Research Article

International Journal of Pharmaceutical Biological and Chemical Sciences

e-ISSN: 2278-5191

# EFFECT OF EXTRACTION TECHNIQUES ON THE EXTRACTABILITY OF ANTIOXIDANT POLYPHENOLICS FROM *C. colocynthis*

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**ABSTRACT:**

***Introduction:*** *Total phenolic contents and antioxidant activity of extracted compounds appreciably influenced by many factors mainly type of solvent and technique. Citrullus colocynthis plant is well known for its medicinal properties.* ***Methods:*** *In current research, antiradical activity of C. colocynthis fruit (dry and fresh) was tested at different concentration by employing various assays. Extracts were prepared with different solvents (Methanol, Methanol 80%, Ethyl acetate, Water, n-hexane) and techniques (Reflux, Soxhlet, Microwave and Mercerization). All extracts were tested for total phenolic contents by standard method and represented as gallic acid equivalents. Extracts having highest phenolic contents were evaluated for antioxidant activity.* ***Results:*** *Findings of current research evidently showed that C. colocynthis dry fruit had more antioxidant potential than fresh fruit. Ethyl acetate proved to be most effective solvent for the extraction of phenolic followed by methanol, water and n-hexane. Soxhlet extracts showed highest antioxidant activity but microwave assisted extraction technique results were also comparable to this technique. Thus microwave assisted extraction technique was proved to be efficient technique due to its time saving nature. Significant positive correlation was found between percentage inhibition and phenolic contents.* ***Conclusions:*** *By this research, it was concluded that C. colocynthis fruit has considerable antioxidant potential confirming its traditional use.*

**KEYWORDS:**

*Dry and Fresh fruit, Microwave-assisted extraction, polyphenols, Correlation.*

## INTRODUCTION

Microwave-assisted extraction is becoming admired among scientists due to its advantageous effects[1]. This technique uses the efficiency of microwaves to heat the solvent and disrupt the hydrogen bonds. As a result, efficiency of solvent infiltration into the matrix increases and leads to more elicitation of desired compounds. Conventional extraction techniques are tedious, time and solvent consuming[2]. Another disadvantage of conservative techniques is the degradation of heat labile compounds. Plants are richer source of compounds that have health benefits but sensitive to heat[3]. They should be extracted with great competency to get profit from them. So the goal is to extract these compounds from plants with greater efficiency and minimum disruption[4].

Natural sources for drug formulation are gaining attention due to deleterious side effects related with synthetic drugs. Natural sources are considered safer than synthetic[5]. Medicinal plants have copious amount of bioactive compounds that are health promoting[6]. These bioactive compounds are secondary plant metabolites. Polyphenolic are largely disseminated in plant kingdom[7]. Commonly they consist of benzene rings which linked to one or more hydroxyl groups[8]. All families of polyphenols have shown a reassuring effect in therapeutic for the treatment of diseases which are commonly caused by oxidative stress. Over production of free radicals direct to the onset of oxidative stress that is responsible for the occurrence of lethal diseases like cancer, atherosclerosis, cardiovascular and CNS diseases[9]. Plants act as antioxidants by scavenging these free radicals and converting then to stable molecules[10]. The antioxidant activity of plants is commonly positively correlated with the total phenolic contents[11]. Previous researches

confirmed the anti-inflammatory[12], anti-viral and anti-carcinogenic activities of polyphenols[13].

*Citrulluscolocynthis* belongs to family Cucurbitaceous. It is a small scabbed herb with mottle yellow fruits and climbing stem. It occurs through the sub-continent and is growing in arid sand and warm tracts. Physicians utilize it extensively for the treatment of purgative, diabetics and jaundice[14]. Previous researches mostly evaluated the antioxidant potential of root of *C. colocynthis*. Due to extensive beneficial effects of *C. colocynthis*, this project had been designed to evaluate the antioxidant potential of its fruit by employing solvent of varying polarities with conventional and microwave assisted extraction technique.

## MATERIALS AND METHODS

### CHEMICALS AND INSTRUMENTS

Chemicals of analytical grade were used in this research: Gallic acid, 2,2-diphenyl-1-picrylhdrazyl (DPPH), Lithium sulphate, Ascorbic acid, Sodium molybdate, Sodium carbonate, Ethylene diamine tetra acetate (EDTA), Phosphoric acid, Nitroblue, tetrazolium regent (NBT), Sodium nitroprusside, Sulphonamide, Tween 80, Linoleic acid, Ammonium thiocyanate, Folin-Ciocalteu reagent, Sodium dihydrogen phosphate, Disodium hydrogen phosphate,Butylatedhydroxytoluene (BHT) etc.

### INSTRUMENTS

Instruments that were used in current research: Spectrophotometer, Rotary evaporator, Digital balance, Incubator, Soxhlet apparatus, Reflux apparatus, Shaker and Microwave oven.

**Plant material:**

The dried fruit of *C.colocynthis* were purchased from local market while fresh fruit of *C.colocynthi,* were collected from the local people of Chakwal, Pakistan. They were washed with distilled water to remove dust. The dried fruit was powdered and the pulp of fresh fruit was wrung to obtain juice.

**Preparation of crude plant extract**

Dry and fresh fruit of *C. colocynthis* (1:10) was elicited with solvents of different polarities and conventional versus innovative microwave assisted extraction technique. After extraction, solvent was evaporated using rotary evaporator and concentrates were stored in refrigerator for further analysis. Over all 30 extracts of *C. colocynthis* plant was prepared. Experimental protocol was shown in **Table 1**.

**Table 1. Experimental protocol**

| Techniques | Solvent | Time |
|---|---|---|
| Reflux | Methanol<br>80 % methanol<br>Water | 1hour |
| Soxhlet | n-hexane<br>Ethyl acetate<br>methanol | 8 hours |
| Mercerization with shaking | Methanol<br>80 % methanol<br>Water | 2 hours |
| Mercerization withoutshaking | Methanol<br>80 % methanol<br>Water | 24 hours |
| Microwave | Methanol<br>water | 2 minutes<br>2 minutes<br>4 minutes |

**TOTAL PHENOLIC CONTENTS**

Total phenolic contents of extracts were evaluated by the method of Nabavi[15]. Plant material (1ml) was mixed with Folin-Ciocalteu reagent (5ml) and sodium carbonate (4ml, 20%). Absorbance of resulting mixture was noted with spectrophotometer at 765nm after an hour. Unknown concentration of plant extract was determined from calibration curve of gallic acid (0.01 - 0.10 mg/ml). Total phenolic contents of plant extract were determined by applying formula (Total phenolics = unknown concentration computed from standard curve × Volume taken in ml / grams of plant extract taken) and expressed as mg GAE/g of plant extract.

**DETERMINATION OF ANTIRADICAL ACTIVITY**

Four different assays were used to determine the antiradical activity of the plant extracts to get reliable results. Extracts with highest phenolic contents were selected for the evaluation of antioxidant activity. Results were reported in term of percentage inhibition and $IC_{50}$ value which is the inhibitory concentration which restricts the formation of 50% radicals.

1. DPPH free radical scavenging activity

Free radical scavenging activity was verified by DPPH assay. DPPH (0.1mM) ethanolic solution (1ml)was mixed with different concentration of plant extract (0.02 - 0.1 mg/ml)and kept in dark for 30 minutes[16]. Absorbance of resulting mixture was noted at 517 nm. BHT and Ascorbic acid were used as standard reference and run in similar way.

2. Superoxide radical scavenging activity

Superoxide radical scavenging activity of plant extracts was evaluated by the method of Mishra[17]. Aliquot (1ml) of various concentrations of plant extract (0.2 - 1 mg/ml) was mixed with sodium carbonate (2 ml, 5%). After that, nitro blue tetrazolium reagent (0.8 ml, 150 µM) was added followed by addition of EDTA (0.6 ml, 0.5%). Consequential solutions absorbance was checked immediately at 560 nm. Standards were also run in similar way.

3. Evaluation of reducing potential

Reducing capability of plant extract was ascertained by reference method[18]with slight modification. Aliquot (1ml) of various concentrations of plant extract (0.2 - 1 mg/ml) was mixed with phosphate buffer (0.2M) and potassium ferricyanide (1%). Reaction mixture was kept for 20 minutes at 50ºC. Later on, trichloroacetic acid (1%) was added and homogenized. Upper layer was diluted with water and mixed with ferric chloride (1%). Resulting solution absorbance was taken at 700nm. Standards were also run in similar way.

4. Antioxidant activity in linoleic acid system

Antiradical activity in linoleic acid system was evaluated by Ammonium thiocyanate method[19]. Extract (5mg) was mixed with phosphate buffer (0.04M) and linoleic acid emulsion. It was kept for 72 hours at $37^0$C. Incubated sample (2ml) was removed after every 24 hour gap and mixed with ammonium thiocyanate (30%) and $Fe_2Cl_3$ (0.02M, 0.5ml). Consequential mixture absorbance was taken at 500nm. Standards were also treated in similar way.

**Statistical analysis:** Results were represented with mean ± S.D. Antioxidant activity was reported in term percentage inhibition and $IC_{50}$ Value. $IC_{50}$ value was calculated from slope equation ($Y = MX+C$). Pearson's correlation coefficient was used in order to establish link between antioxidant activity and total phenolic contents.

**RESULT AND DISCUSSION**

Results of total phenolic contents are depicted in **Table 2**.

**Table 2: Total phenolic contents (mg GAE/g of plant extracts) in *Citrulluscolocynthis*fruit.**

| Total Phenolic Contents (mg GAE/g) | | | | |
|---|---|---|---|---|
| **Techniques** | **Solvent** | **Time** | **Fruit** | |
| | | | Dry fruit | Fresh fruit |
| Reflux | Methanol | 1hour | 103 ± .031 | 65 ± 0.17 |
| | 80 % methanol | | 89 ± 0.17 | 45 ± 0.24 |
| | Water | | 46 ± 0.18 | 40 ± 0.26 |
| Soxhlet | n-hexane | 8 hours | 22 ± 0.16 | 10 ± 0.24 |
| | Ethyl acetate | | 245 ±0. 75 | 85 ± 0.36 |
| | methanol | | 121 ± 0.34 | 55 ± 0.42 |
| Mercerization with shaking | Methanol | 2 hours | 51 ± 0.34 | 39 ± 0.26 |
| | 80 % methanol | | 36 ± 0.43 | 22 ± 0.35 |
| | Water | | 21 ± 0.25 | 35 ± 0.27 |
| Mercerization withoutshaking | Methanol | 24 hours | 39 ± 0.27 | 30 ± 0.19 |
| | 80 % methanol | | 37 ± 0.23 | 22 ± 0.26 |
| | Water | | 30 ± 0.42 | 35 ± 0.42 |
| Microwave | Methanol | 2 minutes | `225 ± 0.24 | 105 ± 0.24 |
| | water | 2 minutes | 105 ± 0.36 | 40 ± 0.16 |
| | | 4 minutes | 185 ± 0.38 | 87 ± 0.56 |

**Effect of solvent on the extraction of polyphenols**

The experimental results showed that the extraction of polyphenols form *C.colocynthis*was greatly influenced by the nature of solvents. According to the results, Ethyl acetate proved best solvent for the extraction of polyphenols. Its extracts showed the presence the highest total polyphenolic contents (245 ± 0.75 mg GAE/g). The extracts prepared with methanol also showed good results but the extracts prepared with n-hexane showed the lowest total polyphenolic contents (10 ± 0.24 mg GAE/g). Methanol and ethyl acetate extracts showed the presence of highest polyphenols due to their polarity and organic nature. As hexane is non polar solvent thus it does not possess the ability to extract polyphenols.

**Effect of extraction technique on the extraction of polyphenols**

The experimental data illustrated that the extraction of phenolic compounds from C. colocynthissignificantly affected by the extraction technique. Soxhlet extraction method yielded highest total polyphenolic contents (245 ± 0.75 mg GAE/g). But it is tedious process and time consuming. Microwave assisted extraction technique yielded polyphenols which are comparable to soxhlet. Thus it is proved to be efficient technique due to its time and solvent saving nature.

**Effect of irradiation time on the elicitation of total phenolic contents**

The results showed that the extraction of polyphenols form *C.colocynthis*obviously influenced by the irradiation time. It was found that TPC increased with increase in time irradiance up to a certain limit. For 2 to 4 minutes irradiance time with water as solvent, TPC were found to increase from 105 to 185 mg GAE/g for dry fruit of *Citrulluscolocynthis*and from 40 to 87 mg GAE/g for fresh fruit of *Citrullus* colocynthis. With methanol as a solvent, 2 minutes irradiance time showed 225± 0.24 mg GAE/g for dry fruit of *Citrulluscolocynthis* and 105± 0.24 mg GAE/g for fresh fruit of *Citrulluscolocynthis*. To practice extracts with 4 minutes irradiance time with methanol as solvent was not achievable due to evaporation of solvent.

**Effect of nature of plant material**

The experimental results clearly indicated that the extracts of dry fruit of *C.colocynthis* contained more total polyphenols than the fresh fruit of medicinal plant *C.colocynthis* in all the extraction methods. Fresh fruit juice was squeezed out from the fruit

*Citrulluscolocynthis*. It does not include the peel. Therefore, the low phenolic contents may be due to absence of peel[20].

## DETERMINATION OF ANTIRADICAL ACTIVITY

### 1. DPPH and superoxide free radical scavenging activity

Antioxidants are very important due to injurious effects of free radicals. Results of inhibitory concentration 50 ($IC_{50}$) value for DPPH and super oxide free radical scavenging assay are depicted in **Table 3 and Figure 1**.

**Table 3.$IC_{50}$ value of *Citrulluscolocynthis* fruit by DPPH and superoxide free radical scavenging assay**

| Plant extracts and standards | $IC_{50}$ value (mg/ml) | |
|---|---|---|
| | DPPH assay | Superoxide assay |
| Dry fruit microwave methanol | 0.171± 0.012 | 1.111± 0.021 |
| Dry fruit soxhlet ethyl acetate | 0.152± 0.035 | 0.944± 0.047 |
| Fresh fruit microwave methanol | 0.309± 0.01 | 1.239± 0.02 |
| Ascorbic acid (Standard) | 0.057± 0.01 | 0.541± 0.01 |
| BHT (Standard) | 0.051± 0.013 | 0.646± 0.03 |

Results of $IC_{50}$ indicated that both standards ascorbic acid and BHT have less $IC_{50}$ value for both assays as compared to tested plant. Thus they have more inhibitory effect at less concentration. Among plant extracts, dry fruit showed less $IC_{50}$ value than fresh fruit thus more inhibitory effect at less concentration. Soxhlet extract exhibited somewhat less $IC_{50}$ value than microwave assisted extraction. But due to its time taking nature to extract bioactive compounds to show this activity, we don't consider it good technique. Thus by considering all aspects, we concluded that microwave assisted extraction technique was best technique for extract preparation. Previous researchers also evaluated the free radical scavenging of *C. colocynthis* seeds compared with ascorbic acid. They also found maximum activity in ethyl acetate extract and found that ascorbic acid possess more antiradical activity than *C. colocynthis* seeds[21].

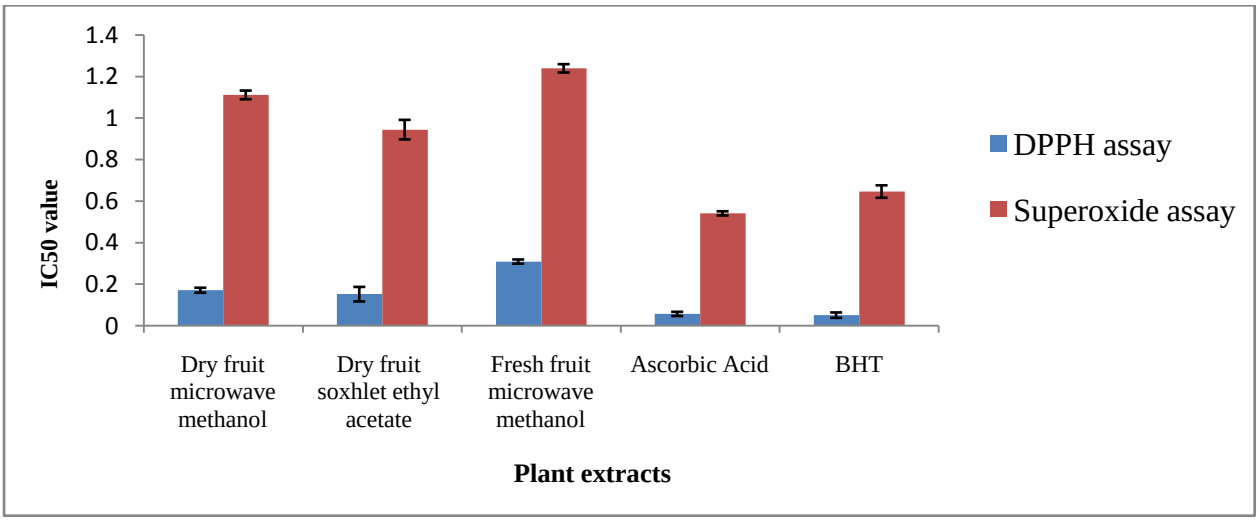
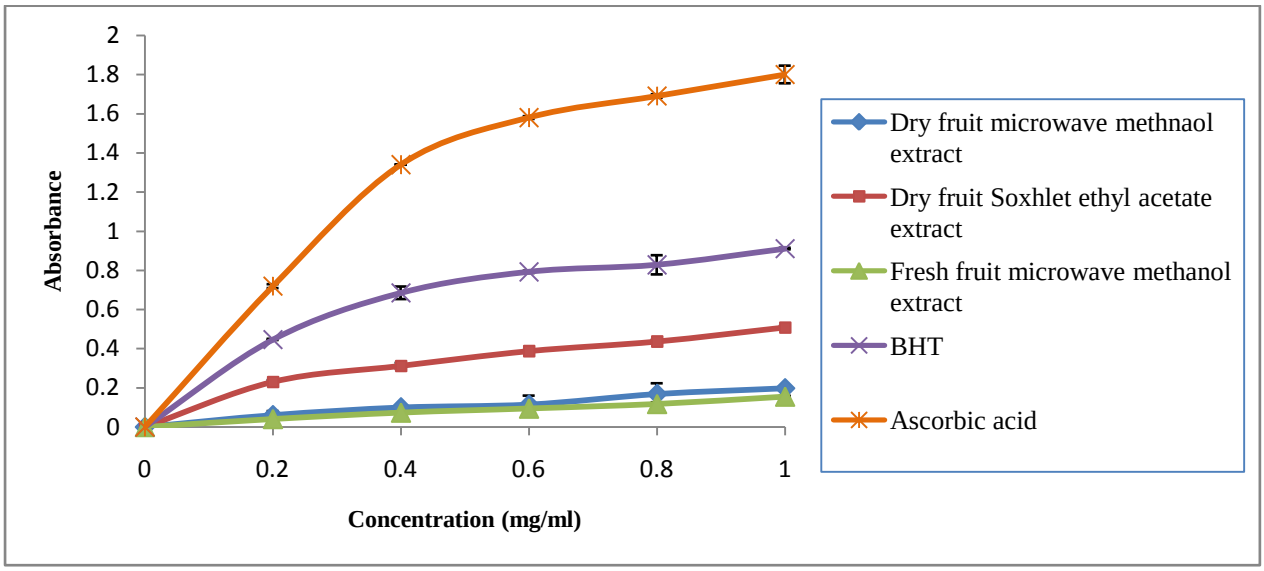



**Figure 1. $IC_{50}$ value of *Citrulluscolocynthis* fruit by DPPH and superoxide free radical scavenging assay**

**2. Measurement of reducing potential**

Results of reducing potential of different extracts of *C. colocynthis* fruit are depicted in figure 2. These results clearly indicate that standard compounds Ascorbic acid and BHT had more reducing potential at all tested concentrations than *C.colocynthis* fruit. Dry fruit extract of *C. colocynthis with* soxhlet and ethyl acetate as solvent showed more reducing potential (0.509± 0.004) than all tested extracts. Previous researchers also ascertained the antioxidant activity of *C.colocynthis* fruit at different concentrations by employing different assays. Reducing potential results were found to be in agreement with this research. They also found than ascorbic acid possess more reducing potential than *C. colocynthis* fruit[22].




Values are expressed in mean ± S.D. (n=3).

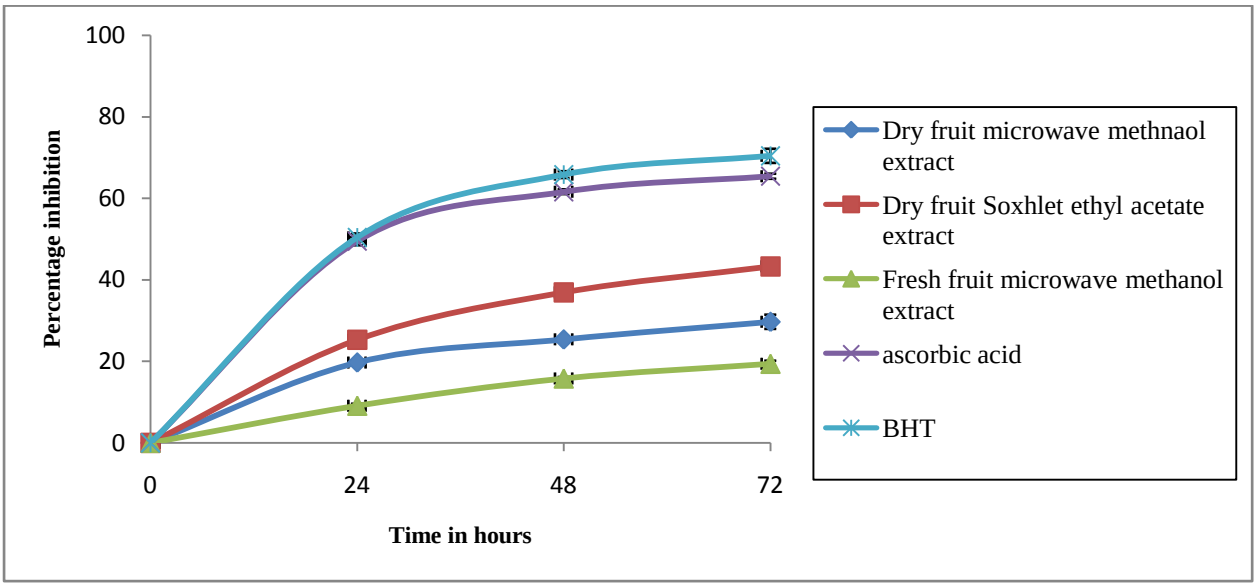
**Figure 2. Reducing potential of *C. colocynthis* fruit at different concentrations**

**3. Antioxidant activity in linoleic acid system**

Results of antioxidant activity in linoleic acid system are depicted in figure 3. These results showed that BHT had maximum percentage inhibition in linoleic acid system followed by ascorbic acid and tested plant extracts. Among plant extract, dry fruit of *C. colocynthis* showed highest percentage inhibition than fresh fruit. Dry fruit extract prepared with soxhlet, ethyl acetate showed 43.3± 0.98% inhibitions followed by its microwave methanol extract (29.7± 1.23%). Fresh fruit extract showed minimum inhibition (19.4± 1.18%).

**Correlation study of phenolic contents with antioxidant activity**

Several studied stated that antioxidant activity is related with total phenolic contents[22]. Correlation between total phenolic contents and antioxidant activity for DPPH assay, superoside assay and Linoleic acid assay was shown in figure 4 and for reducing potential assay in figure 5.All assay confirmed the existence of positive correlation between total pheolic contents and antioxidant activity. Highest positive correlation was found between in DPPH assay with Pearson correlation coefficient r = 0.997 followed by linoleic acid assay (r = 0.923), superoxide assay (r = 0.760) and reducing potential assay (r = 0.692).




**Values are expressed in mean ± S.D. (n=3).**

**Figure 3. Antioxidant activity in linoleic acid system of *C. colocynthis* fruit at different concentrations**

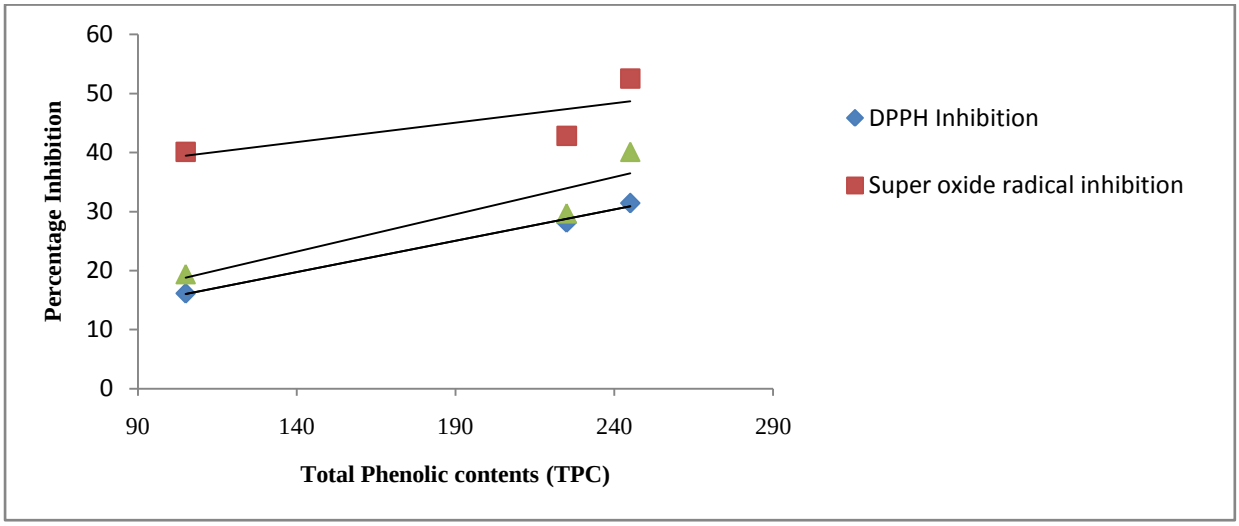



**Figure 4.**

1. **Correlation between TPC and DPPH radical inhibition= $R^2$ = 0.9945, r = 0.997, y = 0.106x + 4.8744.**
2. **Correlation between TPC and superoxide radical inhibition= $R^2$ = 0.5789, r = 0.760, y = 0.0655x + 32.575.**
3. **Correlation between TPC and inhibition in linoleic acid system=$R^2$ = 0.8527, r = 0.923, y = 0.1262x + 5.541**

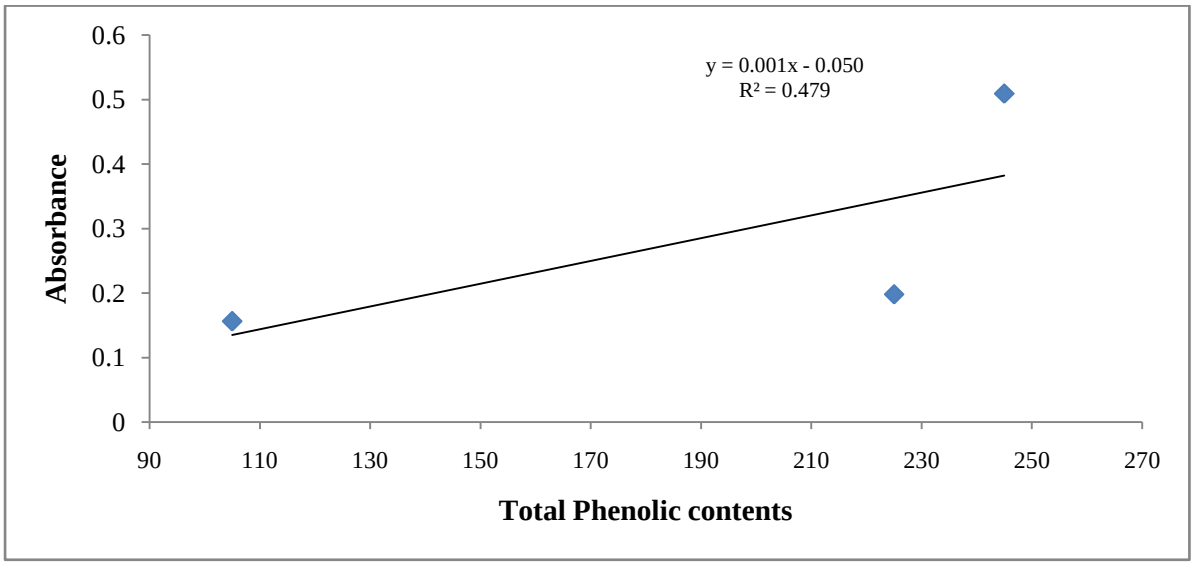



**Figure 5. Correlation between total phenolic contents and reducing potential (r = 0.692)**

## CONCLUSION

Based on the above study it can be concluded dry fruit of *C. colocynthis* has more antioxidant activity than fresh fruit. Microwave assisted extraction technique proved to be most valuable technique. It can extract high polyphenolic contents in less time but optimization of condition is necessary for the effectiveness of results. Ethyl acetate confirmed to be most helpful solvent for the elicitation of phenolic compounds from tested plant. Polarity of solvent plays important role in the extraction of polyphenols. Highest yield of polyphenols can be obtained using polar solvents.

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