EXTRACTION AND BIOANALYSIS OF ULTRASONIC ASSISTED PAKISTANI CULTIVAR PRUNUS DULCIS SEED: AN OPTIMIZATION STUDY

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ABSTRACT: *Prunus dulcis* (almond) is known to produce nutritional oil and the wide range of their food products. Level based experiments were designed for ultrasonic assisted extraction (UAE) technique by using multivariate Taguchi method. Two independent parameters: solvent seed ratio and sonic power were used to study the L9 orthogonal array model and to investigate the detailed effect of independent parameters towards maximized yield effect (MYE) whereas, extracting solvent (hexane), extracting time (30minutes) and extracting temperature (50^0C) were the dependent parameters in the present study. Solvent seed ratio 10:1 and 300W sonic power were found optimal conditions to get 50.70% oil. Ultrasonic assistance methodology was found good for oil extraction and 2.0% more oil fraction were obtained by this method as compared to conventional Soxhlet extraction. The optimization conditions were confirmed by signal to noise ratio (SNR) while adopting larger the better (LTB) expression and ANOVA used for the significant contribution of the independent parameters(91.72% and 8.282%). Antioxidant (IC50 38.2µg/µl) and antimicrobial activities of optimal conditioned UAE almond oil predicted the oil fate as value added special commercial oil.

Keywords: Ultrasonic extraction, taguchi method, signal to noise and *Prunus dulcis.*

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INTRODUCTION

Prunus dulcis commonly known as almond belonging to the Rosacea family is gaining considerable attention as a newly naturally value added edible oils. There have been different view points regarding the use of almond seed as the nut or for the production of edible oil since it is basically change of consumption of the same food source from one form to another. Since both the consumption forms are depending on the demand of consumers due to almond nutritive attributes as well as the health-promoting fatty acid constituents of its lipid (Cristina *et al.,* 2012). Pakistan is the second biggest importer of the edible oil and conventional sources of edible oil is limited, this may lead to explore the new sources. It had been a matter of focus for last ten years to explore rosaceae genus for the production of edible oils .These may include *Prunus* species. *Prunus dulcis* are already evaluated worldwide regarding characterization of oils on basis of physicochemical parameters, profiling of their lipids by conventional industrial adopted extraction technique - Soxhlet extraction with hexane and ether as extracting solvents (Ashraf *et al.,* 2011; Fatima *et al.,* 2012). Besides the consumption of seeds as the edible oil, another appealing aspect is the isolation of their phytochemicals due to the utilization of these vital components as functional food ingredients, food additives, nutraceuticals and food supplements. So new extraction techniques gained the attention of the researchers to get the desire able results. Although, they may not gaining the acceptance by the oil industry for the industrial production of edible oils (Al-Juhaim *et al.,* 2013). Some specific analytical methods like Folch method, Bligh and Dyer method for the extraction of oils of other species of the same genus along with Soxhlet extraction (Iverso*n et al.,* 2001). All these techniques rely on high temperature, the thermal ability of the extracting solvent with a long time of extraction which enforced the chemical (hydrolyse, polymerization, isomerization and oxidation) as well as physical (colour and odour) alterations of the extracted oil. Long time of extraction with high temperature makes conventional methods less effective for modern industrial production of oil due to failure in providing eco-friendly, economically viable, highly efficient and sustainable process to produce high-quality oil (Hisham *et al.,* 2016). Modern extraction techniques are widely used nowadays to meet the pace of technological advancement like green extraction, solvent-free extraction etc. along with the reduction in extraction time and temperature (Filly *et al*., 2014). Although, Soxhlet extraction technique is used commercially to extract the oils. Based on the research of the workers (Leadley *et al* ., 2001); (Ren *et al*., 2001) in which the ultrasound-assisted extraction has been appeared as an improved oil extraction yield technique. Present study focussed to explore the ultrasonic assisted extraction technique to extract the oil due to its minimum temperature and less solvent relying property.

Furthermore, this technique is gaining attention for the ability to extract the bioactive components of some medicinal important plants (Bhaskaracharya *et al.*, 2009; Karim *et al.*, 2012) and not has been used for almond seeds.

Optimization of the extraction process is vital for the transformation of the product into a commercial product and to make the process cost-effective. Different methodologies have been employed to optimize the processes in recent years for better yield (Letellier *et al*., 1999; Taslim *et al*. 2017). Orthogonal array helped to design a number of experiments for the demonstration of the efficiency of each factor adopted in full factorial experiments for Taugchi method selected for UAE. Factors like particle size, extraction temperature, extraction time are dependent variable whereas, the parameters like seed solvent ratio and sonic power considered as the independent variable.

The objective of present research was to develop the improved extraction technique with optimization of process. Antioxidant and antimicrobial potential of UAE almond oil were also determined.

MATERIALS AND METHODS

Almond seed collection: Almond seeds with shells were collected manually from the trees cultivated in Gilgit in the month of April 2015. Almond seeds were heated for 3 hours at 50^0C in a forced hot air Memmert - German oven for a notable hardness of the outer shells. (Piscopo *et al*., 2011). Traditional cracking technique (Hammering) used to collect the seeds. Sodium chloride (20%) solution was used for decortications of seeds to get the seeds for further study (Gupta *et al*., 2009).

Extraction of almond oil (Ultrasonic assisted extraction): Almond seeds were dried in the oven at 105^0C for 3 hours in order to remove moisture. Moisture free seeds were ground in the west point electric grinder. The extraction of oil was carried out batch wise as per DOE (design of experiment) by adjusting solvent seed ratio (mL/gm) and the sonic power (W).n-hexane was used as the extracting solvent. Extracting temperature was set at 50^0 C. Sonication was completed in 30 minutes. After sonication, the mixture was centrifuged at 4000 rpm for 10 minutes. The supernatants of each experiment were poured in separately labelled round bottom flasks. The excess solvent was evaporated with the help of rotary evaporator. The percentage yield of each experiment was determined (Tasliem *et al*., 2017).

Orthogonal array L9 design (Taguchi method): Taguchi method version of the multivariate statistical tool was selected to the design of experiment (DOE) and the L9 orthogonal array was found optimal assay of ultrasonic-assisted extraction of almond oil. Sonic power and solvent seed ratio were the independent variable whereas extraction time, extracting temperature and extracting solvent were the dependent variables. Three levels of sonic power were 300,200 and 100 whereas, 10:1, 5:1 and 2.5:1 mL/gm were the solvent seed ratio.

Table-1: L9 Orthogonal array for DOE with three variables and three levels (3^3) for oil extraction.

Experiment #	Variable parameters and their levels	
	Solvent/seed ratio(ml/gm)	Sonic power (W)
1	+1	+1
2	+1	0
3	+1	-1
4	0	+1
5	0	0
6	0	-1
7	-1	+1
8	-1	0
9	-1	-1

Analysis of variance (ANOVA): Analysis of variance computed for evaluating the individual contribution of each variable parameter. Larger the better (LTB) version was used for the yield determination. Signal to noise ratio (SNR) computed first for every designed experiment by considering the formula mentioned below:

Larger the better – $SNR_i = -10\log \frac{1}{n}\sum_{j=1}^{n}\frac{1}{Y_j^2}$ [1]

Furthermore, signal to noise level mean values (SNRL) of all the three levels of both parameters computed to calculate the overall contribution of each parameter for optimal extraction of oil (Taslim *et al*., 2017).

Antimicrobial activity of hexane-extracted almond oil with ultrasonic assistance: Agar disc diffusion method employed to determine the antimicrobial activities of the oil and its extracted fatty acids (Baydar *et al*., 2004). Some selected pathogenic microorganisms including *Staphylococcus aureus* , *Bacillus subtilis* (Gram positive bacteria) and *Escherichia coli*, *Salmonella enterica*, *Enterobacter aerogenes* (Gram negative bacteria) used to observe the tendency of the oil and its fatty acids to inhibit the microbial activity. Streptomycin a broad spectrum synthetic antibiotic used as the positive control. Microbial activity of samples and control against microbes observed after 24 hours and zone of inhibition was measured.

Total phenolic contents of hexane extracted almond oil with ultrasonic assistance: The Spectrophotometric method based on reagent Folin -Ciocalteu employed to analyse the total phenolic content. Hexane extracted

[1] [1] I,j and n stands for experiment no, trial no and no of experiments

almond oil (1.0 gm) with ultrasonic technique mixed with 1.25 ml reagent (Folin –Ciocalteu) and 2.0ml deionized water. The mixture was left for 10 minutes at room temperature. 3.75 mL out of 20% aqueous sodium carbonate was added to the mixture and incubated at 40^{o}C for 20 minutes. After 20 minutes absorption of the mixture was measured at 755.0 nm on a spectrophotometer. Different Percentages of Gallic acid and their absorbance measured at the same wavelength to prepare the standard curve to calculate the total phenolic content of the oil (Singleton *et al.,* 1965).

DPPH Scavenging Assay of hexane extracted almond oil with ultrasonic assistance: Antioxidant or antiradical activity of ultrasonic hexane extracted almond oil was determined by means of DPPH assay as described by Brand William (Brand William *et al*., 1995). DPPH solution was prepared by taking 0.006gm of DPPH in 100ml hexane. Similarly, oil samples were prepared by taking 15,30,45,60 and 75µg in 1µl hexane. 0.077 to 0.20µg of α-D-tocopherol in 1µl hexane were used to prepare the standard curve for expressing the antioxidant activity of the oil. Vortexed the 3ml of DPPH solution and oil at 25 ^{o}C for 30 minutes left in dark for further 30minute. Measured the absorbance of the oil fractions in DPPH at 517 nm to evaluate the oil ability to scavenge the free radical activity. Hexane used as the blank. The percentage of radical scavenging activity was calculated using the following equation

DPPH Scavenging affect (%) = (Abs.of control at 30 min/abs. of sample at 30 min)] x 100

RESULTS AND DISCUSSION

The edible oil market growth increased rapidly with the focused target of the utilization of different extraction techniques to develop a nutritional industrial product.

Selection of parameters and their level: The success of any newly extraction technique is based on the optimal conditions to get tangible targets. So experiments were designed to achieve the target. Parameters and their levels were selected for the present study.UAE technique was used for the extraction of almond oil for the present study. A no of researchers found UAE an effective process to get valuable food products (Bhaskaracharya *et al*., 2009). Sonic power and solvent seed ratio were selected as the independent variable with their three levels (higher, medium and low). The percentages of almond oil with the three levels of two variables by means of ultrasonic assistance mentioned in table 2 showed that both selected.

parameters yielded almond oil almost equal to the world highly yielded almond oil with the conventional method (Soxhlet extraction technique) reported by the previous researchers (Abdallah *et al*., 1998; Martins *et al*., 2000; Maestri *et al*., 2015). The present results strengthened by the findings of researchers who claimed the effectiveness of the UAE method as compared to others (Vinatoru *et al*., 2011; Sershti *et al*., 2012). Soxhlet method required high temperature which can cause the degradation of oil, loss of essential nutrients and active substances along with the appearance of some undesirable contents i.e. colour and odd aroma (Karim *et al*., 2012). Such matter enforced the posterior requirement of refining of the oil resulted in the increase the cost of the process. Some researcher found the ultrasonic assistance in oil extraction is more effective as compared to Soxhlet method of extraction (Tian *et al*., 2013).

Table-2: Ultrasonic assisted lipid yields of hexane extracted almond seed with two variables.

Extracting solvent	Ultrasonic assistance extraction		
	Sonic power (W)	Solvent seed ratio (mL/gm)	Yield %age
n-Hexane	300	10.0	50.70
n-Hexane	200	5.0	44.10
n-Hexane	100	2.5	29.70

Optimal extraction assay with ultrasonic assistance by Taguchi method: As the maximizing yield effect was the target of the study so optimal assay of the extraction process was the priority of the present study to make the extraction fraction a viable commercial product. Advanced multivariate statistical technique Taguchi method was employed to design the set of experiments. As two parameters with three levels of the values were selected so L9 orthogonal array was the best option to conduct the experiments. Increase the selected parameters forced the selection of other experimental runs. Larger the better was selected to compute the signal to noise ratio of the experiments. Taguchi method was ideal option of the present study because of the selection of LTB for evaluation of the highest yield . All the nine experimental runs with all the possible combinations with the levels of both parameters mentioned in table-3 (in triplicate) with their signal to noise ratio (SNR) and the sum of all the SNR.

Table-3 showed that first level of both the parameters yielded maximum oil fraction (50.70%) with maximum signal to noise ratio (34.09). Optimal conditions cannot be predicted from the yield and SNR values. So level mean signal to noise ratios (SNRL) was calculated. Furthermore, SNRL of level 1, 2, 3 of each parameter computed individually to obtain optimal extraction conditions. The SNR of level 1 was obtained by considering the runs in which solvent seed ratios first level conditions were selected for the extraction of oil. Similarly, SNR of level 2 was computed while

considering experiment 4,5,6 and SNR level 3 value from 7,8,9 experiment. Level 1,2,3 SNRs were calculated the same way while considering the other variable parameter 33.76, 31.83 and 29.67 were the SNR(1), SNR(2) and SNR(3)for solvent seed ratios whereas, 32.43, 31.60 and 31.23 for sonic power for level based SNRs.

The results showed that first level experimental conditions of both the parameters were the optimal conditions for maximum response (percentage yield). The plot of response surface of lipid extraction (Fig 1) confirmed the same result when solvent seed ratio and sonic power were considered together to get the response.

Table-3: Ultrasonic assisted extracted lipid yields of *P. dulcis* with two variables and three levels for L9 orthogonal array.

Experiments	Solvent seed ratio	Sonic power	Responses (yield %)			Mean response (yield %)	Signal to noise (SNR)	Sum of SNR (SNRT)
			1	2	3			
1	10/1	300.0	52.34	50.04	49.74	50.70	34.09	
2	10/1	200.0	47.39	48.25	48.95	48.19	33.65	
3	10/1	100.0	47.01	48.30	47.07	47.46	33.53	
4	5/1	300.0	48.09	46.11	44.32	46.17	33.28	
5	5/1	200.0	44.39	44.0	43.90	44.10	31.44	31.75
6	5/1	100.0	29.93	31.73	30.89	30.85	30.76	
7	2.5/1	300.0	35.80	33.77	34.10	34.56	29.93	
8	2.5/1	200.0	35.13	29.92	29.90	31.65	29.7	
9	2.5/1	100.0	29.00	31.31	28.79	29.70	29.4	

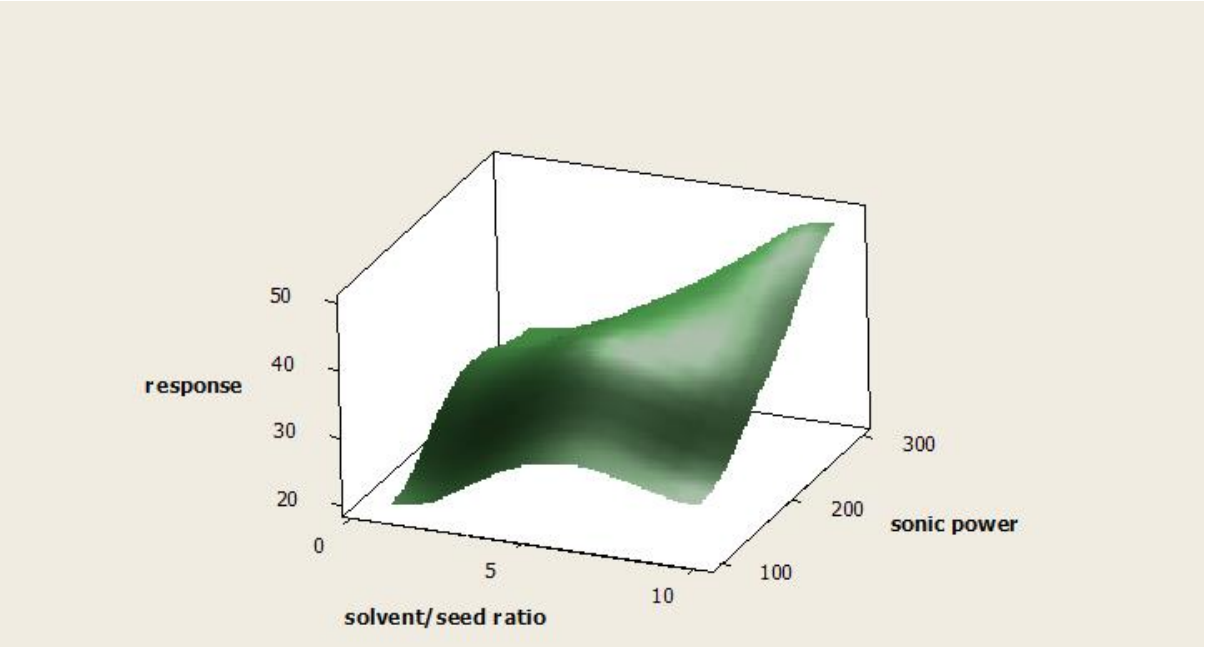

Figure-1: 3-D Structure of response against two variables in hexane solvent

The results showed that optimization was the best solution for the best output of the extraction systems that's why optimization studies was the mandatory part of the research based on the separation, isolation and extraction of oils and bioactive components (Wang *et al*., 2012; Sharazi *et al*., 2015; Khazaei *et al*., 2016). Unlike response surface methodology, Taguchi method showed the significant choice of the researchers seeking higher yield of products ((Taslim *et al*., 2017).

Analysis of variance (ANOVA): Analysis of variance indicated the 91.72 % significant contribution of the solvent seed ratio whereas 8.282% for sonic power.

The behaviour of the selected parameters towards the yield was well in accordance with the previous work (Zhang *et al*., 2008). The results showed that high ultrasonic amplitude and appropriate ratio of solvent and seed increased the yield of oil .The reason might be the increase of temperature and pressure inside

the solvent in short time. The pressure also generate violent wave whose strength was enough to penetrate into the cell tissue and enforced the internal material (oil) to move out in the solvent resulting in the disruption the Dulcis seeds wallsand seaping the oil in the surrounded solvent. Similar findings were reported by other researchers (Li *et al*., 2004; Sivakumar *et al*., 2007).

Antioxidant activity of *Prunus dulcis* in hexane extracted almond oil with ultrasonic assistance technology of almond seeds were tested by DPPH radical scavenging assay. The results showed that oil has the ability to reduce the stable diphenylpicryl hydrazine molecule.The change of DPPH colour from purple to yellow and the absorbance were the indicators of the smooth transfer of hydrogen ion from oil to DPPH. The comparison of the antioxidant activity of oil with α-D-tocopherol revealed its potential to scavenge the free radical. Previous work revealed that most of the studies were reported on the hydroextractor, methanolic extract and essential oils of different Prunus species. UAE almond oil was explored first time against the potent antioxidant as the best our knowledge. The responses of the different concentration of oils against the α-D-tocopherol were mentioned in table-4.

Table-4: Percentage Inhibition of DPPH and $IC_{50}$of hexane extracted UA *P.dulcis* oil against α-D-tocopherol.

UAE almond fractions with different solvents	Concentration in µg/µl	Inhibition of DPPH (%age)	IC_{50} µg/µl
Almond oil extracted with hexane	15	32.25	
	30	45.16	
	45	59.67	38.2
	60	61.29	
	75	74.19	
α-D-tocopherol(positive control)	0.077	22.50	
	0.100	56.45	0.109
	0.150	71.19	
	0.200	93.54	

IC_{50} for oil was calculated graphically (R^2 =0.899). Higher concentration of oil showed higher activity whereas, a low value of IC_{50} indicated high antioxidant activity. Previous work reported that α-tocopherol, phenolic compounds and unsaturated fatty acids polar lipids are the attributes of antioxidant activities in the methanolic extracts of the oil-bearing seeds (Elagbar, 2016). So the antioxidant property of the almond oil may attribute such characteristic of the oil.

UAE Almond Oil showed the tendency to transfer the electrons from phenolic compounds to Folin Ciocalteu reagent.1.84 mg equivalent of gallic acid eq./100g of *Dulcis* hexane extracted UAEAO (ultrasonic assistance extracted almond oil), which may be due to the effectiveness of the extraction technique as well as the selection of extracting solvent (Hexane). The antioxidant property of the oil supported the result of total phenolic content as both the values correlate with each other (Veliglu *et al.*, 1998).

Antimicrobial activity of almond oil itself did not show any antibacterial activity while the extracted fatty acids of almond oil showed intermediate inhibition against microbial activity raised due to gram-positive (*Staphylococcus aureus* and *Bacillus subtilis*) and Gram negative bacteria (*Escherichia coli, Salmonella enterica* and *Enterobacter aerogenes*). The oxide culture media namely GM145 = Staphllo medium 110, CM 27 = Blood ager base, GM 69 = Eosin methylene blue GM 201 = Bismithsulphite ager; CM7 = MacCon key's ager respectively, used with incubation temperature 37˚C for the microbial growth of microbes. Broadspectrum streptomycin used as a positive control. Table -4 showed the mm inhibition zone of fatty acids of hexane extracted ultrasonic treated almond oil, fatty acids of almond oil and potent antibiotic used as the control. Extracted fatty acids of oil showed no inhibition activity against *E.coli* but inhibited intermediately against rest of microbes. Seven mm against *E.aerogens*, 9mm against *S.typi*, 10mm against *S.aureus* and *B.subtilis*. Previously reported microbial activities were against the essential oils and the extracts of seeds in methanol and ethanol (Parveen *et al.,* 2013). No work on the fixed oil was yet reported according to authors best knowledge. But the results were in accordance with the traditional research reviews that mostly oils (fixed) showed no antimicrobial activities but their unsaturated fatty acid exhibited inhibition against microorganism (Kitahara *et al*., 2004; Desbors *et al.,* 2010).

The reason of ultrasonically treated extract of almond fatty acids antimicrobial inhibition against gram positive and gram negative microorganisms might be due to the presence of the appreciable amount of unsaturated fatty acid phenolic compounds and other contents which remained in the natural state due to heat free treatment of the almond seed during the process of extraction.

Table-5: **Antimicrobial Activity of *Prunus dulcis.***

Almond seeds parts	Tested microorganism	Colony Morphology	Incubation temperature ˚C	Culture Media Oxide/ CM	mm Inhibition Zone of almond seed parts at 24 hrs/	mm Inhibition of Zone of Control at 24 hrs	Efficiency
Liberate d Fatty	*Staphylococcus aureus*	Gram +ve cocci	37˚C	145	10	15	I
Acids of	*Bacillus subtilis*	Gram +ve rods	37˚C	271	10	17	I
Oils	*Escherichia coli*	Gram –ve rods	37˚C	69	-	21	-
	Salmonella enterica	Gram –ve rods	37˚C	201	8	20	I
	Enterobacter aerogenes	Gram –ve rods	37˚C	7	7	17	I
UAE	*Staphylococcus aureus*	Gram +ve cocci	37˚C	145	-	15	R
almond oil with	*Bacillus subtilis*	Gram +ve rods	37˚C	271	-	17	R
Hexane	*Escherichia coli*	Gram –ve rods	37˚C	69	-	21	R
	Salmonella enterica	Gram –ve rods	37˚C	201	-	20	R
	Enterobacter aerogenes	Gram –ve rods	37˚C	7	-	17	R

Conclusion: The effectiveness of extraction technique for oil under optimal conditions found solvent seed ratio, sonic power, reaction time and extraction temperature in the present study. Solvent seed ratio showed highest contribution (91.72 %) to get 50.70% oil fraction. IC_{50}µg/µl was 38.2 showed that oil has the antioxidant ability. In the light of the results based on present study, the UAE can be considered as a good replacement of conventional extraction technique for commercial production of edible oil.

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