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Effect of solvent, time and temperature on the some chemical properties of Salep tuber (*Anacamptis Collina*)

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Abstract
To investigate the effects of solvent type (ethanol, methanol, acetone and water), methanol concentration (20-95% v/v) time (2-5 hours) and temperature (37-62 °C) on the extraction of total phenolic compounds (TPC), total flavonoid compounds (TFC), antioxidant capacity and carbohydrate solutes of Salep tuber (*Anacamptis Collina*) using a single factor experiments. all the studied extracting condition showed significant effect ($p<0.05$) on TPC, TFC, antioxidant capacity and carbohydrate solutes. On the basis of the parameters, the best extraction conditions were 95% methanol for 5 hours extraction at 50 °C. According to these optimized condition, high content of TPC, TFC, antioxidant capacity and carbohydrates of Salep tuber extracts were obtained with value of 73.15 GAE/100g, 178 QE/ 100g, 93% and 31.18 mg/g DW respectively. To obtain more data and accuracy of optimization should be investigating another extraction methods and condition for future.

Keywords: plant extraction, phenolic compounds, optimization, antioxidant capacity

Introduction
Anacamptis Collina, one of the plants of the Orchidaceae family, is a flowering plant, commonly known as Salep, Orchis, and is greatly valued in ornament, medicine, conservation and evolutionary research. Root tubers are one of the parts of this herb that are used as food and medicine (Chemical composition and physicochemical properties of tubera salep, 2010) [19].

Salep is made out of the grinding of dried tubers of some Orchidaceae species, which are commonly used as in traditional drinks, as a food additive (Alghamdi, 2019) [25] (Shibao Zhang a, 2018) [29], and in the ice cream industry to prevent freezing and to preserve the taste. These physicochemical properties are determined by the chemical composition of the plant, especially the glucomannan surface of the plant species (Ahmed Kayacier, 2006) [1]. It also has medicinal purposes and is used in traditional medicine for treating some diseases (Bardajee GR, 2014) [4].

High intake of fruits and vegetables has a positive effect on human health due to its antioxidant compounds and plays an important role in the human body (Lila Boulekbache-Makhlouf, 2013) [21]. Consumption of food containing phytochemicals with potent antioxidant properties can reduce the risk of human diseases such as cancer, atherosclerosis, arthritis, diabetes and other aging-associated diseases (Rosana Chirinos, 2007) [24].

Very few studies have been conducted on orchid as an antioxidant source. There have been some studies on the the chemical composition and the antioxidant activity of the leaves and flowers of some orchid species; therefore, orchids are supposed to be potential sources of antioxidants (Truong Ngoc Minh, 2016) [35].

In this regard, it is important to evaluate an extraction process that maximizes the recovery of such compounds for qualitative and quantitative identification. The use of salep tubers as a source of antioxidant and optimization of the extraction and recovery process of these valuable compounds was investigated through an analytical method.

The first step in the separation of phenolic compounds from herbal materials is employing an appropriate extraction method. Solvent Extraction is a process designed to separate soluble phenol compounds by propagating a solid matrix (plant tissue) using a liquid matrix (solvent) (B. Lapornik, 2005) (B.B. Li, 2006). This process is widely used to extract phenol from various herbal materials. Many factors, such as solvent composition, temperature, duration, solvent-to-solid ratio and pressure, affect the extraction. In general, the optimization of a process can be achieved by empirical or statistical methods (Chandrika Liyana-Pathirana, 2005) [11].

Various solvents have been used to extract polyphenols from herbal materials. The antioxidant activity and function of the natural extracts are governed by the solution used for extraction. Aqueous blends of ethanol, methanol, and acetone are commonly used, but according to previous reports, the phenol extraction method differs for plant specimens, and the ideal extraction method for phenolic groups should be designed and optimized individually (Lila Boulekbache-Makhlouf, 2013) [21]. Also, the extraction time and temperature are other important parameters for optimizing the extraction process which is also investigated here (Giorgia Spigno, 2007) [15].

In traditional methods, in which only one factor is considered at a time, the process is time-consuming and optimization is not achieved accurately due to overlooking the interaction of various factors. However, statistical optimization methods can measure different variables by taking interactions of different factors into account (Chandrika Liyana-Pathirana, 2005) [11].

The main objective of this study was to investigate the effect of various solvents (methanol, acetone, and water),

extraction temperature, and extraction time on the extraction capacity of antioxidant compounds such as phenols and total flavonoids from rootstock tubers by using statistical optimization methods.

Material and Methods

Chemicals

Folin–Ciocalteu reagent was provided from Sigma,. Sodium carbonate (Na2CO3), acetone, ethanol and methanol were obtained from Scharlau. gallic acid and quercetin from Biochem-chemopharma (UK). Anthrone and 1, 1- diphenyl-2-picrylhydrazyl (DPPH°) was obtained from Sigma-Aldrich (Steinheim, Germany).

Collection of samples

Salep tubers were collected from the Henderson mountains of Soran District, Erbil, Iraq. To obtain 40-50 g of Salep tuber's powder, about 100 samples of tubers were collected from April to May 2019. Identification of species carried out according to usual methods. In this study Salep species identified based on method that suggested by Sami *et al*. (2019) [26].

Preparation of the samples

To obtain Salep powder, traditional method was performance according to Tekinsen & Tekinsen, 2008 method. At first time tubers washed cold water, after that to inactive enzyme, soften peel and cordex, tubers boiled in milk then dried in the shade for 7-10 days until tubers were hardened. Dried tubers cut in small pieces, then were ground.

Plant material extraction

500 mg of Salep dried powder were weighted and then extracted with 10 mL of extracting solvent. Afterward samples were taken in water bath shaker at different temperature and time. At the end of each extraction process samples were centrifuged at 5000 rpm for 15 min and then filtered with Whatman paper. The obtained extracted samples were used for determination antioxidant capacity, total phenolic compounds (TPC), and total flavonoid compound (TFC) and total carbohydrates measurements.

Experimental design

This study was investigated to determined optimum condition for extracting biochemical properties. A total four parameters were studied as included solvent type (Methanol, Ethanol, Acetone and water), Methanol concentration (20, 40, 80, 95%, v/v), Extraction time (2, 4, 5 h) and extraction temperature (37-62 C). The best extraction condition was selected on basis of antioxidant, TFC, TPC and carbohydrate measurements.

Solvent Extraction

Samples were extracted by use different types of solvents that included: Methanol 60% (v/v), ethanol 60%, Acetone 60% and water. Extraction time and temperature were set to 150 min and 25 C respectively.

Solvent concentration

The best solvent determined selected previously, different concentration solvent (20, 40, 80 and 95% v/v) was used to samples extraction. Extraction time and temperature were fixed at 150 min and 25 C respectively.

Extraction time

Salep samples were extracted with the best solvent concentration that previously was determined. Samples were extracted in various time (2, 4 and 5 Hours). Extraction time fixed at 25 C.

Extraction temperature

Finally after the using the best solvent type, solvent concentration and extraction time samples were extracted with different temperature from 37 to 62 C (37, 50 and 62 C). Extraction time fixed at 150 min.

Total phenolic compound (TPC) determination

The amount of TPC in peach extracts was determined according to the Folin- Ciocalteu method (Singleton *et al*. 1965) [32]. Samples (200 µL) were introduced into test tubes in which 1.0 mL of Folin-Ciocalteu's reagent (previously diluted 10 X with water) and 0.8 mL of sodium carbonate (7.5%, w/v) was added. The tubes were mixed and allowed to stand in darkness at room temperature for 30 min. Absorption at 765 nm against a blank was measured using an UV/VIS Spectrophotometer (Shimadzu UV mini1240, Suzhou Jiangsu, China). The total phenolic content was expressed as mg gallic acid equivalents per 100 g of dry weight (mg GAE/100 g DW) using a calibration curve. All measurements were carried out in triplicate.

Total flavonoid compound (TFC) determination

Total flavonoid content was estimated according to the procedure of Santas *et al*. based on the aluminum chloride complex formation. To 1 mL of peach extract, 1 mL of 2% (w/v) AlCl3 methanolic solution was added. The mixture was allowed to react for 10 min at room temperature and the absorbance was read at 410 nm against a blank. Total flavonoid content was calculated as quercetin from a calibration curve and results were expressed as mg quercetin equivalent per 100g of dry weight (mg QE/100 g DW). All measurements were done in triplicate.

DPPH° Radical-scavenging activity

In the 1, 1-diphenyl-2-picrylhydrazyl (DPPH°) assay, antioxidants were capable to reduce the stable radical DPPHº to the yellow coloured diphenylpicrylhydrazyl (DPPH-H). The test is based on the reduction of an alcoholic solution of DPPH° in the presence of a hydrogen donating antioxidant due to the formation of the non-radical form DPPH-H (Gulcin, 2007) [16]. The DPPH° radical-scavenging activity of the peach fruit extracts was estimated as described by Blois (1958) [6]. Briefly, 0.1 mL of each sample extract was mixed with 0.9 mL of 0.04 mg/mL methanolic solution of DPPH°. The mixtures were left for 20 min at room temperature and its absorbance then

measured at 517 nm against a blank. All measurements were carried out in triplicate. The percentage of DPPH radical-scavenging activity was calculated using the following equation:

% DPPH radical-scavenging = [(Ac - As)] x 100

Where Ac was the absorbance of the negative control (contained extraction solvent instead of the sample), and as was the absorbance of the samples.

Soluble carbohydrates

To measure water soluble carbohydrate was used by Anthrone reagent (Yemm, EW. and Willis, AJ. (1954) [38] the estimation of carbohydrates in plant extracts by anthrone The Biochemical Journal 57, 508–514).

To Make Anthrone reagent: dissolved 1 g of anthrone in 500 mL of 72% sulphuric acid, then Pipette 1.0 mL of test solution into a 10-mL test tube and cool to 0ºC on ice. Also add 5 mL of ice-cold anthrone reagent.

After mix reagent with samples, heat for exactly 11 minutes at 100ºC (in water bath) and cool rapidly to 0ºC on ice. Finally Read in A630 with spectrophotometer.

Results and discussion

Solvent Extraction

In this study a mixture of different solvents and water were used to extract some biochemical compounds from Salep tubers. According to the results methanol was the best solvent for extraction. Results showed that 60% methanol (151.7 mg QE/100g DW) was significantly ($p \leq 0.05$) effects on extraction of TFC while were not significant with water. Ethanol 60% represents lowest value (22.52 mg QE/100g DW) (Fig. 1).

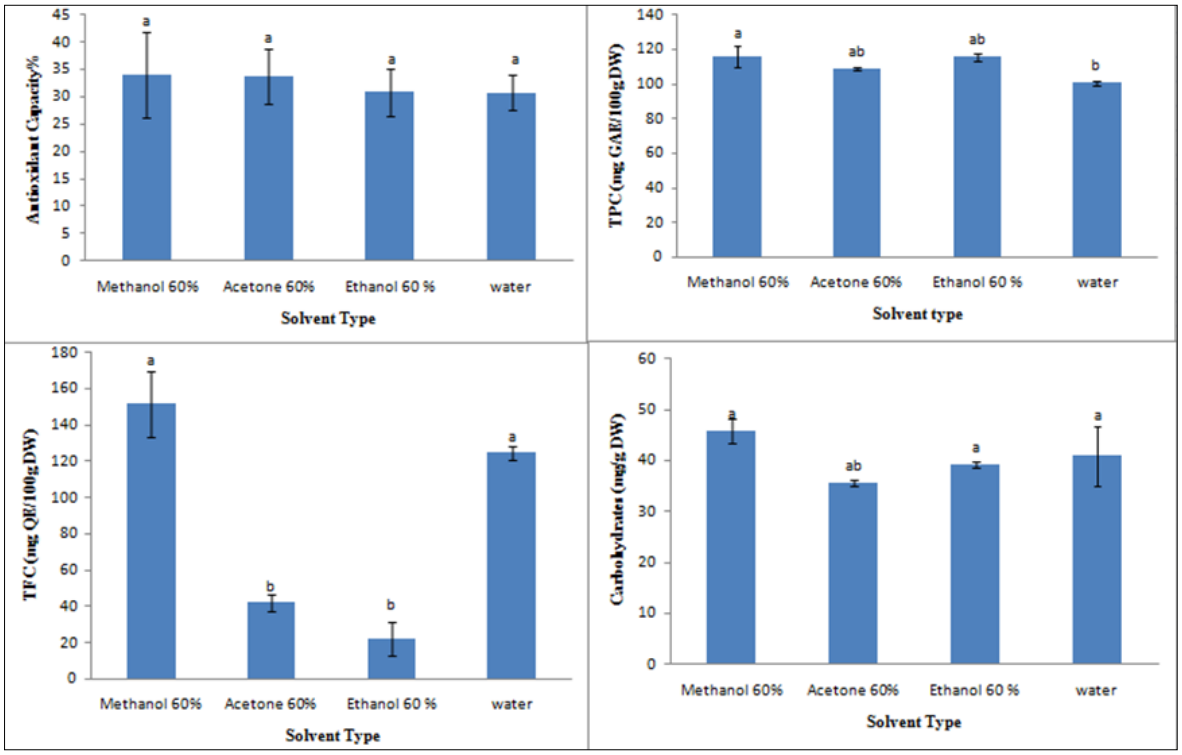

Fig 1: Effect of solvent type on the extraction of TPC, TFC, DPPH (C) and carbohydrates solutes from Salep tuber (n=3). Values are presented as means ± SD. Values marked with the different lower case letters are significantly ($p < 0.05$) different.

Also results showed that to extraction of antioxidant compounds no different significant was observed between solvent types. Although methanol 60% was the highest value (34%) and water the lowest value (30.75%). TPC significantly ($p \leq 0.05$) affected by solvents type. While methanol 60% was the best solvent (116 mg GAE/100 DW) for TPC extraction but no significant different was observed with acetone 60% and ethanol 60%. Water was the lowest value (100.8 mg GAE/100 DW). Methanol also showed more effective on carbohydrate extraction value (45.8 mg/g DW) but no significant different was observed between other solvents. Acetone showed the lowest value (35.63 mg/g DW) in carbohydrates extraction. Methanol is the best solvent to extracting phenolic acid and catechin, whereas ethanol better to flavonoid and catechols extraction. Acetone solvent due to low polarity compare to other is the best solvent to extracting tannins (Tan *et al*. 2013) [33]. Methanol due to has high polarity is able to extracting more polar compounds and increase solubility of non polar compounds. Variation in phenolic compounds in Salep tuber extract could change antioxidant activity present in Salep tuber. Since methanol solvent was the more suitable to extracting TFC, TPC and antioxidant activities. Polyphenol compounds have a range polarity from polar to non-polar, so polarity of solvent has significant effect on polyphenol extraction. Therefore polarity solvent due to results, have more interaction between polar sites of the antioxidant compounds and the solvent than non-polar solvents. Methanol obtains low value of antioxidant due to lower salvation (Jayanthi and Lalitha, 2011) [17]. Acetone solvent just a proton acceptor only while methanol and water are a powerful proton donor.

Solvent concentration

Biochemical compounds were extracted with different concentration of methanol ranging from 20 to 95% (v/v). Results showed that 95% methanol in all determination except TFC was the best concentration of methanol to extracting biochemical compound from Salep tuber. Highest yields of antioxidant activity obtained by 95% methanol (36.6%) but there no different significant to other concentration. Extraction with 95% methanol concentration was gave highest yield of TPC (158 mg GAE/100g DW), more two times the 20% methanol concentration, which showed the lowest value (79.6 mg GAE/100g DW). The highest TFC content was observed in 80% methanol concentration (108.5 mg QE/100g DW) which significantly different to other ($p \leq 0.05$). The lowest value of TFC was obtained by 20% methanol concentration (29.6 mg QE/100g DW). According to the results there is no different significant between 40% and 95% methanol concentration to extracting TFC (Fig. 2).

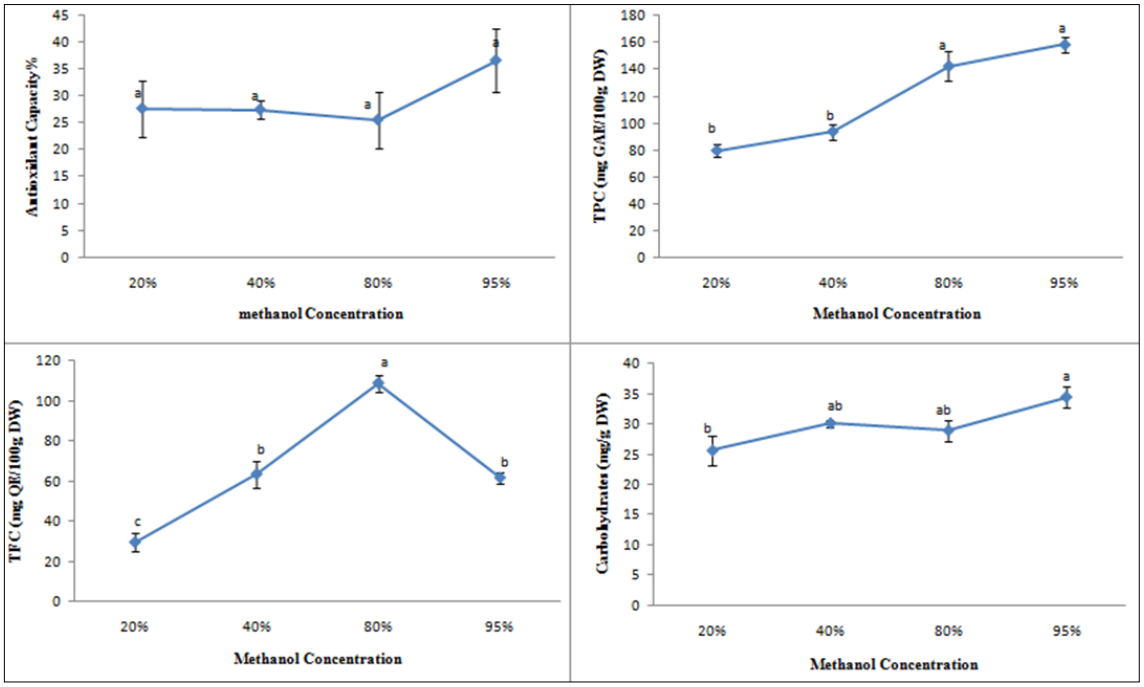

Fig 2: Effect of methanol concentration on the extraction of TPC, TFC, DPPH (C) and carbohydrates solutes from Salep tuber (n=3). Values are presented as means ± SD. Values marked with the different lower case letters are significantly ($p < 0.05$) different.

Also 95% methanol concentration was more efficient for the extraction of carbohydrates from Salep tuber with value of 34.4 mg/g DW, whereas there is no significant different with 40% and 80% methanol concentration. Intermediate value of 30 and 28 mg/g DW was obtained by using 40 and 80% methanol, respectively. The lowest TFC yield was obtained by using 20% methanol (25.6 mg/g DW).

Using aqueous organic solvent, can lead to creation a moderately polar condition which increase the polyphenol extraction. However due to obtained content with high impurities by pure water as extraction solvent, quantification and identification of phenolic compounds could interfere (Chirinos *et al*. 2007) [13]. Bonoli *et al* (2004) [7] reported that aqueous methanol is more effective in extracting of polyphenols and antioxidant activity from rice (Chatha *et al*. 2006) [12], moringa oleifera leaves (Siddhuraju and Becker, 2003) [30]. Extraction antioxidant compounds from different plants including rice bran, wheat bran, oat groats and hull, coffee beans, citrus peel and guava leaves using aqueous 80% methanol were better than other solvents (Anwar *et al*. 2006) [3]. Bushra *et al*. reported that using aqueous solvent (80% methanol, 80% ethanol) to extracting plant materials, showed better antioxidant activity and higher phenolic contents. In another report (amira *et al* 2017) [2] extracting with polar solvent, showed the highest amount of bioactive compounds and however high polyphenol content and high capacity to scavenge radical explain the powerful effects of polar solvent on bioactive extracting.

Extraction time

Results showed that with increasing extraction time from 2 to 5 hours, antioxidant capacity and carbohydrate was increased. After 5 hours extraction, antioxidant capacity was increased to 98 (%) while 2 hours after extraction, antioxidant capacity was obtained at lowest value (71.4%). High value of carbohydrates was obtained at 5 hours after extraction (23.24 mg/g DW). TFC and TPC were decreased when extraction time increased from 2 to 4 hours but an increase value observed from 4 to 5 hours. Highest value of TPC and TFC which obtained with 5 hours extraction time were 73.17 mg GAE/100g DW and 178 mg QE/100g DW respectively (Fig. 3).

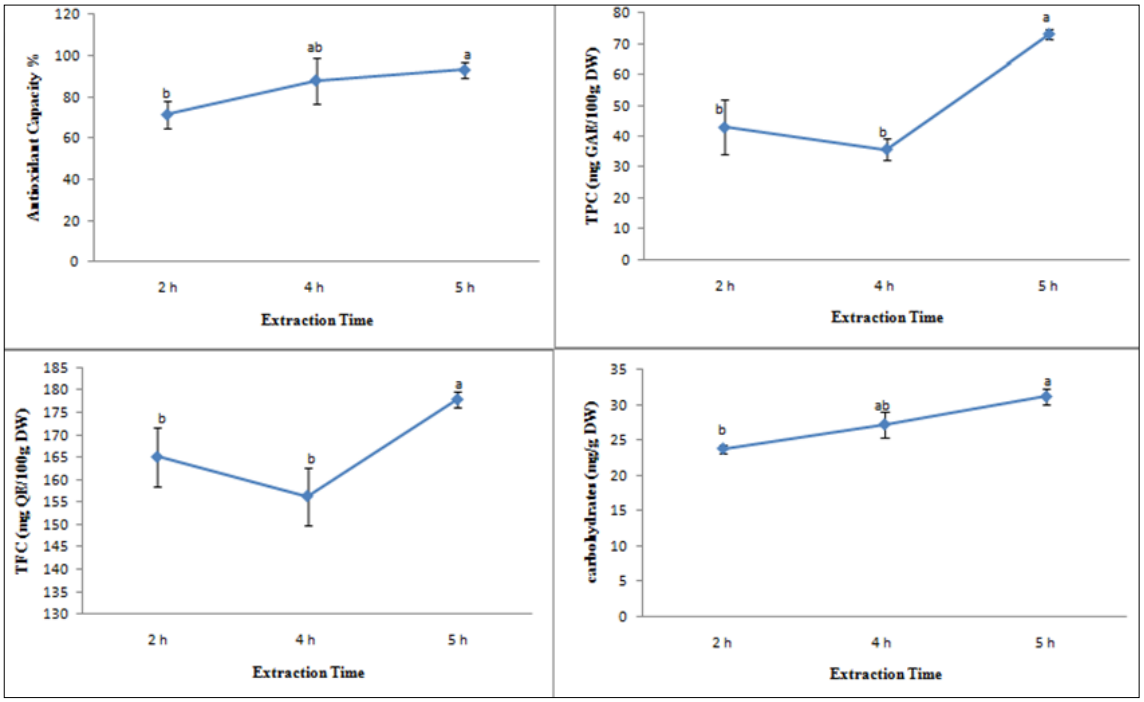

Fig 3: Effect of extraction time on the extraction of TPC, TFC, DPPH (C) and carbohydrates solutes from Salep tuber (n=3). Values are presented as means ± SD. Values marked with the different lower case letters are significantly ($p < 0.05$) different.

According to the law of diffusion that explained by Fick, a final equilibirium between concentration of solute in the solid matrix was predict after a suitable time and no longer time requirement to extract more phenolic compounds (Silva *et al*. 2007) [31]. Yap *et al* (2009) [10] and chan *et al*. (2009) [10] reported that the best time for extracting of phenolic compounds from Ayerrhoa carambola L. and citrus hystrix L. were 180 min after extraction time. Also with increasing extraction time, phenolics oxidation possibility increase (Naczk and shahidi, 2006) [22].

Extraction Temperature

According to the results, applying the best solvent (Methanol 95%) and the best extraction time, the best extraction temperature was obtained by 50 °C. results showed that antioxidant capacity, TPC and TFC content increased when extraction temperature increased from 37°C to 50°C while carbohydrates was decreased. The highest value of antioxidant capacity was obtained by 50°C (90.79%) while this value was decreased in 62°C (44.27%). Results showed that extraction temperature no significantly affected on TPC content but the highest TPC content was obtained by 50°C (83.17 mg GAE/100g DW). Increasing temperature from 50°C to 62°C significantly reduce the TFC content which reduce from 170 (mg QE/100g DW to 139 (mg QE/100g DW respectively. variation in carbohydrates content was significantly ($p \leq 0.05$) affected by different temperature. however, the highest carbohydrates was obtained by 37°C (31.44 mg/g DW) then showed a significant decrease to 50°C (21.29 mg/g DW) (Fig. 4).

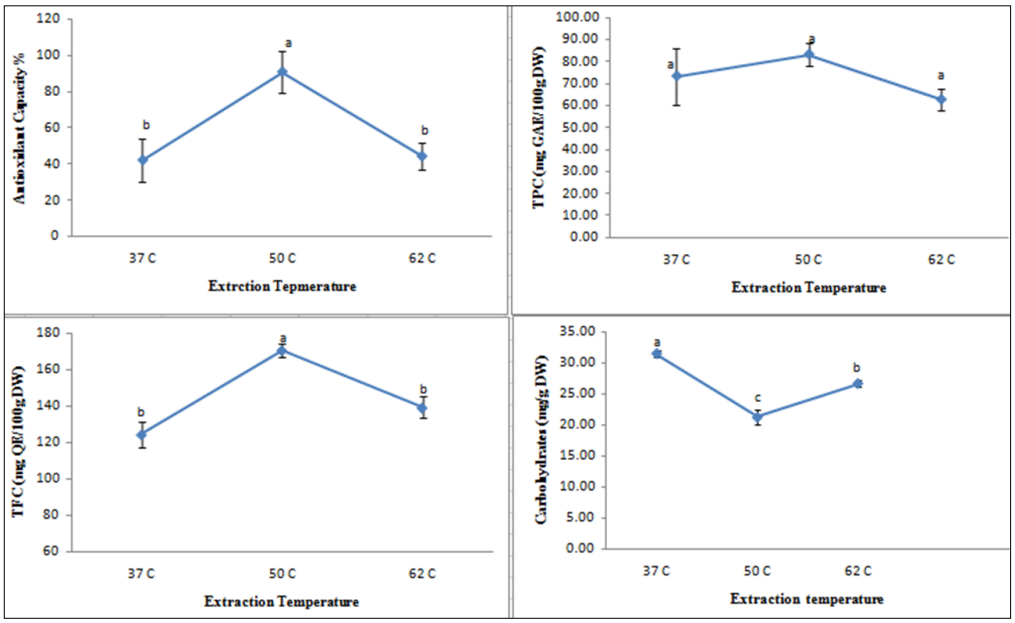

Fig 4: Effect of extraction temperature on the extraction of TPC, TFC, DPPH (C) and carbohydrates solutes from Salep tuber (n=3). Values are presented as means ± SD. Values marked with the different lower case letters are significantly ($p < 0.05$) different.

Plant tissue might soften by heating, also phenol-protein and phenol polysaccharide interaction weaken, therefore polyphenols concentration in solvent was increased (Shi *et al*., 2003) [2]. with this explain an increase in TFC when temperature extraction was increased since flavenoids are founds as glycosides (Garcia-Salas *et al*., 2010) [14]. Enhance of phenolic compounds and antioxidant activity would be improved by heat extraction (Benmeziane *et al*., 2014) [3]. This was probably due to increased phenolic solubility, faster diffusion rate, and better mass transfer extraction yield and reduces solvent viscosity in extraction process (Richter *et al*., 1996) [23]. By the way increasing temperature extraction might degradation of phenol compounds which previously extracted at low temperature.

Furthermore, high temperature may enhance solvent loss through vaporization and augment the extraction process cost from the industrialization point of view (Chan *et al*., 2009) [10]. Xu *et al*. (2007) [36] demonstrated that heat treatment of peel citrus increases the free fraction of phenolic acids and therefore increases the total antioxydant activity of extract. However, ester, glycoside, and ester bound fractions and total flavanone glycosides were decreased.

Shi *et al*. reported that temperature and time have been affected on carbohydrate solute during extraction process. Also another scientific research shows a significant interaction between solvent temperature and carbohydrate solute extracting from soybean (Johansen *et al* 1996) [18]. By increasing extraction temperature when water or 50% and 80% ethanol were used as solvent a higher amount of carbohydrates was observed. Extraction temperature below 50°C had a positive affected in aqueous extraction, but another hand temperature above 50°C decrease the carbohydrates extracting.

Conclusion

To determine the optimization of extraction process of Salep tuber a single experiment approach was used. Some variable such as solvent extraction (solvent type and concentration) extraction time and temperature investigated. Previously no data and information was reported about Salep tuber extraction. Results showed that TPC, TFC, antioxidant capacity and carbohydrate solute content were significantly affected by all the studied parameters. At the end of this investigation, the best condition to extracting was reported thus optimal extraction condition with 95% methanol at 50 °C for 5 hours was obtained. It is interesting to test other extraction methods and condition on extraction phenolic compounds from Salep.

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