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EVALUATION OF THE PLANT ESSENTIAL OILS TO CONTROL OF DRY BUBBLE DISEASE (*LECANICILLIUM FUNGICOLA* (PREUSS) ZARE) IN THE WHITE BUTTON MUSHROOM

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Key words: button mushroom, dry bubble, essential oil, gene expression.

Abstract

Edible mushrooms are not only a rich source of nutrients and proteins, but also some of the species produce medicinal compounds. The white button mushroom (*Agaricus bisporus* Imbach) was first cultivated in France. Dry bubble disease with *Lecanicillium fungicola* (Preuss) Zare and Gams is one of the most important diseases that cause damage to edible fungi. One of the easiest ways to control edible fungal diseases, especially dry blisters, is to use chemical fungicides such as carbendazim. Given that a small number of fungicides are currently available to control edible fungal diseases. However, the total resistance of fungal pathogen to chemical fungicides is due to their frequent use, so the use of plant essential oils can be an alternative method for controlling fungal diseases. In this study, the effect of plant essential oils including fennel, ferula, harmel, thymus and satureja was studied by two methods as the plant essential oils mixing with the culture medium and using the paper disk on the pathogen caused the bubble disease and edible mushroom. In another section of this research, it was investigated the effect of plant essential oils, including thyme and satureja, on the fungal pathogen in the salon and on edible fungi. The results of the mixing essential oils assay with the culture medium showed that the essential oil of thyme and ferula at a concentration of 1000 ppm inhibited the growth of the pathogen at 90.42% and 78.44%, respectively. These plant essential oils showed the highest inhibition of the growth of edible fungi. The results of the paper disk test showed that all the essential oils used in this study were able to reduce the growth rate of the pathogen. 1000 ppm concentration had the highest inhibition of the pathogen growth among all of the concentrations, and thyme essential oil with 85.41% showed the highest inhibition pathogen growth, followed by fennel, ferula and satureja essential oils with values of 74.78, 73.72 and 70.22%, respectively.

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The results of the study in the salon (*in vivo*), showed that the essential oils of thyme and fennel increased the number of healthy fungi by 77.19 and 55.63%, respectively, compared to the control. According to the results of the *in-vitro* and *in-vivo* (salon) of the essential oils of thyme, fennel and ferula showed a high capacity to control edible fungal diseases, especially bubbles.

Introduction

There are about 1.5 million species of fungi in the world. Edible fungi first lived on fossilized wood about 300 million years ago, when early humans collected and used them as food (HAWKSWORTH 2001). Fungal habitats include soil, water, and organisms that may harbor large numbers of understudied fungi, estimated to outnumber plants by at least 6 to 1. More recent estimates based on high-throughput sequencing methods suggest that as many as 5.1 million fungal species exist (BLACKWELL 2011).

Edible mushrooms were first grown in greenhouses in Sweden in 1754 and have since spread around the world. Edible mushrooms are not only a rich source of protein and vitamins, but some species of these fungi also produce medicinal compounds such as *Ganoderma* sp. In China, more than 700 pharmaceutical products are commercially available as the main active ingredient. According to statistics released by the Ministry of Health (USA), there are at least 106 medicinal fungi including *Ganoderma*, *Cordyceps* and *Shitake*. Mushrooms have all the essential amino acids for the body and are in the nutritional value between meat and vegetables. Besides, other non-essential amino acids and amides are present in the mushrooms, and edible mushroom protein can be added to the diet as a valuable supplement. The water content of edible mushrooms is about 97% (MOHAMMADI GOLTEPEH and POURJAM 2010). White button edible mushroom (*Agaricus bisporus* (Lange) Imbach) was first cultivated commercially in France. The methods that led to the development of mushroom cultivation were its cultivation of composted horse manure and then using the multi--spore technique to produce mushroom seeds. In recent years, mushroom production in Iran has undergone significant changes; production has increased from 7 kg to 20 kg per square meter (GOLAFRA 2008). However, edible fungi are exposed to various diseases and pests that can seriously reduce yields. Several of microorganisms, such as fungal pathogen, bacteria, and viruses, attack edible fungi, and it caused the most damage to edible fungi (FLETCHER et al. 1986). Dry blisters is caused by *Lecanicillium fungicola* (Preuss) Zare and Gams and wet bubbles is caused by *Mycogone perniciosa* (Magnus) and spider webs is caused by *Cladobotrium dendroides* (Bull) in edible fungi. The most practical method for the contro-

lling of the fungal diseases is the use of chemical compounds and chemical fungicides such as carbendazim and benomyl, which due to the negative effects on the growth of mycelium of edible fungi, reduced yield and the risk of accumulation of toxins in the fruiting organs of the fungus for the consumer and environmental pollution is often obsolete (ZIOMBRA 2001). Considering that a small number of fungicides are currently available and accepted for use in the cultivation of edible fungi, and on the other hand, the total resistance of the fungal pathogen to fungicides due to their use frequently caused by them, it is difficult to find a suitable fungicide that has no adverse effects on edible fungi as well as the environment. However, edible the agricultural mushrooms are crops that cannot be stored and should be consumed quickly, so if a chemical fungicide is used on them, Karen's period is not applied for them. A very safe and environmentally friendly technique can be the use of plant essential oils to control this disease (GLAMOCLIJA 2005, GHOLAMNEZHAD 2017). Recently, much kinds of researches have been done on the antimicrobial properties of medicinal and aromatic plants. The use of these fungicides is very effective in controlling of the growth and proliferation of the fungal pathogen. Medicinal plants are reservoirs rich in secondary metabolites and are sources of active ingredients and many medicinal substances such as thymol and carvacrol (OMIDBEIGI 2006, GHOLAMNEZHAD 2019). Due to the importance of the button mushroom in terms of nutritional and medicinal value and also because the pathogen *L. fungicola* is considered as one of the most important pathogen limiting the production of button mushroom, so in this study the potential plant essential oils are evaluated to control the disease both in the laboratory (*in vitro*) and in storage (*in vivo*) to find the effective compounds.

Material and Methods

Plant Essential Oils

The plant essential oils used in this research were prepared using a Clevenger method. The plants used in this study were all prepared from the rangelands of Lorestan, Yazd, and Isfahan provinces (Table 1). These pastures are pristine and no chemical treatment has been applied to the plants.

Table 1
Plant essential oils used against button edible fungi and dry bubble disease agent, scientific name, common name and partly used in this study

Plant name	Scientific name	Part used
Fennel	*Foenicelum vulgare*	shoot
Ferula	*Ferula assa-foetida*	shoot
Harmel	*Peganum harmala*	shoot
Thymus	*Thymus daenensis*	shoot
Satureja	*Satureia hortensis*	shoot

Pathogen

White button mushroom production centers were surveyed in different parts of Tehran and Alborz provinces, and after observing the infected and suspected specimens of fungi causing dry bubble disease, sampling of infected fungi was performed.

Isolation and Purification of Pathogen

The collected samples were cultured on the PDA (Potato Dextrose Agar) medium and kept in an incubator at 23°C in the dark for two weeks. After the complete growth of the samples in the culture medium, mycelium plaques were removed from the margins of the culture medium and transferred to the water-agar (WA) culture medium for purification. The cultured pathogen were kept in an incubator at 10°C in the dark for 10 days. After fungal mycelium growth, a layer of fungal mycelium was removed from the culture medium and transferred to the PDA culture medium. After two weeks, the pathogen *L. fungicola* was fully grown in the PDA medium and prepared for laboratory tests by plant extracts.

In Vitro Tests

The Effect of Plant Essential Oils on the Growth of the Pathogen by Mixing with Culture Medium Method

To investigate the antimicrobial effect of essential oil, the method of mixing the essential oil with the culture medium method was used for the investigation the antimicrobial effect of essential oils. The PDA medium was used for this assay. The prepared PDA medium was sterilized at 121°C for 15 minutes in an autoclave. The experiment was performed with five essential oils (fennel, ferula, harmel, thymus and satureja) in four

concentrations (250, 500, 750 and 1000 ppm) and three replications. The essential oil obtained from the Clevenger apparatus was considered as 100% concentration, other concentrations were made using the main and base concentrations. The concentrations including of 100, 150, 500 and 1000 ppm (microliters per liter) were poured in medium, before the culture medium was hardened. The culture medium was made with different concentrations of essential oil according to the active substance was prepared by Tween 20 as a completely uniform suspension and applied to the culture medium by sterile sampler at concentrations of 100, 150, 500, and 1000 ppm of the active ingredient was added. In the control (concentration 0), Tween 20 was used instead of essential oil. The culture medium was then poured into nine-centimeter sterilized Petri-dishes. Active disks of the pathogen (*L. fungicola*) with a diameter of 6 mm were placed in the center of the Petri-dishes containing the culture medium. Then the Petri--dishes were placed in an incubator at 23±2 °C, and the diameter of the fungal colonies on the culture medium was reduced after two weeks of filling of the petri dishes during a period of two days and months. The average growth of the fungal colony diameter in each replication was measured for each treatment using the following formula (1) (GHOLAMNEZHAD et al. 2015):

$$\frac{\text{pathogen conoly diameter in the control} - \text{pathogen colony diameter in the treaments}}{\text{pathogen conoly diameter in the control}} = \text{inhibition percentage of mycelial growth}$$

The Effect of Plant Essential Oils on Fungal Growth by Paper Disk Method

The antifungal activity of the essential oils was performed based on the "Reverse Petri-dishes" method (SINGH et al. 2006). 20 ml of PDA was poured into Petri-dishes (9 diameters), and after coagulation, a 5 mm disk of the five-day culture of the pathogen was removed from the PDA culture medium and placed in the center of the culture medium. Then, volumetric amounts of 10, 750, 17, and 100 microliters of pure essential oil of each plant were poured separately on a square disk with dimensions of 15 mm of Whatman filter paper number one with a sampler, and this disk was placed inside the lid of a Petri-dishes. By dividing the volume value used by the volume of active space inside the Petri-dishes, concentrations of 250, 500, 750 and 1000 ppm, respectively, was obtained. The Petri-dishes was covered with parafilm and the Petri-dishes was placed upside down at room temperature (24°C). After two weeks, when the mycelium of the fungus reached the edge of the Petri-dishes in the control Petri-dishes, the

effect of essential oils was evaluated. This test was performed with three replications for each treatment and the amount of antifungal activity was measured by formula (1) (SUKATTA et al. 2008).

The Effect of Essential Oils on the Pathogen and Edible Fungi in the Salon of Button Mushroom Cultivation

Thyme and fennel extracts (concentration of 500 ppm) were used for this test, which had good results *in-vitro* tests. Carbendazim as a fungicide (900 ppm) was also used as a chemical control. In the next step, a spore's suspensions with a concentration of 10^6 spores per ml was prepared by a hemocytometer slide from seven-day culture of the pathogen. The prepared spore's suspension was then sprayed on the surface two days after topsoil application. Then, the essential oil was sprayed on the cover soil. For this purpose, according to the inhibitory percentage obtained for each essential oil in the laboratory, an emulsion was prepared for each essential oil (500 ppm concentration) and two days after inoculation of each fungus, it was sprayed on the cover soil. This assay was four replications for each plant essential oils. It should be noted that each of the essential oils was treated alone on edible fungi, so that their effect could be investigated alone. This test had a healthy control and an infected mushroom control without using essential oil treatment. After the edible fungal mycelium completely covered the surface of the cover soil, to create stress for the button edible fungus, the temperature was lowered to 16 to 18°C, which was done by aeration of the hall. After a week, the first symptoms of the disease appeared on the surface of the cover soil and in healthy controls, the first pins of edible fungi appeared.

Measured Characters

Weight of Mushrooms

In the first harvest, all mushroom was collected from the surface of each bed and infected and healthy fungi was weighed and counted separately for each bed and the data were recorded. Ten days after the first harvest, the second harvest took place, like the first harvest, all mushroom was collected and weighed separately from the surface of each bed. All assays were carried out in a completely randomized design. Each treatment consisted of three replicates. Statistical significance was assessed at the level $P < 0.01$. When the analysis was statistically significant, Duncan's Multiple Range Test

Results

The Effect of Plant Essential Oils on the Growth of Pathogen by Mixing with Culture Medium

According to the results of the analysis of variance Table 2, there is a significant difference between the treatments of using different plant's essential oils and also the use of carbendazim fungicide in reducing the growth of pathogen on the culture medium mixed with plant essential oils.

Table 2

Analysis of variance of the plant essential oils effect on the growth inhibition of *Lecanicillium fungicola,* at 23 ± 2°C and dark conditions by mixing plant essential oils with culture medium

Source of variation	df	Sum of squares	Mean	*F*
Essential oil	20	25150.73	1257.53	2609.77**
Error	42	20.23	0.48	–
Total	62	25170.19	–	–

**the difference was significant at 99% probability ($P \leq 0.01$); the data were normal, C.V = 1.17%

The results of the antifungal effects of essential oils of fennel, ferula, harmel, thymus, and satureja obtained from the mixing test showed that different concentrations of essential oils had an inhibitory effect on the growth of the pathogen, and there is a direct relationship between increasing the concentration of essential oil and decreasing the growth of fungi (Figure 1).

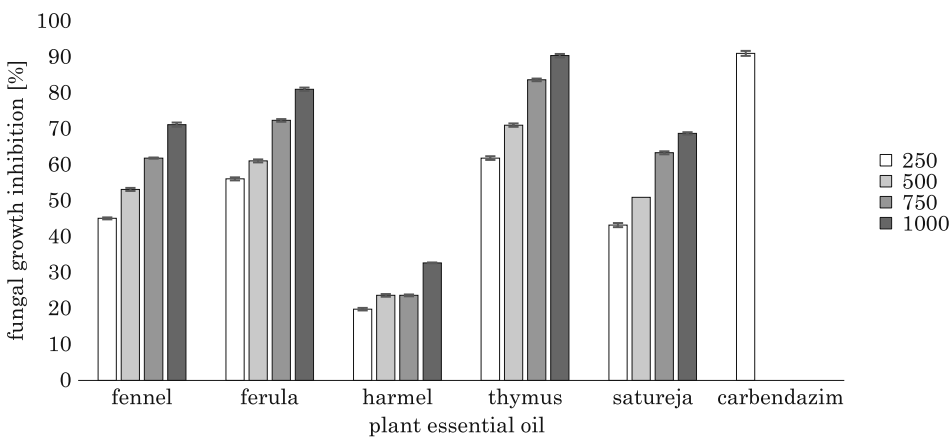

Fig. 1. Effect of plant essential oils on the growth of the pathogen (*Lecanicillium fungicola*) by mixing with culture medium

The highest reduction in fungal colony growth was observed in the treatment of 1000 ppm concentration of thyme essential oil with 90.42%, and two essential oils of ferula and fennel with a concentration of 1000 ppm placed in later levels with values of 81.07 and 71.22%, respectively. However, among the studied treatments, carbandazim treatment with 94.06% had the highest percentage of control, but with 1000 ppm thyme treatment, there was no significant difference. On average, the concentration of 1000 ppm showed the highest control among the concentrations, and this control was observed for all plant essential oils.

The Effect of Plant Essential Oils on the Growth of Button Edible Mushrooms by Mixing with Culture Medium

According to the results of the analysis of variance 2, there is a significant difference between the treatments of using essential oils of different plants and also the use of carbendazim fungicide in reducing the growth of button edible mushrooms on the culture medium mixed with plant essential oils.

The results of studying the antifungal effects of essential oils of fennel, ferula, harmel, thymus, and satureja obtained from the mixing test showed that different concentrations of essential oils have an inhibitory effect on the growth of button edible mushrooms, and there is a direct relationship between increasing the concentration of essential oil and decreasing the growth of fungi (Figure 2). Ferula essential oil showed the greatest effect in reducing the growth of button mushroom at a concentration of 1000 ppm with a value of 78.44%, followed by it was located the satureja and thyme at a concentration of 1000 with values of 73.36 and 69.71%, respectively. Carbendazim fungicide also showed an antifungal effect on edible fungi at 83.26%, which was statistically significant with a concentration of 1000 ppm ferula. Among the studied concentrations, the concentration of 1000 ppm showed the highest inhibition of fungal growth, followed by a concentration of 750 ppm, which always had a significant difference in the inhibition of the growth of fungi with other treatments (Figure 2 and Table 3).

According to this Figure 2, which shows the effect of different concentrations of five essential oils of fennel, ferula, harmel, thymus, and satureja on the average growth of edible mushroom colony growth, the highest inhibition rate was observed for all 1000 essential oils. At all concentrations, Ferula essential oil showed the highest inhibition, and harmel essential oil showed the least inhibition in inhibiting the mycelial growth of edible fungi.

Table 3

Analysis of variance of the plant essential oils effect on the growth inhibition of *Agaricus bisporus,* at 23±2ºC and dark conditions by mixing plant essential oils with culture medium

Source of variation	df	Sum of squares	Mean	*F*
Essential oil	20	15,454.46	1,257.53	1,551.56**
Error	42	20.91	0.48	–
Total	62	15,475.37	–	–

**the difference was significant at 99% probability ($P \leq 0.01$); the data were normal, C.V = 1.25%

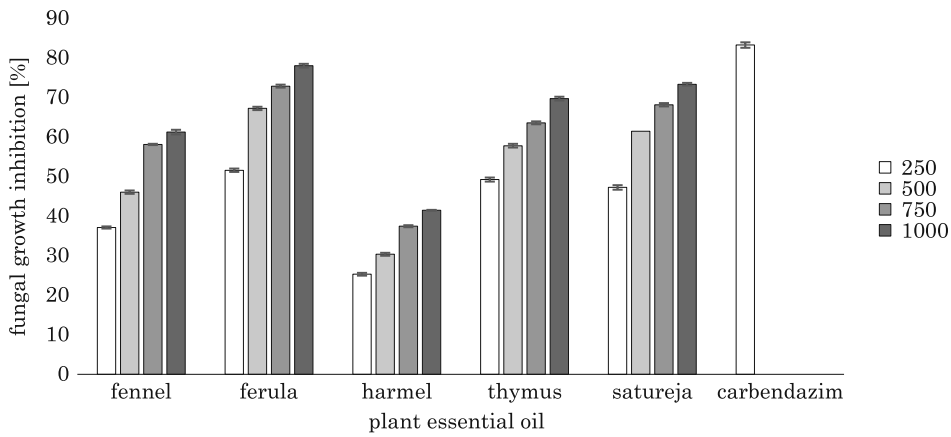

Fig. 2. Effect of plant essential oils on the growth of edible fungus (*Agaricus bisporus*) by mixing with culture medium

The Effect of Plant Essential Oils on the Growth of the Pathogen by Paper Disk Method

The results of the analysis of variance 4 showed that there is a significant difference between the treatments of using essential oils of different plants and also the use of carbendazim fungicide in reducing the growth of the pathogen on the culture medium, in the method of using the paper disk. The results of antifungal effects of essential oils of fennel, ferula, harmel, thyme and satureja obtained from paper disk test showed that different concentrations of essential oils had an inhibitory effect on the growth of edible fungi and between increasing the concentration of essential oil with decreasing Fungal growth has a direct relationship (Figure 3); the highest reduction in the pathogen colony growth was the concentration of 1000 ppm of thyme essential oil of 85.41% and the essential oils of fennel and ferula with a concentration of 1000 ppm were in the next ranks with the values of 74.78 and 73.72%, respectively. Carbendazim fungicide reduced the growth of the pathogen by 44.49%.

Concentrations used in this experiment include 250, 500, 750 and 1000 ppm in the volumetric space of the PDA culture medium. Among the concentrations, the best treatment, for controlling the growth of the pathogen, was the concentration of 1000 ppm, which always showed the highest inhibiting percentage of the pathogen growth, about all of the plant essential oils.

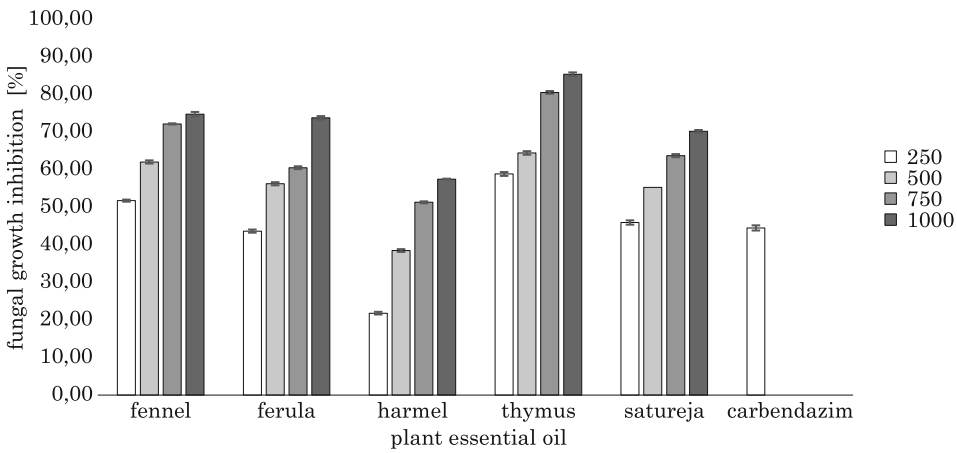

Fig. 3. Effect of plant essential oils on the growth of the pathogen (*Lecanicillium fungicola*) by paper disk method

According to the results of the analysis of variance, there is a significant difference between different treatments at the level of 1 percent probability. The results showed that different concentrations of plant essential oils had an effect on the average growth of the pathogen and reduced the growth of the fungus, which is clearly shown in Figure 3. This figure shows that different concentrations of five plant essential oils have a decreasing effect on the growth of the pathogen's colony and the maximum inhibition for all essential oils was observed in the 1000 ppm concentration. Thyme essential oil had the highest efficiency of 85.41% and harmel had the lowest efficiency of 57.91% in inhibiting mycelial growth of the fungus. After thyme essential oil, fennel, ferula, and saturea essential oils showed 74.71, 73.72, and 70.22% the percentage controlling, respectively.

The Effect of Essential Oils on the Pathogen and Edible Fungi in Button Mushroom Cultivation Salon

In this test, the essential oils of thyme and fennel were used at the 500 ppm concentration and the fungicide carbendazim at 1.5 per thousand was concentration. According to the results of analysis of variance in Table 4,

there is a significant difference between the treatments using these two essential oils and the use of carbendazim fungicide in increasing the percentage of healthy fungi and also increasing the weight of the edible fungi at a probability level of one percent. As shown in the comparison Table 5, the tested treatments include the plant essential oils, carbendazim fungicide, healthy control, infected plant control.

Table 4

Analysis of variance of the plant essential oils effect on the growth inhibition of *Agaricus bisporus*, at 23±2°C and dark conditions by the paper disk method

Source of variation	df	Sum of squares	Mean	*F*
Essential oil	20	13,646.45	682.32	654.30**
Error	42	43.79	1.04	–
Total	62	13,690.24	–	–

**the difference was significant at 99% probability ($P \leq 0.01$); the data were normal, C.V = 1.2573%

Table 5

Analysis of variance of the plant essential oils effect on the number of mushrooms and weight of *Agaricus bisporus*, in button mushroom cultivation salon

Specification		Mean of squares (MS)		Sum of squares (SS)		*F*	
Source of variation	df	mushroom weight	number of mushrooms	mushroom weight	number of mushrooms	mushroom weight	number of mushrooms
Essential oil	6	69479.33	17706.68	17369.83	4426.67	1514.81**	2773.53**
Error	12	114.66	15.96	11.46	1.59	–	–
Total	17	–	–	–	–	–	–

**the difference was significant at 99% probability ($P \leq 0.01$); the data were normal, C.V = 1.39% for mushroom weight and C.V = 2.55% for number of mushrooms

The results of antifungal effects of thyme and fennel essential oils, as well as carbendazim fungicide on the percentage of healthy fungi showed that the use of thyme and fennel essential oils increased the number of healthy fungi by 77.99% and 54.63%, respectively, while the percentage of healthy fungi in the treatment of pathogen use alone (infected control) was 7.30%. The use of carbendazim fungicide alone caused 83.60% of the fungi to remain healthy, which was numerically higher than all the treatments (except 94.16% of the healthy control) and showed a significant difference with the other treatments. Of course, both essential oils of thyme and fennel in the presence of the pathogen were able to increase the number of healthy fungi to 62.12 and 32.25, respectively, compared to the infected control at 7.30%.

The use of different treatments caused a significant difference in the weight of the studied fungi (Table 4–6). In this study, the highest weight of

edible fungus was observed in the control treatment at the rate of 322.66 g, followed by the use of carbendazim fungicide with the amount of 295.66 g. The weight observed in edible fungi treated with thyme and fennel essential oil was 253.33 and 215 g, which showed a significant difference at the level of one percent with the infected control (128.33 g).

Table 6

Comparison of the mean of the test for the effect of plant essential oils and fungicide on the percentage of the number of healthy fungi and the weight of *A. bisporus*

Treatment	Concentrations [ppm]	Weight of *A. bisporus*	Healthy *A. bisporus* [%]
Thymus	500	253.33^{c}	77.99^{c}
Fennel	500	215.00^{d}	54.63^{d}
Thymus + pathogen	500	198.22^{e}	62.12^{e}
Fennel + pathogen	500	156.36^{f}	39.25^{f}
Karbendazim + pathogen	900	295.66^{b}	83.60^{b}
Infected plant (positive control)	–	128.33^{g}	7.30^{g}
Control (healthy plant)	0	322.66^{a}	94.16^{a}

Values are the average of three replicates. Values in the same row followed by the same letter are not statistically different in Duncan's Multiple Range Test.

Discussion

The use of plant essential oils can be used as an alternative to pesticides in mushroom breeding centers. Iran is a country rich in important species of medicinal plants that can be used to control plant diseases and disinfect agricultural products, especially storage products as well as edible mushrooms, against pests and diseases (OMIDBEIGI 1995). Edible fungi can absorb a lot of toxins due to their special texture (lack of woodiness) and also their inability to store them for a long time. Avoid making less on edible mushrooms. Plant essential oils are of plant origin and therefore do not have harmful effects on human health and on the other hand is decomposed very quickly, so they do not leave toxic residues in the environment.

In this study, thyme and fennel essential oils showed a very high ability to inhibit the growth of mycelium of the pathogen compared to other essential oils. The results of this study on the very high antimicrobial effect of thyme essential oil with the results of previous research (LEUSCHNER and ZAMPARINI 2002, AMANLOU 2006, HOSEIN et al. 2009, SOLAIMANI et al. 2009) and the very high antimicrobial effect of essential oil ferula confirmed with the results of other studies (NABIGOL and FARZANEH 2010,

GANDOMI et al. 2009). The results of the gas chromatographic analysis for thyme essential oil show 42% carvacrol and 32.34% thymol. There is a direct relationship between the important chemical structures of the compound in the essential oil and its antimicrobial activity (CACCIONI et al. 1998). The very high antifungal power of these two essential oils is due to the presence of phenolic compounds in them. Thymol and carvacrol are phenol terpenoid compounds that have the highest antimicrobial activity compared to other active compounds such as terpenes and terpenoids with other functional groups (VENTURINI et al. 2002, LIU et al. 2002).

Both essential oils (thyme and fennel) are composed of various compounds, but it seems that the commonality of some of these compounds, in the similarity of the effect of these two essential oils on edible fungi and the fungal pathogen. The presence of phenolic compounds such as thymol and carvacrol as the main compounds of these two essential oils can be effective in obtaining antifungal results. In addition to carvacrol, thyme essential oil also contains thymol, which was not found in saturea essential oil in this study, or its amount was very low, which was not specified in the GC analysis. It seems that the inhibitory effect of thyme essential oil more than fennel essential oil is due to the exacerbating effects of compounds such as thymol and other compounds (BULLERMAN 1977).

In a study, the antifungal role of safflower essential oil on *Alternaria citri* was investigated. The results showed that savory essential oil at 400 ppm concentration and above in the culture medium completely affects the growth of the fungus and inhibits its growth. Lower concentrations of essential oil decrease the growth of the fungus but did not stop its growth (YAZDANPANAH GOHARRIZI et al. 2010).

WHITESIDE (1976) reported that consumption of safflower essential oil in addition to controlling the causative agent of *Alternaria citri* disease increases the marketability and quality of the product and due to the effective concentration of 300 ppm safflower essential oil, its consumption is economically viable (WHITESIDE et al. 1976).

NESLIHAN et al. (2008) concluded that carvacrol has a greater inhibitory effect than thymol on the inhibition of the pathogen. In 2008, MUSCOVY et al. reported that thyme and satureja essential oils could inhibit the growth of fungi that contaminate food as well as horticultural crops and could be used as instead of chemical substitutes. RASOOLI et al. (2009) also reported that plant essential oils can control plant fungal diseases (RASOOLI et al. 2009).

However, the essential oils of medicinal plants used in this study were able to prevent the growth of fungi by mixing with the medium to an acceptable level. The mixing essential oils with the culture medium method

exposes the essential oil permanently to the pathogen, and the antifungal compounds in the essential oil will have ample opportunity to penetrate the wall, membrane and subsequently the depth of the target cell. The mechanism of phenolic compounds is related to their effects on cell membranes, and changes in their function and in some cases changes in membrane structure that increase inflammation and permeability of cell membranes (LIU et al. 2002).

The effect of plant essential oils on the reduction of edible fungi was different from the results of the previous test and the essential oils that had the greatest effect on reducing the growth of the pathogen in this part had less reduction in the growth of edible fungi than other essential oils.

In the mixing the essential oils with the culture medium test, the carbendazim fungicide treatment showed the greatest reduction in fungal growth, followed by the essential oils of ferula and satureja with a concentration of 1000 ppm. Thyme, which had the most effect in the previous test, was ranked third here, and fennel also dropped from third to fourth. This difference in the control of the two fungi can be related to the difference in cell wall compositions between the two pathogenic and edible fungi. In other words, the fungicidal compounds of thyme essential oil in the case of edible fungi did not have a high penetration (compared to the pathogen) into the fungus and as a result could not have a high inhibitory effect on this fungus (ALIZADEH-SALTEH et al. 2010).

The results of the paper disk test showed that the process of the mixing the extract with the culture medium was similar with the paper disk test. In the paper disk test, the concentrations of 1000 ppm of thyme, fennel, ferula, satureja and harmel showed the highest control of the pathogen, respectively (TANOVI et al. 2009).

Differences in the effectiveness of plant extracts can probably be related to the difference in the origin of plant essential oils and consequently the difference in the type and composition of the ingredients of plant essential oils (GHOLAMNEZHAD et al. 2016, GHOLAMNEZHAD 2016); also, with increasing the concentration of plant essential oil, the fungicidal effect in all essential oils used to prevent mycelial growth of fungi has increased, these results are consistent with the results obtained by other researchers. LOTFI et al. (2010) studied on *Fusarium oxysporum* and showed that the essential oils of thyme, trachyspermum and mint inhibited the growth of the fungus. SHAHKARAMI et al. (2006) studied the effect of five plant species including the case, mint, five fingers, thyme and artemisia on mycelial growth of plant pathogens *Rhizoctonia*, *Fusarium* and *Pythium*. The results showed that the essential oils of mint and thyme inhibited 100% mycelial growth of the studied fungi.

In the evaluation of the effect of essential oils on the pathogen and edible fungi, in the button mushroom cultivation salon, two plant essential oils including thyme and fennel were used in one concentrations of 500 ppm. In the healthy control treatment, we saw the highest number and weight of fungi. In this assay, thyme essential oil showed very good performance compared to fungicide in terms of the number and weight of the fungus. In some ways, fennel essential oil, although less effective than thyme, still had acceptable performance.

Increasing the weight of edible fungi treated with thyme and fennel essential oils compared to the control shows that in addition to antifungal compounds, these essential oils may also contain fungal growth stimulants, which significantly increased the weight of infected fungi. The results showed that there are chemical compounds with different percentages in the essential oils of thyme and fennel. The main composition of essential oil of thyme (*Thymus daenensis*) and thyme (*Thymus vulgaris*) is thymol, which is 43.8% and 45.1%, respectively. Thymol is a phenolic chemical compound. Thymol has antibacterial properties and is found in plants such as thyme.

Despite the great effect of these compounds (thymol and carvacrol) in increasing the growth of edible fungi, but there is no report on the effect of essential oil in increasing the growth of edible fungi as well as plants. The results of this study can be used as a starting point that introduces the use of essential oils as well as plant extracts, in addition to being a very good factor for controlling plant diseases. It can be used as a growth stimulant at least for this fungus (*A. bisporus*).

Measurement of defense gene expression in *A. bisporus* treated with thyme essence showed that the thymus extract increased the expression of two genes, peroxidase and catalase, in the presence and absence of the pathogen. Exactly in treatments which the number of healthy edible mushroom as well as the weight of the mushroom was higher, the activity of these two enzymes was also observed. In other words, these results can be interpreted as the effect of thyme essence has not only been a direct fungicide against the pathogen, but also on the defense mechanism of edible fungi and activating the host defense system against the pathogen. Our research results showed that a part of the disease reduction in *A. bisporus* that with essence was due to the induction of defense genes by these natural compounds.

The results of the Study of defense genes expression profile pattern of wheat in response to infection by *Mycosphaerella graminicola* revealed that by increasing the expression levels of defense genes such as catalase, peroxidase and polyphenol oxidase, the resistance of wheat to septoriosis increases (GHOLAMNEZHAD et al. 2016a,b).

The results of GHOLAMNEZHAD (2019) showed that in the storage conditions, the application of aqueous extract of neem (at concentration of 25%) resulted in 89.11% reduction of disease severity compared with the untreated control. Results of enzymes activity showed the plant extracts can increase the activity of peroxidase, phenylalanine ammonia-lyase, β-1,3-glucanase and polyphenol oxidase in the presence of pathogens, in apple fruits (GHOLAMNEZHAD 2019).

This research showed that the plant essence induced the resistant mechanisms and it can be useful to control edible mushroom disease. On the basis of the results obtained during the experiment and reports of success of plant essence in controlling edible mushroom disease, the tested plant essence hold promise for the organic and eco-friendly management of *A. bisporus*. The findings of these studies may become the foundation for the use of natural agents as a safe and cost-effective control method against *A. bisporus* diseases.

Another notable point that was the secondary results of this study, which was not targeted at the beginning of this study, was the flavoring of edible mushrooms. Edible mushrooms treated with the essential oils of thyme and fennel had the taste of these two essential oils, so that even after cooking they had this taste. The results of this study showed that plant extracts have high fungicidal effects, relatively little effect on the growth of edible mushrooms, as well as flavoring edible mushrooms.

Although in recent years the approach to the study of the effects of medicinal plants on animal and plant diseases has increased, for centuries, medicinal plants as well as plant extracts, essential oils and teas of these plants in Iran, have been used to treat diseases in humans and even animals, and at least in our country, the use of these plants and their products is not strange. The results of this study showed that plant essential oils in addition to good fungicidal effects can have a flavoring effect on edible mushrooms.

Conclusions

Edible mushroom diseases are often controlled with chemical compounds as Carbendazim and Sporgon but the perceived harmful effects of synthetic fungicides currently in use on human and the environment no longer make them attractive to use. Based on the findings of this study, there are great potentials in the control of edible mushroom diseases using naturally occurring substances that are both humanly and environmentally friendly and at the same time affordable at less cost to the users than

the procurement and use of chemically formulated fungicides. The results of this study showed that plant essence, in addition to fungicide effects, they can induce resistance in the edible mushroom.

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POLYPHENOL CONTENT AND ANTIOXIDANT ACTIVITY OF AN ENDEMIC PLANT OF ALGERIAN SAHARA: *ANVILLEA RADIATA*

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Key words: *Anvillea radiata,* endemic plant, polyphenol, antioxidant activity.

Abstract

This study was carried out to assess the main secondary metabolites contents of *Anvillea radiata* plant using four solvents of increasing polarities (hexane, dichloromethane, methanol, and water), and three complementary antioxidant tests: total antioxidant capacity assay, DPPH free radical-scavenging ability, and ferric reducing antioxidant power (FRAP) assay. Methanol and Aqueous extracts recorded the highest extract yields (20.49±0.26 % and 11.58±0.23% respectively). Dichloromethane extract showed the highest rate of total phenols, flavonoids, and tannin contents (114.45±0.02 mg GAE/g d.w., 245.21±0.07 mg CE/g d.w., and 101.765±0.014 mg CE/g d.w respectively). Dichloromethane and Methanol extracts recorded a significantly high rate in total antioxidant activity (14.41±0.009 and 9.55±0.0023 mg GAE/g d.w. respectively) and exhibited a significant ability to scavenge DPPH radical (IC50 values = 0.9±0.026 and 1.75±0.051 mg/mL respectively) and a significant iron reducing power (EC50 = 0.98±0.034 and 1.31±0.043 mg/ml

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respectively). These results prove that *A. radiata* could indeed be a potential source of natural antioxidant and could be of considerable interest for the development of new medicines based on local plants to enhance the natural resources of the national heritage.

Introduction

Reactive oxygen species (ROS) are produced by living organisms as a result of normal cellular metabolism and environmental factors, such as air pollutants or cigarette smoke. ROS are highly reactive molecules and can damage cell structural molecules such as carbohydrates, nucleic acids, lipids, and proteins and alter their functions (BIRBEN et al. 2012). In healthy aerobes organisms, the production of ROS is approximately balanced with antioxidant defense systems (HALLIWELL 2007). The shift in the balance between oxidants and antioxidants in favor of oxidants is termed "oxidative stress" (BIRBEN et al. 2012). Oxidative stress is implicated in the progression of several neurodegenerative diseases, including Alzheimer's disease, Parkinson's disease, and Amyotrophic Lateral Sclerosis (ALS) (BARNHAM et al. 2004). Growing evidence indicates that chronic and acute overproduction of ROS under pathophysiologic conditions is integral in the development of metabolic disorders such as diabetes and cardiovascular diseases (MADAMANCHI et al. 2005). It is also known that ROS can induce instability of the cell membrane (MORA et al. 1990), destruction of DNA structures (TAKABE et al. 2001), and induction of mutations (SASTRE et al. 2000), carcinogenic effects (KAWANISHI et al. 2001), and infertility (SHEWEITA et al. 2005). Many synthetic antioxidants, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), tert-butylhydroquinone (TBHQ), and propyl gallate have been widely used in different food products. However, because of the potential health hazards, their use as food additives is under strict regulation in many countries (WANG et al. 2009). Currently, many studies are devoted to exploring and utilizing natural antioxidants to remove excessive free radicals in the human body, thus preventing or treating diseases (LI et al. 2014). The Algerian flora is full of several plant species that offer real pharmacological properties, which is still little or not studied (BENTABET et al. 2014). This diverse flora is a natural reservoir of bioactive molecules (EL GUICHE et al. 2015). Total and perfect control of the different properties of these plants, which involves determining all of the physicochemical groups capable of producing one or more pharmacological effects, is today a goal that occupies an order first place (ABDELWAHED et al. 2007). *Anvillea radiata,* a wild plant included in the Asteraceae family is growing predominantly

in the steppes of North Africa (Algeria and Morocco). *A. radiata* is a small woody shrub, densely branched, 20–50 cm high. The leaves are green-gray, small, and roughly triangular, with a large petiole and strongly toothed limb. The big solitary capitules have a diameter of 3–5 cm, with long ligules. The flowers are all yellow-orange, the outside one is 25 mm lenth. It is usually flowering in spring but can flower throughout the year. It is widely used in traditional medicine for the treatment of dysentery, gastric-intestinal disorders, and chest cold and has been reported to have hypoglycemic activity as well as antifungal activity (BOUKHRIS et al. 2016). This study aims to enhance the local flora by discovering new compounds or active principles with therapeutic interests.

Materials and Methods

Plant Sampling

Phytochemical study and evaluation of antioxidant activity required plant material represented by an endemic plant of Algerian Sahara named *Anvillea radiata* or *"Nugd"* as a vernacular name (BENKHNIGUE et al. 2016), collected in May 2019 in the region of Tamanrasset in Algeria. The plant used was identified by Dr. Bekkouche Assia, a botanist at Salhi Ahmed university center of Naama in Algeria. The aerial part (Figure 1) of the dried plant was ground and used for the preparation of various extracts.

Fig. 1. The dry appearance of the aerial part of *Anvillea radiata*

Chemicals and Reagents

2-(3,4 Dihydroxyphenyl)-3,4-dihydro-2H-chromene-3,5,7-triol (Catechin; C), 2,2-Diphenyl-1-picrylhydrazyl (DPPH), 3,4,5-trihydroxybenzoic acid (Gallic acid; GA), 3-methoxy-4-hydroxybenzaldehyde (Vanillin), 5-(1,2-dihydroxyéthyl)-3,4-dihydroxyfuran-2-one (ascorbic acid), aluminum chloride ($AlCl_3$), ammonium molybdate ($(NH_4)_6Mo_7O_{24}$), Folin-Ciocalteu phenol reagent, hydrochloric acid (HCl), iron chloride ($FeCl_3$), potassium ferricyanide solution $K_3Fe(CN)_6$, sodium carbonate (Na_2CO_3), sodium nitrite ($NaNO_2$), and sulfuric acid (H_2SO_4). All chemicals used were obtained from either Sigma-Aldrich or Merck.

Preparation of Plant Extracts

The aerial part of *Anvillea radiata* (10 g) was weighed into a Soxhlet extractor thimble and placed in the extraction apparatus. Four solvents of increasing polarities (Hexane, Dichloromethane, Methanol, and Water) were measured into a 250 mL conical flask depending on the feed-to--solvent ratio (1:10 g/mL). A heating mantle was used to reflux the mixture for an extraction time of 6 h. After the extraction time has been reached, the extract solution was allowed to cool at room temperature. Then, filtered through a cone of filter paper (Whatman no 1), concentrated to dryness using a rotary evaporator, and stored at 4°C until use (ALARA et al. 2018). The extraction yield was calculated using the following formula:

$$\text{yield of extract [\%]} = \left(\frac{\text{weight of extracts from plant sample}}{\text{weight of dried plant sample}}\right) \cdot 100\%$$

Total Phenolic Content (TPC)

Total phenolic contents were assayed using Folin-Ciocalteu reagent, following the method described by SERAIRI-BEJI et al. (2018). An aliquot of diluted sample fraction was added to 0.5 mL distilled water and 0.125 mL Folin-Ciocalteu reagent. The mixture was shaken and incubated for 6 min before adding 1.25 mL Na_2CO_3 (7%). The solution was then adjusted with distilled water to a final volume of 3 mL and mixed thoroughly. After incubation in the dark, the absorbance was read at 760 nm versus a prepared blank. TPC was expressed as milligrams Gallic acid equivalents per gram dry weight (mg GAE/g d.w.) through the calibration curve with Gallic acid. The calibration curve range was 0–100 μg /mL (R^2 = 0.97). All samples were analyzed in three replications.

Total Flavonoid Content (TFC)

The total flavonoid content of the extracts has been determined using the colorimetric method as described by KIM et al. (2003). 100 µL of the extract was mixed with 0.4 mL of distilled water following with 0.03 mL of sodium nitrite solution 5% $NaNO_2$. After 5 min, 0.02 mL of $AlCl_3$ solution to 10% has been added. 0.2 mL of 1 M Na_2CO_3 solution and 0.25 mL of distilled water after 5 min. The whole is agitated using a vortex and absorbance was measured at 510 nm. TFC was expressed as mg catechin equivalent per gram dry weight (mg CE/g d.w.), through the calibration curve of catechin 0–500 µg/mL range (R^2 = 0.99). Samples were analyzed in triplicate.

Total Condensed Tannin Content (TCT)

Total condensed tannin was measured according to the method described by SERAIRI-BEJI et al. (2018). 200 µL of properly diluted sample was added to (3 mL) of (4%) vanillin solution in methanol, and 1.5 mL of concentrated hydrochloric acid (HCl) was added. After an incubation period of 15 min, the absorption was measured at 500 nm against methanol as a blank. The amount of TCT was expressed as milligrams catechin equivalent per gram dry weight [mg CE/g d.w.]. The calibration curve range of catechin was established between 0 and 400 mg/mL. Samples were analyzed in triplicate.

Total Antioxidant Capacity (TAC)

TAC was evaluated through the method described by SANCHEZ-MORENO et al. (1998). An aliquot (100 µL) of diluted extract fraction was combined with 1 mL reagent solution (0.3 N sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate). Methanol was used instead of a sample for blank. Mixtures were incubated in a boiling water bath for 90 min then cooled to room temperature. Their absorbance was measured at 695 nm against a blank. Antioxidant capacity was expressed as mg Gallic acid equivalent per gram dry weight (mg GAE/g d.w.). All samples were analyzed in triplicate.

DPPH Radical-Scavenging Activity

DPPH radical-scavenging activity was carried out following the method of SANCHEZ-MORENO et al. (1998). Rapidly 50 µL of each extracts at different concentrations (from 0.078 to 5 mg/mL) were added to 1.95 mL

of the methanolic solution of DPPH (0.025 g/L). At the same time, a negative control is prepared by mixing 50 µL of methanol with 1.95 mL of the methanolic solution of DPPH. The mixture was shaken vigorously and left standing at room temperature for 30 min in the dark. The absorbance of the resulting solution was then measured at 515 nm. The scavenging activity was expressed as IC50 [mg/mL], the dose required to cause a 50% DPPH inhibition. A lower IC50 value corresponds to a higher antioxidant activity of plant extract. The percentage of radical-scavenging is calculated according to the following equation:

$$I\%=(A1 - A2/A1 \cdot 100$$

where:
A1 – absorbance of the control (DPPH solution without extract).
A2 – absorbance in the presence of extract.

Ferric Reducing Antioxidant Power Assay (FRAP)

The Ferric reducing antioxidant power assay is determined according to the method described by WU et al. (2014). One milliliter of the extract at different concentrations (from 0.078 to 5 mg/ml) was mixed with 2.5 mL of a 0.2 M phosphate buffer solution (pH 6.6) and 2.5 mL of a potassium ferricyanide solution $K_3Fe\ (CN)_6$ at 1%. The mixture is incubated in a water bath at 50°C for 20 min. thereafter, 2.5 mL of trichloroacetic acid at 10% is added to stop the reaction and the tubes are centrifuged at 3000 rpm for 10 min. An aliquot (2.5 mL) of supernatant is combined with 2.5 mL of water distilled and 0.5 mL of an aqueous 0.1% $FeCl_3$ solution. The absorbance of the reaction medium is read at 700 nm against a similarly prepared blank, replacing the extract with distilled water which allows calibrating the device (UV-VIS spectrophotometer). The positive control is represented by a solution of ascorbic acid and the absorbance was measured under the same conditions as the samples. An increase in absorbance corresponds to an increase in the reducing power of extracts tested.

Statistical Analysis

All assays were carried out in triplicate (n = 3) and their results were expressed as mean ± standard error of the mean and analyzed by SigmaPlot for Windows version 11.0. A comparison between groups was made using the Bonferroni test. Columns not sharing a common letter (a–c) differ significantly at $p < 0.05$ (Bonferroni test).

Results

Extraction Yield, Total Phenol, Flavonoid, and Condensed Tannin Contents

The extraction of the aerial part of *Anvillea radiata* using four solvents of increasing polarity allows us to calculate the extraction yields expressed in percentages relative to the initial dry weight. As shown in Figure 2,

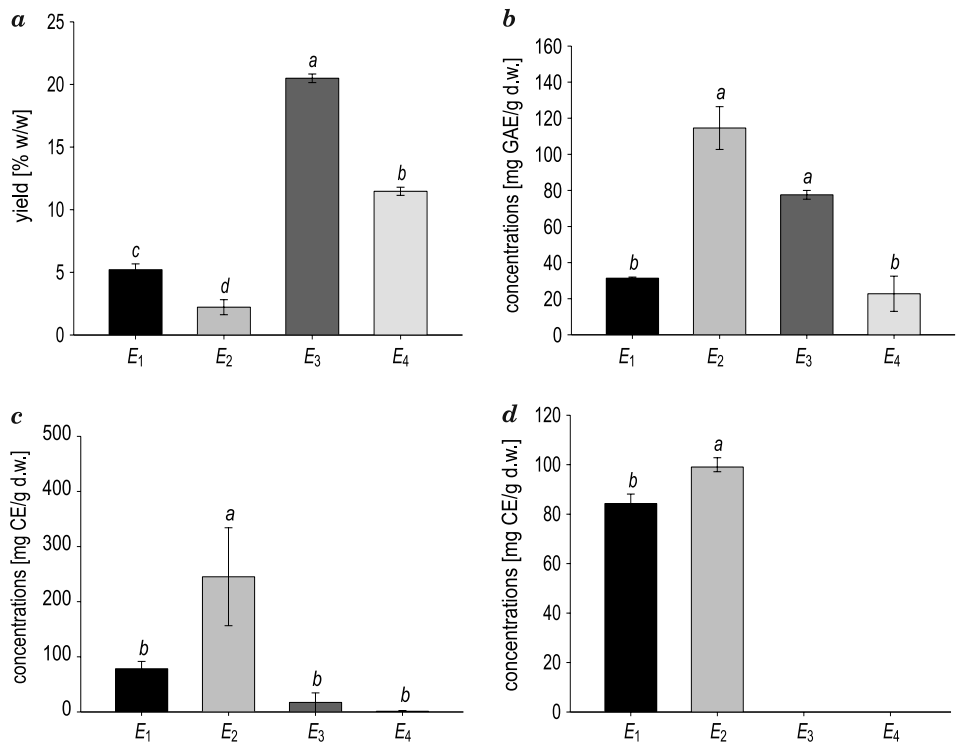

Fig. 2. Different extracts from *Anvillea radiata* (E_1 – hexane, E_2 – dichloromethane, E_3 – methanol, and E_4 – water extracts): *a* – extraction yield; *b* – total phenolic content; *c* – total flavonoid content; *d* – total condensed tannin. Data are expressed as means ± SE ($n = 3$). Comparison between groups was made using the Bonferroni test. Columns not sharing a common letter (*a*–*d*) differ significantly at $p < 0.05$ (Bonferroni test)

the highest yield is obtained in the methanol extract followed by the aqueous extract (20.49±0.26 % and 11.58±0.23 % respectively). Total phenol content was determined as Gallic acid equivalent in milligram per gram dry weight (mg GAE/mg d.w.). While total flavonoid and total condensed tannin were calculated as catechin equivalent in milligram per gram dry weight (mg CE/g d.w.). The total phenol contents of the dichloromethane

and methanol extracts from *Anvillea radiata*'s aerial part were significantly higher than the other extracts and determined to be 114.45±0.025 and 77.56±0.005 mg GAE/mg d.w. However, only the dichloromethane extract shows a significantly high level of flavonoids (245.21±0.074 mg CE/g d.w.) compared to the other extracts. While the highest level of condensed tannin is recorded for the dichloromethane extract (101.76±0.014 mg CE/g d.w.) followed by the hexane extract (86.76±0.003 mg CE/g d.w.).

Antioxidant Activities

Three complimentary tests were used in this study to assess the antioxidant activity of different extracts from *Anvillea radiata* aerial part: Total antioxidant capacity, DPPH free radical-scavenging activity, and reducing power assays. The total antioxidant activity was expressed as the number of Gallic acid equivalent. The study revealed that the dichloromethane extract recorded a significantly high rate in total antioxidant activity (14.41±0.009 mg GAE/g d.w.) compared to the other extracts. Results shows that both the dichloromethane and methanol extracts exhibited a significant ability to quench DPPH radical ($IC_{50\%}$ values = 0.9±0.026 mg/mL and 1.75±0.051 mg/mL respectively) and a significant iron reducing power (EC50 = 0.98±0.034 and 1.31 ± 0.043 mg/mL respectively) compared to the other extracts but that remain significantly lower than that of ascorbic acid ($EC_{50\%}$= 0.005 ± 0.003 mg/ml) (Figure 3 and Table 1).

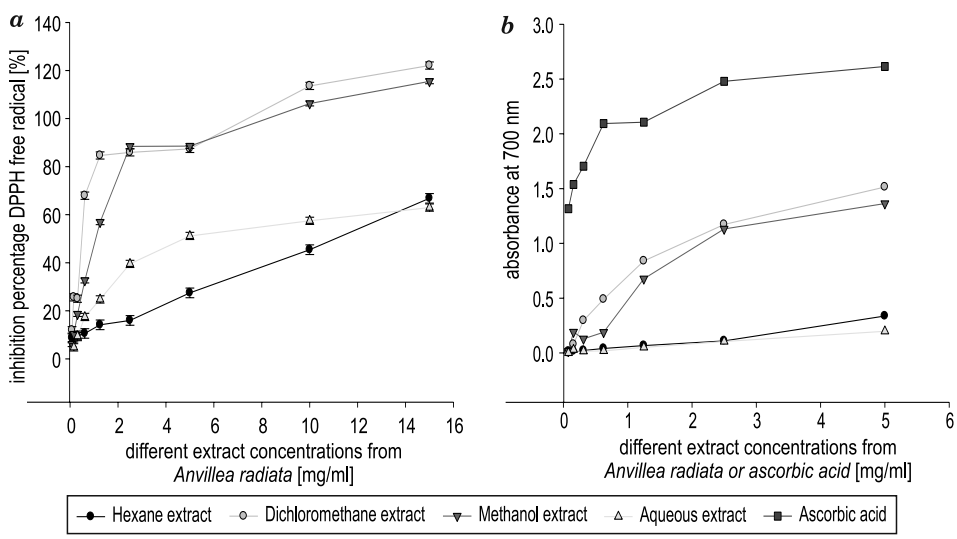

Fig. 3. Different extract concentrations from: *a* – *Anvillea radiata* – DPPH radical-scavenging activity; *b* – *Anvillea radiata* compared to ascorbic acid – ferric reducing antioxidant power assay (FRAP). Data are expressed as means ± SE (n = 3)

Table 1
Total antioxidant activity, DPPH radical-scavenging activity and Ferric reducing antioxidant power assay of the different extracts from *Anvillea radiata*

Extracts	TAC [mg GAE/g d.w.]	IC_{50} value of DPPH [mg/ml]	EC_{50} value of FRAP assay [mg/ml]
E_1	9.55 ± 0.002^b	10.7 ± 0.038^b	7.89 ± 0.060^c
E_2	14.41 ± 0.009^a	0.90 ± 0.028^a	0.98 ± 0.034^b
E_3	6.69 ± 0.0012^c	1.75 ± 0.019^a	1.31 ± 0.043^b
E_4	7.65 ± 0.0010^b	4.23 ± 0.034^b	12.82 ± 0.080^c
Ascorbic acid	–	–	0.005 ± 0.003^a

Explanation: E_1 – hexane; E_2 – dichloromethane; E_3 – methanol; E_4 – water extracts. Data are expressed as means±SE (n = 3). Comparison between groups was made using the Bonferroni test. Column not sharing a common letter (a–d) differ significantly at $p < 0.05$ (Bonferroni test)

Discussion

Nowadays, there is increasing attention to the health benefits of plant phenolic compounds due to their antioxidant activities (SERAIRI-BEJI et al. 2018). This study was conducted to characterize the phenolic profile and antioxidant potential of the extracts obtained from *Anvillea radiata* using four solvents of increasing polarities (hexane, dichloromethane, methanol, and water). Our results show that the highest levels of total phenols and total flavonoids from *A. radiata* were recorded for the dichloromethane and methanol extracts, whereas the most important values of condensed tannin contents were recorded for the dichloromethane and hexane extract. Nevertheless, our results remain higher than the results found by EL GUICHE et al. (2015), who indicate that the methanol extract of *A. radiata* has a total phenols content of 11.54 µg GAE/mg DM. and a total flavonoids content of 30.28 µg CE/mg DM. In the study conducted by HEBI and EDDOUKS (2018) concerning the preliminary phytochemical screening of *A. radiata*, several compounds of chemicals have been found such as polyphenols, flavonoids, tannins, mucilage, sesquiterpenes, terpenoids, and carbohydrates. The total phenolic and flavonoid contents vary according to the plant organ used, the species analyzed, and the choice of solvent (XU and CHANG 2007). SAHREEN et al. (2017), report similar findings and indicate that the methanol extract had illustrated the highest total content of phenolic, whereas the content obtained with ethyl acetate was much lower, and justify that this can be due to the formation of complexes by a part of phenolic compounds with carbohydrates and proteins, which are more extractable in methanol than in other solvents. Our results do not deviate

from those of KANDOULI et al. (2017), who found the same results regarding the levels of total phenols and flavonoids using methanol as solvent (65.8±1.8 mg GAEC/g and 48.4±0.9 mg RUE/g respectively). An imbalance in the oxidant/antioxidant status is mostly accompanied by tissue injury and human disease, creating oxidative stress, which must be amenable to therapeutic intervention with appropriate antioxidants, provided that they can reach the site of damage and are effective in decreasing oxidative damage levels (HALLIWELL 2001). Therefore, because of the potential health hazards of many synthetic antioxidants widely used in different food products, which involve toxic side effects, their use is under strict regulation in many countries (WANG et al. 2009). That is why; actually there is a growing interest in the substitution of synthetic antioxidants used in food preservation with natural ones (SERAIRI-BEJI et al. 2018). Indeed, polyphenols are natural compounds widely distributed in the plant kingdom which have increasing importance in particular thanks to their beneficial effects on health (KOECHLIN-RAMONATXO 2006). According to ADEBIYI et al. (2017), the antioxidant activity of polyphenols is largely due to their redox properties which make them act as reducing agents, hydrogen donors, singlet oxygen quenchers, and as well as potential metal chelators. In this study, three complementary tests were used to assess the antioxidant activity of *Anvillea radiata* (Total antioxidant capacity, DPPH free radical-scavenging activity, and ferric reducing antioxidant power assay). The results showed that both dichloromethane and methanol extracts from *Anvillea radiata* showed strong antioxidant activity. These same extracts had the highest values in polyphenol and flavonoid contents, which confirm the correlation that exists between the content of phenolic compounds of an extract and its antioxidant activity. About that, KSOURI et al. (2009) has pointed out that there is a real positive correlation between antioxidant potential and phenolic content. Other works have shown that many flavonoids and related polyphenols significantly contribute to the total antioxidant activity of many fruits such as red grape (NEGRO et al. 2003).

Conclusion

The results of the present investigation indicate that the Dichloromethane and Methanol extracts of *Anvillea radiata* have the highest levels of the secondary metabolites tested and exhibit an important antioxidant activity in vitro, which confirms the correlation between the content of phenolic compounds and antioxidant activity. This finding provided

a strong evidence that the studied plant might indeed be a potential sources of natural antioxidant which could be of considerable interest to the development of new drugs based on local plants to enhance the natural resources of the national heritage.

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THE RELATIONSHIP BETWEEN SEASONAL AND ENVIRONMENTAL VARIATIONS WITH MORPHOMETRIC CHARACTERISTICS OF *SARGASSUM POLYCYSTUM* (C. AGARDH. 1824) FROM TIDUNG, SEBESI AND BINTAN ISLANDS WATERS, INDONESIA

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Key words: brown seaweed, *Sargassum polycystum*, morphometric characters, environment, small islands.

Abstract

Sargassum brown seaweed is one of the most morphologically complex genera due to environmental adaptation. Therefore, the relationship between seasons and different environments condition with the morphometric characters of *Sargassum polycystum* that grows in Tidung, Sebesi, and Bintan Islands waters is determined. Principal Component Analysis (PCA) is used to determine environmental variables' contribution, and discriminant analysis was employed to differentiate variables between research locations. The results showed that there were variations in morphological characteristics between areas. Tidung Island showed an advantage in terms of the blades' size, while Sebesi Island showed dominance in the diameter of the primary stipe, air bladder, and the branching distance from the holdfast. Additionally, we characterized the thallus's length and the seaweed's total length in the Bintan Islands. PCA shows that the main factors of water quality in ammonia, pH, Se, Fe, and Mn are associated with blade variations. On the other hand, there was a correlation between variations in DO and salinity with the branching distance from the holdfast and presence of nitrates, variations in temperature and

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brightness were correlated with holdfast diameter, thallus diameter, and blade size. This study found that the morphometric variation of *S. polycystum* was significantly influenced by the locations (small islands) and seasons.

Introduction

Brown seaweed is one of the three divisions of macroalgae that have high economic value. Most of them are good sources of alginate (FERTAH 2017, SETYAWIDATI et al. 2018) and potentially used as raw material for nutraceutical and medicine due to its high content antioxidant (BORAZJANI et al. 2017, HERMUND 2018). Moreover, seaweeds also have functioned as a shelter for many kinds of biota and outright can provide nutrition for others in the ecosystem (WILLIAMS and FEAGIN 2010). Its abundance in the marine ecosystem also serves as a primary energy producer that potentially consists of ample metabolite compound and formed the food cycle from phytoplankton and zooplankton (SHAFAY et al. 2016).

Sargassum is one genus from brown macroalgae with a rich diversity of species and one of the most complex taxonomically. The number of *Sargassum* species recorded is more than 500 species known worldwide (GUIRY and GUIRY 2020). There are around 50 species found in Indonesian water, while only 12 are well utilized (Puspita et al. 2020). One type of *Sargassum* which has a wide distribution in Indonesian waters is *Sargassum polycystum* C. Agardh. This type is a potential source of alginate (DHARMAYANTI et al. 2019), and the bioactive compound is promising (PERUMAL et al. 2019).

In general, *S. polycystum* grows well in the littoral and sublittoral zones by sticking to rocks, dead coral, or other substrates. Dead and live corals can provide additional habitat for this species (DICKSON dan SEMBILAN 2015). In general, environmental factors such as season can affect the distribution, growth, and reproduction of *S. polycystum* (NOIRAKSAR et al. (2017), temperature, and salinity (ZOU et al. 2018, CHUNG et al. 2007, FULTON et al. 2017) stated that various environmental parameters, both physical and chemical, can affect the presence and distribution of seaweed in marine ecosystems.

The conventional method can determine seaweed morphology from the shape of the thallus parts such as leaf/blade, stipe, vesicle/air bladder, and holdfast. The presence of stolon-like roots in the holdfast section of *S. polycystum* is an important morphological feature to distinguish it from other types (WONG et al. 2004). Moreover, variations in the shape and size of the thallus from the two different habitats can occur due to their interactions with varying conditions of the environment (WIDYARTINI et al. 2017).

Sometimes in distinguishing species between the genus *Sargassum*, the researcher can use the holdfast form and vesicles' presence (CAMACHO et al. 2015). Changes in seaweed morphology can occur as an adaptation to the changes in environmental conditions such as fluctuations in currents, temperature, salinity, and mineral content in waters (BAWEJA et al. 2016).

Indonesia, which is an archipelago and is dominated by small islands, has high potential seaweed resources. The conditions of the waters between islands can differ from one another so that it impacts the morphometric characters of the seaweed that grows as a form of adaptation (MUTA HARAH et al. 2014). Therefore, environmental and seasonal variations might substantially impact changes in the morphology of *S. polycystum* that grows in the Bintan, Sebesi, and Tidung Islands. Furthermore, the purpose of this study was to determine the relationship between the aquatic environmental conditions of various island types and seasonal variation against the morphometric characteristics of *S. polycystum*.

Materials and Methods

Study Area

The research was done from January to December 2019 in three different locations: Bintan, Sebesi, and Tidung Island. Bintan is part of the Riau Islands province, a monadnock island type formed by the metamorphic rock where the bottom of the coastal dominated by mud, sand, and coral fragments substrates (ASRININGRUM 2009). High agricultural activities and rivers influence the coastal area on this island as freshwater and nutrients. This type of island is characterized by water flow directly goes to the coastal area very fast during the wet season, and it will make the seawater turbid. This phenomenon resulted in highly physio-chemical changes in the coastal zone.

Sebesi Island is part of Lampung province where a hilly topography dominates the island's coast with strong currents and waves from Lampung Bay but weak anthropogenic influence (WIRYAWAN et al. 2002). The condition of hydrodynamic strongly influenced by Lampung gulf activity, and most of the nutrient availability depends on the situation of the gulf waters. This island is characterized by freshwater that abundantly available throughout the year. The tidal type is mixed, which happens twice a day.

According to BMKG (Meteorological, Climatology and Geophysical Agency), all of the research locations included in the same seasonal zone that is closed one to the other, namely the Sumatra and Java area. The seasonal differentiation is also according to weather forecast released by the BMKG. The coordinate points of the sites are as follow: Tidung Island is in 5°47'55.7"S 106°30'26.3"E; 5°47'43.0"S 106°28'45.2"E; 5°47'54.8"S 106°30'22.4"E, Sebesi Island is in 5°58'05.3"S 105°30'04.0"E; 5°58'13.6"S 105°28'56.7"E; 5°55'26.0"S 105°29'02.8"E and Bintan Island is in 1°06'30.6"N 104°13'43.7"E; 1°11'57.2"N 104°34'50.4"E; 1°01'07.9"N 104°39'09.1"E. Furthermore, Figure 1 shows the research location in detail. It consisted of three stations on each Island, and the point determination was based on the Global Positioning System (GPS) by employed Garmin ETrex 10. The map used is downloaded from the Indonesian administrative map available in Google and plotted using a QGIS 2.18 application.

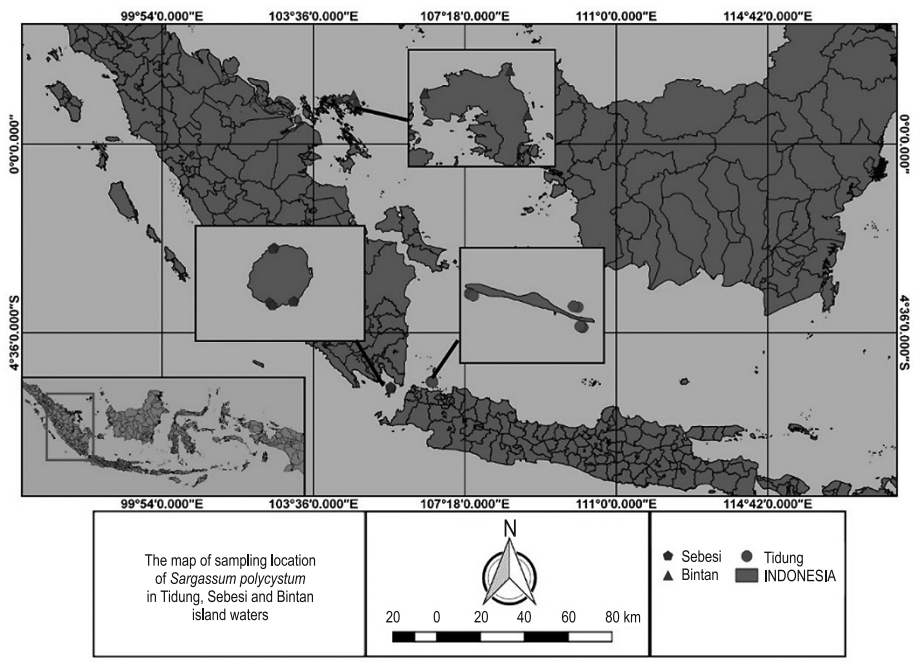

Fig. 1. Three research locations in western part of Indonesian waters
Source: Google named Indonesian administrative map that is available free and that map as a basis to develop the location map by using QGIS app. All of the coordinates points were taken by Garmin GPS

Research Procedure

Samples Collection

We collected the fresh of *S. polycystum* C. Agardh (Phaeophyceae) from the coastal of Tidung, Sebesi, and Bintan Islands waters. The sampling collection was based on a purposive sampling method and divided into three stations on each island. Furthermore, every location consisted of three substations with 50 meters distance of each along the coast.

During the sampling, the water's condition was in low tide, and the depth was about 10–30 cm. The research also considered seasonal variation, which the sampling collections have done in the summer dan wet season. Figure 2 shows the condition of the sampling location in three different islands.

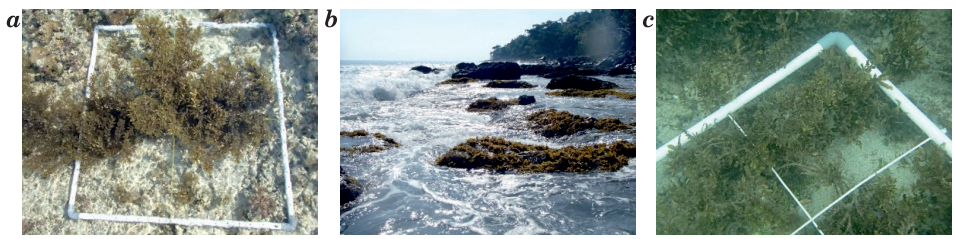

Fig. 2. Research locations: *a* – Tidung; *b* – Sebesi; *c* – Bintan (photo by I Ketut Sumandiarsa)

Environmental Characteristics

The physio-chemical parameters included Nitrate (NO^{3-}), Phosphate (PO_4^{3-}), and ammonia (NH_4^{+}) as well as temperature, brightness, DO, salinity, and pH. The trace elements of the seawaters assessed, which consist of Barium (Ba), Selenium (Se), Iron (Fe), Manganese (Mn), Copper (Cu), Zink (Zn), and Molybdenum (Mo). There are two ways of determining it, namely in situ using a water quality meter, Combo type 8630, and laboratory assessment with *Ultra Violet-Visible Spectro* (UV-VIS) in the Proling Lab IPB University.

The sample preparation was followed by adding 1 ml of H_2SO_4 to 100 ml seawater sample for water quality parameters and preserving fresh seaweed samples done by adding HNO_3 solution to $pH \leq 2$ for trace element analysis. An Atomic Absorption Spectro-photometry (AAS) Perkin Elmer PinAAcle 900H with Flame technique (Acetylene-Air) with a method of APHA, 23rd Edition (RICE et al. 2017) was employed to determine the trace elements.

Data Analysis

The homogeneity of the data variants tested using heterogeneous insignificant variance values ($p > 0.05$) did not need the data transform. Principal Component Analysis (PCA) carried out the data analysis separately on the environmental characteristics and morphological characters. Euclidean biplots plotted to determine the relative contributions of various variables with variations in the data. The determination of which variables differentiate between study locations (islands) is using discriminant analysis. Discriminant analysis carried out using the SPSS program IBM version 24. PCA performed by using the XLSTAT version 2020.1.3.

Results and Discussion

Sargassum polycystum Morphometric Characters

The morphological characteristics of *S. polycystum* on the three islands show distinctive morphometric attributes and features of each island (Table 1). The environmental aspects of the research location and the seasonal variations influence the morphometric variations. There are two seasonal variations in this case, which are wet and dry seasons. Morphometric characteristics, namely the length of the thallus and total length on Bintan Island found to be the longest which are 71.00± 32.78 cm and 77.33±34.01 cm, respectively, and the shortest was in Tidung 32.57 ±15.63 cm and 34.83±14.51 cm, correspondingly, and all of them found in the same season (wet). The most significant size of primary stipe diameter was found in Sebesi (0.46±0.01 cm), whereas the smallest was in Bintan (0.26±0.07 cm), and both of them discovered in the dry season. Based on seasonal variation, the characteristics of primary stipe diameter were not significantly different, but different islands have shown considerably different where the dry season was dominant. The most oversized holdfast diameter in the dry season is part of the Sebesi Island characteristic (2.53±0.45 cm), but it also has shown the smallest during the wet (0.55±0.33 cm). In terms of blade size from the bottom part of the thallus, it has found the longest and the widest was in Tidung during the wet season only (6.40±2.29 cm and 1.57±0.74 cm, respectively). The total length has differed entirely on the island; namely, the shortest found in Tidung, and the longest was in Bintan of about 32.57±15.63cm 77.33±34.01 cm, correspondingly.

Table 1

Morphometric characteristics of *S. polycystum* from different islands

Morphometric [cm]	Tidung Island		Sebesi Island		Bintan Island	
	wet	dry	wet	dry	wet	dry
TL	32.57±15.63	50±25	43.17±18.24	44.33±11.15	71.00±32.78	43.33±6.03
TotL	34.83±14.51	55.33±25	49.67±20.64	48.33±9.71	77.33±34.01	45.33±6.49
DPS	0.35±0.04	0.36±0.11	0.40±0.085	0.46±0.01	0.36±0.03	0.26±0.07
HD	0.66±0.32	0.80±0.2	0.55±0.33	2.53±0.45	1.23±0.05	0.97±0.15
LBL	6.40±2.29	1.80±1.1	4.25±0.40	5.43±1.2	3.00±0.4	4.07±1.6
LBW	1.57±0.74	1.37±0.8	1.33±0.59	0.77±0.2	1.10±0.2	1.53±0.35
MBL	3.40±0.87	4.73±0.7	4.00±1.48	2.80±0.36	3.20±0.3	3.60±1.2
MBW	1.23±0.51	1.03±0.06	1.03±0.25	2.00±1.73	0.83±0.21	0.77±0.15
UBL	3.13±1.79	3.32±0.87	2.87±0.81	2.50±1.25	2.07±0.23	2.07±0.66
UBW	1.20±0.79	0.72±0.19	1.07±0.25	0.60±0.26	0.70±0.2	0.47±0.12
TD	0.27±0.05	0.40±0.13	0.30±0.10	2.60±0.62	0.37±0.03	0.25±0.04
DAB	0.30±0.13	0.21±0.09	0.42±0.13	0.21±0.03	0.31±0.03	0.28±0.03
DFBH1	1.90±0.10	3.17±0.88	2.00±0.9	2.63±0.77	2.23±1.3	2.17±1.6
DFBH 2	3.93±1.62	9.37±5.84	3.13±1.29	13.10±2.62	4.30±1.51	4.50±1.67

Explanation: thallus length (TL), total length (TotL), diameter of primary stipe (DPS), holdfast diameter (HD), lower blade length (LBL), lower blade width (LBW), middle blade length (MBL), middle blade width (MBW), upper blade length (UBL), upper blade width (UBW), thallus diameter (TD), diameter of air bladder (DAB), the distance of the first branch from holdfast (DFBH1), the distance of the second branch from holdfast (DFBH2)

Table 1 shows the variation of *S. polycystum* morphometric characters based on different islands and seasons in the form of the size of thallus parts. Sebesi Island showed the shortest middle blade length, about 2.80±0.36 cm. At the same time, it is the widest of the central blade width (2.00±1.73 cm) during the dry season. Regarding the size of the central blade, the longest was 4.73±0.7 cm, found in Tidung during the dry season, but the shortest was in Bintan throughout the wet (3.20±0.3 cm). It found the tiny upper blade's length and width in Bintan during the dry season of about 2.07±0.66 cm and 0.47±0.12 cm, respectively. On the other hand, the longest and the widest was in the different seasons, namely 3.32±0.87 cm in the dry and 1.20±0.79 cm in the wet season but in the same location, which was in Tidung Island.

Seasonal variations and different locations influenced the dimension of the thallus diameter. It can prove from the finding of the largest thallus diameter in Sebesi with a size of 2.60±0.62 cm and the smallest in Bintan, which is around 0.25 ±0.04 cm in the same period, namely the dry season.

In contrast, the air bladder diameter was only affected by the season. It is indicated by finding the most extensive size during the wet season, which around 0.42±0.13 cm and the smallest occurs in the dry season (0.21±0.03 cm). It found both of the characters only in Sebesi Island. Furthermore, it found the farthest and closest of the first branch's distance from holdfast together in the same location, namely Tidung, which were about 3.17±0.88 cm and 1.9±0.10 cm dry wet season, respectively. The second branch was 13.10±2.62 cm as the furthest in the dry season and was 3.13±1.29 cm in the wet season but only from one location, which was Sebesi.

The macroalgae show a rich variation in size, shape, and structure (Baweja et al. 2016). *Sargassum* has a specific part, mainly consist of a vesicle or air bladder and blade with different adaptive functions as a floating device (Mei et al. 2019). (Mei et al. 2019). Noiraksar dan Ajisaka (2008) reported that *S. polycystum* has secondary holdfast, and some of them spread up or without any thorn in the primary branches. Besides, Wong et al. (2004) stated that secondary holdfast changed into stolon or root-like as the only feature distinguishes this species from *S. baccularia.* Soe-Htun et al. (2012) described that this species' growth influenced by season and optimally grow in intertidal and subtidal zones by sticking to a solid substrate. May-Lin dan Ching-Lee (2013) reported that *S. polycystum* that grow in Teluk Kemang, Malaysia showed the maximum average thallus length was in the dry season of about 228 mm and also in the Thailand waters with average size reach up to < 2.000 mm as maximum (Noiraksa et al. 2006). Rao dan Rao (2002) also described that the growth of *S. polycystum* thallus is varied in different times and seasons in the Visakhapatnam India, with an average length of up to 15.9 cm. The diverse location is also significantly affecting the variation of size and shape of blade and vesicle from the *S. polycystum* grow in the central Java waters (Widyartini et al. 2017).

The research shows the percentage of total length and thallus length of *S. polycystum* reach the utmost condition during the dry season. The primary thallus has several primary branches, the so-called primary lateral. Generally, this species consists of the flat and discoid holdfast. It's also complemented by fine root-like at the bottom of its thallus. The stipe is a roundly cylindrical and commonly has a short distance between the first branch to the holdfast, and it's brown to dark-brown color. Blade/leaf is flat brown to light-brown color with dots in the middle, oval lancet-shape with a tapered tip, and blunt edges. The air bladder or vesicle commonly attached to the blade's bottom has a function to float in the water during immersing. It is a round-shape slightly oval and dark brown. Figure 3 shows the morphological characteristics of *S. polycystum*.

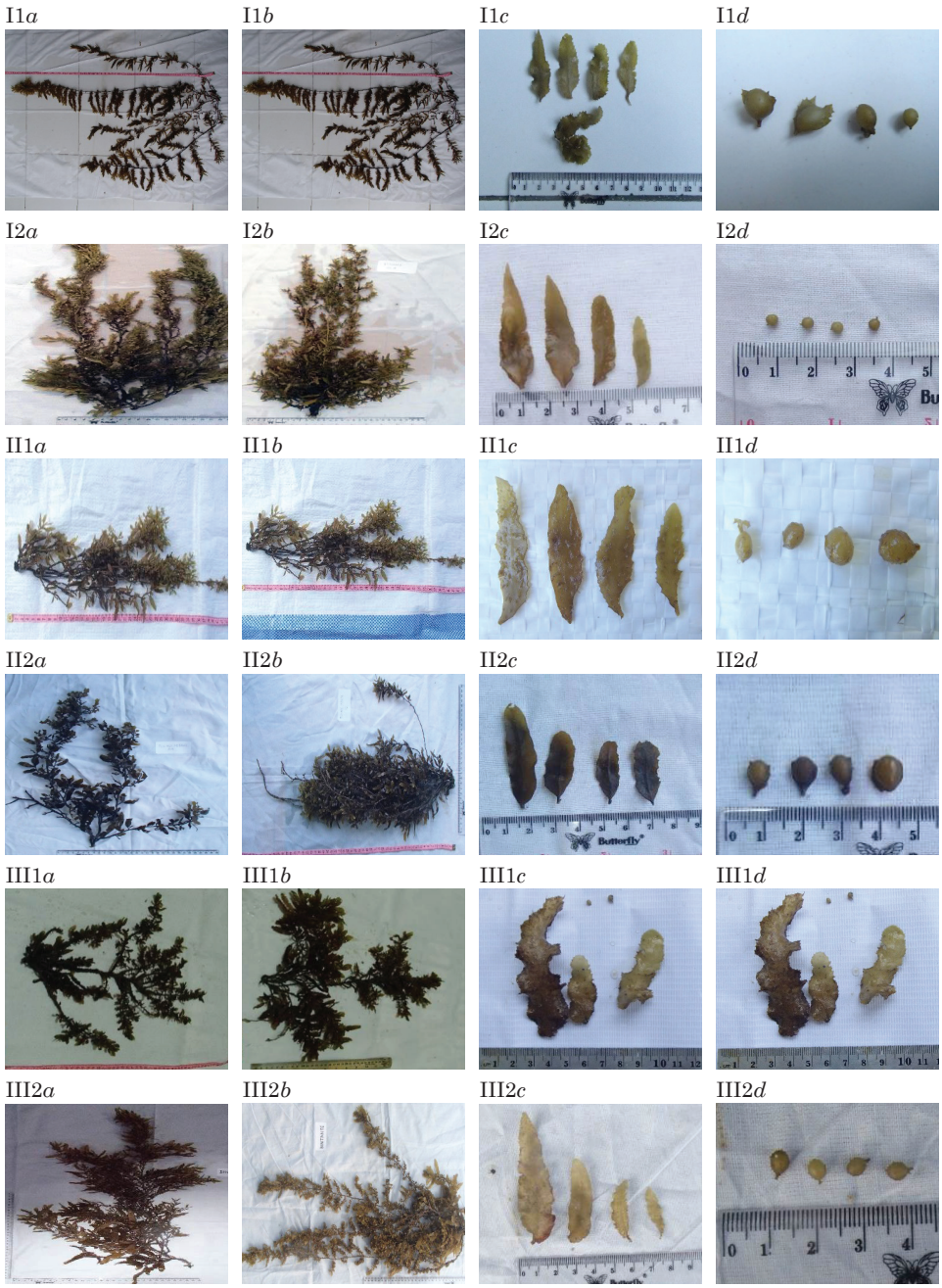

Fig. 3. *Sargassum polycystum* features from 3 different locations and two seasons (photo by I Ketut Sumandiarsa)

Explanation: I – Tidung; II – Sebesi; III – Bintan; 1 – wet season; 2 – dry season; *a* – total length; *b* – thallus length; *c* – lower, middle and upper blade, *d* – air bladder

Moreover, macroalgae can be found easily in shallow water and sticking in rigid substrates such as death coral, coral fragments, stone, and rocky substrate. Furthermore, WONG et al. (2004) stated that the character *of S. polycystum* is the form of the muricate primary stipe; the leaves are oblanceolate with dentate margins. Furthermore, the discoid-shaped holdfast is equipped with dioecious round vesicles. DAN PAYRI (2009) reported that the *Sargassum* population's dispersal could lead to limited genetic differentiation among morphologically different species. The strong holdfast and varying diameter are part of algae's macro adaptation to the constant circulation of seawater carrying nutrients causing mechanical stress. These differences also lead to morphological variations of the branches, leaves, and *Sargassum* vesicles depending on age and habitat (BAWEJA et al. 2016). A research finding by (LIU et al. 2018) concluded that the variation of locations and seasons significantly influences the reproduction part disparity of *Sargassum horneri*.

Trace Elements Content

Figure 4 shows the trace element (TE) content of *S. polycystum* in the three study sites. It found that differences in locations and seasons influence the TE content. Based on the wet season, the Mn content dominates Tidung Island, Sebesi Island, and Bintan Island. On the contrary, the three locations show the fact that Se was the lowest in the wet season, and Molybdenum (Mo) was not detectable in all islands and seasons. The wet season dominated by Mn content in all research locations, and Se content showed the opposite in those sites.

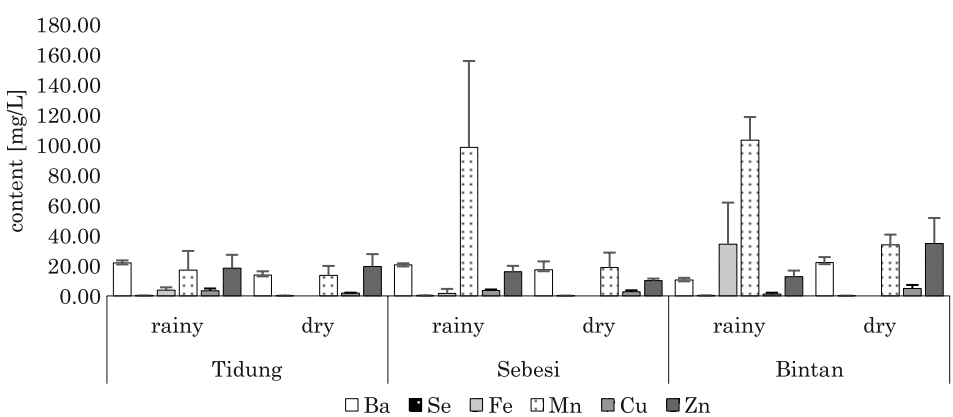

Fig. 4. Trace element composition in different island and season

Regarding the dry season, the content of iron (Fe) was the lowest in all islands (Figure 4). Overall, it can figure out the content of TE from the highest to the lowest in Tidung Island is as the pattern of Ba > Zn> Mn > Cu > Fe > Se during the wet and Zn > Ba > Mn > Cu > Se > Fe in the dry season. On the other hand, Sebesi Island has pattern as Mn > Zn > Fe > Ba > Cu >Se >Fe and Mn > Ba > Zn > Se > Fe during the wet and dry seasons, respectively. A different pattern found in Bintan in the two seasons as well, which are Mn > Fe > Zn > Ba > Cu > Se in the wet and Mn > Zn > Ba > Se > Cu > Fe in the dry.

Overall, the highest content of trace elements found in all locations was Mn with 103.29 mg/L, and Mo content was the lowest with no detection by the AAS. Based on seasonal variations, it found that Mn with 103.29±15.21 mg/L as the highest and 0.2 ± 0.01 mg/L of Se was the lowest detected trace element in the wet season. Meanwhile, Fe content as the lowest, and Mn content during the dry season as the highest with average amounts was 0 mg/L and 33.88 ± 6.76 mg/L. In general, these results indicate that the differences in location and season affect the composition of trace element content in the research locations' waters.

Fe's elemental content in the seaweed is more dominant in Bintan Island; however, in the other two islands, it is few, and there is even no detectable element of iron (Fe). Iron contributes to metabolism and promotes enzyme activity. Se content was detected to stand out from the other content. According to FAIRWEATHER-TAIT et al. (2011), this element is notable, where Se has a significant function in maintaining DNA stability and stopping the cell cycle. The Se content in this study was 0.1–0.3 mg/L. This value is still within the safe threshold value of 0.01–2.0 mg/kg, with an overall mean of 0.4 mg/L (FORDYCE 2013).

Moreover, trace elements on the three islands contain Zn, but Bintan waters have the highest. Zn plays a vital role in growth. Cu and Zn are the main components of various enzymes involved in energy metabolism (OSREDKAR dan SUSTAR 2011). MATANJUN et al. (2009) reported that the Zn content in *S. polycystum* was more dominant than Cu.

MATANJUN et al. (2009) also reported that the Fe content in *S. polycystum* was found to be 68.21±0.03 mg 100 g − 1 DW and Se 1.14±0. 03 mg. 100 g − 1 DW. Fe can function as a component of various metabolic processes, oxygen transport, electron transfer, and oxidase activity. On the other hand, Mn elements can serve as cofactors of metalloenzymes (superoxide dismutase, arginase, and pyruvate carboxylase) and associated with amino acids' metabolism, lipids, and carbohydrates. Se also comes with several functions, namely antioxidants, immunity, and hormone metabolism. Se in seawater shows 0.4–0.5 mg/L (CHAU DAN RILEY 1965). MEHDI et al.

(2013) stated that Se found to vary according to soil type and texture, organic matter content, and rainfall. Assimilation by plants influenced by soil physicochemical factors, including redox status, pH, and microbiological activity.

The Characteristics of the Aquatic Environment

Table 2 shows the indicators of water fertility in the three islands, namely the nitrate content of 0.23–1 mg/L, orthophosphate 0.004–0.047 mg/L, and ammonia 0.09–2.35 mg/L. Nitrate content was increased from 0.23–0.77 mg/L during the wet to 0.24–1 mg/L in the dry season.

Table 2
Seawaters quality of *S. polycystum* habitat in Tidung, Sebesi, and Bintan Islands

Location	Season	Nitrate (NO^{3-}) [mg/L]	Orthophosphate (PO_4^{3-}) [mg/L]	Ammonia (NH^{3-}) [mg/L]
Tidung	wet	0.23±0.1	0.047±0.01	0.09±0.003
	dry	0.24±0.2	0.022±0.004	0.84±0.05
Sebesi	wet	0.27±0.1	0.004±0.003	0.27±0.04
	dry	0.26±0.1	0.027±0.003	0.06±0.01
Bintan	wet	0.77±0.3	0.058±0.009	2.35±0.3
	dry	1.00±0.7	0.008±0.005	0.81±0.1

The wet season's orthophosphate content ranges from 0.004–0.058 mg/L, and the dry season is 0.008–0.027 mg/L. The ammonia content in the wet season is 0.09–2.35 mg/L, and the dry season is 0.06–0.84 mg/L, which means the highest ammonia content found in the wet season. Based on the fertility of the waters in terms of the island, it shows that the highest nitrate concentration found on Bintan Island and the lowest is on Tidung Island, the highest orthophosphate content found on Bintan Island and the lowest is on Sebesi Island. Furthermore, the most increased ammonia found on Bintan Island, and the lowest is on Sebesi Island. It can infer that seasonal and location variations influenced the concentration of these waters' quality indicators. Seaweed growth is mainly depending on the availability of essential elements, which are inorganic carbon, nitrogen, and phosphorus in the environment (ROLEDA and HURD 2019).

Table 3

The physio-chemical characteristics of Tidung, Sebesi, and Bintan waters

Location	Season	DO [mg/L]	pH	Temperature [ºC]	Salinity (PSU)	Brightness [%]
Tidung	wet	5.61±0.1	7.40±0.2	31.6±0.4	34.67±0.6	100±0.0
	dry	6.53±0.8	7.35±0.3	27.53±0.1	35.53±0.4	100±0.0
Sebesi	wet	5.58±0.3	7.55±0.2	30.8±1.6	33.93±1.6	100±0.0
	dry	7.53±1.4	7.26±0.3	28.67±0.7	34.33±1.2	100±0.0
Bintan	wet	5.88±0.5	7.68±0.1	30.47±0.9	32.2±0.9	100±0.0
	dry	7.77±0.6	7.43±0.4	29.8±3.8	34.33±1.15	93.3±11.6

Table 3 shows the water quality of the three islands, which differed by season. The influence of the season on the quality of DO shows a difference; namely, the wet season DO lower than the dry season. As for the overall DO value is 5.61–7.77 mg/L, which indicates it is within normal limits for plant survival. The wet season's pH and temperature conditions are higher than in the dry season, it is 7.26–7.55 and 27.53–31.6ºC, respectively. The salinity in the dry season is higher than the wet season in the three islands. Salinity ranges from 32.2–35 PSU in all sites.

In terms of location in Table 3, we found the highest DO and pH on Bintan Island, and the lowest was on Sebesi Island. The highest and lowest temperatures are on Tidung Island. We found the highest salinity concentration on Tidung Island of about 35.53±0.4 PSU, and the lowest was on Bintan Island in approximately 32.2±0.9 PSU. In general, the brightness shows similarities in season, but only Bintan Island reveals a difference during the dry season. It is due to community activities that affect the level of water transparency. Table 3 shows the temperature conditions of 27.53–31.6ºC and salinity of 32.2–35 PSU. This value has the similarity reported by ZOU et al. (2018), stated that it found that *S. polycystum* grows well at the temperature of 15–25°C and the salinity of 20–40 PSU. HAAS et al. (2014) has stated that increasing seawater temperature can reduce the amount of dissolved oxygen content and cause the photosynthetic process of seaweed.

Figure 5 shows the result of the discriminant analysis of the effect of the island's aquatic conditions on the morphology of *S. polycystum*. F_1 factor 86.07% and F_2 factor 13.93% with a total of 100%. ndBased on the Wilks' Lambda test, it shows a p-value of 0.027 with an alpha of 0.05 ($p < 0.05$) meaning that the three islands have different characteristics. The Wilks' Lambda number is close to 0, so there tends to be a difference within the group. F_1 factors consist of LBL, LBW, MBL, UBL, UBW, DFBH1, and DFBH2. F_2 aspect includes TL and TotL.

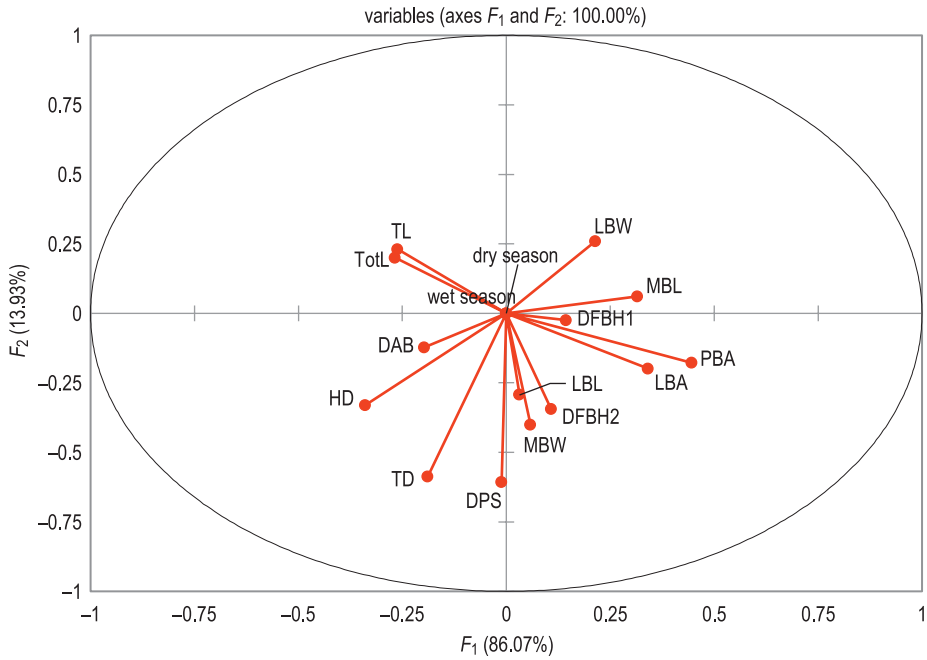

Fig. 5. Morphological characteristics of *S. polycystum* based on different location by using discriminant analysis

Explanation: thallus length (TL); total length (TotL); diameter of primary stipe (DPS); holdfast diameter (HD); lower blade length (LBL); lower blade width (LBW); middle blade length (MBL); middle blade width (MBW); upper blade length (UBL); upper blade width (UBW); thallus diameter (TD); diameter of air bladder (DAB); the distance of the first branch from holdfast (DFBH1); the distance of the second branch from holdfast (DFBH2)

The Relationship Between the Morphology of *S. polycystum* and the Environment Characteristics

The morphological analysis using PCA successfully uncovered the differences in the morphological characteristics of *S. polycystum* as a targeted sample in this study by separating its habitat into three island characteristics (Figure 6). Factors F_1 23.96% and F_2 15.27% with a total of 39.22%. The loading factor (F_1) shows that ammonia and pH have a positive relationship with the content of trace elements Se, Fe, Mn, and morphometric character of the lower blade length and upper blade width. Besides, DO and salinity showed a positive association with the distance of first and second branches to the holdfast (DFBH1 and DFBH2) in *S. polycystum*. The second loading factor (F_2) shows that nitrate, temperature, and brightness correlated with holdfast diameter (HD), central blade length (CBL), upper blade length (UBL), and thallus diameter (TD), but

negatively correlated with the distribution of trace elements of Zn in *S. polycystum* habitat. It means that the higher the water temperature causes HD, CBL, UBL, and TD is getting bigger.

The analysis results based on (Figure 6) show that brightness is the main requirement for the growth and development of *S. polycystum* in the coastal area. The brightness level causes *S. polycystum* to have the ability to absorb CO_2 and produce O_2 in seawater for photosynthesis (KOMATSU DAN KAWAI 1986, KOMATSU 1989). According to BAWEJA et al. (2016), seaweed distribution depends on physical factors like temperature, quality, and quantity of light, dynamic tidal activity, winds, and storms. It is also correlated with chemical elements such as salinity, pH, nutrients, and pollution. Biological factors involve following herbivores, microbes, epiphytes, endophytes, symbionts, parasites, and diseases.

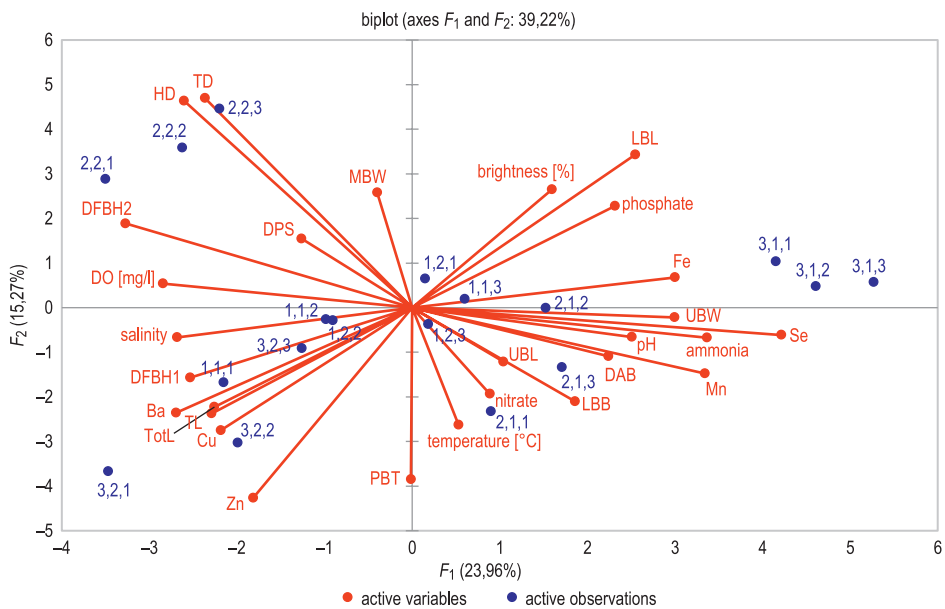

Fig. 6. The relationship between morphological characteristics of *S. polycystum* and environmental conditions

In general, seaweed growth requires an area with sufficient nutrients and light for growth and salinity and temperature (CAMPBELL et al. 2019). Seaweed biomass fluctuation associated with various biotic (competition, herbivory) and abiotic (light, temperature, wave, nutrition) factors (FULTON et al. 2017). HWANG et al. (2004) reported that the maximum growth temperature of *S. polycystum* was at 15–25°C in the south Taiwan waters. The growth of seaweed responds to the ever-changing physical biotic and

abiotic factors (ROLEDA and HURD 2019). Seasonal changes and variations in abiotic factors and temperature, salinity, pH, nutrients, and water movement are also driving *Sargassum*'s annual growth cycle.

According to CHUNG et al. (2007), the temperature has a high impact on marine morphology and geographic distribution of wild seaweed. Furthermore, AGRAWAL (2009) added that weather has a role in regulating reproduction. On the other hand, studies conducted on coral reefs in Southern Taiwan indicated that water temperature impacted abundance for *S. siliquosum* but negatively correlated with *S. polycystum*. The increasing temperature could reduce *S. polycystum* cover (±9.62% coverage) in that region. So, seasonal dynamics, species, temperature limits, and nutrient utilization strategies can all influence *S. polycystum* growth and abundance.

PCA shows that the temperature correlates with holdfast diameter (HD), middle blade length (MBL), upper blade length (UBL), and thallus diameter (TD). It implies that the higher the water temperature causes HD, MBL, UBL, and TD to be bigger or more extended and vice versa. In general, the growth of *S. polycystum* is divided into three periods, namely accretion, reproduction, and degeneration in a year. The typical growth cycle in *Sargassum* species is characterized by a slow accretion phase, a fast-growth phase, and a reproductive phase followed by aging (ANG 2006). RAO AND RAO (2002) reported that the growth pattern of *S. polycystum* was influenced by season and correlated with temperature. The environmental conditions required for seaweed species' growth may vary (KERRISON et al. 2015). *Sargassum* species are controlled by water temperature and related to the wet season because the water temperature is one of the most important factors influencing the Sargassum species' phenological pattern (ANG 2006). Temperature also tends to affect this species' distribution pattern by limiting its growth (KANTACHUMPOO et al. 2014), and the optimal water temperature for the optimal development of tropical *Sargassum* species is 20–25°C (PHANG et al. 2016).

Figure 6 also shows the positive correlation between ammonia and pH, which positively correlates with the trace elements content Se, Fe, Mn, and morphometric PBB and LBA in *S. polycystum*. Thus, this element affects the dissolved oxygen content in seawater, and consequently, the pH distribution through the carbonate equilibrium in seawater changes the absorption and release of CO2 (KOMATSU et al. 1996). The fluctuations of nitrate content in waters due to seasonal changes can affect the distribution and morphology of *S. polycystum*, as reported by MAY-LIN DAN CHING-LEE (2013) and ANDRÉFOUËT et al. (2017).

Sfriso dan Facca (2013) stated that *Sargassum* shows the advantages of broader distribution and forms of dense populations on the side of ledges, rocks, and rigid substrates. Widyartini et al. (2017) reported that environmental factors that influence the talus's variation in vesicle length are salinity and pH. Meanwhile, temperature, salinity, and pH affect the colour of the vesicles, while nitrate and phosphate affect the width of the blade/leaves and the upper and lower surfaces. Phosphate also affects leaf margins and vesicle diameter. Sfriso dan Facca (2013) also stated that *Sargassum* commonly reached a peak growth of 2.32 cm per day. Species growth is mainly regulated by water temperature, nutrient concentration, especially nitrogen and water turbidity. Nitrate is a compound that contains nitrogen and can dissolve in water. The presence of nitrate content in seawater affects seaweed features such as the formation of a delicate root-like part in its holdfast (Connor dan West 1991). On the other hand, the decreased growth decreased photosynthetic activity, and decreased enzymes are involved in carbon metabolism (Collén et al. 2004, Xiao et al. 2019). Reidenbach et al. (2017) reported that decreased pH affects carbon and nitrogen metabolism of seaweed, for example, that happens in *Ulva australis*; however, it did not involve growth changes.

DO, and salinity shows a positive association with the distance of the first and the second branches from holdfast (DFBH1 and DFBH2, respectively) in *S. polycystum*. These features are closely related to the growth of *Sargassum*. Li et al. (2019) reported that the salinity of seawater affects the development of *Sargassum*, precisely 6.40% per day with 30 PSU, and saltiness is also affecting the quality of the biomass (Niwa and Harada 2013). Salinity is an important environmental factor that influences seaweed growth and distribution, and the seaweed spread in different areas shows different ranges of tolerance to salinity Karsten 2012). Fredersdorf et al. (2009) also stated that each growth cycle of brown seaweed provides an overview of differences in salinity conditions. Seasonal variations affect the level of seawater quality, which impacts changes in the structure of the *Sargassum* canopy and affects population density (Rivera dan Scrosati 2006, Ateweberhan et al. 2009). Anthropogenic pressure was also significant to influence water quality into less fertile and decrease organism growth in coral reef area including macroalga (Januar et al. 2015).

Conclusions

In general conclusion, the factors including geographic environment and seawater quality and nutrient availability can affect the morphometrics of *S. polycystum*. Ammonia and pH have a positive relationship with the content of trace elements Se, Fe, Mn, and morphometric lower blade length (LBL) and upper blade width (UBW). Besides, DO, and salinity showed a positive correlation with the distance of first and second branches from holdfast (DFBH1 and DFBH2). Meanwhile, the nitrate, temperature, and brightness correlated with Holdfast diameter (HD), middle blade length (MBL), upper blade length (UBL), and the thallus diameter (DT). The optimum environmental temperature conditions for this species grow in the three different locations are 27.530–31.6ºC. DO, which exceeds the threshold in the waters, around 5.61–7.77 mg/L, has supported the growth and development of *S. polycystum* and correlated with the larger holdfast diameter (HD) and the upper blade length (UBL), which is getting longer.

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THE EFFECTS OF SOAKING TREATMENTS AND FERMENTATION PROCESS ON NUTRITIONAL AND AFLATOXIN CONTENTS OF FERMENTED PEANUT CAKE (*BLACK ONCOM*)

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Abstract

The existing product of fermented peanut cake, namely *black oncom* as a traditional food of West Java contains high aflatoxin and less nutritional contents. This study aimed to evaluate the adaptive processing method using the effect of soaking treatment and fermentation process on nutritional and aflatoxin contents of *black oncom*. Parameters evaluated in this study were the fermentation quality (pH and mold density), nutritional contents (water, ash, protein, lipid, carbohydrate, crude fiber, dietary fiber, and protein digestibility) and aflatoxin (aflatoxin B_1 and total aflatoxin). These parameters were evaluated for peanut cake with unfermented condition and fermented condition such as commercial *black oncom* as a traditional (natural) fermentation and the adaptive processing method of *black oncom* with three soaking treatments of peanut cake, i.e., water (BW), acetic acid 5% (BA) and chitosan 5% w/v (BC) and the use of yeast starter of *Rhizopus oligosporus*. The results showed that growth of mold were spread more homogeneously in BW compared to BC and BA. The adaptive procedure of BW treatment produced *black oncom* with better nutritional value (crude fat 37.46% and protein digestibility 77.91%) and lower aflatoxin content within acceptable level of regulatory standard than the commercial *black oncom* in the market. In conclusion, the adaptive fermentation of *black oncom* processing which used water treatment and the use of yeast starter of *Rhizopus oligosporus* might be applied as a potential solution for *black oncom* production with better nutritional value and acceptable aflatoxin content.

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Introduction

Peanut cake is an industrial residue or main by-product of the peanut oil which can be used as a source of nutrients (SADH et al. 2017, 2018). Peanut cake can be improved in quality and nutritional contents with the solid state fermentation technology (SSF) which is defined as the growth of microorganisms (especially fungi or molds) in moist solid materials without free water (BHARGAV et al. 2008, CHI and CHO 2016) and it can improve the functional properties, nutritional characteristics, good sensory characteristics of a product and and the formation of short chain compounds with lower molecular weight (SADH et al. 2018, XIAO et al. 2015). The SSF can be applied in several cereals, nuts and by-products, as well as other food applications (CHI and CHO 2016).

In Indonesia especially West Java, solid state fermentation technology has also been developed in fermented peanut cake (*black oncom*) as a traditional food in West java which contains fungus *Rhizopus oligosporus* (HO 1986, PANDEY 2003). Fermented peanut cake has functional components, such as phenolic compounds, antioxidant, good nutritional and low aflatoxin contents if it is fermented by microorganism starters, such as *Aspergillus oryzae* (SADH et al. 2017), *A. awamori* (DUHAN et al. 2019), *Bacillus natto* bacteria (YANPING et al. 2017), *Neurospora sitophila*, *Rhizopus oligosporus* (KUMBHARE 2017), and *Zygosaccharomyces rouxii* (ZHOU et al. 2017). The production process of *black oncom* still does not use starters, but a natural fermentation process. This is observed from the low nutritional contents (SLAMET and TARWOTJO 1971) and the high aflatoxin content (ROEDJITO et al. 1972, GINTING et al. 2018).

Therefore, it is necessary to modify the production method of *black oncom* with SSF technology to achieve a product with high nutritional contents and low aflatoxin content. Process modification can be performed using soaking treatments, because the fermentation process is begun with a soaking process. Organic acids such as acetic acid, lactic acid as candidates for the soaking treatment were used to reduce the aflatoxin level. HASSAN et al. (2015) showed that 10% acetic acid has the highest inhibitory effect for the growth of aflatoxin-producing *Aspergillus flavus*, and lactic acid can also inhibit the growth of aflatoxin. In addition, a chitosan treatment can reduce the aflatoxin B_1 content in peanuts (HIDAYAH 2015) and increase nutrients in a fermentation process (GANDRA et al. 2018, NWE et al. 2002).

It has been noticed how the nutritional contents can be increased and the toxin can be decreased in an adaptive processing method with soaking treatments and fermentation process using a yeast starter in *black oncom*

production (HASSAN et al. 2006, SITUNGKIR 2005, JANNAH 2005, PURWIJANTININGSIH 2005, RAHARJANTI 2006). Therefore, the aim of this study was to analyze the adaptive processing method with the effect of soaking treatments (water, acetic acid, chitosan) and the fermentation process using the yeast starter of *Rhizopus oligosporus* on nutritional and aflatoxin contents of *black oncom* product compared to unfermented peanut cake and commercial *black oncom* in the market as a traditional fermentation.

Materials and Methods

The Processing Method of Fermented Peanut Cake (*Black Oncom*)

A completely randomized design was applied in this study. Various materials used for the production of *black oncom* were prepared, i.e., peanut cakes collected from the peanut oil factory in Sukasari, Bogor, West Java, Indonesia. *Black oncom* in the market as a traditional fermentation was collected from craftmens in Bantar Kambing, Bogor, West Java, Indonesia. Three types of soaking solution were prepared, i.e., water, acetic acid 5% w/v, and chitosan 5% w/v. The concentration was applied according to DEWI and NUR (2018) that 2.5% of concentration can reduce aflatoxin as much as 70%, and 10% of concentration generates the highest reduction of aflatoxin. The yeast starter of *Rhizopus oligosporus* (Raprima brand) was used for the fermentation process.

The production process of traditional *black oncom* is shown in Figure 1 (*a*). The production used water for soaking treatments and cassava cake 2% for the fermentation process without the addition of *Rhizopus oligosporus* starter, then it was fermented in a bamboo box for 48 h (ROHIMAH et al. 2021). The adaptive processing method is shown in Figure 1 (*b*). For the adaptive fermentation process, the peanut cake materials were soaked in different soaking treatments (water, acetic acid 5%, and chitosan 5%) for 16 h. After soaking, the cake was washed and drained. Then the cake was steamed for one hour. After cooled down, the starter of *Rhizopus oligosporus* was added as much as 0.2% and was spread by mixing it with the cake. Afterwards, it was molded in a square shape. Then, it was fermented at 25–29°C for 48 h in a anaerobic condition in a laboratory. The three different treatment procedures of *black oncom* production were peanut cake was soaked in water and fermented using *Rhizopus oligosporus* (BW), peanut cake was soaked in acetic acid 5% and fermented using *Rhizopus*

oligosporus (BA), peanut cake was soaked in chitosan 5% and fermented using *Rhizopus oligosporus* (BC). This experiment was analyzed with two replicates.

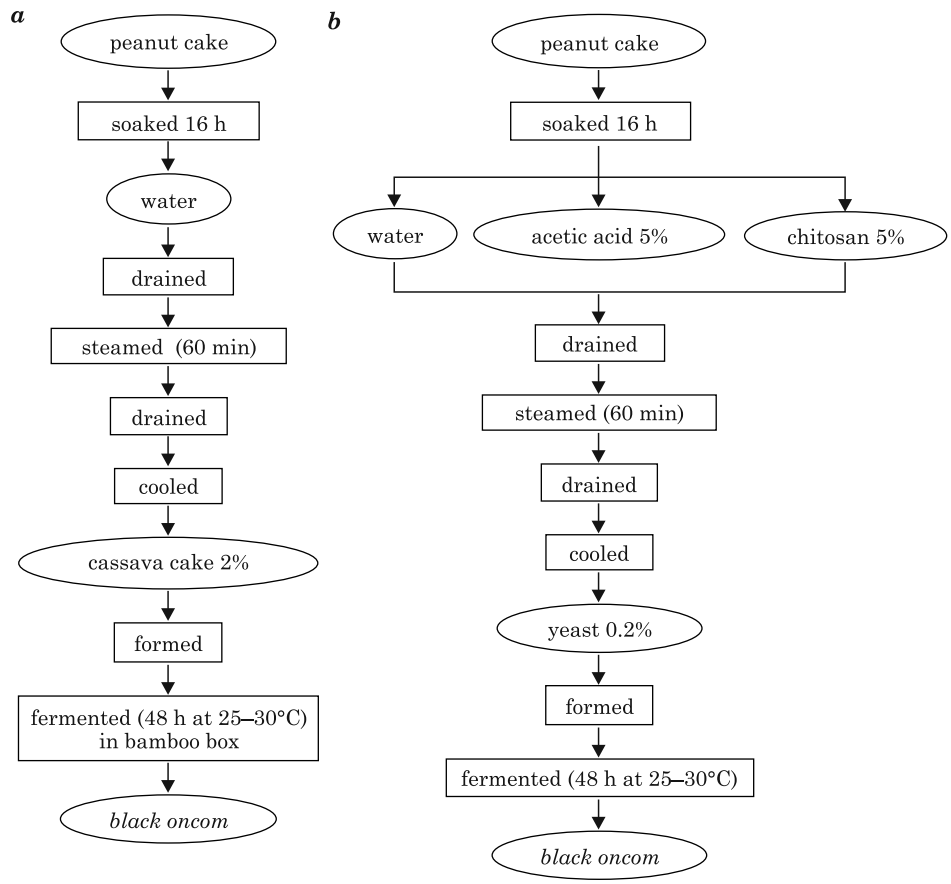

Fig 1. Flow chart of black oncom production processes: *a* – traditional fermentation; *b* – adaptive fermentation

Measurement of Parameters

Determination of Fermentation Quality

Three main parameters were measured in this study, i.e., pH value, mold density, and texture. The level of pH was measured using a OHAUS starter 3100 pH meter. The procedure was as follows: a 10 g sample was prepared, then 10 mL of distilled water was added and stirred until blended. Afterwards, the pH electrode was previously rinsed with distilled

water, then was put in the sample solution. The value of pH was displayed on the device screen. The growth of mold was calculated to determine the mold density that consisted of no mold growth (0); slight mold growth, texture not yet formed (+); sufficient mold growth, the texture is rather compact and soft (++) (SASTRAADMAJA et al. 2002). The spread of mold growth was represented as a percentage. Texture of *black oncom* was examined using a LFRA Stevens texture analyzer with needle-shaped probe, speed of 2.0 mm/s and distance of 10 mm. The data of texture was represented in the equipment as N unit (ISKANDAR et al. 2010).

Determination of Nutritional Content

Nutritional contents determined were the content of water, ash, crude protein, crude fat, carbohydrate, crude fiber, dietary fiber (AOAC 2005), and protein digestibility (SHI et al. 2020). Determination of water content was performed by heating process in an oven at 100 ± 5°C (Eyela, NDO 400, Japan) of 2 g of sample then weighed after being cooled in the desiccator. Water content was determined by the following formula represented as percentage [%].

$$\text{Water content [\%]} = (B/A) \cdot 100$$

where:
A – initial sample weight
B – sample weight after drying.

Ash content was determined by heating of 3 g sample on a hotplate until fume was disappeared. Then, the sample was ashed completely for 5 h at 550°C in a furnace. The ash content was determined as weight of sample after it was ashed compared to weight sample before it was heated and ashed. The ash content was evaluated using the following formula:

$$\text{Ash content [\%]} = (B/A) \cdot 100$$

where:
A – initial sample weight
B – sample weight after ashing

Determination of protein content involved a destruction with 6 mL of H_2SO_4 and 1 g of selenium mix (Foss, DT 208, Denmark). Afterwards, 40 mL of NaOH 40% and 25 mL of aquades were added and the sample was distilled using a KjelDigester instrument (Foss, KT 200, Denmark)

until three times of volume of 4% boric acid. Then, the distillate was titrated using HCl 0.2 N until reached the end point (red colour). The protein content was evaluated using the following formula:

$$\text{Protein content [\%]} = [(V_s - V_b) \cdot N \cdot 1.4007 \cdot F_k\ ]/\ \text{g of sample}$$

where:
V_s – volume of HCl for sample titration
V_b = volume of HCl for blank titration
N = normality of HCl solution
F_k = conversion factor (6.25).

Determination of fat content was performed by hydrolisis method. Two g of sample was added with 30 mL HCl 25 % and 20 mL of aquades, then it was heated at a hotplate for 15 min. The residue was dried at 105°C in an oven for 1 h. Then, the residue was placed in a sample package for fat extraction with hexane using soxhlet equipment that was connected with a boiling flask for 3 h. The boiling flask and residue of fat was dried at 105°C in an oven, and it was weighed after being cooled. The fat content was evaluated using the following formula:

$$\text{Fat content [\%]} = [(C - A)/\ B] \cdot 100$$

where:
A – empty boiling flask weight
B – sample weight
C – boiling flask weight + dried sample.

Based on water, ash, protein and fat contents, carbohydrate content was determined using by difference method. Crude fiber content was determined by firstly removing the fat in the sample that was sieved with 40 mesh of sieve using petroleum ether. Residue of the extraction was refluxed with 100 mL of H_2SO_4 1.25% for 30 min to boil. The filtering process was followed by rinsing using 40 mL hot water for four times. The residue was then rinsed with hot NaOH 1.25% which was collected with an Erlenmeyer. The collected water was refluxed with an upright cooler for 30 min to boil. The solution was then filtered with a filter paper using a vacuum pump. The filter paper containing the residue was rinsed again with 25 mL hot H_2SO_4 1.25%, 25 mL hot water for two times and 25 mL acetone. After rinsing, the filter paper was dried in an oven at 105°C for 2 h. After being heated, the filter paper was cooled and then weighed. If the crude fiber content was above 1%, then the ash content was measured by weighing the heated empty porcelain plate. Paper and residue were char-

red on a hotplate and then was ignited in the furnace (temperature 550°C). After igniting, the plates were cooled in the excicator, then weighed to obtain a fixed weight. Crude fiber content was determined by the gravimetric method.

Determination of dietary fiber was performed by the enzymatic process. A sample of 0.5 g was put into different 50 mL falcon tubes. The sample was extracted with 15 mL of petroleum ether for three times, then was dried in an oven at 100°C. Each sample was transferred to a 500 mL beaker. A total of 40 mL of mes tris buffer was added while stirring until no sample was lumpy. 50 µL *α*-amylase enzyme was added while stirring until homogeneous. The beaker was covered with an aluminum foil and incubated in a waterbath shaker at 100°C for 30 min. The solution was then cooled to 60°C and the walls were rinsed with 10 mL of distilled water. A total of 100 µL of the protease enzyme was added while stirring until there was no clumps of the sample. The solution was closed again with an aluminum foil, then was incubated in a waterbath at 60°C for 30 min. The aluminum was opened and 0.5 M HCl was added. The pH of the sample was adjusted in the range 4.1–4.6 with 1 M HCl or 1 M NaOH. The solution was added with 200 µL of amyloglucosidase enzyme and was incubated again at 60°C for 30 min. 225 ml of 95% ethanol at 60°C was added and stirred until homogeneous. The solution was left to stand for 1 h, then was filtered with a known weight filter paper and rinsed with 15 mL 78% ethanol for two times, 15 mL 95% ethanol for two times and 15 mL acetone for two times. The filter paper was dried in an oven at 103°C overnight. The filter paper containing the residue was weighed, then the weight was determined for ash analysis and the weight for protein analysis. Calculation of dietary fiber content was determined by corrected ash and protein contents of the residue.

Protein digestibility [%] was assessed using enzymatic method (SHI et al. 2020). Protein digestibility was determined based on comparison of the protein content that was digested by enzyme and the protein content in the original sample.

Determination of Aflatoxin

Aflatoxin is one of parameters that can be used for food safety. Aflatoxin B_1 and total aflatoxin was evaluated in the peanut cake and their fermentation. The aflatoxin measurements were performed by using a liquid chromatography/mass spectrophotometry (LC/MS). The column used was Acquity UPLC BEH C18 2.1 · 100 mm; 1.7 µm, mobile phase A of formic acid 0.1% in aquabidest, mobile phase B of formic acid 0.1% in acetonitrile, with

injection volume of 5 μL and column temperature of 30°C. Aflatoxin content in ppb unit was determined using a calibration curve with a line equation (YOUNG et al. 2015, JETTANAJIT and NHUJAK 2016).

Statistical Analysis

The results were statistically analyzed by one-way analysis of variance (ANOVA) using SPSS 16. The assessment of the significance of differences between the means was performed using Duncan test ($p < 0.05$).

Results and Discussions

Fermentation Quality

The quality of fermentation process can be assessed from pH value and mold density (both qualitative and quantitative). Based on the pH indicator, BW treatment had significantly higher pH level compared to other treatments ($p < 0.05$) – Table 1, which mean that the treatment can produce *black oncom* when the pH value was high (pH > 5).

Table 1

The physical characteristics of fermented peanut cakes

Treatment		pH value	Texture (N)	Mold density	
				qualitative (visual)	quantitative [%]
Traditional fermentation	*black oncom* in the market	5.01 ± 0.11^b	n/a	++	100^c
Adaptive fermentation	BW	5.823 ± 0.061^c	64.33–76.67	++	100^c
	BA	3.865 ± 0.022^a	n/a	0	0^a
	BC	5.203 ± 0.30^b	n/a	+	30.83^b

Explanation: data are presented as mean ± sd; in one column; the different superscript (^{a,b,c}) letters mean significantly different ($p < 0.05$); n/a – data can not be measured; 0 – no mold growth; + slight mold growth, texture not yet formed; ++ sufficient mold growth, the texture is rather compact and soft

Mold density of *black oncom* can be observed when the growth of mold produced spread after 48 h fermentation (Fig. 2). Therefore, the treatment that can be measured for the texture was only BW treatment because in other treatments the mold has not been formed. Furthermore, there was a significant correlation between pH and mold density ($p < 0.05$), It means that the higher the pH, the more mold growth. pH has been one of factors which can influence the growth of mold.

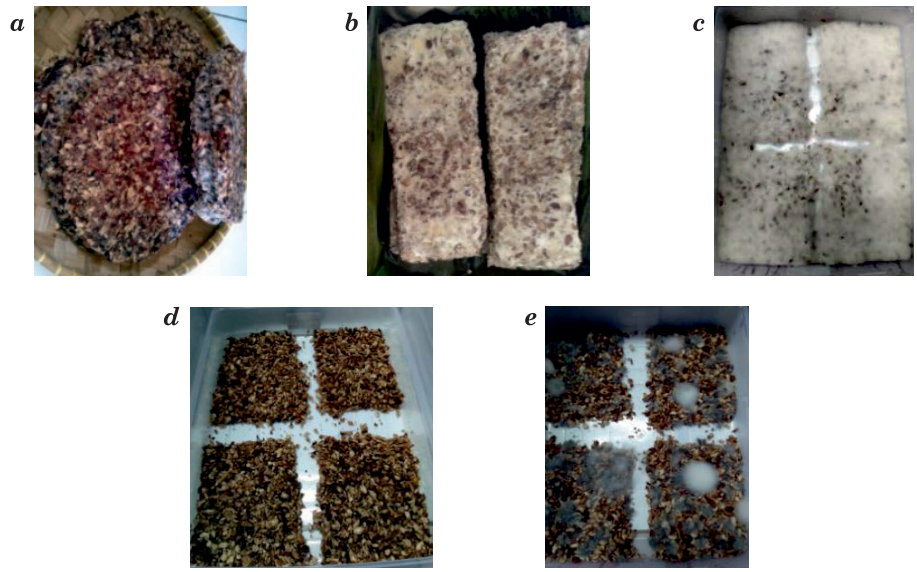

Fig. 2. Appearance of fermented peanut cakes: *a* – peanut cake; *b* – *black oncom* in the market; *c* – *black oncom* produced using adaptive processing methods BW; *d* – BA treatment; *e* – BC treatment

This study showed that peanut cake that can be appropriately fermented was BW in the fermentation process. This was supported by the environment of the fermentation process, i.e., temperature (25–30°C), humidity (70–90%) and pH (KALSUM and SJOFJAN 2008). The water treatment environment was good for the growth of mold, which was shown in the highest pH value. The high pH indicated the activity of extracellular enzymes especially proteolytic enzymes produced by mold, which breaks down proteins into amino acids. Furthermore, the degradation of amino acids produces ammonia so that the pH is high (ISKANDAR et al. 2010).

In the BA adaptive processing treatment, fungi did not grow at all because the concentration of the soaking solution is not optimal, in which the media containing acetic acid more than 7 g/L can stop *Rhizopus* mold growth (ASADOLLAHZADEH et al. 2018). Acetic acid concentration in BA treatment was still too strong in inhibiting mold growth because acetic acid has an inhibition of mold growth (PUNDIR and JAIN 2010, ROGAWANSAMY et al. 2015). Hence, the concentration of acetic acid have to be optimized to support *Rhizopus oligosporus* growth in *black oncom* production.

Meanwhile, the pH value in BC treatment of 5.20, was not significantly different from commercial black oncom. This pH value still supported mold growth, but mold growth was only at certain points with a spread in uneven position. This treatment is still needed to optimize in the

appropriate concentration of treatment so that it can produce *black oncom* with good mold growth. Therefore, the chitosan concentration still have strong activity as an antifungal growth as described by DUTTA et al. (2009), but another study showed the higher biomass production of *Rhizopus oryzae* generates higher chitosan content (TAI et al. 2010).

Nutritional Contents

The nutritional contents are shown in Table 2. The fermentation process experienced a decrease in carbohydrate, total fat, ash, crude protein, and energy in wet basis. Meanwhile, in dry basis, total fat, crude protein, protein digestibility and crude fiber were increased. The differences in the content occurred because a high change of water content in the fermentation product. The highest increase in water and carbohydrate contents was in the commercial *black oncom* where the values between treatments were significantly different ($p < 0.05$). This is because *black oncom* from the market has been added with cassava cake or tapioca. Microbial activity requires energy and nutrients during fermentation so that the carbohydrate content decreased which acts as the main source of energy. This can be confirmed also in the total energy which was decreased after the fermentation compared to raw materials (peanut cake) (YANG et al. 2016).

The adaptive procedure of peanut cake fermentation can significantly increase fat content ($p < 0.05$) than peanut cake and the traditional fermentation. BW treatment had the highest content and the contents were significantly different between treatments ($p < 0.05$). Fat increase occurs due to the activity of fat decomposition by the lipase enzyme produced by mold (WANG et al. 1975). This is supported by a study related to the fermentation process that affects fat distribution and fatty acid composition (ZOU et al. (2018). This study showed in general fungi can synthesis lipids which its lipid changes and composition in solid state fermentation of rice bran. The increasing of fat content is also the result of lipid accumulation from carbon source, such as glucose (ATHENAKI et al. 2018).

Fermentation of peanut cake in the traditional fermentation and adaptive fermentation enhanced the protein content, but the increase of protein content after fermentation in the treatment group tended to be higher than commercial *black oncom* in the market. Protein content of commercial *black oncom* was not significantly different compared to the peanut cake. The increasing of protein content in BW treatment was significantly different compared to peanut cake and commercial *black oncom* in the market.

Table 2

Nutritional contents of fermented peanut cakes

Parameter	Unit	Non-fermentation		Traditional fermentation		Adaptive fermentation					
		peanut cake		commercial *black oncom* in the market		BW		BA		BC	
		wb	db	wb	db	wb	db	wb	db	wb	db
Carbohydrate	%	34.96 ± 3.71^{b}	38.50 ± 4.07^{c}	9.89 ± 0.1^{a}	33.53 ± 0.01^{bc}	8.57 ± 0.15^{a}	22.64 ± 0.38^{a}	10.073 ± 0.08^{a}	25.56 ± 0.23^{ab}	9.21 ± 0.014^{a}	22.67 ± 0.18^{ab}
Total fat	%	27.17 ± 0.31^{c}	29.92 ± 0.35^{a}	9.17 ± 0.02^{a}	31.07 ± 0.01^{a}	14.20 ± 0.32^{b}	37.46 ± 0.37^{c}	13.72 ± 0.26^{b}	34.82 ± 0.63^{b}	15.003 ± 0.80^{b}	36.96 ± 2.22^{b}
Ash content	%	4.89 ± 0.48^{c}	5.37 ± 0.56^{b}	1.93 ± 0.02^{b}	6.53 ± 0.004^{c}	1.47 ± 0.25^{ab}	3.87 ± 0.61^{a}	1.21 ± 0.042^{a}	3.07 ± 0.11^{a}	1.34 ± 0.1^{ab}	3.30 ± 0.22^{a}
Crude protein	%	23.58 ± 2.92^{b}	25.96 ± 3.22^{a}	8.52 ± 0.09^{a}	28.87 ± 0.09^{ab}	14.94 ± 0.39^{a}	36.02 ± 0.95^{bc}	14.39 ± 0.10^{a}	36.54 ± 0.29^{bc}	15.04 ± 0.99^{a}	37.05 ± 2.19^{c}
Protein digestibility	%	39.28 ± 1.47^{a}		57.52 ± 4.05^{b}		77.91 ± 0.80^{c}		44.06 ± 3.08^{a}		55.50 ± 0.40^{b}	
Water content	%	8.76 ± 0.71^{a}	9.61 ± 0.84^{a}	70.51 ± 0.30^{c}	237.31 ± 1.02^{e}	62.117 ± 0.75^{b}	164.06 ± 5.29^{d}	60.6 ± 0.035^{b}	153.82 ± 0.23^{b}	59.42 ± 0.27^{b}	146.38 ± 1.68^{c}
Crude fiber	%	2.99 ± 0.01^{b}	3.33 ± 0.013^{a}	6.81 ± 1.71^{ab}	7.88 ± 0.06^{b}	1.39 ± 0.60^{a}	3.67 ± 1.57^{a}	2.18 ± 0.48^{ab}	5.55 ± 1.22^{ab}	1.06 ± 0.04^{a}	3.16 ± 0.71^{a}
Dietary fiber	%	23.53 ± 1.007^{c}	26.17 ± 1.13^{a}	14.13 ± 2.79^{b}	47.87 ± 8.96^{b}	9.34 ± 2.09^{a}	27.16 ± 4.33^{a}	8.94 ± 0.24^{a}	22.67 ± 0.59^{a}	8.03 ± 1.38^{a}	22.12 ± 0.07^{a}
Energy from fat	kcal/100 g	244.60 ± 2.79^{c}		82.5 ± 0.09^{a}		127.74 ± 2.84^{b}		123.48 ± 2.35^{b}		135.02 ± 7.22^{b}	
Total energy	kcal/100 g	478.81 ± 0.41^{c}		156.11 ± 1.6^{a}		216.62 ± 3.43^{b}		221.36 ± 1.62		232.01 ± 3.30^{b}	

Explanation: data are presented as mean ± sd; in one row; the different superscript (^{a,b,c}) letters mean significantly different ($p < 0.05$); db – dry basis; wb – wet basis

This showed that BW treatment can penetrate and synthesize protein better than traditional fermentation and support extracellular enzyme for protein synthesis process (MANPREET et al. 2005). The protein content of BA treatment was also significantly increased and different compared to peanut cake. However, the increasing protein in BC treatment was significantly different compared to other treatments. The increasing of protein in BC treatment is in agreement with a research by GANDRA et al. (2018) that the addition of chitosan of 5 g/kg sample can significantly increase the protein content.

Fermentation process can decrease the ash content in the traditional fermentation and adaptive fermentation. However, the traditional fermentation had the highest ash content compared to adaptive fermentation. This was due to the addition of cassava cake in black oncom production using traditional fermentation that might be contributed to the total ash content. Decreasing ash content during the fermentation is due to dissolution of water-soluble minerals or the metabolic activity of microorganisms (NNAM 2001) or leaching process which affected the total of ash content (ADEGBEHINGBE et al. 2014, SIMWAKA et al. 2017). The highest ash content was found in the commercial *black oncom,* and the ash contents were significantly different between treatments ($p < 0.05$).

The results in this study is in agreement with STEINKRAUS (2002) that the fermentation process improves nutritional contents. The nutritional contents of *black oncom* production in this study was higher than that of (SLAMET et al. 2002) and similar to SOFYAN (2003) and GINTING et al. (2018), especially in the proximate components such as carbohydrate, water, ash, protein and fat contents. However, the nutritional contents showed a lower value compared to KUMBHARE (2017).

The adaptive fermentation can also increase the protein digestibility especially in BW treatment that achieved the highest protein digestibility ($p < 0.05$) compared to the commercial *black oncom* in the market and other treatments. Commercial *black oncom* as the traditional fermentation process can not effectively increase protein digestibility. Meanwhile, the protein digestibility in BA treatment was lower than BA treatment because there was no growth of *Rhizopus oligosporus* that modified protein to amino acids. The BC treatment had the highest protein, but the protein digestibility was lower than BW treatment. This was due to the addition of chitosan which can add the amine group that is difficult to digest.

This study is in agreement with YANG et al. (2016) that controlled peanut cake fermentation can increase the crude protein and in vitro protein digestibility in plant protein compared to the traditional fermentation

(ALKA et al. 2012, PRANOTO et al. 2013). The process of increasing protein occurs because crude protein can increase in 36 h of fermentation (SIMWAKA et al. 2017). There are also microorganisms activities that use carbohydrates for energy (ONYANGO et al. 2005) and the process of proteolysis during fermentation produces peptides and amino acids, thus producing more soluble proteins (PRANOTO et al. 2013, EL KHALIFA et al. 2005).

Fermentation can be carried out using a starter culture or a natural process. Natural or traditional fermentation is less effective and unpredictable. This study showed that naturally fermented *black oncom* had lower protein digestibility than BW treatment. A previous study has shown that the digestibility of sorghum flour protein fermented using *Lactobacillus plantarum* achieved 92%, whereas the digestibility of the natural fermentation only reached 47% (PRANOTO et al. 2013). The increase in digestibility occurs due to the process of breaking down the food matrix into free nutrients (NKHATA et al. 2018). In BA and BC treatments, the digestibility was not increased because of the soaking treatment factors, i.e., the concentration, and there was no growth of *Rhizopus oligosporus* to perform the fermentation process in order to increase the nutritional contents especially the total fat and protein contents, and the protein digestibility.

Fermentation process significantly increased the crude fiber in the commercial *black oncom*, BW and BA, but there was no increase of crude fiber in the BC treatment. The increase of crude fiber occurred because mold cannot use carbohydrate that is difficult to digest, so it increases the crude fiber content. In addition, dietary fiber was increased in the commercial *black oncom* and BW treatment, but the commercial *black oncom* had the highest dietary fiber than BW treatment. This is because in the commercial *black oncom* production there was an addition of cassava cake as a carbohydrate source for the fungi. This study is in agreement with ZHAO et al. (2017) that solid state fermentation of wheat bran using lactic acid bacteria increases total dietary fiber. Meanwhile, total dietary fiber was decreased in BA and BC treatments. The BA and BC treatments might contain different microorganisms. According to TENG et al. (2017), the use of *Saccharomyces cerevisiae* can increase total dietary fiber, but the use of *Bacillus amyloquefaciens* can decrease total dietary fiber. Based on the nutritional contents, BW treatment was the best adaptive fermentation that could be increased increase nutritional contents especially in content of fat, protein and protein digistibility, so it can be used as food ingredient for product development such as biscuits (SETIAWAN et al. 2020).

Aflatoxin Content

Food safety of *black oncom* can be determined based on the aflatoxin content because the peanut product had a risk of aflatoxin which is produced by *Aspergillus* sp. The adaptive fermentation significantly decreased aflatoxins contents ($p < 0.05$) such as aflatoxin B_1 and total aflatoxin compared to the peanut cake. The aflatoxin content in BC treatment was the lowest with aflatoxin B_1 and total aflatoxin were significantly different within treatments ($p < 0.05$). This decrease occurred because the production process of *black oncom* especially in the soaking and fermentation processes.

Table 3

Aflatoxin content of fermented peanut cakes

Parameter	Non-fermentation	Traditional fermentation	Adaptive fermentation			Standard		
	peanut cake	commercial *black oncom*	BW	BA	BC	ID*	EU**	codex***
Aflatoxin B_1 [µg/kg]	287.27 ± 254.28^b	55.05 ± 69.81^{ab}	3.80 ± 1.002^a	1.47 ± 0.28^a	0.07 ± 0.056^a	15	2	15
Total aflatoxin [µg/kg]	370.79 ± 332.97^b	73.37 ± 93.31^{ab}	6.13 ± 1.55^a	2.35 ± 0.40^a	0.15 ± 0.035^a	20	4	20

Explanation: data are presented as mean ± sd; in one row; the different superscript (^{a,b,c}) letters mean significantly different ($p < 0.05$); *BPOM 2018; **EU (European Commission 2010); ***Codex 2017

The aflatoxin content decreased in the production process of *black oncom* (Table 3). The production process of *black oncom* with adaptive fermentation treatments in this research showed a higher reduction in aflatoxin compared to commercial *black oncom* in the market. The higest reduction of aflatoxins were BC, BA and BW treatment, respectively. The traditional fermentation cannot decrease aflatoxin contents effectively compared to the adaptive fermentation. The decreasing process of aflatoxin can be influenced by the fermentation process including soaking treatments and fermentation by the fungus (*Rhizopus oligosporus*). The aflatoxin content of adaptive fermentation in this study is smaller than other studies (ROEDJITO et al. 1972, JANNAH 2005, GINTING et al. 2018) and is classified as low aflatoxin according to the regulatory standards. The aflatoxin upper limit in Indonesia and codex is 20 ppb for total aflatoxin and 15 ppb for aflatoxin B_1 in processed peanut and corn products (BPOM 2018, CODEX 2017), but the upper limit in EU was lower than other standards (European Commission 2010).

The aflatoxin content in BC treatment was the lowest because chitosan inhibited the growth of pathogenic microorganisms (DUTTA et al. 2009) such as *Aspergillus flavus*. According to (SIMPSON et al. 1997), the interaction between chitosan and pathogenic molds shows that chitosan can resist the mold growth by binding the hosting DNA and damaging its biological membrane. According to KUMAR (2000), chitosan can provide a negative charge on carboxylic group and a positive charge on NH group which will be interact each other which causes expansion of cell surface and changes the cell wall permeability. This will cause the loss of several cell constituents and chitosan will inhibit the metabolisms of pathogenic microorganism (KURNIASIH and KARTIKA 2009). According to HIDAYAH (2015), The level of peanut chemical composition was decreased after 30 days of storage and the addition of 1% chitosan.

BA treatment of *black oncom* production also contributed to the decrease of aflatoxin contents because a concentration of 10 % acetic acid has the highest inhibitory effect on *Aspergillus flavus* of 45.21 (HASSAN et al. 2015). Acetic acid is more effective than lactic acid and has the best inhibition of *Aspergillus flavus* growth (KURNIASIH and KARTIKA 2009, DALIÉ et al. 2010). The mechanism of aflatoxin inhibition by organic acids, such as acetic acid by lowering the media pH is known as the hydrophobic feature which allows free protonized diffusion through cell membranes, and then the cell allocates a part of its energy content to remove the newly formed proton. Therefore, this slows down the growth of the fungus (LEÓN PELÁEZ et al. 2012). In addition, the decrease of aflatoxin contents occurs because a degradation of aflatoxin that formed β-keto acid structure that was catalyzed by acid medium. Lactone ring structure experiences hydrolysis that changes aflatoxin to non toxic components (ALBORES et al. 2005).

Fermentation process, especially in the inoculation process also contributed to the decrease of aflatoxin contents. The degradation mechanism of aflatoxin by *Rhizopus oligosporus* is apparently to occur enzymatically by reducing cyclopentanone bonds, degrading lactone rings, and opening difuran rings (SARDJONO et al. 2004, WU et al. 2009). JANSSEN et al. (1997) stated that the degradation of AFB_1 occurs resulting in aflatoxicol A formation which is 10 times lower in toxicity compared to AFB_1. CHEN et al. (2015) mentioned that there is a biotransformation of aflatoxin to non toxic compounds in the fermented peanut cake. BW treatment also decreased aflatoxins level and the content have no significantly different with BC and BA treatment ($p > 0.05$). Based on this, the adaptive fermentation could be used as alternatife procedure to produce *black oncom* that have low aflatoxin.

Conclusions

The adaptive fermentation method of peanut cake using soaking treatments (water, acetic acid 5%, chitosan 5%) and fermentation process using the yeast starter of *Rhizopus oligosporus* can increase nutritional contents and decrease the aflatoxins content. The adaptive fermentation that produced the best *black oncom* was the BW treatment compared to the commercial *black oncom* in the market, BA, and BC treatments. This was revealed from the pH value, mold density, and the protein digestibility. For the BC treatment, mold growth was only performed at certain points; while for the BA treatment, there was no mold growth. Generally, natural fermentation and adaptive fermentation processes can decrease carbohydrate, energy, and ash contents and can increase water content, total fat, crude protein, protein digestibility. Meanwhile, the crude fiber and dietary fiber showed a different result between natural and adaptive fermentation that might be affected by substrate additions and miroorganisms in the fermentation process. Furthermore, the adaptive fermentation process can decrease the aflatoxin contents below the standard limit set by the Indonesian and Codex standard compared to the commercial *black oncom* in the market. Meanwhile, the aflatoxin content of BC treatment was below the standard limit set by EU. Finally, the adaptive processing BW treatment is potential to produce *black oncom* with high mold growth, fat, protein, protein digestibility and low aflatoxin content compared to the traditional fermentation. BA and BC treatments produced a low aflatoxin profile, but these treatments need a further research especially in optimization of the soaking solution concentration to achieve better nutritional contents.

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MOLECULAR METHODS OF ANIMAL SPECIES IDENTIFICATION

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Abstract

Species identification of animals carried out with molecular biology methods plays an increasingly important role in laboratory practice. Currently, such methods are used, i.e., in phylogenetics, environmental protection, forensic medicine and forensic veterinary medicine, plant and animal breeding. DNA analysis is of particular importance in the identification of protected and endangered animal species being subject to illegal trade. Illicit commerce concerns both live animals and variety of products and objects made of them. In case of such products and objects, identification based on the morphological characteristics is not possible. It is, therefore, necessary to use animal DNA analysis. The paper analyses and presents molecular methods of animal species identification currently in use beginning from qualitative PCR-based methods: random amplification of polymorphic DNA – RAPD-PCR, restriction fragment length polymorphism PCR – RFLP-PCR, single-stranded conformational polymorphism PCR – SSCP-PCR, though quantitative: real-time PCR, high resolution melting PCR – HRM-PCR and finishing DNA barcoding and metabarcoding methods.

Introduction

In the 17th century, Carl Linnaeus began the identification of species on the basis of its morphology. Currently, the number of eukaryotic species is estimated at about 10 million. Identification of organisms on the basis of morphological features is tedious, time-consuming and often sta-

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ge-dependent. This is the reason why only about 1.7 million of species have been described over the course of 300 years. The breakthrough came with the development of the polymerase chain reaction (PCR). Currently, molecular techniques are the most preferred methods in many fields, such as phylogenetics, nature protection, plant and animal breeding, and in – broadly understood – forensics.

Materials and Methods

The aim of this study is to present the methods of animal species identification currently in use. The literature review included data obtained by 2020 and presented in the PubMed and Web of Science databases. The authors employed the following keywords and their combinations concerning: species identification animals, DNA analysis, barcoding, veterinary medicine. The results presented in selected papers were used to describe the methodology of genetic testing for the purposes of individual identification of animals.

Results and Discussion

PCR-based Method

The primary method of the identification of animal species is the polymerase chain reaction. This procedure enables the amplification of any selected DNA sequence. The PCR reaction mix contains four components: a double-stranded DNA template, primers hybridizing with a template, deoxyribonucleotides (dATP, dCTP, dGTP, dTTP) and a thermostable DNA-dependent DNA polymerase. The thermal profile of the reaction consists of three stages: denaturation, primer annealing and synthesis of a new DNA fragment. During denaturation, the double-stranded DNA is split, which allows the primers to attach to complementary sequences on each strand of DNA when the temperature is lowered. Denaturation at a temperature of ca. 95°C takes from several to several dozen seconds. Lowering the temperature to 50–60°C allows for complementary matching and the attachment of primers to a single DNA strand. The temperature range is selected according to the primers used. After primers are attached, a new DNA fragment is synthesized with the help of DNA-dependent DNA polymerase. Each three-step cycle produces two daughter molecules from one parent DNA molecule.

Molecular tests identifying animal species use different variants of PCR reactions:

– random amplification of polymorphic DNA (RAPD-PCR),
– restriction fragment length polymorphism (RFLP-PCR),
– single-stranded conformational polymorphism (SSCP-PCR)
– real-time PCR
– high resolution melting PCR (HRM-PCR).

The technique of random amplification of polymorphic DNA was developed in 1990 (Williams et al. 1990, Welsh and McClelland 1990) and is considered to be one of the simplest methods of species identification.

The discussed method is a modification of the classic PCR and is based on using one primer with a randomly selected sequence (approx. 9–15 bp long). If the primer attaches in the correct orientation to opposite strands of DNA within several thousand base pairs of each other, the PCR reaction takes place (Bardakci 2001). The number and arrangement of these random primer-binding sites varies from species to species. After the separation of the amplification products by electrophoresis, a pattern of bands characteristic for individual species is obtained. The method does not require the knowledge of the DNA nucleotide sequence of the organism in question. This fact provides a great advantage of this method, as it enables the identification of organisms which have never been previously sequenced (Senthil Kumar and Gurusubramanian 2011). The method in question has some disadvantages, the most important of which are: the susceptibility to various changes in the conditions of the amplification reaction (e.g. changes in the concentration and origin of polymerase, fluctuations in the amount of the starting material) and low repeatability of the reaction (Al-Samari and Al-Kazaz 2015).

This method is most commonly used in microbiology (Jarocki et al. 2016, Lin et al. 2018, Mohkam et al. 2016, Tamang et al. 2009) and botany (Abdelsalam et al. 2020, Heubl 2010). In zoology, RAPD-PCR is successfully used to identify beetles of the *Oncocires* genus (Cordeiro et al. 2019), *Bemisia* whiteflies (Jiu et al. 2017), nematodes (Mattos et al. 2019) and others (Ramella et al. 2005, Marmiroli et al. 2007). It is also successfully used in detecting fraud in commerce (Sawicki 2009).

Restriction fragment length polymorphism method is based on amplifying a selected DNA fragment, followed by digesting the amplified fragment with restriction enzymes. Restriction enzymes recognize specific sequences (4–6 bp) and digest DNA specifically. The obtained DNA fragments are separated by electrophoresis. The analysis of the obtained fragments allows to assess their number and size, and to compare them with the patterns characteristic for the given species.

The advantages of the PCR-RFLP include: relatively cheapness, lack of technical overcomplication, suitability for routine analyses. Various regions of DNA are used to identify species, such as satellite – satDNA (GRENIER et al. 1997), mitochondrial – mtDNA (BOWLES et al. 1992, BOWLES et al. 1993) and ribosomal – rDNA (JACOBS et al. 1997, ZHU et al. 1999). Of these DNA regions, the rDNA sequences are often studied. These regions of DNA contain highly conserved sequences, which ensures high reaction sensitivity, while internal transcribed spacer regions – ITS1 and ITS2 rDNA show high interspecies variation and high intraspecific stability (WĘDRYCHOWICZ 2000).

The discussed method found application, e.g. in parasitology for the identification of nematode species. It has been used it to distinguish some species of *Ascaridoidea* – canine and feline parasites, including *Toxocara canis* and *Toxocara cati* (JACOBS et al. 1997). The amplified DNA fragments of the *T. canis* and *T. cati* parasites can be digested with the Rsa I enzyme, while it does not have the sequences recognized by Hinf I and is not cleaved by this enzyme. On the other hand, the DNA fragments of the closely-related nematodes, such as *Toxascaris leonina*, *Baylisascaris procyonis*, *Ascaris suum* and *Ascaris lumbricoides* are fragmented under the influence of the Hinf I enzyme. *Toxocara* spp. can be easily distinguished from other *Ascaridoidea* species that can occur in human tissues. Moreover, *T. canis* and *T. cati* can be distinguished from each other and unambiguously identified – due to the Rsa I enzyme, which cuts their amplified DNA fragments at various sites. Digestion of the *T. canis* amplicons allows 2 fragments (250 and 290 bp) and *T. cati* – 3 fragments (110, 160 and 280 bp) to be obtained. Reaction carried out with primers complementary to the ITS2 sequence of parasites does not amplify human, canine or feline DNA (*Ascaridoidea* larvae can be detected in biopsy material taken from humans and animals without the need to isolate the parasite, specific PCR can be performed directly on the tissue homogenate). RFLP-PCR is used to identify parasitic species such as *Taenia asiatica* (EOM et al. 2020), *Toxoplasma gondii* (IVOVIC et al. 2019), *Trypanosomatidae* (BENTO et al. 2018) and *Phlebotomus* (ZAHRAEI-RAMAZANI et al. 2017).

It has been shown that the PCR-RFLP is effective in the analysis of relatively closely-related species, as well as samples containing different species or subjected to various technological processes, including thermal sterilization (SAWICKI et al. 2009). For these reasons, the PCR-RFLP is often used in research to determine the species of fish and seafood. Over 70% of analyses related to the identification of fish species are performed with the discussed technique (RASMUSSEN et al. 2009, SAWICKI et al. 2009). The cytochrome B gene in mitochondrial DNA is the fragment most

frequently used in the identification of seafood and fish, e.g. sackcloth, cod, salmonids, flatfish (MARMIROLI et al. 2007, TELETCHEA et al. 2006). Although the PCR-RFLP technique is considered to be favorable and often used in species identification, it has also some drawbacks, e.g. the possibility of intra-species variability or the appropriate selection of restriction enzymes.

Single-stranded conformation polymorphism method enables detection of small DNA changes, such as: point mutations, small deletions and insertions, or micro-inversions. This technique is based on the phenomenon that each single-stranded DNA molecule adopts its own conformation during electrophoresis under non-denaturing conditions. This conformation depends, i.a. on its sequence. Replacement of even a single nucleotide in a few-hundred nucleotide strands effects in change of the spatial structure, which leads to differences in electrophoretic mobility of the particle (VORECHOVSKY 2005). The first stage of the SSCP analysis covers thermal denaturation of the double-stranded DNA in a buffer with denaturing agent (most often formamide, however urea or sodium hydroxide can also be used) and then rapid cooling to prevent renaturation of the separated fragments. In the second step of the discussed procedure, the obtained single-stranded molecules are separated electrophoretically in a non-denaturing polyacrylamide gel. The electrophoretic mobility of single-stranded DNA chain fragments depends on their size, charge and spatial structure. Under non-denaturing conditions, individual single-stranded DNA molecules assume a specific conformation determined by intramolecular interactions. The adopted structure depends on the length of the strand, the nucleotide sequence, as well as the location and number of regions within which the internal base pairing has occurred (GASSER et al. 2006).

The PCR-SSCP method is also suitable for testing heat-treated samples and for detection of mixed samples. Its conduct is more demanding than the RFLP-PCR. It can, however, detect differences in the DNA sequence regardless of their location in places recognized by restriction enzymes. PCR-SSCP has been used successfully to identify nematode species, such as *Toxocara vitulorum*, *Toxocara cati*, *Toxocara canis*, *Toxascaris leonina*, *Baylisascaris procyonis*, *Ascaris suum*, *Parascaris equorum* (ZHU and GASSER 1998), *Elaphostrongylinae – Protostrongylidae* (HUBY-CHILTON et al. 2006), *Parelaphostrongylus odocoilei* (BRYAN et al. 2010), protozoa (POWER et al. 2011), fish (REHBEIN et al. 1999, REHBEIN 2002, TISZA et al. 2016, SIVARAMAN et al. 2019), molluscs (ARANCETA-GARZA et al. 2011), mosquitoes (KOEKEMOER et al. 1999) and others (ZHU et al. 2001, GASSER 2013).

Contrary to the classic PCR reaction, which provides information about the presence or absence of the marked species in the tested material, the real-time PCR provides additional information on the quantita-

tive share of individual species. In order to detect sample, fluorochromes or fluorescently labeled probes are used. The level of emitted fluorescence is measured with a spectrofluorimeter coupled with a thermocycler specially adapted for this purpose (the stronger the fluorescence, the greater the number of DNA copies is). Probes may have a fluorescent reporter can be attached at one end of a probe and a fluorescence quenching molecule (TaqMan), a light-emitting molecular beacons (associated with the target DNA sequence), or a LightCycler (which uses two oligonucleotide probes – donor and receptor) at the other end (DORAK 2006). Simultaneous determination of the amount of DNA in a sample of several species is possible since the probes can be labeled with different stains. The combination of the RT-PCR with high resolution melting (HRM) curve analysis enhances the range of possibilities of this method. This combination allowed to identify, e.g., 15 species of *Calliphoridae* (*Diptera*) flies (MALEWSKI et al. 2010), 21 species of fish (BEHRENS-CHAPUIS et al. 2018), 13 species of farm animals (ISHIDA et al. 2018), or 9 species of beetles (MALEWSKI et al. 2019).

The RT-PCR allows short amplicons to be analysed. Therefore, this method can be and is widely used to identify animal species in processed products, e.g. in research on food adulteration, control of illegal trade of wild animals belonging to endangered species. In such trade, prohibited by the particular and international law (CITES 1973), the object of a crime is often a highly processed part of animal body. It is thus impossible to recognize the species on the basis of tradictional keys for species recognition. Illicit trade in live and dead animals is a crucial and emotive problem. According to a report by the World Wide Fund for Nature – WWF, the value of such trade is estimated at $ 19 billion annually. It is the third most-profitable black market in the world, after arms and drug trafficking (MCGARTH 2012).

For example, the RT-PCR method has been successfully applied to detect and quantify material from the elephants (*Loxodonta africana* and *Elephas maximus*) and the woolly mammoth (*Mammuthus primigenius*) in highly processed samples. It provides reproducible results in samples containing only 100 copies of template DNA (WOZNEY and WILSON 2012). There is also a multiplex set of probes and primers developed, which detects nine of the twelve species of sharks listed by the CITES (CARDEÑOSA et al. 2018). It can be also used to determine the species of animals in processed meat products, as even small pieces of DNA left over from heat treatment can be amplified and identified. RT-PCR has been successfully used for species verification and DNA quantification for many species of fish and canned meat products, such as Atlantic cod (*Gadus morhua*) in a mixed sample (HERRERO et al. 2010), hake (*Merluccius merluccius*)

(SÁNCHEZ et al. 2019), or multiplex identification of Atlantic cod, salmon (*Salmo salar*) and flounder (*Pleuronectes platessa*) (HIRD et al. 2012). In addition, RT-PCR is successfully used to quantify DNA from cattle, pigs, horses, sheep (NATONEK-WIŚNIEWSKA and KRZYŚCIN 2015), chickens, ducks and geese (NATONEK-WIŚNIEWSKA and KRZYŚCIN 2016).

DNA marker technology represents a promising means for determining the genetic identity and kinship of an animal. Compared with other types of DNA markers, single nucleotide polymorphisms (SNPs) are attractive because they are abundant, genetically stable, and amenable to high--throughput automated analysis. In cattle, a set of 32 SNPs has sufficient power to identify breeds and crossbred populations (Heaton et al. 2002). SNP arrays were succesfully used in salmonid species identification (Wenne et al. 2016), mussels (Peñarrubia et al. 2019), mule deer, white-tailed deer (Russell et al. 2019) and others (Yang et al. 2013).

DNA Barcoding

PCR-based methods can determine if a sample belongs to a certain species, but cannot determine what the species is. Assignation of a sample to a species is possible by DNA sequencing methods. The most powerful of them is the DNA barcoding.

Paul Herbert from the University of Guelph, Ontario, Canada, inspired by barcodes placed on products in the store, gave rise to the idea of DNA barcoding. This method is based on analysis of a region of DNA, which is present in all organisms and differs sufficiently to distinguish species. The received and processed data are placed in the reference database, which then enables unique identification of an organism and its assignment to certain species.

A genetic marker suitable for DNA barcoding needs to meet a several criteria. Firstly, it needs to be sufficiently variable between species, but sufficiently conserved within than between species. Secondly, priming sites need to be sufficiently conserved to permit a reliable amplification. Thirdly, its amplification and sequencing should be as robust as possible (HEBERT et al. 2003, VENCES et al. 2005).

Different genes can be used to identify different groups of organisms via DNA barcoding. The most commonly used barcode region for animals and some protists is a portion of the cytochrome C oxidase I (COI or COX1) gene. COI have two important advantages: the universal primers for this gene are very robust, enabling recovery of its 5‘ end from representatives of most, if not all, animal phyla (FOLMER et al. 1994; ZHANG and HEWITT 1997); COI appears to possess a greater range of phylogenetic signal than any other mitochondrial gene.

Additionally, with thousands of copies per cell, mitochondrial COI is readily amplified by polymerase chain reaction, even from very small specimens. It was proposed that a 648 bp fragment of COI could permit species-level resolution among all animals. Initial tests of DNA barcoding using COI on animals reported near-100% accuracy (HEBERT et al. 2003, HEBERT et al. 2004a, BARRETT et al. 2005).

Differences in COI sequences between closely related species in birds were 19–24 times greater in magnitude than the differences within species. On the basis of these data it was found that the majority of avian species can be discriminated via DNA barcoding. A standard sequence threshold of ten times the mean intraspecific variation to flag genetically divergent taxa as provisional species can be proposed (HEBERT et al. 2004b). The actual threshold value varies depending upon group of organisms. The presence of this 'barcode gap' (MEYER et al. 2005) has been observed in many animal taxa (WARD et al. 2005, HAJIBABAEI et al. 2006, WAUGH 2007, BUCKLIN et al. 2011). DNA barcoding studies on animals provide an ideal source of data. Analysis of these data have established two important patterns:

– more than 95% of animal species examined possess a diagnostic COI sequence,

– and COI divergences rarely exceed 2% within species, while members of different species typically show higher divergence (RATNASINGHAM et al. 2013).

One of goals of the DNA barcoding is to build a publicly accessible reference database with species-specific DNA barcode sequences. This goal has been achieved by creation of the Barcode of Life DataSystems – BOLD (RATNASINGHAM et al. 2007). BOLD, established in 2005, is a web platform that provides an integrated 'environment' for assembling and usage of the DNA barcode data. The on-line database for the collection and management of specimens covers distributional and molecular data, as well as analytical tools to support their validation. Currently BOLD contains sequences for ~317,000 formally described species (~8.8 million specimens).

The possibilities and merits of the DNA barcoding can be well illustrated by entomofauna studies conducted in Germany. During two major DNA barcoding campaigns starting at 2009: the 'Barcoding Fauna Bavarica' and the 'German Barcode of Life', the DNA barcode library for 5,200 German flies and midges (*Insecta – Diptera*) has been assembled (MORINIÈRE et al. 2019). It should be noted that – despite this impressive achievement – the barcode coverage varies from only about 10% in marine invertebrates to 70% freshwater fish (WEIGAND et al. 2019).

DNA Metabarcoding

The DNA barcoding simplifies only the taxonomic aspect by establishing the list of species present in an ecosystem. It does not help to reduce the sampling effort. The development of next generation sequencing – NGS offers an opportunity to solve this problem.

Next generation sequencing technologies allow considerably greater numbers of nucleotides to be characterized, The DNA barcodes can be, therefore, generated for thousands of taxa in parallel (SHOKRALLA et al. 2015). DNA metabarcoding refers to an automated identification of multiple species from a single environmental sample containing degraded DNA (soil, water, faeces, etc.). It can be implemented for both modern and ancient environmental samples. By relaxing the need for an intensive sampling effort, the DNA metabarcoding opens the door to high--throughput biodiversity assessment for plants and animals (TABERLET et al. 2012).

It should be observed, that the COI region (used for the DNA barcoding) is too long to be applied for most commonly used NGS platforms, such as Illumina, or Ion Torrent. Instead of COI, mini-barcodes must be used (BRANDON-MONG et al. 2015). Nevertheless, research has shown that COI barcode of smaller size, such as 250 bp (MEUSNIER et al. 2008), and even 135 bp (HAJIBABAEI et al. 2006), can distinguish majority of animal species. Ability to identify species by mini-barcodes opens the opportunity to work with environmental samples in which DNA is usually degraded.

The metabarcoding approach provides a significant step-change in the analysis of foodwebs. For a diet analysis it is necessary to identify not only animals, but also plants, fungi and microorganisms. While the COI is an almost perfect marker for animals, it is not preferable for plants and fungi, and not applicable for bacteria. For fungi identification an internal transcribed spacer – ITS of the ribosomal DNA is recommended as a barcode (SCHOCH et al. 2012). For plants – it is the ribulose 1,5-bisphosphate carboxylase gene (*rbcL*), combined with a fragment of the maturase gene (*matK*) (CBOL PLANT WORKING GROUP 2009). For identification of microorganisms 16S ribosomal DNA is widely used (KLINDWORTH et al. 2013).

The metabarcoding can be used not only to identify species. It can also be applied to estimate relative abundances within a sample (HARRISON et al. 2020, LIN et al. 2019; LOU et al. 2018). Several aforementioned advantages provide the multifaceted application of the metabarcoding in biological research (COBLE et al. 2019, DREINER et al. 2017, DUARTE et al 2020, POPESCU et al. 2018, SACCÒ et al. 2019, WILLIS and GABALDÓN 2020).

Several short-read NGS platforms are currently available, each having unique pros and cons, allowing one to generate increasing quantities of data at decreasing costs (REUTER et al. 2015). Although the Illumina MiSeq instrument is capable of sequencing maximum 2 x 300 bp, it is the most expensive platform for short-read sequencing and still insufficient to cover the entire 16S rRNA gene which is approximately 1500 bp long. More recently, a new generation of NGS technologies was revealed, capable of producing much longer reads. One of them has been offered by Pacific Biosciences since 2010 (EID et al. 2009, KORLACH et al. 2010) and successfully been used to generate long-read DNA barcode data (HEBERT et al. 2018, TEDERSOO et al. 2018). It was reported that the long-read DNA metabarcoding provides better taxonomic resolution than any single marker (HEEGER et al. 2018). Prohibitive expensiveness of PacBio instruments hampers broad introduction of this sequencing platform for species identification.

A highly promising alternative solution for long read sequencing has been offered by nanopore-based sequencing technologies (ONT – Oxford Nanopore Technologies). In this technology, single-strand of DNA passes through a protein nanopore, resulting in changes in the electric current that can be measured. The DNA polymer complex consists of double-stranded DNA and an enzyme that unwinds the double-strand and passes the single-stranded DNA through the nanopore. As the DNA bases pass through the pore, there is a detectable disruption in the electric current, and this allows the identification of the bases on the DNA strand (LU et al. 2016). ONT sequencer is of a small size, lightweight, inexpensive and allows for sequencing of several gigabases of DNA on a single flow cell.

Quality of nanopore sequencing significantly increased over the last few years. Together with the development of appropriate software, consecutive sequence error rates decreased to 0.1% (ZHANG et al. 2020). Moreover, nanopore sequencing has been recently used for metagenomics in clinical, environmental and agricultural settings (LATORRE-PÉREZ et al. 2020, PETERSEN et al. 2019, POMERANTZ et al. 2018, STEWART et al. 2019), as well as in monitoring of the current state of genetic biodiversity in higher Eukaryotes (KREHENWINKEL et al. 2019).

Existing molecular methods offers many possibilities of animal species identification. Simple PCR-based methods (RAPD-PCR, RFLP-PCR) can easily be implemented in testing laboratories. More demanding - real-time PCR, high resolution melting PCR, DNA barcoding are today widely used in everyday scientific work. Appearance of Next Generation Sequencing platforms revolutionised animal identification. It has become possible not only to identify individual specimens but also to determine what species are present in soil, water or other environmental samples. Pendrive size

NGS sequencer of Oxford Nanopore Technologies democratized metabarcoding and made it possible to perform in every laboratory as well as in non-laboratory environment in field studies.

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EFFECT OF DIETARY SUPPLEMENTATION OF *MORINGA OLEIFERA* LEAF MEAL ON BLOOD PROFILE, HAEMAGGLUTINATION POTENTIAL AND TESTICULAR ACTIVITIES OF RABBITS

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Abstract

Forty-eight unsexed weaner rabbits of mixed breeds, with initial live weight of 710–780 g were randomly allocated into four experimental dietary treatments (0%, 15%, 30% and 45% *Moringa oleifera* leaf meal (MOLM)) and replicated three times with four rabbits per replicate for ten weeks. Data were collected on blood profile (haematology: packed cell volume – PCV, red blood cell counts – RBC, white blood cell counts – WBC, haemoglobin – Hb, lymphocytes, mean corpuscular and haemoglobin concentration – MCHC; serum biochemical indices: total serum protein, albumin, globulin, glucose, cholesterol, alkaline phosphate – ALP and aspartate transferase – AST) and testicular activities while haemagglutination assay was also carried out. All data generated were subjected to one-way analysis of variance in a completely randomized design. White blood cell concentration, alkaline phosphatase, aspartate aminotransferase and glucose were significantly ($p < 0.05$) influenced by MOLM. Highest titre was obtained at 45% MOLM while spermatogenic activities were supported by Moringa supplementation at 15% and 30% inclusion level. The study concluded that supplementation of MOLM in the diets of weanar rabbits up to 45% boost the immune systems of the rabbits as shown by increased in haemagglutination titre and normal range of blood values but decreased testicular activities.

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Introduction

Livestock producers are facing much difficulty with availability and higher prices of feed ingredients (KHATUN et al. 2003). Feeding ingredients are getting expensive and scarce due to high competition between human and animals resulting in the escalating cost of these ingredients. The cost of feed constitutes the major proportion of between 60–75% of the total cost of livestock production and protein cost account for over 15% of the total feed cost in livestock farming (BHUIYAN 1998, AWONIYI et al. 2004, OJEWOLA et al. 2005). The price of conventional protein feeds resources such as groundnut cake, fish meal and soybean meal, is on the high side and cannot permit profit maximization in livestock ventures. In view of this, current research interest in the livestock industry is aimed at finding alternatives to this elusive feed ingredient. One of such unconventional animal feed ingredient worth considering is moringa leaves. *Moringa oleifera* leaf meal (MOLM) is promising as a food source in the tropics because the tree is covered with green leaves during the dry season when other foods are scarce (MELESSE et al. 2009). However, ODURO et. al. (2008) gave details of the composition of this plant to be very rich in crude protein among others. There is no doubt that it can be used as rabbit feed.

The production of rabbits imposes no competition directly with man for most conventional cereals and legumes. It has ability to utilize diverse forages and is also favoured because of its high fecundity, short generation interval, as well as low cost of investment (TAIWO et al. 2004). However, it is imperative to give consideration to the health status of the animals subjected to this feeding trial; this is because the overall deficiency of a feed can be determined by examining the haematological component of the blood and this would provide a way to prevent nutrient deficient feed to farm animals. ISAAC et al. (2013) stated that haematological components are valuable in monitoring feed toxicity, especially with feed constituents that affect the blood as well as the health status of the animal. Serum protein had been used as an indirect measure of protein utilization. TERZUNGWE et al. (2013) reported that MOLM can be included in weaner rabbits' diets at up to 15% dietary level without adverse effect on the haematological and serum biochemical indices of rabbits EWUOLA et al. (2012) reported that the chemical composition of MOLM contains 27.53% crude protein – CP, 9.93% ether extract – EE, 14.05% crude fibre – CF, 7.98% ash and 40.51% nitrogen free extract – NFE. It was also documented to have multiple antioxidants with high levels such as phenolic acids (ellagic, chlorogennic, gallic and ferulic acid), glucosinolate and flavonoids such as kaempferol, quercetin and rutin (MBIKAY 2012). Hence, this study was carried

out with the aim of evaluating the effect of MOLM at higher inclusion levels on the haematological, biochemical indices, haemagglutination potential of erythrocytes and testicular activities of weaner rabbits.

Materials and Methods

The experiment was carried out at the Directorate of University Farms, Federal University of Agriculture, Abeokuta. It is located in 76 m above sea level and falls within Latitude 7ºN and Longitudes 3ºE. The climate is humid and located in the forest zone of South Western Nigeria. The mean precipitation and the temperature are 1,112.7 mm and 23.5ºC respectively. Relative humidity averaged 81.5% throughout the year. Seasonal distribution of rainfall is approximately 110.9 mm (9.97%) in the late dry season (January – March), 462.1 mm (41.53%) in the early wet season (April – June), 376.6 mm (33.85) in the late wet season (July – September) and 163.1 mm (14.66%) in the early dry season (October – December) (ORBDA 2011).

The study protocol was approved and conducted in line with the Animal Ethics Committee guidelines of Federal University of Agriculture, Abeokuta, Nigeria (FUNAAB 2013). The experiment was carried out using 48 weaner rabbits, and these were divided into 4 dietary treatments having 12 rabbits per treatment. Each treatment was replicated three times containing 4 rabbits per replicate. The experiment was carried out for a period of 10 weeks. Weaner rabbits, weighing between 710–780 grams were procured from a reliable farm in Abeokuta metropolis. The rabbits were fed with experimental diet after a week of adaptation supplemented with forages. Fresh water was provided for them in the morning and late in the evening. All other routine management such as disinfection of the hutches and stable, cleaning of feeders and drinkers, etc. were observed.

Moringa oleifera leaves were harvested by hand-plucking the leaves from the trees within Abeokuta metropolis. The leaves were air-dried and milled to form leaf meal while other feed ingredients were sourced from a reputable feed mill. After this, experimental diets were formulated such that MOLM were included at 0% (control), 15%, 30% and 45% levels as a replacement of soybean for diets T_1, T_2, T_3 and T_4 respectively as shown on Table 1.

Table 1

Percentage composition of experimental diets

Specification	T_1	T_2	T_3	T_4
Level of inclusion of MOLM [%]				
Ingredients	0	15	30	45
Maize	39.00	39.00	39.00	39.00
Rice bran	20.00	20.00	20.00	20.00
MOLM	0.00	1.50	3.00	4.50
Soya bean meal	10.0	8.50	7.00	5.50
GNC	10.0	10.00	10.00	10.00
PKC	10.0	10.00	10.00	10.00
Wheat offal	4.80	4.80	4.80	4.80
Bone meal	3.00	3.00	3.00	3.00
Oyster shell	2.00	2.00	2.00	2.00
Salt	0.30	0.30	0.30	0.30
Vit. prix	0.30	0.30	0.30	0.30
Lysine	0.30	0.30	0.30	0.30
Methionine	0.30	0.30	0.30	0.30
Total	100.00	100.00	100.00	100.00
Calculated analysis				
ME [MJ/kg]	2507	2533	2559	2585
Crude protein [%]	17.60	17.35	17.10	16.86
Fibre [%]	12.19	12.36	12.54	12.72

At the 10th week of the experiment, 2.5 ml of blood sample was collected from 3 rabbits per treatment into ethylene diamine tetra acetic acid (EDTA) bottle for haematological parameters which include PCV, RBC, WBC, Hb and were determined as described by JAIN (1986). The procedure of CAMPBELL (2012) was used in calculating the mean corpuscular volume, mean corpuscular Hb and mean corpuscular Hb concentration. For the determination of biochemical constituents of the blood, plain bottle was used for blood sample collection. Total serum protein was determined using the Biuret method as described by KOHN and ALLEN (1995). Albumin was determined using Bromocresol Green (BCG) method while globulin was estimated by subtracting albumin values from total protein. Serum cholesterol was determined spectrophotometrically using commercial Bio--La-Tests, and serum glucose was estimated using a commercial glucose colorimetric assay kit.

Haemolymph was collected from the mantle (oxygenated) cavity region of the snail (*Archachatina marginata*), and temporarily stored in universal bottle before the commencement of haemagglutination assay for agglutinin evaluation.

Three millilitres of blood was collected aseptically from 3 rabbits per treatment into EDTA bottles. Blood was centrifuged at 900 g for 5 minutes to harvest erythrocytes. Erythrocytes were washed three times in phosphate buffer saline (PBS), diluted to 2%v/v and stored at 4°C.

Serial dilution of haemolymph was prepared using 0.85% Phosphate Buffer Saline (PBS – pH 7.2). Diluted haemolymph was aliquoted into wells of microtitre plates at 100 µl per well. Equal volumes of rabbits' red-blood cells suspension were then added. The plates were covered, mixed gently and incubated at 30, 60, 90, 120, 150 and 180 minutes at room temperature to determine the reaction time, after which titre values were recorded. Each test consisted of eight replicates. Red-blood cell in PBS served as the control.

At the end of ten weeks, testes were harvested from three bucks that were randomly selected per treatment for morphometric analysis and histology. The length and weight of each testis were measured with vernier caliper and electronic scale, respectively. The testes were then fixed in 10% formalin, dehydrated in series of alcohol (70%, 90%, 100%), cleared in xylene, embedded in paraffin wax after which the tissue were sectioned (5 µm) and stained with haematoxylin and eosin (HE).

Data generated were subjected to least square analysis of variance in a completely randomized design using the statistical package (SAS 2007) while the significant means among treatments were separated using Duncan Multiple Range Test of the statistical package.

$$Y_{ij} = \mu + M_i + \sum_{ij}$$

where:

Y_{ij} – dependent variables

μ – population mean

M_i – Effect of i^{th} concentrate with MOLM supplementation at different levels (j = 0%, 15%, 30%, 45%)

$\sum_{ij}$ – residual error.

Results

The effect of MOLM on the haematological parameters of weaner rabbits is presented in Table 2. No significant effect ($p > 0.05$) was found in all the parameters measured except WBC which was significantly ($p < 0.05$) influenced by the experimental diets. The WBC values at 0% and 30% inclusion levels of MOLM were statistically the same while 15% MOLM recorded the least value.

Table 2

Haematological parameters of weaner rabbits fed *Moringa oleifera* leaf meal diets

Parameter	0%	15%	30%	45%	SEM
Pack Cell Volume [%]	40.33	36.00	40.00	31.33	3.09
Haemoglobin [g/dl]	13.43	12.00	13.33	10.43	1.03
Red Blood Cell ($\cdot 10^{12}$/L]	3.63	3.16	3.60	2.82	0.26
MCH [pg]	37.03	37.16	37.01	36.99	0.10
MCHC [g/dl]	33.30	33.32	33.34	33.30	0.41
MCV [Fl]	11.12	11.35	11.10	11.11	0.09
White Blood Cell [$\cdot 10^3$/l]	7.33^a	4.27^b	6.53^a	5.40^{ab}	0.65
Neutrophil [%]	41.33	42.00	38.67	43.00	5.19
Lymphocyte [%]	55.00	54.33	58.00	53.67	5.47
Eosinophil [%]	3.33	3.00	3.00	2.67	0.24
Monocytes [%]	0.67	0.67	1.00	0.67	0.29
Basophil [%]	0.00	0.00	0.00	0.00	0.00

Explanation: ^{a,b} means with different superscripts along the same row are significantly ($p < 0.05$) different; MCH – corpuscular haemoglobin; MCHC – corpuscular haemoglobin concentration, MCV – corpuscular volume

The result obtained on the effect of MOLM on the serum component of rabbit is shown on Table 3 . Significant effect of diet was not observed on all the parameters studied except serum glucose, AST and ALP. The values of ALP were significantly ($p < 0.05$) increased in rabbits fed 0%, 30% and 45% dietary supplementation of MOLM but decreased in those fed diet containing 15% dietary supplementation of MOLM.

Table 3

Serum biochemical indices of Weaner Rabbits fed *Moringa oleifera* leaf meal diets

Level of MOLM	0%	15%	30%	45%	SEM
Parameter					
Total protein [g/dl]	6.20	6.40	6.50	6.63	0.45
Albumin [g/dl]	3.37	3.87	3.93	4.07	0.20
Globulin [g/dl]	2.83	2.53	2.67	2.67	0.35
Alakaline phosphate [U/L]	143.00^a	102.33^b	141.33^a	140.67^a	10.63
Alkaline aminotransefrase [U/L]	55.33	55.33	74.00	70.67	6.78
Aspartate aminotransferase [U/L]	41.67^b	23.00^c	43.00^b	66.67^a	4.54
Cholesterol [Mg/dl]	77.87	84.17	82.03	84.33	7.07
Glucose [Mg/dl]	129.73^a	99.17^b	78.37^b	81.20^b	6.51

Explanation: $^{a, b, c;}$ means with different superscripts along the same row are significantly ($p < 0.05$) different

The haemagglutination potential of *Moringa oleifera* leaf meal on the erythrocytes of the experimental animals is shown on Table 4. The result shows that the best haemagglutination titre was obtained at 45% level of MOLM inclusion while the titre values were statistically similar at 0% and 15% MOLM levels of inclusion.

Table 4

Haemagglutination potential of *Moringa oleifera* leaf meal on erythrocytes of weaner rabbits

Treatment	Titre
T_1 (0%)	8.50 ± 0.290^{b}
T_2 (15%)	8.67 ± 0.290^{b}
T_3 (30%)	9.33 ± 0.290^{ab}
T_4 (45%)	9.83 ± 0.290^{a}

Table 5 shows the effect of timing on haemagglutination titre. At reaction time of 30, 60 and 90 minutes, haemagglutination titres were not significantly ($p > 0.05$) different for the four levels of inclusions of MOLM. However, there was significant ($p < 0.05$) variation observed at 120, 150 and 180 minutes with 45% inclusion level of MOLM recording the highest haemagglutination titre (9.00±0.204) at 180 minutes.

Table 5

Effect of timing on haemagglutination titre of erythrocytes of weaner rabbits fed diets containing graded levels of *Moringa oleifera* leaf meal

Reaction time (minute)	Treatment (titre)			
	T_1 (0%)	T_2 (15%)	T_3 (30%)	T_4 (45%)
30	10.00± 0.204	10.00±0.204	10.00±0.204	10.00±0.204
60	10.00±0.204	10.00±0.204	10.00±0.204	10.00 ±0.204
90	10.00±0.204	10.00±0.204	10.00±0.204	10.00±0.204
120	10.00 ± 0.204^{a}	9.00 ± 0.204^{b}	10.00 ± 0.204^{a}	10.00 ± 0.204^{a}
150	6.00 ± 0.204^{c}	9.00 ± 0.204^{b}	10.00 ± 0.204^{a}	10.00 ± 0.204^{a}
180	5.00 ± 0.204^{c}	4.00 ± 0.204^{d}	6.00 ± 0.204^{b}	9.00 ± 0.204^{a}

Explanation: $^{a, b, c, d}$ means along the same row with different superscript differs significantly ($p < 0.05$)

Table 6 shows the result obtained on the effect of MOLM on the testicular and epididymal weight of rabbit. Significant effect of diet was only observed in T_3 (30% MOLM) where the weight of the testis and epididymis was significantly reduced.

Table 6
Least-square means of testicular weight of weaner rabbits fed diets containing graded levels of *Moringa oleifera* leaf meal

Treatment	Testis + Epididymis [g] left	Testis + Epididymis [g] right
T_1 (0%)	2.385±0.87^a	2.310±0.523
T_2 (15%)	2.100±0.87^a	1.925±0.523
T_3 (30%)	0.890±0.87^b	1.495±0.523
T_4 (45%)	2.170±0.87^a	2.305±0.523

Explanation: $^{a, b}$ means on the same column with different superscript differs significantly ($p < 0.05$)

HE stained sections of testes tissue of rabbit bucks fed dietary supplementation of MOLM at 0%, 15%, 30% and 45% (T_1, T_2, T_3 and T_4, respectively) showing the histological appearance are shown in Figure 1.

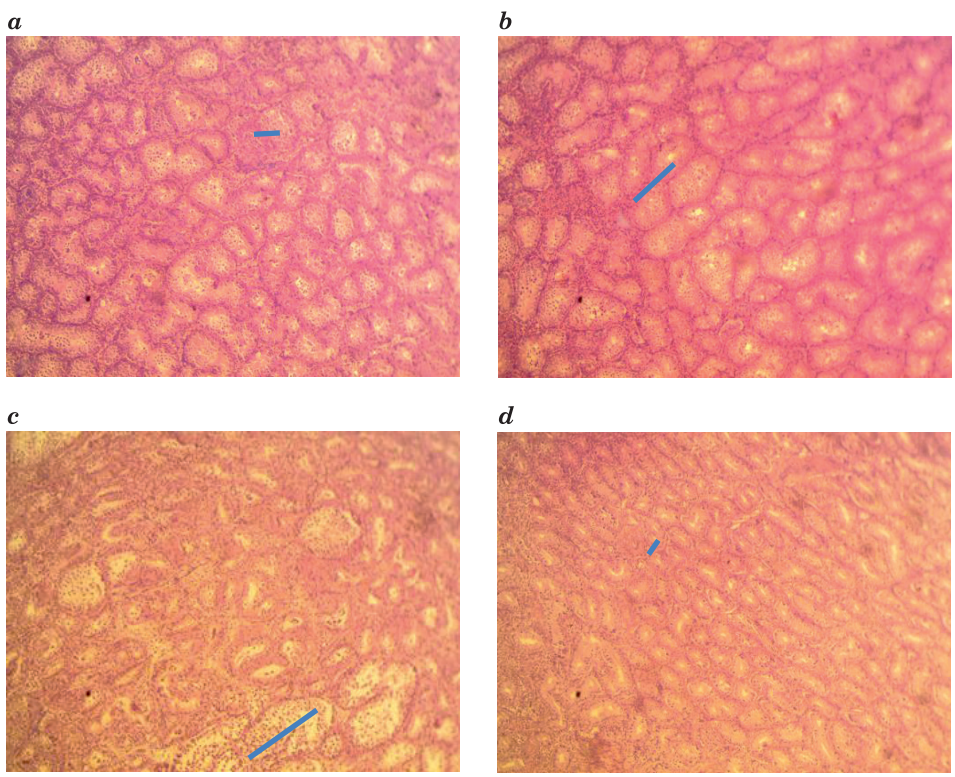

Fig. 1. Photomicrographs of testes tissue sections of rabbit bucks: a – T_1: control rabbit showing normal testes architecture; b – T_2: treated with 15% *Moringa oleifera* leaf meal; c – T_3: treated with 30% *Moringa oleifera* leaf meal; d – T_4: treated with 45% *Moringa oleifera* leaf meal. Magn. × 400

Discussion

Some of the haematological parameters measured in the present experiment were within the normal physiological ranges reported for rabbits most especially haemoglobin, MCH, MCV, MCHC, neutrophils, lymphocytes and eosinophils. BAKER and SILVERTON (1982) reported that a measure of the relative mass of blood is referred to as Packed cell volume (PCV). The observed PCV of rabbits which ranged from 31.33–40.33% in this study were within the normal range reported by MEDIRABBIT (2011) who considered the normal PCV of a healthy rabbit to be between 30–50%. These values suggested adequate nutritional status of the rabbits (CHURCH et al. 1984). This result is also in agreement with the findings of OKEKE et al. (2009) who observed no significant ($p > 0.05$) effect of feeding *Moringa oleifera* leaf meal in the diet on PCV of Rabbit. The values of RBC obtained in this study were in line with report of OKEKE et al. (2009), who found no significant influence of diet on RBC of rabbit fed *Moringa oleifera* leaf meal and reported lower values (2.82–3.63 · 106 mm^3). The values of haemoglobin, MCV and MCHC obtained suggest that the animals responded positively to the test diet. The lower ($p < 0.05$) value of WBC observed in 15% MOLM (4.27 · 10^3/l) shows that these animals were predisposed to high risk of disease infection; however, the immunity levels of the rabbits were not challenged as the values still fell within the recommended normal range reported by MITRUKA and RAWNSLEY (1977). This is however contrary to the report of TERZUNGWE et al. (2013) who reported no significant mean value in WBC of rabbits fed 15% moringa leaf meal diet.

The total protein values obtained fall within the normal range (6.00–8.30 g/dl) recommended by MITRUKA and RAWNSLEY (1977). Since total protein, albumin and globulin are generally influenced by the quality and quantity of protein intake (ONIFADE and TEWE 1993), the values obtained in the study indicates nutritional adequacy of the dietary protein. Rabbits fed the control diet had higher ($p < 0.05$) mean serum glucose level than those on the test diets. The reduced serum glucose values in the test diets gave an indication that the control will supply more energy for the animals than the test diets. It is also promising that moringa inclusion into the diets may decrease excess glucose thus preventing the animal of being diabetic. The serum ALP value of the rabbits fed T_2 was significantly ($p < 0.05$) lower than the mean serum ALP of those fed the control diet (T_1), which was significantly ($p > 0.05$) similar to the serum ALP of those on T_3 and T_4, while the serum AST activities of the rabbits fed the T_1 and T_3 were significantly ($p < 0.05$) lower than those on T_4 but significantly higher than those on T_2. CHAMPE et al. (2007) stated that aminotransferases are normally intracel-

lular enzymes, with low levels found in the plasma representing the release of cellular contents during normal cell turnover. The serum ALT and AST levels, according to CHAMPE et al. (2007) are elevated in nearly all liver diseases and are particularly high in conditions that cause extensive cell necrosis, including severe viral hepatitis or toxic liver injury.

The result on haemagglutination potential of *Moringa oleifera* showed that the highest haemagglutination titre was at 45% MOLM inclusion, which indicated that this inclusion level boost the immune system best. This result corroborates the findings of ABIONA et al. (2012) which reported higher haemagglutination titre for layer birds raised on legumes compared to concentrate feeding. Haemagglutination titres were not significantly ($p > 0.05$) different for the four levels of inclusions of MOLM; however, the significant ($p < 0.05$) variation observed at 120, 150 and 180 minutes with 45% inclusion level of MOLM recording the highest haemagglutination titre (9.00± 0.204) at 180 minutes is an indication that inclusion at this level modulates the immune function most. This observation is in line with the work of NUHU (2010), where MOLM was used to achieve similar result at 25% level of inclusion in rabbit's feed. The reason for this observation may be as a result of over-reactivity at these levels. On the overall basis, immune boosting effect of *Moringa oleifera* may be as a result of the presence of lectins which are found mostly in legumes.

The significant reduction observed in the weight of the testis and epididymis at 30% inclusion level of MOLM is contrary to the works of ABIONA et al. (2012) and NUHU (2010) who reported insignificant effect of MOLM on the morphometry performances of the male reproductive organs in the diets fed to African giant snails and rabbits respectively.

Figure 1 shows the photomicrographs of testis tissue sections of rabbit bucks fed different levels of MOLM. Considering the testicular architecture, seminiferous epitheliums were seen to be intact in treatments 1, 2, and 3. Tubular diameter of the seminiferous tubule were also seen to consistently increase in treatments 2 and 3, but decreased in treatment 4. This observation is an indication that spermatogenic activities were supported by Moringa inclusion at 15% and 30% inclusion level since spermatozoa produced are flushed via the lumen. Decreased in seminiferous diameter is an indication of arrest of spermatogenesis as shown by 45% supplementation of MOLM. This further showed this amount is in excess and could affect the normal spermatogenesis. The supportive role of this plant may be attributed to the fact that it contains some substances that could stimulate testicular androgenesis that are responsible for testicular differentiation, integrity and steroidogenic functions (DAWSON et al. 1990, LUCK 1995, EL-MISSIRY 1999, GHOSH et al. 2002, KUJO 2004).

Conclusion

The supplementation of *Moringa oleifera* leaf meal in the diets of weaner rabbits up to 45% boost the immune systems of the rabbits as shown by increased in haemagglutination titre and normal range of blood values but decreased testicular activities.

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THE ROLE OF NEUTROPHIL ON SUBCLINICAL MASTITIS IN COWS

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Key words: cows, mastitis, neutrophils, antimicrobial potential, apoptosis.

Abstract

It has been reported that the neutrophils are actively involved in the local udder immune defense. The aim of this study was to investigate the role of milk neutrophils in subclinical mastitis in cows. The research carried out on 70 cows at the farms of the Khmelnitsky Region Ukraine. Cows were divided into experimental (n = 37) and control (n = 33) groups. The indicators of the cytochemical reactivity of oxygen-independent and oxygen-dependent factors of protection on milk phagocytes, cell migration activity index and correlation were evaluated. The results were expressed as mean ± SD and percentage. Changes in the composition of cells were observed: increase of neutrophils ($P < 0.01$), monocytes ($P < 0.01$), and lymphocytes ($P < 0.05$). The indicators like cationic proteins (CP), myeloperoxidase (MPO), reaction neutrophils with nitro-blue tetrazolium (NBT), activation of neutrophils index (ANI) and cytological index (CLI) differed significantly in subclinical mastitis in cows in relation to healthy control group. The neutrophils activation occurred by initiation of respiratory burst, which triggered apoptosis induced by antimicrobial activity. Alterations on the cells migration activity index were observed in subclinical mastitis in cows. Based on results we can suggest that determination of the parameters related to milk neutrophils and the behavior of cell migration could contribute more information to subclinical mastitis diagnosis.

Introduction

Cow's mastitis is the inflammatory pathology of udder dairy cattle, causing significant economic damage (HEIKKILÄ et al. 2018). An important role in this belongs to the body's immune system, which supports homeostasis and responds first to all changes in physiological constants (GARZONI and KELLEY 2009, GÜNTHER et al. 2016). Moreover pathogenesis

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of mastitis depends on numerous etiological factors (MUSHTAQ et al. 2018, RABE et al. 2020). Biological mechanisms of homeostasis. which carry out the control, regulation and adaptation of the organism to changes in environmental conditions (BROZ et al. 2016, YOUSSIF et al. 2019).

The pathogenesis of mastitis and the manifestation of immune responses depend on numerous etiological factors (DERVISHI et al. 2015). The leading etiological factor of mastitis is the microbial factor, which by its virulent and pathogenic properties determines the nature of manifestation and course of the pathological process (GUERRERO et al. 2015). Thus in the body there is a functional restructuring in the local immune protection of the udder (GUO et al. 2015, ZHELAVSKYI 2017). Immunocompetent cells (neutrophils, monocytes, lymphocytes and histiocytes) is involved primarily in the fight against pathogenic microorganisms (JORGENSEN et al. 2016, DERAKHSHANI et al. 2018). The occurrence of mastitis depends on the functional state of the mammary gland, innate resistance and the state of organism (PETZL et al. 2018).

Phagocytes have a variety of defense mechanisms (KRUGER et al. 2015). Particular attention should be paid to the investigation of extracellular factors (oxygen-dependent, oxygen-independent, NETosis) and the investigation of apoptosis cells (LIU et al. 2014, SHI et al. 2015, ZHELAVSKYI 2019).

The study of immunological mechanisms of cow mast development in the future will become the basis of new diagnostic tests and effective methods of treatment. Therefore, the study aim was to investigations the functional of neutrophil of milk in cow's which subclinical mastitis.

Materials and Methods

The research carried out on 70 cows of *Ukrainian black-and-white milk breeding* at the farms of the Khmelnitsky Region Ukraine. Part of the experiments were carried out in a specialized laboratory of mammalian reproductive immunology of the Faculty of Veterinary Medicine of the State Agrarian and Engendering University in Podilya.

Animals' Criteria

All cows were divided into experimental and control (healthy animals, $n = 33$) groups. Experimental group ($n = 37$) formed from cows with subclinical mastitis (SCM). Diagnosis of mastitis made on the basis of cytological diagnosis of milk (Lactoscan SCC, Milkotronic Ltd, Bulgaria). This investigation approved according to the Law of Ukraine "On the Protec-

tion of Animals from Cruel Treatment" (No. 3447-IV of February 21, 2006) and according to the requirements of the European Convention for the Protection of Pet Animals (ETS No. 125, Strasbourg, 13/11/1987). All experiments were carried out with the Ethical Permit at the State Agrarian and Engineering University in Podilya, Ukraine. All animal manipulations were performed in accordance with the European Convention for the Protection of Vertebrate Animals used for experimental and scientific purposes (Strasbourg, 18 March 1986).

Immunology Analysis

In the cytochemical investigation of phagocyte reactivity in milk, the state of Oxygen-dependent phagocytic protection factors. Determined: myeloperoxidase (MPO) activity and reaction neutrophils with nitro-blue tetrazolium (NBT). Oxygen-dependent cellular protection mechanisms were determined by the activity of cationic proteins (CP) (SNOW and LENARDO 2019). In the experiment, it was determined the of activation of neutrophils index, ANI), index cytological (ICL), lymphocyte migration activity index (LMAI), neutrophils migration activity index (NMAI), index of macrophage transformation (IMT) and lymphocyte/granulocyte ratio – LMR (ZHELAVSKYI 2019). Apoptosis of neutrophilic granulocytes was studied for characteristic changes in the nucleus (pyknosis, rexis, vacuolization, fragmentation), cytoplasm (vacuolization and toxic vacuolation), cytolysis (cell disintegration) and size reduction (TAYLOR et al. 2007).

Statistical Analysis

The values in this investigation are presented as mean ± SD. The data in this investigation are given as the mean ± SD. Data were analyzed by one-way analysis of variance (MANOVA). Differences were considered statistically significant at a *P*-value of less than 0.05. Biometric analysis and interpretation of the obtained results were performed using statistical software Stastistica v. 12.6 (StatSoft, Inc., USA, 2015).

Results and Discussion

In Table 1 shows the cytogramme of the secret mammary gland of cows which subclinical mastitis. Inflammation udder manifested by an increase in SCC ($P < 0.01$). Changes in the composition of individual cell populations were also notes: increased the population of neutrophils

($P < 0.01$), monocytes ($P < 0.01$) and lymphocytes ($P < 0.05$). This due to the increase in neutrophil migration activity ($P < 0.01$) and the activation of macrophage transformation processes (Figure 1). Such changes occurred as a result of a microbial attack. Neutrophils of the peripheral blood began to was active in the inflammation zone (Figure 2).

Table 1

Changes in the cytologic composition of the mammary gland of cow's at subclinical mastitis (mean ± SD)

Value	Milk somatic cells						
	Somatic cell counts ($\cdot 10^3$)	leucocytes [%]				histiocytes [%]	epithelial cells [%]
		neutrophils	lympho-cytes	monocytes	total		
Healthy animals (control, n = 33)	255.72±17.5	44.69±0.58	3.87±0.33	1.93±0.24	50.51±1.05	4.18±0.10	45.50±0.89
Subclinical mastitis (n = 37)	3819.23±76.36**	50.73±1.34**	2.33±0.43*	4.28±0.51**	57.26±1.22**	8.2±0.21**	34.51±1.38**

Explanation: n – number; * – $P < 0.05$; ** – $P < 0.01$

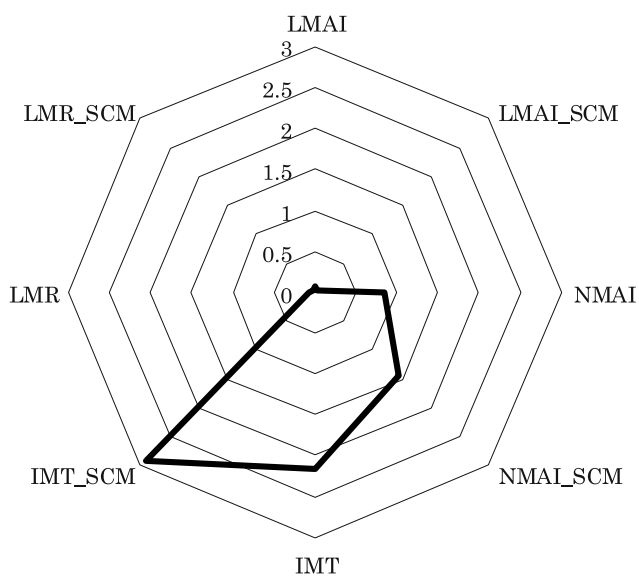

Fig. 1. Changes in cytological parameters in subclinical mastitis (SCM) of cows: LMAI – lymphocyte migration activity index; NMAI – neutrophils migration activity index; IMT – index of macrophage transformation; LMR – lymphocyte/granulocyte ratio

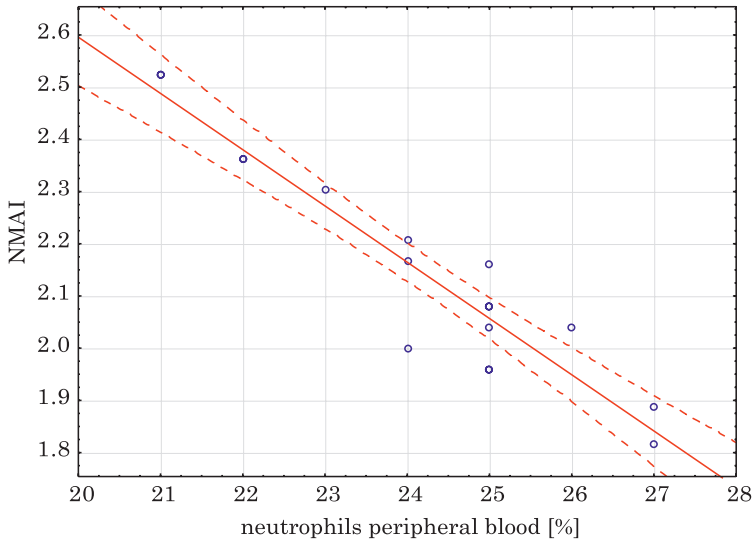

Fig. 2. The plot shows a correlation (r = 0.94) between NMAI – neutrophils migration activity index of cows which subclinical mastitis and the number neutrophils of peripheral blood

Antimicrobial reactivity oxygen-independent factors of protection of local immunity of a mammary gland are shows in Table 2. In the milk of cows at subclinical mastitis increases the activity of the cationic protein of neutrophils ($P < 0.001$).

Table 2

Changes in cytochemical reactivity oxygen-independent factors of protection of cow's breast phagocytes at subclinical mastitis (mean ± SD)

Value	Cytochemical reactivity CP		
	CP [%]	ANI	CLI (%)
Healthy animals (control, n = 33)	57.06±0.65	1.28±0.06	2.88±0.08
Subclinical mastitis (n = 37)	64.63±0.48***	1.04±0.04*	3.01±0.04**

Explanation: n – number; CP – cationic proteins; ANI – activation of neutrophils index; CLI – cytological index; * – $P < 0.05$; ** – $P < 0.01$; *** – $P < 0.001$ vs healthy animals

The pathogenesis of inflammation also noted the activation of oxygen--dependent mechanisms of phagocytes (Table 3). Antimicrobial mechanisms of phagocyte protection (MPO, $P < 0.05$, NBT, $P < 0.001$) were also activated in the milk of experimental animals. In the pathogenesis of SCM was clearly dominated by induction of respiratory burst (NBT – test).

Table 3

Indicators of cytochemical reactivity of oxygen-dependent factors of protection of cow's mammary gland phagocytes in subclinical mastitis (mean ± SD)

Value	MPO			NBT − test		
	MPO [%]	ANI	CLI [%]	NBT [%]	ANI	CLI [%]
Healthy animals (control, n = 33)	66.81±0.68	2.80±0.17	2.16±0.07	19.12±0.89	0.44±0.02	2.24±0.08
Subclinical mastitis (n = 37)	77.36±0.94**	1.52±0.72***	3.7±0.05***	74.18±0.69***	1.46±0.05***	3.4±0.06**

Explanation: n – number; MPO – myeloperoxidase; NBT − reaction neutrophils with nitro--blue tetrazolium; ANI − activation of neutrophils index; CLI − cytological index; * − $P < 0.05$; ** − $P < 0.01$; *** − $P < 0.001$ vs healthy animals

Triggering the antimicrobial activity of neutrophils can also affect cell apoptosis (TAYLOR et al. 2007). We also found a significant increase in the number of neutrophils with signs of apoptosis (apoptosis index 42.09±0.83% vs 35.39±0.57% in control). The number of neutrophils granulocytes cells with the vacuolization of the nucleus, zeiosis and the toxic vacuolization of the cytoplasm increased. Showed signs of cytolysis (plasmolysis) of cells.

The researchers are constantly faced with problems of interpretation of the obtained results (GEORGIEV 2008, DERVISHI et al. 2015). This which is related to the dynamic changes somatic cells, periods of lactation, daily fluctuations in their number, breed and individual characteristics of animals (BORTOLAMI et al. 2015, HEIKKILÄ et al. 2018). Thus, cytochemical studies will certainly give researchers a new search for the diagnosis of subclinical mastitis in cows (HEIKKILÄ et al. 2018).

The role of innate neutrophils in immunity and mastitis development is only the beginning to evaluate the physiological functions of apoptosis (RONGVAUX et al. 2014, MALTEZ and MIAO 2016, ZHELAVSKYI 2019). The investigation of the cascade of immune responses and the role of apoptosis is important in determining the physiological constants of immunity (GUERRERO et al. 2015, YOUSSIF et al. 2019). This fact clearly confirmed that inflammatory reaction occurring and against the background of activation of neutrophil migration (ZHELAVSKYI 2017). Obviously, neutrophilic granulocytes that migrated into the udder parenchyma isolated a number of cytokines, enzymes.

The presented investigation shows the process of attraction and phagocytosis of apoptotic neutrophil granulocytes by activated macrophages of mammary gland (LIU et al. 2016, PETZL et al. 2018). Changes occur

together with metamorphoses of the nucleus (pycnosis, rexis, vacuolization), cytoplasm (wrinkles), when the cells lose their specific granularity. Zeiosis of the membrane is one of the metamorphic signs of cell apoptosis. It is manifested by the projection (deformation) of the cell wall. Such projections of the membrane further form apoptotic bodies (vesicles), which are the remains of organoids surrounded by the membrane. Macrophages recognize and phagocytize apoptotic cells through special receptors that interact with external phosphadylserine (RONGVAUX et al. 2014, KRUGER et al. 2015). Thus, it can be concluded that under subclinical mastitis is active migration from the bloodstream to the area of pathological process of neutrophils, which actively destroy pathogenic microorganisms, while being exposed to inflammatory mediators, cytokines, microbial toxins and a number of other substances that reduce their cytochemical reactivity (BROZ et al. 2016).

Migration from the bloodstream of neutrophils leads to and activation of antimicrobial defense mechanisms has led to the release of inflammatory mediators. This including also active excretion into the extracellular space of the active forms of Oxygen (GUO et al. 2015). This made it possible to launch a program of self-destruction of neutrophils (apoptosis) in the parenchyma of the mammary gland (MUSHTAQ et al. 2018, ZHELAVSKYI 2019). Probably microphages also transmit certain signals to the cells of the peripheral bloodstream, so a certain proportion of neutrophils already undergo apoptosis without reaching the pathological process.

Conclusion

The results obtained study indicate that subclinical mastitis of cow's accompanied by intense antimicrobial reactivity of neutrophils. The neutrophils activation occurred by initiation of respiratory burst, which trigger apoptosis induced by antimicrobial activity. Based on results we can suggest that determination of the parameters related to milk neutrophils and the behavior of cell migration could be contribute with more information to subclinical mastitis diagnosis. Correction of the immune responses of local mammary gland immunity may be useful in the development of adequate methods of treatment of cows with mastitis.

Conflict of interest. The author declare that there is no conflict of interest.

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