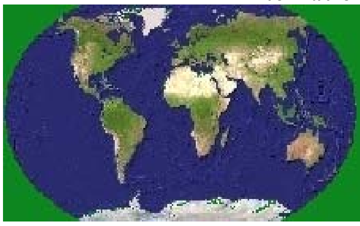

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Extraction of polyphenol in bitter melon (M*omordica charantia*)

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Abstract
Bitter melon (*Momordica charantia*) is traditionally known for its medicinal properties such as antidiabetic, anticancer, anti-inflammation, antivirus, and cholesterol lowering effects. It contains many phenolic compounds that may have the potential as antioxidant and antimutagen. We evaluate and select bitter melon to extract polyphenol. Main technical factors for extraction: ethanol 70^o, material/solvent 1/15, temperature 70 ^{o}C, time 120 minutes, pH 3.5, two step extraction recovery 87.27%. Main technical factors for acid treatment: ratio of extract/water 1/15, temperature 70 ^{o}C, time 45 minutes, deposit 36 hours. Main technical factors for purification: polyphenol/ethyl acetate 2/1, three steps of purification.

Keywords: Bitter melon, extraction, acid treatment, purification, polyphenol.

1. Introduction

Phenolic compounds are categorized as secondary metabolites essential for growth and reproduction of plants. They are known as hydrophilic antioxidants, and are produced as a response for defending injured plants against pathogens. They potentially show antioxidant, antimutagen, antitumor, antiinflammatory, and anticarcinogenic properties (Lee *at al.*, 2003) [6].

Bitter melon or bitter gourd (*Momordica charantia*) or commonly as Kho Qua, is an herbal plant grown in Vietnam. Bitter gourd is harvested as a physiologically ripening stage, before the true onset of ripening. It is important, if the fruit is to be transported overland (taking from 1-5 days from origin to market place), that fruit be selected that harvest started the physiologically process of ripening, for they will produce ethylene that hastens improving at store fruit (Aminah *et al.*, 2011; Morgan and Midmore, 2002) [2, 8]. It is traditionally known for its medicinal properties such as antidiabetic, antitumorous, anticancer, anti-inflamatory, antiviral, and cholesterol lowering effects etc. (Parichat Budrat and Artiwan Shotipruk, 2008; Ahmed *et al*, 2001; Grover *et al.*, 2004; Taylor, 2002) [9, 1,4, 12]. The main constituents of bitter melon which are responsible for these effects are such as triterpene, proteid, steroid, alkaloid, inorganic, lipid, and phenolic compounds (Grover *et al.*, 2004) [4]. The protein in bitter melon including protein MAP-30, alpha–momorcharin, and beta–momorcharin were shown to have the ability for fighting against HIV (Luetrakul, 1998) [7]. A steroid, charantin, contained mainly in the aerial parts, has been proven for its antidiabetic activity (Chanchai *et al.*, 2003) [3]. The phenolic compounds from bitter melon extracted by solvent extraction were reported to exhibit antioxidant activity (Horax *et al.*, 2005) [5].

The Bitter gourds have many health benefits and medicinal properties. These are such as kills bacteria, reduce inflammation, kill viruses, fights free radicals, kills cancer cells, kills leukemia cells, prevents tumors, cleanses blood, reduces blood sugar and balance hormones. Beside these stem and leaf of bitter gourd is used in cancer treatment, in vital infections (HIV, herpes, Epstein Barr, hepatitis, influenza, and measles), in bacterial infections (*Staphylococcus, Streptococcus*, and *Salmonella*), as a bitter digestive aid (for dyspepsia and sluggish digestion) and in diabetes.

The Bitter gourds have many health benefits and medicinal properties. These are such as kills bacteria, reduce inflammation, kill viruses, fights free radicals, kills cancer cells, kills leukemia cells, prevents tumors, cleanses blood, reduces blood sugar and balance hormones. The bitter gourd has natural benefits and curative properties. It has excellent medicinal value. It is antidotal, antipyretic tonic, appetizing, stomachic, antibilious and laxative. Bitter gourd being rich in all essential vitamins and minerals, especially vit. A, B1, B2, C and Iron, its regular use prevents many complications such as hypertension, eye complications, neuritis and defective metabolism of carbohydrates. It increases body's resistance against infection. But the main problem in its consumption is its bitter taste (Tanuja Srivastava and D.C. Saxena, 2012) [11]. Purpose of our research is to investigate some main technical factors affecting to polyphenol extraction in bitter melon so that we can get the best extraction recovery.

2. Material & Method
2.1 Material
Bitter gourd material is collected on farms of Tra Vinh and Soc Trang province, Vietnam.

Fig 1: Bitter melon

2.2 Research method
2.2.1 Effect of extraction
2.2.1.1 Effect of solvent extraction

Each sample has 10 gram. Prepare 6 samples to extract by different solvents: H_2O, ethanol 50^0, 60^0, 70^0, 80^0, 96^0.

2.2.1.2 Effect of solvent ratio

Each sample has 10 gram. Prepare 6 samples to extract by different solvent ratios such as 1:5; 1:10; 1:15; 1:20; 1:25; 1:30.

2.2.1.3 Effect of extraction temperature

Each sample has 10 gram. Prepare 6 samples to extract by different extraction temperatures: 30, 40, 50, 60, 70, and 80 (^{0}C).

2.2.1.4 Effect of extraction time

Each sample has 10 gram. Prepare 6 samples to extract by different extraction times: 30, 60, 90, 120, 150, and 180 (minutes).

2.2.1.5 Effect of extraction pH

Each sample has 10 gram. Prepare 6 samples to extract by different pH values: 5.5, 5.0, 4.5, 4.0, 3.5, 3. 0.

2.2.1.6 Extraction recovery

Extraction is executed through 4 step, measured OD value after each step to compare to the initial so that extraction recovery can be calculated.

2.2.2 Acid treatment
2.2.2.1 Effect of extract/solvent

Each extract sample has 5 gram. Prepare 4 samples with different ratios of extract/solvent: 1/5; 1/10; 1/15; 1/20.

2.2.2.2 Effect of acid treatment temperature

Each extract sample has 5 gram. Prepare 6 samples to treat with different temperatures: 30, 40, 50, 60, 70, 80 (^{0}C).

2.2.2.3 Effect of acid treatment time

Each extract sample has 5 gram. Prepare 5 samples to treat with different times: 15, 30, 45, 60, and 75 (minutes).

2.2.2.4 Effect of deposit time

Each extract sample has 5 gram. Prepare 4 samples to treat with different deposit times: 12, 24, 36, 48(hours).

2.2.3 Purification
2.2.3.1 Effect of the acidified extract/ ethyl acetate

After acid treatment, the extract is finely filtered and collected 100 ml for each testing. Prepare 5 samples with different ratios of polyphenol extract/ ethyl acetate as follows 1/1, 2/1, 3/1, 4/1, 5/1.

2.2.3.2 Effect of purification time

Prepare 5 samples to purify in different times: 30, 45, 60, 75, 90(minutes)

2.2.3.3 Purification efficiency

Purification is executed through 5 steps by ethyl acetate. After each purification step, we measure its OD to compare with the initial amount so that we can calculate the purification efficiency.

2.3 Statistical analysis
All data are processed by ANOVA (Startgraphics) to check the significant difference via LSD.

3. Result & Discussion
3.1 Polyphenol in different bitter melon maturities

Table 1: Polyphenol content in bitter melon

Bitter melon	*OD*	*C (mg/ml)*	*Dilution*	*$V_{extract}$ (ml)*	*∑Polyphenol (mg/∑$V_{extract}$ from 10 gram material)*
Unripe (dark-green)	0.800	$85.802.10^{-3}$	10	185	158.734
Ripe (green-yellowish)	0.938	$90.802.10^{-3}$	10	186	168.892
Overripe (yellow-orange)	1.056	$103.094.10^{-3}$	10	186	191.755

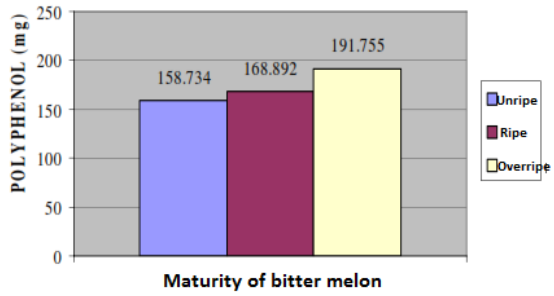

Fig 2: Polyphenol in bitter melon at different maturity stages

From table 1 and figure 2, we notify that the polyphenol in bitter melon at override maturity is higher than the unripe and ripe maturity. So we choose the overripen maturity for further experiements.

Table 2: Comparison of polyphenol in bitter melon with other materials

Material	OD	C (mg/ml)	Dilution	$V_{extract}$ (ml)	∑Polyphenol (mg/∑$V_{extract}$ from 10 gram material)
Bitter melon	1.056	$103.094.10^{-3}$	10	183	188.662
Green tea leaf	0.845	$81.115.10^{-3}$	10	185	150.063
Dried tea	1.063	$103.823.10^{-3}$	10	185	384.145

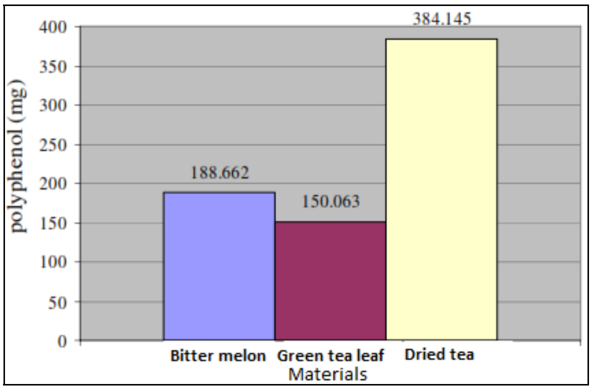

Fig 3: Comparison of polyphenol in bitter melon with other materials

From table 2 and figure 3, we can see the polyphenol content in bitter melon is 1.25 times higher than then green tea leaf but 2.04 times lower than the dried tea. So it's very ideal to choose bitter melon for polyphenol extraction.

3.2 Polyphenol extraction in bitter melon
3.2.1 Effect of extraction solvent

Table 3: Effect of extraction solvent

Solvent (ethanol)	OD	C (mg/ml)	Dilution	$V_{extract}$ (ml)	∑Polyphenol (mg/∑$V_{extract}$ from 10 gram material)
0^o (H_2O)	0.498	$44.97.10^{-3}$	20	185	166.389
50^o	0.673	$63.19.10^{-3}$	20	185	233.803
60^o	0.778	$74.146.10^{-3}$	20	187	277.306
70^o	0.755	$84.236.10^{-3}$	20	187	315.043
80^o	0.886	$85.594.10^{-3}$	20	187	320.122
90^o	0.759	$75.912.10^{-3}$	20	187	283.911

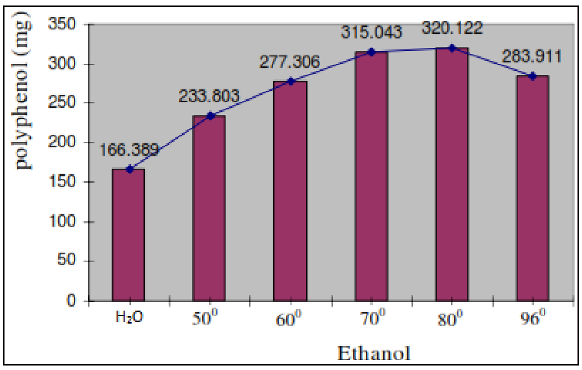

Fig 4: Effect of extraction solvent

From the experimental data shown in table 3 and figure 4, we can see the changing of the concentration of aqueous ethanol from 0o (water) up to 80o with total polyphenol in the ascendant. Ethanol solvent enables to extract many compounds with different polarization and even the non-polar substances, the more of ethanol concentration is, the more of the osmotic potential and diffusion for active elements are. Therefore the total polyphenol extracted will be increased if the concentration of polyphenol increases.

The total polyphenol will be increased significantly when changing solvent concentration from 600 to 700 (1.137 time). From solvent concentration increases from 700 to 800, the polyphenol content changes insignificantly (1.015 times). Moreover, if inceasing ethanol concentration to 960 the polyphenol will be decreased from 320 to 283 (mg/∑V extract in 10g raw material). This phenomenon is explained by high ethanol concentration, the vegetable cell will be denatured and hindered the extraction. So we choose ethanol 70o for further experiments.

3.2.2 Effect of extraction solvent ratio

Table 4: Effect of extraction solvent ratio

Solvent ratio	OD	C (mg/ml)	Dilution	$V_{extract}$ (ml)	∑Polyphenol (mg/∑$V_{extract}$ from 10 gram material)
1:5	0.673	$63.192.10^{-3}$	20	34	42.971
1:10	0.752	$71.435.10^{-3}$	20	86	122.868
1:15	1.102	$107.89.10^{-3}$	20	135	291.303
1:20	0.899	$86.741.10^{-3}$	20	187	324.411
1:25	0.890	$86.01.10^{-3}$	20	230	395.646
1:30	0.788	$75.188.10^{-3}$	20	278	418.045

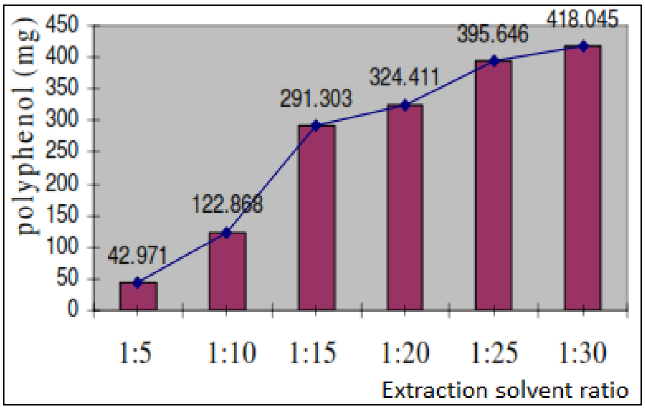

Fig 5: Effect of extraction solvent ratio

When increasing solvent ratio, the extraction efficiency also increase significantly, especially from 1:5 to 1:10, 1:10 to 1:15; but slightly change at 1:15 to 1:20, 1:25 to 1:30. During extraction by solvent, the more solvent we use, the more extraction efficiency we receive owing the solvent gradient.

Table 5: Extraction efficiency by solvent ratio

Solvent ratio	Solvent gradient	Polyphenol increasing
1: 5 to 1: 10	100%	190%
1: 10 to 1: 15	50%	138%
1: 15 to 1: 20	33%	11.3%
1: 20 to 1: 25	25%	21.9%
1: 25 to 1: 30	20%	5.8%

In table 5, when we can see the solvent ratio 1:15 (g/ml) is optimal to extract polyphenol.

2.2.3 Effect of extraction temperature

Table 6: Effect of extraction temperature

Temperature (°C)	OD	C (mg/ml)	Dilution	$V_{extract}$ (ml)	∑Polyphenol (mg/∑$V_{extract}$ from 10 gram material)
30	1.162	$114.135.10^{-3}$	20	115	262.511
40	1.201	$118.198.10^{-3}$	20	115	271.855
50	1.230	$121.219.10^{-3}$	20	115	278.804
60	1.264	$124.760.10^{-3}$	20	115	286.948
70	1.312	$129.761.10^{-3}$	20	115	298.450
80	1.284	$126.844.10^{-3}$	20	115	291.741

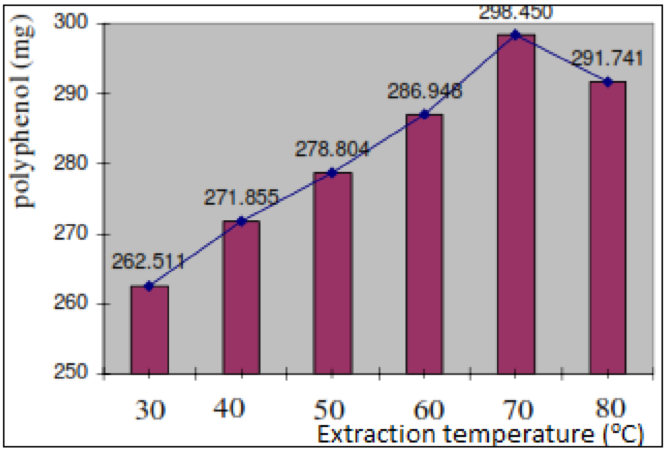

Fig 6: Effect of extraction temperature

From table 6 and figure 6, we see the polyphenol extracted increases when temperature increases from 30°C to 70°C and decreases at 80 ^{0}C. At 70 ^{0}C, the extraction efficiency is optimal.

3.2.4 Effect of extraction time

Table 7: Effect of extraction time

Extraction time (minutes)	OD	C (mg/ml)	Dilution	$V_{extract}$ (ml)	∑Polyphenol (mg/∑$V_{extract}$ from 10 gram material)
30	0.620	57.677.10^{-3}	20	115	132.675
60	0.796	76.011.10^{-3}	20	115	174.825
90	1.142	112.052.10^{-3}	20	115	257.720
120	1.291	127.573.10^{-3}	20	115	293.418
150	1.298	128.302.10^{-3}	20	115	295.095
180	1.303	128.823.10^{-3}	20	115	296.293

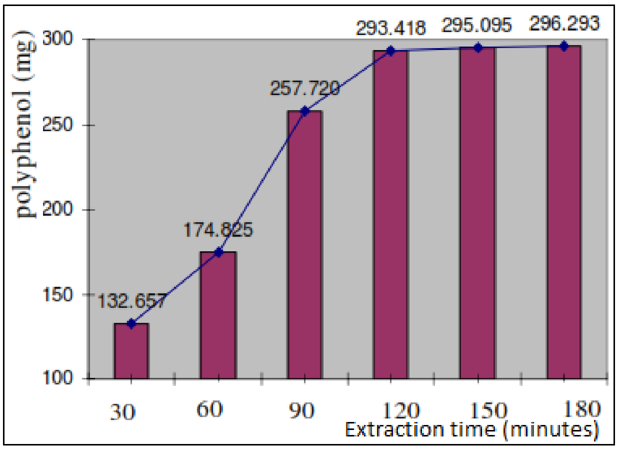

Fig 7: Effect of extraction time

From table 7 and figure 7, when we increase the extraction time from 30 minutes to 180 minutes, the total polyphenol will be increased step by step and clearly noticed at 90 minutes. So we choose extraction time 90 minutes for further experiments.

3.2 5 Effect of extraction pH (using Citric acid 5%)

Table 8: Effect of extraction pH

Extraction pH	OD	C (mg/ml)	Dilution	$V_{extract}$ (ml)	∑Polyphenol (mg/∑$V_{extract}$ from 10 gram material)
5.5	1.310	129.552.10^{-3}	20	115	297.970
5.0	1.318	130.385.10^{-3}	20	115	299.886
4.5	1.325	131.115.10^{-3}	20	115	301.565
4.0	1.347	133.406.10^{-3}	20	115	306.834
3.5	1.407	139.656.10^{-3}	20	115	321.209
3.0	1.338	132.469.10^{-3}	20	115	304.679

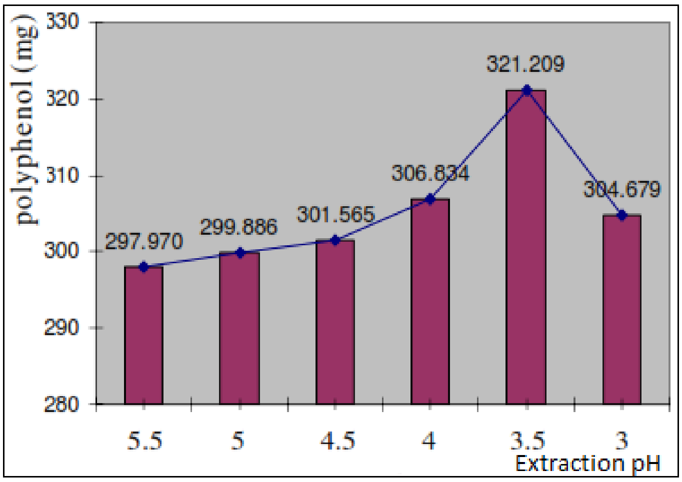

Fig 8: Effect of extraction pH

From table 8 and figure 8, when we decrease pH from 5.5 to 3.5, we get more polyphenol because at low pH the oxidation happens slowly. However if the pH is too low, the extraction is also limited by tissue shrinkage. At pH 3.5, we get the optimal polyphenol so we choose this value for further experiments.

3.2.4 Extraction efficiency

Table 9: Polyphenol extraction efficiency

Replication	OD	C (mg/ml)	Dilution	$V_{extract}$ (ml)	∑Polyphenol (mg/∑$V_{extract}$ from 10 gram material)
1	1.318	$130.385.10^{-3}$	20	115	299.886
2	0.622	$57.885.10^{-3}$	20	115	133.136
3	0.328	$27.260.10^{-3}$	20	115	62.699
4	0.112	$4.760.10^{-3}$	20	115	10.949

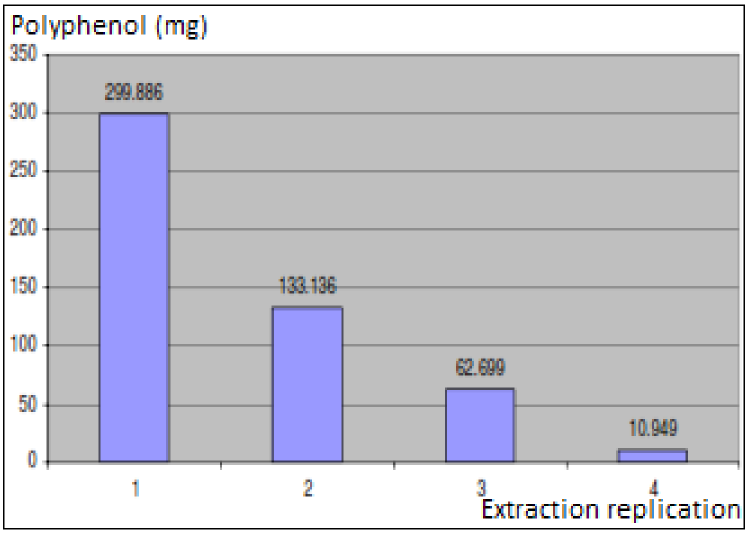

Fig 9: Polyphenol extraction efficiency

After 4 replications of extraction, the residual polyphenol is insignificant, so the total polyphenol extracted in bitter melon is 506.67 mg.

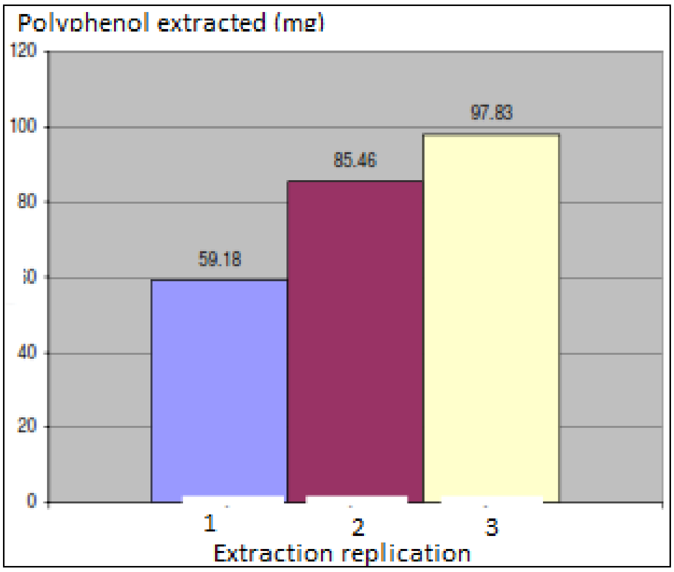

Fig 10: Polyphenol extracted after replications

After 1st extraction, the residual polyphenol is 206.784 mg. After 2nd extraction, the residual polyphenol is 73.647 mg. After 3rd extraction, the residual polyphenol is 10.948 mg. So after 2nd extraction, we get polyphenol 433.022 mg/10g raw material equivalent to 16.96% dry matter.

3.3 Effect of acid treatment to polyphenol

Using 100 gram raw material passed 2nd extraction; the extracted compound is concentrated to get the extrate in paste form (31.5g).

3.3.1 Effect of ratio extract:solvent

Table 10: Effect of ratio extract:solvent

Ratio	OD	C (mg/ml)	Dilution	V (ml)	∑P (mg/∑Vextract)
1:5	1.823	$182.989.10^{-3}$	100	18	329.38
1:10	0.839	$80.489.10^{-3}$	100	41	330.007
1:15	0.569	$52.364.10^{-3}$	100	63	329.896
1:20	0.429	$37.781.10^{-3}$	100	87	328.696

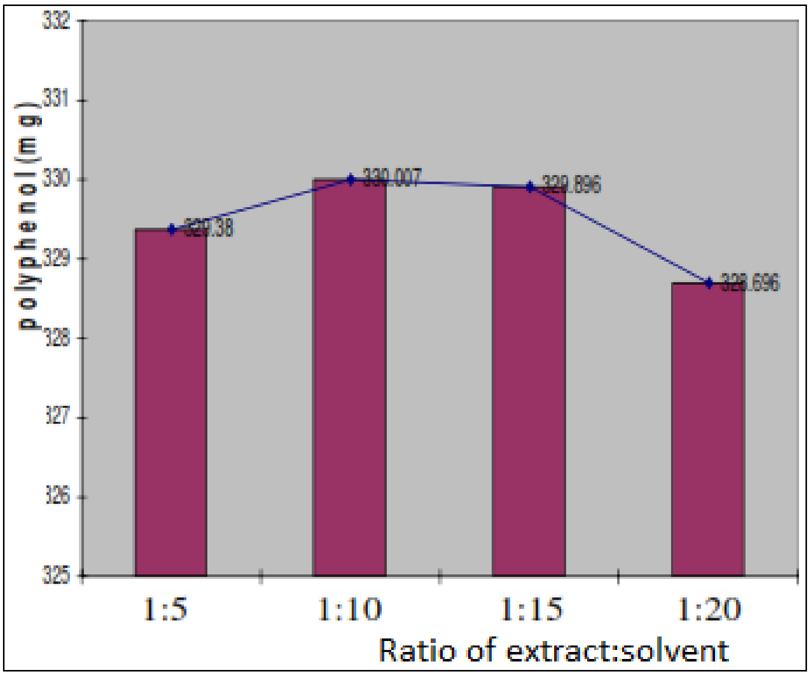

Fig 11: Effect of ratio extract:solvent

At ratio extracte:solvent 1:15, we get the optimal polyphenol

3.3.2 Effect of acid treatment temperature

Table 11: Effect of acid treatment temperature

Temperature (°C)	OD	C (mg/ml)	Dilution	V (ml)	∑P (mg/∑Vextract)
30	0.568	$52.260.10^{-3}$	100	63	329.241
40	0.570	$52.469.10^{-3}$	100	63	330.553
50	0.572	$52.677.10^{-3}$	100	63	331.865
60	0.575	$52.990.10^{-3}$	100	63	333.834
70	0.578	$53.302.10^{-3}$	100	63	335.803
80	0.578	$53.302.10^{-3}$	100	63	335.803

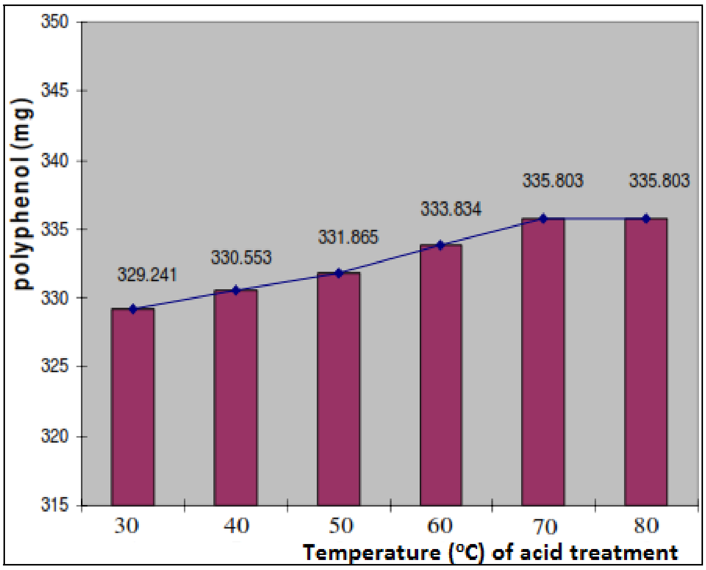

Fig 12: Effect of acid treatment temperature

From table 11 and figure 12, when the acid treatment temperature increases, the polyphenol increases forward because of its dissolution to speed up the hydrolysis. At 60°C, the deposit happens more easily than in 70°C, 80°C.

3.3.3 Effect of deposit time

Table 12: Effect of deposit time

Deposit time (hours)	OD	C (mg/ml)	Dilution	V (ml)	∑P (mg/∑Vextract)
12	0.405	$35.281.10^{-3}$	100	63	222.271
24	0.421	$36.948.10^{-3}$	100	63	232.772
36	0.458	$40.802.10^{-3}$	100	63	257.053
48	0.461	$41.115.10^{-3}$	100	63	259.022

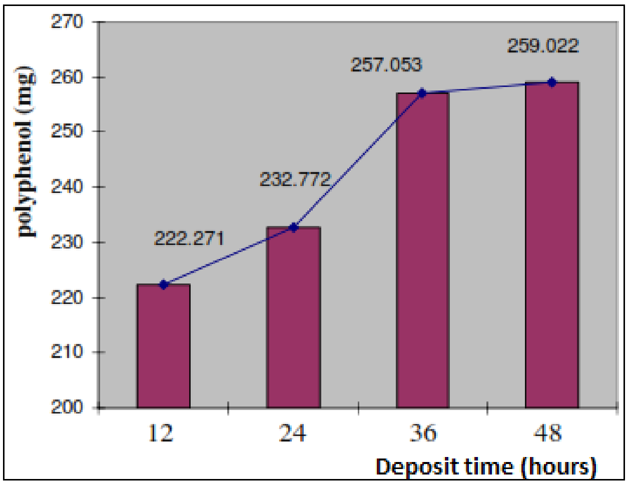

Fig 13: Effect of deposit time

From table 12 and figure 13, the longer deposit time is, the more polyphenol we receive. From 12h to 36h the polyphenol receiving is significant (24.281mg), but insignificant (1.969 mg) from 36h to 48h. However, if the deposit is too long, the oxidation of polyphenol will create dark color so 36 hours is optimal.

3.4 Purification
3.4.1 Effect of solvent ratio for purification

Acidified deposited polyphenol is then purified by ethyl acetate.

Table 13: Effect of ratio polyphenol/ethyl acetate

Ratio	OD	C (mg/ml)	Dilution	V (ml)	∑P (mg/∑Vextract)
1:1	0.240	$18.094.10^{-3}$	100	71	128.467
2:1	0.340	$28.510.10^{-3}$	100	35	99.785
3:1	0.371	$31.739.10^{-3}$	100	22	69.826
4:1	0.236	$17.677.10^{-3}$	100	17	30.051
5:1	0.162	10.10^{-3}	100	12	12

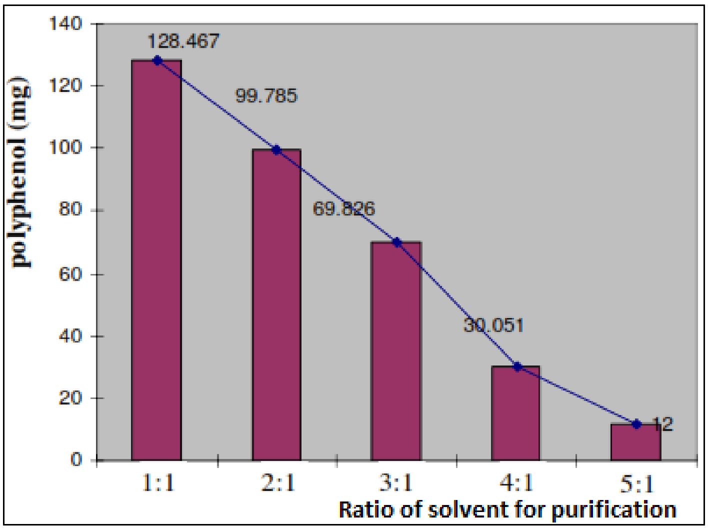

Fig 14: Effect of solvent ratio for purification

The more ethyl acetate we use, the more polyphenol we get. At ratio $V_{extract}$:$V_{solvent}$ = 2:1, polyphenol purified is optimal.

3.4.2 Effect of purification time

Table 14: Effect of purification time

Purification time	OD	C (mg/ml)	Dilution	V (ml)	∑P (mg/∑V ethyl acetate)
30	0.242	$18.302.10^{-3}$	100	35	64.057
45	0.279	$22.156.10^{-3}$	100	35	77.546
60	0.339	$28.406.10^{-3}$	100	35	99.421
75	0.337	$28.198.10^{-3}$	100	35	98.693
90	0.328	$27.260.10^{-3}$	100	35	95.41

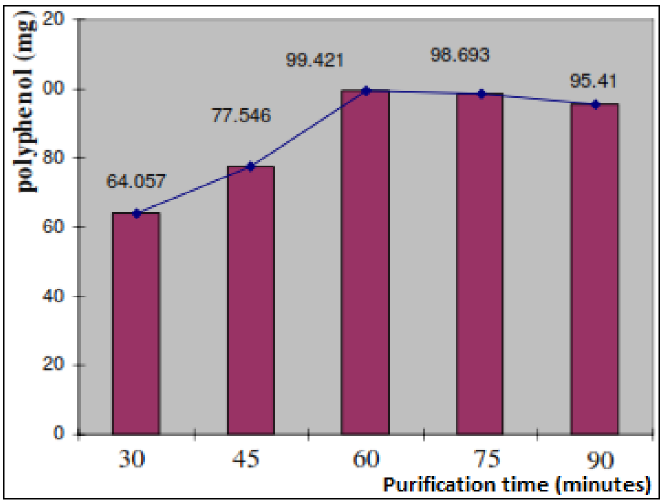

Fig 15: Effect of purification time

When we increase the purification time, the more purified polyphenol we receive, especially from 45 minutes to 60 minutes the purified polyphenol increases 21.875 mg (28.21). From 60 minutes to 75 minutes and from 75 minutes to 90 minutes, the purified polyphenol doesn't increase respectively, even decrease owing to oxidation.

3.4.3 Purification efficiency

With 5 gram of initial extract, we dilute, filter and acidify to get 63 ml solution having polyphenol 257.053 mg. If we use 100ml solution, we get acidified solution having polyphenol 408.02 mg. After 3 purification steps by ethyl acetate, we receive 0.243 g of purified extract.

So total purified extract from 31.5 gram of the initial extract will be 1.53 gram.

Table 15: Residual polyphenol after 5 steps

Step	OD	C (mg/ml)	Dilution	$V_{extract}$	∑P (mg/∑V ethyl acetate)
1st	0.468	$41.843.10^{-3}$	100	35	146.45
2nd	0.395	34.23910^{-3}	100	35	119.836
3rd	0.154	$8.823.\ 10^{-3}$	100	35	30.88
4th	0.099	$3.406.10^{-3}$	100	35	11.921
5th	0.082	$1.635.\ 10^{-3}$	100	35	5.722
TOTAL POLYPHENOL					314.809

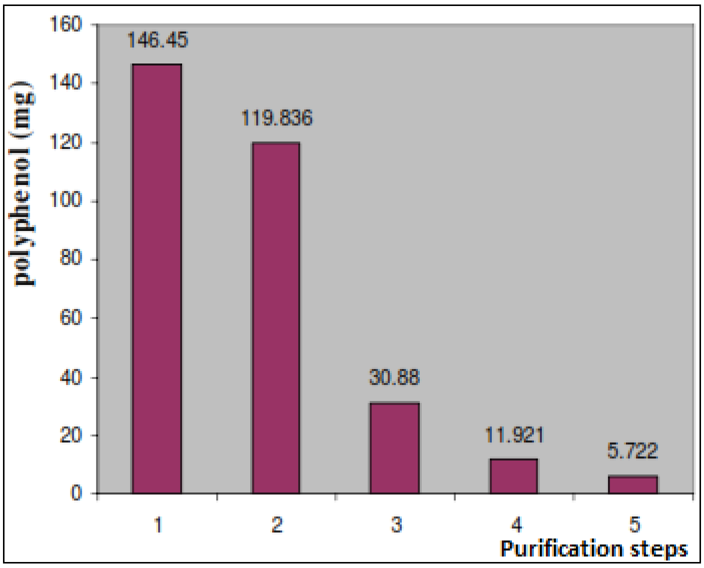

Fig 16: Residual polyphenol after 5 steps

After 5 purification steps, the residual polyphenol in the extract is insignificant. So total purified polyphenol in the extract is 314.809 mg.

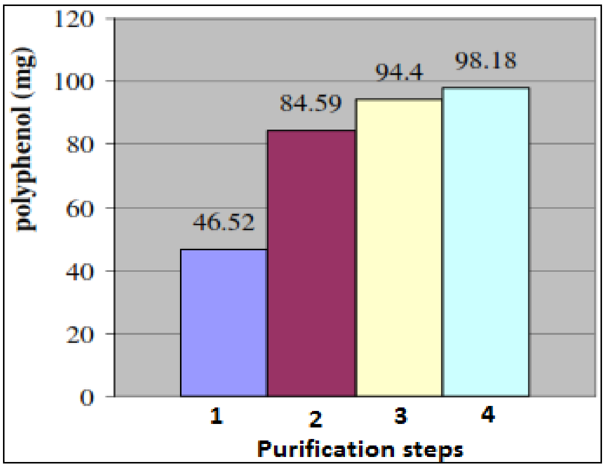

Fig 17: Purification efficiency after 4 steps

From figure 17, the polyphenol purification efficiency is 84, 59% after 2nd purification, 94.4% after 3rd purification, 98. 18% after 4th purification. If we prolong the purification time, we will use more solvent, labor and time so it's not economic. So we choose purification by ethyl acetate after 2nd steps.

3.4.4 Purified polyphenol

3.4.4.1 In case of acid treatment

The polyphenol receiving in different stages as follows:

100(g) raw material, ∑polyphenol = 5.07(g)

Extraction by ethanol 70^0, ∑polyphenol = 4.33(g)

Extraction by acid HCl, ∑polyphenol = 1.62(g)

Purification by ethyl acetate, ∑polyphenol = 1.53(g)

3.4.4.2 In case of non- acid treatment

The polyphenol receiving in different stages as follows:
100(g) raw material, ∑polyphenol = 5.07(g)
Extraction by ethanol 70^0, ∑polyphenol = 4.33(g)
Dilution with distilled water, ∑polyphenol = 4.33(g)
Purification by ethyl acetate, ∑polyphenol = 4.09(g)

4. Conclusion

Bitter gourd (*Momordica charantia*) is one of the popular vegetables in Asia. It is used for the preparation of several dishes. It can be fried, deep-fried, boiled, pickled, juices and dried to drink as tea. Bitter melon is a good source of phenolic compounds which possess potent antioxidant activity. We have successfully found out technical factors affecting to extraction, acid treatment and purification of polyphenol in bitter melon.

5. Reference

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