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Analysis of the Efficiency of Precipitation on the Evolution of Agricultural Production in Upper-Casamance (South Senegal) between 1985 and 2018

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Abstract: In Senegal, rainfall is the main factor affecting agricultural production. In Upper-Casamance, it is a major equator for the development of agriculture, makes farmers vulnerable, and leads to changes in farming systems. The objective of this study is to analyze the evolution of agricultural production over the last 34 years in Upper-Casamance and the impact of rainfall variability on yields. To this end, data on cereal production (millet, sorghum, corn, rice and fonio) and industrial production (groundnut, cotton and cassava) in tons between 1985-1986 and 2018-2019 from the Regional Directorate of Rural Development (RDRD) of Kolda and precipitation data over the same period acquired at ANACIM was used. Production data are processed on the basis of statistical tests, including descriptive statistics, Pearson correlation, and simple linear regression. The calculation of rainfall data is based on the calculation of the annual average rainfall, the Standardized Precipitation Index (SPI), the number of rainy days per year, the average precipitation per day of rain, and the start and end date of the "agronomic" monsoon, and its duration. Analysis of the results showed an increase in agricultural production as rainfall conditions are good. The simple linear regression calculation for each product at the $p<0.05$ threshold indicated that rice, corn, groundnuts and cassava are the crops that depend significantly on the annual rainfall period. Analysis of the start and end dates showed that the length of the agronomic season (on average 112 days) appears to be favorable for the production of millet and groundnut.

Keywords: Agricultural production, Rainfall variability, Agronomic monsoon, Statistical analysis, Upper-Casamance

Introduction

Climate is the most important factor governing food production and causes inter-annual variability in socio-economic and environmental systems related to the availability of water resources (Djaman et al., 2017) and their use. In West Africa, agriculture is a major economic sector and is most vulnerable to climate change (Roudier et al., 2011). For the West African monsoon, which rhythms the agricultural calendar, is becoming shorter as we move north, and its abundance is becoming smaller (Descroix et al., 2015); those, despite a return to wet rainfall conditions noted since 1999 (Bodian, 2014) and wetter from 2008 (Nouaceur, 2020). In Senegal, particularly in Upper-Casamance, our area of interest, the monsoon represents a major equation for the development of agriculture. It makes farmers vulnerable, especially since the main part of agricultural production is during the rainy season, and leads to changes in farming systems.

Senegal and other UN member countries have committed themselves to the 2030 Sustainable Development Goals, of which food security and sustainable agriculture are the main priorities (FAO, 2011). Moreover, the agricultural sector contributes 17% to the gross domestic product (GDP) (MEFS, 2011), provides 15.3% of the country's exports, and employs more than 60% of the labor force in 2014 (Ndiaye, 2018).

In Upper-Casamance, the agricultural sector mobilizes about 80% of the assets over a period of 8 to 9 months of the year (ANSD, 2017). Its importance is mainly due to the fact that the region has long hosted migrants of various origins, who have come in search of agricultural land (Sidibé, 2005; Fanchette, 1999). The production is

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mainly based on industrial crops (groundnuts, cotton and cassava) and food crops (millet, sorghum, corn, rice and fonio). However, in addition to the variability of rainfall conditions, the sector faces land degradation, water erosion, and the seeding of rice fields (Mballo et al., 2019). In addition, there is a lack of control of water by farmers, crop cycles, varieties to be used, dates of the beginning and end of the rainy season and duration. These latter variables are, however, the most important at the agronomic and agro-climatic level (Balme et al., 2005); Descroix et al., 2015) for the development of the sector.

For a better understanding of the evolution of agricultural production and its constraints, this study attempts to: 1) analyze the evolution of cereal and industrial production over the last thirty-four years in Upper-Casamance; 2) and analyze the evolution of the average annual rainfall and its impact on yields.

Materials and Method

Study Area

Upper-Casamance is located in the south of Senegal between 12 ° 20 and 13 ° 40 latitude North, and between 13 ° and 16 ° longitude West. It is limited to the East by the region of Tambacounda, to the West by the region of Sedhiou (Middle Casamance), to the North by the Republic of Gambia and to the South by the Republics of Guinea and Guinea-Bissau (Figure. 1). It corresponds administratively to the Kolda region according to Law N 2008-14 of 18 March 2008 modifying the contours of the administrative division of the national territory. It covers an area of 13,721 km² or 7% of the national territory.

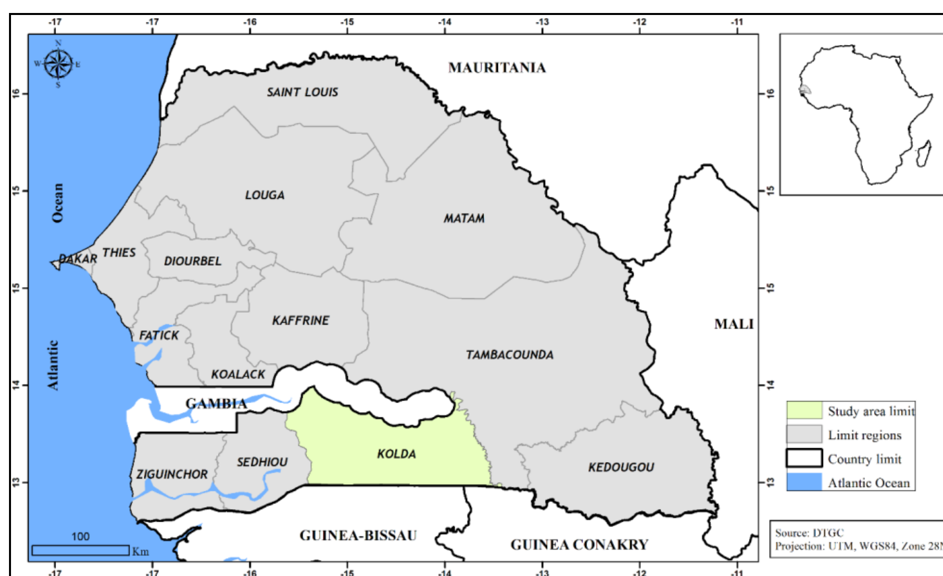


Figure 1. Localization of study area

From a climatic point of view, it belongs to the Sudanese domain. One part is in the northern zone and another in the southern zone (Sagna, 2005). Precipitation varies between 500 and 1500 mm. It has two distinct seasons. A dry season characterized by the presence of the maritime trade winds and a rainy season influenced by the arrival of the monsoon. The duration of the rainy season is six months (May to October).

Data Used

Two types of data are used in this study. First, we have data on cereal and industrial production in tons of Upper-Casamance between 1985-1986 and 2018-2019. These data come from the Regional Directorate for Rural Development (RDRD) of Kolda. Next, we have precipitation data from the stations of Kolda, Vélingara, Medina Yoro Foulah, Dabo, Kounkane, and Bonconto for the period 1985-2018. These data are provided by the National Civil and Military Aviation Agency (NCMAA). The role of temperature on agricultural production will not be studied in this study, as it is a major role (Sarr and Traoré, 2010; Sultan, 2012).

Methods

Statistical Processing of Agricultural Production Data

Data on agricultural production were processed on the basis of statistical processes using the SPSS software. Processing began by organizing the data and dividing the series into three periods: 1985-1998, 1999-2007 and 2008-2019. The first period corresponds to the great drought of the years 1970-1980 and is characterized by a general weakness of precipitation in Senegal. The second period corresponds to the return to wet rainfall conditions. The third period corresponds to the return to wetter conditions (Nouaceur, 2020). Thus, this subdivision of the series allows us to see the different trends in agricultural production depending on whether the period is dry (1985-1998), wet (1999-2007) or very wet (2008-2018).

The second step was to calculate the descriptive statistics. These include trend measures (mean, median and mode), dispersion measures (standard deviation, variance, range, and percentiles), minimum and maximum. Similarly, correlation and simple linear regression were calculated for each product. Correlation measures the linear relationship between two variables. It is essential to verify the linearity of the relationship. It can take values between -1 and 1. When it is close to -1, there is a strong negative relationship. When it's close to zero, there is no linear relationship. When it's close to 1, we're talking about a strong positive linear relationship.

Simple linear regression not only measures the linear relationship between two continuous variables, but also the effect of an independent variable on a dependent variable, depending on the number of samples and the nature of the variables studied. In this study, the dependent variable is products; while the independent variable is rainfall. For this, we used Adjusted R Square and ANOVA. The threshold of significance is 0.05 or a confidence threshold of 95%.

Rainfall Data Processing

Rainfall data processing is based on the calculation of: the evolution of annual average rainfall, the Standardized Precipitation Index (SPI) (McKee et al., 1993), the number of rainy days per year, and the average rainfall per rainy day.

The standardized precipitation index is used by several authors to characterize rainfall in many parts of West Africa (Bodian, 2014; Descroix et al., 2015). It measures weather droughts and quantifies precipitation deficits at multiple time scales that reflect impacts on the availability of different types of water resources. It is obtained on the basis of the difference between the precipitation during a year and the series average, on the standard deviation of the series, which is the formula:

$$SPI = (Pi - Pmoy)/\sigma$$

Where; Po= rain of the year i; Pmoy= average rain of the series; σp= Standard deviation of the series

The SPI values were interpreted according to the criteria given by WMO (2012). The average precipitation per rainy day was calculated by dividing the annual average precipitation by the number of rainy days in the year. Knowing that the number of rainy days of the year corresponds to the total sum of each rainy day. In addition to these calculations, we determined the start and end date of the "agronomic" monsoon, as well as the duration. This is based on the work of Balme et al., (2005), and Sivakumar (1988). In agronomic terms, the start of wintering corresponds to the first precipitation or a set of events producing more than 20 mm in less than three days and not followed by a dry phase of more than 7 days in the following month (Balme et al., 2005); whereas the end of wintering is the last event of more than 10 mm not preceded by a dry episode of more than 20 days (Sivakumar, 1988). The length of the rainy season corresponds to the difference in days between the end and beginning dates of the rains. All the treatments carried out were spatialized by the Thiessen polygon method.

Results and Discussions

Analysis of the Evolution of Cereal Production from 1985-1986 to 2018-2019 in Upper-Casamance

The evolution of cereal production over the various periods is shown in Figure 2. The result generally indicates a change in production as we move away from the dry period. Indeed, R^2 indicated a significant trend in

agricultural production during the wetter period (R^2=0.4205), and not significant during the intermediate period (R^2=-0.076). It is even less so during the dry period (R^2=-5,747). However, it should be recalled that, despite these encouraging productions in recent years, projections from climate models all foresee decreases in crop yields (in the range of 11 to 18%) in West Africa mainly due to warming (Roudier et al., 2011), increased sweating and decreased soil water content (Léauthaud et al., 2011).

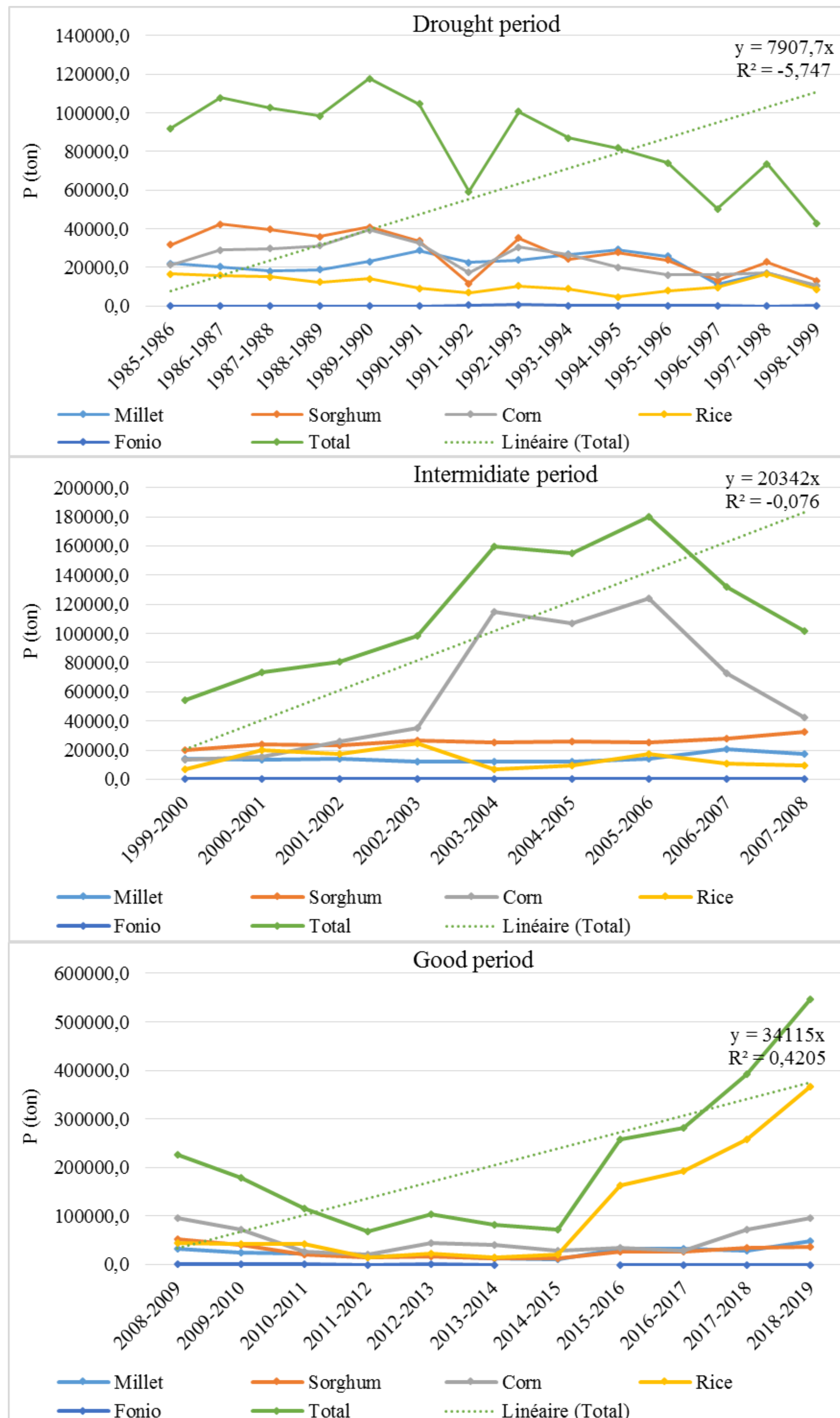


Figure 2. Evolution of cereal production (in tons) during the periods 1985-1998, 1999-2007 and 2008-2018 in Upper-Casamance

The results of the descriptive statistics for each product are shown in Table 1. They show that during these three periods, the minimum production of millet is 10,472.3 tons and the maximum production is 47,732 tons. The average production is 20 999 497 tons. For sorghum, corn and rice, the minimum production is 11,596.7 tons, 10,567.5 tons and 4,659.7 tons respectively; whereas the maximum is 51,568.8 tons, 123,816.5 tons and 366,081 tons. The average production is 27,134,071 tons, 42,631,826 tons, and 42,984,162 tons, respectively. The results also indicate that, over the last 34 years, fonio has been the lowest producing product; rice, the highest production, especially since the 2014-2015 crop year.

Table 1. Descriptive statistics of the different cereal crops

	N Statistic	Range Statistic	Minimum Statistic	Maximum Statistic	Mean Statistic	Std. Error
YEARLY-PERIOD	34	2	1	3	1.91	.148
Millet	34	37259.7	10472.3	47732.0	20999.497	1428.0642
Sorghum	34	39972.1	11596.7	51568.8	27134.071	1689.2600
Corn	34	113249.0	10567.5	123816.5	42631.826	5432.2510
Rice	34	361421.3	4659.7	366081.0	42984.162	13752.5336
Fonio	34	1766.9	.0	1766.9	214.576	53.6262
Valid N (listwise)	34					

	Std. Deviation Statistic	Variance Statistic
YEARLY-PERIOD	.866	.750
Millet	8326.9734	69338486.240
Sorghum	9849.9939	97022379.246
Corn	31675.1942	1003317929.236
Rice	80190.3620	6430494163.486
Fonio	312.6918	97776.193
Valid N (listwise)		

The Pearson correlation calculation yielded the following results: 0.213 for millet, -0.071 for sorghum, 0.388 for corn, 0.503 for rice, and 0.040 for fonio. These results indicate that there is a strong positive linear relationship between rice production and rainfall, which is the dependent variable. For corn, the result indicates a weak positive linear relationship. However, for sorghum, there is a strong negative linear relationship. For mil, the result indicates that there is a relationship, but it is not linear. For the fonio, there is no relationship, indicates the result.

The simple linear regression equation applied for each product at the $p<0.05$ threshold indicated that rice and corn are cereal crops that are significantly dependent on the annual rainfall period (Table 2 and 3).

Table 2. Result of the simple linear regression applied to rice

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate	Change Statistics R Square Change	F Change	df1
1	.503[a]	.253	.230	70383.8861	.253	10.836	1

ANOVA[a]

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	53681781940.624	1	53681781940.624	10.836	.002[b]
	Residual	158524525454.416	32	4953891420.451		
	Total	212206307395.040	33			

Coefficients[a]

Model		Unstandardized Coefficients B	Std. Error	Standardized Coefficients Beta	t	Sig.
1	(Constant)	-46077.206	29625.683		-1.555	.130
	YEARLY-PERIOD	46585.946	14151.898	.503	3.292	.002

For rice, a significant linear regression equation was found with b=0.503, (1,32)=-1.555, $p<0.05$. Adjusted R^2 gives us 23%. This means that rainwater quantities in Upper-Casamance explain almost the variance in rice production. For corn, the regression equation was found to be significant with b=0.38, (1,32)=1.24, $p<0.05$. Adjusted R^2 is 12.4%. The results also indicate that the more rainfall conditions improve, the more rice and corn production.

Table 3. Result of the simple linear regression applied to corn

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate	Change Statistics		
					R Square Change	F Change	df1
1	.388[a]	.151	.124	29642.4738	.151	5.681	1

ANOVA

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	4991851604.583	1	4991851604.583	5.681	.023[b]
	Residual	28117640060.204	32	878676251.881		
	Total	33109491664.786	33			

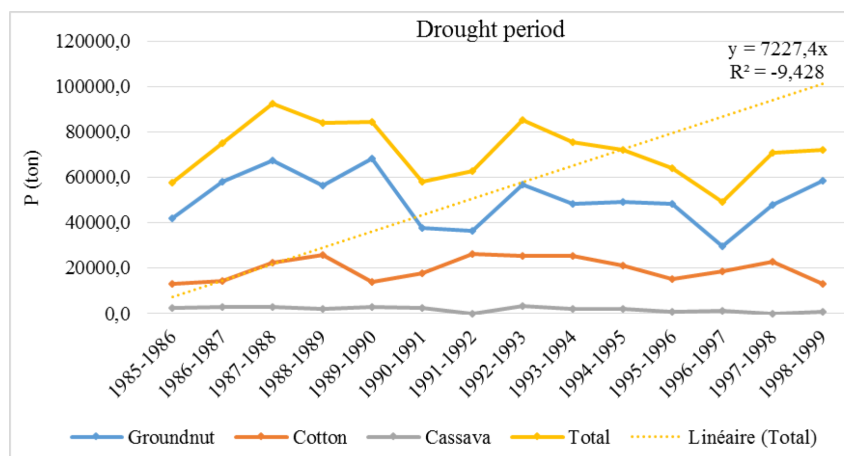
Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta		
1	(Constant)	15473.267	12476.983		1.240	.224
	YEARLY-PERIOD	14206.016	5960.132	.388	2.384	.023

For millet, sorghum, and fonio, the simple linear regression equation was found to be non-significant ($p>0.05$). P is 0.227, 0.691, and 0.821, respectively. Adjusted R^2 is 1.5%, -2.6%, and -3%, respectively.

Analysis of industrial production evolution from 1985-1986 to 2018-2019 in Upper-Casamance

The evolution of industrial production over the various periods is shown in Figure 3. Like cereal production, industrial production is also dependent on rainfall conditions. Through R^2, we can see a change in production as we move away from the dry period.



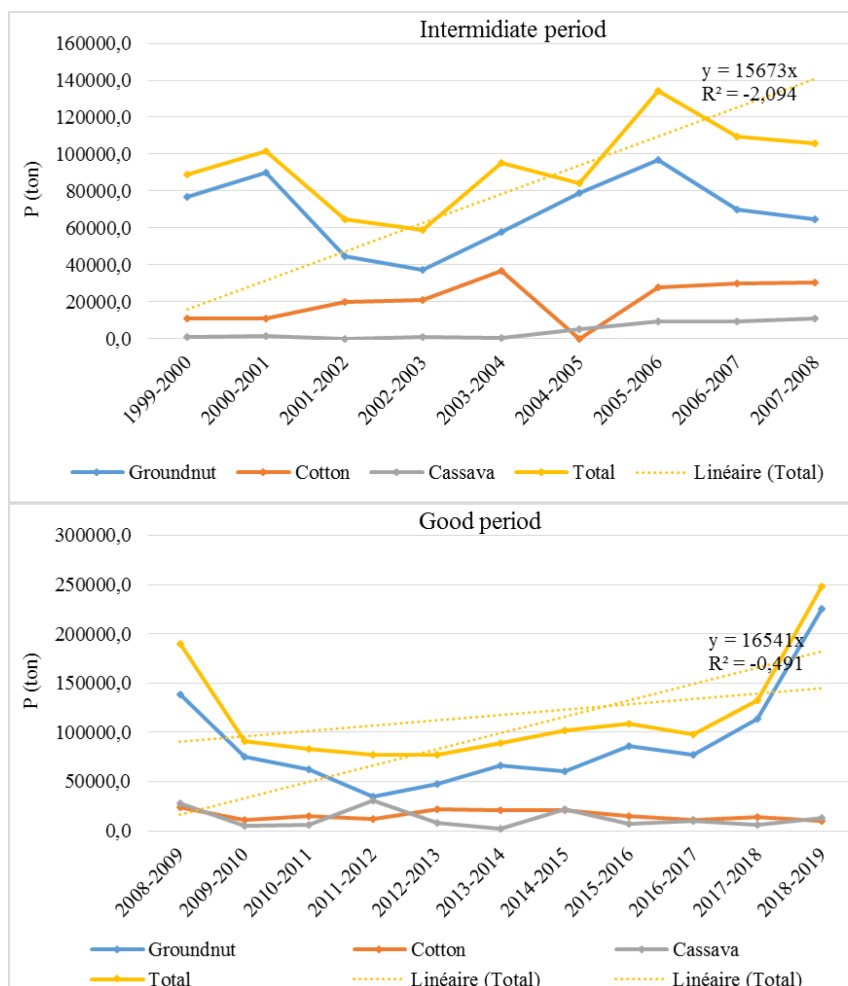


Figure 3. Evolution of industrial production (in tons) during the periods 1985-1998, 1999-2007 and 2008-2018 in Upper-Casamance

The results of the descriptive statistics indicate high production for groundnuts, followed by cotton and cassava. The maximum production is 225,654 tons, 36,855 tons and 30,538 tons respectively for the three speculations. The average is 67,840.93 tons for groundnuts, 18,661.07 tons for cotton, and 5,903.47 tons for cassava.

Table 4. Descriptive statistics of the different industrial crops

		YEARLY-PERIOD	Groundnut	Cotton	Cassava
N	Valid	34	34	34	34
	Missing	0	0	0	0
Mean		17.50	67840.93	18661.07	5903.47
Median		17.50	59472.81	19139.68	2671.44
Mode		1[a]	29561[a]	25407	0
Std. Deviation		9.958	36157.245	7465.983	7491.783
Variance		99.167	1307346354.144	55740905.591	56126808.143
Minimum		1	29561	0	0
Maximum		34	225654	36855	30538
Sum		595	2306592	634477	200718
	25	8.75	47687.95	13008.97	1159.38
Percentiles	50	17.50	59472.81	19139.68	2671.44
	75	26.25	76838.98	23820.51	8161.32

The simple linear regression equation applied to different products shows that groundnuts and cassava are the industrial crops that depend significantly on the annual rainfall period (Table 5 and 6). For groundnut, the regression equation was found to be significant with b=0.471, (1,32)=2.21, $p<0.05$. Adjusted R^2 is 19.7%. This

also shows that rainfall explains the variance of groundnut production in Upper-Casamance; although, during the dry period, it still met the water needs of groundnuts (Sene, 2007).

For cassava, the linear regression equation gave b=0.606, (1,32)=-1.620, with $p<0.05$. Adjusted R^2 is 34.8%. An increase in production is predicted, if trends continue.

Table 5. Result of simple linear regression applied to groundnuts

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate	Change Statistics R Square Change	F Change	df1
1	.471[a]	.222	.197	32392.860	.222	9.116	1

ANOVA[a]

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	9564913660.757	1	9564913660.757	9.116	.005[b]
	Residual	33577516026.002	32	1049297375.813		
	Total	43142429686.759	33			

Coefficients[a]

Model		Unstandardized Coefficients B	Std. Error	Standardized Coefficients Beta	t	Sig.
1	(Constant)	30247.112	13634.664		2.218	.034
	Y PERIOD	19664.458	6513.145	.471	3.019	.005

Table 6. Result of simple linear regression applied to cassava

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate	Change Statistics R Square Change	F Change	df1
1	.606[a]	.368	.348	6050.580	.368	18.593	1

ANOVA[a]

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	680680218.909	1	680680218.909	18.593	.000[b]
	Residual	1171504449.814	32	36609514.057		
	Total	1852184668.722	33			

Coefficients[a]

Model		Unstandardized Coefficients B	Std. Error	Standardized Coefficients Beta	t	Sig.
1	(Constant)	-4125.294	2546.784		-1.620	.115
	Y PERIOD	5245.816	1216.574	.606	4.312	.000

For cotton, on the other hand, the regression equation was found not significant with b=-0.214, (1,32)=7.116, $p>0.05$. Adjusted R^2 is 0.16%; which is very small. This means that cotton production does not depend on rainfall conditions. In fact, it depends on the policies of reimbursement to SODEFITEX (which finances its activities) and on the perception of the population on the product used for fertilization of the land (which is dangerous for their health and contributes to the degradation of their land).

Evolution of average annual rainfall and its impact on yields

The evolution of average annual rainfall between 1985 and 2018 indicates significant variation with values that are both above average (946.7 mm) and below (Figure 4a). This is especially the case for the period 1985-1999, during which we recorded both 7 rainy and 7 dry years; with two years (1990 and 1991) with moderately dry indices (Figure. 4b). The cumulative effects of the drought on the availability of water reserves of the various dams in the Anambe basin that could not fill up (Dacosta et al., 2002) would explain by extension the low production of rice during this period. Because, compared to other speculations and outside the fonio, it is the one that recorded the lowest productions during this period. The year 1999, which indicates the return to wet conditions in Senegal (Bodian, 2014), was more rainy after 2003 with an index considered to be very wet. Between these two dates, the quantities of precipitated water were worrying, as they were well below average.

The years 2001 and 2002 were the most deficit in the series studied. The period from 2003 to 2010 was the most rainy with indices ranging from near normal to moderately wet. Only 2006 had a negative index, however close to normal. During this period, all productions experienced almost an increase compared to the dry period. The period from 2011 to 2018, on the other hand, recorded overall precipitation below average. However, all positive and negative indices are close to normal. This would explain the results sometimes good, sometimes bad of production; but not to the values of the previous two periods.

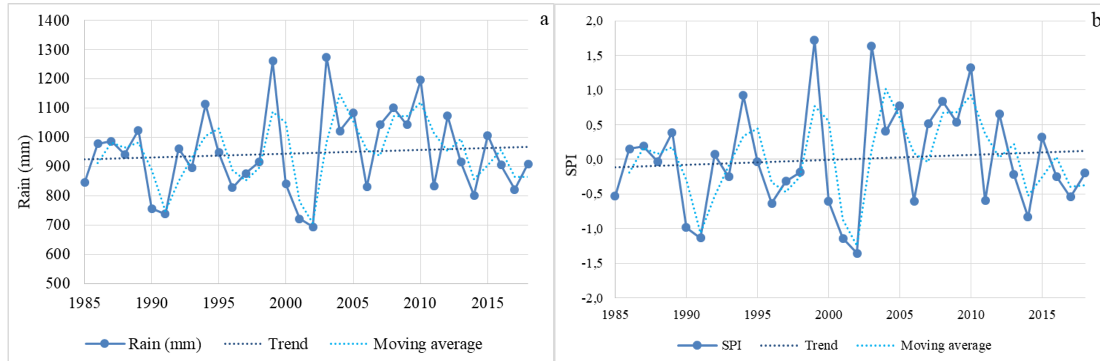


Figure 4. a) Evolution of average annual rainfall and b) Standardized Precipitation Index in Upper-Casamance between 1985 and 2018

Calculating the number of rainy days per year indicated that rainfall ranged from 81 in 1999 to 43 in 2017 (Figure 5), an average of 60 days on the series studied. Compared to the results of Sane et al., (2008), which recorded an average of 79 days of rain in Kolda and 60 days in Vélingara between 1951 and 2000, or an average of 69 days, our results show a continuous decline in the number of days of rain The average rainfall per day ranged from 12.4 mm in 2001 to 19.1 mm in 2017. Unlike the number of rainy days that are declining, the average rainfall per rainy day appears to be increasing since 2003; or since 2009 in Senegal (Descroix et al., 2015). This indicates a tightening of precipitated quantities over the duration of the season.

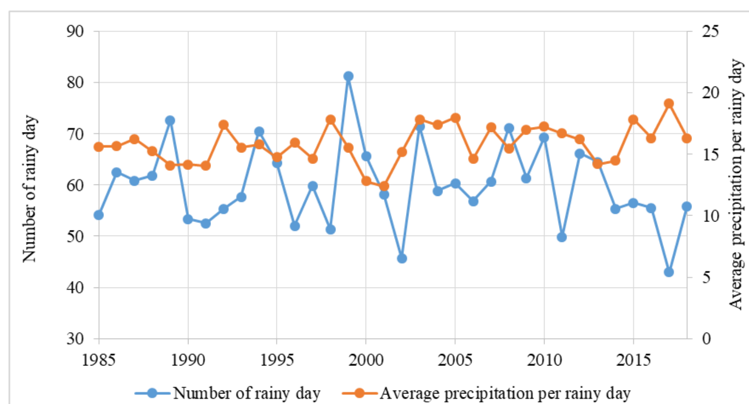


Figure 5. Change in the number of rainy days per year and the average rainfall per rainy day in Upper-Casamance between 1985 and 2017

In terms of the length of the agronomic season, analysis of the start and end dates shows a relatively early start compared to the dry period (Balme et al., 2005) and a relatively late end. Indeed, over 33 years, nine showed that the beginning of the agronomic season begins in the third decade of June (178th day), and six years showed that it begins in the second decade of the same month. For the end, 12 out of 33 years reported the second decade of October (290th day), and 11 years reported the third decade of the same month (Figure 6). The duration of the season is on average 112 days. However, for the beginning of the season, Sane et al., (2008) had recorded an 80% start in the second decade of July. For the duration, Balme et al., (2005) had averaged 105 days. Compared to the millet growth cycle (90-120 days for constant-cycle varieties), the duration of the current agronomic season in Upper-Casamance seems favorable to production. However, this does not seem to be perceived by farmers who tend to blame the rain for the loss of millet production. According to Descroix et al., (2012), the fact that the return of rain, which is generally very positive for rural societies, is not always perceived or recognized may be linked to the degradation suffered by soil and vegetation during the drought phase. For, our results are similar to those of Descroix et al., (2015) They noted that in reality the rainy season is now significantly longer than during the dry phase, although in recent years in the Central Sahel (even in Upper-

Casamance) there has been a return of "bad" wintering in the agronomic sense of the term. In addition, they noted that Upper-Casamance had no "failed" agronomic season; in other words, these winters, which have become relatively longer in recent years, have experienced a good distribution of the rains during the season.

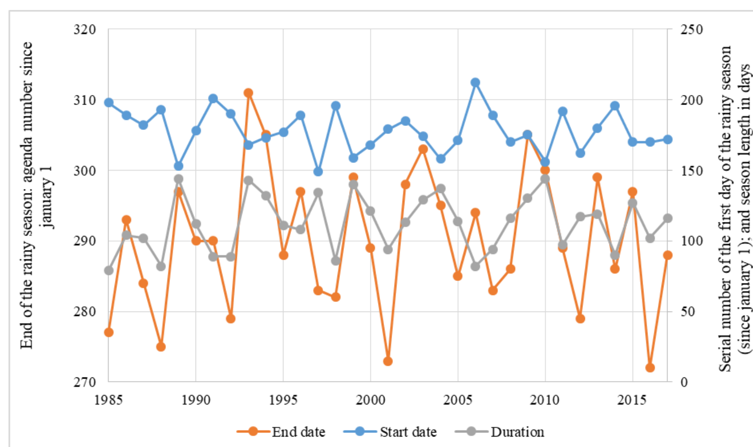


Figure 6. Start and end date and duration of the rainy season in Upper-Casamance between 1985 and 2018

However, for millet culture, Balme et al., (2005) proposed the use of faster maturing varieties (constant cycle mil of 90 days or less, photoperiodic mil) if the season starts late. For their part, Sane et al., (2008) proposed that the farmers of Upper-Casamance should match their farming activities between the second half of July and the second half of October to minimize the risks associated with the late start and early end of wintering. The risk of climate hazards to agricultural activities is lower during this period.

Conclusion

The purpose of this study was to analyze the evolution of agricultural production over the last thirty-four years in Upper-Casamance. It was coupled with the analysis of the annual average change in precipitation to determine their impact on yields. The results showed that agricultural production is increasing as we move away from the dry period. The Pearson correlation calculation showed that there is a positive linear relationship between rice, corn, groundnut and cassava production at rainfall. The simple linear regression applied to the different products at the $p<0.05$ threshold indicated that these four speculations are significantly dependent on the annual rainfall period; this is not the case for millet, sorghum, fonio and cotton. The analysis of the evolution of rainfall indicated favorable conditions for good agricultural production. The "agronomic" season, which averages 112 days, appears to be sufficient for the cultivation of millet and groundnut, which require an average of 4 months of good rainfall. For rice and corn, the results also indicated that the better the rainfall conditions, the better the production.

These results are important to farmers, development actors, and the branches and programs responsible for the country’s agricultural production and the achievement of national food self-sufficiency. However, the study did not take into account certain parameters that also influence production, such as: agricultural policies, technical constraints, and organizational constraints. The study therefore recommends that these parameters be included in future analyzes.

References

ANSD. (2017). *2013 General Census of Population and Habitat, Agriculture and Livestock*. Kolda Final Report, Edict. National Agency for Statistics and Demography, Dakar (Senegal), 90.

Balme, M., Galle, S., & Lebel, T. (2005). Start of the rainy season in the Sahel: variability at the hydrological and agronomic scales, analysis based on EPSAT Niger data. *Secheresse*, *16*(1), pp. 15-22

Bodian, A. (2014). Characterization of recent temporal variability in annual precipitation in Senegal (West Africa). *Physio-Géo*, *8*, pp. 297-312.

Dacosta, H., Coly, A., & Soumare P.O. (2002). Adequacy of water supply and demand: hydrological dynamics of the Anambé (Senegal). In: Orange Didier (ed.), Arfi Robert (ed.), Kuper M. (ed.), Morand Pierre (ed.), Poncet Yveline (ed.), Témé B. (pref.) *Integrated management of natural resources in tropical*

flood zones. Paris (FRA); Bamako: IRD; CNRST, 395-410. (Colloquia and Seminars). GIRN-ZIT: Integrated Management of Natural Resources in Tropical Flood Zones: International Seminar, Bamako (MLI), 2000/06/20-23. ISBN 2-7099-1480-8

Descroix, L., Diongue Niang, A., Panthou, G., Bodian, A., Sane, Y., Dacosta, H., Malam Abdou, M., Vandervaere, J-P., & Quantin, G. (2015). Recent rainfall trends in West Africa across two regions: Senegal and the Middle Niger Basin. *Climatology, 12*, pp. 25-43.

Descroix, L., Genthon, P., Amogu, O., Rajot, J-L., Sighomnou, D., & Vauclin, M. (2012). Change in Sahelian Rivers hydrograph: The case of recent red floods of the Niger River in the Niamey region. *Global Planetary Change, 98,* 18-30.

Djaman, K., Balde, AB., Rudnick, DR., Ndiaye, O., & Irmak, S. (2017). Long-term trend analysis in climate variables and agricultural adaptation strategies to climate change in the Senegal River Basin. *Int. J. Climatol. 37*, pp. 2873–2888.

FAO. (2011). Overview of rice development in Senegal. http://www.fao.org/fileadmin/user_upload/spid/docs/Senegal/Riziculture_etatdeslieux_SN.pdf

Fanchette, S. (1999). Colonization of sylvo-pastoral lands and land conflicts in Upper-Casamance. *IIED, Coll. Pastoral Land Tenure, 13*, 41.

Léauthaud, C., Demarty, J., Cappelaere, B., Grippa, M., Kergoat, L., Velluet, C., Guichard, F., Mougin, E., Chelbi, S., & Sultan, B., (2015). Revisiting historical climatic signals to better explore the future: prospects of water cycle changes in Central Sahel. *Proc. IAHS*, *371*, pp.195–201.

Mballo, I., Sy, O., & Faye, C. (2019). Climatic variability and food production in Upper-Casamance (South-Senegal). *Espace Géographique et Société Marocaine*, pp.161-178.

Mckee, TB., Doesken, NJ., & Kleist, J. (1993). The relationship of drought frequency and duration to time scales. *Proceedings of the eight conference on applied climatology, Anaheim, California, Boston, American Meteorological Society*, pp.179–184.

MEFS. (2011). 2011 Finance Act. Rapport, Ministry of Economy and Finance of Senegal, Dakar (Senegal), 134.

Ndiaye, O. (2018). Analysis of agricultural and trade policies in Senegal: food security and sovereignty compromised? *Master in Environment, Université de Sherbrooke*, 88.

Nouaceur, Z. (2020). The resumption of rains and the resurgence of floods in West Africa. *Physio-Géo, 15*, pp. 89-109.

WMO. (2012). Standard Precipitation Index User's Guide. *Weather-Climate-Water*, WMO-N°1090, 25p

Roudier, P., Sultan, B., Quirion, P., & Berg, A. (2011). The impact of future climate change on West Africa crop yields: What does the recent literature say? *Global Environment Change, 21*, pp. 1073-1083.

Sagna, P. (2005). *Climate dynamics and recent developments in western Africa*. Thèse de Doctorat d'Etat Es Lettres, Département de Géographie, Université Cheikh Anta Diop, Dakar, Tome 1, 270.

Sane, T., Diop, M., & Sagna, P. (2008). Study of the quality of the rainy season in Upper-Casamance (South Senegal). *Sécheresse, 19*(1), pp. 23-28.

Sarr, B., & Traoré, S. (2010). Impacts of climate change on agriculture. In Le Sahel face au changement climatique, *monthly bulletin of the AGRHYMET Regional Centre, special issue*, pp. 22-24

Sene, I.M. (2007). *Impacts of climate change on agriculture in Senegal: climate dynamics, economics, adaptations, water balance modelling of groundnuts and millet*. Doctoral thesis in Geography. University Cheikh Anta Diop of Dakar, 301 p.

Sidibe, M. (2005). *Groundnut migrants. The conquest of the Pata Classified Forest*. Casamance, Senegal. IRD Editions, Paris, 301.

Sivakumar, M.V.K. (1988). Predicting rainy season potential from the onset of rains in southern Sahelian and Sudanian climatic zones of West Africa. *Agric. For. Meteorol*, *42*, 295–305.

Sultan, B. (2012). Global warming threatens agricultural productivity in Africa and South Asia. *Environ. Res. Lett., 7*, 041001.

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Evaluation of Pistacia vera Sap Waste Sections and Its Potential Role on Treatment

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Abstract: It is estimated that 1.3 billion waste is produced annually in the world and this amount is expected to increase to 38-67% by the end of 2025. Considering the waste production of pistachio, it is seen that around 132 165 thousand tons of waste products have been accumulated after the product processing phase due to excessive consumption and wide usage area. However, it has been reported that there are important secondary metabolites such as "masticadienonic acid", "masticadienolic acid", "tirucallol" and "pistasionic acid" in the extract content of unused waste stem parts. Masticadienonic acid has been shown in the literature to have anti-proliferative and apoptotic activity. It has been determined that masticadienolic acid component has cytotoxic activity on five different cancer cell lines (Leukemia, Breast, Prostate, Colon and CNS). Examining the studies on the Tirukallol component, it was determined that it inhibits adhesion molecules in human endothelial cells. Pistacionic acid is a newly purified and characterized compound and only anticholinesterase and antidiabetic activities have been studied. The aim of our study is to determine whether these components obtained from stem parts have any cytotoxic effect on Mcf7 cells. Within the scope of the study, the stems were obtained from pistachio processing plants and kept at room temperature, in a cool environment. In order to obtain the compounds from sap extracts, column chromatography, ion layer chromatography (TLC) and crystallization methods which are moving phase systems were used. Mcf7 cells were exposed to different concentrations of the purified components and their cytotoxic activity was evaluated using the MTT test. Mcf7 cells were exposed to different concentrations of the purified components and their cytotoxic activity was evaluated using the MTT test. The results of our study are important preliminary data in the literature and we believe that they may contribute to further studies.

Keywords: Waste Solid, Pistacia vera, Bioactive Metabolite, Antitumoral activity, Phytotherapy

Introduction

Pistacia vera L. is the only edible fruit species among 12 different tree species that is included in the Pistacia genus (Mannino vd., 2019). This fruit contains an edible long seed. This seed part consists of a lilac-colored skin and a greenish flesh with a pleasant taste and aroma (Fabani vd., 2013). Pistachio, when we look at the amount of production, equals to 551 307 tonnes in Iran, 447 700 tonnes in the US, it is reported that the production of Pistachio in Turkey equals to 240,000 tons and 74 828 tons in China (FAO, 2018). The latest data in our country show that its production has tripled in the last 50 years (Tüik, 2019). The reason why the

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production is so high is that it is used in various industrial areas such as cake, ice cream, chocolate, salami, tahini halva and baklava making. An average of 2 kg biomass waste accumulates for every 3 kg of pistachio with a wide range of usage areas. (Mehrnejad, 2001). Biomass wastes generally consist of shell, root, leaf or stem parts and it has been determined that these parts have valuable bioactive components. It has been determined that terpenes, essential oils, phenolics, fatty acids and sterols, which are among these bioactive components, play a role in various biological activities through ethnopharmacological studies (Bozorgi vd., 2013). Unfortunately, in phytochemical and pharmacological studies, mainly the shell, fruit, resin and leaf parts are used, while the stem parts are mostly ignored. However, it has been documented by several studies that these parts are also rich in phytochemical components and fulfill important pharmacological activities. Among these studies, Dambagı and her team determined that there are important secondary metabolites such as masticadienolic acid, masticadienolic acid, tirucallol and pistacionic acid in the waste stems. In addition, in the continuation of the study, it was also shown that pistacionic acid did not have any inhibitory effect on acetylcholinesterase, although these components had inhibitory effects on α-glucosidase, α-amylase, acetylcholinesterase and butyrylcholinesterase (Dambagı, 2019). It has also been determined that masticadienonic acid reduces blood sugar and serum fatty acid concentration by inhibiting the 11β-HSD1 enzyme and has anti-proliferative activity on prostate cancer cells (Vuorinen vd., 2015; Sânchez-Monroy vd., 2017). In the study with experimental mouse models, it has been proven that masticadienolic acid has an anti-inflammatory effect and this component has an antifungal effect (Giner-Larza vd., 2001; Johann vd., 2010). Although Tirukallol inhibits adhesion molecules such as VCAM-1 and ICAM-1 on Human Aortic Endothelial Cell (HAEC), it has been determined that it has no cytotoxic effect. In addition, it has been shown to inhibit critical events such as edema formation and migration of polymorph nuclear leukocytes caused by Tissue Plasminogen Activator (TPA) (Loizou vd., 2009; Fernandez-Arche vd., 2010). A new compound with an organic structure different from the existing phytochemical components was synthesized and characterized as pistacionic acid (Dambagı, 2019). There is no cytotoxic activity study on breast cancer cells in other components, including pistacionic acid, whose pharmacologically only anticholinesterase and antidiabetic effects have been determined.

Our aim in this study is to analyze the cytotoxic activities of major compounds to be purified from the waste stalks of Pistacia vera fruit on MCF-7 cell line by molecular approaches to determine which concentration is toxic and how it affects cell proliferation.

Method

Obtaining Pistachio Extracts

The stem part of the Pistachio (P. vera L.) we used as the study material was taken from the Pistachio processing facilities as waste material and the waste stem part was ground using a blender. Plant samples were macerated with n-Hexane, chloroform and ethyl alcohol and extracts were obtained. Solvents were removed by a rotary evaporator and the extracts were concentrated. Column Chromatography, Ion Layer Chromatography (TLC) and crystallization methods, which are moving phase systems, were used to obtain compounds from the extracts. Subsequently, masticadienonic acid, masticadienolic acid and triquallol compounds from chlorofome extracts, pistacionic acid compound from ethyl alcohol extract were purified. The organic structure of these components has been illuminated using FTIR, 1H-NMR, 13C-NMR, 1D- and 2D-NMR spectroscopic methods. (Dambagı, 2019).

Cell Culture

The Michigan Cancer Foundation-7 (Mcf-7) cell line was used as the experimental group and the human umbilical vein endothelial cell (HUVEC) cell line was used as the control. Cells were grown in DMEM (Dulbecco's Modified Eagle Medium) medium enriched with FBS (Fetal Bovine Serum), Penicillin-Streptomycin and L-glutamine. For the duplication of the cells, the cryovials were taken from -80 ^{o}C and dissolved quickly by using double boiler. Then, DMEM was taken into the medium and after centrifuging at 800 rpm for 5 minutes, the supernatant was removed and the medium was added to the remaining cell pellet. Finally, the cell suspension was taken into flasks of 75 cm^2 and left to incubation at 37 ^{o}C in an environment containing 5% CO^2, allowing it to duplicate (Cevatemre, 2012).

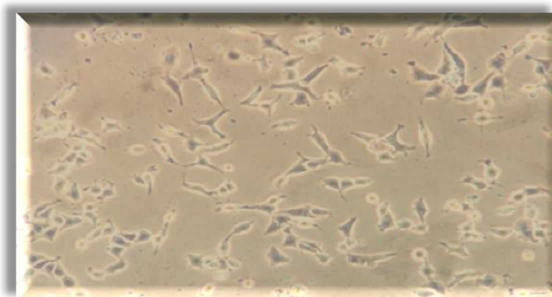

Figure 1. MCF-7 celss (X20)

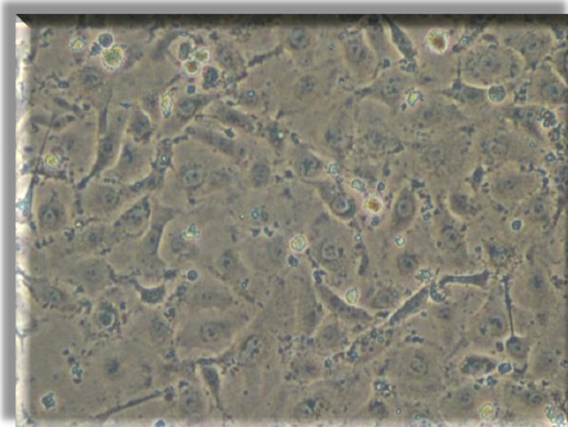

Figure 2. HUVEC cells (X40)

Cytotoxicity Test

Cytotoxic activity is determined by the reduction of "yellow tetrazolium (MTT)" compound of mitochondrial dehydrogenase enzyme activity in living cells. This method is based on measuring the color change colorimetrically. Cells were first planted in a 96-well plate with 5 × 10^3 cells per well and incubated for 24 hours at 37 °C, 5% CO^2. After incubation, pistacionic acid compound was added to the cells in 5 different concentrations (100 µm / ml, 50 µm / ml, 25 µm / ml, 12, 50 µm / ml and 6, 25 µm / ml) and incubated for 48 hours. In cells, the wells were completed by placing medium and cell as positive control (maximum viability, MO), medium and Triton X-100 as negative control, and cell-free medium for blank. After incubation, 40 µl of MTT dye was added to each well and incubated again at 37 ^{o}C for 4 hours. DMSO was added to each well to make the formazan crystals formed after incubation soluble. The resulting color intensity was measured in a spectrophotometer at a wavelength of 570 nm. (Cevatemre, 2012). The% viability rates of the cells were determined by using the absorbance values read in the calculation of the viability equation.In this equation, non-extracted control cell (MO) viability was assumed as 100%, and the viability rates of cells treated with extract were calculated using the formula.

% Viability = [100 × (mean of Compound-treated cell absorbance / Drug-treated control cell (MO) viability)
In the experiment, each concentration was repeated in three separate wells.

Findings

Findings of Cytotoxic Activity

Cytotoxicity of pistacionic acid on the MCF-7 breast cancer cell line was determined by the MTT (3- [4,5-dimethylthiazol-2-yl] -2,5 diphenyltetrazolium bromide) method and the absorbance and concentration graph is given in Figure 3.

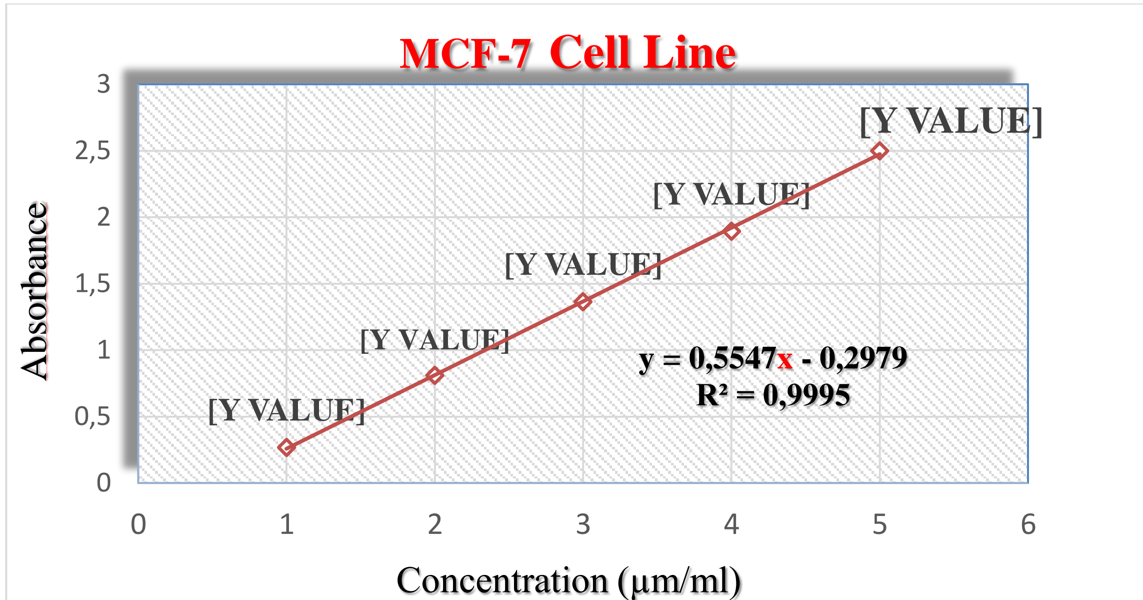

Figure 3. Absorbance and concentration graph of pistacionic acid compound in Mcf-7 cells line.

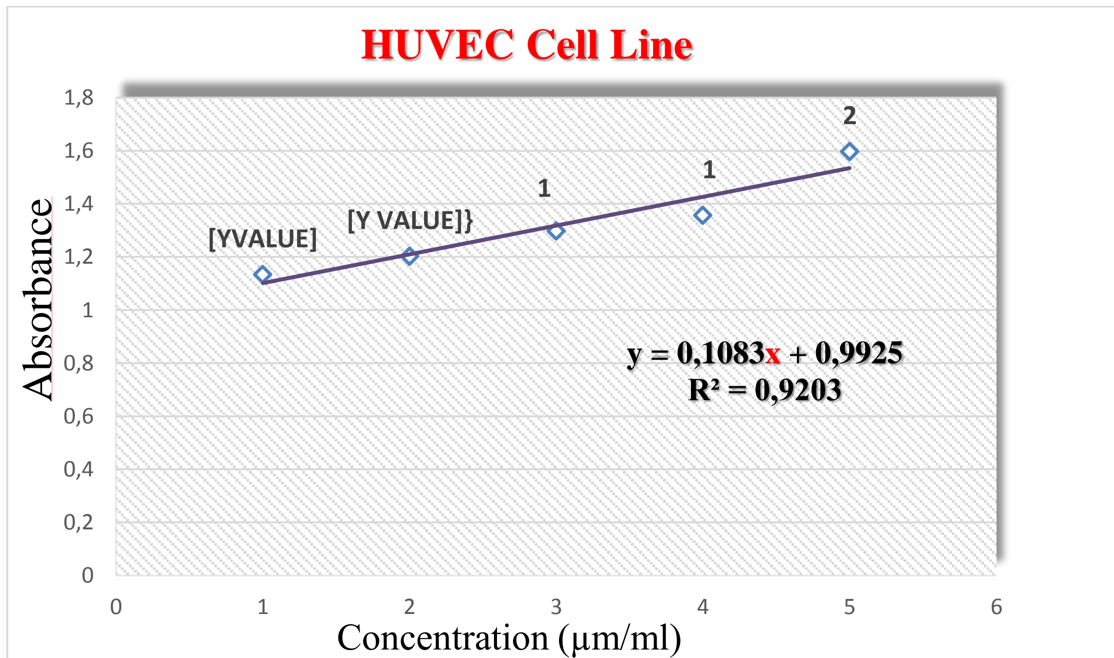

Figure 4. Absorbance and concentration graph of pistacionic acid Compound in HUVEC cells line.

Result and Discussion

Pistacionic acid is a newly synthesized and purified compound. For this reason, it has been found in the literature that it only inhibits α-glucosidase and α-amylase enzyme activity and shows antidiabetic activity. Anticholinesterase activity was determined by determining BchE (Butyrylcholine Esterase) enzyme activity and AchE (Acetylcholine Esterase) enzyme activity.

The results confirmed that Pistasionic acid exhibited a cytotoxic effect in Mcf-7 cells but was less toxic in HUVEC cells. In the remaining stages of our study, we think that determine the cytotoxic activities in other major compounds we purified. Then, how the compound affects apoptosis by determining the activity of the caspase 3 enzyme involved in the apoptosis process by immunocytochemical method. We anticipate that it has many biological activities waiting to be discovered in further studies and that its cytotoxic activity may be used for an advanced new drug design.

Reference

Bozorgi, M., Memariani, Z., Mobli, M., Salehi Surmaghi, M. H., Shams-Ardekani, M. R., & Rahimi, R. (2013). Five Pistacia species (P. vera, P. atlantica, P. terebinthus, P. khinjuk, and P. lentiscus): a review of their traditional uses, phytochemistry, and pharmacology. *The Scientific World Journal*, *2013*.

Cevatemre, B. (2012). *Fenretinid ve İndol-3-Karbinol Kombinasyonunun Meme Kanseri Hücre Soyları Üzerindeki Sitotoksik/Apoptotik Etkilerinin Araştırılması*, Yüksek lisans tezi (Master Thesis).

Dambagı, L.Y. (2019). *Antepfıstığı (Pistacia vera L.) Meyve Sap Ekstreleri ve Saf Metabolitlerinin Antidiyabetik ve Antikolinesteraz Özellikleri*. Yüksek Lisans Tezi (Master Thesis).

Fabani, M.P., Luna, L., Baroni, M.V., Monferran, M.V., Ighani, M., Tapia, A., Wunderlin, D.A., Feresin, G.E., 2013. Pistachio (Pistacia vera var Kerman) from Argentinean cultivars. A natural product with potential to improve human health. *J. Funct. Foods 5*, 1347–1356.

FAO. (2018). World Fertilizer Trends and Outlook to 2018. Data available at. http://www.fao.org/3/a-i4324e.pdf.

Fernandez-Arche, A., Saenz, M. T., Arroyo, M., De la Puerta, R., & Garcia, M. D. (2010). Topical anti-inflammatory effect of tirucallol, a triterpene isolated from Euphorbia lactea latex. *Phytomedicine*, *17*(2), 146-148.

Giner-Larza, E. M., Máñez, S., Giner, R. M., Recio, M. C., Prieto, J. M., Cerdá-Nicolás, M., & Ríos, J. (2002). Anti-inflammatory triterpenes from Pistacia terebinthus galls. *Planta Medica*, *68*(04), 311-315.

Johann, S., Sá, N. P., Lima, L. A., Cisalpino, P. S., Cota, B. B., Alves, T. M., ... & Zani, C. L. (2010). Antifungal activity of schinol and a new biphenyl compound isolated from Schinus terebinthifolius against the pathogenic fungus Paracoccidioides brasiliensis. *Annals of clinical microbiology and antimicrobials*, *9*(1), 30.

Loizou, S., Paraschos, S., Mitakou, S., Chrousos, G. P., Lekakis, I., & Moutsatsou, P. (2009). Chios mastic gum extract and isolated phytosterol tirucallol exhibit anti-inflammatory activity in human aortic endothelial cells. *Experimental biology and medicine*, *234*(5), 553-561.

Mannino, G., Gentile, C., & Maffei, M. E. (2019). Chemical partitioning and DNA fingerprinting of some pistachio (Pistacia vera L.) varieties of different geographical origin. *Phytochemistry*, *160*, 40-47.

Mehrnejad, M. R. (2001). The current status of pistachio pests in Iran. *Cahiers options méditerranéennes*, *56*, 315-322.

Sánchez-Monroy, M., Jacobo-Herrera, N. J., Zentella-Dehesa, A., Hernández-Téllez, B., & Martínez-Vázquez, M. (2017). Masticadienonic and 3α-OH masticadienoic acids induce apoptosis and inhibit cell proliferation and tumor growth in prostate cancer xenografts in vivo. *Molecules*, *22*(9), 1479.

TÜİK (2019). Bitkisel Üretim İstatistikleri [Basın Bülteni]. Erişim adresi: http://www.tuik.gov.tr/PreTablo.do?alt_id=1001.

Vuorinen, A., Seibert, J., Papageorgiou, V. P., Rollinger, J. M., Odermatt, A., Schuster, D., & Assimopoulou, A. N. (2015). Pistacia lentiscus oleoresin: Virtual screening and identification of masticadienonic and isomasticadienonic acids as inhibitors of 11β-hydroxysteroid dehydrogenase 1. *Planta medica*, *81*(06), 525-532.

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Evaluation of the Effect of RhoB Inhibition on Epithelial to Mesenchymal Transition Properties in NSCLC A549 Cells

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Abstract: Epithelial to mesenchymal transition (EMT) is a naturally occurring process during embryonic development and wound healing that is implicated in cancer progression when aberrantly reactivated. EMT has also been linked to chemotherapy resistance, anti-apoptosis, migration, and invasion of cells. It is thought to endow cancer cells with the ability to self-renew and mesenchymal properties that promote dissemination and metastasis. RhoB, a protein that binds GTP, regulates cellular processes such as cell survival, tumorigenesis, angiogenesis, migration, and metastasis. RhoB down-regulation is correlated to higher degrees of tumor progression and invasiveness. The loss of RhoB during cancer progression is thought to induce EMT by regulating the expression of certain EMT-associated transcription factors. This study aims to investigate the possible relationship between RhoB expression and the EMT characteristics of non-small cell lung cancer cells, such as in vitro migration proprieties. RhoB siRNA or All Stars siRNA negative control was transfected into A549 cells to determine whether RhoB depletion could promote EMT properties. The wound-healing assay was used for evaluating cell migration. The results demonstrate that the knockdown of RhoB promoted the migration ability of A549 cells. The current study may help to better understand the role of RhoB in EMT.

Keywords: EMT, RhoB siRNA, Migration, Wound healing assay.

Introduction

Lung cancer is a malignant disease that often does not show obvious symptoms until its advanced stages. In the late 20th century, lung cancer has become the leading cause of cancer-related deaths worldwide (Molina et al., 2008), with 1.76 million deaths in 2018, according to WHO (World Health Organization, 2018). Lung cancer accounts for 31% of cancer-related deaths in women and approximately 26% of cancer-related deaths in men (Viani et al., 2012). The 5-year survival rate of lung cancer patients is 36-73% (Padda et al., 2014). Lung cancer is divided into four main types: squamous cell carcinoma, adenocarcinoma, large cell carcinoma, which are included in the non-small cell lung cancer, and small-cell carcinoma. (Travis et al., 2012). Non-small cell lung cancer (NSCLC) accounts for about 80% of lung cancer cases. Although new therapies have been developed, the 5-year survival rate of patients with NSCLC is as low as 15% (Gao et al., 2014).

Epithelial- to- mesenchymal transition (EMT) is a reversible mechanism in which epithelial cells lose characteristic epithelial proprieties while simultaneously gaining mesenchymal stem cell characteristics (Acloque et al.,2009; Maier et al.,2010; Stone et al.,2016; Skrypek et al., 2017). Epithelial-mesenchymal transition plays a role in normal physiological functions such as embryonic development during embryogenesis as part of tissue remodeling and during wound healing, but can also be recapitulated by cancers (Hay,1968; Thiery 2003; Lee at al.,2006; Thiery et al.,2006; Visvader and Lindeman,2008; Micalizzi et al.,2010). It contributes to pathological conditions such as fibrosis and tumorigenesis (Aroeira et al.,2007; Angadi and Kale, 2015; Stone et al.,2016). Cancer cells undergo a change in phenotype to make them more mobile and lose

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polarity (Angadi and Kale, 2015). EMT allows cancer cells to be more invasive and metastasize ("walk out" of the primary tumor) and contributes to chemoresistance (Thiery et al., 2002; Thiery et al., 2006; Christiansen and Rajasekaran., 2006). Once metastatic cells reach a secondary site, they must regain the proliferative characteristics of epithelial cells to form a secondary tumor (Acloque et al.,2009; Stone et al.,2016; Skrypek et al., 2017).

Migration is a key process that enables cells to modify and reach their proper location in a given environment to perform their function (Te Boekhorst et al., 2016). This phenomenon plays an important role in various processes in multicellular organisms such as gastrulation, embryogenesis, nervous system development, tissue homeostasis, and trafficking of immune cells. However, if cell migration is deregulated, it may lead to many pathological processes, including inflammation and cancer metastasis (Charras and Sahai, 2014; Mayor and Etienne-Manneville,2016; Van Helvert et al., 2018). In the development and progression of cancer, metastasis occurs as tumor cells migrate through the circulatory and lymphatic systems from the primary tumor, invade the basement membranes and endothelial walls and eventually colonize distant organs (Friedl and Wolf, 2003; Friedl and Alexander, 2011).

RhoB, a Rho GTPase, is different from other Rho family members in terms of various properties such as intracellular localization, short half-life at both mRNA and protein levels in cells, and isoprenylation of the -COOH terminal (Adamson et al., 1992). Several studies have reported that in many cancer types, RhoB expression is downregulated, while RhoA and RhoC expressions are upregulated (Chen et al., 2000; Abraham, 2001; Kamai et al., 2001; Forget et al., 2002; Adnane et al., 2002; Kamai et al., 2003; Horiuchi et al., 2003; Jiang et al., 2004; Mazieres et al., 2004; Mazières et al., 2005; Sato et al., 2007; Zhou et al., 2011). As the tumor progresses towards advanced invasive carcinoma, RhoB remains localized in the cytoplasm, loses its ability to translocate to the nucleus, and its expression decreases dramatically in the middle and poorly differentiated regions of the tumor (Adnane et al., 2002).

Increasing evidence suggests a relationship between EMT induction and RhoB downregulation (Bousquet et al., 2009; Vega et al., 2015; Bousquet et al., 2016; Calvayrac et al., 2017). Data from various studies have shown that RhoB inhibition is accompanied by cytoskeletal rearrangements such as modulation of the cytoskeleton of actin and vimentin and altering the expression of vinculin and cadherins, which are the main features of the EMT process (Bousquet et al., 2009). During cancer progression, RhoB loss causes EMT through overexpression of mRNA levels of SLUG but not SNAIL transcription factor, and a decrease in E-cadherin mRNA and protein levels (Bousquet et al., 2009; Vega et al., 2015; Bousquet et al., 2016; Calvayrac et al., 2017). This suggests that RhoB downregulation has an effect only on specific EMT-inducing transcription factors (Shih and Yang., 2011; Baldwin et al., 2014; Wang et al., 2016).

This study aims to investigate the contribution of RhoB expression to the in vitro EMT properties of non-small cell lung cancer cells, such as cell migration.

Materials and Methods:

Cell Culture:

A549 cells were grown in RPMI-1640 medium supplemented with 10% FBS, 1% L-glutamine, and 1% penicillin/streptomycin at 37°C and 5% CO_2 in a humidified incubator. Cells were seeded in 25 cm^2 tissue culture flasks and passaged when 80% confluence was reached.

Transfection with RhoB siRNA

Before transfection, 6×10^4 cells were seeded in 24-well plates in an appropriate culture medium containing serum and antibiotics. In the short time until transfection, the cells were incubated under normal growth conditions (typically 37°C and 5% CO_2). RhoB siRNA or AllStars siRNA negative control (used as RhoB siRNA negative control) was diluted in Opti-MEM I medium without serum. To dilute HiPerfect, Opti-MEM I was added and gently mixed by inverting the tube 2-3 times. Diluted HiPerFect was added to the diluted RhoB siRNA and mixed by vortexing. RhoB siRNA or RhoB siRNA negative control (Qiagen) were allowed to form transfection complexes with HiperFect (Qiagen) for 20 minutes at room temperature (15−25°C) in serum-free Opti-MEM I (Invitrogen) at 25 nM final concentration according to the manufacturer's instructions. Then,

transfection complexes were added drop-wise onto A549 cells. The plate was swirled gently to ensure uniform distribution of the transfection complexes. The cells were incubated for 24 hours with the transfection complexes under their normal growth conditions. RhoB knockdown was monitored 24 h after transfection. Transfected cells were harvested for total RNA isolation. The relative expression of RhoB was evaluated by qRT-PCR.

RT-PCR:

Total RNA was isolated from cells using the miRNeasy Kit (Qiagen) according to the manufacturer's protocol. Reverse transcription reaction was carried out using the High-Capacity RNA to cDNA ™ kit (Applied Biosystems). SYBR green-based real-time RT-PCR was carried out using the Power SYBR® Green PCR Master Mix kit (Applied Biosystems) to measure the expression of RhoB in cells by the StepOnePlus Real-Time PCR System (Applied Biosystems). GAPDH was used as an endogenous control.

Scratch Wound Healing Assay:

A549 cells transfected with RhoB siRNA or AllStars siRNA negative control were seeded at a density of $2x10^5$ cells/well in 24-well plates. After the cells reached 90-100% confluence, they were serum starved overnight in media before starting the experiment. The confluent cell monolayer was then wounded by scraping the monolayer using a 10µl pipette to generate scratch wounds and washed with PBS twice to eliminate cell debris. Then, cells were allowed to migrate for 72 hours in the 1% FBS RPMI-1640 medium at 37°C. Wound closure or cell migration images were photographed when the scrape wound was introduced at 0 h and 24, 48, and 72 h after initial wounding. Six locations in each transfection group were visualized and photographed using a 10X objective lens under a Nikon ECLIPSE TS100 phase-contrast inverted microscope equipped with a DS-Fi1 Camera. The migration areas were measured by calculating the difference between the wound area at the indicated time points and the initial wound area using the Image J- NIH (U. S. National Institutes of Health, Bethesda, MA, USA).

Statistical Analysis:

All data are expressed as mean ± SD. Student's *t*-test for the comparison between groups was performed using GraphPad Prism version 8.0.2 (GraphPad Software, San Diego, California USA). Those with a *p*-value equal to 0.05 or less were considered statistically significant.

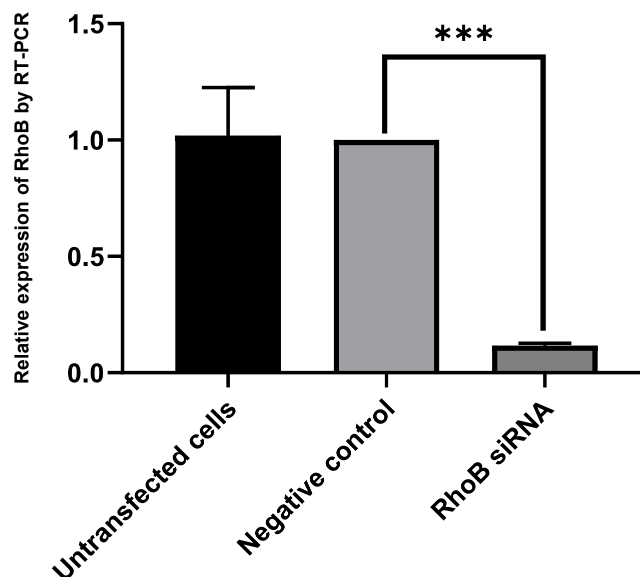

Figure 1. Relative expression of RhoB siRNA by RT-PCR. (*** indicates $p<0.001$).

Results and Discussion

RT-PCR:

To explore whether RhoB siRNA can decrease the expression of RhoB, A549 cells were transfected with RhoB siRNA or RhoB siRNA negative control (AllStars siRNA negative control) at a final concentration of 25 nM for 24 h, then the relative expression of RhoB was measured by qRT-PCR. The relative expression of RhoB was 0.12 ± 0.01 in A549 cells transfected with RhoB siRNA, which was significantly decreased, as compared to 1.00 ± 0.00 in A549 cells transfected with RhoB siRNA negative control cell group ($p< 0.001$; Fig.1) when analyzed by RT-PCR. The results suggest that RhoB siRNA could decrease RhoB expression in A549 cells.

Scratch Wound Healing Assay:

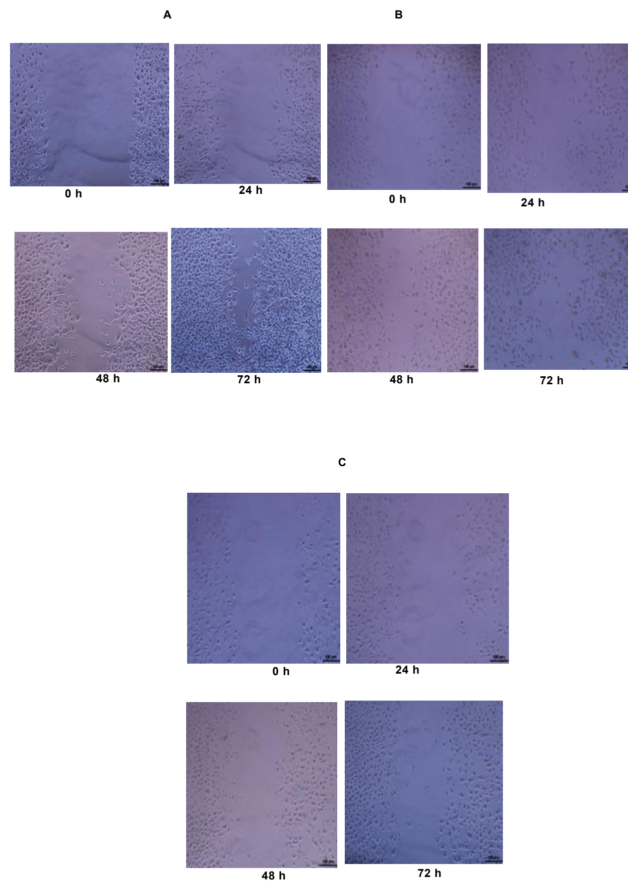

Figure 2. Phase-contrast photographs of the cultures taken at 0 h and at the indicated time intervals. Scale bar= 100 µm.

To explore the effects of RhoB siRNA on the migration of A549 cells, an in vitro scratch assay was performed on A549 cells transfected with RhoB siRNA (Fig. 2A) or RhoB siRNA negative control (Fig. 2B) and untransfected cells (Fig. 2C) and images were taken at 0h, and 24,48 and 72 h incubation times after initial wound scratching using a phase-contrast microscope. The migration rate was calculated by measuring the total distance that A549 cells migrated towards the center of the wound from the edge of the wound.

Wound closure% at different time points was represented as the percentage of wounded area at time 0. The capacity of wound healing in A549 cells transfected with RhoB siRNA was significantly enhanced compared to control cells at 24 h (p= 0.013; Fig. 3A), at 48 h (p=0.030; Fig. 3B), and at 72 h (p= 0.025; Fig. 3C) after scratching, suggesting that RhoB siRNA could promote cell migration in A549 cells, which further emphasize the function of RhoB in the migration and metastasis of non-small cell lung cancer cells.

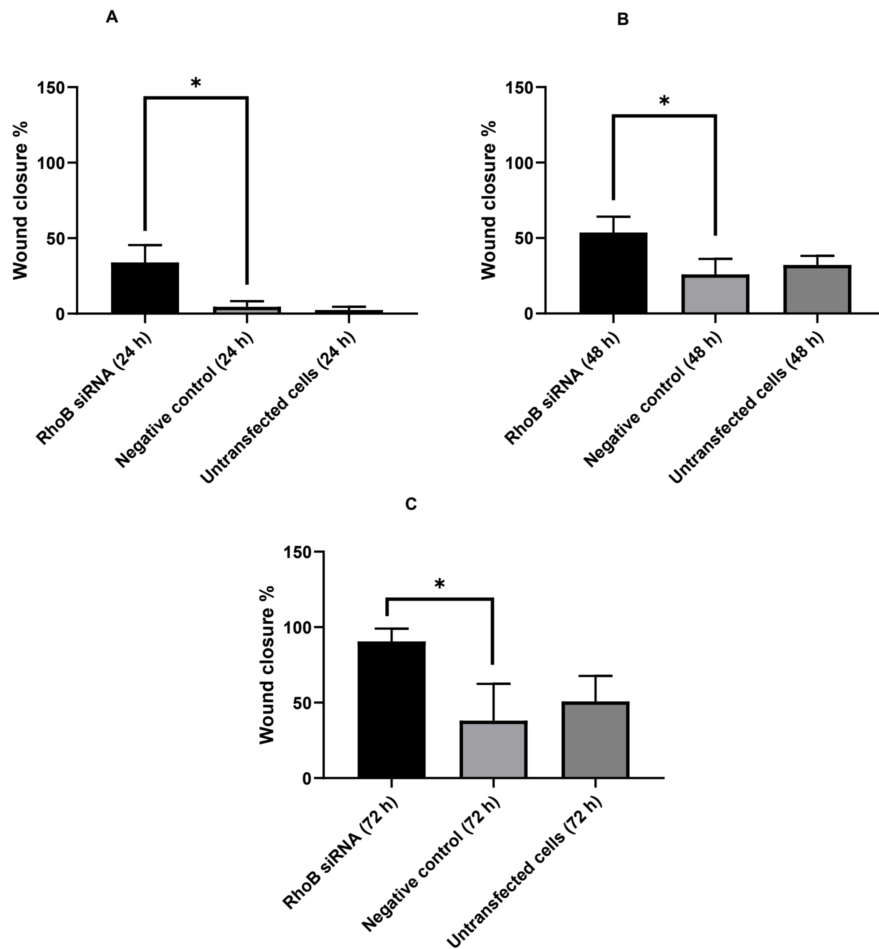

Figure 3. Analysis of the wound area closure of A549 cells at different time points. (* indicates $p < 0.05$).

Discussion and Conclusion:

RhoB, a protein that binds GTP, is involved in regulating many cellular processes such as apoptosis, tumorigenesis, angiogenesis, migration, and metastasis (Howe and Addison,2012). RhoB down-regulation is correlated to higher degrees of lung tumor progression and invasiveness (Adnane et al.,2002; Wang et al.,2003; Mazieres et al.,2004; Sato et al.,2007; Chen et al.,2016). The loss of RhoB during cancer progression is thought to induce EMT by regulating the expression of certain EMT-inducing transcription factors (Shih and Yang., 2011; Baldwin et al., 2014; Wang et al., 2016). This study aimed to investigate the relationship between RhoB expression and the in vitro EMT functional properties of non-small cell lung cancer cells, such as cell motility and migration. For this purpose, RhoB siRNA or RhoB siRNA negative control was transfected into A549 cells to determine whether RhoB depletion could promote EMT properties. The scratch wound-healing assay was used for evaluating cell migration. Results revealed that RhoB knockdown significantly enhanced the migration of NSCLC A549 cells. Our results are in accordance with previous studies, reporting that low expression of RhoB promoted EMT, migration, and invasion (Bousquet et al.,2009; Ma et al.,2019). Taken together, these results indicate that RhoB plays an important role as a tumor suppressor in tumorigenesis, cancer cell motility, EMT, and metastasis in NSCLC and could be a potential target for cancer treatment.

References

Abraham, R. T. (2001). Cell cycle checkpoint signaling through the ATM and ATR kinases. *Genes & development*, *15*(17), 2177-2196.

Acloque, H., Adams, M. S., Fishwick, K., Bronner-Fraser, M., & Nieto, M. A. (2009). Epithelial-mesenchymal transitions: the importance of changing cell state in development and disease. *The Journal of clinical investigation*, *119*(6), 1438-1449

Adamson, P., Paterson, H. F., & Hall, A. (1992). Intracellular localization of the P21rho proteins. *The Journal of cell biology*, *119*(3), 617-627.

Adnane, J., Muro-Cacho, C., Mathews, L., Sebti, S. M., & Munoz-Antonia, T. (2002). Suppression of rho B expression in invasive carcinoma from head and neck cancer patients. *Clinical Cancer Research, 8*(7), 2225-2232.

Angadi, P. V., & Kale, A. D. (2015). Epithelial-mesenchymal transition-A fundamental mechanism in cancer progression: An overview. *Indian Journal of Health Sciences and Biomedical Research (KLEU), 8*(2), 77.

Aroeira, L. S., Aguilera, A., Sánchez-Tomero, J. A., Bajo, M. A., del Peso, G., Jiménez-Heffernan, J. A., ... & López-Cabrera, M. (2007). Epithelial to mesenchymal transition and peritoneal membrane failure in peritoneal dialysis patients: pathologic significance and potential therapeutic interventions. *Journal of the American Society of Nephrology, 18*(7), 2004-2013.

Baldwin, L.A., Hoff, J.T., Lefringhouse, J., Zhang, M., Jia, C., Liu, Z., Erfani, S., Jin, H., Xu, M., She, Q.B. and Van Nagell, J.R. (2014). CD151-α3β1 integrin complexes suppress ovarian tumor growth by repressing slug-mediated EMT and canonical Wnt signaling. *Oncotarget, 5*(23), p.12203.

Bousquet, E., Mazières, J., Privat, M., Rizzati, V., Casanova, A., Ledoux, A., ... & Pradines, A. (2009). Loss of RhoB expression promotes migration and invasion of human bronchial cells via activation of AKT1. *Cancer research, 69*(15), 6092-6099.

Bousquet, E., Calvayrac, O., Mazieres, J., Lajoie-Mazenc, I., Boubekeur, N., Favre, G., & Pradines, A. (2016). RhoB loss induces Rac1-dependent mesenchymal cell invasion in lung cells through PP2A inhibition. *Oncogene, 35*(14), 1760-1769.

Calvayrac, O., Mazières, J., Figarol, S., Marty- Detraves, C., Raymond- Letron, I., Bousquet, E., ... & Guibert, N. (2017). The RAS- related GTP ase RHOB confers resistance to EGFR- tyrosine kinase inhibitors in non- small- cell lung cancer via an AKT- dependent mechanism. *EMBO molecular medicine, 9*(2), 238-250.

Charras, G., and Sahai, E. (2014). Physical influences of the extracellular environment on cell migration. *Nature reviews Molecular cell biology, 15*(12), 813-824.

Chen, Z., Sun, J., Pradines, A., Favre, G., Adnane, J., & Sebti, S. M. (2000). Both farnesylated and geranylgeranylated RhoB inhibit malignant transformation and suppress human tumor growth in nude mice. *Journal of Biological Chemistry*, *275*(24), 17974-17978.

Chen, W., Niu, S., Ma, X., Zhang, P., Gao, Y., Fan, Y., ... & Zhang, Y. (2016). RhoB acts as a tumor suppressor that inhibits malignancy of clear cell renal cell carcinoma. *PLoS One, 11*(7), e0157599.

Christiansen, J. J., & Rajasekaran, A. K. (2006). Reassessing epithelial to mesenchymal transition as a prerequisite for carcinoma invasion and metastasis. *Cancer research*, *66*(17), 8319-8326.

Forget, M. A., Desrosiers, R. R., Del Maestro, R. F., Moumdjian, R., Shedid, D., Berthelet, F., & Béliveau, R. (2002). The expression of rho proteins decreases with human brain tumor progression: potential tumor markers. *Clinical & experimental metastasis*, *19*(1), 9-15.

Friedl, P., & Wolf, K. (2003). Tumour-cell invasion and migration: diversity and escape mechanisms. *Nature reviews cancer*, *3*(5), 362-374.

Friedl, P., & Alexander, S. (2011). Cancer invasion and the microenvironment: plasticity and reciprocity. *Cell*, *147*(5), 992-1009.

Gao, Y., Fan, X., Li, W., Ping, W., Deng, Y., & Fu, X. (2014). miR-138-5p reverses gefitinib resistance in non-small cell lung cancer cells via negatively regulating G protein-coupled receptor 124. *Biochemical and biophysical research communications*, *446*(1), 179-186.

Hay, E. D. (1968). Organization and fine structure of epithelium and mesenchyme in the developing chick embryo. In *Epithelial-Mesenchymal Interactions; 18th Hahnemann Symposium, 1968*. Williams & Wilkins.

Horiuchi, A., Imai, T., Wang, C., Ohira, S., Feng, Y., Nikaido, T., & Konishi, I. (2003). Up-regulation of small GTPases, RhoA and RhoC, is associated with tumor progression in ovarian carcinoma. *Laboratory investigation*, *83*(6), 861-870.

Howe, G. A., & Addison, C. L. (2012). RhoB controls endothelial cell morphogenesis in part via negative regulation of RhoA. *Vascular cell, 4*(1), 1.

Jiang, K., Sun, J., Cheng, J., Djeu, J. Y., Wei, S., & Sebti, S. (2004). Akt mediates Ras downregulation of RhoB, a suppressor of transformation, invasion, and metastasis. *Molecular and cellular biology*, *24*(12), 5565-5576.

Kamai, T., Arai, K., Tsujii, T., Honda, M., & Yoshida, K. (2001). Overexpression of RhoA mRNA is associated with advanced stage in testicular germ cell tumour. *BJU international*, *87*(3), 227-231.

Kamai, T., Kawakami, S., Koga, F., Arai, G., Takagi, K., Arai, K., ... & Yoshida, K. I. (2003). RhoA is associated with invasion and lymph node metastasis in upper urinary tract cancer. *BJU international*, *91*(3), 234-238.

Lee, J. M., Dedhar, S., Kalluri, R., & Thompson, E. W. (2006). The epithelial–mesenchymal transition: new insights in signaling, development, and disease. *Journal of Cell Biology*, *172*(7), 973-981.

Ma, Q., Gao, Y., Xu, P., Li, K., Xu, X., Gao, J., ... & He, X. (2019). Atorvastatin inhibits breast cancer cells by downregulating PTEN/AKT pathway via promoting ras homolog family member B (RhoB). *BioMed research international*, *2019*.

Maier, H. J., Wirth, T., & Beug, H. (2010). Epithelial-mesenchymal transition in pancreatic carcinoma. *Cancers, 2*(4), 2058-2083.

Mayor, R., & Etienne-Manneville, S. (2016). The front and rear of collective cell migration. *Nature reviews Molecular cell biology*, *17*(2), 97.

Mazieres, J., Antonia, T., Daste, G., Muro-Cacho, C., Berchery, D., Tillement, V., ... & Favre, G. (2004). Loss of RhoB expression in human lung cancer progression. *Clinical cancer research*, *10*(8), 2742-2750.

Mazières, J., Tillement, V., Allal, C., Clanet, C., Bobin, L., Chen, Z., ... & Pradines, A. (2005). Geranylgeranylated, but not farnesylated, RhoB suppresses Ras transformation of NIH-3T3 cells. *Experimental cell research*, *304*(2), 354-364.

Micalizzi, D. S., Farabaugh, S. M., & Ford, H. L. (2010). Epithelial-mesenchymal transition in cancer: parallels between normal development and tumor progression. *Journal of mammary gland biology and neoplasia*, *15*(2), 117-134.

Molina, J. R., Yang, P., Cassivi, S. D., Schild, S. E., & Adjei, A. A. (2008, May). Non-small cell lung cancer: epidemiology, risk factors, treatment, and survivorship. In *Mayo clinic proceedings* (Vol. 83, No. 5, pp. 584-594). Elsevier.

Padda, S. K., Burt, B. M., Trakul, N., & Wakelee, H. A. (2014, February). Early-stage non-small cell lung cancer: surgery, stereotactic radiosurgery, and individualized adjuvant therapy. In *Seminars in oncology* (Vol. 41, No. 1, pp. 40-56). WB Saunders.

Sato, N., Fukui, T., Taniguchi, T., Yokoyama, T., Kondo, M., Nagasaka, T., ... & Minna, J. D. (2007). RhoB is frequently downregulated in non- small- cell lung cancer and resides in the 2p24 homozygous deletion region of a lung cancer cell line. *International journal of cancer*, *120*(3), 543-551.

Shih, J. Y., & Yang, P. C. (2011). The EMT regulator slug and lung carcinogenesis. *Carcinogenesis*, *32*(9), 1299-1304.

Skrypek, N., Goossens, S., De Smedt, E., Vandamme, N., & Berx, G. (2017). Epithelial-to-mesenchymal transition: epigenetic reprogramming driving cellular plasticity. *Trends in Genetics*, *33*(12), 943-959.

Stone, R. C., Pastar, I., Ojeh, N., Chen, V., Liu, S., Garzon, K. I., & Tomic-Canic, M. (2016). Epithelial-mesenchymal transition in tissue repair and fibrosis. *Cell and tissue research*, *365*(3), 495-506.

Te Boekhorst, V., Preziosi, L., & Friedl, P. (2016). Plasticity of cell migration in vivo and in silico. *Annual review of cell and developmental biology*, *32*, 491-526.

Thiery, J. P. (2002). Epithelial–mesenchymal transitions in tumour progression. *Nature Reviews Cancer*, *2*(6), 442-454.

Thiery, J. P. (2003). Epithelial–mesenchymal transitions in development and pathologies. *Current opinion in cell biology*, *15*(6), 740-746.

Thiery, J. P., & Sleeman, J. P. (2006). Complex networks orchestrate epithelial–mesenchymal transitions. *Nature reviews Molecular cell biology*, *7*(2), 131-142.

Travis, W. D. (2012). Update on small cell carcinoma and its differentiation from squamous cell carcinoma and other non-small cell carcinomas. *Modern Pathology*, *25*(1), S18-S30.

Van Helvert, S., Storm, C., & Friedl, P. (2018). Mechanoreciprocity in cell migration. *Nature cell biology*, *20*(1), 8.

Vega, F. M., Thomas, M., Reymond, N., & Ridley, A. J. (2015). The Rho GTPase RhoB regulates cadherin expression and epithelial cell-cell interaction. *Cell Communication and Signaling, 13*(1), 6.

Viani, G.A., Boin, A.C., Ikeda, V.Y., Vianna, B.S., Silva, R.S., & Santanella, F. (2012). Thirty years of prophylactic cranial irradiation in patients with small cell lung cancer: a meta-analysis of randomized clinical trials. *J Bras Pneumol, 38*(3), pp.372-381.

Visvader, J. E., & Lindeman, G. J. (2008). Cancer stem cells in solid tumours: accumulating evidence and unresolved questions. *Nature reviews cancer*, *8*(10), 755-768.

Wang, S., Yan-Neale, Y., Fischer, D., Zeremski, M., Cai, R., Zhu, J., ... & Cohen, D. (2003). Histone deacetylase 1 represses the small GTPase RhoB expression in human non-small lung carcinoma cell line. *Oncogene, 22*(40), 6204-6213.

Wang, W., Yi, M., Chen, S., Li, J., Li, G., Yang, J., ... & Li, G. (2016). Significance of the NOR1-FOXA1/HDAC2-Slug regulatory network in epithelial-mesenchymal transition of tumor cells. *Oncotarget*, *7*(13), 16745.

World Health Organization. (2018, September 12). *Cancer*. https://www.who.int/news-room/fact-sheets/detail/cancer.

Zhou, J., Li, K., Gu, Y., Feng, B., Ren, G., Zhang, L., ... & Fan, D. (2011). Transcriptional up-regulation of RhoE by hypoxia-inducible factor (HIF)-1 promotes epithelial to mesenchymal transition of gastric cancer cells during hypoxia. *Biochemical and biophysical research communications*, *415*(2), 348-354.

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Extraction and Purification of the Potential Allergen Proteins from Mucor Mucedo

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Abstract: Allergy is an important health problem affecting public health. Allergic diseases occur when the immune system reacts to non-harmful substances with the effect of genetic predisposition and environmental factors. According to the data of the World Allergy Organization (WAO), the prevalence of allergies in different countries varies between 10-40%. Pollen, mold, animal hair, house dust mite, medicines, and foods are the most common allergen agents. Common mushrooms in nature have the potential to produce allergenic proteins. Penicillium, Aspergillus, Rhizopus, and Mucor species, which are allergic fungi, are widely found in nature. In our country, 614 patients with respiratory allergy have been reported to develop allergic reactions against Aspergillus fumigatus, Trichophyton rubrum, Mucor, Penicillium notatum, Aspergillus niger, and Alternaria tenius. In recent years, the cases of allergies caused by molds have increased significantly and studies to determine the causing allergens have accelerated. Mucor mucedo (brown bread mold) was used in our study. Mucor mucedo produced in our laboratory was collected and allergen fungus protein was extracted by 2 different extraction methods. By preparing protein samples from prepared mushroom extracts, the total concentration of potential allergen proteins was determined by the BCA method. According to the data obtained, it was determined that the protein concentration of the mushroom samples dried by that were subjected to dialysis was higher than ethanol. As a result, Mucor mucedo was found to have a high protein concentration and revealed basic data for further analysis.

Keywords: Allergy, Fungal allergy, Mucor mucedo, Allergen protein, BCA

Introduction

Allergy is one of the diseases that affect public health. Hypersensitivity of the immune system is called allergy. Allergic disease is a mistargeted immune reaction that occurs after the body is exposed to a certain antigen known as an allergen and when re-stimulated by the same antigen causes clinical symptoms and transient or chronic organ dysfunction. Allergic diseases usually affect the skin and mucosal tissues such as sinuses, lungs, and intestines (Tao & Raz, 2015).

According to the data of the World Allergy Organization, the prevalence of allergies in different countries varies between 10-40%. (Pawankar, 2013) It is possible to encounter allergic diseases seasonally or throughout the year. Seasonal allergic reactions are caused by fungal spores, pollen, insecticides, indoor and outdoor mold fungi, house dust, and animal hairs that persist throughout the year (Şimşekli, 1994).

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In recent years, many researches have been carried out in the field of aeromicology, which is very popular in the world, sports calendars of many cities in our country and abroad have been published, and atmospheric sports concentrations are announced and announced to the public through meteorological bulletins (Çeter & Pınar, 2008).

Pollen, fungi, and house dust mites are the most common allergens. Fungi or fungal spores that can be found in the other environment can hang in the environment for a long time due to the effect of the airflow from their location (Simon-Nobbe et al., 2008).

Approximately 80% of fungi whose spores and micellar cells have been known to cause health problems for years are associated with diseases related to the respiratory tract. Even though the allergen spores are small in number, they enter the body by means such as eye conjunctiva, skin, respiratory and nasal mucosa and cause symptoms such as asthma, allergic rhinitis, conjunctivitis (Tatlıdil et al., 2001).

Mushrooms, which have the most species after insects, have the potential to produce allergenic proteins. These organisms, which have a wide distribution area, are estimated to constitute more than 90% of the biomass in the world (Kendrick, 2000).

Mushrooms, which are among the most harmful organisms for humans, are equally useful organisms due to their use in different areas such as the decay of organic substances in the ecosystem, the production of species consumed as food, and the development of biotechnology, such as the synthesis of biofuels, enzymes and drug active substances (Kendrick, 2000; Esch, 2017).

More than 80 types of fungi have been associated with respiratory allergies. (Çetinkaya et al., 2005). Penicillium, Aspergillus, Rhizopus, and Mucor species, which are allergic fungi, are widely found in nature. In our country, it has been reported that 614 patients with respiratory tract allergies develop allergic reactions against Aspergillus fumigatus, Trichophyton rubrum, Mucor, Penicillium notatum, Aspergillus niger, and Alternaria tenius (Güneser et al., 1994).

Mucor is a very common breed that causes mold on foodstuffs. Mucor mucedo, an allergy-associated saprophytic fungus, is abundant in soil, plants, decaying fruits, and vegetables. Especially, they cause mold on bread, cheese, and other foods by forming a white then browning mycelium on bread, cheese, and other foods. The mycelium's formed do not have transverse walls. The sporangium is formed at the ends of the vertically growing hyphae, which later turn brown. It produces many spores within the sporangia (Actor, 2011; Money, 2016).

Method

Preparation of *M. mucedo* Extracts

The mushroom samples used in our study were purchased commercially (CECT 2653) and reproduced in our laboratory. Mucor mucedo spores, which were cultivated on PDA medium, were left to incubate for 7-14 days at +25°C. It was collected after morphological examination by staining with cotton blue. The resulting mushrooms were treated in chloroform-methanol and ethanol on a magnetic stirrer for 24 hours. The dried mold samples were digested in PBS and dialyzed. The extraction method described by Ziwei Li et al. (2018) was used to extract the active ingredients of the samples lyophilized after dialysis. Protein concentrations were determined by extracting the obtained extracts into 50mM SDS Buffer.

Determination of Total Protein Concentration

The total protein concentration of the mushroom extracts was made using the bisinconic acid (BCA) method proposed by Smith et al. (1985). Commercially purchased BCA Macro Assay Kit (Serva Electrophoresis GmbH) was used to determine protein concentration. BCA analysis was performed following the protocol suggested by the manufacturer (Walker, 2002).

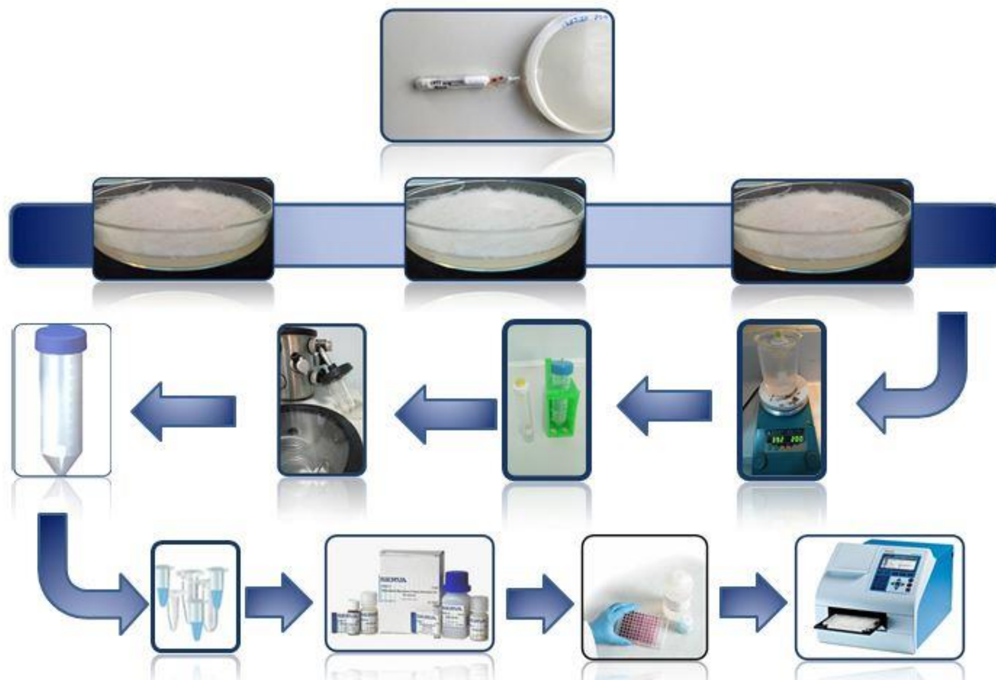

Figure 1. BCA working diagram

Results and Discussion

In our study, proteins of M. mucedo, one of the allergenic fungi, were extracted with 2 different extraction protocols. The amount of extracted proteins was measured by the BCA method. The protein amounts of the mushroom extracts prepared in the study were determined as 0,117 mg/mL for ethanol and 0,157 mg/mL for chloroform-methanol. Similar results were obtained with the amount of protein stated in previous studies (Wójcicka., 2014), and 1.19 times more protein was obtained as a result of chloroform-methanol extraction (Table 1).

Tablo 1. Total protein concentration values of *M. mucedo* extracts measured by BCA assay

Allergen name	Protein concentration (mg/mL)
M. mucedo (ethanol)	0,117
M. mucedo (chloroform-methanol)	0,157

Recommendations

In recent years, allergy cases caused by molds have been increasing. For this reason, studies to determine the allergen proteins of fungi commonly found in nature have gained importance. In our study, protein concentrations were determined by preparing M. mucedo extracts, which is one of the allergen fungi and used in allergen kits. The data obtained from this study form the basis for the production of alternative domestic kits to imported kits used in the diagnosis and treatment of allergy patients with advanced studies.

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References

Actor, J. K. (2011). Elsevier's Integrated Review Immunology and Microbiology (pp. 139-146) E-Book: with Student Consult Online Access. Elsevier Health Sciences. http://www.sciencedirect.com/science /article/pii/B9780323074476000156

Çeter, T. Y., & Pinar, N. M. (2008). *The concentration of atmospheric pollen and spores of Kastamonu province (Central Region) and effects of meteorological factors on this concentration* (January 2006-December 2007) (Doctoral dissertation, Ankara University Graduate School of Natural and Applied Sciences Department of Biology.

Esch, R. E., & Codina, R. (2017). Fungal raw materials used to produce allergen extracts. *Annals of allergy, asthma & immunology : Official publication of the American College of Allergy, Asthma, & Immunology, 118*(4), 399–405. https://doi.org/10.1016/j.anai.2016.05.012

Güneser, S., Atici, A., Köksal, F., & Yaman, A. (1994). Mold allergy in Adana, Turkey. *Allergologia et immunopathologia, 22*(2), 52–54.

Kendrick B. (2000). *The Fifth Kingdom* (pp. 227-247). Hackett Publishing

Money, N. P. (2016). *Fungal cell biology and development. In The fungi* (pp. 37-66). Academic Press.

Pawankar, R., Canonica, G. W., Holgate, S. T., Lockey, R. F., & Blaiss, M. S. (2011). *WAO white book on allergy* (pp. 102-110). Milwaukee, WI: World Allergy Organization, 3, 156-157.

Simon-Nobbe, B., Denk, U., Pöll, V., Rid, R., & Breitenbach, M. (2008). The spectrum of fungal allergy. *International archives of allergy and immunology, 145*(1), 58–86. https://doi.org/10.1159/000107578

Şimşekli, Y. (1994). *The fungus exist in the air outside the hauses in the different regions of Bursa*. (Master's thesis, Uludağ University.

Tao, A., & Raz, E. (Eds.). (2015). *Allergy bioinformatics* (8nd ed., pp.1-14). Springer.

Tatlıdil, S., Bıçakçı, A., Akkaya, A, & Malyer, H. (2001). Allergen cladosporium sp. and alternaria sp. spores in the atmosphere of Burdur. *Süleyman Demirel University Medical Faculty Journal:8*(4), 1-3.

Walker J M. 2002. *The Protein Protocols Handbook* (pp. 11-14). Second edition. Humana Press.

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Evaluation of the Effect of RhoB Inhibition on Cancer Stem Cell Properties in NSCLC A549 Cells

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Abstract: The cancer stem cells model suggests that a small subgroup of cancer cells can self-renew and substantially leads to the recurrence of tumors. Such cells are thought to be a reservoir for the regeneration of tumor-initiating mutant cells, which are insensitive to the chemotherapy currently being used. RhoB GTPase is implicated in regulating cell survival, tumorigenesis, angiogenesis, migration, and metastasis. Higher degrees of tumor progression and invasiveness are associated with RhoB expression. The contribution of Rho GTPases, in particular RhoB, in cancer stem cell properties, has not yet been fully explored. This study aims to explore the possible relationship between RhoB expression and the in vitro cancer stem cell-like characteristics of non-small cell lung cancer cells such as spheroid formation and self-renewal. To determine whether the depletion of RhoB could promote CSC-like characteristics, RhoB siRNA or AllStars siRNA negative control were transfected into A549 cells. The spheroid formation assay was used to evaluate the ability of cells to grow in anchorage-independent conditions to form spheroids. The results demonstrated that RhoB knockdown did not have a statistically significant effect in promoting cancer stem-like properties in A549 cells. This study may lead to further understanding of the contribution of RhoB to in vitro cancer stem cell features such as, the formation of tumorspheroids.

Keywords: CSCs, RhoB siRNA, Spheroid formation, Self-renewal.

Introduction

Lung cancer is the main cause of cancer deaths worldwide with both high mortality and morbidity rates due to the limited therapeutic options (Kalemkerian et al., 2013; Siegel et al., 2016). Approximately 80% of lung cancers are non-small cell lung cancers, which are non-neuroendocrine tumors including adenocarcinomas, large cell carcinoma and squamous cell carcinoma (Parsons et al., 2010; Siegel et al., 2016). Even with the most advanced imaging, staging, surgical strategies, chemotherapy, radiotherapy, as well as personalized treatments with targeted therapies such as EGFR tyrosine kinase inhibitors and ALK inhibitors in specific patient groups, the five-year survival rate for patients is only 5–15% (Kato et al., 2004; Winton et al., 2005; Leon et al.,2016).

The stem cell model of carcinogenesis suggests that cancers arise from cells with stem-like characteristics as a result of the impaired regulation of self-renewal pathways. Such dysregulation stimulates the expansion of this cell population that then may undergo additional genetic or epigenetic modifications to turn into a fully transformed clone (Wicha et al., 2006).

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Tumor heterogeneity and the presence of small subsets of cells with stem-like properties called cancer stem cells (CSCs) have been reported in almost all malignancies during the past decade (Leon et al.,2016). CSCs typically exhibit many features of embryonic or tissue stem cells including slow growth rates and insensitivity to chemotherapy and/or radiation therapy, therefore new treatment modalities that selectively target these cells are needed to control stem cell survival, proliferation, and differentiation (Jordan et al., 2006).

Increasing evidence proposes that lung cancer incorporates a cancer stem cell subpopulation that is responsible for the onset, spread, and metastasis of the tumor (Ho et al.,2007; Eramo et al.,2008; Levina et al.,2008; Bertolini et al.,2009; Jiang et al.,2009, O'Flaherty et al.,2012; Wang et al.,2013). Since CSCs cells have been implicated in tumorigenicity, cancer progression, and therapeutic resistance of lung cancer, their study would provide important insights into the understanding of their biology, expansion, and maintenance of, as well as to the development of novel diagnostic and prognostic tests and exploring novel therapeutic targets (Pine et al.,2008; Peacock and Watkins ,2008; Miyata et al.,2015; Zakaria et al.,2017). However, each type of tumor comprises different progenitor or stem cell types that are controlled by various molecular pathways, which results in variation in the expression of markers in lung cancer subtypes making these cells more difficult to be identified and targeted (Zakaria et al.,2017).

The propensity to self-renew and produce differentiated progeny constitutes the basic features of cancer stem cells (Ponti et al., 2005; Eramo et al., 2008). Tumorspheroids are a typical descriptor of CSCs that can maintain stemness profiles in proliferation, differentiation and under serum-free culturing conditions (Weiswald et al., 2015). Sphere formation assay has been widely used as a surrogate assay for the enrichment of CSCs or cancer cells with stem-like characteristics from solid tumors. Tumorspheroids culture systems are based on the ability of stem/progenitor cells to survive and form floating 3-dimensional spheroid bodies in semisolid media, such as collagen or Matrigel or in low attachment plates, in serum-free growth medium containing epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) (Ishiguro et al.,2017). Spheroid cultures as a method of enrichment for cancer stem cells have been used in many human cancers and cancer cell lines including glioma, melanoma, sarcoma, lung cancer, renal cancer, and rhabdomyosarcoma (Singh et al., 2003; Fang et al., 2005; Gibbs et al., 2005; Eramo et al., 2008; Zhong et al.,2010; Walter et al., 2011).

RhoA, RhoB, and RhoC are a subgroup of GTPases belonging to the family of GTP binding proteins involved in the regulation of various cellular processes, including cytoskeletal regulation, cell morphology, adhesion, motility, cell survival, cell cycle progression and gene expression (Bishop and Hall, 2000; Bar-Sagi and Hall, 2000; Aznar and Lacal, 2001; Sahai and Marshall, 2002; Calvayrac et al., 2016). RhoB is associated with cell survival, migration, angiogenesis, tumor formation, and metastasis (Huang and Prendergast, 2006; Karlsson et al., 2009; Ridley, 2013). Several factors, including growth factors such as PDGF, EGF, and TGF-β, UV radiation, and DNA damaging substances, can induce RhoB expression (Jähner and Hunter, 1991; De Cremoux et al., 1994; Fritz et al., 1995; Engel et al., 1998).

Downregulation of RhoB has been associated with higher degrees of tumor progression and invasiveness, ranging from reduced expression in superficially invasive carcinoma to near disappearance of RhoB expression in deeply infiltrating carcinoma (Adnane et al., 2002). Several studies have reported that lung cancer progression is accompanied by a lack of RhoB expression (Wang et al., 2003; Mazieres et al., 2004; Sato et al., 2007) and plays an important role in the acquisition of a more aggressive adenocarcinoma phenotype. Based on this, it can be used as a prognostic factor in NSCLC (Calvayrac et al., 2014). Furthermore, RhoB loss has been found to increase the migration and invasion of bronchial cells in vitro and in vivo by a mechanism related to AKT (Bousquet et al., 2009; Bousquet et al., 2016).

In view of all that has been mentioned so far, this study aims to assess cancer stem-like properties in A549 and investigate the contribution of RhoB expression to the in vitro cancer stem cell-like characteristics such as spheroid formation.

Materials and Methods:

Cell Culture:

A549 cells was grown in RPMI-1640 medium supplemented with 10% FBS, 1% L-glutamine and 1% penicillin/streptomycin at 37°C and 5% CO_2 in a humidified incubator. Cells were seeded in 25 cm^2 tissue culture flasks and passaged when 80% confluence was reached.

Transfection with RhoB siRNA

Before transfection, 6×10^4 cells were seeded in 24-well plates in an appropriate culture medium containing serum and antibiotics. For the short time until transfection, the cells were incubated under normal growth conditions (typically 37°C and 5% CO_2). RhoB siRNA or AllStars siRNA negative control (used as RhoB siRNA negative control) was diluted in Opti-MEM I medium without serum. To dilute HiPerfect, Opti-MEM I was added and gently mixed by inverting the tube 2-3 times. Diluted HiPerFect Transfection Reagent was added to the diluted RhoB siRNA and mixed by vortexing. RhoB siRNA or RhoB siRNA negative control (Qiagen) were allowed to form transfection complexes with HiperFect transfection reagent (Qiagen) for 20 minutes at room temperature (15−25°C) in serum-free Opti-MEM I (Invitrogen) at 25 nM final concentration according to the manufacturer's instructions. Then, transfection complexes were added drop-wise onto A549 cells. The plate was swirled gently to ensure uniform distribution of the transfection complexes. The cells were incubated for 24 hours with the transfection complexes under their normal growth conditions. RhoB knockdown was monitored 24 hours after transfection. Transfected cells were harvested for total RNA isolation. The relative expression of RhoB was evaluated by qRT-PCR.

Tumorspheroids Formation Assay:

A549 cells transfected with RHoB siRNA, RhoB siRNA negative control, and untransfected cells were plated at 1000 cells per well in ultra-low attachment 96-well plates (Corning, New York, USA) in spheroid culture medium containing serum-free medium DMEM-F12K (1:1) supplemented with 20 ng/ml EGF, 10 ng/ml bFGF, 1x B27 supplement, 1x insulin, 0.4% Bovine Serum Albumin and 1 % PenStrep. To prevent cell aggregation, methylcellulose was added at a concentration of 1.5 % to the culture medium. The cells were incubated at 37°C for 10 days. At the end of the incubation period; spheroids were counted using an inverted microscope. The diameters of the spheres were analyzed using the Image J-NIH (U. S. National Institutes of Health, Bethesda, MA, USA).

RT-PCR:

Total RNA was isolated from cells using the miRNeasy Kit (Qiagen) according to the manufacturer's protocol. Reverse transscription reaction was carried out using the High-Capacity RNA to cDNA kit (Applied Biosystems). SYBR green-based real time RT-PCR was performed using the Power SYBR Green PCR Master Mix kit (Applied Biosystems) to measure the expression of RhoB in cells by the StepOnePlusTM Real-Time PCR System (Applied Biosystems). GAPDH was used as endogenous control.

Statistical Analysis:

All data are expressed as mean ± SD. Student's *t* test for the comparison between groups was performed using GraphPad Prism version 8.0.2 (GraphPad Software, San Diego, California USA). Those with a *P* value equal 0.05 or less were considered statistically significant.

Results

RT-PCR:

To explore whether RhoB siRNA can decrease the expression of RhoB, A549 cells were transfected with RhoB siRNA or RhoB siRNA negative control (AllStars siRNA negative control) at a final concentration of 25 nM for 24 h, then the relative expression of RhoB was measured by qRT-PCR. The relative expression of RhoB was 0.12 ± 0.01 in A549 cells transfected with RhoB siRNA, which was significantly decreased, as compared to 1.00 ± 0.00 in A549 cells transfected with RhoB siRNA negative control cell group ($p< 0.001$; Fig.1), when analyzed by RT-PCR. The results suggestes that RhoB siRNA could decrease RhoB expression in A549 cells.

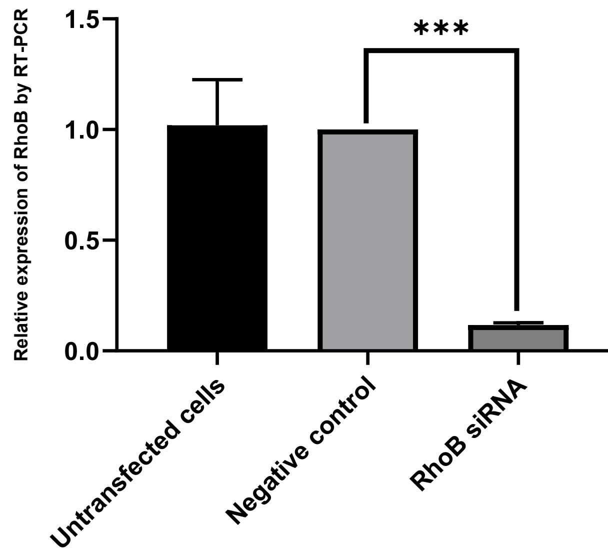

Figure 1. Relative expression of RhoB siRNA by RT-PCR. (*** indicates $p<0.001$).

Tumorsphere Formation Assay Results:

To explore the effects of RhoB siRNA on the growth potential of floating spheroids of A549 stem-like cells, tumorspheroids formation assay was performed. A549 cells were seeded at a density of 1000 cells/well in a total of 24 wells from an ultra-low adherent 96 well plates to obtain spheroids. The medium was changed four times a week during the 10 days incubation period. After 10 days of culture, ball-like spheres were observed. Images of tumor spheroids derived from A549 cells transfected with RhoB SiRNA (Fig. 2A), negative control (Fig. 2B) and untransfected cells (Fig. 2C), respectively were taken with an inverted microscope using a 10x objective lens to observe and analyze spheroids. The spheroid analysis was performed using image processing algorithms of the ImageJ-NIH software program. Spheroids with diameters > 40µm were counted from a total of 10 wells per cel group. The percentage of the spheroid formation efficiency (SFE%) was calculated by dividing the total number of formed spheroids by the total number of seeded living cells and multiplying by 100.

The sphere forming efficiency of A549 transfected with RhoB siRNA (2.20 ± 0.92%; Fig. 3A) was statistically insignificent ($p > 0.05$; Fig. 3A) as compared to A549 cells transfected with negative control (1.78 ± 0.89 %; Fig. 3A) and untransfected cells (2.44 ± 0.85 %; Fig. 3A).

The mean diameter of spheroids formed by A549 cells transfected with RhoB siRNA (52.28 ± 18.74 µm; Table 1; Fig.3B) was statistically insignificent (p > 0.05; Table 1; Fig. 3B) as compared to A549 cells transfected with negative control (52.35 ± 10.48 µm; Table 1; Fig.3B) and untransfected cells (52.76 ± 11.00 µm; Table 1; Fig.3B).

Table 1. The diameters of spheroids formed by A549 cells transfected with RhoB siRNA, negative control and untransfected cells.

	A549 cells transfected with RhoB siRNA	A549 cells transfected with negative control	Untransfected cells
Diameter Mean (µm)	52.28 ± 18.74	52.35 ± 10.48	52.76 ± 11.00
P value	0.96		

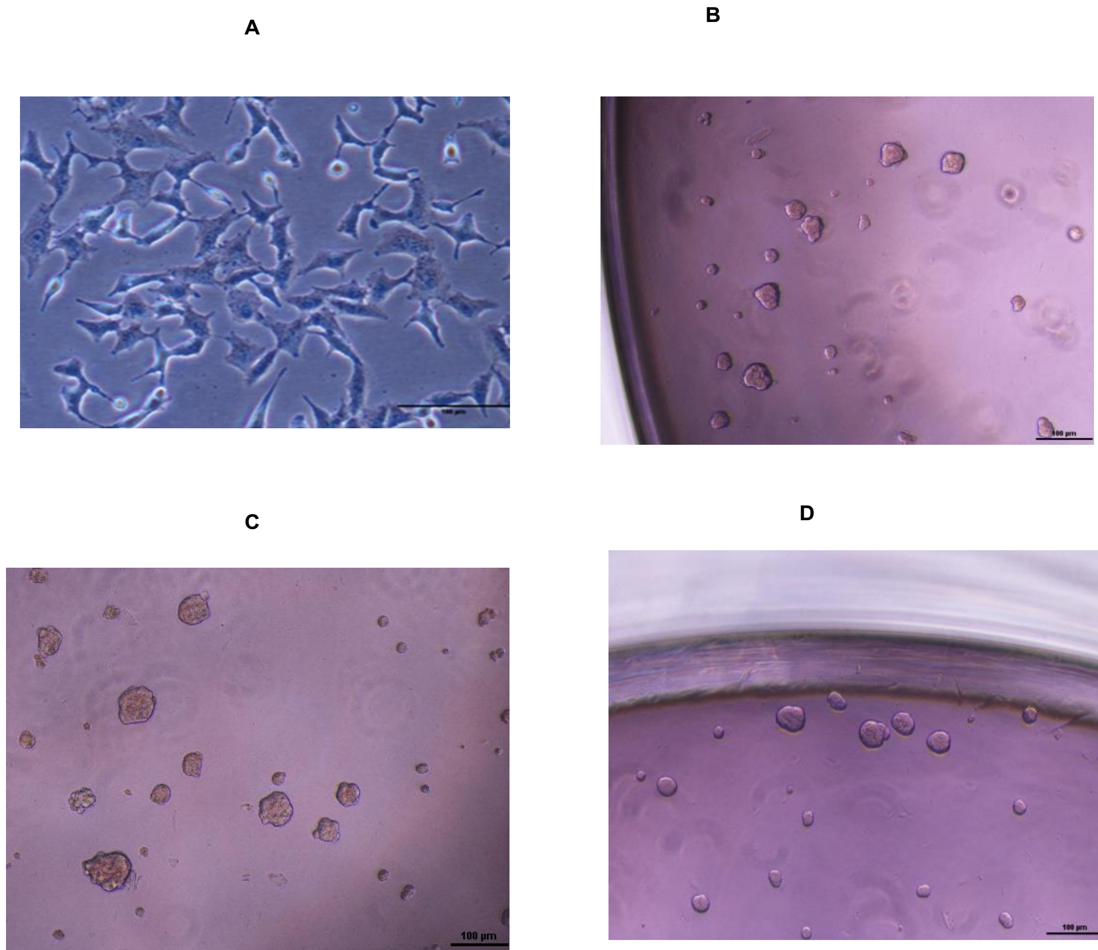

Figure 2. (A) A549 adherent monolayer parental cells. Scale bar is 100µm. Original magnification 20x for A and 10x for (B), (C) and (D). (B), (C) and (D) Tumor spheroids derived from A549 cells transfected with RhoB SiRNA, negative control and untransfected cells, respectively.

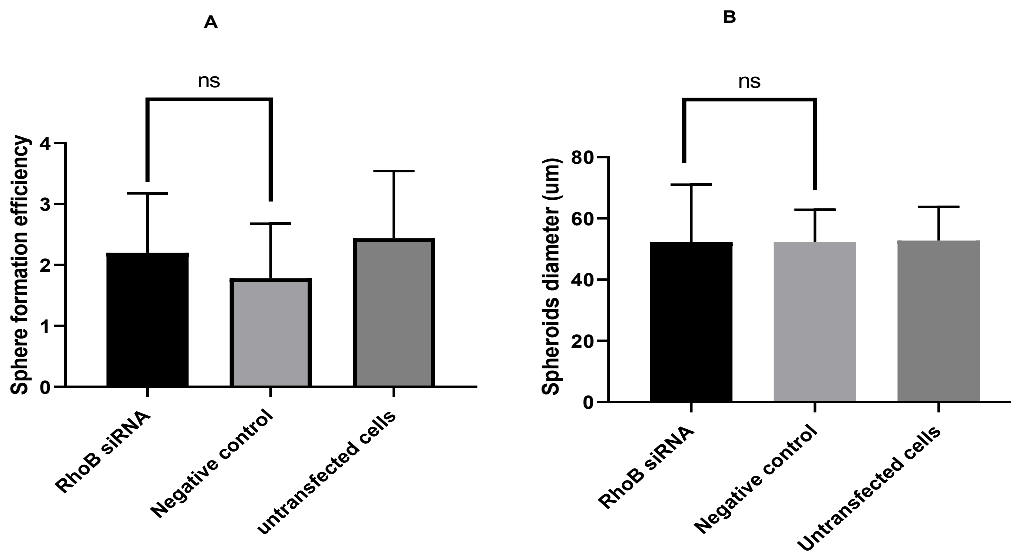

Figure 3. (A) Sphere-forming efficiency of A549 transfected with RhoB siRNA, negative control, and untransfected cells. (ns indicates non-significance, $p > 0.05$). (B) The diameters of spheroids formed by A549 cells transfected with RhoB siRNA, negative control, and untransfected cells. (ns indicates non-significance, $p > 0.05$).

Discussion

Increasing evidence has shown that NSCLC enhanced tumorigenicity, as in other solid cancer types, is driven by a tumor cell population that exhibits stem cell-like characteristics such as self-renewal, differentiation, cell mobility and therapy resistance, and referred to as cancer stem cells (CSC) or tumor-initiating cells (TIC) (Eramo et al.,2008; Bertolini et al.,2009; Magee et al.,2012;Visvader et al.,2012). Further investigation of the CSCs population would provide a deeper understanding of the regulatory mechanisms involved in their maintenance and facilitate the development of therapeutic strategies to control lung cancer in the long term (Liu et al.,2013). To study cancer stem-like properities in NSCLC A549 cells, we used the sphere formation assay, which is a marker-independent approach.

Loss of RhoB expression has been detected in numerous lung cancer cell lines and tumor tissues (Wang et al.,2003; Mazieres et al.,2004; Sato et .,2007; Mazières et al.,2007). In this study, we aimed to assess the effects of RhoB knockdown on cancer stem-like properties, such as tumorspheres formation in A549 NSCLC cells. The inhibition of RhoB did not enhance the capability of A549 cells to form spheroids, suggesting that RhoB loss in cancer cells might be required for other aspects of malignancy, such as acquiring of a motile and invasive phenotype, but not cancer stem-like properties, such as tumorspheres formation.

Recommendations

Recently the cancer stem cells are implicated in tumor initiation, progression, invasion, metastasis, relapse and resistance to chemotherapy and radiotherapy. Since the resistance to conventional chemotherapeutic agents is becoming a growing phenomenon, there is an urgent need to develop novel drugs that specifically target the cancer stem cell populations in tumors. Thus, it is of great value to conduct future studies to broaden our current knowledge of the biology of cancer stem cells and the underlying molecular regulatory mechanisms of their behavior.

References

Adnane, J., Seijo, E., Chen, Z., Bizouarn, F., Leal, M., Sebti, S. M., & Muñoz-Antonia, T. (2002). RhoB, not RhoA, represses the transcription of the transforming growth factor β type II receptor by a mechanism involving activator protein 1. *Journal of Biological Chemistry*, *277*(10), 8500-8507.

Aznar, S., & Lacal, J. C. (2001). Rho signals to cell growth and apoptosis. *Cancer letters*, *165*(1), 1-10.

Bar-Sagi, D., & Hall, A. (2000). Ras and Rho GTPases: a family reunion. *Cell*, *103*(2), 227-238.

Bertolini, G., Roz, L., Perego, P., Tortoreto, M., Fontanella, E., Gatti, L., ... & Roz, E. (2009). Highly tumorigenic lung cancer CD133+ cells display stem-like features and are spared by cisplatin treatment. *Proceedings of the National Academy of Sciences*, *106*(38), 16281-16286.

Bishop, A. L., & Hall, A. (2000). Rho GTPases and their effector proteins. *Biochemical Journal*, *348*(2), 241-255.

Bousquet, E., Mazières, J., Privat, M., Rizzati, V., Casanova, A., Ledoux, A., ... & Pradines, A. (2009). Loss of RhoB expression promotes migration and invasion of human bronchial cells via activation of AKT1. *Cancer research*, *69*(15), 6092-6099.

Bousquet, E., Calvayrac, O., Mazieres, J., Lajoie-Mazenc, I., Boubekeur, N., Favre, G., & Pradines, A. (2016). RhoB loss induces Rac1-dependent mesenchymal cell invasion in lung cells through PP2A inhibition. *Oncogene*, *35*(14), 1760-1769.

Calvayrac, O., Pradines, A., Raymond-Letron, I., Rouquette, I., Bousquet, E., Lauwers-Cances, V., ... & Milia, J. (2014). RhoB determines tumor aggressiveness in a murine EGFRL858R-induced adenocarcinoma model and is a potential prognostic biomarker for lepidic lung cancer. *Clinical Cancer Research*, *20*(24), 6541-6550.

Calvayrac, O., Mazières, J., Figarol, S., Marty‐ Detraves, C., Raymond‐ Letron, I., Bousquet, E., ... & Guibert, N. (2017). The RAS‐ related GTP ase RHOB confers resistance to EGFR‐ tyrosine kinase inhibitors in non‐ small‐ cell lung cancer via an AKT‐ dependent mechanism. *EMBO molecular medicine*, *9*(2), 238-250.

De Cremoux, P., Gauville, C., Closson, V., Linares, G., Calvo, F., Tavitian, A., & Olofsson, B. (1994). EGF modulation of the ras‐ related rhoB gene expression in human breast‐ cancer cell lines. *International journal of cancer*, *59*(3), 408-415.

Engel, M. E., Datta, P. K., & Moses, H. L. (1998). Signal transduction by transforming growth factor‐ β: A cooperative paradigm with extensive negative regulation. *Journal of Cellular Biochemistry*, *72*(S30–31), 111-122.

Eramo, A., Lotti, F., Sette, G., Pilozzi, E., Biffoni, M., Di Virgilio, A., ... & De Maria, R. (2008). Identification and expansion of the tumorigenic lung cancer stem cell population. *Cell death and differentiation*, *15*(3), 504.

Fang, D., Nguyen, T. K., Leishear, K., Finko, R., Kulp, A. N., Hotz, S., ... & Herlyn, M. (2005). A tumorigenic subpopulation with stem cell properties in melanomas. *Cancer research*, *65*(20), 9328-9337.

Fritz, G., Kaina, B., & Aktories, K. (1995). The ras-related small GTP-binding protein RhoB is immediate-early inducible by DNA damaging treatments. *Journal of Biological Chemistry*, *270*(42), 25172-25177.

Gibbs, C. P., Kukekov, V. G., Reith, J. D., Tchigrinova, O., Suslov, O. N., Scott, E. W., ... & Steindler, D. A. (2005). Stem-like cells in bone sarcomas: implications for tumorigenesis. *Neoplasia*, *7*(11), 967-976.

Ho, M. M., Ng, A. V., Lam, S., & Hung, J. Y. (2007). Side population in human lung cancer cell lines and tumors is enriched with stem-like cancer cells. *Cancer research*, *67*(10), 4827-4833.

Huang, M., & Prendergast, G. C. (2006). RhoB in cancer suppression. *Histology and histopathology*.

Ishiguro, T., Ohata, H., Sato, A., Yamawaki, K., Enomoto, T., & Okamoto, K. (2017). Tumor‐ derived spheroids: relevance to cancer stem cells and clinical applications. *Cancer science*, *108*(3), 283-289.

Jähner, D., & Hunter, T. O. N. Y. (1991). The ras-related gene rhoB is an immediate-early gene inducible by v-Fps, epidermal growth factor, and platelet-derived growth factor in rat fibroblasts. *Molecular and Cellular Biology*, *11*(7), 3682-3690.

Jiang, F., Qiu, Q., Khanna, A., Todd, N. W., Deepak, J., Xing, L., ... & Katz, R. L. (2009). Aldehyde dehydrogenase 1 is a tumor stem cell-associated marker in lung cancer. *Molecular cancer research*, *7*(3), 330-338.

Jordan, C. T., Guzman, M. L., & Noble, M. (2006). Cancer stem cells. *New England Journal of Medicine*, *355*(12), 1253-1261.

Kalemkerian, G. P., Akerley, W., Bogner, P., Borghaei, H., Chow, L. Q., Downey, R. J., ... & Hayman, J. (2013). Small cell lung cancer. *Journal of the National Comprehensive Cancer Network*, *11*(1), 78-98.

Karlsson, R., Pedersen, E. D., Wang, Z., & Brakebusch, C. (2009). Rho GTPase function in tumorigenesis. *Biochimica et Biophysica Acta (BBA)-Reviews on Cancer*, *1796*(2), 91-98.

Kato, H., Ichinose, Y., Ohta, M., Hata, E., Tsubota, N., Tada, H., ... & Ohta, M. (2004). A randomized trial of adjuvant chemotherapy with uracil–tegafur for adenocarcinoma of the lung. *New England Journal of Medicine*, *350*(17), 1713-1721.

Leon, G., MacDonagh, L., Finn, S. P., Cuffe, S., & Barr, M. P. (2016). Cancer stem cells in drug resistant lung cancer: Targeting cell surface markers and signaling pathways. *Pharmacology & therapeutics*, *158*, 71-90.

Levina, V., Marrangoni, A. M., DeMarco, R., Gorelik, E., & Lokshin, A. E. (2008). Drug-selected human lung cancer stem cells: cytokine network, tumorigenic and metastatic properties. *PloS one*, *3*(8), e3077.

Liu, J., Xiao, Z., Wong, S. K. M., Tin, V. P. C., Ho, K. Y., Wang, J., ... & Wong, M. P. (2013). Lung cancer tumorigenicity and drug resistance are maintained through ALDHhiCD44hi tumor initiating cells. *Oncotarget*, *4*(10), 1698.

Magee, J. A., Piskounova, E., & Morrison, S. J. (2012). Cancer stem cells: impact, heterogeneity, and uncertainty. *Cancer cell*, *21*(3), 283-296.

Mazieres, J., Antonia, T., Daste, G., Muro-Cacho, C., Berchery, D., Tillement, V., ... & Favre, G. (2004). Loss of RhoB expression in human lung cancer progression. *Clinical cancer research*, *10*(8), 2742-2750.

Mazières, J., Tovar, D., He, B., Nieto-Acosta, J., Marty-Detraves, C., Clanet, C., ... & Favre, G. (2007). Epigenetic regulation of RhoB loss of expression in lung cancer. *BMC cancer*, *7*(1), 220.

Miyata, T., Yoshimatsu, T., So, T., Oyama, T., Uramoto, H., Osaki, T., ... & Gotoh, A. (2015). Cancer stem cell markers in lung cancer. *Personalized Medicine Universe*, *4*, 40-45.

O'Flaherty, J. D., Barr, M., Fennell, D., Richard, D., Reynolds, J., O'Leary, J., & O'Byrne, K. (2012). The cancer stem-cell hypothesis: its emerging role in lung cancer biology and its relevance for future therapy. *Journal of thoracic oncology*, *7*(12), 1880-1890.

Parsons, A., Daley, A., Begh, R., & Aveyard, P. (2010). Influence of smoking cessation after diagnosis of early stage lung cancer on prognosis: systematic review of observational studies with meta-analysis. *Bmj*, *340*, b5569.

Peacock, C. D., & Watkins, D. N. (2008). Cancer stem cells and the ontogeny of lung cancer. *Journal of clinical oncology: official journal of the American Society of Clinical Oncology*, *26*(17), 2883.

Pine, S. R., Marshall, B., & Varticovski, L. (2008). Lung cancer stem cells. *Disease markers*, *24*(4-5), 257-266.

Ponti, D., Costa, A., Zaffaroni, N., Pratesi, G., Petrangolini, G., Coradini, D., ... & Daidone, M. G. (2005). Isolation and in vitro propagation of tumorigenic breast cancer cells with stem/progenitor cell properties. *Cancer research*, *65*(13), 5506-5511.

Ridley, A. J. (2013). RhoA, RhoB and RhoC have different roles in cancer cell migration. *Journal of microscopy*, *251*(3), 242-249.

Sahai, E., & Marshall, C. J. (2002). RHO–GTPases and cancer. *Nature Reviews Cancer*, *2*(2), 133-142.

Sato, N., Fukui, T., Taniguchi, T., Yokoyama, T., Kondo, M., Nagasaka, T., ... & Minna, J. D. (2007). RhoB is frequently downregulated in non- small- cell lung cancer and resides in the 2p24 homozygous deletion region of a lung cancer cell line. *International journal of cancer*, *120*(3), 543-551.

Siegel, R. L., Miller, K. D., & Jemal, A. (2016). Cancer statistics, 2016. *CA: a cancer journal for clinicians*, *66*(1), 7-30.

Singh, S. K., Clarke, I. D., Terasaki, M., Bonn, V. E., Hawkins, C., Squire, J., & Dirks, P. B. (2003). Identification of a cancer stem cell in human brain tumors. *Cancer research*, *63*(18), 5821-5828.

Visvader, J. E., & Lindeman, G. J. (2012). Cancer stem cells: current status and evolving complexities. *Cell stem cell*, *10*(6), 717-728.

Walter, D., Satheesha, S., Albrecht, P., Bornhauser, B. C., D'Alessandro, V., Oesch, S. M., ... & Moch, H. (2011). CD133 positive embryonal rhabdomyosarcoma stem-like cell population is enriched in rhabdospheres. *PloS one*, *6*(5), e19506

Wang, S., Yan-Neale, Y., Fischer, D., Zeremski, M., Cai, R., Zhu, J., ... & Cohen, D. (2003). Histone deacetylase 1 represses the small GTPase RhoB expression in human nonsmall lung carcinoma cell line. *Oncogene*, *22*(40), 6204-6213.

Wang, P., Gao, Q., Suo, Z., Munthe, E., Solberg, S., Ma, L., ... & Gaudernack, G. (2013). Identification and characterization of cells with cancer stem cell properties in human primary lung cancer cell lines. *PLoS one*, *8*(3), e57020.

Weiswald, L. B., Bellet, D., & Dangles-Marie, V. (2015). Spherical cancer models in tumor biology. *Neoplasia*, *17*(1), 1-15.

Wicha, M. S., Liu, S., & Dontu, G. (2006). Cancer stem cells: an old idea—a paradigm shift. *Cancer research*, *66*(4), 1883-1890.

Winton, T., Livingston, R., Johnson, D., Rigas, J., Johnston, M., Butts, C., ... & Fry, W. (2005). Vinorelbine plus cisplatin vs. observation in resected non–small-cell lung cancer. *New England Journal of Medicine*, *352*(25), 2589-2597.

Zakaria, N., Satar, N. A., Halim, A., Hanis, N., Ngalim, S. H., Yusoff, N. M., ... & Yahaya, B. H. (2017). Targeting lung cancer stem cells: research and clinical impacts. *Frontiers in oncology*, *7*, 80.

Zhong, Y., Guan, K., Guo, S., Zhou, C., Wang, D., Ma, W., ... & Zhang, S. (2010). Spheres derived from the human SK-RC-42 renal cell carcinoma cell line are enriched in cancer stem cells. *Cancer letters*, *299*(2), 150-160.

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Development of Protein-Rich Fish Feed by Using *Gammarus sp.* and Chitosan

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Abstract: Aquaculture is the cultivation of aquatic animals and plants under controlled or semi-controlled conditions. Aquaculture meets most of the world's fish needs. Due to the high nutritional value of fish and meeting the nutritional needs, interest in aquaculture has increased. In aquaculture, the most spending is made on fish feed. By reducing this cost, an increase in fish production can be achieved and incentives for fish farming can be supported. The main protein source used in fish farming is fish meal. However, it has been getting harder to supply fishmeal recently and its price is increasing rapidly. It is of great importance to investigate high nutritional and protein-rich sources in fish feed. The target with quality and balanced feed is to ensure that the fish reach the market weight as soon as possible. In this study, Gammarus and chitosan were used as feed additives. The effect of gammarus, which is rich in protein, on growth and development performance of rainbow trout juveniles was investigated by using it as a feed additive together with commercial feed. It was aimed to achieve this goal by coating with non-toxic, biocompatible chitosan in order to ensure that the feed composition has a high nutritional value, sufficient and balanced, as well as providing resistance against diseases. As a result, it was determined that the coating of fish feeds with Gammarus and chitosan have a growth-supporting effect and, together with this, they provide resistance against diseases in fish. In this regard, we believe that our work has the potential to bring innovation to the fish feed industry.

Keywords: Gammarus, chitosan, fish feed.

Introduction

In aquaculture, it is a wide and important field of production and science that includes the principles of aquaculture, by taking the development of aquatic organisms under the most suitable environmental conditions, without disturbing the ecological structure of water resources, by taking the natural environment and natural game stocks under control. In the field of production, technological and scientific developments have made significant contributions to aquaculture in the last 60 years (Bostock, 2011).

One of the main problems of the developing world and increasing population is nutrition. The animal protein needs of the rapidly growing population cannot be met. On the other hand, the need for protein is constantly increasing. In order to meet this need, aquaculture fishing and cultivation are carried out. However, it is not sufficient for animal protein needs. In our country, especially in landlocked inland fish consumption is well

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below the average in Turkey with red meat are to meet the demand for animal protein. Considering the difficulties in terrestrial animal husbandry and the increasing costs to meet the animal nutritional needs, it should be considered that we should give due importance to aquaculture. Aquaculture has been started to meet the animal protein needs of fishery products, which have important nutritional value for human health (Korkut et al., 2002).

In addition to the continuous development in aquaculture, it has brought various problems. Intensive production in aquaculture has caused a significant increase in fish diseases. Diseases spread and huge economic losses were experienced due to wrong drug use and wrong information. The use of chemical drugs in the fight against diseases has increased costs and caused immunity to drugs over time. A solution was sought against this problem with the additives of natural products, not chemicals, in fish diseases. It is aimed to increase resistance to diseases, strengthen the immune system and accelerate growth by adding natural products to fish meal. The fact that natural additives do not leave residues on fish and do not threaten human health has made it inevitable to carry out different studies in aquaculture (Kabak, 2009).

In this study, we worked on two feed additive materials to solve the problem and contribute to healthy and efficient fish production activities. In our study, the protein-rich *Gammarus sp.* and we will describe the immunostimulating and immune-supportive chitosan.

Gammarus sp.

Gammarids are commonly used organisms for risk assessment of freshwater quality criteria (Rinderhagen et al., 2000; Serdar et al., 2018). Generally, they are found in the source parts of rivers and are an important food source for fish, birds, and amphibians (MacNeil et al., 2002), and leaf litter plays an important role in the breakdown process (Forrow & Maltby, 2000).

Table 1. Chemical composition of Gammarus sp.

%	Taleb et al, 2020-Abo	Harlıoğlu & Farhadi, 2018
Protein	40	40-45
Lipid	5,5	5-10
Carbohydrate	27,4	6-15
Ash	21,4	25-35

It has been reported in previous studies that *Gammarus sp.* consists of 40-45% protein and chitosan consists of 12% protein.

Chitosan

Chitin, a natural polysaccharide that is the most abundant in nature after cellulose, is the main component of the exoskeletons of the cell walls of crab, insect, fungus and some fungi and green algae. Chitosan is one of the best known non-toxic polymers that can be broken down naturally, adapt to our body. Chitosan [poly-β- (1 → 4) N-acetyl-D-glucosamine] differs from the chitin molecule is the deacetylated [N-deacetylated] form of chitin (Jikakis, 1984; Chhabra, 1999).

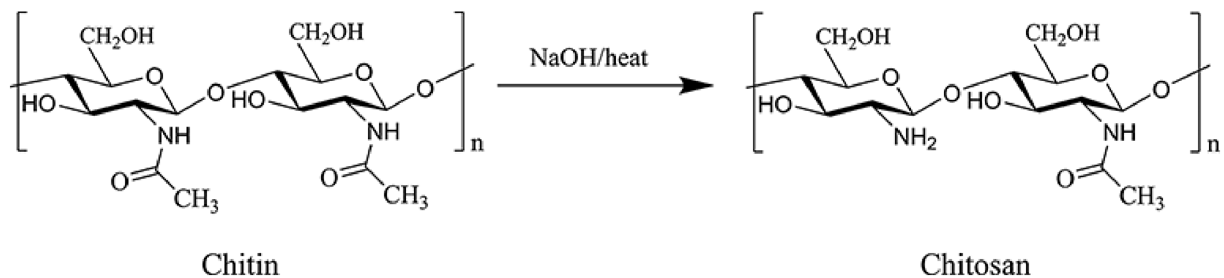

Figure 1. Chitin deacetylation process to produce chitosan.

The deacetylation degree (DD) of chitosan is calculated by the ratio of 2-acetamido-2-deoxy-D-glucopyranose units to 2-amino-2-deoxy-D-glucopyranose units. The degree of deacetylation has been found to have significant antimictrobial effects on some bacterial species (Tsai et al., 2006).

Method

Preparation of Gammarus sp.

Gammarus sp. were collected from the wetland in Gaziantep province Karkamış district. *Gammarus sp.* to be used as feed material were cleaned after being collected and shocked at -80°C.
After shocking, *Gammarus sp.* were homogenized (Figure 2) and distributed in sterile sample containers with cover (Figure 3). Later, the samples were kept at -40°C for 2 days and the excess water was taken and freeze dried in the lyophilizer (Figure 4).

Figure 2. Grinding of *Gammarus sp.*

Figure 3. Sharing of *Gammarus sp*. to sterile sample containers

Figure 4. Freeze drying in lyophilizer

Figure 5. *Gammarus sp.* to be used as feed covering material.

Preparation of chitosan

Commercially available crayfish *(Astacus leptodactylus)* were washed cleaned and dried (Figure 6).

Figure 6. Cleaning and drying Freshwater Lobsters *(Astacus leptodactylus)*

The dried crayfish were ground into powder (Figure 7) and stored at + 4°C until use (Figure 8).

Figure 7. Grinding Freshwater Lobsters

Figure 8. Keeping Freshwater Lobsters at + 4°C

Chitosan Synthesis from Chitin

The chitosan synthesis from chitin consists of 4 steps:
Deproteinization: Proteins are removed.
Demineralization: Minerals are removed.
Decoloration: Bleaching is done.
Deacetylation: Acetyl groups are removed and chitosan is synthesized from chitin.

For deprotenization, the sample was treated in 3.5% NaOH [1:10, (w / v)] solution at 65oC for 2.5 hours. At the end of the process, the sample was washed several times with distilled water and dried in the oven. For demineralization, the sample was treated in 1N HCl [1:15, (w / v)] solution at room temperature for 30 minutes under continuous stirring. At the end of the process, the sample was washed several times with distilled water and dried in the oven. For decoloration, acetone & 0.315% NaOCl [1:10, (w / v)] was used (No et al., 1989).

The chemical production of chitosan from chitin is based on the removal of acetyl groups in chitin using high alkaline solutions. For deacetylation, Koçer (2015) performed the deacetylation process in an autoclave (121°C; 15 minutes) in his master's thesis. He stated that the use of autoclaves increased the deacetylation efficiency (18%). In our study, we performed the deacetylation process in an autoclave. At the end of the process, the sample was washed with distilled water and dried in an oven.

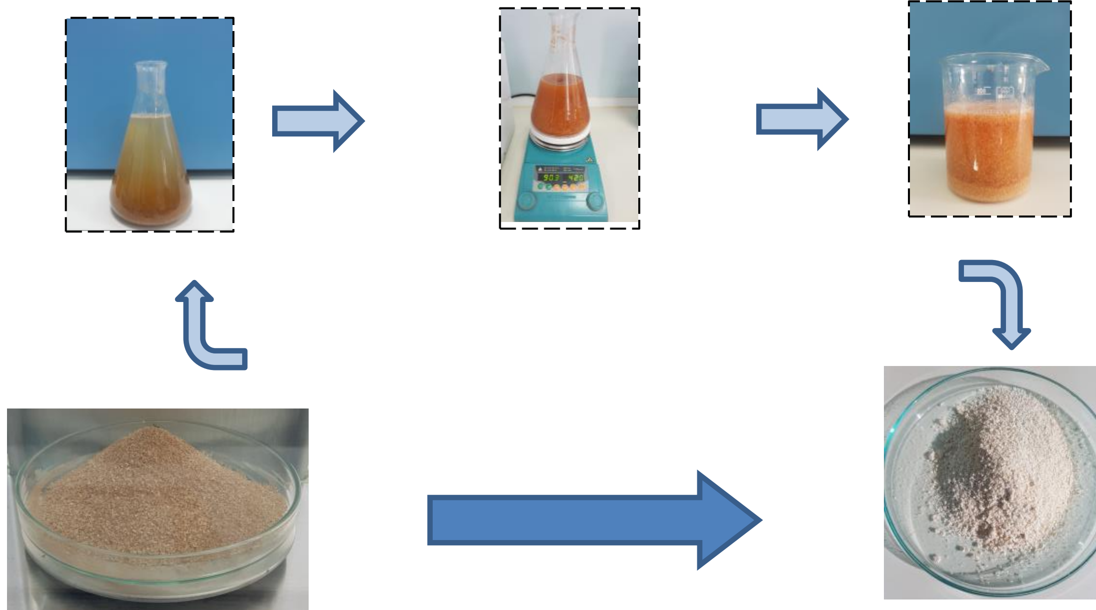

Figure 9. Chitin

Figure 10. Chitosan

Figure 11. Scheme showing chitosan synthesis from chitin

Findings

FT-IR Analysis and Determination of Deacetylation Degree

FT-IR peaks of chitin and chitosan are given in figure 12. chitin is shown in red and chitosan in green. Here, it is seen that chitin in the spectrum of 3550-3230 (OH band) gives a peak at 3259.19 cm^{-1}. Chitosan is observed to give two sharp peaks in the OH band interval. In the spectrum of 1660-1600 (amide I band), it is seen that chitin gives a peak at 1629.41 cm^{-1}. Chitosan is observed to give two sharp peaks in the amide I band range. This indicates the presence of bonds and purification in chitosan.

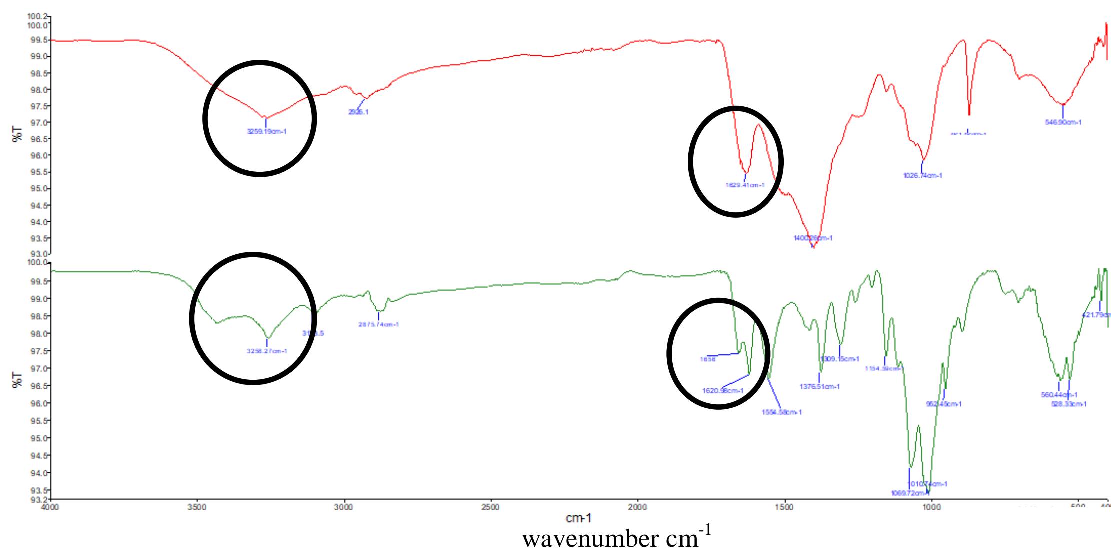

Figure 12: FT-IR analysis result

$$*DA(\%)=[(A_{1655}/A_{3450})x100/1,33]$$

Kitosan DA= **%85,705**

*Domszy & Roberts, 1985

FT-IR analysis results were used to calculate the degree of deacetylation. The calculation was made using the equation suggested by Domszy & Roberts (1985). The deacetylation degree of chitosan that we purified was found to be 85.705%. The protein amounts of Chitosan and *Gammarus sp.* are shown in the table (Table 2). BCA method was used for protein determination. Two solvents are used; PBS, ammonium bicarbonate. While the protein amount of *Gammarus sp.* was found to be 800,217 µg/mL in PBS, it was found 738,673 µg/mL in ammonium bi carbonate. The amount of protein was determined mostly in PBS. While the protein PBS of chitosan was found to be 577,143 µg/mL, it was found as 632,063 µg/mL in ammonium bicarbonate. The amount of protein was determined mostly in ammonium bicarbonate. When the data obtained are examined, it is seen that high amounts of protein are detected.

Table 2. Gammarus sp. and chitosan crude protein amounts

	µg/mL
Gammarus sp. (PBS)	800,217
Gammarus sp. (Ammonium bicarbonate)	738,673
Kitosan (PBS)	577,143
Kitosan (Ammonium bicarbonate)	632,063

Conclusion

In this study, synthesis was carried out using the method recommended in the literature for chitosan extraction from chitin (No et al., Koçer, 2015). In previous studies, the deacetylation degree of chitosan was reported as 69.4-77%. The deacetylation degree of chitosan we obtained in our study is 86%. We used the same method in our study and were able to obtain purer chitosan. This indicates that chitosan may be more effective in our study.

Note: We shared the work we did from Kadriye GUNBATTI's ongoing Master Thesis in this study.

Acknowledgment

This study is supported by Gaziantep University Scientific Research Projects Coordination Unit with the project number FEF.YLT.20.07.

This study was approved by Gaziantep University Animal Experiments Local Ethics Committee. GAÜN-HADHEK approval was obtained. In the continuation of our work, Fırat Su Ürünleri Ltd. We will do it in Şti. Thank you to this business for giving us the opportunity.

Reference

A., Abo-Taleb, H., F Zeina, A., Ashour, M., M Mabrouk, M., E Sallam, A., & MM El-feky, M. (2020). Isolation and cultivation of the freshwater amphipod *Gammarus pulex* (Linnaeus, 1758), with an evaluation of its chemical and nutritional content. *Egyptian Journal of Aquatic Biology and Fisheries, 24*(2), 69-82.

Bostock, J. (2011). The application of science and technology development in shaping current and future aquaculture production systems. *Journal of Aqricultural Science*, *149,* 133-141.

Chhabra, P. (2004). Antimicrobial and antioxidant properties of chitosan. Unpublished Doctoral Dissertation, University of Georgia.

Domszy, J. G., & Roberts, G. A. (1985). Evaluation of infrared spectroscopic techniques for analysing chitosan. *Die Makromolekulare Chemie: Macromolecular Chemistry and Physics, 186*(8), 1671-1677.

Forrow, D. M., & Maltby, L. (2000). Toward a mechanistic understanding of contaminant‐ induced changes in detritus processing in streams: Direct and indirect effects on detritivore feeding. *Environmental Toxicology and Chemistry: An International Journal, 19*(8), 2100-2106.

Harlıoğlu, M. and Farhadi, A. (2018*): Importance of Gammarus in aquaculture. Aquac. Int., 26*: 1327–1338. J. P. Jikakis (Ed.) (1984), Chitin, Chitosan and Related Enzymes, *Academic, Orlando, FL, pp. XVII–XVIV.*

Kabak, T., (2009). *Gökkuşağı alabalık (Oncorhynchus mykiss)'larının spesifik olmayan immün sistemi üzerine bazı geofit bitki ekstraktlarının etkilerinin incelenmesi.* Süleyman Demirel Üniversitesi Fen Bilimleri Enstitüsü Su Ürünleri Yetiştiriciliği Ana Bilim Dalı Yüksek Lisans Tezi.

Koçer, İ. (2015). *Farklı yöntemlerle kitosan eldesi ve karakterizasyonu.* Hacettepe Üniversitesi Fen Bilimler Enstitüsü Kimya Mühendisliği Anabilim Dalı Yüksek Lisans Tezi.

Korkut, A. Y., Hoşsu, B., Gültepe, N., (2002). Balıklarda Beslenmeye Bağlı Hastalıklar. *E.U. Journal of Fisheries & Aquatic Sciences*. *19,* 555–564.

MacNeil, C., Dick, J. T., Bigsby, E., Elwood, R. W., Montgomery, W. I., Gibbins, C. N., & Kelly, D. W. (2002). The validity of the Gammarus: Asellus ratio as an index of organic pollution: abiotic and biotic influences. *Water Research, 36*(1), 75-84.

No, H. K., Meyers, S. P., & Lee, K. S. (1989). Isolation and characterization of chitin from crawfish shell waste. *Journal of Agricultural and Food Chemistry*, *37*(3), 575-579.

Rinderhagen, M., Ritterhoff, J., & Zauke, G. P. (2000). Crustaceans as bioindicators. *In Biomonitoring of Polluted Water-Reviews on Actual Topics. Trans Tech Publications-Scitech Publications, Environmental Research Forum, 9,*161-194.

Ruiz, G. A. M., & Corrales, H. F. Z. (2017). Chitosan, Chitosan Derivatives and their Biomedical Applications, *Biological Activities and Application of Marine Polysaccharides, Emad A. Shalaby, IntechOpen,* DOI: 10.5772/66527.

Serdar, O. (2019). The effect of dimethoate pesticide on some biochemical biomarkers in *Gammarus pulex. Environmental Science and Pollution Research, 26*, 1–10.

Tsai, G. J., Tsai, M. T., Lee, J. M. ve Zhong, M. Z. (2006). Effects of Chitosan and a Low-Molecular-Weight Chitosan on Bacillus Cereus and Application in the Preservation of Cooked Rice. J. *Food Protect. 69,* pp. 2168-2175.

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Zoonotic Epidemics of Coronaviruses, Emergence of Covid-19, and Future Perspective for Zoonotic Diseases

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Abstract: Infectious emerging and re-emerging zoonotic epidemics are of greater threat for public health. Coronaviruses are group of related viruses that are mainly considered to cause diseases in mammals, birds and humans. During last two decades, it spilled over three times by zoonotic pathways with genetic modification and emerged as severe acute respiratory syndrome coronavirus 1 (SARS-CoV-1), middle-east respiratory syndrome coronavirus (MERS-CoV) and severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). In December 2019, pneumonia of unknown origin currently known as COVID-19 was reported which is linked to the wet market of Wuhan, China, has drained the attention of the world as global pandemic with 34,495,176 cases, 1,025,729 deaths from all territories of world (WHO, Weekly Epidemiological Update 2 October 2020). Complete genomic sequencing and phylogenetic studies revealed that SARS-CoV-2, a beta coronavirus, originated from bats with remaining codons of an intermediate host, which is still under research. SARS-CoV-2 is highly pathogenic and human to human spread makes it more virulent. Government institutions and private organizations have boosted the research for drug, vaccine and immunoglobulin development which are now under clinical trial phases. AI helped a lot in this panic situation and advancement in such technologies can help in combating such baffling battles. Therefore, collaborative world response and investment to reinforce research for technological advancements and strategically development in various disciplines to control future pandemics is perquisite.

Keywords: Global Pandemic, COVID-19, SARS-CoV-1, MERS-CoV, Zoonotic epidemics

Introduction

Emerging and re-emerging zoonotic outbreaks are a significant threat to public health like Hantavirus pulmonary syndrome, Henipa and Ebola viral diseases. Coronaviruses are positive sense, single stranded enveloped large RNA molecule with a molecular weight ranging between 29 to 32 kilobases virus having crown-like spikes and broad distribution in humans, mammals, and birds, causing respiratory, neurological, and enteric diseases (Lai et al., 2007). Since the first zoonotic emergence of Severe Acute Respiratory Syndrome (SARS-CoV-1) in 2002, coronaviruses have become a global public threat (Zhong et al., 2003). The continued evolution resulted in a second zoonotic epidemic, Middle East Respiratory Syndrome (MARS-CoV), in 2012, which caused a high fatality and gained the world's attention (Zaki et al., 2012). Given the wider distribution and prevalence, recombination of genetic material, an adaptation of new host through evolution, cross-species

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transmission makes the coronavirus emerge periodically with fatal outbreaks (Zhu et al., 2020). At present, the world is concerned about the third major zoonotic epidemic of a newly emerged coronavirus disease.

At the end of December 2019, pneumonia of unknown origin was reported which is linked to the wet market of Wuhan, China (P. Zhou et al., 2020). The epidemiological and etiological investigation was done on 31 December 2019 by the Center for Disease Control (CDC). Similar to other CoVs, the newly emerged novel coronavirus also affected the respiratory system, and clinical investigation of the affected patients showed symptoms of fever, cough, and rarely gastrointestinal infection (Guo et al., 2020; Kim et al., 2020). The genetic sequencing of the sampled virus revealed the novel coronavirus, an etiological microbe for the unknown disease. Phylogenetic studies showed that this is a different virus from SARS and MERS CoVs, originated from bats with an intermediate host, which is still unknown (Lu et al., 2020; P. Zhou et al., 2020; Zhu et al., 2020). World Health Organization (WHO) named SARS-CoV-2 to this newly emerged novel coronavirus and COVID-19 to the disease caused by this virus. Due to a large number of cases and wide spreading of conditions across the countries, on 30 January 2020, WHO declared COVID-19 a public health emergency of international concern. SARS-CoV-2 is highly pathogenic, with 34,495,176 cases, 1,025,729 deaths from all territories of the world (Weekly Operational Update 2 Oct, 2020). Further epidemiological surveillance, phylogenetic studies, medicine for treatment and active and passive immunization strategies for control are under investigation.

This review is focused on present insight of zoonotic epidemics caused by coronaviruses, their structure, phylogenetic origin, organization, and divergence of SARS-CoV-2 genome and to summaries the epidemiology, pathogenesis, clinical features of the COVID-19 along with prevention strategies to combat the disease on the bases of advances in previous research, with the aim to provide the brief recent research progress that will be helpful for epidemiologist, biologist and clinicians for disease investigation, drug and biologics development, and future planning for control of zoonotic epidemics.

Coronavirus Taxonomy

Nidovirales order contains three families of RNA viruses, including Roniviridae, Arteriviridae, and Coronaviridae (Figure 1). Coronaviridae is subdivided into two subfamilies, Torovirinae and coronavirinae respectively. Torovirinae mainly affects gastrointestinal tract of goats, pigs, horses, cats, and cattle. Up till now, this subfamily is not involved in infections, disturbing human health as they don’t have zoonotic propagation (Burrell et al., 2016). Coronavirinae is further subdivided in to four genera. Alpha-CoV infects humans and animals, Beta-CoV infects humans like severe acute respiratory syndrome corona virus (SARS-CoV) and middle east respiratory syndrome coronavirus (MERS-CoV) and animals, Gamma-CoV infects birds and whales, and Delta-CoV infects birds and pigs. Hibecovirus, Sarbecovirus, Embecovirus, Nobecovirus and Merbecovirus are subgenera of Beta-CoV (Cui et al., 2019). The CoV affects the respiratory system and mainly infect bats, dogs, mice, cattle and birds (Perlman and Netland, 2009). Saif (2004) reported that during the 20-century coronavirus was considered to cause only a minor respiratory disorder like 229E, NL63, HKU1 and OC43. In 21th century the old concept about the coronavirus changed entirely after the SARS-CoV attack in china (Peiris et al., 2003a) and the MERS-CoV report in Saudi Arabia (Zaki et al., 2012). To et al. (2013) stated that almost all coronaviruses are transmitted through animal (zoonotic).

Severe Acute Respiratory Syndrome Coronavirus 1 (SARS-Cov-1)

The SARS was transmitted through close contact and droplets being produced during cough. It was a febrile syndrome that mainly affects the respiratory system leading to pneumonia. In November 2002, it was the first time reported in the Chinese province Guangdong (Woodhead et al., 2003). Later in 2003 February, it spreads to Hong Kong and five continents infecting approximately 30 other countries (Control and Prevention, 2003). Peiris et al. (2003b) stated that SARS-CoV spread as epidemically affecting 8000 people out of which 774 people died. The etiological agent for SARS was isolated and named novel coronavirus (SARS-CoV), categorized as Beta-coronavirus (Drosten et al., 2003; Ksiazek et al., 2003). The case fatality rate (CFR) proposed during the SARS-CoV outbreak was 9.6% (Leung et al., 2004). The significant sign appears in humans during severe condition is acute respiratory distress syndrome, its proportion is 16% but it increases the CFR from 9.6% to 50% (Fowler et al., 2003; Lew et al., 2003). Masked palm civet was considered the reservoirs of SARS 2003 and 2004 epidemics from zoonotic point of view (Guan et al., 2003; Kan et al., 2005; Song et al., 2005). Bats, belonging to mammalian family are also considered as reservoir of SARS-CoV (Figure 2) as it is also involved in many virus transmissions in last 20 years like Nipah, Rabies, Ebola, Hendra and

Menangle (Leroy et al., 2005; Mackenzie et al., 2001). Shi and Hu (2008) stated that SARS-CoV mainly found in Horseshoe bats but civet cats are carrier host of the virus. It can be transmitted zoonotically to humans and can cause massive pathogenicity.

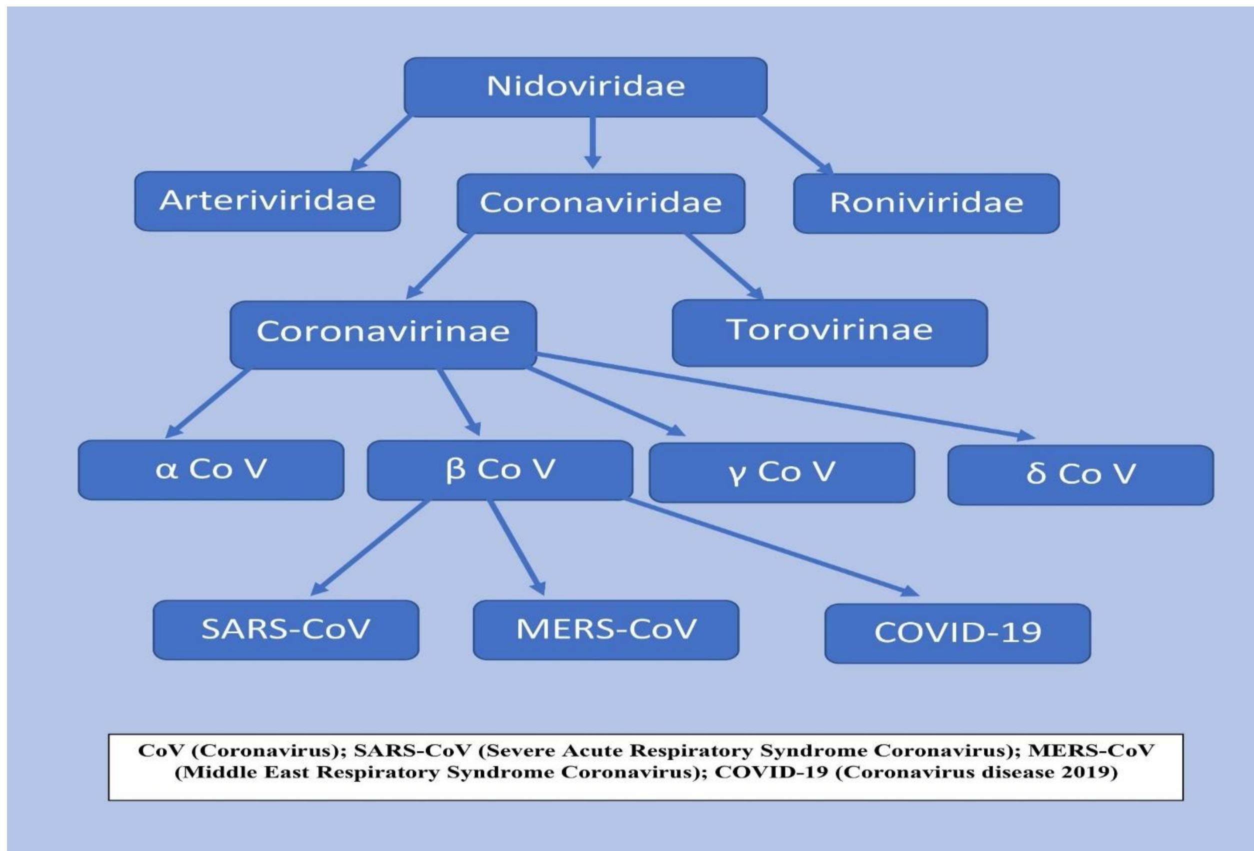

Figure 1. Flow chart diagram of Nidoviridae

Middle East Respiratory Syndrome Coronavirus (MERS-Cov)

In June 2012, MERS-CoV was firstly reported in Saudi Arabia (Zaki et al., 2012). Earlier, author named it as human coronavirus, but the International Committee on Taxonomy of Viruses later named it as MERS-CoV (Murphy et al., 2012). It was ranked as second coronavirus zoonotic disease propagating through bats to humans (Sharif-Yakan and Kanj, 2014). The first infected person was died due to respiratory disorder leading to renal failure. The MERS-CoV was genetically studied and found to be most similar to bat CoV (Cui et al., 2019). The total no. of infected people in 27 countries with MERS-CoV was reported as 2468 in December 2019 (Baharoon and Memish, 2019) with a mortality rate of 36% (Arabi et al., 2017). The later authors stated that most recently 186 new cases reported in South Korea, out of which 35 died. About 80% of all infected people with MERS-CoV belongs to Saudi Arabia. Camel was considered as a reservoir of MERS-CoV (Figure 2) but the genetic sequence matches with bat virus (Corman et al., 2014; Omrani et al., 2015; Sabir et al., 2016). Generally human contact with bats is rear, but camels are in constant contact with humans since ancient times. MERS-CoV transferred to camels from bats and spread to Africa and middle east territories rich with camel population (Hemida et al., 2014). Transmission of MERS-CoV to humans from camels has been reported in Africa, the Arabian Peninsula and Pakistan (Memish et al., 2014; Omrani et al., 2015; Zheng et al., 2019). The coronavirus strain isolated from camels and humans are genetically as well as phonetically identical (Farag et al., 2015). For diagnosis of infection sample from throat swab tested at nucleic acid level, but chest image and symptoms help in clinical diagnosis.

Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-Cov-2)

In the start of January 2020, rapidly propagating human pathogenic disease spreading zoonotically has been ranked third beta-CoV disease (Cui et al., 2019; Huang et al., 2020). The novel 2019 CoV noted as 2019nCoV

resembles with previous out breaks of SARS-CoV-1 and MERS-CoV having the same etiological agent beta-CoV with alterations (Rodriguez-Morales et al., 2020). The first case was reported in 12th of December, 2019 in china (F. Wu et al., 2020) in Hubei province Wuhan city, showing resembling signs with pneumonia named SARS-CoV-2 (Q. Li et al., 2020; Zhu et al., 2020). Within a few weeks, this infection spreads all over the world, crossing border of different countries from China (Giovanetti et al., 2020; Harries and Takarinda, 2020; Phan et al., 2020). The Chinese scientists and medial focal persons quickly studied the genomic sequence of virus and share it with world to warn them of the dangerous condition (F. Wu et al., 2020). P. Zhang et al. (2020) stated that SARS-Cov-2 genome sequence resembles with bats or having slight modification due to involvement of other mammals in wet market of China. Later on, it started spreading swiftly from human to human with super spreading phenomenon (Riou and Althaus, 2020).

Epidemiology

Novel Coronavirus pneumonia later called COVID-19 was first reported from China in December. Chinese scientist team sent an alert to WHO on 31 December 2019 regarding large no of cases showing signs of flu. Later on, the 5th of January, WHO has press released to warn the world to stop travel movement from China to other countries. This unknown agent causing a large no of infection throughout the public was identified as 2019 novel coronavirus (2019nCoV) on the 7th of January. The cruelty of this virus was showed in front of all world after first death due to 2019nCoV on 11th of January. After this kind of response, first committee on this emergency condition was organized by WHO to tackle and investigate this 2019nCoV on 22th of January. Later on, it spreads rapidly all over the world across border affecting all regions of the world. It was declared as a pandemic by WHO on 11 March, 2020 (Organization, Situation Report – 51, 2020). According to the latest update of WHO 26 March 2020 report, COVID19 has been confirmed in 201 territories (Organization, Situation Report – 66, 2020). It was stated in COVID-19 report no. 59 that the first 10000 cases take round about three months, but the next 100000 cases only take 12 days (Organization, Situation Report – 59, 2020). This swiftly increasing pattern shows the pandemic outbreak of COVID-19. To take a better step for the prevention of such kind of pandemic disease accurate database regarding laboratory data, epidemiological figures, and the clinical records should be provided (Morgan, 2019). Jin et al. (2020) reported that people with older age are more prone to COVID-19, whereas there is no gender discrimination for COVID-19 susceptibility. The later author further stated that female patients are less prone to COVID-19 irrespective of age. The CFR of patients older than 80 years was 14.8% as compared to 40-49 years patients having 0.4% in China (Gao et al., 2020). The ISS (2020) stated that CFR on 13, March in Italy was 21.1% in the age group of more than 90, 17.5% in the age group of 80-89, 10.8% in the age group of 70-79 and 6% for less than 50 years of age. According to CDC report 2.3% was overall Case Fertility Rate (CFR), cases with 70-79 years of age had 8.0% CFR and cases with 80 years of age and older had 14.8% CFR but no death was reported among group of 9 years old and younger ones (Surveillances, 2020). Critical cases had 49.0% CFR while mild and severe cases had no death (P. Wu et al., 2020). The later authors reported that among patients with previous comorbid conditions CFR was raised as following, 6.3% for chronic respiratory diseases, 10.5% for cardiovascular disease, 5.6% for cancer, 7.3% for diabetes and 6.0% for hypertension.

Nioi and Napoli (2020) conducted a trial on malaria and COVID-19 paradox epidemiology. The later authors concluded that under developed countries, which under goes malarial threat in history are less prone to COVID-19 as compared to developed countries. The possible reason for such kind of trend can be linked with treatment of malaria with anti-malarial drugs against virus, leads to development of immunity against viral attacks. To update public regarding latest updates on COVID-19, WHO has launched a service with collaboration with Facebook and WhatsApp. To utilize this portal just send a “Hi” message on +41 798931892 and the user will receive recent updates regarding COVID-19 as well as precautionary measures. Solidarity Trial study plan has been launched by WHO to find more suitable treatment plan with collaboration of keenly working organization on COVID-19.

Phylogenetic Origin of SARS-Cov-2

The epidemic existence of the COVID-19 is not mosaic. The evolution of the virus has a relation to the sea market of Wuhan, China, and this sea market might be the critical reservoir of SARS-CoV-2 (Giovanetti et al., 2020). But still, there is no evidential existence relating to the origin of SARS-CoV-2 to the sea market. Recent complete evolutionary researches refer bat to be an ancestor host of SARS-CoV-2 (Ashour et al., 2020; Lake, 2020; Phan, 2020; P. Zhou et al., 2020). But there is evidence that bats were not being sold in the market, and

only 1.18%of patients had exposure to wildlife (Z. Liu et al., 2020; P. Zhou et al., 2020). Complete genomic sequence interpretation of the SARS-CoV-2 with pangolin and human as host showed higher similarity than rhinolophine and human as host. Pangolin genomic sequences are not just similar but also homologous to SARS-CoV-2. Phylogenetic analysis of the data reveals the similarity of receptors residues from different species like snakes, rabbits and turtles that might be the intermediate host in the emergence of the newly discovered coronavirus (figure 2) (Z. Liu et al., 2020). SARS-CoV-2 is significantly congregated with bat SARS-CoV and different from SARS-CoV-1 (Benvenuto et al., 2020). Recombination in the S glycoprotein of SARS-CoV-2 on the bases of bias usage of codon suggests cross-species transfer from snake as compared to other species (Ji et al., 2020). But some recent studies omitted the snake as an intermediate host of SARS-CoV-2. Further studies are still required to investigate intermediate host responsible for viral transfer from bats to humans. Genomic sequencing of SARS-CoV-2 revealed similarity ranges from 55.6% to 56.1% to the other flu viruses, which shows that they are totally

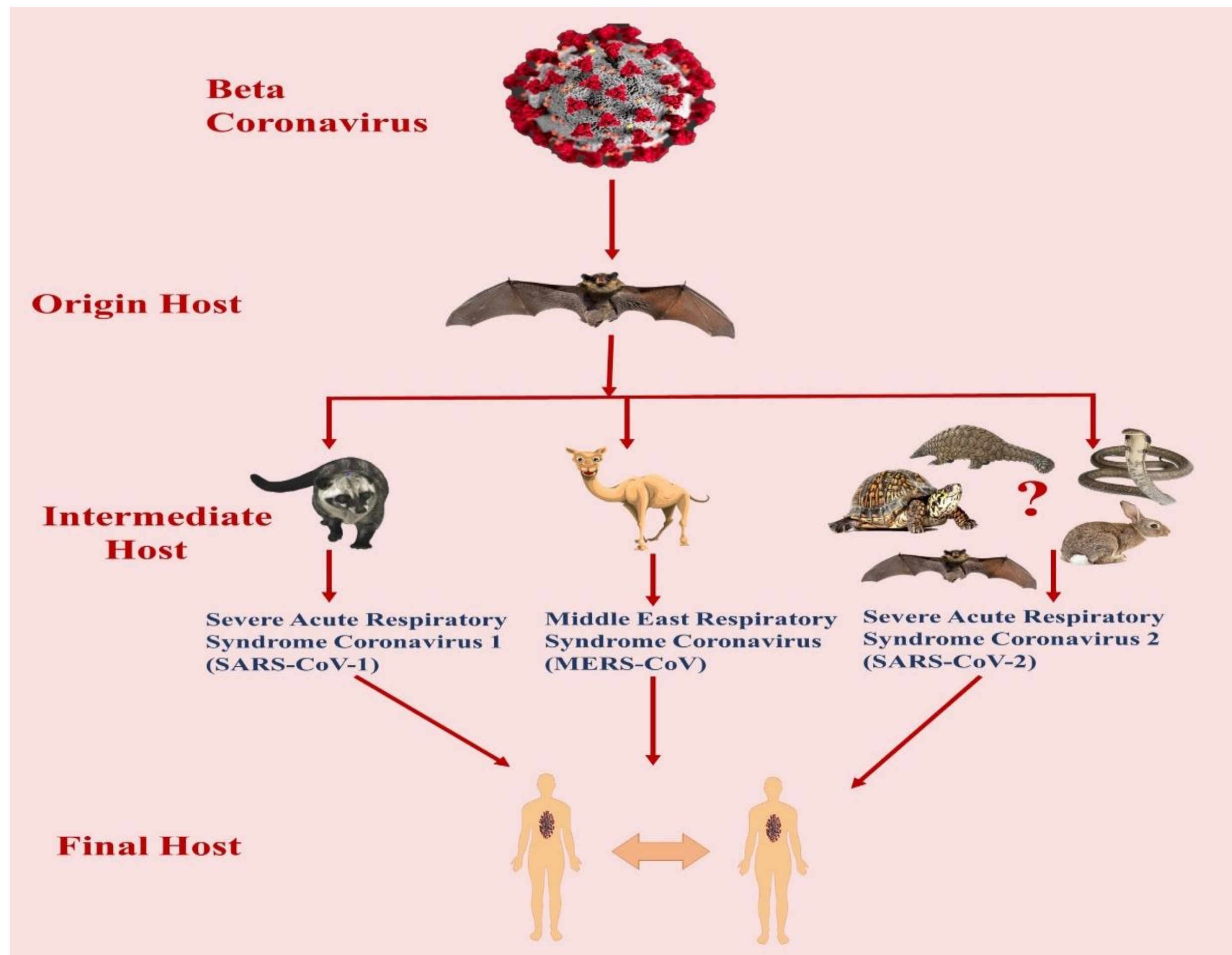

Figure 2. Schematic diagram showing origin of recent zoonotic epidemics from Beta Coronavirus: a serious health threat

different from SARS-CoV-2. For understanding the genomic similarity between previous human host zoonotic CoVs like SARS and MERS, researchers studied the complete genomics of these CoVs. The SARS genome showed a similarity of 82.6%, which was also higher than the similarity of MERS CoV (Guo et al., 2020; Kim et al., 2020). The Phylogenetic tree of SARS-CoV-2 and other beta CoVs, is shown in figure 3. Sequence Identity results of Lu shows 88%, 79% and 50% identity to bat SARS, SARS and MERS COVs respectively, which is lower than the other studies that might be due to the usage of different strains genomic analysis which has undergone a little mutation (Lu et al., 2020). Computational Complete genomic analysis of environmental and body SARS-CoV-2 are homologous with the highest similarity of 99.7%. Twenty-one strains from various countries are homologous and share the same origin that is not astounding because of global traveling (Li et al., 2020). TheSARS-CoV-2 strains from various countries undergo small mutation but are highly similar to each other. Tang classified the SARS-CoV-2 into L and S type based on the population genetic evaluation of SARS-

CoV-2 (Tang et al., 2020). The L type is emerged from the S type and is more contagious. Epidemiologists are required to practically observe and study the virulence and transmission of both types.

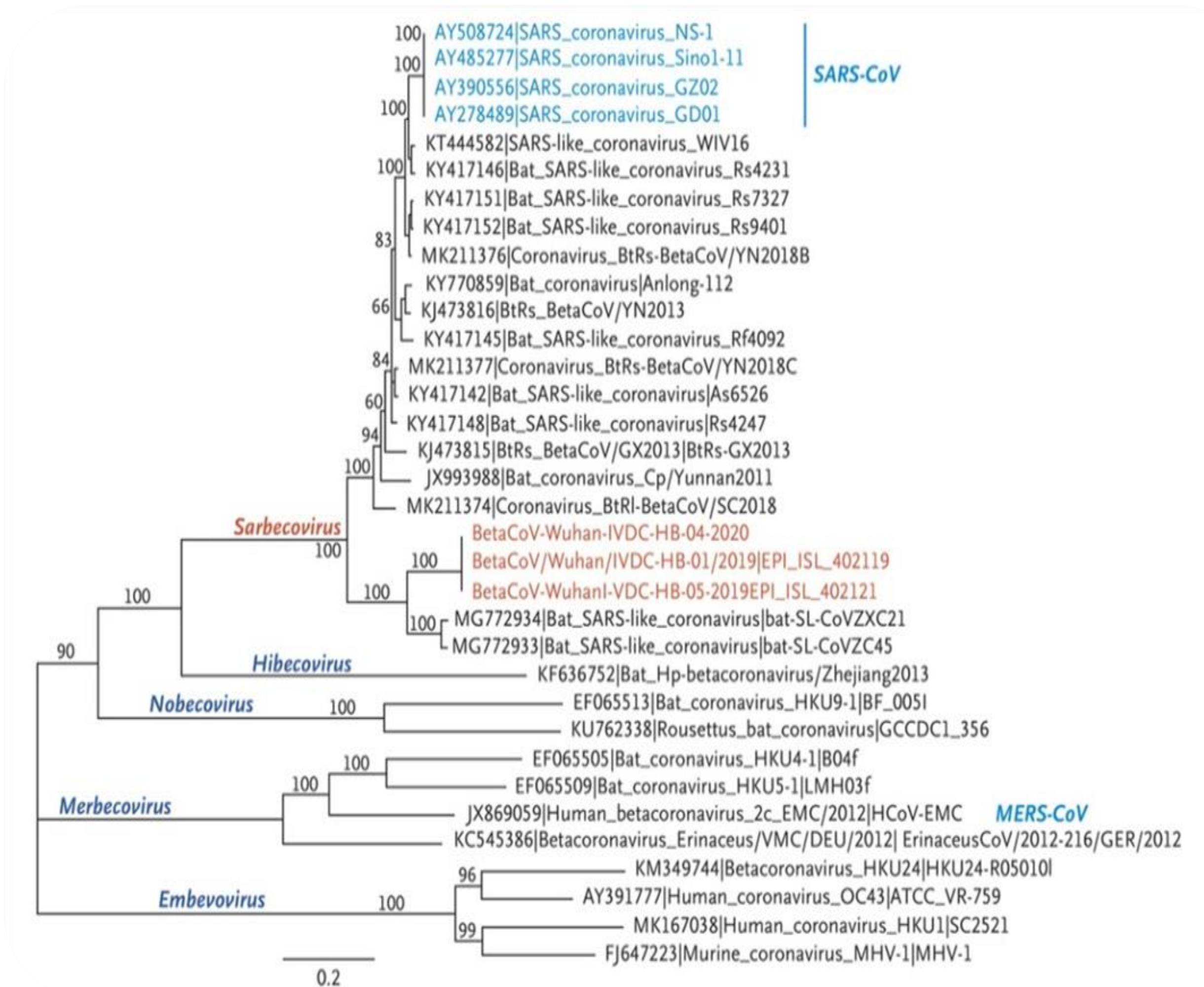

Figure 3. Phylogenetic origin tree between SARS-CoV-2 and other beta corona viruses (Zhou *et al*., 2020)

General Morphology of SARS-CoV-2

SARS-Cov-2 is a spherical, positive sense, single-stranded enveloped RNA virus having crown-like projecting spike proteins from the surface of virion which are also characteristic structure of other CoVs (figure 4). The particle size of the virus ranges from 70 to 90 nm (Kim et al., 2020). The Viral structure is composed of nucleocapsid proteins (N) and structural proteins comprising of an envelope (E), spikes(S) and membrane (M) proteins. The structural and the nucleocapsid proteins are associated with their specific functions. During assembly of RNA into particles N protein interacts with viral RNA (Chang et al., 2006). The S protein comprised of S1 and S2 subunits are highly glycosylated protein that aids for entry of the virus into host cells (Siu et al., 2008). Abundant amount of the M protein in the virus is responsible for giving shape to the virus. The E protein is involved in mature envelope development during the assembly process of the virus.

SARS-CoV-2 Genomic Organization and diversification

SARS-CoV-2 like the other beta coronaviruses shares the similar genomic structure constitute of 5′ and 3′ un-translated regions, six Open–Reading–Frames (ORFs), S (spike), M (membrane), E (envelope) and N (nucleocapsid) genes as shown in figure 5 (Cui et al., 2019; Phan, 2020; P. Zhou et al., 2020). Zhang analysis revealed mutation in viral genome isolated from different pneumonic patients in China (L. Zhang et al., 2020).

Complete genomic analysis showed deletion in genome isolated from Australia, Japan, and America (Phan, 2020). The mutation degree in the SARS-CoV-2 genome was lower as compared to mutation diversity in the avian influenza virus (H7N9) (Wu et al., 2015). Open –Reading –Frames (ORF1ab) polyproteins showed two deletions at three and twenty-four nucleotides and one deletion at 3′end genome. The entire genomic sequence of the SARS-CoV-2 revealed 93 mutations and except E protein, 42 mutations are recognized at structural and non-structural proteins. In ORF1ab polyproteins, S protein, N protein, and M protein twenty-nine, eight, four and one mis-sense mutations are identified. On N region at 380th and 410th amino-acidic position Glutamine is replaced by Asparagine and Threonine residue is replaced by Alanine respectively in viral genome isolated from a pneumonia patient at Wuhan, China. Asparagine residue is replaced by Glutamine and Alanine residue is replaced by Threonine at 409th and 380th amino-acidic positions respectively at clade-II of SARS-CoV-2 strain isolated in Wuhan. Asparagine residue replaced the Aspartic residue and Threonine residue replaced the Alanine residue at 536th and 644th amino-acidic position on S region of Wuhan strain of SARS-CoV-2. At 380th and 309th amino-acidic position SARS-CoV-2 and bat CoV share identical amino-acidic sequence which is different in SARS-CoV-1. S protein amino-acidic sequence of SARS-CoV-2, bat CoV and SARS-CoV-1 at 536th and 644th amino-acidic position is different in all three types of these viruses (Benvenuto et al., 2020).

Conserved classification replicase domains amino acid sequences at ORF1ab among SARS-CoV-1 and SARS-CoV- 2 are 94.4% identical, showing both viruses from same specie origin (P. Zhou et al., 2020). Paraskevis et al. (2020) rejected the emergence theory of SARS-CoV-2 through recombination of the previous COVs and proposed that SARS-CoV-2 come into existence through clustering of bat-SARS-CoV genome. Lu et al. (2020) demonstrated that E gene has highest similarity (98.7%) with two bat- SARS – like CoVs at 13 regions. But codon base analyses of S proteins suggest that SARS-CoV-2 might the result of recombination originated from the coronavirus which is still unknown and un-sampled (Ji et al., 2020). SARS-CoV-2 has higher percentage of pyrimidine than purines. Lower effective numbers of codons make SARS-CoV-2 higher codon bias and highly gene expressive than SARS-CoV-1 and MERS (Kandeel et al., 2020). The addition of the several glycoproteins encodings, adding properties of hemagglutination and acetyl esterase in SARS-CoV-2, make it different from other related beta coronaviruses (Wu and McGoogan, 2020). The S protein is disparate from other SARS-CoVs with nucleotide similarity less than 70%. Furthermore, S gene of SARS-CoV-2 is also larger than SARS-CoV-1. Three insertions at N-terminal of S gene and four key remains in receptor–binding motif divaricate the SARS-CoV-2 spike genomic sequence from other SARS-CoVs spike genomic sequence. Spike protein is responsible for virus host interaction and main target for neutralizing antibodies (Fung and Liu, 2019; Yu et al., 2020). Any alteration in this protein can change the antigenicity and host tropism of the virus. Still no detailed information in the amino acid configuration of the S proteins is not available that must be investigated.

Preventive measures for such zoonotic outbreaks in the future

Genetic spillover of viruses with the passage of time and rapid spread to a massive number of populations is a problem of the hour. Marvelous struggle of the scientists and the governments resulted in reduction in the severity of the outbreak in some countries but some countries are still under big threat. Scientists are working continually in order to control the present situation. A great millstone has been covered with studying the virus, testing drugs and biologics which are under clinical trials. But still there is a gap at molecular level in the origin, cross-species transmission, mutation, pathogenicity, early screening of the suffering patients and epidemiological spread. Re-emergence of the more virulent coronavirus from animals to human is the indication for the future threats to public health. It is therefore recommended to ban legal or illegal farming of wildlife and their trade should also be banned anywhere in the world except Zoo and Safari Parks. To tackle such kinds of infectious epidemic issues in the future, it is highly recommended to test out virus profiles of all the wild animal species encounter directly or indirectly with the human. In-vitro experiments of genetic modification of these viruses and their outcomes with effective treatment and control should be proposed projects for well-established genomic labs. There should be a global backup for such kind of new information under the supervision of the World Health Organization. General public awareness courses should be launched to educate people to handle such type of emergency.

The learned lesson from this outbreak is if a new virus outbreak occurs in any country than all countries should make the risk analysis on emergency basis for prevention and control. National disaster authorities with epidemiologists from medical and veterinary sciences (One Health) interlinking with information technologist can speed up innovation and technology for current and upcoming possible disasters. Surveillance among wildlife to identify the high-risk pathogens they carry and among people who have contact with wildlife to identify early spillover events is the time need. Improvement of market bio security regarding the wildlife trade is an essential component for prevention of emerging zoonotic outbreaks. Priority should be given to the

research regarding future prevention of such outbreaks and transmission of zoonotic diseases. The future of such zoonotic outbreaks is not just dependent on the mutation and re-emergence of pathogens but also based on the strategies, measures and technologies we develop for the future of public health.

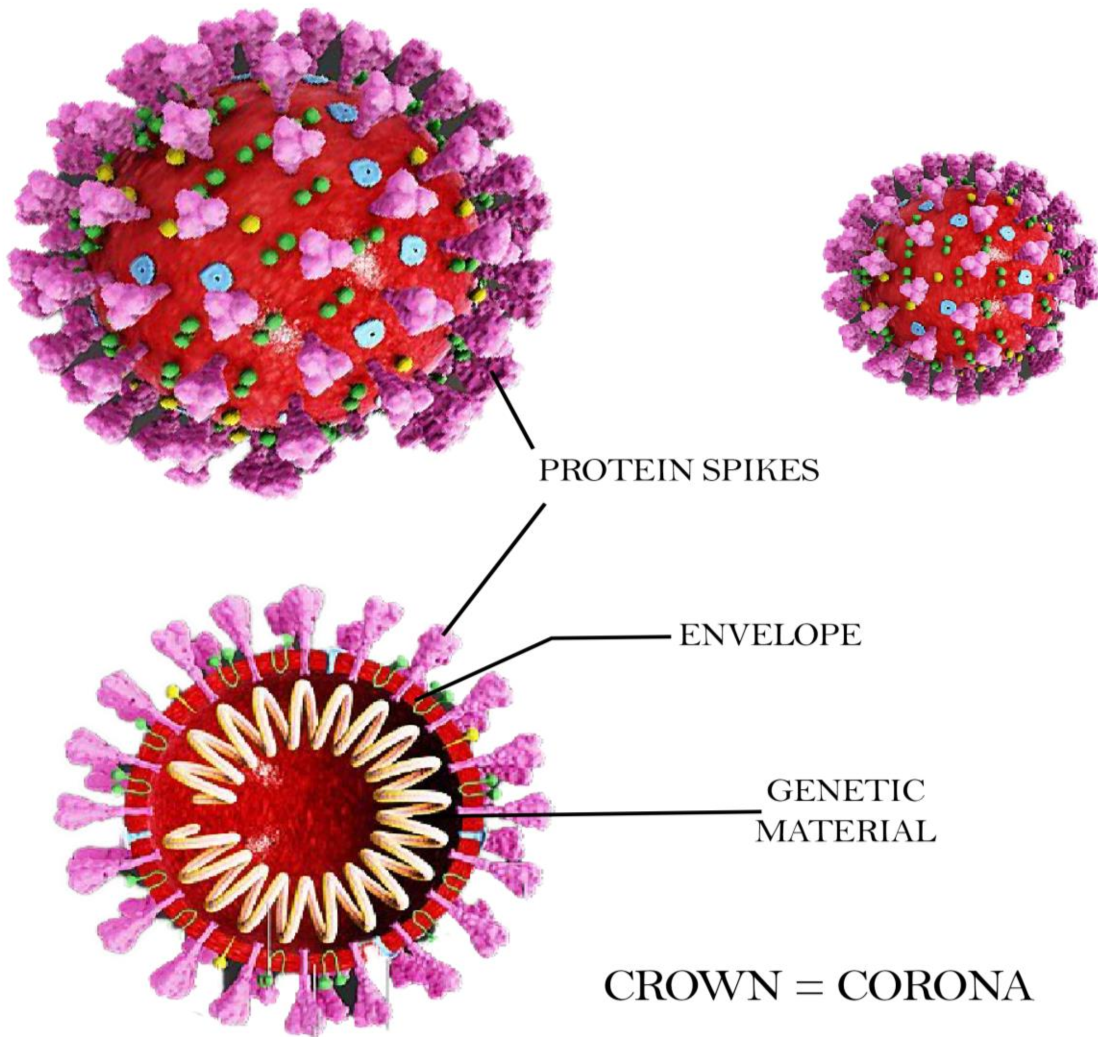

Figure 4. Illustration showing general structure of the newly emerged corona virus SARS-CoV-2

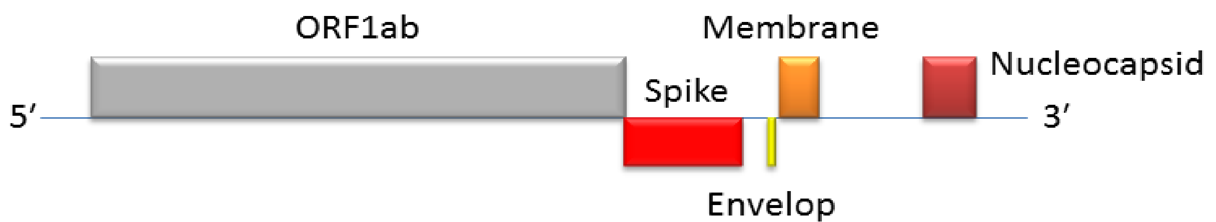

Figure 5. SARS-CoV-2 genomic arrangement demonstrating 5′ and 3′ ends, and various structural

Conclusion

Zoonotic diseases outbreak from coronavirus is still a matter of concern, as it spilled over 3rd time from the wild. In December 2019, COVID-19, has drained the attention of the world. SARS CoV-2 the responsible etiological agent enters to host cell by binding to ACE2 of the host cell and leads to multiple organ failure with most obvious respiratory symptoms (coughing, sneezing, shortness of breath and fever). SARS-CoV-2 most likely has a zoonotic origin (mammals). We should have to focus on three areas specifically to control and prevent such further epidemics and pandemics as following; Monitoring of wildlife animals to identify if they carry any dangerous pathogens, monitoring of people in contact with wildlife animals to diagnose early spillover of disease and biosecurity of wildlife markets should be improved to control and prevent pathogens related to wildlife animals.

References

Arabi, Y. M., Balkhy, H. H., Hayden, F. G., Bouchama, A., Luke, T., Baillie, J. K., Fowler, R. A. (2017). Middle east respiratory syndrome. *New England Journal of Medicine, 37*6(6), 584–594. http://doi:10.1056/nejmsr1408795

Ashour, H. M., Elkhatib, W. F., Rahman, M. M., & Elshabrawy, H. A. (2020). Insights into the recent 2019 novel coronavirus (SARS-CoV-2) in light of past human coronavirus outbreaks. *Pathogens, 9*(3), 186. http://doi:10.3390/pathogens9030186

Baharoon, S., & Memish, Z. A. (2019). MERS-CoV as an emerging respiratory illness: A review of prevention methods. *Travel Medicine and Infectious Disease, 32*, 101520. http://doi:10.1016/j.tmaid.2019.101520

Benvenuto, D., Giovanetti, M., Ciccozzi, A., Spoto, S., Angeletti, S., & Ciccozzi, M. (2020). The 2019-new coronavirus epidemic: Evidence for virus evolution. *Journal of Medical Virology, 92*(4), 455–459. http://doi:10.1002/jmv.25688

Burrell, C. J., Howard, C. R., & Murphy, F. A. (2016). *Fenner and White's medical virology*, Academic Press. Pp.355–365. http://doi:10.1016/b978-0-12-375156-0.00025-4

Chang, C., Sue, S.-C., Yu, T., Hsieh, C.-M., Tsai, C.-K., Chiang, Y.-C., Huang, T. (2005). Modular organization of SARS coronavirus nucleocapsid protein. *Journal of Biomedical Science, 13*(1), 59–72. http://doi:10.1007/s11373-005-9035-9

Corman, V. M., Ithete, N. L., Richards, L. R., Schoeman, M. C., Preiser, W., Drosten, C., & Drexler, J. F. (2014). Rooting the Phylogenetic Tree of Middle East Respiratory Syndrome Coronavirus by Characterization of a conspecific virus from an African bat. *Journal of Virology, 88*(19), 11297–11303. http://doi:10.1128/jvi.01498-14

Cui, J., Li, F., & Shi, Z.-L. (2019). Origin and evolution of pathogenic coronaviruses. *Nature Reviews Microbiology, 17*(3), 181–192. http://doi:10.1038/s41579-018-0118-9

Drosten, C., Günther, S., Preiser, W., van der Werf, S., Brodt, H.-R., Becker, S., Doerr, H. W. (2003). Identification of a Novel Coronavirus in Patients with Severe Acute Respiratory Syndrome. New England. *Journal of Medicine, 348*(20), 1967–1976. http://doi:10.1056/nejmoa030747

Farag, E. A. B. A., Reusken, C. B. E. M., Haagmans, B. L., Mohran, K. A., Raj, V. S., Pas, S. D., Koopmans, M. P. G. (2015). High proportion of MERS-CoV shedding dromedaries at slaughterhouse with a potential epidemiological link to human cases, Qatar 2014. *Infection Ecology & Epidemiology, 5*(1), 28305. http://doi:10.3402/iee.v5.28305

Fowler, R. A. (2003). Critically Ill Patients With Severe Acute Respiratory Syndrome. *JAMA, 290*(3), 367. http://doi:10.1001/jama.290.3.367

Fung, T. S., & Liu, D. X. (2019). Human coronavirus: Host-pathogen interaction. *Annual Review of Microbiology, 73*(1), 529–557. http://doi:10.1146/annurev-micro-020518-115759

Gao, Q., Hu, Y., Dai, Z., Xiao, F., Wang, J., & Wu, J. (2020). The epidemiological characteristics of 2019 novel coronavirus diseases (COVID-19) in Jingmen, Hubei, China. *Medicine, 99*(23), e20605.

Giovanetti, M., Benvenuto, D., Angeletti, S., & Ciccozzi, M. (2020). The first two cases of 2019-nCoV in Italy: Where they come from? *Journal of Medical Virology, 92*(5), 518–521. http://doi:10.1002/jmv.25699

Guan, Y. (2003). Isolation and Characterization of Viruses Related to the SARS Coronavirus from Animals in Southern China. Science, 302(5643), 276–278. http://doi:10.1126/science.1087139

Guo, Y.-R., Cao, Q.-D., Hong, Z.-S., Tan, Y.-Y., Chen, S.-D., Jin, H.-J., Yan, Y. (2020). The origin, transmission and clinical therapies on coronavirus disease 2019 (COVID-19) outbreak – an update on the status. *Military Medical Research, 7*(1). http://doi:10.1186/s40779-020-002400

Hemida, M. G., Chu, D. K. W., Poon, L. L. M., Perera, R. A. P. M., Alhammadi, M. A., Ng, H., Peiris, M. (2014). MERS Coronavirus in Dromedary Camel Herd, Saudi Arabia. *Emerging Infectious Diseases, 20*(7), 1231. http://doi:10.3201/eid2007.140571

Harries, A., & Takarinda, K. C. (2020). F1000Prime recommendation of first case of 2019 novel coronavirus in the United States. F1000 - Post-Publication Peer Review of the Biomedical Literature. http://doi:10.3410/f.737289584.793572167

Huang, C., Wang, Y., Li, X., Ren, L., Zhao, J., Hu, Y., Cao, B. (2020). Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. *The Lancet, 395*(10223), 497–506. http://doi:10.1016/s0140-6736(20)30183-5

ISS, I. S. d. S. (2020). Istituto Superiore di Sanità (Higher Institute of Health) official website 13 March.

Ji, W., Wang, W., Zhao, X., Zai, J., & Li, X. (2020). Cross-species transmission of the newly identified coronavirus 2019-nCoV. *Journal of Medical Virology, 92*(4), 433–440. http://doi:10.1002/jmv.25682

Jin, J. M., Bai, P., He, W., Wu, F., Liu, X. F., Han, D. M., ... & Yang, J. K. (2020). Gender differences in patients with COVID-19: Focus on severity and mortality. *Frontiers in Public Health, 8*, 152.

Kan, B., Wang, M., Jing, H., Xu, H., Jiang, X., Yan, M., Xu, J. (2005). Molecular evolution analysis and geographic ınvestigation of severe acute respiratory syndrome coronavirus-like virus in palm civets at an animal market and on farms. *Journal of Virology, 79*(18), 11892–11900. http://doi:10.1128/jvi.79.18.11892-11900.2005

Kandeel, M., Ibrahim, A., Fayez, M., & Al-Nazawi, M. (2020). From SARS and MERS CoVs to SARS-CoV-2: Moving toward more biased codon usage in viral structural and nonstructural genes. *Journal of Medical Virology, 92*(6), 660-666. http://doi:10.1002/jmv.25754

Kim, J.-M., Chung, Y.-S., Jo, H. J., Lee, N.-J., Kim, M. S., Woo, S. H., Han, M.-G. (2020). Identification of coronavirus isolated from a patient in Korea with COVID-19. *Osong Public Health and Research Perspectives, 11*(1), 3–7. http://doi:10.24171/j.phrp.2020.11.1.02

Ksiazek, T. G., Erdman, D., Goldsmith, C. S., Zaki, S. R., Peret, T., Emery, S., Anderson, L. J. (2003). A novel coronavirus associated with severe acute respiratory syndrome. *New England Journal of Medicine, 348*(20), 1953–1966. http://doi:10.1056/nejmoa030781

Lai, M. M. C., Perlman, S., & Anderson, L. J. (2007). *Coronaviridae,* p 1305–1335. Fields virology, 5th ed. Lippincott Williams & Wilkins, Philadelphia, PA.

Lake, M. A. (2020). What we know so far: COVID-19 current clinical knowledge and research. *Clinical Medicine, 20*(2), 124–127. http://doi:10.7861/clinmed.2019-coron

Leroy, E. M., Kumulungui, B., Pourrut, X., Rouquet, P., Hassanin, A., Yaba, P., Swanepoel, R. (2005). Fruit bats as reservoirs of Ebola virus. *Nature, 438*(7068), 575–576. http://doi:10.1038/438575a

Leung, G. M., Hedley, A. J., Ho, L.-M., Chau, P., Wong, I. O. L., Thach, T. Q. Lam, T.-H. (2004). The epidemiology of severe acute respiratory syndrome in the 2003 hong kong epidemic: an analysis of all 1755 patients. *Annals of Internal Medicine, 141*(9), 662. http://doi:10.7326/0003-4819-141-9-200411020-00006

Lew, T. W. K. (2003). Acute respiratory distress syndrome in critically ıll patients with severe acute respiratory syndrome. *JAMA, 290*(3), 374. http://doi:10.1001/jama.290.3.374

Li, Q., Guan, X., Wu, P., Wang, X., Zhou, L., Tong, Y., Feng, Z. (2020). Early Transmission Dynamics in Wuhan, China, of Novel Coronavirus–Infected Pneumonia. *New England Journal of Medicine, 382*(13), 1199–1207. http://doi:10.1056/nejmoa2001316

Liu, Z., Xiao, X., Wei, X., Li, J., Yang, J., Tan, H., Liu, L. (2020). Composition and divergence of coronavirus spike proteins and host ACE2 receptors predict potential intermediate hosts of SARS-CoV-2. *Journal of Medical Virology. 92*(6),595-601. http://doi:10.1002/jmv.25726

Lu, H., Stratton, C. W., & Tang, Y. (2020). Outbreak of pneumonia of unknown etiology in Wuhan, China: The mystery and the miracle. *Journal of Medical Virology, 92*(4), 401–402. http://doi:10.1002/jmv.25678

Lu, R., Zhao, X., Li, J., Niu, P., Yang, B., Wu, H., Tan, W. (2020). Genomic characterisation and epidemiology of 2019 novel coronavirus: implications for virus origins and receptor binding. *The Lancet, 395*(10224), 565–574. http://doi:10.1016/s0140-6736(20)30251-8

Mackenzie, J. S., Chua, K. B., Daniels, P. W., Eaton, B. T., Field, H. E., Hall, R. A., Williams, D. T. (2001). Emerging Viral Diseases of Southeast Asia and the Western Pacific. *Emerging Infectious Diseases, 7*(7), 497–504. http://doi:10.3201/eid0707.017703

Memish, Z. A., Cotten, M., Meyer, B., Watson, S. J., Alsahafi, A. J., Al Rabeeah, A. A., Drosten, C. (2014). Human Infection with MERS Coronavirus after Exposure to Infected Camels, Saudi Arabia, 2013. *Emerging Infectious Diseases, 20*(6), 1012–1015. http://doi:10.3201/eid2006.140402

Morgan, O. (2019). How decision makers can use quantitative approaches to guide outbreak responses. *Philosophical Transactions of the Royal Society B: Biological Sciences, 374*(1776), 20180365. http://doi:10.1098/rstb.2018.0365

Murphy, Frederik A., Claude M. Fauquet, David HL Bishop, Said A. Ghabrial, Audrey W. Jarvis, Giovanni P. Martelli, Mike A. Mayo, and Max D. Summers. (2012) eds. *Virus taxonomy: classification and nomenclature of viruses. Vol. 10*. Springer Science & Business Media.

Nioi, M., & Napoli, P. E. (2020). Global Spread of Coronavirus Disease 2019 and Malaria: An Epidemiological Paradox. *SSRN Electronic Journal*. http://doi:10.2139/ssrn.3554389

Omrani, A. S., Al-Tawfiq, J. A., & Memish, Z. A. (2015). Middle East respiratory syndrome coronavirus (MERS-CoV): animal to human interaction. *Pathogens and Global Health, 109*(8), 354–362. http://doi:10.1080/20477724.2015.1122852

Organization, W. H. (2020). Coronavirus disease 2019 (COVID-19): situation report, 51.

Organization, W. H. (2020). Coronavirus disease 2019 (COVID-19): situation report, 59.

Organization, W. H. (2020d). Coronavirus disease 2019 (COVID-19): situation report, 66.

Organization, W. H. (2020e). (COVID-19): Weekly Operational Update, 2 October, 2020.

Paraskevis, D., Kostaki, E. G., Magiorkinis, G., Panayiotakopoulos, G., Sourvinos, G., & Tsiodras, S. (2020). Full-genome evolutionary analysis of the novel corona virus (2019-nCoV) rejects the hypothesis of emergence as a result of a recent recombination event. *Infection, Genetics and Evolution, 79*, 104212. http://doi:10.1016/j.meegid.2020.104212

Peiris, J., Lai, S., Poon, L., Guan, Y., Yam, L., Lim, W., Yuen, K. (2003a). Coronavirus as a possible cause of severe acute respiratory syndrome. *The Lancet, 361*(9366), 1319–1325. http://doi:10.1016/s0140-6736(03)13077-2

Peiris, J., Chu, C., Cheng, V., Chan, K., Hung, I., Poon, L., Yuen, K. (2003b). Clinical progression and viral load in a community outbreak of coronavirus-associated SARS pneumonia: a prospective study. *The Lancet, 361*(9371), 1767–1772. http://doi:10.1016/s0140-6736(03)13412-5

Perlman, S., & Netland, J. (2009). Coronaviruses post-SARS: update on replication and pathogenesis. *Nature Reviews Microbiology, 7*(6), 439–450. http://doi:10.1038/nrmicro2147

Phan, L. T., Nguyen, T. V., Luong, Q. C., Nguyen, T. V., Nguyen, H. T., Le, H. Q., Pham, Q. D. (2020). Importation and Human-to-Human Transmission of a Novel Coronavirus in Vietnam. *New England Journal of Medicine, 382*(9), 872–874. http://doi:10.1056/nejmc2001272

Phan, T. (2020). Genetic diversity and evolution of SARS-CoV-2. *Infection, Genetics and Evolution, 81*, 104260. http://doi:10.1016/j.meegid.2020.104260

Riou, J., & Althaus, C. L. (2020). Pattern of early human-to-human transmission of Wuhan 2019 novel coronavirus (2019-nCoV), December 2019 to January 2020. *Eurosurveillance, 25*(4). http://doi:10.2807/1560-7917.es.2020.25.4.2000058

Rodriguez-Morales, A. J., Bonilla-Aldana, D. K., Balbin-Ramon, G. J., Rabaan, A. A., Sah, R., Paniz-Mondolfi, A., Pagliano, P., & Esposito, S. (2020). History is repeating itself: probable zoonotic spillover as the cause of the 2019 novel Coronavirus Epidemic. *Infez Med, 28*(1), 3-5.

Sabir, J. S. M., Lam, T. T.-Y., Ahmed, M. M. M., Li, L., Shen, Y., E. M. Abo-Aba, S., Guan, Y. (2016). Co-circulation of three camel coronavirus species and recombination of MERS-CoVs in Saudi Arabia. *Science, 351*(6268), 81–84. http://doi:10.1126/science.aac8608

SAIF, L. J. (2004). Animal coronaviruses : what can they teach us about the severe acute respiratory syndrome? *Revue Scientifique et Technique de l'OIE, 23*(2), 643–660. http://doi:10.20506/rst.23.2.1513

Sharif-Yakan, A., & Kanj, S. S. (2014). Emergence of MERS-CoV in the Middle East: Origins, Transmission, Treatment, and Perspectives. *PLoS Pathogens, 10*(12), e1004457. http://doi:10.1371/journal.ppat.1004457

Shi, Z., & Hu, Z. (2008). A review of studies on animal reservoirs of the SARS coronavirus. *Virus Research, 133*(1), 74–87. http://doi:10.1016/j.virusres.2007.03.012

Siu, Y. L., Teoh, K. T., Lo, J., Chan, C. M., Kien, F., Escriou, N., Nal, B. (2008). The M, E, and N Structural Proteins of the Severe Acute Respiratory Syndrome Coronavirus Are Required for Efficient Assembly, Trafficking, and Release of Virus-Like Particles. *Journal of Virology, 82*(22), 11318–11330. http://doi:10.1128/jvi.01052-08

Song, H.-D., Tu, C.-C., Zhang, G.-W., Wang, S.-Y., Zheng, K., Lei, L.-C., Xiang, H. (2005). Cross-host evolution of severe acute respiratory syndrome coronavirus in palm civet and human. *Proceedings of the National Academy of Sciences, 102*(7), 2430–2435. http://doi:10.1073/pnas.0409608102

Surveillances, V. (2020). The Epidemiological Characteristics of an Outbreak of 2019 Novel Coronavirus Diseases (COVID-19)—China, 2020. *China CDC Weekly, 2*(8), 113-122.

Tang, X., Wu, C., Li, X., Song, Y., Yao, X., Wu, X., Lu, J. (2020). On the origin and continuing evolution of SARS-CoV-2. *National Science Review, 7*(6), 1012–102. http://doi:10.1093/nsr/nwaa036

To, K. K., Hung, I. F., Chan, J. F., & Yuen, K.-Y. (2013). From SARS coronavirus to novel animal and human coronaviruses. *Journal of Thoracic Disease, 5*(Suppl 2), S103.

Woodhead, M., Ewig, S., & Torres, A. (2003). Severe acute respiratory syndrome (SARS). *European Respiratory Journal, 21*(5), 739–740. http://doi:10.1183/09031936.03.00035403

Wu, D., Zou, S., Bai, T., Li, J., Zhao, X., Yang, L., Shu, Y. (2015). Poultry farms as a source of avian influenza A (H7N9) virus reassortment and human infection. *Scientific Reports, 5*(1). http://doi:10.1038/srep07630

Wu, F., Zhao, S., Yu, B., Chen, Y.-M., Wang, W., Song, Z.-G., Zhang, Y.-Z. (2020). A new coronavirus associated with human respiratory disease in China. *Nature, 579*(7798), 265–269. http://doi:10.1038/s41586-020-2008-3

Wu, P., Hao, X., Lau, E. H. Y., Wong, J. Y., Leung, K. S. M., Wu, J. T., Leung, G. M. (2020). Real-time tentative assessment of the epidemiological characteristics of novel coronavirus infections in Wuhan, China, as at 22 January 2020. *Eurosurveillance, 25*(3). http://doi:10.2807/1560-7917.es.2020.25.3.2000044

Wu, Z., & McGoogan, J. M. (2020). Characteristics of and Important Lessons From the Coronavirus Disease 2019 (COVID-19) Outbreak in China. *JAMA, 323*(13), 1239. http://doi:10.1001/jama.2020.2648

Yu, F., Du, L., Ojcius, D. M., Pan, C., & Jiang, S. (2020). Measures for diagnosing and treating infections by a novel coronavirus responsible for a pneumonia outbreak originating in Wuhan, China. *Microbes and Infection, 22*(2), 74–79. http://doi:10.1016/j.micinf.2020.01.003

Zaki, A. M., van Boheemen, S., Bestebroer, T. M., Osterhaus, A. D. M. E., & Fouchier, R. A. M. (2012). Isolation of a Novel Coronavirus from a Man with Pneumonia in Saudi Arabia. *New England Journal of Medicine, 367*(19), 1814–1820. http://doi:10.1056/nejmoa1211721

Zhang, L., Shen, F., Chen, F., & Lin, Z. (2020). Origin and Evolution of the 2019 Novel Coronavirus. Clinical Infectious Diseases. *Clin Infect Dis*., 28;71(15):882-883. doi: 10.1093/cid/ciaa112. PMID: 32011673; PMCID: PMC7108176. http://doi:10.1093/cid/ciaa112

Zhang, P., Gao, Q., Wang, T., Ke, Y., Mo, F., Jia, R., Zhao, Y. (2020). Evaluation of recombinant nucleocapsid and spike proteins for serological diagnosis of novel coronavirus disease 2019 (COVID-19). MedRxiv 2020. http://doi:10.1101/2020.03.17.20036954

Zheng, J., Hassan, S., Alagaili, A. N., Alshukairi, A. N., Amor, N. M. S., Mukhtar, N., Yaqub, T. (2019). Middle East Respiratory Syndrome Coronavirus Seropositivity in Camel Handlers and Their Families, Pakistan. *Emerging Infectious Diseases, 25*(12), 2307.. http://doi:10.3201/eid2512.191169

Zhong, N., Zheng, B., Li, Y., Poon, L., Xie, Z., Chan, K., Li, P., Tan, S., Chang, Q., & Xie, J. (2003). Epidemiology and cause of severe acute respiratory syndrome (SARS) in Guangdong, People's Republic of China, in February, 2003. *The Lancet, 362*(9393), 1353-1358. http://doi.org/10.1016/s0140-6736(03)14630-2

Zhou, P., Yang, X.-L., Wang, X.-G., Hu, B., Zhang, L., Zhang, W., Shi, Z.-L. (2020). A pneumonia outbreak associated with a new coronavirus of probable bat origin. *Nature, 579*(7798), 270–273. http://doi:10.1038/s41586-020-2012-7

Zhu, N., Zhang, D., Wang, W., Li, X., Yang, B., Song, J., Tan, W. (2020). A Novel Coronavirus from Patients with Pneumonia in China, 2019. *New England Journal of Medicine, 382*(8), 727–733. http://doi:10.1056/nejmoa2001017

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Heavy Metal Removal from Ore Processing Plant Wastewater by Electrocoagulation Process

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Abstract: Discharging wastewater originating from industries to the receiving environment without any purification causes environmental pollution and endangers biotic life. When the literature is examined it seems that there are studies on acid mine drainage but purification of ore processing wastewater by electrocoagulation (EC) is not seen much. In this study the heavy metal removal from wastewater of ore processing facility (magnesite crushing, screening and washing facility) by using electrocoagulation process was investigated. At the EC process Ferrum-Ferrum electrot was used in parallel. The removed heavy metal parameters have been identified as: Ni^{2+}, Zn^{2+} ve Cr (VI). In this research study as a result: At removal of Nickel it has been obtained %98,14 efficiency by 21,3mA/cm^2 current density, 20 minute contact time and 8,5 pH value, at removal of Zinc, it has been obtained %97,80 efficiency by 16mA/cm^2 current density, 40 minute contact time and 8,5 pH value, at removal of Chrome^{+6}, it has been obtained %98,20 efficiency by 16mA/cm2 current density, 20 minute contact time and 7 pH value.

Keywords: Electrocoagulation, Magnesite, Wastewater, Removal, Heavy Metal

Introduction

As a result of the rapid population growth in the world and in our country, the needs have also increased. The supply required to meet the demands indirectly caused the increase of industrial facilities and the development of production (Bayar, 2014). With industrialization, environmental pollution has occurred and living life has been threatened. Discharge of solid, liquid and gas wastes before and after industrial plants to receiving environments without treatment has left impaired impacts on the environment that are difficult to repair. These environmental pollution has exceeded nature's absorption capacity and adversely affected living life. In order to minimize these negative effects, it is of great importance to determine the wastes causing pollution and to carry out studies on how to eliminate them (Kasap, 2017; Yılmaz, 2009).

Underdeveloped and developing countries can only treat 5% of waste water. Since the remaining industrial and domestic wastewater is supplied to the environment uncontrolled, underground and aboveground supplies are polluted day by day. As a result of this pollution, the effects of global climate change are being felt intensely. These kinds of effects cause floods, drought and irregularities in annual precipitation rate. These kinds of effects cause floods, drought and irregularities in annual rainfall rate. Water quality is affected by environmental conditions. One of the most important problems of today is the decrease in drinking water resources (Bayar, 2014; Şık, 2015). According to the UNESCO report, it is stated that one billion eight hundred thousand people will experience water shortage in 2025. It is stated that 40% of the population in approximately 80 countries will

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be insufficient to meet their water needs due to the population density and the uneven distribution of water resources in the last decade. By 2025, the amount of water per capita is expected to decrease by half (Öztürk & Çelik, 2008). The need for clean water is an important issue, especially in third world countries. Developed countries like America think that wastewater treatment is a "critical need" due to urbanization, ever-increasing population and climate changes, and they are doing intensive studies on this subject (Güney, 2013). The need for economical, fast and highly transformable, feasible wastewater treatment systems has become a necessity in terms of being integrated into the rapidly developing world in order to ensure a healthy environment in our country's accession and harmonization process to the European Union (Bayar, 2014). With the increase of regulations on wastewater treatment, great efforts are being made to develop new technologies and procedures for water treatment alongside traditional treatment methods (Çebi, 2018). Among the industries that cause environmental pollution, one of the industries with great strategic importance is the mining industry. Due to the different geological structures and tectonic structures in our country, there are a wide variety of mineral ores. Mining, which is one of the important sectors for the economy, includes all activities that provide basic raw materials in sectors such as industry and energy for national development. Mines are among our natural resources and it is inevitable to be used for demands. As a result of these mining activities, wastewater containing high amounts of metal occurs. The fact that these waters reach lakes and rivers without treatment poses a threat to water resources (Yücesoy, 2011). In addition, geological wastes containing heavy metals can cause great damage to the environment and the health of the living creatures around them when they mix into the soil or water resources uncontrolled (İşler, 2019).

The reasons for entering the mining sector in research such as electrocoagulation: 1. Reusing the water it needs during production to deal with the danger of drought as well as environmental pollution. 2. Dewatering the final product. 3. To recover fine ores and by-products released as a result of ore preparation and enrichment processes (Atay, 2019). Electrocoagulation, used as a wastewater treatment technology, is a long-standing method used to remove a variety of pollutants. In the early days, electrocoagulation (EC) was not developed in terms of electrode reliability (especially passivation of electrodes over time) and lack of a systematic approach for reactor design and operation. Thanks to the latest technical developments and the increasing need for small-scale water treatment plants, EC has been brought to the agenda again (Holt et al, 2005). EC is a simple and effective method for wastewater treatment. This method; It is a low cost and environmentally friendly treatment method that can be used in industrial wastewater containing heavy metals, wastewater containing suspended solids, wastewater containing oil, etc. (Karagözoğlu & Malkoç, 2017; Gülyaşar, 2019).

EC process is a method of wastewater treatment with an electrolysis system using a metal electrode and a double-sided (anode and cathode) mechanism. It is based on the principle of forming coagulants by electrically dissolving the electrode material used during the process (Yılmaz & Karagözoğlu, 2019). In this study, the raw wastewater generated in the ore preparation phase (crushing and screening plant) of the Chrome-Magnesite plant operating in the Konya region was taken. It was transported according to standard methods and stored in the laboratory. Heavy metal removal efficiency has been investigated in the EC process. The pH value of the wastewater is 8.5 and its electrical conductivity is 35 µS/cm. Electrocoagulation application was carried out by rotating iron-iron (Fe-Fe) electrodes to treat the ore washing wastewater of the Chrome-Magnesite crushing and screening plant.

In the electrocoagulation (EC) process, the heavy metal removal efficiency of the raw wastewater was investigated by optimizing the solution environment pH, current density and reaction contact time. The characterization of ore washing wastewater taken from the Chrome-Magnesite crushing and screening plant is given in Table 1.

Table 1. Crushing and screening plant ore washing wastewater characterization

Parameters	Measurement Value
pH	8, 5
Conductivity	395 µS/cm
COD	103 mg/L
Chromium (IV)	10 mg/L
Zinc	5,74 mg/L

In the study, the removal efficiencies of Nickel, Zinc, Chromium (VI) with different electrodes, different pH values, different currents and different durations were examined.

Material and Method

The studies were carried out in a glass plexiglass (mica) reactor at room temperature and Fe-Fe electrode pairs were used. Rectangular electrodes with side lengths of approximately 6x12 cm^2 were used in the reactor, and the average weight of the electrodes used was measured around 100 grams. During the EC process, 2 plexiglass reactors were used simultaneously and the wastewater volume used was approximately 500 mL and the mixing speed was 100 rpm. While pH adjustment was made in the reactor, solutions of HCl and NaOH (0.1 N-1 N) were used, while the required doses of NaCl were used for conductivity adjustment. The EC reactor assembly used is shown in Figure 1.

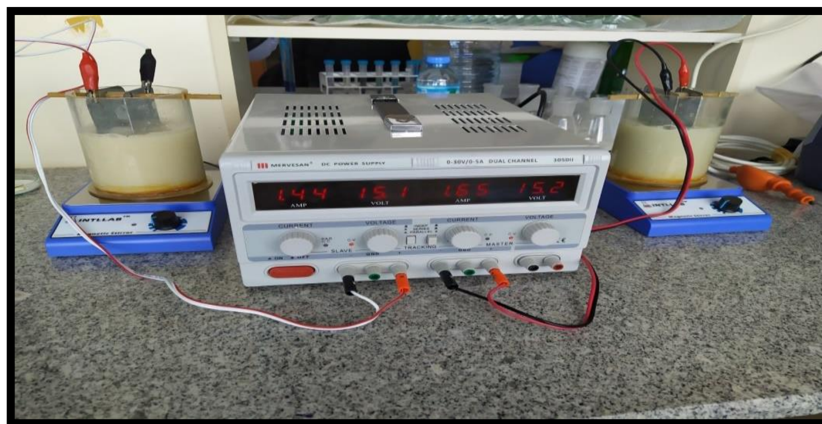

Figure 1. Visual presentation of EC process in the laboratory

DC power supply with 0-30 V / 0-5 A current density was used in the reactor, and the effects of pH, reaction time, current density and electrode types on removal efficiency were investigated. Coagulant precipitation process: At the end of the reaction, the treated waste water was taken into 500 mL measure and it was waited for sludge to settle for 1 hour. Precipitated sludge amounts were recorded by reading, and pollutant removal efficiencies were calculated in the samples taken from the supernatant after treatment.

Results and Discussion

Within the scope of the study, firstly, pH optimization, Current optimization and Time Optimization were carried out by using Fe-Fe electrodes and Nickel, Chromium (VI) and Zinc removal efficiencies at different pH, current density and contact times were examined.

pH Optimization and Pollution Removal Using Fe-Fe Electrode

At this stage of the study, Nickel, Chromium (VI) and Zinc removal efficiencies at pH 3, 5, 7 and 8.5 (original pH) were investigated by using Fe-Fe electrodes for 20 minutes of contact time, current density of 10.67 mA/cm^2. The graphic below was created as a result of this experiment.

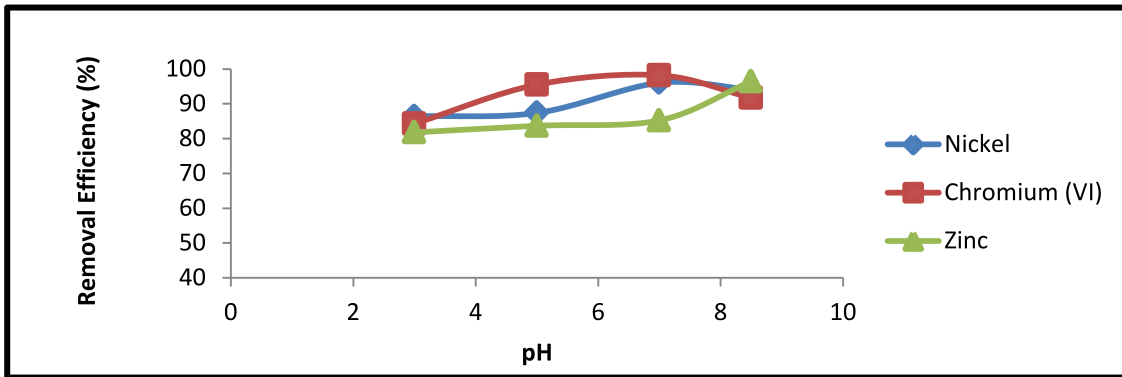

Figure 2. Nickel, Chromium (VI) and Zinc Removal Efficiency (pH Optimization)

Maximum nickel removal efficiency was obtained as 96.02% at pH 7. Removal efficiency was measured as 86.4% at pH 3, 87.3% at pH 5 and 94.08% at pH 8.5 (original pH). Maximum chromium (VI) removal efficiency was obtained as 98.20% at pH 7. Removal efficiency was measured as 84.30% at pH 3, 95.50% at pH 5, and 91.70% at pH 8.5 (original pH). Maximum zinc removal efficiency was 96.46% at pH 8.5 (original pH). Removal efficiency was measured as 81.77% at pH 3, 83.68% at pH 5 and 85.24% at pH 7. Considering the heavy metal removal efficiencies after pH optimization, it has been determined that the optimum pH is 8.5 (original pH).

Current Density Optimization And Pollution Removal Using Fe-Fe Electrodes

At this stage of the study, using Fe-Fe electrodes for 20 minutes contact time, pH 8.5 (original pH), current density 10.67 mA/cm^2, 16 mA/cm^2, 21.3 mA/cm^2, 26.67 mA/cm^2, 32 mA/cm^2, 37.33 mA/cm^2, 42 mA/cm^2 Nickel, Chromium (VI) and Zinc removal were investigated. The graphic below was created as a result of this experiment.

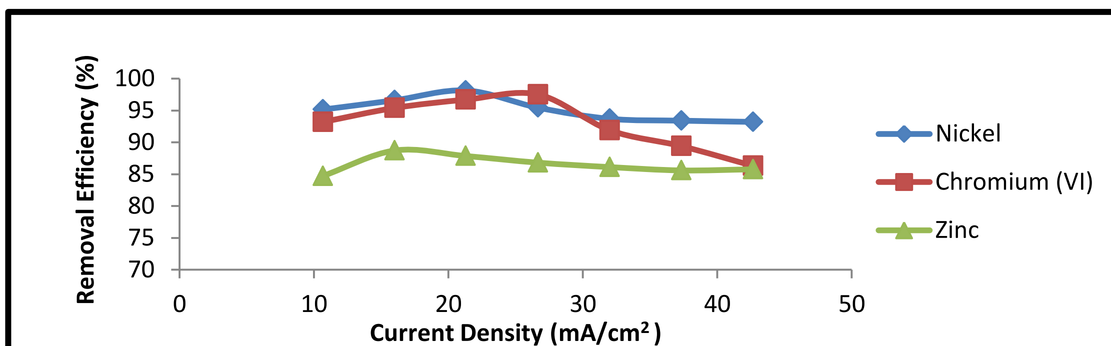

Figure 3. Nickel, Chromium (VI) and Zinc removal efficiency (current density optimization)

Maximum nickel removal efficiency was obtained as 98.14% and current density at 21.3 mA/cm^2. The current density is 95.15% at 10.67 mA/cm^2, 93.69% at 32 mA/cm^2, and the removal efficiency 93.20% at 42.67 mA/cm^2. Maximum chromium (VI) removal efficiency was obtained as 97.50% and current density at 26.67 mA/cm^2. The current density is 93.20% at 10.67 mA/cm^2, 91.90% at 32 mA/cm^2, and the removal efficiency 86.30% at 42.67 mA/cm^2. Maximum zinc removal efficiency efficiency was obtained as 88.72% and current density at 16 mA/cm^2. The current density is 87.85% at 21.3 mA/cm^2, 86.11% at 32 mA/cm^2, and the removal efficiency 85.76% at 42.67 mA/cm^2. After the current density optimization, considering the heavy metal removal efficiencies, it was determined that the optimum current density is 16 mA/cm^2.

Time Optimization and Pollution Removal Using Fe-Fe Electrodes

At this stage of the study, nickel, chromium (VI) and zinc removal efficiencies were investigated at the original pH value of 8.5, constant current density of 16 mA/cm^2 and contact time between 0-40 minutes using Fe-Fe electrodes. The graphic below was created as a result of this experiment.

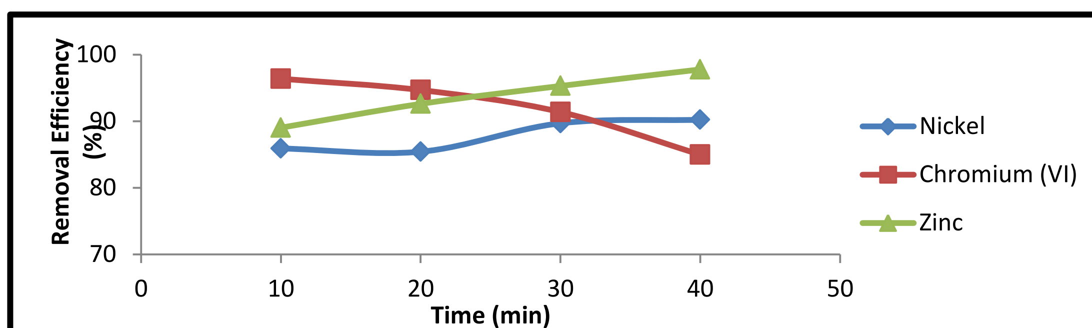

Figure 4. Nickel, Chromium (VI) and Zinc removal efficiency (eperation contact time optimization)

The maximum nickel removal efficiency was 90.23% and contact time was obtained in 40 minutes. If the contact time was 85.92% in 10 minutes, the contact time was 85.44% in 20 minutes and the contact time was 30 minutes, removal efficiency was measured as 89.71%. Maximum chromium (VI) removal efficiency was 96.40% and contact time was obtained in 10 minutes. If the contact time was 94.70% in 20 minutes, the contact time was 91.40% in 30 minutes and the contact time was 40 minutes, removal efficiency was measured as 85%. The maximum zinc removal efficiency was 97.80% and the contact time was obtained in 40 minutes. If the contact time was 89.05% in 10 minutes, the contact time was 92.62% in 20 minutes and the contact time was 30 minutes, removal efficiency was measured as 95.31%. As the time increased, an increase in removal efficiency was observed. Considering the heavy metal removal efficiencies after contact time optimization, it was determined that the optimum contact time was 20 minutes.

Conclusions

pH optimization: It was determined that the optimum pH (original pH) in pH optimization is 8.5. At this pH value, nickel removal efficiency 94.08%, chromium (VI) removal efficiency 91.70%, zinc removal efficiency 96.46% was measured. $Fe(OH)_3$ formation has the highest removal efficiency in the pH range of 8,0–8,5. Because the solubility of Fe $(OH)_3$ formed in this pH range is very low and flocs occur in the environment (Çebi, 2018). In the study where chromium (VI) removal was performed using iron electrodes, it was stated that besides chromium (VI) reduction of iron electrodes, an additional reduction reaction occurred on the cathode electrode surface to the EC process. It was stated that as a result of this reaction, $Cr(OH)_3$ was formed and precipitation was observed (Aygün, 2015). Maximum nickel (96.2%), Maximum chromium (VI) (98.20%) was obtained at pH 7 as removal efficiency. Maximum zinc (96.46%) was obtained at pH 8.5 as removal efficiency. Nevertheless, optimum pH (original) 8.5 was preferred to adjust the pH, as it would give an additional pollution to the water with the use of chemicals and due to the high removal efficiency. In a study, similar results were obtained in Chromium (III) measurements, and a decrease in removal efficiency was observed after pH: 7 (Gülyaşar, 2019). In another study, when the pH value is 5-9, it was observed that the zinc removal efficiency increased as the time increased (over 90%) (Türk, 2016).

Current density optimization: In the current density optimization, it was determined that the optimum current density is 16 mA/cm^2. At this current density, nickel removal efficiency 96.60%, chromium (VI) removal efficiency 95.40%, zinc removal efficiency 88.72% were measured. Maximum nickel (98.14%) removal efficiency at 21.3 mA/cm^2, Maximum chromium (VI) (97.50%) removal efficiency at 26.67 mA/cm^2 and maximum zinc (88.72%) removal efficiency at 16 mA/cm^2 ıt was obtained. Despite this, 16 reasons for choosing the optimum current density: 1. Adequate amount of expense has been provided 2. The more basic the wastewater environment, the higher the potential difference (voltage) value. In this case, it also increases the amount of electrical energy consumption, depending on the Faraday Law (Türk, 2016). With this, energy consumption will also be reduced.

Time optimization: It has been determined that the optimum contact time in time optimization is 20 minutes. During this contact period, Nickel removal efficiency was measured as 85.44%, chromium (VI) removal efficiency 94.70%, zinc removal efficiency 92.62%. Maximum nickel (90.23%) removal efficiency contact time was achieved in 40 minutes, Maximum chromium (VI) (96.40%) removal efficiency contact time was 10 minutes, Maximum zinc (97.80%) removal efficiency contact time was obtained in 40 minutes.

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References

Atay, Ö. (2019). *Removal of colloidal clay particles in water with electroconagulation method. Master of Science* (Master Thesis). Muğla Sıtkı Koçman University, Graduate School of Natural and Applied Sciences, Department of Mining Engineering, Muğla.

Aygün, A. (2012). Treatment of wastewater form reactive and dispers dye bathes in textile industry by electrocoagulation process: optimization by response surface methodology. Unpublished Doctoral Dissertation. Selçuk University, Natural and Applied Sciences Department of Environmental Engineering, Konya

Bayar, S. (2014). Investigation of treatment of pistachio processing industry wastewaters by fenton and electrocoagulation methods. Unpublished Doctoral Dissertation. Atatürk University, Graduate School of Natural and Applied Sciences Department of Environmental Engineering, Erzurum.

Çebi, Ş.E. (2018). *Investigation of food industry waste water treatment with electrocoagulation process by using Al electrod*. Unpublished Master Thesis. Kocaeli University, Graduate School of Natural and Applied Sciences Department of Environmental Engineering, Kocaeli.

Gülyaşar H. C. (2019). *Treatment of metal plating wastewater by electrocoagulation process.* Unpublished Master Thesis. Konya Technical University, Institute of Graduate Studies, Department of Enviromental Engineering, Konya.

Güney, S. (2013). *Investigation of applications of electrocoagulation process in environmental engineering*. Unpublished Master's thesis, Uludağ University. Graduate School Of Natural And Applied Sciences, Department of Chemistry, Bursa.

Holt, P. K., Barton G. W., & Mitchell C. A. (2005). The future for electrocoagulation as a localised water treatment technology. *Chemosphere, 59*(3), 355-367.

İşler, E. (2019). *Biotreatment of arsenic containing acid mine drainage water in anaerobic membran bioreactor and investigation of membrane fouling properties*. Unpublished Master Thesis. İstanbul Medeniyet University, Graduate School of Natural, Environmental and Energy Systems Engineering, İstanbul.

Karagözoğlu, B., & Malkoç R. (2017). The Investigation of the Removal of Reactive Orange 16 DYE From Textile Wastewater by Using Electrocoagulation Process. *Cumhuriyet Science Journal, 38*(3). 544-556.

Kasap, T. (2017). *Process optimization of paper industry wastewater treatment by electrocoagulation with peroxite using response surface methodology*. Unpublished Master Thesis. Yildiz Technical University Graduate School of Natural and Applied Sciences, İstanbul.

Öztürk, M., & Çelik, R. (2008). Determination of Diyarbakır Plain's groundwater maps with geographic information system (Cbs). *TMMOB , 2* , 125-134.

Şık, E. (2015). *Removal of arsenic from ground water using iron ball anodes by electrocoagulation process*. Unpublished Doctoral Dissertation. Gebze Tecnical University, Graduate School Of Natural And Applied Sciences, Department of Chemistry, Kocaeli.

Türk, B. (2016). *Aluminyum - research on refinability of aluminum – zinc metal working plant waste water with electrocoagulation method*. Unpublished Master Thesis. Ondokuz Mayıs University, Graduate School of Natural and Applied Sciences Department of Environmental Engineering, Samsun.

Yılmaz Z., & Karagözoğlu M.B. (2019). Electrocoagulation Applications in Wastewater Treatment. *BEU Journal of Science, 8*(1), 319-334.

Yılmaz, A. (2009). *Electrocoagulation method boron removal from industrial wastewater*. Unpublished Doctoral Dissertation. Atatürk Universitey, Graduate School of Natural and Applied Sciences Department of Environmental Engineering, Erzurum.

Yücesoy, Z. (2011). *Biotreatment of copper and zinc containig acid mine drainage water in anaerobic baffled reactor (ABR) and metal recovery*. Unpublished Master Thesis. Harran University, Graduate School of Natural and Applied Sciences Department of Environmental Engineering, Şanlıurfa.

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Extraction and Purification of the Potential Allergen Proteins from Candida Albicans

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Abstract: A commercial strain of *Candida albicans*10231 was grown and isolated using Sabouraud's Dextrose Agar Media. The samples were then subjected to lyophilization before protein extraction. TCA/acetone/methanol/phenol method has been used for protein extraction. For estimation of the percentage of extracted protein, BCA Protein Macro Assay Kit was applied. The samples were then transferred to the spectrophotometer for reading at wave length of 562 nm. The results show that the profile of the quantity of protein concentrations obtained upon supplementation of the following chemicals: PBS, ammonium bicarbonate, and 50mM of SDS buffer, were 0.133mg/mL, 0.080mg/mL, and 0.068mg/mL respectively.

Keywords: Allergy, Fungal allergy, *Candida albicans*, protein extraction

Introduction

Allergy can be described as an abnormal response of the immune system. The immune system of individuals with allergies develops an extreme reaction to substances (allergens) that are usually harmless, such as surrounding pollen granis, molds, animal hairs. Allergic skin diseases are more common in all age, ranges from children to the elderly, due to excessive urbanization today, rapid increase in negative environmental factors, increasingly moving away from natural nutrition (Günenç, 2020). About 20-30% of the world's population is affected by different allergic problems, and about 30% of them are caused by fungal spores (Grinn, 2011). More than 80 species of fungi allergic more than 100 species with respiratory symptoms have been associated with serious infections in humans and animals, while other species cause serious diseases in plants. Allergic effects on atopic individuals are important because of their opportunistic pathogenic effects in immunocompromised/suppressed individuals (Fang et al., 2005; Chakrabarti et al., 2012). When air as a medium is examined, it appears to contain viruses, bacteria, protozoans, algae, fungal spores and pollen (Schillinger et al., 1999). As a result of mycological research, which has a history of about 150 years, it has been found that so far there are more than 100,000 yeast and mold fungi, and only about 100 of them cause diseases in humans and animals (Töre, 1996). Meterological factors such as wind, humidity, temperature, precipitation, geographical location, air pollution, vegetation and human studies influence the concentration and type of fungal spores in the atmosphere (Bezerra et. al., 2014; Aydoğdu & Asan, 2008). Fungi and fungal spores may remain suspended in the air for long time , among these fungi are many genus of family *Saccharomycetaceae*, for example *Candida albicans*.

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Habitat of Candida Albicans:

Mcosal membranes of human and other warm blooded animals. Also found in the gut, in the vagina and also on the surface of the skin. Found in the digestive tract of birds. Isolated from soil, animal, hospitals, in-animate objects and food. Small, oval, measuring 2-4 µm in diameter. Yeast form, unicellular, reproduce by budding. Single budding of the cells may be seen. Both yeasts and pseudo-hyphae are gram positive. Encapsulated and diploid, also form true hyphae. Dimorphic fungai (yeast and pseudohyphal form) Can form biofilms. Normal condition: Yeast. Special condition (pH, Temperature): Pseudohyphae 80-90% of cell wall is carbohydrate (Carlile et. al., 2001).

The cell wall is formed of an inner chitin layer, a β-1,3-glucan layer, a β-1,6-glucan layer, and an outer mannan layer (Kapteyn et. al., 2000). In *C. albicans*, there are two Pir proteins: Pir1 and Pir32. Pir1 was found to be an essential protein, required for the stability and rigidity of the cell wall (Martı´nez et. al., 2004). Pir32 is a 422 amino acid long protein (Bahnan et. al., 2012). In laboratory, *C. albicans* usually grown on Sabouraud's Dextrose Agar Media at incubation temperature between 26-37°C and incubation time between 24-48 hrs.

The present work deals with the application of new method for the isolation and purification of *C. albicans* proteins in an attempt to obtain a high yield of protein from this fungus. Such a protein may serve as a drug for the treatment of many diseases, in addition to suggest recommendations that may be helpful in the reconsidering or changing of prescription strategy and dosing protocol.

Method

Preparation of C. albicans 10231 culture

C. albicans 10231 strain was purchased from local market. It was cultured on cooled sterilized SDA agar medium in petri dishes and kept in in incubator, previously adjusted at 37°C, for 48 hrs incubation.

Extraction of total proteins

Total protein extraction procedure based on the method described by. Pure culture of *C. albicans* was then transferred to eppendrof tubes, followed by the addition of chloroform and 70% of methanol (2:1 v/v). The mixture in, eppendrof tubes, was well mixed by vortex, and then kept in the shaker at 4°C for 24 hrs. After 24 hrs the mixtures in the tubes were subjected to mixing by vortex. The samples in eppendrof were then subjected co centrifugation at 15000rpm for 15 min. The samples kept in the incubator for 48 hrs at 37°C. 40mL of PBS was added and the samples then mixed well by vortex and followed by addition of phenol to the samples and kept in the incubator for 3 days at 4°C. The samples were then freezed at -20°C, and subjected to lyopholization for 24hrs. 10% of TCA was then added, and the samples left at room temperature for 15 min and the centrifuged at 13000rpm for 3min. The supernatant was discarded. To the precipitate, ammonium acetate solution (7.7mg ammonium acetate in 1 liter methanol) was added. The sample mixed well by vortex, then centrifuged at 13000rpm for 3min.The supernatant was discarded and 80% of cold acetone was added to the precipitate, then centrifuged The supernatant was discarded. The precipitate kept in the incubator for 24 hrs. at 37°C, then 0.8 ml phenol and 0.4 ml of SDS buffer (5% at pH 8) were added, mixed by vortex, then centrifuged at 15000rpm for 3min. The supernatant was discarded; while10% methanol and 1 M ammonium acetate were added to the precipitate. The sample kept at -20°C for 24hrs. Then centrifuged at 13000rpm for 3min. The supernatant was discarded; the precipitate was washed by 8% acetone (for seven times) by centrifugation at 13000rpm for 20min. and incubated at 37°C for 2 days, then stored at -20°C for 2 days. The sample is divided in to three aliquots, each aliquot contained 0.025mg of the sample, and to each aliquot the following chemicals were added: 50mM SDS, PBS, and ammonium bicarbonate. These 3 samples were then put in an orbital shaker previously adjusted at 250rpm and 4°C for 72hrs.

Detection of total protein

Determination of Total Protein Concentration Total protein concentration of mushroom extracts Smith et al. (1985) by using the bicinchoninic acid (BCA) method. Commercially purchased BCA Macro Assay Kit (Serva Electrophoresis GmbH) was used to determine protein concentration. BCA analysis was performed in

accordance with the protocol suggested by the manufacturer. All above experiments were done in triplicate and the average value of the data was considered.

Results and Discussion

The table below represents the results of the current study (Table 1). It shows that the profile of the quantity of protein concentrations obtained upon supplementation of the following chemicals: PBS, ammonium bicarbonate, and 50mM of SDS buffer, were 0.133 mg/mL, 0.080mg / mL, and 0.068 mg/mL respectively. This means that the highest yield of total protein obtained is 0.133 mg/mL developed upon supplementation of PBS. However, Kustrzeba-Wojcicka, et.al.(2009) have shown that the highest concentration of the total protein extracted from *C. albicans* was 0.095mg / mL. This means that the increment in the amount of total protein of the present work is 0.098 mg/mL (Kustzeba, 2009), compared to Kustrzeba-Wojcicka, et al study. Since it become well known that *C.albicans* protein is considered as an allergen and source of allergic reaction, therefore finding of new technique for improvement of the production of a such protein (as immunogen) and other drugs may help to improve the current medication status especially in those patients who suffering from severe allergic response and reactions to the common allergen. Therefore, the future work should be concentrated on these aspects since little or no work concern this field.

Tablo 1. Total protein concentration values of *C. albicans* extracts measured by BCA assay

Alerjen adı	Absorbance Measurements	Absorbance Average	Protein concentration (mg/mL)
Candida albicans (Ammonium bicarbonate)	0,812/0,787/0,834	0,811	0,080 mg/mL
Candida albicans (PBS)	0,978/0,893/0,876	0,916	0,133 mg/mL
Candida albicans (50mM SDS Buffer)	0,808/0,738/0,706	0,750	0,068 mg/mL

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References

Aydoğdu, H, & Asan A. (2008). Airborne fungi in child day care centers in Edirne City, Turkey. *Environ Monit Assess; 147*, 423-44. http://dx.doi.org/10.1007/s10661-007-0130-4

Bahnan, W., Koussa, J., Younes, S., Abi Rizk, M., Khalil, B., El Sitt, S., et al (2012). Deletion of the Candida albicans PIR32 results in increased virulence, stress response, and upregulation of cell wall chitin deposition. *Mycopathologia, 174*, 107–19. https://doi.org/10.1007/s11046-012-9533-z PMID: 22391823

Bezerra, G. F. D. B., Gomes, S. M., Silva, M. A. C. N. D., Santos, R. M. D., Muniz Filho, W. E., Viana, G. M. D. C., & Nascimento, M. D. D. S. B. (2014). Diversity and dynamics of airborne fungi in São Luis, State of Maranhão, Brazil. *Revista da Sociedade Brasileira de Medicina Tropical, 47*(1), 69-73.

Carlile, M. J., Watkinson, S. C., & Gooday, G. W. (2001). *The fungi*. Gulf Professional Publishing.

Chakrabarti, H. S., Das, S., & Gupta-Bhattacharya, S. (2012). Outdoor airborne fungal spora load in a suburb of Kolkata, India: its variation, meteorological determinants and health impact. *International Journal of Environmental Health Research, 22*(1), 37-50.

Fang, Z., Ouyang, Z., Hu, L., & Wang, X. Z. H, LX. (2005). Culturable airborne fungi in outdoor environments in Beijing. *China. Science and Total Environment, 350*, 47-58.

Grinn-Gofroń, A. (2011). Airborne Aspergillus and Penicillium in the atmosphere of Szczecin, (Poland) (2004-2009). *Aerobiologia, 27*(1), 67–76. https://doi.org/10.1007/s10453-010-9177-8

Günenç, M.Z. (2020, 06 May). What is a vaginal yeast infection?. https://www.florence.com.tr/vajinal-mantar

Kapteyn, J. C., Hoyer, L. L., Hecht, J. E., Müller, W. H., Andel, A., Verkleij, A. J., ... & Klis, F. M. (2000). The cell wall architecture of Candida albicans wild-type cells and cell wall-defective mutants. *Molecular Microbiology, 35*(3), 601-611.

Kustrzeba-Wójcicka, I., Siwak, E., Terlecki, G., Wolańczyk-Mędrala, A., & Mędrala, W. (2014). Alternaria alternata and its allergens: a comprehensive review. *Clinical Reviews in Allergy & Immunology, 47*(3), 354–365. https://doi.org/10.1007/s12016-014-8447-6

Martínez, A. I., Castillo, L., Garcera, A., Elorza, M. V., Valentín, E., & Sentandreu, R. (2004). Role of Pir1 in the construction of the Candida albicans cell wall. *Microbiology, 150*(10), 3151-3161.

Schillinger, J. E., Vu, T., & Bellin, P. (1999). Airborne fungi and bacteria background levels in office buildings. *Journal of Environmental Health, 62*(2), p. 9–14.

Töre, O., (1996). *Basic microbiology and parasitology, chapter 14.general mycology* (Editor: Kılıçturgay, K.) Güneş & Nobel Medical Bookstores, Bursa-İstanbul.

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Virome Methodology in Animal Diseases

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Abstract: Virus discovery is a novel and ascending field in science for the last decade. This field is important because of changing host interactions and novel viruses in animals is necessary both for understanding the origin of different virus species that can infect human hosts and for preventing new zoonotic infections. The COVID-19 discovery was shown all around the world, viruses can easily adopt other hosts. The goal of this presentation is to explain the state-of-the-art in virome studies in various animals and in different specimens with different methodologies such as next-generation sequencing with different taqmentation reagents and analyses total raw data in different pipeline. Specifically Chaphamaparvoviruses which belongs to Parvoviridae family have recently been detected in dogs, cats, pigs, rats, red-crowned cranes, turkeys and chickens, macaques, bats, Tasmanian devils, murine and fish. Another chaphamaparvovirus was recently found in the serum of a febrile Brazilian. Some chaphamaparvovirus members have been shown to cause nephropathy in laboratory mice and in tilapia fish. As a result virus discovery has important role in animal and human science and to learn methods in this area give privilege to researchers as well as countries, which may eventually become the epicenter of a new and unpredicted novel virus someday.

Keywords: Next-generation sequencing, virome, metagenomic

Introduction

Virus discovery is a novel and ascending field in science for the last decade. This field is important because of changing host interactions and novel viruses in animals is necessary both for understanding the origin of different virus species that can infect human hosts and for preventing new zoonotic infections. The COVID-19 discovery was shown all around the world, viruses can easily adopt other hosts (Huang et al, 2020). The goal of this presentation is to explain the state-of-the-art in virome studies in various animals and in different specimens with different methodologies such as next-generation sequencing with different taqmentation reagents and analyses total raw data in different pipeline.

Classical methods for detection of known viruses are cell culture, immunological assays, and PCR (Chan J.F et al, 2017) all methods design according to the known viral structure but these methods are not enough for the detection of divergent unknown viruses. The growing rate of virus discovery, caused detailed steadily improved bioinformatic analysis pipeline for each virus discovery groups and public databases such as IDseq. These programs allow to researcher to discovery so divergent viruses base on protein database (Gorbalenya et al,2019).

There are different methodology for virus discovery such as tagmentation and ligation. Traditional library preparation methods include three-step first step is fragmentation for 200~400 bp length DNA and the second step is ligation adaptors and the last step is ligated product amplification. Tagmentation methods do not have fragmentation and ligation step, this method uses transposases for these two-step, will cut the DNA and attach the adaptors (Feng K et al, 2018). This study was compared two different library preparation methods for a novel virus discovery.

Method

Animal and Post-Mortem Examination and PCR

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A young, adult female Northern paradise tanager (*Tangara chilensis paradisea)* housed in a large aviary at a zoological institution was found dead with no premonitory signs of disease. Brain tissue was found negative for general protozoa, paramyxovirus, bornavirus, and herpesvirus by PCR. Used all brain tissue sample used for PCR analyses because of that liver tisue was used for viral metagenomic (next generation sequencing -NGS).

Viral Metagenomics

Same extraction and RT-PCR method followed for two different library preparation. Liver which had been frozen at -70C were thawed and homogenized and after centrifugation for 10 min in a table-top microfuge (15 000 × g, 4°C), supernatant was collected and filtered through a 0.45 µm filter (Millipore). Filtered supernatant treated with enzyme coctail to enrich for particle-protected viral nucleic acids. Total nucleic acids were then extracted (MagMAX Viral RNA Isolation Kit, Ambion, Inc, Austin, Tx, USA) (Li L et al, 2015) and amplified by random RT-PCR (Li L et al, 2015).

Amplified PCR product separated 2 for library prepation for tagmentation followed by use of the Nextera™ XT Sample Preparation Kit (Illumina) to generate a library for Illumina MiSeq (2 × 250 bases) with dual barcoding as previously described (Li L et al, 2015). For ligation medhodology was used Ovation® Ultralow System V2 DNA-Seq Library Preparation Kit (Nugen) and followed kit instruction for library prepartion. All two libraries runned in Illumina Miseq NGS machine. An in-house analysis pipeline was used to analyze sequence data. Before analyzing, raw data were pre-processed by subtracting human and bacterial sequences, duplicate sequences, and low quality reads. Following de novo assembly using the Ensemble program (Deng X et al, 2015), both contigs and singlets viral sequences were then analyzed using translated protein sequence similarity search (BLASTx v.2.2.7) to all annotated viral proteins available in GenBank. Candidate viral hits were then compared to an in-house non-virus non-redundant (nr) protein database to remove false positive viral hits. To align reads and contigs to reference viral genomes from GenBank and generate complete or partial genome sequences the Geneious R10 program was used.

Phylogenetic Analysis

Paradise Parvovirus NS1 and VP1 protein sequences were aligned using MAFFT in Geneious v10.1.3. and the aa phylogenetic trees of parvoviruses were constructed using the Maximum likelihood method with two substitution models: Le_Gascule_2008 model (LG) with Freqs and gamma distributed, invariant sites (G + I) MEGA software ver. X (Le SQ et al, 2008). Positions containing gaps and missing data were removed. Evolutionary analysis was conducted in MEGAX (Tamura K et al, 2013).

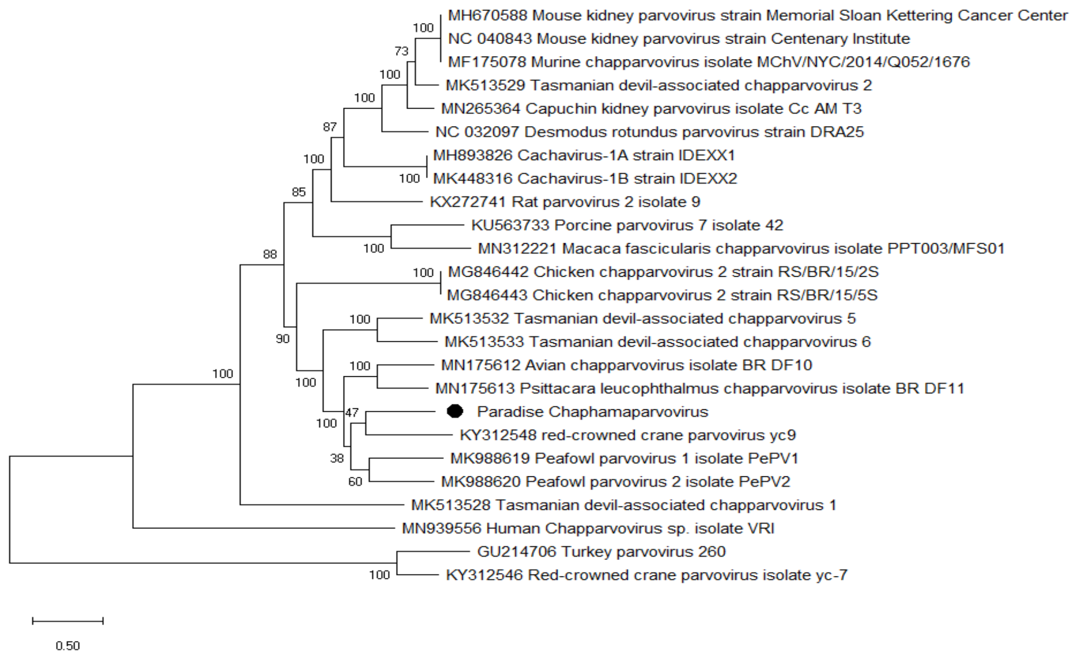
Conclusion

Viral Metagenomics

There wasn’t any evident cause of death at necropsy and no gross lesions diagnosed. After all process with two different library preparation had not seen any significant differences in the two methodologies. Both libraries have given the same result and shown the same virus which the length of the paradise bird chaphamaparvovirus genome obtained was 4196 nucleotide (nt) long with typical genome organization of four major ORFs (MT764779).

In phylogenetic analyses the bird of paradise chaphamaparvovirus proteins clustered with other bird-associated chaphamaparvoviruses: Peafowl parvovirus 1 and 2 (MK988619-20), red-crowned crane parvovirus (KY312548), Avian chapparvovirus (MN175612), and Psittacara leucophthalmus chapparvovirus (MN175613) (Figure 1A-B). The nonstructural protein (NS1) and the predicted capsid proteins (VP1) proteins showed closest aa identity of 49.85% and 49.78% to the corresponding proteins of Peafowl parvovirus 1 (MK988619).

A



B

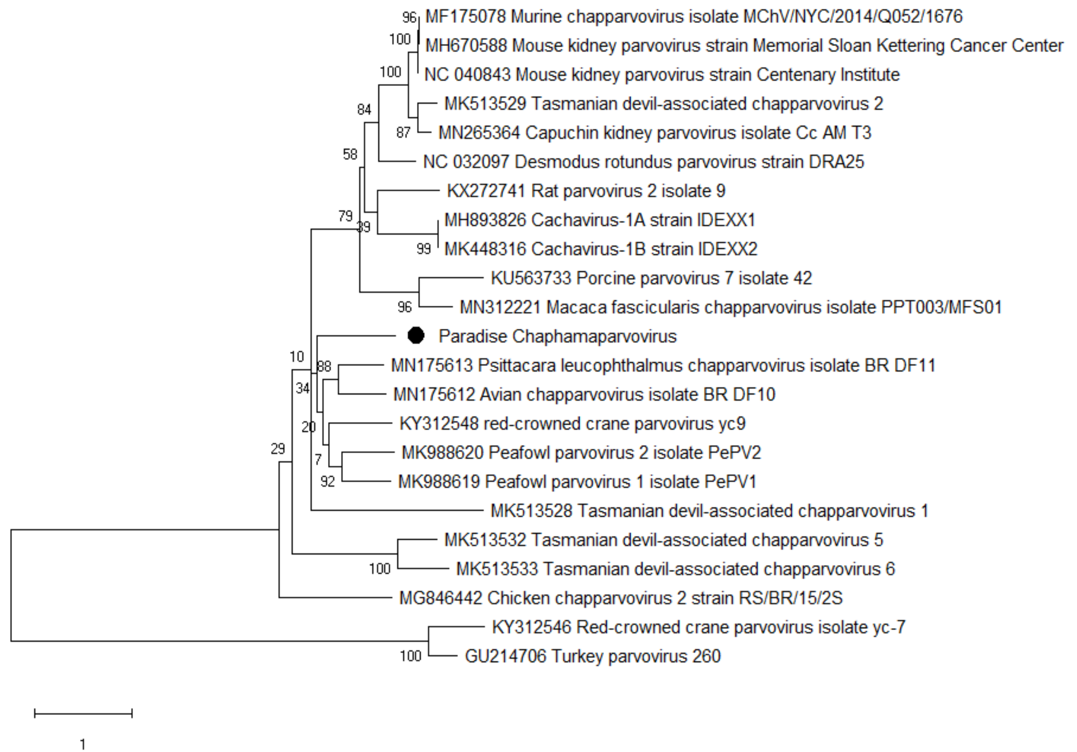


Figure 1. Phylogenetic trees (**A; NS1 and B; VP1**) were constructed using the maximum likelihood method with two substitution models: Le_Gascule_2008 model (LG) with freqs and gamma distributed, invariant sites (G + I) model MEGA software version X.

Recommendations

All countries must be ready for any kind of viral epidemic. Because of that laboratories working virus discovery are necessary for all countries. Specifically working on animal viruses can help to be on alert for any other zoonotic virus.

References

Chan, J.F., Sridhar, S, Yip, C.C., Lau, S.K., Woo, P.C. (2017). The role of laboratory diagnostics in emerging viral infections: the example of the Middle East respiratory syndrome epidemic. *J. Microbiol. 55*(3):172-82.

Deng, X., Naccache, S. N., Ng, T., Federman, S., Li, L., Chiu, C. Y., & Delwart, E. L. (2015). An ensemble strategy that significantly improves de novo assembly of microbial genomes from metagenomic next-generation sequencing data. *Nucleic acids research, 43*(7), e46-e46.

Feng, K., Costa, J., & Edwards, J.S. (2018). Next-generation sequencing library construction on a surface. BMC *Genomics, 19*(1):416.

Gorbalenya, A. E., Lauber, C. & Siddell, S. (2019). Taxonomy of Viruses. Virus Taxonomy and Computational Virology: Experts vs Algorithms. *Reference Module in Biomedical Sciences*. Elsevier https://doi.org/10.1016/B978-0-12-801238-3.99237-7.

Huang, C, Wang, Y, Li, X, Ren, L, Zhao, J, Hu, Y., et al. (2020). Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. *Lancet, 395*(10223):497-506.

Le, S.Q., Gascuel, O. (2008). An improved general amino acid replacement matrix. *Mol Biol Evol., 25*(7):1307-20.

Li, L, Deng, X, Mee, E.T, Collot-Teixeira, S, Anderson, R., Schepelmann, S., et al. (2015). Comparing viral metagenomics methods using a highly multiplexed human viral pathogens reagent. *J Virol Methods. 213*, 139-46.

Tamura, K, Stecher, G, Peterson, D, Filipski, A, Kumar, S. (2013). MEGA6: molecular evolutionary genetics analysis version 6.0. *Mol Biol Evol.30*(12):2725-9.

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Allergenic Proteins of Tilia Cordata

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Abstract: There are a lot of environmental allergenic factors; pollens, fungal spores, house dust mites, some fooods and drinks. These factors are harmless for many people in despite of making hypersensitivity any people. This sensitivity is calling “allergy’’. Allergens generally has a protein structure and when Charles Blakey’s discovered of pollens making allergenic diseases in 1873 after that pollens have determined a pivotal role to allergenic diseases. The pollens allergenic qualifications have known orginated layers of exine and intine including of glycoproteins, lipoproteins, polysaccaride and proteins. In this study, we investigated that allergenic proteins of *Tilia cordata* collected from Gaziantep. *T. cordata* pollen samples were collected during dissemination period and pollen extract prepared. We used Wodehouse method and prepared slides for determination of pollen morfology and were taken photos under light microscopy and measured morphological parameters. Total concentration of pollen proteins was determined using by BCA method. The amount of *T.cordata* allergen proteins was calculated as standard graph obtained from BSA. The average diameter of the *T. cordata* pollens diameter was measured as 88,83 µm and allergenic proteins from *T. cordata* was determined as significantly high. 1259.28 µg/ml concentration of *T. cordata* protein was measured. We suggested that this study provides important data to the literature by determining the amount of allergenic protein of *T. cordata* in this region because of differences about the content of allergenic proteins among regions.

Keywords: Allergy, Allergen, Pollen, T. cordata

Introduction

The term “allergy” was introduced in 1906 by von Pirquet, who recognized that in both protective immunity and hypersensitivity reactions, antigens had induced changes in reactivity. There are a lot of environmental allergenic factors; pollens, fungal spores, house dust mites, some fooods and drinks. These factors are harmless for many people in despite of making hypersensitivity any people. This sensitivity is calling “allergy’’. Allergic reaction can be cell or antibody mediated. Gell and Combs categorized allergic reaction to 4 groups (Type I, Type II, Type III, TypeIV). The Ige ısotype is responsible for allergic reactions in many patients and it’s calling IgE mediated allergy. The term “atopy” is often used to describe IgE-mediated diseases. Person with atopy is calling “atopic’’. Persons with atopy, by contrast, have an exaggerated response characterized by the production of allergen-specific IgE antibodies; they have elevated serum levels of IgE antibodies and positive reactions to extracts of common aeroallergens on skin-prick tests. T cells from their blood respond to allergens in vitro by inducing cytokines produced by type 2 helper T (Th2) cells. In the nose allergens are processed by antigen-presenting cells (dendritic cells expressing CD1a and CD11c and macrophages) in the nasal epithelial mucosa, with subsequent presentation of allergenic peptides by MHC class II molecules to T-cell receptors on resting CD41 T lymphocytes in regional lymph nodes. With costimulatory signals, allergen-stimulated T cells

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proliferate into TH2-biased cells that release IL-3, IL-4, IL-5, IL-13, and other cytokines. Inhalation of allergen in sensitized subjects, deposited allergens are recognized by IgE antibody bound to mast cells and basophils, causing degranulation and release of preformed mediators, such as histamine and tryptase, and the rapid de novo generation of mediators, including cysteinyl leukotrienes (leukotrienes C4, D4, and E4) and prostaglandin D2 (Dykewicz ve Hamilos, 2010). Rhinitis and sinusitis are among the most common medical conditions and are frequently associated. In Western societies an estimated 10% to 25% of the population have allergic rhinitis, with 30 to 60 million persons being affected annually in the United States. Pollens are cause of type I allergenic disease around the world and they are called aeroallergens most important facts of allergy (Boral and at al., 2004; Kaneko and at al., 2005). A lots of trees, grasses and weeds are during their pollination, the air pollen consantration is going higher and thats make hypersensitive pepople rhinit, alergic rhinit, asthma and bronchial asthma (Rauder ve Breiteneder, 2006). Pollen allergy is a seasonal disease, typically due to the individual's sensitivity to pollen during thebloom period of plants. However hypersensitive allergic people can be observed allergic rhinits sypmtoms all year around (Weerd and at al., 2002). Pollens are the most common of all outdoor allergens and part of the life cycle. Pollen is a natural, biologically active substance composed of the male reproductive cells of many plants. Because it is used as a source material to manufacture allergen extracts used to diagnose and treat allergic diseases, it is considered an active pharmaceutical ingredient. A pollen grain contains vegetative and generative nucleus, endoplasmic reticulum, mitocondria and intine, exine (Knox, 1984). The intine wall creates a barrier to contain the pollen components, while the openings in the exine allow the formation of the germination-dependent tube and the movement of water and proteins due to hydration (Singh and at al., 1991). Pollen proteins that don't interact with the immune system are harmless. In other words, protein that doesn't interact with IgE antibodies in the immune system is not considered an allergen. Linden is a large tree of variable form. In woodland up to 30 m (rarely to 37 m) high, with a cylindrical trunk up to about 1 m in diameter at breast height, tapering gradually and unbranched to two-thirds of its height. Lower branches of the first-order horizontal and arching; branches of the second-order horizontal, ascending or vertical: upper branches ascending or vertica. This description applies to the species in the strict sense but *T. cordata* may also be regarded as a collective species, which extends from western Europe to eastern Asia and includes at least seven species or subspecies (d.piggot) Variation in *T. cordata* in central and western Europe can also arise as a consequence of hybridization with *T. platyphyllos* (Pigott 1969). Analysis of hybrid populations shows that the majority of morphological characters, which normally separate the two species, can exist in all combinations and all degrees of intermediacy. The small-leaved linden – *T. cordata* - is found throughout Europe and most parts of North America. The dried leaves are used as herbal tea. *T.cordata's* height can reach up to 30m. Its leaves are half-heart-shaped, dark green, 4-8cm long. Pollen dissemination continues from June to July. Sometimes it can continue until August.

Method

In this study, we collected *T.cordata* pollens in Gaziantep University at first step. Collected pollen for extraction had to be fresh as stated in the literature (Aytuğ and Peremeci, 1987). The most suitable period for this is the phase immediately after the anthers are opened. Considering these, pollens were collected by appropriate methods during the dissemination period of the plant to be used. We dried the our sample in dry and sterile laboratory. Pollen was poured onto a clean blotter by hitting the dried flowers. The spilled pollen was placed in dark glass bottles and kept in a desiccator for 24 hours to dry. After drying, the flowers were separated from their pollen by sieving with 3 different pore diameters (180, 90, 63 µm). Then, washing with acetone was performed to separate the pollen from foreign materials such as plant parts. The pollens were then dried in a climate cabinet at 20-37 ℃. Then the drying process was continued in the vacuum desiccator, thus preventing mold growth. Finally, the pollens were placed in dark glass bottles, capped with paraffin and put in the refrigerator, so the pollens became suitable for study.

Preparing Pollen Extract

For extraction of *T.cordata* pollens procured from Gaziantep University campus. It was mixed in a 1:12 (weight: volume) 125mM NH_4HCO_3 (ammonium bicarbonate) solution at + 4 ° C for 12 hours in a low speed magnetic stirrer. Then the pollen residues will be removed by settling in a centrifuge (13000xg, + 4 ° C, 1 hour). The upper liquid phase was first passed through the 125mm thick whatman paper and then through the filtration system. The filtrate obtained was transferred to the dialysis tube. Dialysis was performed at + 4 ° C for 48 hours in a shaker against pure water.

Determination of Protein Concentration In Pollen Extracts

The protein concentration in the pollen extracts was determined according to the bicinchoninic acid (BCA) method by BCA Protein Assay Macro Kit (Serva, Germany). This method is based on the processing of proteins in alkaline solution with biurea reagent, reduction of Cu (II) ions to Cu (I) ions and spectrophotometric measurement of the complex formed by Cu (I) ions with BCA. First of all, the bovine serum albumin (BSA) was dissolved in water and diluted in appropriate proportions, and standards were prepared in certain concentrations. The BCA indicator was obtained by mixing the reagent solutions in the kit in a certain ratio. 200µL reagent was added onto the protein samples, standards and blank. Then it was incubated for 30 minutes in a 37°C incubator. After the incubation process, absorbance values at 562 nm were measured in the spectrophotometer. The protein concentrations were determined by placing the absorbance values of the protein values into the line equation.

Result and Discussion

In this study,we used BCA method at the first step, prepared the BCA according to the method suggested by Walker (2002). After we calculated the protein concentration as 4.193,32 µg / mL in the measurements we made at 562nm. In addition, as a result of morphological examinations, it was determined that the diameter of pollen was approximately 83 micrometers.

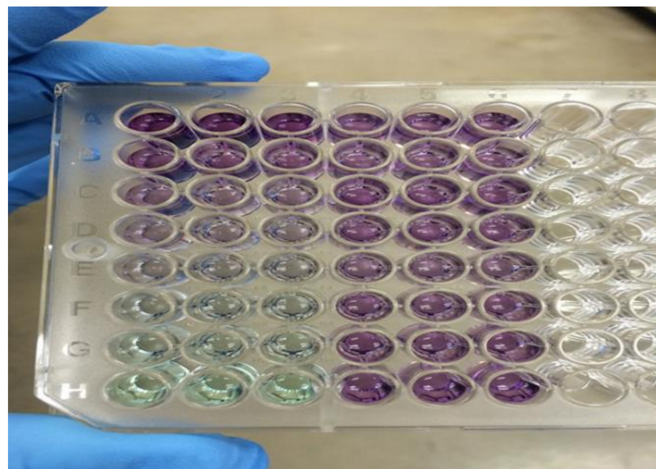

Figure 1. BCA method for calculate protein concentration.

Figure 2. Pollen images of T.cordata under microscope

A lot of studies show that allergy is important health problem in recent years. Pollens are the one of the important sour of allergen and allergenic disease. Pollens are aeroallergenic allergens and they enter the body

through respiration, causing allergic rhinitis and asthma. Allergenicty of proteins varies depending on size of molecule, affect mucosal barriers and solubility.

We could not find extensive research on *T.cordata* among the studies conducted so far. Against *T. cordata* in a small number of publications have been described allergic rhinitis, rhinoconjunctivitis, cough and allergic contact dermatitis. Mur et al. in their 2001 study, they reported allergic reactions to *T.cordata* in a 21-year-old woman. In laboratory examinations, the total Ig E level is high and the skin against *T.cordata* pollen extract They detected that the prick test (5% w / v) as positive. Several bands were identified by Ig E immune detection after SDS-PAGE on *T. cordata* extract. Non-specific ones at the molecular weight of ~ 20 kDa and ~ 21 kDa and essentially a specific allergen protein band at the ~ 50 kDa level were detected. This observation show that *T.cordata* has diferrent allergenic pollens. In the future, pollen allergies can be treated with specific immunotherapies using vaccines containing recombinant allergens to replace the ordinary simple pollen extracts used today. Therefore, in our further studies, we intend to characterize the tilia cordata pollen proteins in our region and observe the level of ig E in humans.

References

Behrendt, H. & Becker, W.M., (2001). Localization, release and bioaviability of pollen allergens: the influence of enviromental factors, *Journal Current Opinion in Immunology, 13*,709-715

Boral, D., Chatterjee, S. & Bhattacharya, K. (2004). The occurrence and allergengising potential of airborne pollen in West Bengal. *India, Annals of Agricultural and Enviromental Medicine, 11,* 45-52.

Celik, A., Guvensen, A., Uysal, I. & Ozturk, M., (2005), Differences in concentrations of allergenic pollens at different heights in Denizli, Turkey, *Pakistan Journal of Botany, 37*(3), 519-530.

Dykewicz, M.S., & Hamilios, D.L. (2010). Rhinitis and sinusitis, *Journal of Allergy and Clinical Immunology, 125*, 103-15

Heptt, W., Dinh, Q.T., Cryer, A., Zweng, M., Noga, O., Peiser, C.,Melvan, M., Witt, C., Fincher, A. & Gronberg, D.A. (2004). Phenotypic alteration of neuropeptide-containing nervefibres in seasonal intermittent allergic rhinitis, *Clinical and Experimental Allergy, 34*, 1105-10.

Knox, R.B. (1979), *Pollen and allergy*, London, Edward arnold limited, 60p.

Knox R.B. (1984). *The pollen grain*. Embryology of angiosperms. Springer, Berlin,pp. 197-271

Ring, J., Przybilla, B., & Ruzicka, T., (2006). *Atopy: Condition, Disease, or Syndrome?*, Handbook of Atopic Eczama, 2th ed. Springer, Germany, ISBN 3-540-23133-1

Rauder, C & Breiteneder, H. (2006), Pollen allergens are restricted to few proteins familles and show distinct patterns of species distribution, *Journal of Allergy and Clinical Immunology, 117*, 141-7.

Spieksma, F.T.M., (1991). Regional Europen pollen calenders. In: D'Amato G, Spieksma, F.T.M, Bonini S Editors, *Allergenic pollen and polinosis in Europe*. Oxford: Blackwell Sci. Publ., 49-65

Weerd, N.A., Bhalla, P.L & Singh, M.B., (2002). Aeroallergens and polinosis: molecular and immunological characteristics of cloned pollen allergens, *Aerobiologia 18,* 87-106

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