Bulgarian Journal of Agricultural Science, 24 (No 6) 2018, 1129–1136

Evaluation of sequential extraction of some biological materials from orange fruits peel (*Citrus sinensis*)

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Abstract

Ramadan, K., Nader, S., & Ibrahim, A. (2018). Evaluation of sequential extraction of some biological materials from orange fruits peel (*Citrus sinensis*). *Bulgarian Journal of Agricultural Science*, *24*(6), 1129–1136

The possibility of sequential extraction and determination of some biological material from orange fruits peel (Valencia variety which is obtained from two areas S and G) has been verified. Sequential extraction of essential oil, phenols, fixed oil, pigments, proteins consequently, and the impact of each stage of extraction stages on properties of the extracted materials in the other stages has been measured. The chemical composition of Essential oil has been identified by GC-MS. α-Limonene, β-Linalool, and β-Myrcene, major compounds are presence in the two regions oil. When sequential extrication was used the yield of water extract increased nearly 27% and the total phenolic compound increased higher than 5%, also yield of fixed oil raised by 8-38%, acid value and pigments of the fixed oil raised higher than 30%. Finally, the total content of proteins has been calculated and increased by 28-19%. Some of the microorganisms were growth in Bushnell Haas Broth when orange peel was added as carbon source. The results indicate the ability of getting benefit from the sequential extraction stages to the same sample, using various polar solvents; the absence of impact of extraction stages among them; significant increase in the extracted material in quantity and quality, and thus can be benefit from the biomass, especially if the biomass quantity is low to extract a number of active compounds, and many bioactive materials can be produced by bioconversion using microorganisms from orange peel, so this stages and methods can be used in industrial and economical field.

Keywords: orange peel; sequential extraction; bioactive materials; bioconversion

Introduction

Citrus are well known as one of the world's major fruit, that are produced in many tropical or subtropical climate countries, Brazil, USA, Japan, China, Mexico, Pakistan, countries of the Mediterranean region and South Asia are the major citrus producers (Kamal et al., 2011; Rashid et al., 2013). Orange tree is often cultivated, rarely found in the forests, it was planted for the first time in southern China, Northeast India and Burma, and it's in China since 2500 BC (Spiegel-Roy and Goldschmidt, 1996; Nicolosi et al., 2000).

Citrus Global production reached 48 million metric ton (Foreign Agricultural Service/USDA, 2017), which confirms the importance of this crop. Orange fruits are one of the most important food and medical source, due to the presence of many materials and chemical elements (USDA Nutrient Database, 2014).

It is the most important natural source of vitamin C (ascorbic acid), folic acid, vitamin E, and an excellent source of fibers (Matthaus and Özcan, 2012; Rashid et al., 2013). Juice production is the main objective of citrus industry, which leads to accumulation of abundant amount of waste,

which includes peels, seeds and pulp. Many studies show the importance of these residues, especially in the food industry, medical, cosmetic, and biodiesel production (Schulz et al., 2002; Silalahi, 2002; Saïdani et al., 2004; Rashid et al., 2013); that's for high content of many bioactive compounds such as essential oil and phenols, especially against bacteria, fungi, *Leishmania* and *Trypanosoma* (Graebin et al., 2010; Milind and Dev, 2012), and anti-oxidant materials (Anagnostopoulou et al., 2006; Li et al., 2006). Orange fruits are a natural integrated carotenoids source (Giuffrida et al., 2010). Orange peel contain fixed oil, which has been characterized with its fatty acids content (Islam et al., 2012; Khan et al., 2013), and it does not contain a high proteins content within limits 1% (USDA Nutrient Database, 2014).

The ability of *C. reticulata* fruits residue to absorb heavy metals such as chromium has been studied (Shafqat et al., 2008). In addition, the waste can be used as a source to produce animal fodder, bio-ethanol, citric acid, and many enzymes by peel bioconversion (Mamma and Christakopoulos, 2008).

The importance of this study is to find a method to extract the maximum amount of biomaterials from the same biomass – especially the orange peel considered as industrial residues or agriculture waste – by different extraction stages and using of solvents with different polarity, then studying of those materials characteristics, to estimate the effect of each extraction stages on other stages. The aim of this study is to extract from the same biomass essential oil, phenols by water and determination of the total phenolic compound, fixed oil extraction and determination of acid value and pigments presented in it, finally calculate the total content of proteins and using peels as a carbon source for the growth of some microorganisms.

Materials and Methods

Fruits of *Citrus sinensis* "Valencia" had been collected from two areas {Al-ssisanyah (S): (34º46′46″N 36º8′31″E), Al-jma'ashyah (G): (34º56′48″N 35º59′51″E)} in Tartous countryside (Syria), which are different in environmental factors due to the different topographic site. Collecting samples from two areas wasn't for studying the effect of environmental factors on extracted materials, that is just to confirm the result from two areas instead of one area. The study has been carried out on the first and second layer of the fruit (Flavedo: the orange layer and Albedo: spongy white layer), without pulp, dried and moist peel. When moist peel was used, it was cut to small pieces 1-2 cm^2 then used in extraction stages. When dried peel was used, it was dried in Hot Air Incubator (JSGI-250DT–JSR) at 50°C for 3 days, then used in extraction stages.

Extraction stages

1 – Essential oil extraction

1-1 Essential oil was extracted from moist peel of each area by steam distillation using Clevenger apparatus, 200 g of moist peel and 400 ml of water for two hours (Clevenger, 1928). Essential oil has been preserved at 6ºC, while water extraction has been preserved – after treatment described in Stage (2-1) – at 6ºC, until use it to determine the total phenolic content; it was expected that part of the phenols extracted during this stage; because they are well soluble in water. Some of pigments extracted in this stage and separated by centrifuging, then collected from the upper layer of the supernatant, and they were determined by Spectrophotometer (Spitzen) (Wellburn, 1994).

1-2 Essential oil was analyzed by Gas Chromatography (Agilent Technologies 7890A), 1 µg of Essential oil and *n*-Hexane (1:9 v/v) was injected, thermal program: 50°C to 250°C, at rate of 10°C/min, processing time 30 min, carrier gas (helium) at a flow rate 1 ml/min, pressure 7.6522 psi, column (HP-5MS 5% Phenyl Methyl Silox: 1785.43335 / C: 30 m × 250 µm × 0.25 µm 325), and Mass Spectrometry (Agilent Technologies 5975C – Ei), compounds were identified using mass spectrum and matching with mass spectral library (NIST).

2 – Phenols extraction and quantity determination

2-1 Phenols was extracted by water through the essential oil extraction stage, supernatant was obtained after sample centrifuging at 5000 rpm for 10 min by centrifuge (BOECO-Type 1610-13), then water was evaporated by Rotary Evaporator (RV 10 digital IKA), the crude extract was placed at 6ºC until the measurement.

2-2 Phenols was extracted by maceration from dried peel which resulted from the essential oil extraction stage, it was divided in two samples: (A) ungrinded, (B) grinded, pure water (8:1) has been used at room temperature for 24 hours (Rehman, 2006). Supernatant was obtained after sample centrifuging, the water has been evaporated by rotary evaporator, then placed at 6°C until the measurement. This stage aims to see if there is a need to extract phenols after they have been extracted during the essential oil extraction stage (2-1), and the effect of grinding peel process (samples A and B) in the yield and the total phenolic content.

2-3 Phenols were extracted from moist peel without grinding and without essential oil extraction, as first positive control, extraction method was the same in (2-2) stage.

2-4 Phenols were extracted from dried grinded peel and without essential oil extraction, as second positive control, extraction method was the same in (2-2) stage.

2-5 Folin–Ciocalteu method was used to determine total phenolic compounds by a linear standard curve of Gallic

acid solution and results were estimated by equivalents of 1 g of gallic acid to dried water extraction (Shaghaghi et al., 2008; Al Hafez et al., 2014). All total phenoilc compounds determination were performed in duplicate and results are presented as mean ± standard deviation.

3 – Fixed oil extraction

Fixed oil was extracted from dried-grinded peel (which resulted from (2-2-B) stage after essential oil and phenolic compound maceration extraction), Soxhlet Extractor with *n*-Hexane was used for 6 hours (Agarry et al., 2013), and for positive control dried-grinded peel (without essential oil and phenolic compound extraction) was used. Fixed oil was separated from the solvent using rotary evaporator, and placed at 6ºC until the measurement.

3-1 Fatty acid determination of Fixed oil according to (Hasuntree et al., 2011) by NaOH solution, and free fatty acid from formula (FFA% = AV × 0.502).

3-2 In this stage some of pigments were also extracted, and they were determined in fixed oil by Spectrophotometer (Wellburn, 1994).

Fatty Acid and pigment experiments were performed in triplicate and results are presented mean ± standard deviation.

3-3 Pigments were isolated by activated carbon, which activated by drying at 150°C for 24 hours, to confirm there is no water in carbon particles (Ferreira-Dias et al., 2000). 10 ml of n-Hexane and 1 g of activated carbon were added to 1 g of oil with rotating for 4 hours at 20°C (Ribeiro et al., 2001). The mixture was filtered using 0.2 µm filtering paper with Büchner funnel to isolate the carbon which contains pigments. *n*-Hexane was evaporated by rotary evaporator, then the residual pigments concentrations in oil was determined using Spectrophotometer (Wellburn, 1994).

4 – Extraction of total proteins and quantity determination

Total proteins were extracted from peels which resulted from (1, 2-2-B, 3) stage – after essential oil, phenolic compound, fixed oil and pigments extraction – by alkali method using KOH solution (Scopes, 1994), and were extracted from dried and moist peels as two positive control.

Total proteins content was determined using a linear standard curve of Casein solution by Coomassie blue detector (Bradford, 1976). This stage performed in duplicate and results are presented as mean ± standard deviation.

5 – Bioconversion of peel as source of carbon by microorganisms

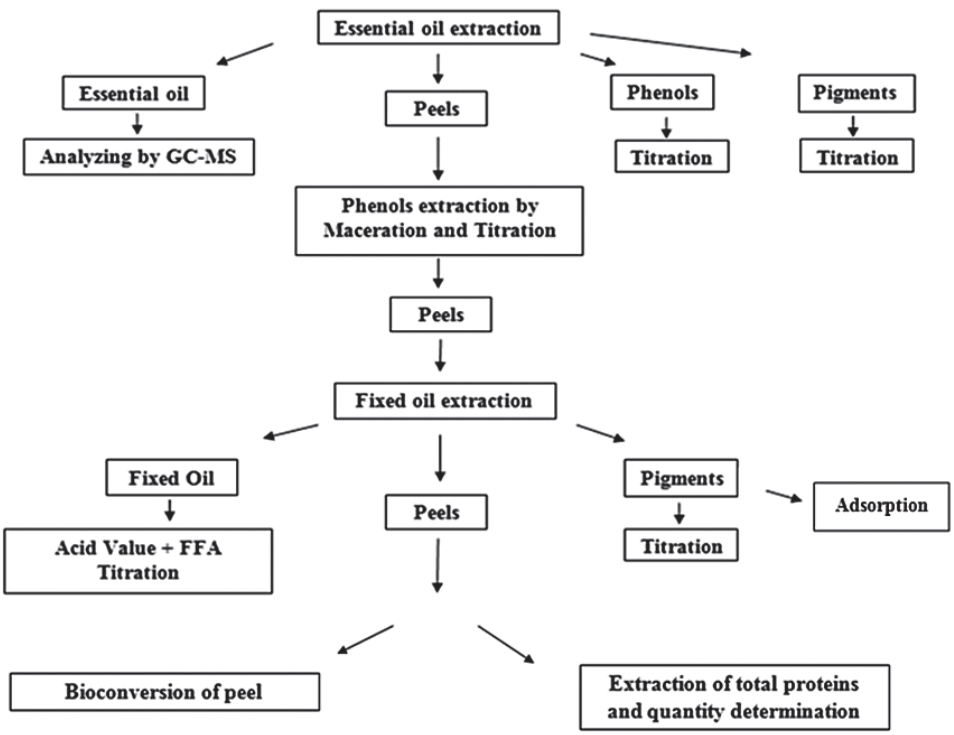

Fig. 1. The general outline of the extraction stages

Peels which resulted from (1, 2-2-B, 3) stage was added to the medium Bushnell Haas Broth by 1%, and 1% glucose as a positive control. *Bacillus subtilis, Aspergillus flavus, and Saccharomyces cerevisiae,* were used in this stage. The samples were incubated for 72 hours, at 26ºC for fungi and yeast, and 37ºC for bacteria (Ajao et al., 2011). Fig.1 shows the general outline of the stages of samples processing, and the experiments, which were performed at each stage.

6 – Statistical analysis

SPSS V20 software was used to calculate the significant differences (sig < 0.05) between mean values, One-way ANOVA procedure was used to perform the analysis of variance between sequential extraction and positive control in all stages.

Results and Discussion

1-1 Moist peel was used to increase the yield of active compounds especially D-Limonene **(**Kamal et al., 2011). Water is one of the most used solvents, and it is used instead of organic solvents which are used in essential oil extraction such as ethanol or methanol and to avoid fixed oils extraction, especially polar compound (mono and diglycerides), and it was used to increase stage economy. Extraction time is 2 to 6 hours referentially, and in this work two hours to avoid phenols heating, with considering that the presence of heating in phenols extraction increases its quantity, which increases the anti-oxidant activity (Li et al., 2006). In addition, there is no obvious increase in essential oil yield beyond two hour of extraction. The essential oil was extracted firstly, because it maybe evaporated in the next stages.

Table 1 shows that moist peel composed 40% of fruit weight, this percentage didn't reach referential percentage (50%) because the third layer of fruit (pulp) wasn't used. There is no significant difference in peel moisture for the two areas. Regarding the difference in essential oil yield between the two areas peels, due to the different environmental factors in the cultivation samples areas, and the result was in accordance with Siddiqi (2005), who indicated 0.2-0.5%. There is no significant difference in volumetric mass in the two samples, the result was in accordance with Njoku and Evbuomwan (2014), who indicated 0.84 g/ml.

Table 1. Orange peel properties

	S	G
Peel percentage %	36.75	42.86
Moisture %	76.86	78.81
Essential oil yield %	0.42	0.51
Volumetric mass of essential oil g/ml	0.8406 ±0.0026	0.8406 ±0.0005

1-2 There is a slight difference between the percentages of the essential oil components for the two areas samples, on the other hand Table 2 shows the difference between essential oil components and referential oils, that maybe due to the difference in varieties and environmental factors of cultivation areas.

Table 3 shows that pigments which was extracted in essential oil extraction stage weren't extracted by water, and was extracted by essential oil due to its ability to dissolve in non-polar compounds like essential oil, then it wasn't evaporated with essential oil, so pigments remained in water.

2 – Phenols were extracted in this stage (before the fixed oil extraction) to avoid heating of the fixed oil extraction by *n*-Hexane within a relative long period of time.

Table 2. Major components in essential oil of two samples and some of referential studies

%	Present study		Referential studies				
	S	G	1	2	3	4	5
α-Limonene	81.99	83.23	88.4	84.2	80.9	96.62	86.18–96.80
β-Linalool	2.76	3.79	3.49	4.4	1.52	–	0.31–2.56
β-Myrcene	2.89	2.40	0.25	4.1	4.19	1.72	0.93–2.05
Octanal	1.69	1.59	–	–	–	–	0.09–0.68
1-Terpinen-4-ol	1.94	1.44	–	–	–	–	0-0.31
α-Terpineol	1.08	0.99	1.01	0.8	–	–	0-0.25
1R-α-Pinene	0.68	0.55	0.36	0.9	1.65	0.47	0.28–0.55
α-Citral	0.46	0.53	–	0.5	–	0.31	–
Sabinene	0.52	0.32	–	–	0.37	–	0.13–0.93
Valencene	0.21	0.29	–	–	1.20	–	0-0.31

1: (Lin et al., 2010); 2: (Sharma and Tripathi, 2008); 3: (Kamal et al., 2011); 4: (Velázquez-Nuñez et al., 2013); 5: (Dugo and Mondello, 2011); (–): it's not existing or not determined

Table 3. Pigments concentration in orange peel water extract (µg/100 g dried peel)

Pigments	S	G
Chlorophyll A	18.32 ±1.40	13.26 ±0.87
Chlorophyll B	14.53 ±1.51	23.44 ±0.59
Carotenoid	580.15 ±62	496.41 ±43

Stage (2-3) was a positive control to determine the phenols content without any treatment. Drying and grinded was applied on another peel in stage (2-4) the second positive control.

Table 4 shows that, it's necessary to complete the extraction by maceration in stage (2-2) after the extraction of phenols in Essential oil extraction stage (2-1). About half of the yield resulted from stage (2-2), and the first half resulted from stage (2-1); due to the presence of heating and short time compared with the stage (2-2) where no heating was applied, but the extraction time was long. Stage (2-2) shows that, peels should be dried and grinded that increase yield ratio slightly.

Comparison between the two positive control, moist peel (2-3) give lower yields; but if its biomass was increased to reach – without moisture – biomass of dried peel (2-4), the yield in (2-3) would be higher than (2-4); because drying process (with heating and time) degrade some of phenols, and in dried peel all the components (membranes and organelles) in the cell adhere together in the absence of water; that makes the extraction more difficult (Li et al., 2006), conversely presence of water in moist peel facilitate phenols extraction; so if the yield was calculated in (2-3) by weight of dried peel, it would be (42.38% in S) and (43.02% in G).

Regarding total phenolic content in 1 g of dried water extract, there was no obvious difference in (2-2) and (2-4), but the slight increase in (2-1) and (2-3) maybe due to extract carbohydrate and organic acids in moist peel more than dried peel, which is titrated with phenols by Folin detector. So water in moist peel facilitate extraction of carbohydrate and organic acid addition to phenolic compound (Kim and Lee, 2002).

Grinding process in stage (2-2-B) also raises the total phenoilc content relatively, and there is a direct relation between phenols content and anti-oxidant activity (Liu et al., 2002). So, the yield of water extraction for the orange peel increased significantly nearly 27% and the total phenolic compound increased significantly higher than 5% for two samples when sequential extraction was used.

It's better not to use the water left out from stage (2-1) in stage (2-2), and use pure water to raise extraction efficiency; due to the possibility of decreasing the extraction efficiency if water that contains part of phenols – through the essential oil extraction stage (2-1) – was used.

3 – Extraction stages (essential oil and phenols) increased fixed oil yield in treated peel which was passed in stages (1, and 2-2-B: Essential oil and phenols extraction), in comparison with a positive control (untreated; wasn't passed in stages 1, and 2-2-B) (Table 5). Acid value was determined and shown in Table 5, because it is one of the most important indicators of oil disintegration (rancidity).

Table 5. Fixed oil yield in orange dried peel (%)

	S	G
Untreated	1.075	1.233
Treated	1.49	1.342

The increasing in yield of the treated sample may due to the absence of some extracted compounds (essential oil, phenols) after stages (1, 2-2-B), or by increasing the extraction efficiency resulted from changes in the cell wall structure, which facilitated the fixed oil dissolution in the solvent (*n*-Hexane).

Table 6 shows the effect of extraction stages (1, 2-2-B) in acid value and free fatty acid of fixed oil.

Table 6. Acid value and free fatty acid of fixed oil orange peel

	S		G	
	AV	FFA%	AV	FFA%
Untreated	23.4 ±0.86	11.74	26.449 ± 0.87	13.27
Treated	27.87 ±0.78	13.99	34.66 ±0.97	17.39

Table 4. Yield and total phenol content in orange peels for two samples

Phenolic extract		Extraction yield %		Total phenol content mg/g dried extract	
		S	G	S	G
Water was used in essential oil extraction		20.15	22.16	45.60 ±0.2	47.45 ±0.04
Peel was used in essential oil extraction and phenols was extracted again	A-Ungrinded	18.56	20.07	41.68 ±0.6	43.86 ±0.5
	B- Grinded	22.03	24.19	40.26 ±0.4	41.86 ±2.08
3- Moist peel without grinded		7.9	7.43	49.18 ±0.5	47.26 ±0.5
Dried peel with grinded		33.45	28	38.19 ±0.1	37.38 ±0.09

The relative increasing in the free fatty acid proportion in the treated peel is due to oil exposure to high temperature during the first stage – essential oil extraction – which caused an increase in the proportion of glycerides oxidation (Gupta, 2005).

Table 7 shows the concentration of pigments in fixed oil for two treated samples which was passed in stages (1, and 2-2-B), and compared with untreated sample (dried peel wasn't passed in 1 and 2-2-B stages). The rising in Chlorophyll A + B concentration in the two treated samples maybe was caused by changes in the cell wall, that allowed the solvent to extract more pigments amount. In addition, the yield of fixed oil extraction was raised in treated samples (Table 4), while the reduction in carotenoids concentration is due to its low content in these peel, because a part of carotenoids was extracted in essential oil extraction stage (Table 3).

Table 7. Pigments concentration in orange peel fixed oil (µg/100 g peels)

Pigments		S	G
Chlorophyll A	Untreated	48.32 ±1.10	29.26 ±0.33
	Treated	68.04 ±2.09	86.86 ±1.94
Chlorophyll B	Untreated	38.53 ±1.51	55.34 ±0.66
	Treated	105.83 ±3.1	200.46 ±3.41
Carotenoids	Untreated	903.15 ±121.3	1026.59 ±96.10
	Treated	277.31 ±37.17	437.22 ±13.47

So when sequential extraction was used, fixed oil extraction from orange peel increased significantly 38% for S and 8% for G, and the acid value increased 17% for S and 30% for G, also Chlorophyll A and B raised higher than 41% for two samples, and carotenoids decreased nearly 30–42% for two samples.

Pigments which extracted from orange peels with fixed oil were isolated by activated carbon. Table 8 shows that, there is obvious decrease in pigments concentrations especially carotenoid. Pigments have important characteristics in the food industry as colorants, dairy products, confectionery margarine and in medicine due to possessing several important functional properties, mainly antioxidant activity,

Table 8. Pigments concentration in orange peel fixed oil before and after adsorption (µg/100 g peels)

Pigments		S	G
Chlorophyll A	Before	48.32 ±1.10	29.26 ±0.33
	After	11.16 ±0.49	8.8 ±0.75
Chlorophyll B	Before	38.53 ±1.51	55.34 ±0.66
	After	8.3 ±0.37	18.4 ±0.8
Carotenoids	Before	903.15 ±121.3	1026.59 ±96.10
	After	204.67 ±55.9	356.68 ±57.4

as well as prevention of cardiovascular diseases, cancer and macular degeneration, and in some cases, provitamin A activity (Giuffrida et al., 2010; El-Sharnouby et al., 2013).

Pigments could be isolated from activated carbon by desorption process using displacement method by water or methanol which have higher polarity with presence of heating (Chern and Wu, 2001).

4 – Proteins were extracted at this stage (after 1, 2-2-B, 3: Essential oil, phenols, fixed oil and pigments extraction) to ensure proteins presence by determination of total proteins content in these peels. Table 9 shows the total content of proteins in the orange peel of the two areas, in addition to the two positive controls.

Table 9. Total proteins content in orange peels (mg/g)

	S	G
Moist Peel	3.88 ±0.13	3.83 ±0.07
Dried Peel	7.81 ±1.68	8.31 ±0.07
Treated	10.58 ±0.38	9.95 ±1.0

The increasing in yield of the treated samples maybe due to the absence of some extracted compound (essential oil, phenols, fixed oil, pigments) after stages (1, 2-2-B, 3), that facilitated proteins extraction. Comparison between protein percentage in moist and dried peels, protein content reduced in moist peel due to the low biomass, while in the case of calculating proteins proportion in moist peel according to its dried weight, the extraction yield rises in moist peel of two samples, and it was 14.63 mg/g in (S), and 20.67 mg/g in (G), so water in moist peel facilitates proteins extraction. Total proteins content also increased significantly 19-28% for two samples when sequential extraction was used.

5 – Bushnell Haas Broth is recommended for the examination the ability of organisms to use the source of carbon which added to a medium. Due to the low proteins content in orange peels, it's preferred to use this sample (peel after 1, 2-2-B, 3 stages) as a source of sugars rather than using it as a source of proteins. Table 10 shows that, Growing fungi and bacteria in this medium, and that is an evidence to produce bioactive materials such as Protease by *Bacillus subtilis,* bio-ethanol by *Saccharomyces cerevisiae* and citric acid by *Aspergillus flavus* (Mamma and Christakopoulos, 2008).

Table 10. Growth of microorganisms by orange peel as carbon source

	S	G	Glucose
Bacillus subtilis	+	+	++
Aspergillus flavus	+	+	++
Saccharomyces cerevisiae	+	+	++

Regarding fruit pulp, it can be used as a source for beverage clouding agents or used in animal feeding in animal feeding (Sreenath et al., 1995).

Conclusion

The results indicate the ability of getting benefit from the sequential extraction stages to the same sample – from orange peel which consider as agriculture waste – by using various polar solvents; the absence of negative impact of extraction stages among them, with relative increase in the extracted material in quantity and quality, and thus can be benefit from the biomass, especially if the biomass quantity is low to extract a number of active compounds. Many bioactive materials can be produced by bioconversion using microorganisms from orange peel after sequential extraction, so this stages and methods can be used in industrial and economical field.

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Received: January 22; *Accepted*: March 9; *Published*: December 31