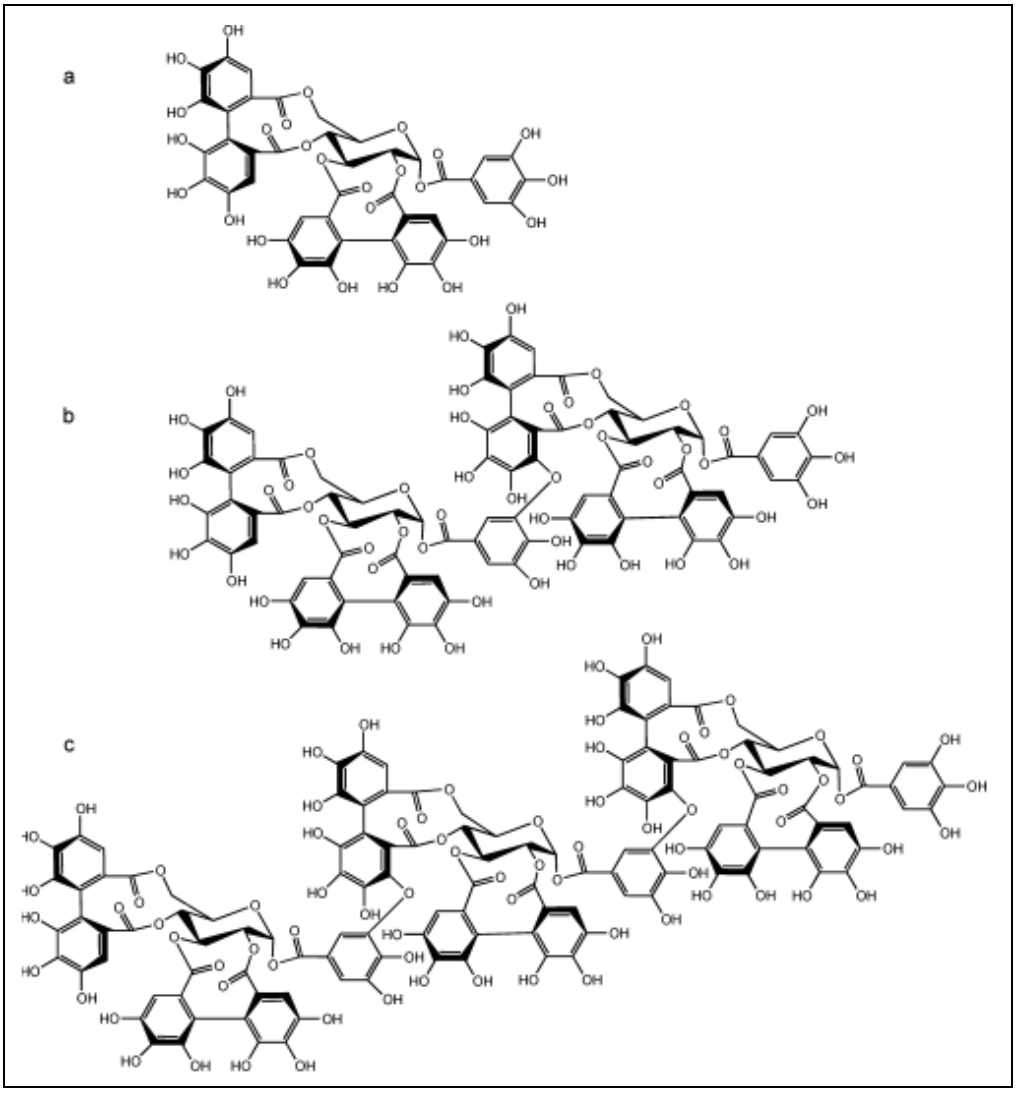

Figure 3. Structures of the major ellagitannins in raspberries and raspberries pomace: casuarictin (a), sanguiin H-6 (b), lambertianin C (c).

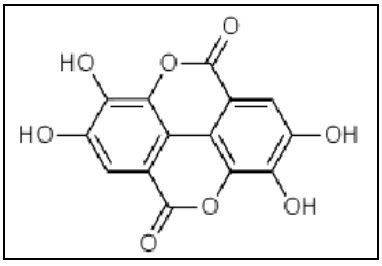

Figure 4. Ellagic acid - the hydrolysis product of ellagitannins.

4. ANTIOXIDANT ACTIVITY

Bioactive compounds, described above, display potential health-promoting effects such as antioxidant, anticancer, anti-inflammatory, and anti-neurodegenerative biological properties. Therefore, the identification of antioxidant activity of raspberry pomace is necessary. Vulić et al. determined the

berry pomace extracts (bilberry, strawberry, raspberry and blackberry) using methods such as DPPH free radical scavenging assay and reducing power. The IC_{50} values were determined using the RSC_{DPPH}. The IC_{50} value is a parameter used to measure the free radical scavenging activity, and can be defined as the extract concentration required for 50% inhibition of DPPH radicals under experimental conditions. The results show that the IC_{50} of the obtained raspberry pomace extract was 0.040 mg/ml. Also, there was observed a high linear correlation between the IC_{50} and the content of anthocyanins, polyphenols and flavonoids. Thus, there is a great importance of phenolic compounds in the radical scavenging activities [23]. The results obtained by Vulić et al. indicate that the reducing power of berry pomace extracts increased with increasing concentration. Berry fruits pomaces are a good source of antioxidant compounds and can be used as a potential value-added ingredient in the food, cosmetic and pharmaceutical industry [23].

4. GENERATION OF RASPBERRY POMACE

In the horticulture, there has been observed a growth in acreage as well as in agricultural production to fulfill the requirements of global food demand. It is estimated that the average worldwide production of fresh fruits and vegetables is 800,000 tons per year [21, 38]. However, in Poland annually about 1.5 million tons of fruit are being produced. Most of them (around 60%) is used for wine, juice and beverage production, 15% for frozen products, and around 15% for marmalade and jams production [39].

During processing of plant materials basic products and by-products are obtained. The latter can be divided into wastes generated during storage, production and manufacturing. A disposal of raspberry pomace, as well as other fruit pomaces, usually represent a serious ecological and environmental problem due to the low pH value. Other emergingproblems are the legal waste stream restrictions which must not be exceeded. Wastes can impede the proper conduct of production due to spoilage, which has to be avoided because of the possibility of microbiological contamination of the process. In the processing of raspberry juice or wines, the pomace becomes a by-product which is currently underexploited. Raspberry waste is prone to microbial spoilage; therefore, drying is necessary before further exploitation. However, the cost of drying, storage, and transport possesses additional economical limitations to waste utilization. Thus, agroindustrial waste is very often utilized as feed or fertilizer [5]. However, there appears some new aspects concerning the use of berry wastes.

5. POSSIBLE USES OF RASPBERRY POMACE IN THE VARIOUS FIELDS OF INDUSTRY

Several potential uses can be considered for raspberry by-products, covering various fields of industry: food, pharmaceutical, medical, cosmetic, composting as well as chemical industry [21].

5.1. Raspberry pomace as antimicrobial agent

In the past few years, due to concerns regarding the safety of synthetic antimicrobial agents, an increase in consumer demand for naturally processed food is observed. It has resulted in a huge increase in the use of naturally derived compounds such as plant extracts as antimicrobials in food. What is more, natural antimicrobial compounds can be an alternative to food preservation [21]. Studies confirmed that phenolics (ellagitannins) which occur in berry pomace, including raspberry, display a very effective role in inhibiting the growth of the pathogenic bacteria: *Clostridium, Enterococcus, Escherichia, Mycobacterium, Salmonella* and *Staphylococcus* species as well as some Gram-positive and Gram-negative bacteria [22, 40]. Puupponen-Pimiä et al. reported that isolated ellagitannin fractions from raspberry were highly efficient against Gram-negative bacteria such as *Staphylococcus aureus* and *Salmonella*, but with no effect on Gram-positive lactic acid bacteria. Raspberry anthocyanins were found to exhibit the strong inhibiting effects on the growth of *L. acidophilus*, a Gram-positive bacterium. It can be important when raspberry anthocyanins are consumed in high concentrations because *L. acidophilusis* is commonly used in fermented milk products [11]. In addition, raspberry can inhibit

the growth of *Bacillus subtilis* and *Micrococcus luteus* [41].

Also, it has been reported that solidstate bioprocessing of cranberry pomace, using food-grade fungus results in an enrichment of the total soluble phenolics and of ellagic acid. Also, it has been confirmed that bioprocessing improved the antimicrobial activities of the extracts against important foodborne pathogens *L. monocytogenes*, *Vibrio parahaemolyticus* and *E. coli* O157:H7. Microorganisms, used in studies, showed different sensitivities to various functional properties of the extracts, which may indicate that different mechanisms of action in the antimicrobial activity exist. Therefore, bioprocessing of berry pomace may offer an innovative solution to produce a broad spectrum of antimicrobials against important pathogens [11].

In this context, raspberry by-products are promising new sources of phenolic antimicrobial compounds [21].

5.2. Raspberry pomace as dietary fibre additive

Until recently, people believed that non-digestible components of plant products belonged to ballast substances. Nowadays beneficial physiological properties of these substances on human health are appreciated. Numerous studies on dietary fibre proved that this component can prevent and treat some diseases. Diet enrichment in fibre reduces risk of certain cancers (large intestine), coronary heart disease (CHD), atherosclerosis, diabetes and obesity. Additionally, dietary fibre increases the faecal bulk, and stimulates intestinal peristalsis, lowers the levels of total cholesterol and low-density lipoprotein cholesterol in the serum. Due to that fact the addition of dietary fibres to food becomes more and more popular [17]. It is well-known that fruit processing waste (raspberry pomace) represents an important source of dietary fibre [42]. Dietary fibre is not only desirable for its technological properties, but also for its nutritional and functional properties. It can be used in order to modify the texture and enhance the stability of the food during production and storage, and to upgrade agricultural products and by-products for the use as a food ingredient [17]. The investigations by Górecka et al. show that the addition of raspberry pomace to shortcrust cookies increase their fruity smell and taste, as well as crumbliness [1].

Due to numerous health benefits of dietary fibre, it can be used for many applications in food and pharmaceutical industry. Dietary fibre fractions from raspberry processing waste can create functional food products. A wide range of fibre-enriched foods included, for instance bakery products, biscuits, cereals, snacks, sauces, dairy products, meat products, drinks [17].

Moreover, it may supplement the daily diet as a prebiotic which is defined as a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improves host health [43]. There are dietary fibre supplements available on the market, both fruit- and vegetable-based, but these mostly contain apple, peach or carrot fibre rather than raspberry fibre. Raspberry pomace fibre may be of a great interest to food technologists. According to literature the exotic fruits such as guava, carambola, mamey, mango, sapodilla and raspberries possess a significant dietary fibre content. Besides, fibre from raspberry waste can be incorporated into food products as inexpensive, non-caloric bulking agents for partial replacement of flour, fat or sugar, as enhancers of water and oil retention and to improve emulsion or oxidative stabilities [21].

5.3. Raspberry pomace as a source of natural colorants

The colour of a food has a major impact on the consumer's behaviour. It influences the priority of purchase and is therefore of great economic value. Increasing consciousness of consumers about healthy lifestyle causes that they prefer natural colorants isolated from fruits, vegetables, herbs and spices rather than unwholesome synthetic ones.

Raspberry pomace has become a significant source of those pigments and colours, mainly anthocyanins and carotenoids which demonstrate high colour stability, good availability, high yield and low price. Currently, natural colorants are received from wastes such as chokeberry, cherry, elderberry, blackberry, red cabbage, red radish, black carrot, and purple sweet potato [21].

5.4. Raspberry pomace as cosmetic and pharmaceutical component

Raspberry seed oil from raspberry by-product is very important for its potential application in food, pharmaceutical as well as cosmetic products. The addition of raspberry seed oil in cosmetics and pharmaceutical products has been patented. Therefore, the unique fatty acid composition and the high tocopherol content, as well as the protective effect against oxidative stress and relatively good shelf life makes oil of raspberry pomace desirable for uses as dietary supplements, in toothpastes, bath oil, shampoos, creams for prevention of skin irritations, aftershave cream, lipsticks, antiperspirants, etc. [9].

5.5. Raspberry pomace as metal-binding agent

It has been noticed that fruit pomaces have the potential for binding heavy metal ions. In particular, fractions from dietary fibre of pomace are able to bind heavy metals [42, 44]. According to literature, hemicellulose and pectins have better binding capacity than cellulose and lignin. Studies report that the stability of metal-dietary fibre complexes differs according to the metal involved and fibre source [45, 46]. In the study conducted by Nawirska pectins were found to be the most effective metal ion binders, and lignins the least effective metal ion binders. As it has been mentioned before, dietary fibre of raspberry pomace consists of 63.16% of lignins, thus has a smaller binding ability. However, it has been noticed that polyphenols bind considerable amounts of lead ions in chokeberry, pear, apple, and rosehip pomace (34.8, 34.0, 35.2, and 26.5%, respectively) [44]. Therefore, raspberry pomace may also be an effective ion binder due to the rich source of polyphenols. It was also reported that tannin compounds (proanthocyanidins or the galloyl ester of glucose) of *Rubus* berries are chelating agents for metal ions such as aluminium, iron, and cooper. These polyphenols at neutral pH form complexes with metal ions and precipitate easily at neutral pH through the gut barrier [22].

6. CONCLUSIONS

To conclude this review, raspberry pomace represents a potential source of natural food ingredients. No major exploitation of this source is happening today, although there is a great opportunity for the food industry in this area.

The exploitation of raspberry pomace during fruit processing as a source of functional compounds and their application in food is a promising field which requires interdisciplinary research. Due to the high nutritional value of raspberry by-products, it can be exploited as food additives or supplements providing the high-valuable products which may be economically attractive for consumers. Raspberry pomace has a great potential as a source of antioxidants and may have important applications in the future. By presenting antibacterial activity, it can be used as a natural antimicrobial agent in the future. Some components of raspberry pomace can be isolated and may be the goal of prospective findings in medicine (therapy) as well as in the food industry.

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TRANSPARENCY DECLARATION

The author declares no conflicts of interest.

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Research Article

Efficiency of consortium for *in-situ* bioremediation and CO_2 evolution method of refines petroleum oil in microcosms study

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ABSTRACT

An *in-situ* bioremediation study was conducted in a laboratory by using mixed microbial consortium. An indigenous microbial consortium was developed by assemble of two *Pseudomonas* spp. and two *Aspergillus* spp. which were isolated from various oil contaminated sites of India. The laboratory feasibility study was conducted in a 225 m^2 block. Six treatment options: Oil alone, Oil+Best remediater, Oil+Bacterial consortium, Oil+Fungal consortium, Oil+Mixed microbial consortium, Oil+Indigenous microflora. Out of five treatments, the mixed microbial consortium (Block 5) degraded 55.12% refine petroleum oil compare to degradation of bacterial (Block 3) and Fungal consortium (Block 4) (i.e, degradation rate were 19.88% and 18.07% correspondingly) after the end of treatment (60 days). Bioremediation ability of this consortium was confirmed by CO_2 evolution method. The result showed that 136.36 % CO_2 release after 12 days incubation. 16r DNA sequencing showed that two bacterial species were *Pseudomonas aeruginosa* and morph taxonomical examination of fungus were *Aspergillus terrus* (At) and *Aspergillus flavus* (Af).The ability of degradation of synthetic mixture of refine petroleum oils makes the consortium potentially useful for bioremediation and microbial enhanced oil recovery.

Keywords: *In-situ* bioremediation; CO_2 evolution method; Microbial consortium; *Pseudomonas aeruginosa*; *Aspergillus terrus*; *Aspergillus flavus*.

1. INTRODUCTION

Large amounts of hydrocarbon contaminants are released into environment as a result of human activities. While release like industrial emission can be controlled and carefully regulated, catastrophic release like major spillage from tankers, pipelines and storage tanks are largely accidental and unavoidable and occur frequently in present times [1]. Oil sludge is carcinogenic and a potent immune-toxicant. Due to industrialization and over use of petroleum hydrocarbon based refinery products are one of the most prevalent pollutants. Oil contamination is a severe threat for our environment and therefore invites general concern. Consequently, the remediation of oil-polluted sites has become an important issue worldwide [2] Bioremediation, the degradation or stabilization of contaminants by microorganisms, is claimed as a safe, effective and economic alternative method of environmental clean-up [3]. In biological treatments it is always necessary to perform laboratory

feasibility tests to determine the microbial potential to degrade the pollutants and to evaluate strategies to optimize the degradation rates before the design of real scale *in-situ* or *ex-situ* (bioreactors, land farming and others) treatments [4, 5]. Thus, the purpose of the present study was to investigate possible methods to enhance the rate of aerobic biodegradation (*ex-situ* treatments) of refines petroleum oil. In this work, the bioremediation processes were applied to a sandy loam soil of Haridwar region, India contaminated by Synthetic Mixture of Refine Petroleum Oil (SMRP Oil) and biodegradation was performed by bioaugmentation (treatment with inoculation of mixed microbial consortium) and study the efficiency of that consortium.

2. MATERIAL AND METHOD

2.1. Source of soil sample

Soil samples were obtained from depths of 0.5 and 1 m as well as form ground surface in a contaminated area close to the storage and Distribution Centre of Oily Products in Indian Oil Refinary, Haldia. (West Bengal) and local garages, refinery waste, petrol pumps, service stations of Kolkata, West Bengal as well as Haridwar city, Uttrakhjand.The petroleum contaminated soil samples were collected in duly labelled sterile container from the depth of 0.5 to 1.0 cm surface and subsurface. Then all samples were transported in ice to the laboratory and stored at 4^0C for further analysis.

2.2. Isolation and screening of indigenous microorganisms

Soil sample were sieved moist using a 2 mm mesh screen and thoroughly mixed. 10 g of soil was added to 95 ml deionised water containing 2 drops of Tween 80 and then was incubated and shake (150 rpm) for 30 min at room temperature. The mixture prepared was called soil solution. A 100 ml Erlenmeyer flask (flask 1) was prepared containing 2.5 ml soil solution and 95 ml of MSM (Mineral Salt Medium) and 2.5 ml of synthetic mixture of refine petroleum hydrocarbons (SMRP) (Petrol, Diesel and Kerosene; 1:1:1) as a sole source of carbon. The flask was incubated at 37^0C. After 15 days, 2.5 ml of Flask 1 was transferred to a second flask (flask 2) with same condition as flask 1. The incubating-transferring were repeated 4 times and at final stage (fourth period) pure hydrocarbon degrading strains were isolated on petroleum agarose plates. In the preparation of petroleum agarose plates 1-2 drops of sterile SMRP oil was evenly spread with glass spreader, so that a film of SMRP oil got absorb over the entire agarose surface of mineral medium in the petriplate and then inoculums was spread on the medium. The plate was incubated at 37^0 C for one week in an incubator. Pure and representative colonies were transferred to slant for preservation.

2.3. Preservation and subculture of the strains

The isolated strains were preserved in 25% v/v glycerol solution at -70^0C. For day-to-day experimentation strains were maintained on nutrient agar slants at 4^0 C in refrigerator and sub-cultured at an interval of 30 days.

2.4. Preparation of consortia inoculum

The bacterial isolates were grown separately in NB and processed to yield separate suspension with an absorbance reading of 0.5 at 550nm. Specific aliquots of bacterial inoculums were then separately added into normal saline solution to give final combined inoculums concentration of 10% (v/v) according to Mukred et al. [6] and used as bacterium consortium. The fungal isolates were cultivated in slant tubes at 30^0C for 6-7 days. The conidia of each strain were suspended in sterile distilled water according to Lemos et al. [7], and produced fungal consortium. All combined bacterial inoculums and conidia-suspension of fungal isolate were thoroughly mixed to prepare final combined inoculums concentration was 10% v/v according to Malik et al. [8].

2.5. *In-situ* microcosms studies

2.5.1. Bioremediation setup

The total area (225 m^2) of the feasibility study was divided into 25 Blocks (four replicate Block

for each treatment and one block was remain undisturbed to check the physic-chemical properties of soil) of a tin vessel. 5 kg of soil (sieved with 2 mm mesh size) from Kanya Gurukula Campus was taken as a normal soil without oil and added 200 g soil in each block separately. In each block 2% SMRP oil was added, thoroughly mixed and left undisturbed for 24 hours to allow the volatilization of the oil. The initial soil pH, temperature, moisture level, organic carbon were also determined over a period of 60 days. The soil used for microcosm study had a pH of 7.78, which was well within the range of optimal degradation. Therefore, no treatment for pH control was needed. The experiment was conducted in the premises of Kanya Gurukul Campus, Gurukul kangri University, Haridwar. The experiment was conducted during March to May months, in the year 2014 and followed the method of Pritchard & Bourquin [9] and Mittal & Singh [10].

2.5.2. Experimental design

The experimental design chosen was a completely randomized block design. The treatment were as follows: (i) oil alone; control where no treatment was done, (ii) Oil+Bacterial consortium, (iv) Oil+Fungal consortium, (v) Oil+Developed consortium, (vi) Oil+Indigenous micro flora.

2.5.3. Extraction of SMRP oil sample

For extraction of SMRP oil broth culture was first taken out and the culture activities were stopped by adding 1% 1N HCl and then the extracted SMRP oil broth was mixed with 50 mL petroleum ether: acetone (1:1) in a separating funnel and was shaken vigorously to get a single emulsified layer. Acetone was then added and shaken gently to break the emulsification, which resulted in three layers. Top layer was a mixture of petroleum ether, SMRP oil and acetone; clumping cells make the middle layer and the bottom aqueous layer contains acetone, water in soluble form. The lower two layers were separated out while top layer containing petroleum ether mixed with SMRP oil and acetone was taken out in a clean bottle.

2.5.4. To assess the hydrocarbon degradation

To assess the rate at which the SMRP oil was being degraded, sample were collected at time zero (just before initiating the bioremediation), 15 days later, 30 days later, 45 days later and at the end of the study (60 days after initiating the process). After evaporation the residual oil content was determined by the absorbance of the extract at 420 nm in a spectrophotometer.

2.5.5. Fraction of SMRP oil and analysis of fractions

For isolation of various compound class fractions (saturate, aromatic and NSOs), column chromatographic technique was used. The separation was carried out on packed activated silica-gel columns by successive elution with petroleum ether for saturates, benzene for aromatics and methanol for NSO compounds. Silica gel (60-120 mesh) was activated at 150^0C for 24 hours and cooled in desiccators. The glass column (Internal diameter 1.1 cm, length 65 cm and reservoir capacity 100 ml) was packed by placing a thin cotton plug at the bottom. The slurry of 20 g activated silica gel was filled. The column was washed with petroleum ether. The extracted oil sample was dissolved in chloroform, absorbed on silica gel. The adsorbed sample was charged at the top and eluted saturates with 10 ml of petroleum ether (40^0-60^0C), aromatics with 10 ml benzene and NSO with 10 mL methanol, respectively. Each fraction was transferred in air tight bottle and taking O.D at 420 nm in a spectrophotometer [11].

2.6. Laboratory scale experiment on the bioremediation of refined petroleum hydrocarbon by using CO_2 evolution method (Standardized Biodegradability Tests-ASTM D-5864)

A specially equipped 150 ml Erlenmeyer flask contains 50 ml of optimized BH broth and 5 ml of SMRP oil. A reservoir holding 10 ml of barium hydroxide solution was suspended out of the flask to trap CO_2. After inoculation(10% v/v), the test flasks were sparged with CO_2 free air (flasks were aerated with compressed air that had been scrubbed free of CO_2 by passage through a series of

three 250 ml bottles each containing 200 ml of 5N NaOH) to ensure aerobic conditions and that CO_2 was trapped only from microorganism's metabolizing the test substrate. The flasks were sealed and incubated with shaking in a dark room for 0, 4, 8, 12, 16, 20, 24, 28 days under normal temperature (32 to 37^0C), pH (7) condition because as per ASTM method [12], the test shall continue for at least 28 days or until the CO_2 evolution has reached a plateau. Non inoculated flasks were included as control for abiotic losses [13].

2.7. Measurement of CO_2 evolution

Periodically, the 10 ml of $Ba(OH)_2$ a plus 10 ml rinsing water (DW) was removed for CO_2 measurement by titration with 0.1 N HCl to the phenolphthalein end point. All the samples were analyzed at time zero and at least a 28 day time period to allow for a smooth biodegradation plot for test system (ASTM D-5864). 3 ml of 20% H_2SO_4, were added on the day prior to terminating periodically.

The percentage CO_2 evolution-was based on the following formula:

$$\% \, CO_2 \text{ evolution} = \frac{TF\text{-}CF}{C} \times 100\%$$

Where,

TF = ml of 0.1 N HCI required to titrate Ba $(OH)_2$, samples from the test flask;

CF = ml of 0.1 N HCl required to titrate Ba $(OH)_2$ samples from the control flask;

C = A constant which is equal to the theoretical amount of 0.1N HCl required to titrate the CO_2 evolved from metabolizing total carbons in the test substrate by bacteria. For example, for 10 mg carbon: C = 16.67 ml of 0.1N HCl.

2.8. Molecular characterization of best petroleum remediating microorganisms

Bacterial strains of the consortium were identified by 16S rDNA sequence structure performed by Royal Life Sciences Pvt. Ltd. (Affiliated to MIDI Sherlock, USA) and fungal cultures were identified as morph taxonomically from Agharkar Research Institute, Pune, India (National Fungal Culture Collection of India).

3. RESULT AND DISCUSSION

The first and foremost criterion for designing a bioremediation program was to study the native micro flora of the system and to analyses the physico-chemical composition of soil. The soil sample taken for feasibility study (garden soil of Kanya Gurukula Mahavidyalaya, Haridwar) was analysed for detection of different treatment boxes. The soil contains $6x10^8$/ g of total heterotrophs and $1.2x10^3$/ g of fungi. The pure hydrocarbon utilizing bacteria as well as fungus were identified in presence of SMRP Oil as a carbon source. Two bacterial and fungal strains were identified by using standard procedures. The experimental outcome of cultural, morphological and biochemical characterization proved that both bacterial species were *Pseudomonas aeruginosa* (Ps-I and Ps-II) (Tables 1 and 2, Fig. 1) and two fungal species were identified as *Aspergillus flavus* (Af) and *Aspergillus terrus* (At) (Table 3, Fig. 2). All were used to make the consortium. *In situ* bioremediation approach was adopted in laboratory conditions. Physical and chemical properties of soil sample taken were analysed for pH, temperature, and moisture level, organic carbon (Table 4) in remaining block at zero time intervals i.e, initially and after 60 days of optimal value of pH was 7 and temperature 32^0C to 35^0C for maximum degradation.The soil used was sandy loam in texture and its pH was 7.78. Moisture content of soil was 5 %, while water holding capacity was 50. The temperature recorded during the study varied from 32^0C to 35^0C.

Since maintenance of temperature in open soil system was not feasible, the bioremediation efforts should be concentrated during such a period of year when the temperature was suitable for treatment. Verstraete et al. [14] reported that a doubling rate of biodegradation of gasoline in an acidic (pH 4.5) soil by adjusting the pH to 7.4. Extremes in pH were shown to have negative influence as well as at low temperature the viscosity of oil was increased, volatilization of alkanes reduced, so the degradation was affected. Rhaman et al. [15] reported 30^0C to 40^0C was the normal temperature for petroleum hydrocarbon degradation.

Table 1. Cultural and morphological characteristics of selected bacterial isolates.

Code no of isolate	Size	Shape	Elevation	Margin	Opacity	Texture	Pigment	Gram's reaction	Motility
Ps-I	Big	Irregular	Slightly raised	Irregular	Opaque	Smooth	Deep Green	-ve rod	motile
Ps-II	Big	Irregular	Slightly raised	Irregular	Opaque	Smooth	Brownish green	-ve rod	motile

Table 2. Biochemical characterisation of bacteria.

Name of biochemical test	Code number of isolates	
	Ps-I	Ps-II
Citrate utilization	+	+
Urease production	+	+
Nitrate reduction	+	+
Oxidase	+	+
Catalase production	+	+
Gelatin Utilization	+	+
Starch Hydrolysis	-	-
Indole production	-	-
M.R test	+	+
V.P test	-	-
Lipid hydrolysis	+	+
Glucose utilization	+	+
Sucrose utilization	_	_
Mannitol utilization	+	+
Lactose utilization	+	+
Maltose utilization	+	+

Table 3. Cultural and size and shape of spore of fungal isolates.

Code no of isolate	Colour of the colony	Appearance of the colony
At	Brown	Brownish in colour and gets darker as it ages on culture media
Af	Green	Conidial heads were typically radiate, later splitting to form loose columns, biseriate but hading some heads with phialides borne directly on the vesicle.

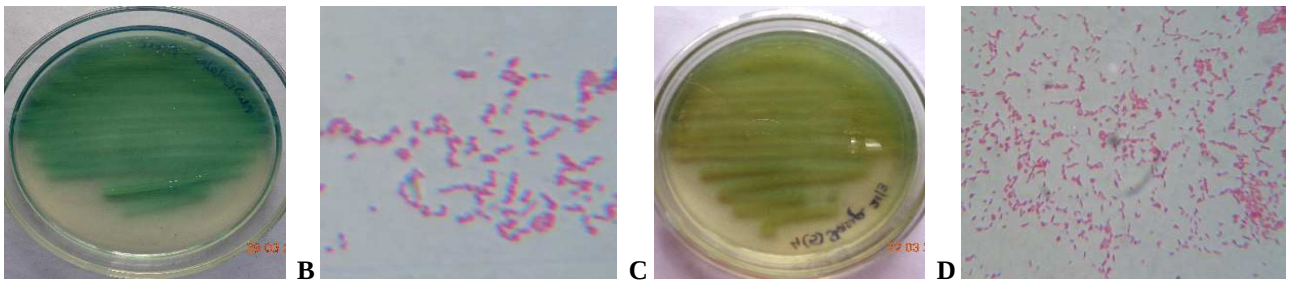

Figure 1. Showing colony colour on plates and microscopic view of Ps-I (A and B) and Ps-II (C and D).

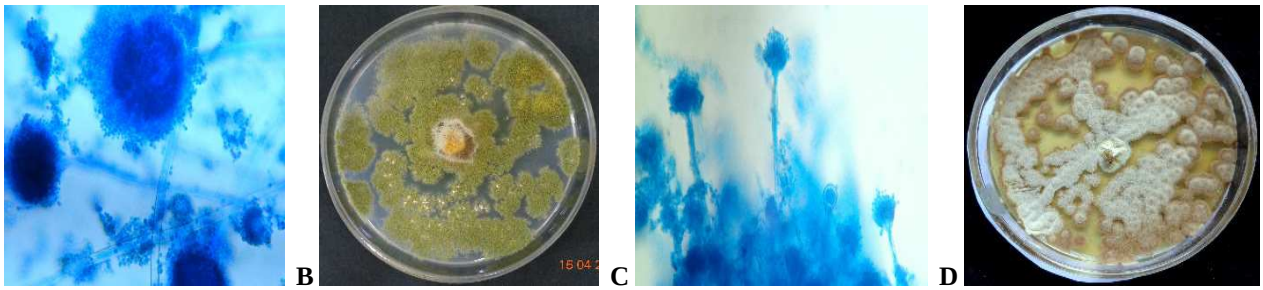

Figure 2. Macroscopic and microscopic appearance of At (A and B) and Af (C and D).

Table 4. Physico-chemical parameters of soil of *in situ* bioremediation process.

Days	Time zero*	After 60 days*
Temperature	32^0C to 35^0C	32^0C to 35^0C
pH	7.7	7.30
Moisture contain	5%	5.6
Organic carbon	2.35	2.43

*= Average of triplicates

SMRP Oil was extracted from six treatment boxes- Oil alone, Oil and best remediating microorganisms, Oil+ bacterial consortia, Oil+fungal consortia, Oil+microbial consortium, Oil+indegenious micro-flora and assessment the degradation rate.

3.1. Block 1: Oil alone

The hydrocarbon utilizing bacteria count during the study was also found nil, though some heterotrophic bacterial activity in an open environment was not possible. It was because the soil was sterilized and 2% $HgCl_2$ treatment also arrested the soil microbial activity. The minimum loss of SMRP Oil (6.02%) can be attributed to the abiotic losses like evaporation of low volatile fraction of SMRP Oil and photo-oxidation etc. (Table 5).

3.2. Block 2: Oil+ best remediater

The Box 2 which shows the bioremediation of best remediater (Ps-I). The absorbance of SMRP Oil decreases from 0.332±0.002 to 0.292±0.004 O.D value which means 12.05% extracted SMRP Oil was degraded within 60 days. Initially within 15 days rapid degradation was occurred (i.e 6.93%), then degradation rate was decreased (Table 5).

3.3. Block 3: Oil+Bacterial consortium

The Box 3 containing bacterial species of Ps-I +Ps-II. Both *Pseudomonas* spp. degraded extracted SMRP oil 19.88% within 60 days which means that presence of Ps-II enhanced the degradation rate. After 30 days degradation rate was moderately increased (Table 5).

3.4. Block 4: Oil+ Fungal consortium

At and Af were formed fungal consortium; they degrade extracted SMRP Oil near about same as bacteria consortium. They degraded extracted SMRP Oil 18.07% within 60 days. But in case of fungal consortium till 45 days degradation rate was increased equal proportion but after 45 days (12.65) degradation rate was increased rapidly (18.07) (Table 5).

3.5. Block 5: Oil+ Mixed consortium

Both fungus and bacteria degraded extracted SMRP Oil 55.12% within 60 days which means mixed microbial consortium degrade more, than individual consortium. (Table 5) In case of mixed microbial consortium rapid degradation occurred within very short period of time (i.e 15 days) (43.07%) but after then the degradation rate moderately increased.

3.6. Block 6: Oil+ Indigenous microflora

Box 6 shows degradation of indigenous microflora from 2.17 to 6.63 % within 60 days which means that all soil contain hydrocarbon degrading microorganisms but a mixed microbial consortium enhanced the degradation rate that why it was essential to make a consortium which was

needed for effective bioremediation (Table 5).

3.7. Fraction of SMRP Oil and analysis of fractions

Effect of biodegradation on alkane, aromatics and NSO = asphaltene fractions by best remediator, bacterial consortium and fungal consortium and mixed microbial consortium had been studied for 60 days. Effect was seen at time interval of 15 days (Table 6). These fractions were separated by column chromatography. At the end of 60 days it was observed that mixed microbial consortium metabolized 40.42% alkanes, 36.15% aromatics fraction whereas Ps-I degraded 9.04% alkane and 4.22% aromatic hydrocarbon, Bacterial consortium degraded 12.23% alkane and 8.45% aromatic hydrocarbon and fungal consortium degraded 7.98% alkane and 7.04 % aromatic fraction. From above result it was cleared that fungal consortium degraded near about equal proportion of alkane and aromatic fraction as bacterial consortium.

Assessment of CO_2 production by consortium confirmed that the utilization of refine petroleum hydrocarbon fraction as a source of carbon and energy by the microbial community. It was a Standardized biodegradability test (ASTM 5864) which also previously used by various scientists to calculate biodegradation efficiency [5, 13, 16]. Carbon di oxide production in SMRP oil of the control ranged from 0% to 30.30% while % of CO_2 evolves in SMRP oil ranged from 0.05% to 136.36% shown in Table 7. There was a progressive increase in the amount of CO_2 produced for the first 12 days, after which CO_2 production decreased. Large amounts of CO_2 were liberated in SMRP oil than in control oil. The progressive increased in the amount of CO_2 evolved in the incubated oil in the first 12 days was an indication of the utilization of petroleum hydrocarbon fractions as a source of carbon and energy by the microbial community. Respiration of microbes occurred very rapidly during the initial period of incubation when the lighter and more readily degraded fractions were degraded but slowed down as the residue become more difficult to degrade on account of the increase of the heavier fractions.

Table 5. Percent (%) of degradation of SMRP Oil through microcosms study.

Treatment	SMRP Oil (Synthetic Mixture of Refine Petroleum Oil)										P-value Prob> F$
	Initial O.D at 420 n.m		Final O.D at 420 n.m								
	0		15*		30*		45*		60*		
	O.D	% of D	O.D	% of D	O.D	% of D	O.D	% of D	O.D	% of D	
Control	0.332 ±0.002	0	0.325 ±0.007	2.10	0.322 ±0.012	3.61	0.314 ±0.018	5.42	0.312 ±0.02	6.02	0.0029
Ps-I (Best remediator)	0.332 ±0.002	0	0.309 ±0.002	6.93	0.302 ±0.003	9.04	0.299 ±0.002	9.94	0.292 ±0.004	12.05	0.0001
Ps-I+Ps-II (Bacterial Consortium)	0.332 ±0.002	0	0.298 ±0.003	10.24	0.292 ±0.002	12.05	0.276 ±0.052	16.87	0.266 ±0.002	19.88	0.0005
At+Af (Fungal consortium)	0.332 ±0.002	0	0.302 ±0.007	9.04	0.297 ±0.001	10.54	0.290 ±0.002	12.65	0.272 ±0.006	18.07	0.0007
Ps-I+Ps-II+At+Af (Mixed Microbial Consortium)	0.332 ±0.002	0	0.189	43.07	0.172 ±0.008	48.19	0.166 ±0.107	50	0.149 ±0.002	55.12	1.0952e-06
Ind Microbs.	0.332 ±0.002	0	0.323 ±0.004	2.71	0.260 ±0.005	4.22	0.25 ±0.10	6.02	0.242 ±0.002	6.63	0.0140

***=** Average of triplicate, **$=** significant only when the calculated F value was greater than the table F value at P is less than or equal to 0.05.

Table 6. Effect of microbial degradation on various fraction of SMRP Oil (Synthetic Mixture of Refine Petroleum Oil).*=Average of triplicates.

Treatment	Incubation Period (Days)	SMRP Oil (Synthetic Mixture of Refine Petroleum Oil)				
		Alkanes		Aromatic		NSO+Asphalt
		R*	D	R*	D	R*
***Pseudomonas* sp.-I**	**0**	0.188	0	0.213	0	0.112
	15	0.180	4.25	0.209	1.88	0.109
	30	0.176	6.38	0.208	2.35	0.105
	45	0.173	7.98	0.206	3.29	0.102
	60	0.171	9.04	0.204	4.22	0.101
***Pseudomonas* sp.-I+*Pseudomonas* sp.-II**	**15**	0.178	5.32	0.204	4.23	0.110
	30	0.172	8.51	0.200	6.10	0.111
	45	0.171	9.04	0.198	7.04	0.109
	60	0.165	12.23	0.195	8.45	0.109
Aspergillus terrus* +*Aspergillus flavus	**15**	0.180	4.25	0.208	2.35	0.111
	30	0.178	5.32	0.204	4.23	0.110
	45	0.174	7.45	0.200	6.10	0.110
	60	0.173	7.98	0.198	7.04	0.101
Consortium (Bacteria+ fungus)	**15**	0.146	22.34	0.170	20.19	0.105
	30	0.133	29.25	0.159	25.35	0.098
	45	0.122	35.10	0.151	29.11	0.088
	60	0.112	40.42	0.136	36.15	0.085

*=Average of triplicate

Table 7. Percent (%) of CO_2 evolves from SMRP (Synthetic Mixture of Refine Petroleum Oil) oil by fungus-bacterium consortium. *=Average of triplicate.

Treatment period (days)	% of CO_2 evolves in Control*	% of CO_2 evolves in Consortium*	P value Prob> F[@]
0	0	0.05	0.0160
4	4.32	47.62	
8	6.49	106.06	
12	12.98	136.36	
16	15.15	134.19	
20	30.30	32.46	
24	6.49	28.13	
28	2.16	19.48	

The use of autochthonous microorganisms inhabiting hydrocarbon polluted niches for bio-degradation and bioremediation has been widely accepted as a formidable approach due to avalanche of successes recorded by various researchers. The mechanisms of adaptation employed by the autochthonous microorganisms to achieve this feat includes synthesis of inducible enzyme, mutations such as single nucleotide change or DNA-rearrangement that results in degradation of the

compound and acquisition of genetic information from closely related or phylogenetically distinct population within the hydrocarbon-challenged community through horizontal gene transfer [17]. Here in this study the mix microbial consortium degrade 40.42% saturated and 36.15% aromatic hydrocarbon present in the refine petroleum oil. Refine petroleum oil contains many kinds of hydrocarbon, resins and asphaltenes. Muthuswamy et al. [18] reported that mixed population had a broad enzymatic capacities which enhanced petroleum degradation. This mixed culture had a metabolic versatility over to pure culture. Due to presence of bacterial and fungal species which synthesize the degradative enzymes for different parts of the decomposition pathway is considered to be well suited to the refine petroleum degradation. Vasudevan [19] and Rahman et al. [15] had illustrated the ability of mixed microbial consortia to degrade 28 to 51% saturated and 0 to 18% of aromatic present in crude oil or upto 78% crude oil. Microorganisms not directly involved in the degradation process also probably play a role by producing micronutrients or surface-active agents for the solubilization of aromatic hydrocarbons [20]. Various organisms had the capability of degrading various forms of hydrocarbons and thus when a consortium of these microbes was applied to degrade various forms of hydrocarbons in a single source like refine oil; the total degradation was more effective This result made it obvious that the metabolic capability of the consortium was not restricted to one type of refine oil. But such type of result could not expect from pure cultures which were substrate specific. Hasanuzzaman et al. [21] observed 75 and 85% degradation of total crude oil by *Pseudomonas aeruginosa* strain at 20 and 30^0C, respectively. Since in this study all isolates were mesophilic in nature, they all exhibited optimum activity at 32^0C-35^0C. Increase in crude oil concentration decreased the percent degradation but an increase in the quantity of crude oil degradation was noticed. Zhang et al. [22] reported 58 and 60% degradation of crude oil with the initial concentration of 0.7 g/l in mineral salt medium by *P. aeruginosa* in the presence of 1 g/l glycerol and 0.22 g/l rhamnolipids, respectively, used as emulsifiers. Tzarkova and Groudeva [23] reported that compounds such as saturates, aromatics, and polar compounds present in different crude oil samples were degraded to different degrees by the same organisms. The degradability was not solely determined by the chemical structure but other factors as well. The bioavailability of these compounds in different crude oil samples might differ. Saturated compounds with molecular weight larger than 500 might not be degraded by the organisms, because this size corresponds to the exclusion size for passage through the outer membrane of Gram-negative bacteria [24]. Generally, it was believed that microbes preferably degrade/metabolize C_8–C_{15} n-alkanes followed by C_{16}–C_{36} n-alkanes due to the simplicity of these hydrocarbons. Saturated, cyclic high-molecular weight compounds like hopanes are usually not attacked by the microbes due to their complexity. Although it was not possible to specifically emphasize the metabolic pathway of degradation by individual microbes and microbial consortium without complete characterization of the refine oil before and after degradation, from the percent degradation of the total refine oil content it was conclude that the bacterial consortium and fungal consortium and mixed microbial consortium had the capability of degrading a wide range of hydrocarbons. Due to the highly complex nature of the refine oil, it was very difficult to understand the degradation mechanism especially for aromatics. The effectiveness of bio-augmentation (i.e mixed microbial consortium such as bacterial and fungal) was observed in this study. Before there were very little research [25] was conducted with the consortium that was made with bacteria and fungus. After performing 16sr DNA sequencing, sequence aligned with NCBI database gave 98% similarity of Ps-I and 92.6% similarity with Ps-II with Pseudomonas *aeruginosa* and morphologically Af & At identified as *Aspergillus flavus* and *Aspergillus terrus*, correspondingly. The result presented here will be particularly useful in choosing strains for environmental application involving the implantation of microorganism in the soil matrix (bioaugmentation). As contaminated sites usually contain heterogeneous hydrocarbon, it is promising to use for bioaugmented clean-up strains with broad abilities to grow on different hydrocarbons. For this purpose a model consortium including isolates *Pseudomonas aeruginosa* I and II

and *Aspergillus terrus* and *Aspergillus flavus* were proposed for refine petroleum hydrocarbon waste treatment of soil environment. Hence it was suggested that the use of above mixed microbial consortium would be an effective and eco-friendly technology for degradation of refine hydrocarbons.

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AUTHORS' CONTRIBUTION

All the authors contributed equally for the success of this research. The final manuscript has been read and approved by all the authors.

TRANSPARENCY DECLARATION

The authors declare no conflicts of interest.

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Review Article

Natural flavonoids: classification, potential role, and application of flavonoid analogues

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ABSTRACT

Nowadays, it is assumed that natural flavonoids occurring in fruits and plant derived-foods are relevant, not only for organoleptic properties or technological reasons, but also because of their potential health-promoting effects, as suggested by the available experimental and epidemiological studies. This large group of phenolic plant constituents can be divided into several classes: flavanols, flavanones, flavonols, isoflavones, flavones and anthocyanins depending on the differences in their structures.The beneficial biological effects are also attributed to flavonoid analogues and their metal complexes. These compounds are characterized by antioxidant, pharmacological, anti-inflammatory, anti-allergic, antiviral, anticarcinogenic, as well as therapeutic and cytotoxic properties. Furthermore, they possess a wide range of applications including various fields of industry.

Keywords: Flavonoids; Flavonoid analogues; Application; Properties; Medicine.

1. INTRODUCTION

For many years, increasing attention is paid to the presence of bioactive compounds in the diet favorably affecting the human body [1]. Biologically active substances contained in the food considerably reduce the risk of lifestyle diseases (diabetes, arteriosclerosis, cataracts, Alzheimer's disease, Parkinson's disease) [2]. These substances include polyphenolic compounds, which are characterized by high antioxidant activity, and hence antiviral, anti-inflammatory and anticancer [3]. Polyphenols are substances commonly found in plants and belong to the basic elements of the diet. One of the most famous groups of polyphenols are flavonoids [4, 5]. These compounds are mainly accumulated in the edible parts of plants, particularly in fruits and vegetables. Flavonoids are responsible for red and dark blue color of berries, as well as orange and yellow coloring citrus fruits. In the human body they play a similar role as vitamins [6, 7].

Flavonoids with biological activity are often called bioflavonoids. They possess the ability to capture superoxide, hydroxyl and lipid radicals [8]. Flavonoids have a long history of medicinal use, mainly for support of healthy capillary and blood vessel function. They are marketed as anti-inflammatory and anti-spasmodic remedies [9]. What is more, flavonoid analogues and their metal complexes play a significant role in agriculture, industrial and pharmaceutical chemistry [10].

Flavonoids are divided to several subgroups, and it is important and should be mentioned that the biological and chemical properties of flavonoids

belonging to different subgroups can be quite different [11].

This review will present the most important and valuable properties of natural flavonoids and its analogues, namely: antioxidant, pharmacological, anti-inflammatory, anti-allergic, antiviral, anti-carcinogenic, as well as therapeutic and cytotoxic properties. What is more, the most noteworthy applications of flavonoids will be also included. These applications will contain various branches of industry, including agriculture, skin protection, potential clinical applications, as well as prospects for the metabolic engineering of bioactive flavonoids.

2. FLAVONOIDS AND ITS POTENTIAL ROLE

2.1. Classification and structure

Flavonoids belong to a large group of phenolic plant constituents [11]. They are presented as derivatives of 2-phenyl-benzo-γ-pyrone. The carbon atoms in flavonoid molecules are assembled in two benzene rings, commonly denoted as A and B, which are connected by an oxygen containing pyrene ring (C). A common part in the chemical structure of all flavonoids is carbon skeleton based on flavan system (C_6-C_3-C_6) (Fig. 1) [12]. Condensation of A and B ring leads to the formation of chalcone, which undergoes cyclization involving isomerase and formed flavanone - initial compound for the synthesis of flavonoids other groups.

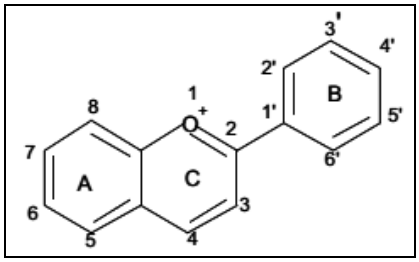


Figure 1. The structure of flavylium cation.

Due to the differences in the structure of flavonoid compounds, flavonoids are classified as flavanols, flavanones, flavonols, isoflavones, flavones and anthocyanins (Fig. 2). Among other flavonoid compounds can be also included compounds such as biflavonoids (e.g. ginkgetin), prenylflavonoids, flavonolignans (e.g. silybin), glycosidic ester flavonoids,chalcones and proanthocyanins [13].

2.2. Flavanols

Flavanols constitute a greatly complex group of polyphenols in the range from the monomeric flavan-3-ols (e.g. catechin, epicatechin, gallocatechin) to polymeric procyanidins known as condensed tannins [14]. Flavanols mainly occur in fruits and derived products, for example fruit juices or jams. This group also appears in tea, red wine, cocoa, apples, kiwi and cereals. However, they almost do not exist in vegetables and legumes except lentils and broad beans. Flavanols can be found in peels or seeds of fruits and vegetables as well, which are often removed during eating or processing, therefore their intake is also limited [15].

It is confirmed that flavanols can stimulate the levels of nitric oxide in the blood of smokers and reverse some of their smoking-related impairment in blood vessel function. Researchers from Germany have shown significant increases in circulating nitric oxide and flow-mediated dilation after ingestion of drinks containing 176-185 milligrams of flavanols (dose potentially exerting maximal effects). These changes are correlated with growth in flavanol metabolites. Dr. Heissfrom American College of Cardiology strongly believed that chronic consumption of flavanol-rich foods leads to sustained increases in endothelial function or the prevention of future cardiovascular ailments [16].
Catechin is the most important representative of the group of flavanols. Catechins are known as the major building blocks of tannins. These compounds may be found in the seeds and skins of fruits which are not fully ripened. Several types of catechins can be distinguished: catechin, gallocatechin, catechin 3-gallate, gallocatechin 3-gallate, epicatechin, epigallocatechin, epicatechin 3-gallate, epigallocatechin 3-gallate (Fig. 3). The main sources of catechin are green and black tea, red wine, chocolate, apricot, apples, peach, red raspberry, and blackberry [17].

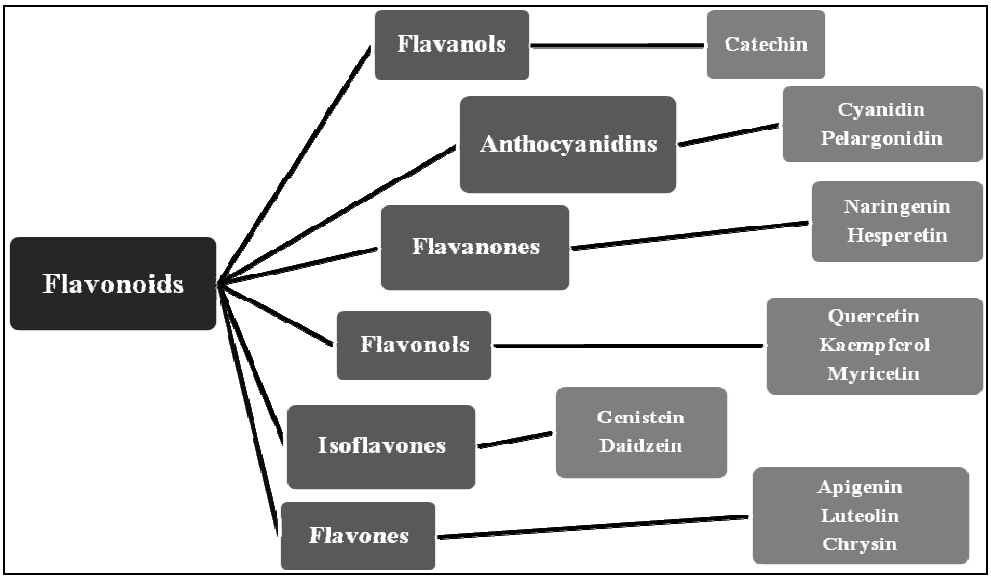


Figure 2. Distribution of flavonoids commonly occurring in plants.

Catechin prevents protein oxidation by its free radical scavenging capacity. Furthermore, it possesses ability to reduce covalent modification of protein induced by ROS or by-products of oxidative stress [18].

Additionally, catechin exhibits anti-atherosclerotic properties. It has been shown the inhibition of the oxidation of low-density lipoprotein (LDL), endothelin reduction and block the platelet aggregation [19, 20]. Catechins have also revealed anti-carcinogenic activity. Epigallocatechin 3-gallate may inhibit urokinase which is u-plasminogen activator. This enzyme is often expressed in human cancer cells. Catechins can restrain cell proliferation and induce apoptosis, as well as modulate and inhibit the NFKB activity [17].

Likewise, catechin polyphenol seems to be an effective promoter of thermogenesis [21]. Furthermore, catechin and epicatechincan act as enzymes. They also play a crucial role in defense against pathogens of tea. [22]. In turn, green tea catechins have been presented to possess antibiotic effects because of their function in disrupt during the bacterial DNA replication process [23]. Black tea catechins have antidiabetic properties. It is well-known that black tea have the highest α-amylase and α-glucosidase inhibitory activity [24]. It has been also suggested that catechin causes the increase of insulin activity, but there is no evidence enough to confirm this state [25].

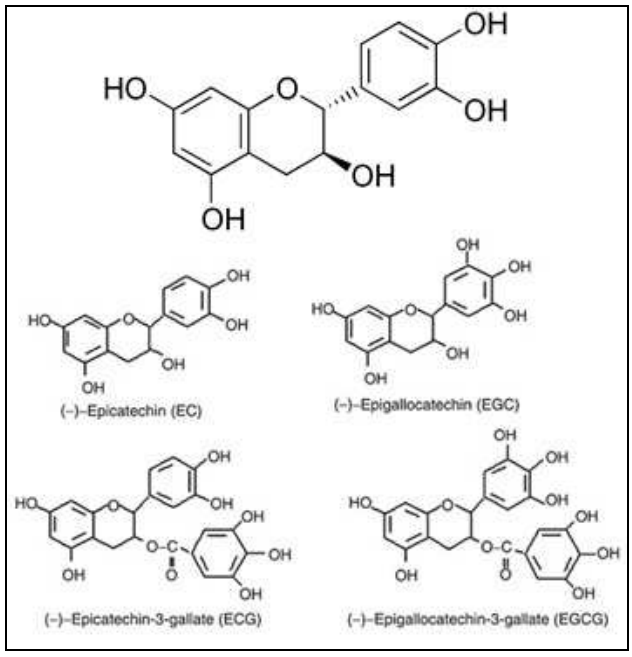


Figure 3. Chemical structure of green tea catechin.

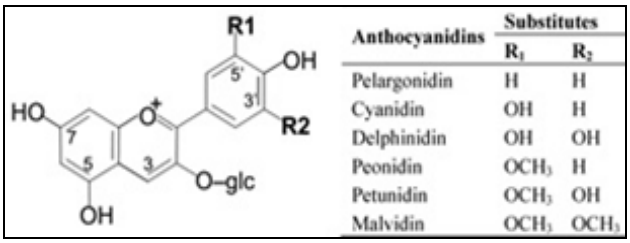
2.3. Anthocyanidins

Anthocyanidins are a group of phyto-chemicals, as natural pigments are responsible for blue, red, purple and orange colors present in many fruits and vegetables, as well as in many fruit- and vegetable-based food products. Over and above 500 different anthocyanidins are known and have been described in literature [14, 26]. This flavonoid group dominates in teas, honey, fruits, vegetables, nuts, olive oil, cocoa and cereals. They can be also found in berries (e.g. black currant, blueberries,

strawberries, elderberries), their juices, as well as red wine [27].

Anthocyanidins have appeared as aglycone form which is structurally based on the flavylium or 2-phenylbenzopyrilium cation possessing hydroxyl and methoxyl groups present at different positions of the basic structure [28].

The most common anthocyanidins occurring in fruits and vegetables are: cyanidin, pelargonidin, delphinidin, malvidin, petunidin and peonidin. These compounds depend on the number and position of the hydroxyl and methoxyl groups as substituents (Fig. 4) [29].



Anthocyanidins	Substitutes	
	R_1	R_2
Pelargonidin	H	H
Cyanidin	OH	H
Delphinidin	OH	OH
Peonidin	OCH_3	H
Petunidin	OCH_3	OH
Malvidin	OCH_3	OCH_3

Figure 4. Structures of the most well-known anthocyanidins in plant-derived foods.

Anthocyanidins have been revealed to play an essential role in cardiovascular disease, cholesterol decomposition, visual acuity, as well as antioxidant efficacy, and cytotoxicity [30].

Anthocyanidins are able to act on different cells participating in the development of atherosclerosis. These compounds have been demonstrated to have protective effect against TNF-α induced MCP-1 (chemokine monocyte chemotactic protein 1) excretion in primary human endothelial cells. MCP-1 is one of the direct reasons of atherogenesis [31]. Anthocyanidins, mainly delphinidin and cyanidin have been proved to prevent expression of vascular endothelial growth factor (VEGF), which is stimulated by platelet derived growth factor in vascular smooth muscle cells by preventing activation of p38 mitogen-activated protein kinases (p38 MAPK) and c-Jun N-terminal kinase (JNK) [32].

Edible berries are supposed to have anti-angiogenic properties. Angiogenesis is a term used to describe formation of new blood vessels. It is especially undesirable in situations including tumor formation or varicose veins due to the fact that it provides food for tumor growth and cancer metastases [33].

It should be mentioned that anthocyanidins possess influence on cholesterol distribution through protection of endothelial cells from CD40-induced proinflammatory signalling [34].

Many studies strongly suggested a significant relationship between improved visual acuity with anthocyanin consumption. Particular attention should be paid to enhancement of rhodopsin regeneration by which anthocyanidins enhance visual acuity. The greatest effects are assigned to compounds from black currant [35].

Furthermore, it has been demonstrated that addition of bilberry anthocyanidins is able to dissolve the toxic intermediates and fibrils, and, thus the toxicity of the intermediates was hence neutralized [36].

Principal therapeutic benefits attributable to anthocyanidins include antioxidant protection. Free radicals damage lipids and proteins and menace DNA integrity. Antioxidants are intense scavengers of free radicals and correspond to inhibition of neoplastic processes. Anthocyanidins protect DNA integrity and bolster tissue antioxidant levels [30].

2.4. Flavanones

Flavanones are extensively disseminated in around 42 larger plant families, especially in *Compositae*, *Leguminosae* and *Rutaceae*. Depending on the type of plants, flavanones can be discovered in all parts of plants - above and below ground, from vegetative part to generative organs: branches, bark, stem, leaves, roots, flowers, fruits, seeds, rhizomes, peels etc. Due to the high spread of flavanones in foods, naringenin and hesperetin-aglycones (Fig. 5) seem to be of particular interest [37]. Hesperetin (4'-methoxy-5,7,3'-trihydroxy-flavanone) is distinctive flavanone of lemon, orange, lime and tangelo [38]. Naringenin (5,7,4'-trihydroxyflavanone) can be found in grapefruit and sour orange. Tomatoes and their products are also rich in this flavonoid. Naringenin can be described both as aglycone or glycosides [39].

Flavanones belong to the flavonoid compounds frequently found in the plant world, constituting the daily human diet, as well as medicinal plant materials [41]. The main directions

of the pharmacological activity of flavanones are: radical scavenging, anti-inflammatory, anticancer, cardiovascular, and antiviral effects [37].

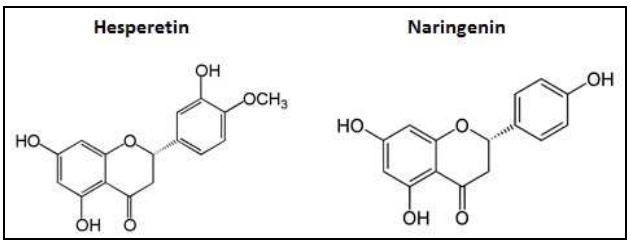


Figure 5. Structure of flavanones in the aglycone forms.

The antioxidant activity of flavanones depends on the number and spatial location of phenolic OH groups. Flavanones show a higher antioxidant activity in a hydrophilic environment. This environment causes the reduction of antioxidant potential by some flavanones (hesperetin, neohesperidin) while others (narirutin, naringin, naringenin) become pro-oxidant. Generally, widespread dietary flavanones which do not possess catechol nucleus are classified as weak antioxidants and their metabolites are supposed to be even less strong. Thereby, the most meaningful mechanisms involved in their health effects must be unrelated to their antioxidant activity [42].

Naringenin flavanones are very efficient in inhibition of pro-inflammatory cytokines induced by lipopolysaccharide in macrophages and reduced production of nitrate and nitrite which are indicators of inflammatory process to control the formation of intestinal edema [43, 44].

Flavanones have not been extensively studied for their anticancer properties. However, the major citrus flavanones may have potential in working against carcinogenesis by minimizing DNA damage, tumor proliferation and development [45]. Flavanones, mainly naringenin, show antimutagenic activity manifested in the protection against DNA damage by their capacity to absorb UV light. The moderate antioxidant capacity of flavanones is found helpful in protecting against mutation by free radicals generated nearly DNA. It is confirmed that naringenin participates in presenting antimutagenic changes by stimulating DNA repair, following oxidative damage in human prostate cancer cells [46]. The pharmacological importance of flavanones may also be estimated by their effect against tumor development. It is confirmed the influence of hesperetin and naringenin on the development of breast cancer induced by 7,12-dimethylbenz-anthracene in female rats [47]. Furthermore, flavanones present an important antiproliferative activity against prostate, breast, colon, lung and melanoma cancerous cell lines [48].

Flavanones are believed to have anti-atherosclerosis potential. The studies demonstrated the reduction of atherosclerosis in mice fed with high fat-high cholesterol diet using naringenin supplementation at nutritionally relevant level. This result could be exerted to improve dyslipidemia and biomarkers of endothelial dysfunction, as well as changes in gene expression. Thus, flavanones may prevent from cardiovascular disease [49].

2.5. Flavonols

Flavonols (3-hydroxyflavones) are one the most analyzed subgroup of flavonoids due to the importance referring to their antioxidant properties and other biological activities. This class of polyphenolic phytochemicals occurs in commonly consumed vegetables, fruits and plant based beverages. Major sources of these compounds are part of grape berries, apple, tomato, onion, broccoli and red lettuce. In addition to fruits and vegetables, beverages such as green tea, black tea and red wine constitute also a significant source of flavonols [50]. Among major flavonols can be distinguished quercetin, kaempferol or myricetin. The structures of the most common flavonol aglycones are presented below (Fig. 6) [51].

Flavonols ensure plentiful health benefits. For instance, the intake of flavonols in increased quantities is related to reduced risk of cardiovascular diseases. This can imputed to their antioxidant properties which have been of interest for considerable time [51].

The efficacy of flavonols as antioxidant agents mostly depends on their chemical structure. There are three structural attributes constituting the most significant determinants: the catechol structure in the B ring, which is a radical target site; the 2,3-double bond in conjugation with a 4-keto function, which are responsible for electron delocalization from the B ring and the additional presence

of both 3- and 5-hydroxyl groups for maximal radical-scavenging potential and strongest radical absorption [50].

Antioxidant activity of flavonols may protect against oxidative damage to cells, lipids or DNA. Furthermore, these properties are the result of the presence of aromatic rings of the flavonoid molecule, which permit the donation and acceptance of electrons from free radical species. This aids in suppressing free radicals. Moreover, the consumption of flavonols is connected with reduced risk of stroke and cancer. Additionally, some of these compounds are believed to prevent osteoporosis and possess anti-inflammatory or neuroprotective properties [51].

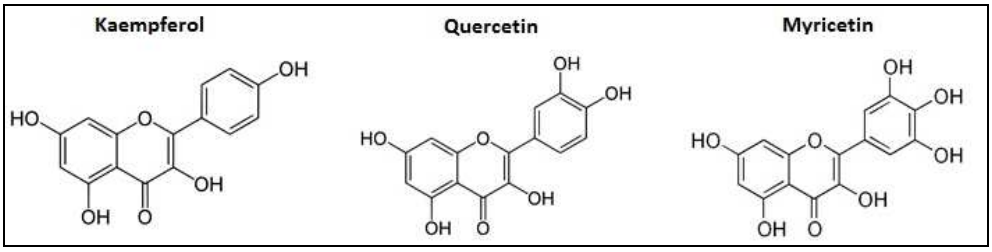


Figure 6. Structures of the major flavonol aglycones.

Quercetin is the major representative of the flavonol subclass which as powerful antioxidant prevents from oxidation of low density lipoproteins in vitro. It is a water-soluble plant pigment commonly found in green tea, red wine, apples, onions, leafy vegetables. Quercetin protects cellular structures and blood vessels from the damaging effects of free radicals (antioxidant and anti-inflammatory activity). What is more, this flavonol improves blood vessel strength and stems the activity of catechol-O-methyltransferase that suppress the neurotransmitter norepinephrine. This action may lead to elevated levels of norepinephrine, thermogenesis, and fat oxidation. Furthermore, quercetin acts as antihistamine agent preventing from allergies or asthma. Antioxidant properties of quercetin have evinced in LDL cholesterol reduction and heart disease protection. It can also block an enzyme resulting in sorbitol accumulation which has been associated with nerve, kidney or eye damage in diabetics. Quercetin may protect against cataract formation. It could be also examined as phytoestrogen [11].

Kaempferol is a flavonol antioxidant occurring in fruits and vegetables, mainly in broccoli. Many studies have presented the advantageous effects of dietary kaempferol in decreasing the risk of chronic diseases, particularly cancer. Furthermore, it can strengthen the antioxidant defense against free radicals, which support the cancer development. Kaempferol has been investigated to modulate a number of key elements in cellular signal transduction pathways related to angiogenesis, apoptosis, metastasis, and inflammation. It is confirmed that kaempferol meaningfully inhibits cancer cell growth and angiogenesis, as well as generates cancer cell apoptosis. However, this flavonol seems to maintain normal cell viability, usually exerting a protective effect [52].

Myricetin is succeeding natural flavonol, commonly consumed through human diets such as vegetables, fruits tea, red wine, and berries. Significantly, myricetin may ameliorate insulin resistance. In addition, this flavonol performs activity including antioxidative stress, anti-non-enzymatic glycation, anti-hyperlipidemia, anti-inflammation, anti-aldose reductase [53]. Myricetin appears to be an effective agent to quit smoking [54].

2.6. Isoflavones

Isoflavones are distinctive and very important subclass of flavonoid compounds. Their structures constitute the 3-phenylchromen skeleton which is chemically derived from the 2-phenylchromen skeleton by an aryl-migration mechanism. Isoflavones are mostly found in legumes, especially in soy. However, their presence has been also

reported in green split peas, split peas, chickpeas, black beans, lima beans, clover sprouts, and sunflower seeds. Furthermore, these compounds are included in the composition of several foods, vegetarian formulations, soy products in infant foods etc. [55]. The major isoflavones in human diet are genistein and daidzein (Fig. 7), which exist in four related chemical structures, namely aglycones, the 7-O-glucosides, the 6'-O-acetylglucosides and the 6'-O-malonylglucosides [56].

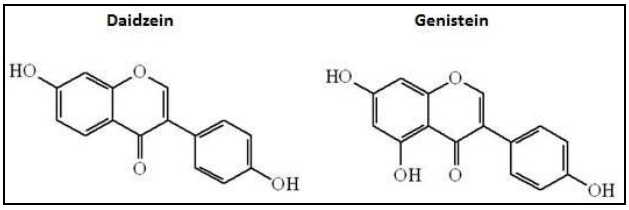


Figure 7. Chemical structures of major isoflavones.

Isoflavone compounds appear to possess effects on cardiovascular and menopausal health, and even are able to prevent cancer. They are considered as natural products that may be salutary to postmenopausal women in cardiovascular health. Furthermore, isoflavones are supposed to be responsible for the lipid lowering effects. They can also simply bind estrogen receptors - beta which are essential receptors in the central nervous, as well as cardiovascular systems. Isoflavones may also have antioxidant effects on blood vessels [57].

Soy isoflavones are promising dietary supplements for prevention of breast cancer. Isoflavones connect estrogen receptors (ER) and can variably work as either estrogen agonists or antagonists which depends on estrogen environment. It has been reported that the highest isoflavone dose brought to significantly lower breast proliferation and uterine size in the high-estrogen environment. Moreover, demographic and epidemiologic studies demonstrate that high dietary intake of soy isoflavones can reduce breast cancer risk [58].

Isoflavones, often determined as dietary phytoestrogens are used as food additives to preclude menopause-related disorders [56]. It is confirmed that diet containing soy protein rich in isoflavones has influence on the hormonal status and regulation of the menstrual cycle [59].

One of the major isoflavone - daidzein inhibits the class I isoenzymes of human alcohol dehydrogenase (ADH) and the human mitochondrial aldehyde dehydrogenase (ALDH-2), which may extinguish alcohol consumption in humans. Furthermore, daidzein shows antioxidant effect [60]. Another well-known isoflavone, genistein is a potential chemopreventive (therapeutic) agent in the treatment or prevention of various kinds of cancer. Genistein is supposed to possess an anabolic effect on bone by acting directly on osteoblasts, and prevent bone loss [61, 62]. Furthermore, genistein intake has been connected with decreased BMI, waist circumference, weight, and total body fat mass in postmenopausal women [63].

2.7. Flavones

Flavones are very similar structurally to flavonol compounds, having an extra hydroxyl substitution at the carbon 3-position. The major flavones are included apigenin and luteolin (Fig. 8). Luteolin occurs in vegetables and fruits such as broccoli, celery, carrots, parsley, onion leaves, cabbages, peppers, chrysanthemum flowers, and apple skins [64]. While apigenin can be found in onions, parsley, wheat sprouts, tea, oranges, chamomile, and in some seasonings [65].

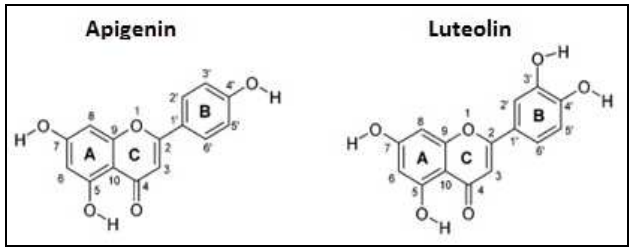


Figure 8. The major structures of flavones.

Apigenin is a principal component of chamomile, which is responsible for antibacterial, antiphlogistic, and antispasmodic effects. Recently, apigenin has captured the interest as beneficial and health promoting agent because of its low internal toxicity and differential results in normal against cancer cells relative to other structurally related flavonoids. It has been reported that apigenin possesses prominent anti-inflammatory, anti-carcinogenic and antioxidant properties. Apigenin

has been demonstrated to inhibit benzo[a]pyrene and 2-aminoanthracene-induced bacterial mutagenesis [65]. Furthermore, the studies have proved that apigenin supports metal chelation, scavenges free radicals and stimulates phase II detoxification enzymes in cell culture and in *in vivo* tumor models. Apigenin may act as severe inhibitor of ornithine decarboxylase, an enzyme playing an essential role in tumor promotion. The anti-carcinogenic effects of this flavone is indicated in a skin carcinogenesis model [67].

Plants rich in luteolin have been used as Chinese traditional medicine for hypertension, inflammatory diseases, and cancer treatment. Luteolin possesses multiple biological effects such as anticancer, anti-allergy, and anti-inflammation [68]. Thus, luteolin behaves as either antioxidant or prooxidant biochemically [69]. These biological effects can be related to each other, for example the anti-inflammatory properties are associated with its anticancer activity. Its anticancer properties are related to the induction of apoptosis, including DNA damage, redox regulation and protein kinases, inhibition of cell metastasis, proliferation, and angiogenesis. Furthermore, luteolin may sensitize diversity of cancer cells to therapeutically induced cytotoxicity through damping cell survival pathways and stimulating apoptosis pathways. Luteolin is also called blood-brain barrier permeable. This statement makes it suitable to the therapy of central nerve system diseases, including brain cancer [68]. Additionally, luteolin is examined as antioxidant - it has been reported that luteolin is able to inhibit ROS-induced damage of lipids, protein and DNA. Luteolin may show its antioxidant properties through protecting or extending endogenous antioxidants such as: glutathione reductase, glutathione-S-transferase, superoxide dismutase [69]. Luteolin presents its anti-inflammatory effect by damping the production of these cytokines and their signal transduction pathways [64].

3. FLAVONOID ANALOGUES - ITS BIOLOGICAL ACTIVITY AND HEALTH EFFECTS

Many studies have reported that flavonoid analogues possess plentiful intrinsic properties for human being, supposing even more than flavonoids. The examples of the most important flavonoid derivatives are shown below.

The most extensively distributed glycosides of hesperetin are hesperidin and neohesperidin (Fig. 9). Hesperidin (hesperetin-7-rutinoside) occurs in higher contents in sweet oranges, lemons, limes, tangerine and tangor species of citrus fruits, while neohesperidin (hesperetin-7-neohesperidoside) is present in tangelo and sour orange. Hesperetin glycosides are more predominant in nature than the aglycone form [38].

The most abundant naringenin glycosides are naringin and narirutin (Fig. 10). Naringin (naringenin-7-neohesperidoside) gives bitter taste because of its glucose moiety. Naringin mostly occurs in grapefruits and sour oranges. Another greater naringenin glycoside is narirutin (naringenin-7-rutinoside) which is detected in higher levels in tangerine, tangor, tangelo and sweet orange [39].

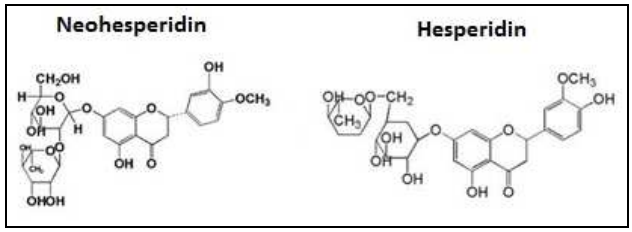


Figure 9. Structure of hesperetin derivatives - glucoside forms: neohesperidin (neohesperidoside) and hesperidin (rutinoside).

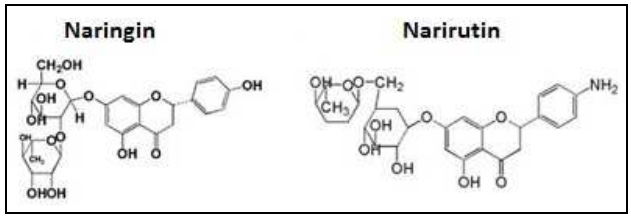


Figure 10. Structure of naringenin derivatives - glucoside forms: naringin (neohesperidoside) and narirutin (rutinoside).

Inflammation is the most obvious diagnostic of immune defense. The most common symptoms are: pain, swelling, and redness in the affected tissues [70]. Due to a dysfunctioning of the immune response many chronic diseases are noticed in human populations. It is believed that hesperidin is able to inhibit kinases and phosphodiesterases which

are responsible for cellular signal transduction and activation over an inflammation response. Hesperidin is considered as gently anti-inflammatory agent because of the reduction in the volume of exudates and the number of migrating leucocytes by 48% and 34%, respectively [71]. Furthermore, hesperidin is supposed to decrease yeast-induced hyperthermia in rats and inhibit lipopolysaccharide-induced overexpression of cyclooxygenase-2, inducible nitric oxide synthase, overproduction of prostaglandin E2, and nitric oxide [72, 73]. In addition, it has been also demonstrated the antiviral activity of hesperidin against parainfluenza, polio, herpes simples,and syncytial viral infections [74].

The main directions of pharmacological activity of naringenin derivatives include: estrogenic activity, cholesterol-lowering properties, anti-inflammatory, antiulcer, antispasmodic, and anticancerogenic activity. In contrast, the prenylated derivatives of naringenin (6-PIN and 8-PN), present in hops and beer, revealed antioxidant properties [41].

Anthocyanins are classified as anthocyanidin derivatives. Anthocyanins are glycosylated polyhydroxy and polymethoxy derivatives of flavilium salts and are members of the flavonoid family, possessing a characteristic C_3-C_6-C_3 carbon structure [28]. Anthocyanin pigments are supposed to be used in folk medicine all over the world. For instance, bilberry anthocyanins have been exploited in the treatment of microbial infections, diarrhea or vision disorders for many years. In recent years, studies have shown the particular properties of isolated anthocyanin pigments. Some reports indicate that anthocyanin activity is getting up when delivered in mixtures, in contrast to isolates [75]. Studies have presented that dietary supplementation with berries rich in anthocyanins were efficient in reducing oxidative stress [30]. It is confirmed that anthocyanin extracts from bilberry, chokeberry and elderberry have exhibited endothelium-dependent relaxation capacity in porcine coronary arteries [76]. Furthermore, chronic intake of anthocyanins increased cardiac glutathione concentrations in rats [77].

Another examples of natural flavonoid analogues are few 7-hydroxy-8-formylchromones (A) and their partially hydrogenated derivatives, lavinal (B), and 4,7-dihydroxy-6-methyl-5-methoxy-8-formylflavan (C) which have been discovered by large class of natural flavonoids (Fig. 11) [78]. These first two compounds protrude together in plants of the family Annonaceae, like *Desmoschinensis*, *Desmoscochinchinensis*, *Dasymaschalonrostratum*, and *Unonalawii*. The last one can be found in roots of *Desmoscochinchinensis*. The composition of these three flavonoids isolated from *D. cochinchinensis* showed antimalarial activity while lavinal itself was seen as virtual AIDS agent.Moreover, the analogs obtained through the synthesis of compounds similar to angular α-pyrono[2,3-*f*]chromones are very promising that they have activity against *S. aureus* and *E. coli* [78]. On the other hand, Lewis and Shaw in their study shows that either leucocyanidin as natural flavonoid or its hydroxyethylated and tetraallyl derivatives are able to protect the gastric mucosa against aspirin challenge. Furthermore, leucocyanidin and its synthetic analogues significantly increased mucus thickness [79]. Additionally, Verghese et al. presented that flavone-based analogues obtained from complex natural product simocyclinone D8 can inhibit DNA gyrase, and may act as topoisomerase poisons and DNA intercalators [80].

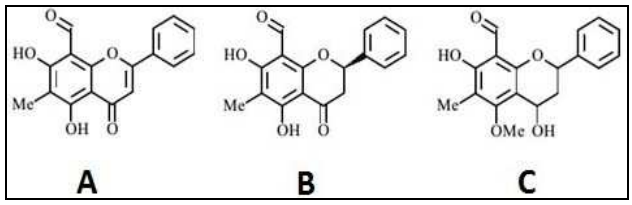


Figure 11. Analogues of natural flavonoids.

Silybin and its analogues have been reported as chemopreventive agents for certain cancers. They are also able to fast repair DNA bases from oxidative damage by pulse radiolysis method. Moreover, silybin and its analogues preserve DNA from radiation damage at micromolar concentrations [81].

Synthesized a novel series of N1-(flavon-7-yl)amidrazones incorporating N-piperazines and related congeners through the reaction of the hydrazonoyl chloride derived from 7-aminoflavone and 7-amino-2-methylchromen-4-one with the appropriate piperazine are supposed to be anticancer agents and possess antitumor activity against breast cancer [82].

4. APPLICATION OF FLAVONOIDS AND ITS ANALOGUES

4.1. Medicinal uses

Natural flavonoids and their derivatives have been a beneficial source of bioactive molecules in medicines much before the advancement of other modern therapeutics in the post-genomic period [83].

Flavonoids possess various applications in food industry. Some compounds seem to be widely used as sweetening agents or food colors, while many others play a significant role as flower pigments - thus they are found useful in horticulture and cut flower industry. These compounds have been reported to show antifungal, antibacterial or antiviral properties [84]. Plentiful flavonoids and its derivatives, both natural and synthetic, have been studied as potential medicinal agents which prevent human diseases including malaria and HIV. For example, the alleviation of toothache by chewing on a willow twig which is based on the presence of salicylic acid derivatives, thus it gives an opportunity to use acetyl salicylic acid and its many synthetic variants to alleviate minor pain. Naturally occurring, as well as synthetic flavonoid derivatives have demonstrated many medicinal uses. Seeds of the milk thistle *Silybum marianum*, which are rich in active flavanolignans have been used as a remedy for liver disease for long time. Well-known chalconediglucoside and the isomeric flavanonediglucoside are responsible for the antihepatotoxic activity of *Butea* extracts. Furthermore, it has been confirmed that flavonoids are active against HIV. Besides, 5,6,7-trihydroxyflavone 7-*O*-glucoside presented the ability of inhibition the human T-cell leukemia virus type 1 (HTLV-1). Another flavonoids: quercetin and fistein (5-deoxyquercetin) have shown activities similar to that of the drug Adriamycin. Moreover, phenolic compounds (e.g. proanthocyanidins and gallic or ellagic acid derivatives) have been suggested to inhibit specific ligands at 16 receptors sites [85].

Flavonoids and their derivatives may act as efficient agents against plants viruses, mainly potato virus X (PVX) and tobacco mosaic virus (TMV) [86]. As antibacterial agents may participate in the field of strains of pathogenic bacteria to routinely used antibiotics. Furthermore, there are many reports in the literature documenting antifungal activity of flavonoids. Thus, they may be used as potential biocides (e.g. phytoalexin analogues). Alkyl derivatives of flavonoids prevent from variety of wood-destroying fungi and Gram-positive, as well as Gram-negative bacteria. On the other hand, flavan-3-ols play an important role as antiscorbutic elements of foods [84].

4.2. Food uses

The characteristic flavor of *Citrus* species is related to flavonoids as sweetening agents. Moreover, the taste of common beverages, such as wine, beer or tea is also caused by flavonoid features. The structure of the diglycoside plays an essential role in determining taste properties. It has been reported that loss of rhamnose from naringin or neohesperidin, leaving only the 7-*O*-glucosides, did not cause the decline of bitterness. Otherwise, it is observed that the movement of rhamnose to position 3 or 4 of glucose gives compounds gently bitter taste while the movement of rhamnose substituent from position 2 on the glucose to position 6 results in loss of bitterness. Removal of both sugars caused a complete loss of taste, thus a sugar group at position 7 specifies structural requirements. On the other hand, the sweet taste is due to the presence of dihydrochalcones. For sweetness in tea is probably responsible the compound, called aspalathin. Surprisingly, the replacement of the rhamnose with glucose to provide the 2'-*O*-β-D-glucoside eliminates any element of sweetness from the compound. This glucoside - phloridzin possesses quite bitter taste. Moreover, rhizomes have been the subject of studies to determine the chemical nature of their sweet-bitter components [84, 87, 88].

Furthermore, flavonoids play a greatly important role in the production and pleasure of several well-known and commonly used beverages, especially tea, wine and beer. It is confirmed that flavonoids in grapes have a huge influence on the quality of wine. Two main groups of flavonoids are inherent in wine: anthocyanins which are responsible for the color of grapes and proanthocyanidins related to astringency. The anthocyanin chemistry of grapes constitutes a complex with five aglycones (cyanidin, petunidin, peonidin, malvidin

and delphinidin) occurring in various mixtures of 3-*O*-mono- and 3,5-di-*O*-monoglucosides some of which are acylated. The most general acid is *p*-coumaric. In addition, the acetaldehyde molecule plays a significant role in the aging of wine [84].

The most common problem of plagues brewers is the haze generation in their products. Hazes can be endless or they may occur during product's chilling. The principal collaborators of haze problem constitute polyphenolic compounds most of which are proanthocyanidins. In order to solve the problem is the removal of these compounds at source instead of having to resort to some physical methods of separation at a larger stage of production [89].

Honey is a natural product well-known due to its high alimentary and preventive-medicinal quality. From the chemical point of view, honey is extremely concentrated solution of a complex mixture of sugars. Aside from sugar groups, it possesses a wide range of smaller compounds, majority of which, containing polyphenols, are noted to have antioxidant properties. Because of beneficial effects of antioxidants on human health honey may be considered as a biomarker for environmental pollution and may cumulatively determine the level of water, air, plant and soil contamination over the forage area of the bees. Due to the importance of natural polyphenols, interest in their identification and quantification has significantly increased in recent years. Furthermore, the medical application of honeybee products, called apitheraphy has aroused an interest as popular and prophylactic medicine for diseases treatment as well as supporting overall health and well-being. Honey characteristics, such as sweetness, flavor and color, cause that honey is often used as a sugar substitute, a natural preservative or ingredient in plentiful of manufactured food products [90].

4.3. Leather tanning uses

One of the oldest processes involving polyphenolic compounds is the conversion of animal hides and skins into leather. The tanning process concerns handling hides with substances that protect the molecular form of the collagen fibers of which the hides are composed [84]. Vegetable tannins are supposed to be the earliest used agents in this transformation. Apart from the vegetable tannins, other reagents commonly used in tanning include salts of aluminium(III) and chromium(III), and the bifunctional organic reagent glutaraldehyde. Characterization of the nature of intermediates and the final product in any tanning process would clearly be of importance in mechanism-based attempts to enhance tanning efficiency or finished leather quality. Nowadays, the industry applies certain standards based on the physical characteristics of intermediate and final product, such as shrinkage and shrinkage temperature, and thermal properties, which do not give clear view to any underlying chemical and physicochemical transformations [91]. Vegetable tannins are important as retanning agent in the leather production and have been recognized as an important tanning agent in non-chrome tanning. Commercial vegetable tannins are not capable of radically changing the quality of the usual leather products, so that the appearance of a new vegetable tannin is of great importance. Tannins leave a distinctive spectroscopic signature in the tanned leather product by which the origin and type of the tannin used may be inferred. It is to be expected that processes using mixtures of tanning reagents will leave equally distinctive fingerprints in the leather product. The fingerprint reflects not only the process chemistry but also underlying molecular mechanisms whereby tanning converts unprocessed leather into a commercial product [92].

4.4. Natural pigments uses

Flavonoids constitute one of the largest groups of plant pigments, and some of them are often found in many kinds of plants. Nowadays, because of ingenious manipulations of genetic flavonoid material, it is possible to modify the flavonoid biosynthetic pathway in such way that plants may be prompted to produce novel compounds. There are some pros of this innovative method, namely it is likely to place the normal range of plant colors in the best practicable genetic background, that can possess such features as stature, cold hardiness, and disease resistance. Besides, it appears an opportunity to engineer novel flower colors. Flower color in majority plants requires the interaction of anthocyanins with flavone

or flavonol glycoside co-pigments. It has been reported that modification of flower color may be obtained by altering the genetic control of vacuolar pH [84].

Furthermore, natural plant dyes containing flavonoids are often used as mordant-dyes, except for catechins being considered as direct dyes. As mordants are considered substances combined with dyes (flavonoids) in order to define dyes on fibers. The example of mordant commonly used with flavonoids is a soluble aluminium salt, such as alum. The green color is usually created by the combination of the flavonoid-dye and indigo [93]. Many dyes present in plants are glycosides. The dye process is followed by the glycosidic bond breaking and formation of new bonds between the fiber and the dye. Thus, a water insoluble and washable coloration is produced. The major flavonoids occurring in yellow dyes are: quercetin O-galactoside (hyperoside), quercetin O-glucoside (isoquercetin), quercetin O-apioside, kaempferol O-galactoside, isorhamnetin O-glucoside or galacto-side and kaempferol O-glucoside (astragalin). On the other hand, tannins are commonly used in connection with other dyes, as a pretreatment to the fiber and produce mostly brown to black colors [94].

5. CONCLUSIONS

The biological properties of dietary flavonoids and their analogues have been indicated to be due to multiple mechanisms of actions including free radical scavenging, activation of survival genes and signaling pathways, transition metal ion chelation, regulation of mitochondrial function and bioenergetics, modulation of inflammation response, and even interactions with micro biota. Nevertheless, activity of flavonoids are not limited to their health promoting benefits but spread to a wide range of ecological interactions of plants, such as acting as a signal and defense molecule. Their applications in industry are beyond the limit of nutraceuticals and drug candidate molecules. The heterogeneous biological activities of flavonoid compounds and its derivatives depend on their structural diversity.

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TRANSPARENCY DECLARATION

The author declares no conflicts of interest.

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Research Article

Evaluation of antiplasmodial effects of the ethanolic leaf extract of *Salacia lehmbachii* on *Plasmodium berghei* infected mice

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ABSTRACT

Salacia lehmbachii leaves are used in Nigerian traditional medicine for the treatment of malaria and other diseases. The ethanolic extract was tested for its activities against suppressive, prophylactic and established infections in *Plasmodium berghei* infected albino mice at dose levels of 100, 200 and 400 mg/kg; while chloroquine (10 mg/kg) was used as positive control. The extract exhibited significant dose-related antiplasmodial activities on parasites with the used-dose levels, showing significant mean survival time. The results, therefore, co-relate with claims by traditional users for the treatment of malaria and other feverish conditions; and could serve as source of potential new antimalarial agents.

Keywords: Malaria; *Salacia lehmbachii*; Mice; Suppressive; Prophylactic; Curative.

1. INTRODUCTION

Malaria is mosquito-borne plasmodial infection. It is a global killing parasitic disease, causing approximately up to 2 -3 million deaths annually, and still causing major setback to health in Sub-Sahara Africa and other endemic areas [1, 2]. The annual occurrence of 400-500 million clinically newly-manifested cases portrays the severity of the disease, making it a global burden [3, 4]. It is of necessity to circumvent this global burden by widening research into new potential potent compounds with antimalarial activity, that are not based on existing synthetic antimalarial agents [5]. Plants are widely accepted to contribute major parts of medications globally used by traditional healers. Some plants are notably known to be used against malaria [6], thus leading to increased scientific authentication of medicinal plants used in Nigeria as claimed by traditional users.

Salacia lehmbachii which belongs to Celastraceae family and genus of *Salacia* is one of such plants. It is a shrub-like to small tree of about three meters high, richly found in the tropical rain forest of Central, west and East Africa [7]. The leaves are seasonally evergreen, firm and difficult to slice. There are diverse therapeutic applications of

S. lehmbachii justifying its folkloric background; the leaf extract as an antipyretic [8] anti-diarrheal, antimotility and anti-ulcer properties [9], while the root extract exhibits analgesic/anti-inflammatory effect, anticholinergic property and anti-infertility in male [10-12]. The wet pad from the root is used in hemorrhoids [13].

2. MATERIALS AND METHODS

2.1. Collection and preparation of plant materials

The fresh leaves of *S. lehmbachii* Loes were collected in November, 2014 from fully grown plant in a local farm forest in Ukanafun, Akwa Ibom State, Nigeria. The plant materials were identified and authenticated by a taxonomist, Department of Botany, University of Calabar, where a voucher specimen (No. 688) is maintained. Internationally, the plant is indexed as "*Salacia lehmbachii* Loes. Bot. Jahrb.Syst.XLIV.(2-3) 173 (22/03/1910)". The leaves were cleaned, cut into smaller pieces, air-dried at room temperature (28-30^{o}C) for 15 days and pulverized to dry powder with the help of mortar and pestle.

2.2. Extraction of plant (leaf) material

Five hundred grams (500 g) of the dried-leaf powder was extracted in ethanol (BDH chemicals Ltd, England) using a Soxhlet extractor (Friedrich Polzine, England) and the filtrate were dried on a water bath at a controllable temperature. The yield was 12.5% w/w. The leave extract was subsequently reconstituted in normal saline for routine use during the study.

2.3. Phytochemical analysis

The phytochemical screening of ethanolic leaf extract of *S. lehmbachii* was carried out for various secondary metabolites such as tannins (ferric chloride test), alkaloids (Mayer's and Draggendorff reagents), saponins (Froth test), steroids (Liebermann-Burchard test), terpenoids (Salkowski test), flavonoids (ammonia and sulphuric acid test) and anthraquinones (Borntrager's test) [14, 15].

2.4. Animals

The albino mice (18-22 g) of both sexes were obtained from animal house, Department of Pharmacology, College of Medical Sciences, University of Calabar, Nigeria. The animals were housed in cages under standard laboratory conditions, with naturally illuminated environment of 12 hrs dark and 12 hrs light cycles. They were fed on standard pellet diet and had free access to water. Care according to recommendation by Helsinki Declaration was implemented. Approval for study was obtained from the Research and Ethical Committee of Faculty of Basic Medical Sciences of University of Calabar.

2.5. Acute toxicity study of the extract

The LD_{50} of the ethanolic extract of the leaf to authenticate the safety of the extract was determined as described by Lorke [16] method. All the doses were administered orally. During the two phases, the mice were observed for signs of toxicity for 48 hours. There was no overt evidence of toxicity at the dose above 5000 mg/kg.

2.6. Malaria parasites (donor)

The chloroquine sensitive *P. berghei* (NK65) was sourced from National Institute for Medical Research, Lagos Nigeria. Parasites are maintained in the Animal House of the Department of Pharmacology, College of Medical Science, University of Calabar by continuous re-inoculation of mice and affirmation of concentration of the parasites.

2.7. Inocula

Parasitized erythrocytes for this study were obtained from a donor infected mouse by cardiac puncture, and prepared according to methods of Akuodor et al. [17] and David-Oku et al. [18]. To each mouse was administered intraperitoneally with infected blood suspension (0.2 ml) containing $1x10^{7}$ *P. berghei* parasitized red blood cells.

2.8. Suppressive test

This study was carried out according to methods described by Akuodor et al. [19]. Thirty albino mice of both sexes were selected and passaged. After three hours, the infected mice were randomly divided into 5 groups, each cage containing 6 mice. Animals in each group were treated orally for four consecutive days (D_0-D_3) with 100, 200, and 400 mg/kg of the ethanolic leaf extract, chloroquine diphosphate (10 mg/kg) and Normal saline (20 ml/kg) for positive and negative controls respectively. On day five (D_4), the films were prepared from tail blood of each mouse, and parasite concentration examined microscopically, counting the parasitized red blood cells on 1000 red blood cells in 10 different fields.

2.9. Prophylactic study

This study was carried out according to the methods described by Peters et al. [20]. Thirty albino mice of both sexes selected for this study were grouped into 5 of 6 mice per cage. Groups 2-4 were treated orally with graded doses of 100, 200, and 400 mg/kg of ethanolic extract of the leaf for four days (D_0-D_3); whilst group 1 and 5 received 20 ml/kg of normal saline and 10 mg/kg of chloroquine diphosphate respectively. On the last day of treatment, mice in all groups were injected intraperitioneally with constituted *P. berghei* erythrocyte suspension. After 72 hours, films were prepared (as previously described) and examined microscopically.

2.10. Curative test

On the first day, thirty Swiss albino mice were inoculated with *P. berghei* infected erythrocytes. After 72 hrs, the mice were randomly grouped into 5 groups of 6 mice, and treated daily (groups 2-4) for four days with the extract (100, 200, and 400 mg/kg); while the animals in group 1 and 5, were given 20 ml/kg of normal saline and chloroquine diphosphate (10 mg/kg). Thereafter, films were made and viewed to determine the parasite density. Mortality was monitored daily for mean survival time (MST), and the number of days from the time of inoculation of the parasite up to death was recorded for each mouse in the extract treated and control groups throughout the follow up period (D_0-D_{29}) [21].

2.11. Statistical analysis

The obtained results were expressed as mean ± SEM. Data were analyzed using One-way ANOVA and differences between the means were considered significant at $P< 0.05$.

3. RESULTS

3.1. Phytochemical test

Phytochemical results of the screened ethanolic extract of the leaf of *S. lehmbachii* revealed the presence of alkaloids, saponins tannins, terpenoides, flavonoids, phenols, steroids and anthraquinones while resin is absent (Table 1).

Table 1. Phytochemical constituents of the ethanolic leaf extract of *Salacia lehmbachii.*

Alkaloids	++
Saponins	++
Tannins	+
Terpenoids	++
Flavonoids	+
Phenols	+
Steroids	+
Anthraquinones	+
Balsam	-

Key: (+) = presence, (−) = absence

3.2. Acute toxicity study of the extract

The acute toxicity test of the ethanolic leaf extract was negative. The LD_{50} was greater than 5000 mg/kg orally, in tested mice.

3.3. Suppressive effect

The ethanolic extract of the leaf showed a dose-related effect at different graded doses used. Doses of 200 and 400 mg/kg significantly ($P < 0.05$) produced 66.5% and 80.1% inhibition

of parasitaemia respectively, compared to 89.8% exhibited by 10 mg/kg of chloroquine (Table 2).

Table 2. Suppressive effect of ethanolic extract of the leaf of *S. lehmbachii* against *P. berghei* in mice.

Drug	Dose (mg/kg)	Mean parasitemia density	% suppression
Control	20 ml/kg	41.20±1.17	-
S. lehmbachii	100	25.00±1.12	39
	200	13.80±0.67*	67
	400	8.20±0.53*	81
Chloroquine	10	4.20±0.53*	90

Values represent the mean ± SEM (n=6), *significantly different from control at P< 0.05.

Table 3. Prophylactic effect of ethanolic extract of the leaf of *S. lehmbachii* against *P. berghei* in mice.

Drug	Dose (mg/kg)	Mean parasitemia density	% suppression
Control	20 ml/kg	40.40±1.18	-
S. lehmbachii	100	26.00±1.18	36
	200	11.40±0.47*	72
	400	5.80±0.53*	86
Chloroquine	10	4.00±0.50*	90

Values represent the mean ± SEM (n=6), *significantly different from control at P< 0.05

3.4. Prophylactic effect

The leaf extract exhibited a dose-related effect at different doses used. Doses of 200 and 400 mg/kg significantly (P< 0.05) prevented the replication of the invaded parasites by producing 71.1% and 82.1% inhibition of parasitemia respectively, compared 88.6% exhibited by 10 mg/kg of chloroquine (Table 3).

3.5. Curative effect

The extract exhibited a significant dose-dependent reduction in parasitemia density. The doses of 200 and 400 mg/kg produced significant (P< 0.05) effect comparable to the effect exhibited in chloroquine treated group; whilst in the negative group, there was a consistent increase in the blood parasites. The survival rate among the mice also reflected dose-dependent response and showed that the extract significantly (P< 0.05) destroyed the invaded parasites at (71.3 and 82.3 % for 200 and 400 mg/kg respectively) of the established infection (Table 4). In the negative control group, from the 9^{th} day mice started dying, and by the 12^{th} day, no mouse survived, whereas, in the positive control group (chloroquine treated), there was no death observed, Table 4. It is worthy to note that some mice which received 200 and 400 mg/kg survived the 30-day observation period. Photomicrographs of thin blood smears are on the Fig. 1.

4. DISCUSSION

In this study, suppressive, prophylactic and curative antiplasmodial activities of *S. lehmbachii* were investigated in albino mice infected by *P. berghei*, which produces disease similar to those of human plasmodium infections, for the prediction of treatment outcomes [22].

Table 4. Data on curative effect of ethanolic extract of the leaf of *S. lehmbachii* against *P. berghe*i in mice.

Drug	Dose (mg/kg)	Mean parasitemia density		% suppression
		Pre-treatment	Post-treatment	
Control	20 ml/kg	38.60±0.62	41.40±1.18	-
S. lehmbachii	100	40.20±1.86	25.53±0.37	37
	200	40.60±1.84	11.65±0.62*	71
	400	39.40±0.94	6.97±0.88*	82
Chloroquine	10	39.00±1.04	2.26±0.34*	94

Values represent the mean ± SEM (n=6), *significantly different from control at P< 0.05.

These parameters are accepted scientific methods for evaluating and identifying new potential antiplasmodial agents [5, 18]. This usually determines the level of destruction of parasites in blood, hence a mean parasitaemia levels of about ninety percent mock-treated control animals indicates that the test agent is potent in standard screening studies [23]. Therefore, the result could be used to show that *S. lehmbachii* leaf extract is capable of destroying or suppressing plasmodial growth to near non-detectable levels in the infected erythrocytes.

Table 5. Mean survival time (days) of ethanolic extract of the leaf of *S. lehmbachii* against *P. berghei* in mice during curative study.

Drug	Dose (mg/kg)	Mean survival time (days)
Control	20 ml/kg	11.00±0.96
S. lehmbachii	100	20.80±1.65
	200	27.60±0.98*
	400	29.80±0.18*
Chloroquine	10	30.00±0.00*

Values represent the mean ± SEM (n=6), *significantly different from control at P< 0.05.

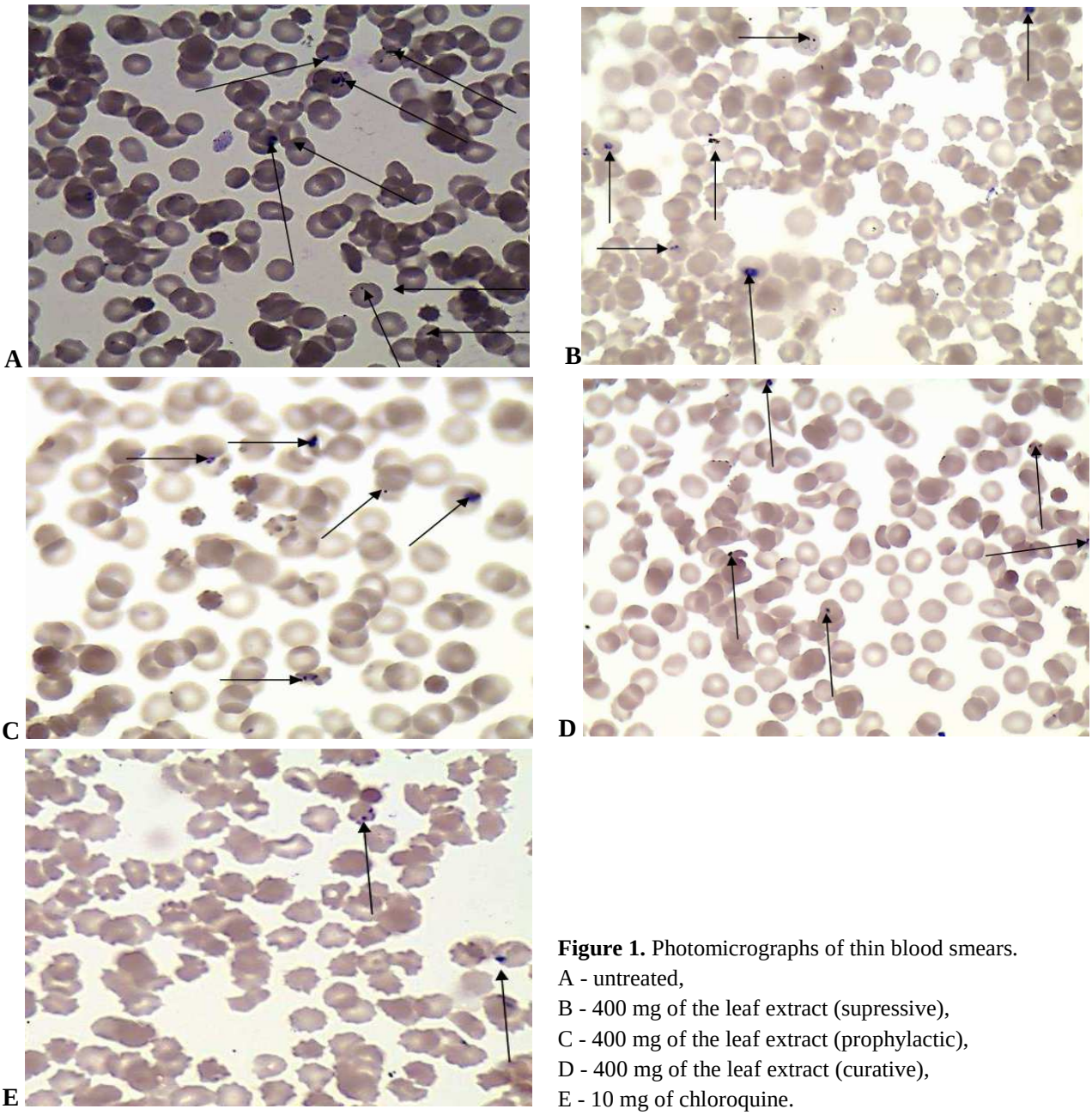

Figure 1. Photomicrographs of thin blood smears.
A - untreated,
B - 400 mg of the leaf extract (supressive),
C - 400 mg of the leaf extract (prophylactic),
D - 400 mg of the leaf extract (curative),
E - 10 mg of chloroquine.

The leaf extract, apart from exhibiting suppressive and prophylactic effects, also exerted significant curative activity during established infections. This could be seen in high percentage inhibition of parasites in blood as well as mean survival time especially in 200 and 400 mg/kg extract treated groups. It was recorded that some mice in these groups survived the 30 days of observation. However, the traditional use of *S. lehmbachii* by herbalists could be attributed to the presence of certain phytochemicals identified in the leaf extract. Most medicinal plants possess a wide variety of important phytoconstituents such as: anthraquinones, flavonoids, alkaloids and terpenoids as their bioactive compounds. The activities of these compounds have been proved against plasmodial infections [24-26]. Thus, the exhibited antimalarial activities of *S. lehmbachii* leaf extract might be due to the presence of these compounds. *S. lehmbachii* leaf also possess phenols known for their anti-oxidant and other diverse physiological properties: anti-carcinogenic, anti-inflammatory and anti-parasitic activities [27].

A standard antimalarial drug suppresses parasitemia significantly [28] which is in agreement with the effect of chloroquine in this study. Chloroquine is both clinically used for suppressive, prophylactic and curative, treatment of malaria, except for resistant strains of *Plasmodium falciparum* [29]. It destroys plasmodia by preventing the digestion of hemoglobin, and blocking the parasites source of amino acids, or by inhibiting hem polymerase to prevent the production of hemozoin, a protective medium against autolysis.

5. CONCLUSION

The results obtained from the present work have scientifically justified the reasons for the folkloric use of this local plant in the treatment of malaria attack in Nigerian traditional herbal practice. *S. lehmbachii* leaf extract has also proved to be a potential source of lead molecule(s) for the development of a new antimalarial agent. Further studies are however recommended to isolate and characterize the active ingredients responsible for the observed antimalarial activities.

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AUTHORS' CONTRIBUTION

EAD and AGC designed and carried out the experiments, EGA wrote the first draft of the manuscript, NNN did extensive literature review, and AJL performed the statistical analysis, while USJ supervised the study. All authors read and approved the final manuscript.

TRANSPARENCY DECLARATION

The author declares no conflicts of interest.

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Research Article

Alteration in biochemical indices following administration of seafood (*Thais coronata*) extract

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ABSTRACT

Seafood consumption has been a way of life to most people especially those that leave in riverine areas, because seafoods are known to contain many nutrients that are essential for healthy living. Consequently, this research therefore seeks to investigate the effect of these nutritive components of *Thais coronata* on biochemical indices of albino Wistar rats. Fourty five male albino Wister rats weighing between 180-220 g were assigned into 3 groups of fifteen rats each in metabolic cages and were given rat feed and drinking water *ad libitum*. Two test doses (low dose 7.0 mg protein/ml and high dose 52 mg protein/ml) were selected and administered to two groups of rats orally and daily for six weeks, while a third group of rats served as the control, n = 15. At the expiration of the feeding period, blood samples were obtained from all the rats via cardiac puncture for the analysis of the various biochemical indices. Both the low and high doses of the extract produced significant increases in HDLc ($P<0.001$) compared with control. k ($P<0.001$), HCO_3^- ($P<0.01$) and Ca^{2+} ($P<0.001$) were also significantly increased in the extract treated groups. The extract groups had significant reductions in ALT ($P<0.001$), ALP ($P<0.001$), Na^+ ($P<0.001$) and Cl^- ($P<0.001$) compared with control. Also Tc ($P<0.001$), TG ($P<0.001$), LDL ($P<0.001$) and VLDLc ($P<0.001$) were significantly decreased in the extract treated group. In conclusion seafood consumption is of immense benefit to health because it serves to regulate the lipid profile, electrolytes and enzyme concentrations in blood.

Keywords: Rock snail; *Thais coronata*; Biochemical indices; Vitamin; Omega-3 fatty acid.

1. INTRODUCTION

Seafood consumption is a way of life to those that cherish it and it is yet to be discovered by those who don't know about it. Seafood constitutes important and readily available sources of edible nutrients and is found in different kind of waters. There exist different types of Seafoods but one of the most common one is *Thais coronata* (Rock snail) a strong member of the mollusca phylum [1] from the family Muricidae. *Thais coronata* the world's largest fresh water snails are locally known as Nkonko by the Efiks in Nigeria. They occur basically in tropical and subtropical localities in different parts of the world including Nigeria (Calabar) Cuba, Brazil, Central America, USA (California), Philippines, Hawaii, Taiwan, Japan and Indonesia. *Thais coronata* is very rich in iron, iodine, selenium, Vit. A, Vit. D, Vit. E, Vit. B12,

Vit. B6, proteins and essential fatty acid. They are essential for human consumption and their shell is used in making jewelry [2-5].

Nutrition evaluation of seafood in Nigeria indicates that it has high protein content and elemental composition [6]. Moreover, it has been reported that seafood consumption enhance blood production [7] and serves as a rich source of essential fatty acids like the omega-3 fatty acid, which is important in reducing the incidence of coronary heart disease [8] and preventing other diseases [9-11]. Seafoods are known to have antioxidant property which is essential in lowering of arterial blood pressure; they were shown to elevate HDL-c and lower LDL-c levels in blood [12, 13] and they also enhance tissue lipoprotein lipase activities. Seafood also provides negligible amounts of trans-fats, dietary fibres and sugars [14-19].

2. MATERIALS AND METHODS

2.1. Experimental animals

Forty-five (45) male albino Wistar rats weighing initially between 180 to 220 g obtained from the animal house of the Department of Physiology, University of Calabar, Nigeria were employed for this study for 6 weeks. The animals were allowed free access to their feed and drinking water. The rats were weighed before commencement of the feeding experiment and thereafter were weighed daily. Ethical approval was obtained from the Ethics Committee of the Faculty of Basic Medical Sciences, University of Calabar, Nigeria. They were nursed under control of environmental conditions in accordance with international standard [43].

2.2. Collection of Rock snail sample

Fresh samples of the Rock snail were purchased from the local markets (watt market) in Calabar.

2.3. Preparation of the aqueous extract

The preparation of aqueous extract was done according to the method described by Walker [20] and Aldeen et al. [21] as used by Archibong et al. [22]. Fresh Rock snail was obtained from Watt Market Calabar and was rinsed in water to remove leaves and debris on different occasions. One hundred grams of the fresh rock snail was weighed out respectively and homogenised for 5 minutes using tissue blender. The homogenate was then dissolved in 100 ml of saline (0.9% NaCl). After dissolving the homogenate, it was then centrifuged for 10 minutes using 10,000 revolutions per minute. The supernatant was then poured into a clean container via filter paper fitted funnel, and this formed the stock solution of 1 g/ml.

2.4. Experimental design

Fourty five (45) male albino Wistar rats were randomly selected and assigned to three groups thus the control, low dose (LD) and high dose (HD) groups of fifteen (15) rats each. The test doses were selected based on pre-determined LD_{50} values and on serial dilution of the stock solution. The extract was added into a small amount of the feed based on the weight of each rat. The low dose groups received 7 mg/ml of the extracts daily while the high dose groups received 50 mg/ml of the extracts daily. The control group received 0.6 ml of normal saline daily. All the animals had free access to food and drinking water and the experiment lasted for six weeks.

2.5. Collection of blood plasma samples

The animals were made unconscious using chloroform anesthesia. The blood samples were collected via cardiac puncture, a method modified by Ohwada [23]. A 5 ml syringe, attached to a sterilized needle was used to collect the blood samples from the heart and then emptied into plane sample bottles. The blood samples were then used for the estimation of various levels of plasma constituents.

2.6. Preparation and extraction of serum

About 4-5 ml blood was collected from each rat into separate sample bottles and allowed to stay for 30 minutes to enhance clotting. It was then centrifuged at 2,500 revolutions/min for 15 minutes with the help of the micro hematocrite centrifuge. The serums were collected into clean test tubes for

the analysis of the various biochemical indices.

2.7. Determination of liver enzymes

2.7.1. Determination of alkaline phosphatase (ALP)

ALP was analyzed according to the method as described by Bowers and McComb [24]. The p-nitrophenyl phosphate was hydrolyzed to phosphate and p-nitrophenol in the presence of ALP. A calculated amount of sample 0.01 ml in a test tube was mixed with reagent (0.5 ml) containing the substrate p-nitrophenyl phosphate and kept at room temperature. The solution was mixed and initial absorbance read after 1 min. The reaction was allowed to stand for 3 min and the absorbance read again at 405 nm [24]. Alkaline phosphate activity was calculated from the following formular:

$$UL = \frac{2760 \times A\ nm}{min\ micro}$$

Where: UL = Unit of alkaline phosphatase affinity, A = Change in absorbance.

2.7.2. Determination of aspartate transferase (AST) and alanine transferase (ALT)

Serum AST and ALT levels were determined, using endpoint colorimetric-diagnostic kit from Randox Laboratories, UK [25]. The pyruvate produced by transamination reaction between L-alanine and ketoglutarate reacts with 2,4-dinitrophenyl hydrazine to give a colored hydrazine and was used to measure alanine aminotransferase activity. The oxaloacetate hydrazone formed with 2,4-dinitrophenyl hydrazine was used to measure aspartate aminotransferase (AST). Both ALT and AST were read at 540 nm wavelength.

2.8. Determination of serum lipids (lipid profile)

2.8.1. Determination of total cholesterol

The determination of total cholesterol was carried out as demonstrated by Siedel et al. [26]. Cholesterol esters were hydrolyzed by cholesterol esterase to produce cholesterol and fatty acids. The cholesterol was oxidized by cholesterol oxidase to cholesterone and hydrogen peroxide. The H_2O_2 was later hydrolyzed by peroxidase to form water and oxygen. The oxygen then reacted with 4-amino-antipyrine, which is the chromogen to form quino-neimine. The color intensity of the solution was proportional to the concentration of cholesterol in the sample. The samples were mixed and incubated for 10 min in a water bath at 37°C. The color produced was read colorimetrically at 540 nm [26].

Calculation:

$$\frac{\text{Absorbance of test} \times \text{Concentration of standard (5.2 mmol } l^{-1})}{\text{Absorbance of standard}}$$

2.8.2. Determination of triglyceride

The determination of triglyceride was analyzed as demonstrated by Negele et al. [27]. Triglyceride in the sample was hydrolysed by lipoprotein lipase to glycerol and free fatty acids. Glycerol was phosphorylated by the kinase to form glycerol-3-phosphate and ATP. The glycerol phosphate was then oxidized by glycerol phosphate oxidase to dihydroxyacetone phosphate and H_2O_2. The H_2O_2 was hydrolysed by peroxidase to form H_2O and O_2. The O_2 then reacted with 4-amino-antipyrine and phenol to form the color complex quinoneimine. The samples were mixed and incu-bated for 10 min in a water bath at 37°C. The color produce was read colorimetrically at 540 nm [27].

Calculation:

$$\frac{\text{Absorbance of test} \times \text{Concentration of standard (2.3 mmol } l^{-})}{\text{Absorbance of standard}}$$

2.8.3. Determination of high density lipoprotein

The determination of high density lipoprotein cholesterol was analysed as demonstrated by [26]. The HDL-cholesterol is a precipitate off apoprotein B-containing lipoprotein using a mixture of sodium phosphotungstic acid and magnesium chloride. The samples were mixed thoroughly and allowed to stand at room temperature for 15 min and later centrifuged at 3000 revolutions per min. The samples were mixed and incubated for 10 min in a water bath at 37°C [26].

Calculations:

$$\frac{\text{Absorbance of test} \times \text{Concentration of standard (1.3 mmol } l^{-})}{\text{Absorbance of standard}}$$

Final result was multiplied by the dilution factor 3.0.

2.8.4. Determination of low and very low density lipoprotein

Low and very low density lipoprotein concentrations were calculated using the Friedwald formular [28]:

LDL_c = Total cholesterol - (HDL_c+$VLDL_c$)

VLDL = Triglyceride/2.22

2.9. Determination of serum electrolytes

Serum Na^+ and K^+ concentrations were determined using a flame photometer (Model 410C, Petracourt Ltd, England). Serum Cl^- concentration was determined using the end point calorimetric titration method [29]. Serum bicarbonate (HCO_3) concentration was measured using the modified method [30].

2.10. Statistical analysis

Data was presented as Mean ± SEM. The student's t test was employed to compare two sets of data. Three or more variables were compared with one-way analysis of variance (ANOVA). The $p<0.05$ and $p<0.001$ were considered statistically significant.

3. RESULTS

3.1. Analysis of serum enzymes

As shown in Table 1 the alanine transferase enzyme concentration was significantly lower ($p<0.05$ and 0.001) in the low dose (59.0 ± 2.1*) and high dose (43.1±2.81) groups than the control (77.8± 0.37) group, respectively.

The alkaline phosphatase enzyme concentration was significantly lower ($p<0.05$ and 0.001) in the low dose (75.0±1.20) and high dose (71.0±1.41) groups than the control (86.6±0.75) group, respectively.

The difference in aspartate transferase enzyme concentration was of no statistical significance among the three groups.

Table 1. Serum enzymes in the different experimental groups.

	ALT (IU/L)	ALP (IU/L)	AST (IU/L)
Control	77.8±0.37	86.6±0.75	105.0±0.23
Low dose	59.0±2.1*	75.01±1.20	104.2±0.14
High dose	43.1±2.81***	71.0±1.41*	103.2±0.43

Values are represented as Mean ± SEM. *P < 0.05, ***P < 0.001 vs Control.

3.2. Analysis of lipid profile

As shown in Table 2 the total cholesterol concentration was significantly lower ($p<0.001$) in the low dose (1.15±0.04) and high dose (1.03±0.03) extract treated groups than the control (1.32±0.04) group, respectively.

The triglyceride concentration was significantly lower ($p<0.001$) in the low dose (0.31±0.01) and high dose (0.30±0.01) extract treated groups than the control (0.65±0.02) group, respectively.

The high density lipoprotein cholesterol concentration was significantly higher ($p<0.001$) in the low dose (0.67±0.04) and high dose (0.69±0.01) extract treated groups than the control (0.64±0.02) group, respectively.

The low density lipoprotein cholesterol concentration was significantly lower ($p<0.001$) in the low dose (0.63±0.01) and high dose (0.47±0.01) extract treated groups than the control (1.97±0.03) group, respectively.

The very low density lipoprotein cholesterol concentration was significantly lower ($p<0.001$) in the low dose (0.15±0.03) and high dose (0.13±0.05) extract treated groups than the control (0.29±0.01) group, respectively.

3.3. Analysis of serum electrolytes

As shown in Table 3 the sodium (Na) concentration was significantly lower ($p<0.001$) in the low dose (125.6±0.75) and high dose (123.6± 0.45) groups than the control (136.2±1.00) group, respectively.

The pottassium (K) concentration was significantly higher ($p<0.001$) in the low dose (7.0±0.14) and high dose (7.10±0.21) groups than the control (5.66±0.07) group, respectively.

Table 2. Lipid profile in the different experimental groups.

	Tc(mg/dL)	TG (mg/dL)	HDLc (mg/dL)	LDLc (mg/dL)	VLDLc (mg/dL)
Control	1.32±0.04	0.65±0.02	0.64±0.02	1.97±0.03	0.29±0.01
Low dose	1.15±0.04***	0.33±0.01***	0.67±0.04**	0.63±0.01***	0.15±0.03***
High dose	1.03±0.03***	0.31±0.01***	0.69±0.01***	0.47±0.01***	0.13±0.05***

Values are represented as Mean ± SEM. **P < 0.01, ***P < 0.001 vs Control.

Table 3. Serum electrolyte in the different experimental groups.

	Na^+ mmol/L	K^+ mmol/L	Cl^- mmol/L	HCO_3 mmol/L	Ca^{2+} mmol/L
Control	136.2±1.00	5.66±0.07	101.4±0.75	25.1±0.37	0.94±0.04
Low dose	125.6±0.75***	7.0±0.14***	95.0±0.75***	27.2±0.43**	1.35±0.04***
High dose	123.6±0.45***	7.10±0.21***	80.0±0.63***	29.0±0.37**	1.75±0.02***

Values are represented as Mean ± SEM. **p<0.01, ***p<0.001 vs Control. Na^+: Sodium, K^+: Potassium, Cl^-: Chlorine, HCO_3^-: Bicarbonate, Ca^{2+}: Calcium.

The chloride (Cl) concentration was significantly lower (p<0.001) in the low dose (95.0±0.75) and high dose (80.0±0.63) groups than the control (101.4±0.75) group, respectively.

The bicarbonate (HCO_3) concentration was significantly higher (p<0.001) in the low dose (27.2±0.43) and high dose (29.0±0.37) groups than the control (25.1±0.37) group, respectively.

The serum calcium (Ca^{2+}) concentration was significantly higher (p<0.001 and 0.01) in the low dose (1.35±0.04) and high dose (1.75±0.02) groups than the control (0.94±0.04) group, respectively.

4. DISCUSSION

This study was meant to investigate the effect of crude extract of *Thais coronata* on some biochemical indices of albino Wister rats and the results we got were quite amazing. The serum enzyme result revealed that the crude extract was able to reduce the level of ALT and ALP in a dose dependent manner. This is an indication that the hepatocytes or liver tissues in general benefited from the extract administration and ALT is a more specific and stronger indicator of liver cell damage than AST, also ALT is found primarily in the liver and AST is found in many other organs of the body besides the liver [31, 32]. Therefore, lowered serum ALP confirms that the extracts may not have damaging effects on the liver cells and bone.

Consumption of edible seafood was found to be of immense benefit to health because of its high content of unsaturated fatty acid and polyunsaturated fatty acid especially omega-3 fatty acid [33]. Here administration of *Thais coronata* extract was shown to cause significant reduction in total cholesterol, triglyceride and low density lipoprotein level and an increase in high density lipoprotein level in albino Wistar rats.

This result conforms with various studies previously carried out [13] which revealed that edible seafood are capable of boosting high density lipoprotein level, [34] which revealed that administration of fenofibrate therapy decreased TG level and also ameliorate system oxidation and inflammation [35] which revealed that extract of saffron and crocin administration reduced TC and TG levels and are useful in the prevention of dyslipidemia and obesity.

Raised levels of serum total cholesterol, triglycerides and low density lipoprotein cholesterol are possible indicators of coronary heart attack, risk of heart disease and stroke [36]. The ability of the extract of rock snail to reduce these bad cholesterols in the blood shows that their consumption would be beneficial to health. Rock snail extract has been reported to contain omega-3 fatty acid which is believed to mediate the decrease in the concentrations of these bad cholesterol [37] and this is useful in promoting the clearance of triglyceride from blood [38] also the mechanism of action of

nutraceuticals on lipid profile is further being reviewed [39].

The increase in HDL-c observed in the rock snail extract fed groups could also be attributed to omega-3 component of the extract [12], which is equally important because HDL-c is the good cholesterol that function in preventing the accumulation of bad cholesterol and ameliorating the risk of heart disease

The serum electrolyte result has revealed that there was a reduction of sodium ion concentration following the administration of rock snail extract, this may be due to the low concentration of sodium in the extract, or possibly due to the ability of the extract to potentiate excretion of sodium ions from the body. This was followed by a decrease in chloride concentration since sodium and chloride ions are always transported alongside [40]. This result is also very important because elevated Na^+ concentration predisposes one to high blood pressure [41], it therefore means that consumption of *Thais coronata* extract may be important in preventing high blood pressure. The extract treated group also had a significant increase in potassium ion concentration, this may be brought about by the decrease in serum sodium ions occasion by it excretion and reabsorption of potassium ions, since sodium and potassium ions are always exchanged in alternate manner by the Na^+/K^+ pump along the cell membrane [42]

There was an increase in HCO_3 concentration in the rock snail extract treated group. It is well known that bicarbonate is essential in neutralizing the acidic pH produce by the acid in the gastrointestinal tract [41]. Bicarbonate ions also maintain the acid-base buffering system of the blood. Finally, extract treated group also produced elevated plasma Ca^{2+}, this may be useful in preventing bone resorption and other related conditions associated with calcium deficiency.

5. CONCLUSION

Seafood consumption is of immense benefit to health because it serves to regulate the lipid profile, electrolytes and enzyme concentrations in blood.

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AUTHORS' CONTRIBUTION

AAN wrote the initial draft of the manuscript; AEO and AAA designed the study, OEO did the statistical analysis while, IOB and SUK proof read, and edited the word. All authors were involved in the execution of the research plan. The final manuscript has been read and approved by all authors.

TRANSPARENCY DECLARATION

The authors declare no conflicts of interest.

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Research Article

Occurrence and characteristics of the migrating myoelectric complex in ovine gallbladder and its relationships to the small intestinal motility

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ABSTRACT

An attempt has been made to identify the migrating motility complex in the ovine gallbladder and to span it with the small-intestinal pattern. For this purpose, four rams underwent surgical implantation of bipolar electrodes into the abomasal antrum, entire small bowel and gallbladder infundibulum, corpus and fundus. The strain gauge force transducer was also mounted in the gallbladder fundus, near the electrode. In the course of chronic experiments, the myoelectrical and motor activity was recorded in fasted and non-fasted rams, with or without feeding. Cyclic myoelectrical and motor activity pattern was found in the gallbladder. It resembled the migrating myoelectric complex present in the small bowel. The gallbladder pattern was well correlated with the intestinal migrating complex. Three or four phases of this pattern could be identified in all gallbladder regions. The most characteristic phase 3-like activity was longer and more intense in the gallbladder fundus as compared with the upper gallbladder regions. In both the small bowel and gallbladder, motility alterations caused by various feeding conditions were comparable. Therefore, the migrating motility complex occurs in the ovine gallbladder, albeit its putative role can be different from that in the small bowel, at least in part.

Keywords: Sheep; Gallbladder; Myoelectrical activity; Mechanical activity; Migrating motility complex.

1. INTRODUCTION

The principal roles of the gallbladder are to store and concentrate bile and to deliver it periodically towards the duodenum [1, 2]. Thus it exhibits the composed motor function. Permanent gallbladder filling and emptying facilitates normal enterohepatic circulation of bile acids [3]. The gallbladder exhibits phasic and tonic contractions being the strongest after feeding and evacuating almost all the gallbladder bile into the duodenum. In monogastrics, during the interdigestive period, gallbladder motility is also intense, particularly when phase 2 or phase 3 of the migrating motility complex (MMC) arrives in the small bowel. Therefore, cyclic gallbladder motor activity and cyclic bile evacuation into the duodenum occurs during this period [4]. In sheep, the presence of phasic and tonic gallbladder contractions has also been reported, except during short quiescent periods

in the course of phase 1 of the duodenal MMC cycles [5]. Feeding enhanced gallbladder contractility also in this species [6]. In scanty studies on sheep, cyclic character of gallbladder myoelectrical activity, few differences between the gallbladder neck and fundus and presence of the 'minute rhythm' were demonstrated as well [7, 8]. When the 'minute rhythm' occurs regularly in the ovine gallbladder, as in the upper small bowel, the presence of the MMC could also be expected, like in the proximal small bowel, as suggested in the dog [9] and in brief report in sheep [10]. Thus, the aim of this study was to identify and characterize the MMC in ovine gallbladder and to span it with the duodenal MMC.

2. MATERIALS AND METHODS

2.1. Experimental animals

Four adult rams of Polish Merino breed, each weighing 42 kg (range 39-43 kg), were used. Animals were clinically healthy and were not used previously for other types of the experiments. Before the surgery, they were kept in the spacious, clean and dry cages in small groups at the natural daily light rhythm. They were fed with a good quality hay and grain mixture (CJ mixture for calves and lambs, Dolpasz, Wrocław, Poland) according to the appropriate daily intake. Drinking water was not limited except in the course of the experiments.

2.2. Animal preparation

The bipolar platinum serosal electrodes and strain gauge force transducers were used for the recording of electrical and mechanical activity. The strain gauge force transducers (RB Products, Madison, WI, USA) were calibrated before implantation. In 24 h fasted rams, after 10-12 cm laparotomy, each animal was fitted with ten electrodes and one strain gauge force transducer under general and local anesthesia [11].

The electrodes were located as follows:

1 - the abomasal antrum, 4 cm proximally to the mid of the pyloric ring;

2 - the duodenal bulb, 6 cm distally to the mid of the pyloric ring;

3 - the duodenum, 50 cm distally to the bulbar electrode;

4 - the jejunum 1, 200 cm distally to the duodenal electrode;

5 - the jejunum 2, spaced 100 cm distally from the jejunal 1 electrode;

6 - the ileum 1, located 110 cm proximally to its termination, i.e. before the ileocecal junction;

7 - the ileum 2, located 10 cm proximally to the ileocecal junction;

8 - gallbladder infundibulum, 1 cm distally to the gallbladder neck;

9 - gallbladder corpus, 4 cm distally to the upper gallbladder electrode;

10 - gallbladder fundus, 4 cm distally to the mid of gallbladder electrodes.

The strain gauge force transducer was attached near gallbladder fundic electrode. After the surgery, animals were kept in single cages and feeding was gradually started from the second postsurgical day. Further details of the experimental protocol are available elsewhere [11, 12].

2.3. Experimental design

The total of 32 randomized experiments were conducted. Chronic experiments, lasting 6-8 h each, were started at least ten days following the surgery.

Four types of the experiments were carried out: [a] in 48 h fasted rams without feeding (control group), [b] in 48 h fasted rams with feeding during phase 2b of duodenal MMC (250 g of the grain mixture), [c] in non-fasted rams without feeding, [d] in non-fasted rams with feeding during phase 2b of the MMC identified in the duodenum (250 g of the grain mixture). Each type of the experiment was preformed twice: with or without mechanical activity recording (the myoelectrical activity recorded with ten electrodes). The mechanical activity was thus recorded from the fundic strain gauge force transducer replacing the ileal 2 electrode and the myoelectrical activity was recorded simultaneously from nine remaining electrodes. The data were derived from these experiments. The fodder was removed from the cage 1-2 h before each experiment performed in non-fasted rams. The myoelectrical and mechanical activity was recorded throughout the experiments using the 10-channel electroencephalograph (Reega) equipped additionally with the Wheatstone bridge for the recordings

of mechanical activity. After the initial period, lasting 15-40 min, at least two full small intestinal MMC cycles were recorded and then the recording was continued until the arrival of phase 2 of the subsequent MMC cycle.

2.4. Myoelectrical and mechanical recordings

Typical spike bursts along with the myo-electrical patterns were recorded in the abomasal antrum and entire small bowel. The phasic contractions and their myoelectrical correlates were principally recorded in the gallbladder fundus while in the remaining gallbladder regions, mostly the short spike bursts as the myoelectrical correlates of phasic contractions, were identified on the recordings. These events were principally organized in the cycles closely resembling the small-intestinal MMCs and were observed most clearly in the myoelectrical recordings. The subsequent MMC phase-like activity, i.e. the phases 1-3, were identi-fied in the gallbladder as well. Phase 4 was not always observed. Several parameters characterizing the duodenal and gallbladder MMC were calculated from the tracings: the MMC cycle duration, duration of the MMC phase, expressed both in minutes and in percentage of total cycle duration, coordination of the gallbladder phase 3 of the MMC with those in the duodenum, the propagation (migration) velocity of phase 3, the amplitude and duration of the phase 3-spike bursts and the contractions forming phase 3 of the MMC. The propagation velocity of phase 3 of the MMC was expressed as the overall parameter (for the whole gallbladder). Additionally, its posi-tive values (aboral migration) and negative values (oral migration also called the retropropagated event) were presented separately. The representative figures illustrating the gallbladder MMC were also shown.

2.5. Statistical data elaboration

The mean values and standard deviations were calculated where appropriate. Then, the Student *t*-test for paired values, preceded by analysis of variance, was used [13]. Statistical significances ($P<0.05$, $P<0.01$ and $P<0.001$) were introduced into the tables.

2.6. Ethical approval

Protocol of the study and informed consent were in compliance with the Helsinki convention and were approved by local Ethics Committee.

3. RESULTS

In the recordings of electrical activity, the myoelectric correlates of phasic contractions (short-lasting spike bursts of various duration, lasting usually 0.2-1.5 s) were observed in all the regions examined. Mechanical recordings divulged the presence of both phasic and tonic contractions in the gallbladder fundus. The MMC was conclusively identified in the entire small bowel and gallbladder in all the experiments performed. In the abomasal antrum, the MMC was absent and in the duodenal bulb it was usually greatly reduced. Duration of the MMC cycle in the small bowel was very similar to that in the gallbladder regardless of feeding conditions (Table 1). It was longer after feeding, but changes were not statistically significant. The MMC phases 1-4 were identified both in the small bowel and the gallbladder (Fig. 1, 2). Duration of phase 1 of the MMC in the gallbladder was similar in the various gallbladder regions regardless of feeding conditions (Table 1). Duration of phase 2 of the MMC was significantly longer after feeding than in not fed rams. Similar difference was observed between fasted and non-fasted rams. Duration of phase 3 of the MMC was significantly longer in the gallbladder fundus than in the infundibulum and corpus (Table 1, see also Figs. 1, 2). After feeding, duration of phase 3 in the gallbladder fundus was significantly shorter than in not fed animals. Phase 4 of the MMC was often present, but in few cases it was virtually absent. Phase 4, despite its variable duration, was significantly longer in the gallbladder corpus after feeding than in not fed animals (Table 1). Phase 3 of the MMC in the gallbladder was well coordinated with that in the duodenum (Table 2, Fig. 3). However, coordination of phase 3 of the MMC in the gallbladder infundibulum and corpus with duodenal phase 3 was different. Sometimes the onset of phase 3 in the gallbladder preceded the onset of phase 3 in the duodenum. In other cases, it was slightly delayed.

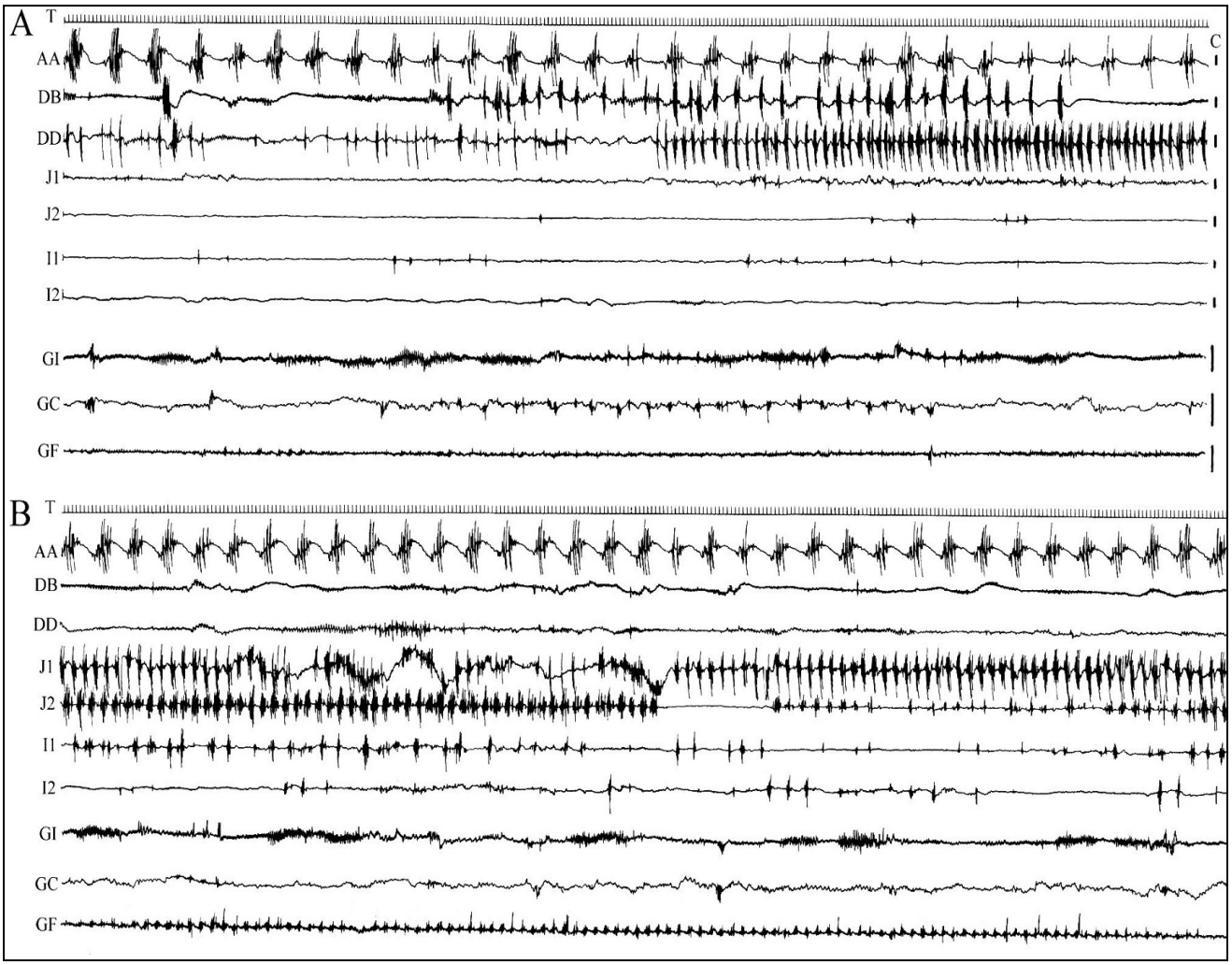

Figure 1. The presence of the migrating motility complex (MMC) in small intestine and the gallbladder of non-fasted ram. Two five-minute electromyographical recording fragments separated with 2.5 min. brake are shown.
Panel A: Phase 3 in the duodenal bulb (shortened) and phase 3 of the MMC present at the same time in the gallbladder infundibulum slightly preceded by phase 3 in the gallbladder corpus. In the gallbladder fundus, phase 2 of the MMC is noticeable. In the gallbladder infundibulum, the short-lasting spike bursts are alternated with the long-lasting spike bursts. Note that phase 3 in the abomasal antrum cannot be identified because of the continuous maximal spike bursts.
Panel B: Phase 3 is present in the jejunum and in the gallbladder fundus where it is followed by very short phase 4 of the MMC. In the gallbladder infundibulum and corpus, phase 1 of the MMC is visible.
Explanations of symbols: T, time in seconds. Electrodes: AA, abomasal antrum; DB, duodenal bulb; DD, duodenum; J1, jejunum 1; J2, jejunum 2; I1, ileum 1; I2, ileum 2; GI, gallbladder infundibulum; GC, gallbladder corpus; GF, gallbladder fundus. C, calibration, 100 μV. Other explanations as in the section Materials and methods.

In the gallbladder fundus, phase 3 of the MMC arrived few minutes later than in the upper gallbladder (Table 2). The propagation velocity of phase 3 of the MMC, observed in the gallbladder, differed substantially. While measured between gallbladder infundibulum and corpus, the values were sometimes negative due to retropropagation of phase 3 in the upper gallbladder region. Therefore, the overall values were fairly dispersed since they contained both positive and negative values. Furthermore, no marked differences related to feeding conditions were denoted in the gallbladder, except markedly and significantly lower negative values in not fed animals, studied either with or without feeding, as compared with the fasted animals (Table 2). In the lower gallbladder region, i.e. when the propagation velocity was measured between gallbladder corpus and fundus, the values were much lower than the values obtained from the upper gallbladder region (Table 2).

Table 1. Characteristics of the migrating motility complex-like activity in the ovine gallbladder in various feeding conditions.

			MMC cycle duration (min)		Duration of gallbladder MMC phases (min)											
					Phase 1			Phase 2			Phase 3			Phase 4		
			duod.	gallbl.	infund.	corpus	fund.	infund.	corpus	fund.	infund.	corpus	fund.	infund.	corpus	fund.
Fasted	not fed	n=	4	4	4	4	4	4	4	4	4	4	4	3	3	2
		mean	61.3	61.8	19.8	19.8	18.3	39.8	40.3	38.3	1.9	1.6	5.3^{z}	0.4	0.3	0.5
		±S.D.	25.4	25.1	6.7	9.1	8.0	18.1	16.7	18.5	0.5	0.9	0.7	0.2	0.2	0.3
		%	-	100.0	32.0	32.0	29.6	64.4	65.2	61.9	3.0	2.6	8.5	0.6	0.5	0.8
	fed	n=	4	4	4	4	4	4	4	4	4	4	4	4	4	4
		mean	94.3	94.3	21.8	20.8	19.0	70.0^{a}	69.3^{a}	70.3^{a}	1.5	1.9	3.9^{az}	1.0	1.5^{a}	1.0
		±S.D.	17.0	18.1	8.7	7.9	7.6	13.1	13.9	13.8	0.5	1.1	0.7	0.5	0.9	0.7
		%	-	100.0	23.1	22.0	20.2	74.3	74.0	74.5	1.6	2.0	4.1	1.0	1.6	1.0
Not fasted	not fed	n=	4	4	4	4	4	4	4	4	4	4	4	4	2	3
		mean	80.0	79.5	24.5	25.3	20.8	53.0	52.0	53.5	1.8	1.8	4.7^{z}	0.4	0.5	0.6
		±S.D.	22.7	24.0	7.7	8.8	10.9	15.9	17.8	14.2	0.4	0.4	0.9	0.3	0.1	0.5
		%	-	100.0	30.8	31.8	26.1	66.7	65.4	67.3	2.2	2.2	5.9	0.4	0.6	0.8
	fed	n=	4	4	4	4	4	4	4	4	4	4	4	4	4	4
		mean	100.3	100.5	26.0	26.0	26.3	72.0^{a}	72.0^{a}	69.5^{a}	1.6	1.5	4.0^{az}	0.9	1.2^{b}	0.8
		±S.D.	19.1	19.2	7.5	7.6	5.1	11.5	12.8	14.0	0.3	0.7	0.6	0.6	0.5	0.5
		%	-	100.0	25.9	25.9	26.1	71.6	71.6	69.2	1.6	1.5	4.0	0.9	1.1	0.8

Explanations: %, percent of the total MMC cycle duration, ^{a}, P<0.05; ^{b}, P<0.01 vs. relevant value in fasted animals, ^{z}, P<0.001 vs. relevant value in gallbladder indundibulum. Other explanations as in the section Materials and methods.

Table 2. Characteristics of phase 3 of the migrating motility complex-like activity in the ovine gallbladder in various feeding conditions.

			Duodenum-gallbladder phase 3 coordination (min)			Propagation velocity of gallbladder phase 3 (cm/min)				Spike burst amplitude of gallbladder phase 3 (μV)			Contract. amplit. (g)	Spike burst duration of gallbladder phase 3 (s)			Contract. duration (g)
			infund.	corpus	fund.	infundib.-corpus *total*	infundib.-corpus *posit.*	infundib.-corpus *negat.*	corp.-fund. *(posit.)*	infund.	corpus	fund.	fundus	infund.	corpus	fund.	fundus
Fasted	not fed	n=	4	4	4	4	1	3	4	4	4	4	4	4	4	4	4
		mean	0.5	-0.15	5.0	22.7	5.0	28.6	0.8	55.3	55.3	55.0	2.1	1.3	1.2	1.1	5.1
		±S.D.	0.8	0.4	2.2	20.0	0.0	18.1	0.5	7.1	7.5	8.8	0.3	0.4	0.2	0.4	0.4
	fed	n=	4	4	4	4	3	1	4	4	4	4	4	4	4	4	4
		mean	0.0	0.8	9.0	15.7	7.5	40.0	0.6	30.8^{c}	28.0^{c}	28.5^{c}	1.5^{a}	1.6	1.6	1.5	4.2^{a}
		±S.D.	0.4	0.3	4.0	16.8	5.1	0.0	0.3	8.2	8.3	6.1	0.3	0.3	0.2	0.3	0.3
Not fasted	not fed	n=	4	4	4	4	2	2	4	4	4	4	4	4	4	4	4
		mean	-0.7	-1.0	5.3	6.5	8.5	4.5^{b}	1.0	41.8	39.0^{a}	41.0	1.6^{a}	1.1	1.1	1.1	4.7
		±S.D.	-0.4	0.7	1.9	2.6	2.1	0.7	0.4	9.5	10.7	10.6	0.2	0.3	0.6	0.4	0.3
	fed	n=	4	4	4	4	3	1	4	4	4	4	4	4	4	4	4
		mean	0.1	0.8	9.3	2.7	2.9	2.3^{c}	0.8^{a}	37.8^{c}	38.0^{b}	36.5^{b}	1.2^{b}	1.0	0.9	1.0	3.8^{b}
		±S.D.	0.4	1.5	5.8	0.9	1.0	0.0	0.6	5.1	5.2	4.0	0.2	0.4	0.3	0.3	0.2

Explanations: *posit.*, positive values only (phase 3 propagated); *negat.*, negative values only (phase 3 retropropagated) ^{a}, P<0.05; ^{b}, P<0.01, ^{c}, P<0.001 vs. relevant value in fasted animals. Other explanations in the section Materials and methods.

The amplitude of the spike bursts of phase 3 of the MMC observed in all the gallbladder regions, was significantly lowered after feeding when compared with fasted animals. In the non-fasted, not fed rams, the lowering tendency of this parameter in the gallbladder infundibulum and fundus was observed, while in the gallbladder corpus it achieved the level of statistical significance when compared with fasted rams (Table 2). The amplitude of phase 3 contractions in the gallbladder fundus was

significantly higher in fasted - not fed animals as compared with other experimental groups (Table 2). Duration of the spike bursts forming phase 3 of the gallbladder MMC was higher in fasted animals after feeding than in the control group and than in non-fasted rams (Table 2).

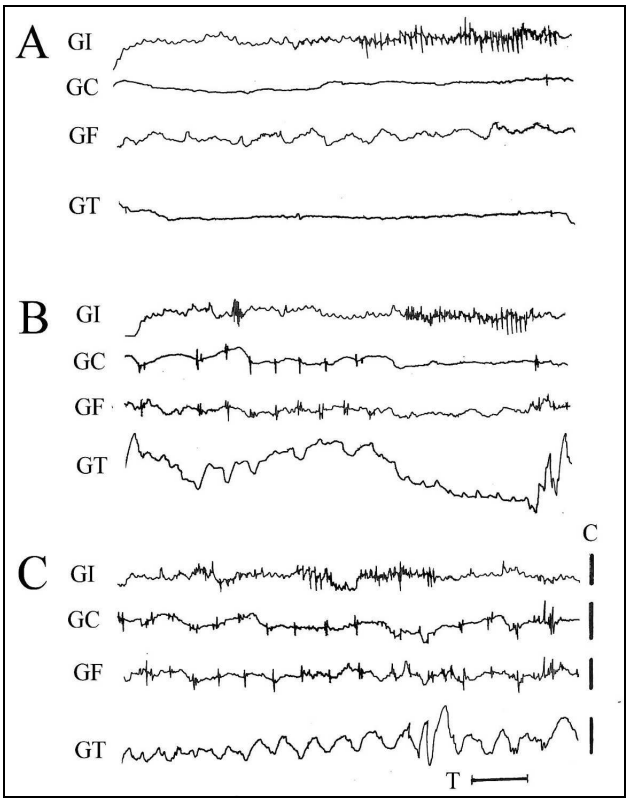

Figure 2. The subsequent phases of the migrating motility complex (MMC) in the gallbladder of non-fasted ram, recorded during the same MMC cycle. Note the presence of the long-lasting spike bursts in the gallbladder infundibulum during all the MMC phases.
Panel A, phase 1 of the MMC. Panel B, phase 2 of the MMC. Panel C, phase 3 of the MMC.
Explanations of symbols: GT, strain gauge force transduced in gallbladder fundus. C, calibration, 100 μV. T - time, 10 s, 2.5 g. Other explanations as in the legend to Fig. 1.

Duration of phase 3-related contractions in the gallbladder fundus was shortened in both groups studied with feeding procedure when compared with the relevant data obtained from the experiments performed in fasted animals (Table 2). Fig. 4 presents the fragments of well-developed phase 3 of the MMC in the gallbladder fundus of fasted rams. In the gallbladder infundibulum and also frequently in the gallbladder corpus, the so-called long-lasting spike bursts were observed during all the MMC phases (see Fig. 1).

In the course of additional experiments the recordings were similar to those obtained from proper experiments and phase 3 of the MMC was also observed in all recording channels, except the abomasal antrum, including the recordings from the ileal 2 electrode.

4. DISCUSSION

The MMC cycles were observed both in the small bowel and gallbladder in all the experiments performed. The MMC pattern in the ovine gallbladder was more evident in the electromyographical than in mechanical recordings because of the character of gallbladder motor function. Analysis of the mechanical recordings showed that short-lasting contractions were often combined with the relatively frequent long-lasting contractions and made the analysis of the short-lasting (phasic) contractions unclear. There are several similarities between gallbladder motility in sheep [7, 8, 14, 15] and in monogastric species [16-19] comprising its complexity, relations to the interdigestive motility of the small bowel and character of gallbladder emptying. The long-lasting contractions including giant cotractions are known to be present both in the gallbladder and the gut [20-22]. Tonic contractions are also present both in the small bowel and gallbladder although no their myoelectric correlates have been described. In the gallbladder, tonic contractions occur mostly after feeding as the slow, smooth contractions that are responsible for gallbladder emptying [1]. Phasic contractions are often super-imposed on tonic contractions [17]. Since no myoelectric correlates of tonic contractions were observed in the gallbladder, the MMC was more evident in the myoelectrical recordings than in the mechanical recordings. Its wall is relatively thin and it is not easy to obtain the good quality myoelectrical recordings. Since in the earlier report Ludwick and Bass [23] did not find the electrical activity in the gallbladder of the dog and monkey, technical difficulties were probably the reason. This is improbable because the muscle layer is present in the gallbladder and Matsumoto et al. [24] obtained positive results in the dog.

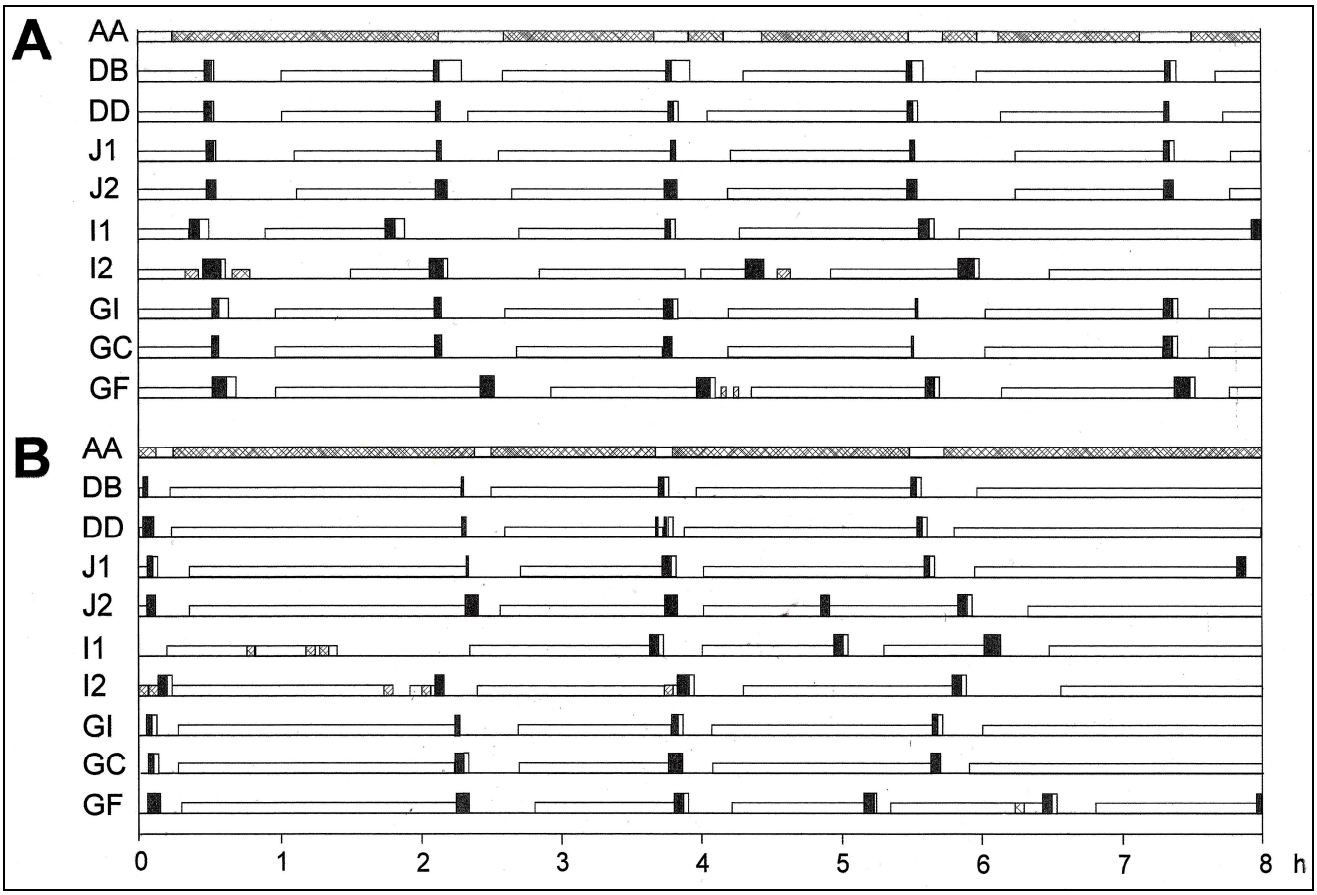

Figure 3. Scheme of the gastrointestinal and gallbladder migrating motility complex (MMC) based upon the eight-hour myoelectrical recordings. Upper panel, fasted rams; lower panel, not fasted rams; scheme of the experiments performed in the same animal.

Bars: crossed bars, maximal myoelectric activity observed in the abomasal antrum, ileum and gallbladder fundus, not interpreted as phase 3 of the MMC; open low bars, phase 2 of the MMC; closed high bars, phase 3 of the MMC, open high bars, phase 4 of the MMC; no bar, phase 1 of the MMC. Other explanations as in the legend to Fig. 1.

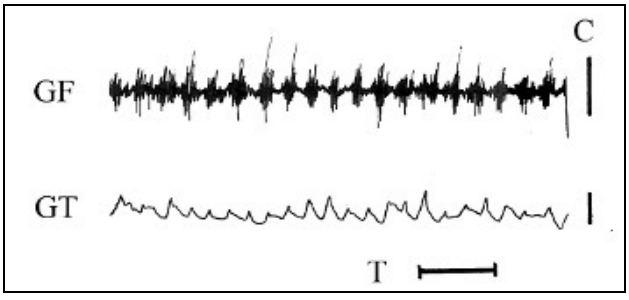

Figure 4. Fragments of phase 3 of the migrating motility complex in gallbladder fundus of fasted ram.

Explanations: GF, the myoelectrical activity recording from gallbladder fundus; GT, motor activity recording from gallbladder fundus. C, calibration, 50 μV and 2.5 g, T, time, 10 s.

Only few full reports clearly presenting the gallbladder myoelectrical activity are available in sheep [7, 8, 11, 15] and in pigs [25]. None of them described the MMC in the gallbladder. The studies utilizing the mechanical recording methods of gallbladder motility examination demonstrated periodic alterations of gallbladder motor function in concert with the duodenal MMC, but not visualized the MMC there [16, 17]. There were, however, few suggestions that the MMC is present in the gallbladder [9, 10].

This report presents the evidence and further confirmation that the MMC occurs in animal gallbladder. Although the MMC demonstration appeared clear enough, marked regional differences may accomplish the interpretation of the gallbladder myoelectrical and motor recordings. The coordination of phase 3 of the MMC between the upper (gallbladder infundibulum and corpus) and lower part of the organ (gallbladder fundus) is different than the coordination between the gallbladder

infundibulum and gallbladder corpus. Thus in the gallbladder, the 'cleaved phase 3' of the MMC is present. The apparent role of shorter propagated phase 3 of the MMC in the gallbladder infundibulum, and also in the gallbladder corpus, is to facilitate the gallbladder bile inflow and perhaps also mixing of bile. Sometimes the direction of propagation of phase 3 in the upper gallbladder can be reversed (retropropagated phase 3) which, along with tonic contractions, may promote gallbladder bile evacuation. In the gallbladder fundus, periodic arrival of the longer phase 3 of the MMC may guarantee good mixing and stirring of the gallbladder content preventing crystallization of biliary sediment. Its role in bile transport seems doubtful. The mixing in the gallbladder fundus appears more important than in its proximal parts.

Furthermore, in the ruminants, more continuous than that in monogastrics, evacuation of the bile into the duodenum is probable since in these species rather uninterrupted digestive processes occur in the intestinal lumen. Therefore, intense gallbladder motility represents its specific character being easily adaptative to the situation in the bowel.

Longer myoelectrical events, observed mostly in the gallbladder infundibulum, but also in other gallbladder regions, resembled to some extent the long-lasting spike bursts described in the ovine colon [26]. They were not related to the MMC. Bueno and Praddaude [7] suggested the presence of clusters of spike potentials occurring at regular intervals in the ovine gallbladder. They could serve as the myoelectric correlates of long-lasting contractions and contribute to bile transport.

The role, character and functioning of ovine gallbladder motility does not appear to be much different from that in monogastric species including man [6, 14, 15, 27, 28]. The results obtained from the studies on ovine gallbladder motility can be similar to those in monogastrics and the question arises whether the ovine gallbladder motility model can serve as the relevant model for monogastrics.

By virtue of obtained results it can be concluded that the MMC occurs in ovine gallbladder and is correlated with the MMC pattern observed in the small bowel. Therefore, it can be expected that in other species the MMC in the gallbladder will also be described and this report makes the first base.

TRANSPARENCY DECLARATION

The author declares no conflicts of interest.

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Research Article

In silico sequence analysis of predicted beta-amylase 7-like protein in *Juglans regia* L.

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ABSTRACT

Walnut (*Juglans regia* L.) is a deciduous tree of the Juglandaceae family. Beta-amylase (β-amylase, EC 3.2.1.2) is an enzyme that catalyses hydrolysis of glycosidic bonds in polysaccharides. In this study; sequence, physicochemical, and three-dimensional analyses of predicted β-amylase 7-like protein in *Juglans regia* using various bioinformatic tools were conducted. The physicochemical properties of the predict β-amylase 7-like protein were analyzed by using ExPASy ProtParam tool that revealed the molecular weight (MW), Isoelectric Points (*pI*), total number of negatively charged residues (Asp + Glu), total number of positively charged residues (Arg + Lys), instability index, aliphatic index, and GRAVY (Grand Average of Hydropathy) values. Subcellular localization using CELLO v.2.5, putative phosphorylation sites using NetPhos 3.1 server, domain analysis using Pfam, and secondary structure prediction using SOPMA were accomplished. To predict the 3D structure of the predict β-amylase 7-like protein, homology models were applied using PSIPRED, RAMPAGE, and PyMOL programs. The results of our study provide insight into fundamental characteristics of the predicted β-amylase 7-like protein in *Juglans regia.*

Keywords: *Juglans regia*; β-amylase 7-like; In silico.

1. INTRODUCTION

The genus *Juglans* (family Juglandaceae) comprises 7 to 45 species depending on the taxonomic study. The genus is distributed mostly across the temperate and subtropical regions of the Northern Hemisphere, with several species also found in Central America and along the Andes Mountains in Western South America [1]. Walnut (*Juglans regia* L.) is a species of deciduous tree of the family Juglandaceae. Its leaves, husks, bark, and fruits are used as a raw herb. In the literature, antibacterial and antifungal properties of the fruit extracts of walnut have been described [2]. There are also reports of antioxidant [3, 4], and insecticidal [5] properties of extracts of walnut green husk. Walnuts are mostly consumed in the form of dried fruits.The tree bark of walnut, fruit bark, green fruit bark and leaf parts are widely used in the pharmaceutical and cosmetic industries, and as a stain in the carpet and textile industry [6].

Amylase is a kind of enzyme that catalyses the breakdown of starch into sugars. This enzyme is found in plants and in some bacteria. All types of amylases belong to glycoside hydrolases with α-1,4-glycosidic bonds in polysaccharides, including amylose, amylopectin, glycogen, or their degradation products [7]. Beta-amylase is an exoamylase that attacks the nonreducing ends of starch molecules, producing, β-maltose and β-limit

dextrin as products. β-amylases are strictly plant enzymes that have been reported in ungerminated wheat and soybean seeds; germinating barley, rice, sorghum, and wheat seeds; sweet potato roots; broad bean leaves; and pea seedling roots [8]. Additionally, β-amylase has widespread applications in many industries such as foods, brewing, textiles, adhesives, detergents, pharmaceuticals, and sewage treatments [9].

The aim of this study was to generate predicted 3D structure of β-amylase 7-like protein by using comparative homology modeling. Also, primary and secondary structure analyses were performed utilizing various bioinformatics tools.

2. MATERIALS AND METHODS

The protein sequence of β-amylase 7-like (acession no: XP_018859154.1) in *Juglans regia* was retrieved from NCBI (https://www.ncbi.nlm.nih.gov/protein). The physicochemical analysis and amino acid contents of the pro-teins were analyzed by ExPASy'sProtParam (http://web.expasy.org/protparam/) that is also used to determine isoelectric point (*pI*), mole-cular weight (MW), total number of positive (+R) and negative (-R) residues, extinction coefficient (EC), instability index (II), aliphatic index (Al), and GRAVY values. The putative phosphorylation sites of the β-amylase 7-like protein were determined by NetPhos 3.1 (http://www.cbs.dtu.dk/services/NetPhos/). Secondary structure prediction was performed using SOPMA server (http://npsa-pbil.ibcp.fr/). For domain analysis, Pfam (http://pfam.xfam. org/) was used. Subcellular localization was predicted using CELLO v.2.5 (http://cello.life.nctu.edu.tw/). Motif Scan (http://myhits.isb-sib.ch/cgi-bin/motif_scan) was used to identify known motifs in the sequence. The average amino acid rates were determined by MEGA 6.0 [10]. To predict the 3D structure of the β-amylase 7-like protein, homology model was performed using PSIPRED v3.3 (http://bioinf.cs.ucl.ac.uk/psipred/). The results were checked and verified by a Ramachandran plot analysis in RAMPAGE (http://mordred.bioc.cam.ac.uk/~rapper/rampage.php), which determined the best predicted models. Finally, 3D comparative analysis was performed using PyMOL (https://www.pymol.org/) (TM) (Schrodinger, LLC).

3. RESULTS AND DISCUSSION

The physicochemical analysis of the predicted β-amylase 7-like was performed using ExPASy ProtParam (the results were shown in Table 1). This protein had 693 amino acids with a molecular weight of 78124.14 Daltons and a *pI* of 5.73. The total number of negati-vely charged residues (Asp + Glu, 94) was found higher than the total number of positively charged residues (Arg + Lys, 76). Instability index (43.93), aliphatic index (78.48) and GRAVY value (-0.389) were also determined. The subcellular localization prediction of unknown proteins contributes to understanding of their functions [11].

Table 1. The physiochemical properties of the predicted β-amylase 7-like protein.

Parameters	Value
Molecular weight	78124.14
Theoretical *pI*	5.73
Total number of negatively charged residues (Asp + Glu)	94
Total number of positively charged residues (Arg + Lys)	76
Instability index	43.93
Aliphatic index	78.48
GRAVY	-0.389

Table 2. Secondary structure of the predicted β-amylase 7-like protein.

Parameters	Number of amino acids	Amino acids (%)
Alpha helix (Hh)	243	34.81
3_{10} helix (Gg)	0	0.00
Pi helix (Ii)	0	0.00
Beta bridge (Bb)	0	0.00
Extended strand (Ee)	122	17.48
Beta turn (Tt)	69	9.89
Bend region (Ss)	0	0.00
Random coil (Cc)	264	37.82
Ambiguous states	0	0.00
Other states	0	0.00

The subcellular localization was performed using CELLO v.2.5, and the protein was found to be localized in cytoplasmic, chloroplast and nuclear. The secondary structure of the protein was predicted using SOPMA (Table 2). It was observed that random coil was predominant (37.82%) followed by an alpha helix (34.81%) and an extended strand (17.48%). Also, a beta turn was predicted (9.89%). Random coils have important functions in proteins for flexibility and conformational changes such as enzymatic turnover [7].

Our findings could be related with the enzymatic function of the protein.The domain analysis was conducted using Pfam database and glycosyl hydrolase family 14 was detected. Glycoside hydrolases (GHs), a widely distributed group of enzymes, cleave glycosidic bonds in glycosides, glycans and glycoconjugates, and they can play key roles in the development of biofuels and in disease research. GHs such as cellulases, xylanases, and other glycosidases are being used to produce sugars from pretreated biomass substrates, which are then fermented to produce ethanol or butanol as renewable alternatives to gasoline [12, 13]. The Motif scan tool was used to determine different motifs (Table 3).

The seven types of motifs were observed while the highest number of motifs were N-myristoylation site and casein kinase II phosphorylation site with 11 times, and the lowest number of motifs were amidation site, β-amylase active site 1, and glycosyl hydrolase family 14 as once. Myristoylation is an irreversible, post-translational protein modification found in fungi, higher eukaryotes and viruses. Myristoylation can influence the conformational stability of individual proteins as well as their ability to interact with membranes or the hydrophobic domains of other proteins. Myristoylation plays a critical role in many cellular pathways, especially in the areas of signal transduction, apoptosis, and extracellular export of proteins [14].

Casein kinase II (CKII) is a multifunctional protein kinase that has been implicated in the regulation of central cellular functions, such as cell division and growth, mitosis, signal transduction, gene expression, and DNA replication [15, 16]. The most abundant amino acid composition in the predicted β-amylase 7-like protein Gly (9.1%), while the minimum amino acid ratio in the predicted β-amylase 7-like protein were Cys and Trp (1.8%) (Fig. 1). Phosphorylation processes are important mechanisms regulating cellular functions. Phosphorylation serves to effect critical post-translational modification of proteins having profound effects on their functions, which in turn governs the metabolic processes in a cell and tissue [17]. NetPhos 3.1 server was used to detect the putative phosphorylation sites (Fig. 2). The confidence rate that these were true Phosphorylation sites was above the threshold (0.5) and the output score was given in a 0.0-1.0 range.

ERRAT is a protein structure verification algorithm that analyzes statistics of non-bonded interactions between different atom types based on characteristic atomic interaction [18]. The overall quality factor was found as 66.121 (Fig. 3). The stereochemical quality of the modeled protein was analyzed by RAMPAGE (Fig. 4).

Table 3. The motifs of the predicted β-amylase 7-like protein by Motif Scan.

Motif information	No. of sites	Amino acid residues
Amidation site	1	376-379
N-glycosylation site	2	317-320, 588-591
Casein kinase II phosphorylation site	11	11-14, 184-187, 202-205, 237-240, 312-315, 340-343, 366-369, 373-376, 398-401, 424-427, 692-695
N-myristoylation site	11	46-51, 69-74, 152-157, 177-182, 232-237, 306-311, 345-350, 429-434, 486-491, 537-542, 584-589
Protein kinase C phosphorylation site	8	75-77, 98-100, 174-176, 209-211, 312-314, 373-375, 409-411, 465-467
Beta-amylase active site 1	1	342-350
Glycosyl hydrolase family 14	1	264-684

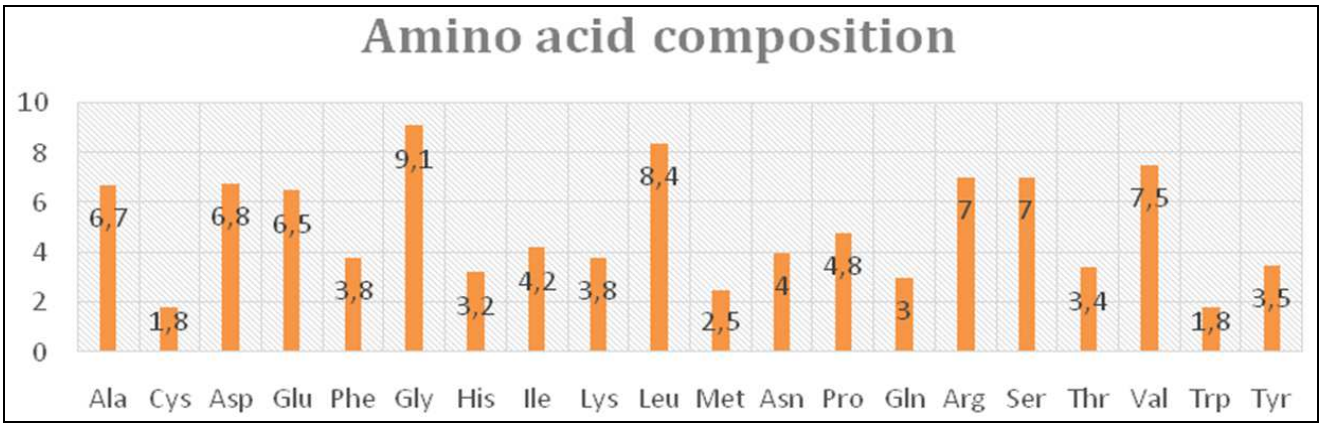


Figure 1. The average amino acid composition of predicted β-amylase 7-like protein from *Juglans regia*.

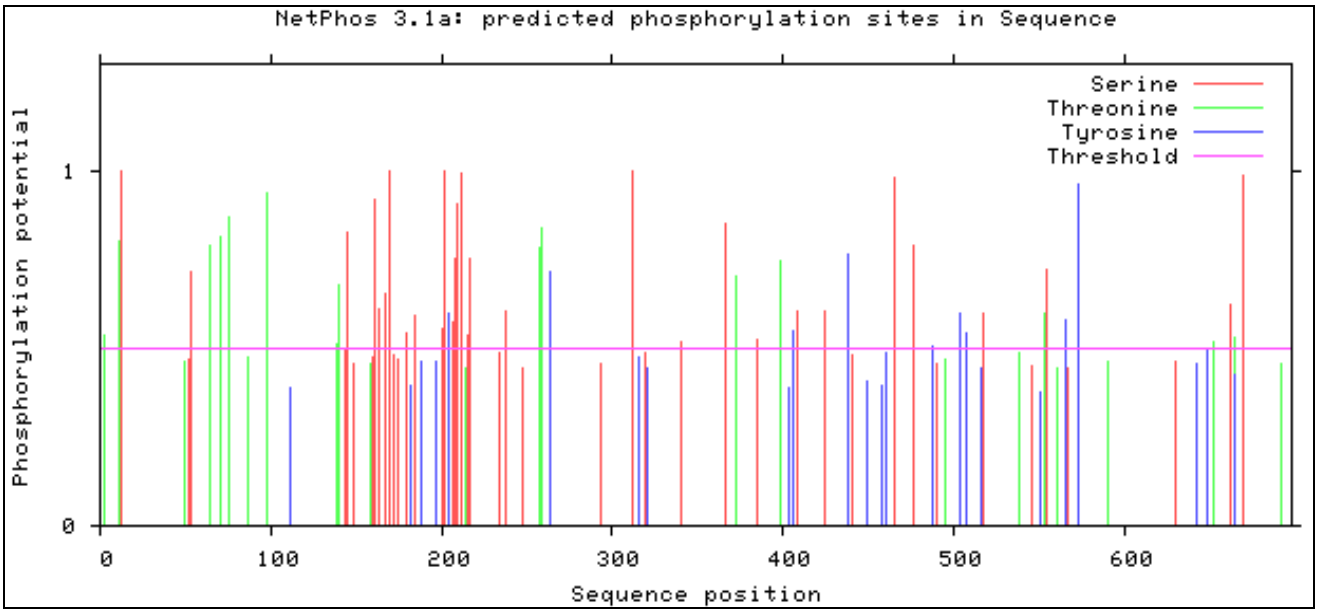


Figure 2. Putative phosphorylation sites of the predicted β-amylase 7-like protein determined with a score above a threshold of 0.5.

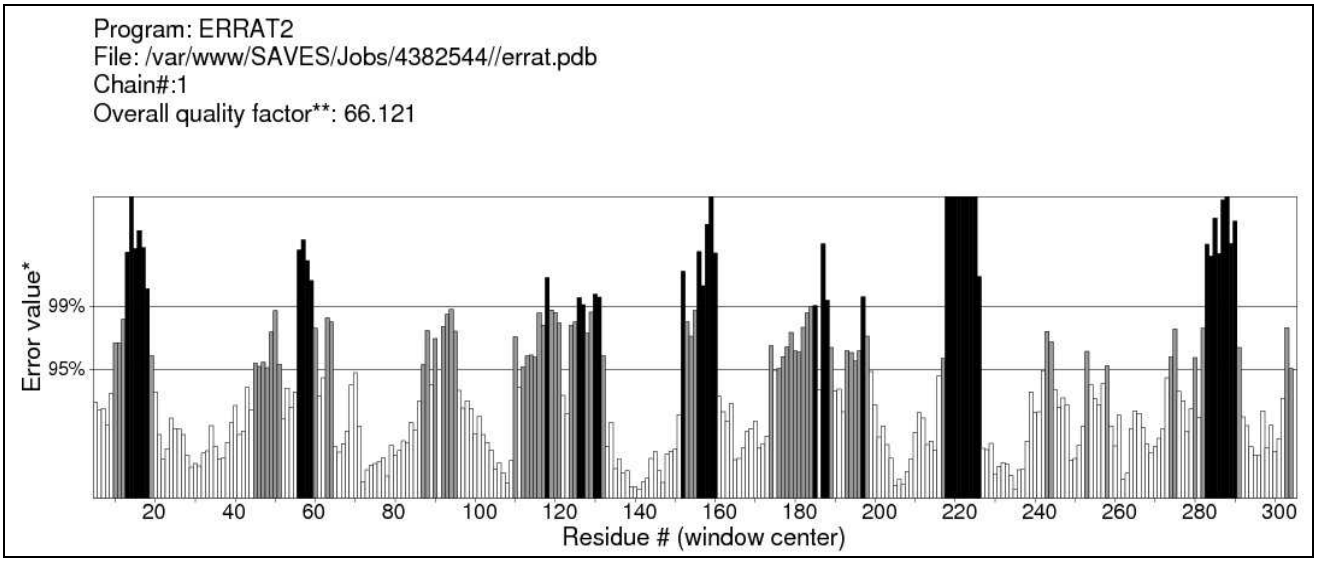
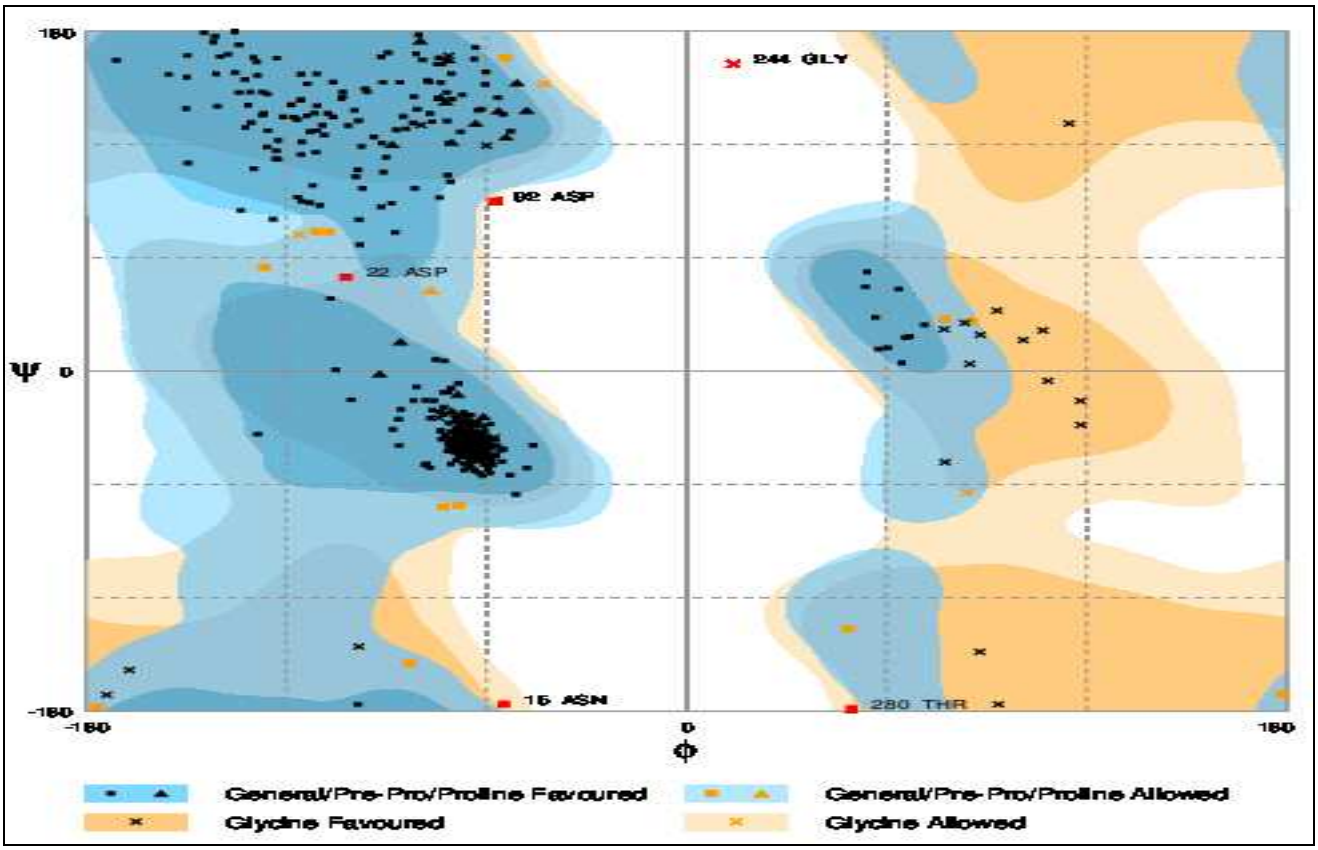


Figure 3. Overall quality factor evaluated by ERRAT.



Number of residues in favoured region (~98.0% expected) : 414 (95.2%)
Number of residues in allowed region (~2.0% expected) : 16 (3.7%)
Number of residues in outlier region : 5 (1.1%)

Figure 4. RAMPAGE values for indicating the number of residues in favored, allowed, and outlier regions.

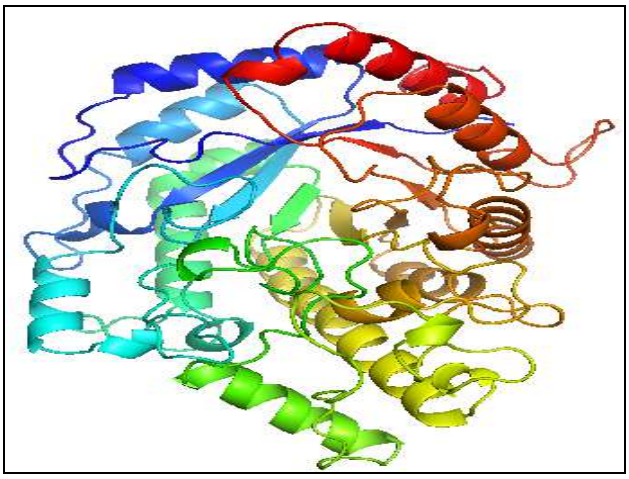


Figure 5. The 3D structure of the predicted β-amylase 7-like protein of *Juglans regia* by PyMOL.

Ramachandran plot analysis showed only 1.1% residues in outlier region, 3.7% allowed region and 95.2% in favored region, indicating that the models were of reliable and good quality. The three-dimensional structure of the predict β-amylase 7-like protein was constructed using the PyMOL program. The alpha helix and beta helix structures were demonstrated (Fig. 5). The three-dimensional structure of the proteins contributes to the understanding of protein function and active regions, and facilitates drug design [7].

4. CONCLUSION

In this study, in silico analysis was carried out using bioinformatic tools such as ExPASy ProtParam, CELLO v.2.5., MEGA 6.0, SOPMA, Pfam, NetPhos 3.1, ERRAT, PSIPRED v3.3, RAMPAGE and PyMOL for β-amylase 7-like protein in walnut. The results of this study will pave the way for further research on β-amylase 7-like protein in different plant species and will illuminate the future in silico studies.

TRANSPARENCY DECLARATION

The author declares no conflicts of interest.

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