IJCRT.ORG ISSN : 2320-2882

INTERNATIONAL JOURNAL OF CREATIVE RESEARCH THOUGHTS (IJCRT)

An International Open Access, Peer-reviewed, Refereed Journal

REVIEW ON EFFECTS OF PRE-TREATMENT ON DETERMINATION OF TOTAL ALKALOID, FLAVONOID, AND PHENOLIC CONTENT IN PLANT-BASED MATERIALS.

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Abstract: Plants are considered a valuable source for phytoconstituents, active pharmaceutical ingredients, pigments, flavours, colorants, enzymes, fibers, etc. in pharmaceutical industries. Using synthetic chemical-based compounds being the main concern of consumers for their health sake. Thus, extraction from plant-based material gain increasing popularity. Various methods like soxhlet extraction, maceration, hydro distillation, etc. are involved in the extraction process. Prior to extraction, pre-treatment leads to enhanced yield, increased stability, highly pure extraction of compounds from plants. Different pre-treatment methods are listed as ultrasonic pretreatment, temperature drying pre-treatment, pulsed electric field pre-treatment, enzymatic pre-treatment, etc. The presented paper is a systematic review of the different pre-treatment methods adopted prior to the extraction process which shows the number of folds, increase in bioactive compounds and also determining pretreatment effects on total alkaloid, flavonoid, and phenolic contents from the plant-based materials.

Keywords: Pretreatment; ultrasonic pretreatment; pulse electric field pretreatment; enzymatic pretreatment; temperature drying pretreatment.

Abbreviation: TAC-Total Alkaloid Content; TFC-Total Flavonoid Content; PEF-Pulsed Electric Field; HPLC-High performance liquid chromatography; TPC-Total Phenolic Content; TOF-Time of flight; MS-Mass spectroscopy; DC-Direct current; DNSA-Dinitrosalysilic acid; ESI-Electrospray Ionization; GAE-Gallic acid equivalent; BCG-Bromocresol green.

I. Introduction

Plants are considered as a valuable source for various phytoconstituents, active pharmaceutical ingredients, pigments, flavours, colorants, enzymes, fibers, etc. in pharmaceutical industries. Use of synthetic chemical-based compounds being the main concern of consumers for their health's sake due to various allergic reactions and side effects. Thus, nowadays plant-based Phytoconstituents are highly preferred. Therapeutically active Phytoconstituents are extracted from plants and their parts with the help of conventional methods of extraction.

Maceration, percolation, reflux extraction, supercritical fluid extraction, Soxhlet extraction, pressurized liquid extraction, microwave-assisted extraction, etc are the conventional extraction methods and have been applied in natural products extraction. This technique usually requires a large volume of organic solvents and high extraction time[1]. Difficulty with choice of solvent for these conventional methods includes various factors like the qualitative extraction of phytochemicals, variety of different compounds extracted and inhibitory compounds extracted, the toxicity of solvent, probable health hazard of extractants[2]. To overcome these difficulties, pre-treatment methods were introduced. Prior to extraction, pre-treatment leads to enhanced yield, increased stability also highly pure

extraction of compounds from plants[3]. Pre-treatment means, treating the sample physically, chemically, biologically, etc prior to the extraction process. Pre-treatment methods mainly include physical, chemical, biological and/or a combination of these methods. Compared with physical and biological pre-treatment methods, chemical pretreatment methods are most likely used because of their inexpensiveness and are effectiveness to enhance the biodegradation of complex materials[4].

II. Results and discussion

1.UltrasonicPretreatment

Ultrasounds are the vibration having ultrasonic frequency exceed than 20 kHz and are mostly used for medical imaging. Ultrasounds are the vibrating body that makes the surrounding medium vibrate and then the transfer of energy takes place between ultrasound waves and neighboring particles[5]. It had been studied that ultrasound works very fruitfully during extraction bearing an improved mechanical effect and thereby producing reliable cavitations in the solvent[6,7]. According to a study conducted on black currant fruit, the mixtures of solvent and sample were steadily irradiated for three different pre-determined extraction times (3, 6, and 10min) with ultrasonic amplitudes (10, 40 and 70%). Prior to ultrasonic pre-treatment the sample was freeze dried using a lyophilizer at –50 °C and 0.04 mbar, until 5% moisture was retained. Another set of Sample was oven air dried using a convection oven at 45°C with a fan speed of 100% until 6.115% moisture was retained. After complete extraction, all samples were centrifuged at 8000rpm at temperature 4°C for 10 mins using the refrigerated centrifuge. Table1: explains the experimental design for the ultrasonic pretrearment purpose. The obtained rudimentary extracts were used for the determination of total phenolic content.

Table1: Experimental data with different ultrasonic extraction time and ultrasonic amplitude.

Experimental run	E_1	E_2	E_3	E_4	E_5	E_6	E_7	E_8	E_9	E_{10}
Ultrasonic extraction time (mins)	3	3	3	6	6	6	10	10	10	-
Ultrasonic amplitude (%)	10	40	70	10	40	70	10	40	70	-

Effect of pretreatment

The total phenolic contentwas increased by approximately 4% as compared to control when sample under went to ultrasonic pretreatment (40 kHz frequency, 60% ethanol with 0.15% HCl, solvent-to-sample ratio 15/1, ultrasonic probe) in all three cases of sample preparation (frozen, freeze drying, oven air-drying) was applied for 10 mins at high amplitude (70%). The total phenolics content was found to be increased with an increase in extraction time and amplitude[8].

2. Temperature drying pretreatment

Dryopteris erythrosora contains bioactive compounds such as flavonoids[9], polyphenols, terpenoids,etc. and are mostly found in China. Scientific evidence has suggested that dietary flavonoids play a major role in averting and coping with diseases such as cancer, diabetes, and cardiac diseases[10]. According to a study conducted on *Dryopteris erythrosora* leaves in which fresh leaves were randomly divided into four different groups and then given fourdifferent pre-treatments. The groups were named as groups A, B, C and D respectively.

Group A- Cleaned and liquid nitrogen frozen leaves were pulverized.

Group B-Leaves were dried at first in the shade for one day and then oven-dried at 75°C for 2 days, and ground to a fine powder.

Group C-At first, leaves were dried in sun on newspaper, then oven-dried at 75°C for two days and then grounded to a powder.

Group D- leaves were cleaned and oven-dried at 75°C for 48 h prior to grinding. The grounded powdered sample was passed through an 80-mesh screen.

Procedure

One gram powder from Group B, C and D of above described, were separately added to 25 ml of 60% ethanol except for Group A which were taken 3.3gm to obtain the same dry weight and exposed with ultrasound machine-assist for about 20 mins and then water-bath at 50˚C for 2 hr. The process was performed twice, and the mixture was filtered using a vacuum suction filter pump. One portion of the extract used to determine the total flavonoid content and antioxidant activity and the other portion was extracted with the help of petroleum ether, dichloromethane, ethyl acetate, and n-butanol for HPLC-ESI-TOF-MS analysis of flavonoids[11].

Effect of pretreatment

Table2 and fig1: explains the effect of temperature drying pretreatment on total flavonoid content on the leaves of *Dryopteris erythrosora.*

Table2: Effect of temperature drying pretreatment on total flavonoid content.

	GROUP –A	GROUP-B	GROUP –C	GROUP –D
	Frozen in liquid nitrogen and pulverized	Shade dried for one day then oven-dried at 75°C for two days	Sun driedand then oven-dried at 75°C for two days	Oven-dried at 75°C for two days
POWDERED SAMPLE	3.3gm (To obtain same dry wt)	1gm	1gm	1gm
PROCEDURE	added to 25ml, 60% ethanol,exposed to ultrasound machine-assist for 20 min and then water-bath at 50°C for 2 h. The extraction procedure was repeated twice, and the mixture was filtered using a vacuum suction filter pump.			
TOTAL FLAVONOID CONTENT EXAMINED	7.38%	7.60% (HIGHEST)	6.41%	2.17% (LOWEST)

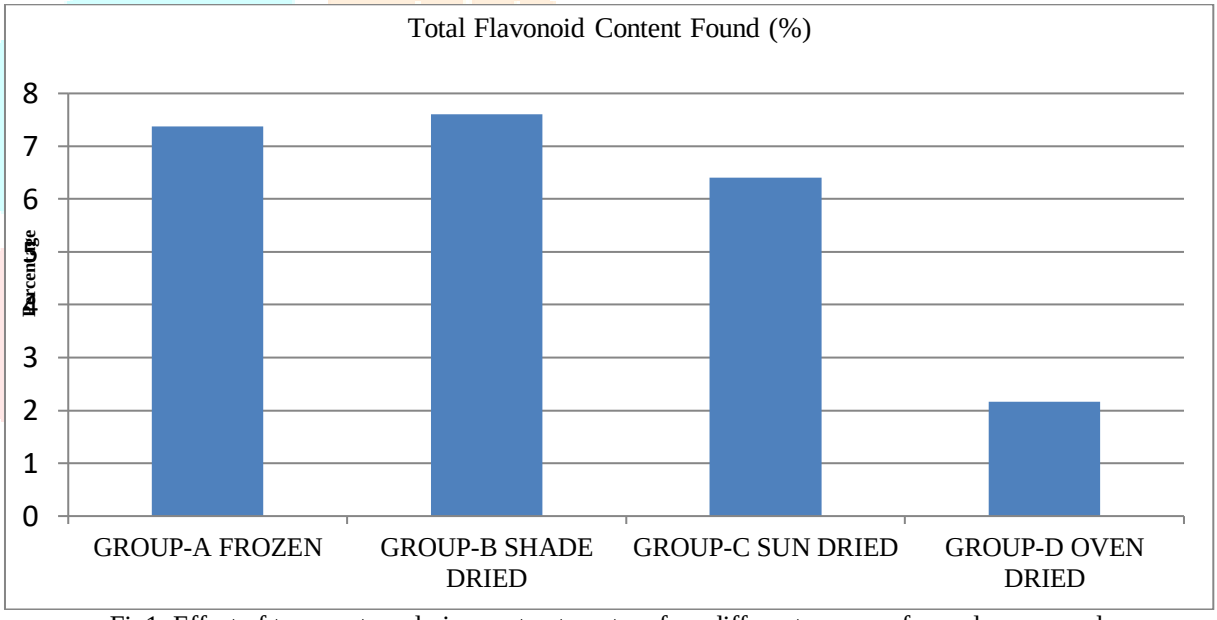

Fig1: Effect of temperature drying pretreatment on four different groups of samples prepared.

3. Pulsed electric field pretreatment

Basil leaves contain bioactive compounds such as flavonoid and phenols having antimicrobial and antioxidant properties[12]. Basil leaves are also used in the treatment of ailments like headaches, coughs, diarrhea, constipation, warts, worms, and kidney malfunction, etc[13]. To increase the extractable amount of bioactive compounds from the leaf of basils, they were exposed to PEF pretreatment. PEF initiates a transmembrane potential difference across the cell membrane[14]. The basic principle of the PEF process is that a certain amount of energy coming from the direct current (DC) power supply is channeled through the charging resistor and stored in a series of capacitors that convert the energy into high-voltage pulses. When the switch is connected, the high-voltage pulse generated will be applied to the electrode. Further, the electrodes carry a high-intensity electrical pulse on the sample located between two electrodes. The sample will experience an electric field that is responsible for cell membrane damage. PEF applies high-voltage electrical shock (kV cm-1) in the form of short pulses to break up cell membranes, called electroporation phenomena[15]. Electroporation results in a charge shift in the atom or molecule, so that the positively charged molecule shifts to the negative electrode and vice versa, forming a dipole. This shift increases the transmembrane potential, so the cell membrane will be thinning. The transmembrane potential exceeding the critical threshold will allow for the destruction of the cell membranes leading to the formation of pores. These pores would facilitate the rate of transfer of the compound from the cell to the solvent. The level of the resulting

compound increased with the increase of applied electric field strength, and the proper selection of duration would affect the alterations of cell structure. The pulsed electric field of mid range intensity and specific less duration accelerates the flow kinetics of phenolic compounds through the cell membranes[16].

According to a study conducted on *Ocimum Americanum*, the fresh leaves were collected and separated from other parts of the plants. They were washed and cleaned with running water and then dried for eight hours using the oven (50ºC). The dried basil leaves were blended and sieved to obtain 40-mesh basil leaf powder. A 40 mesh 20 grams basil leaf powder and distilled water as much as 300 ml were homogenized in a glass beaker. After reaching the homogeneous state, mixed basil leaves were treated using PEF with an electric field strength of 2, 3, and 4 kV cm-1 and different pre-treatment durations of 1, 2, and 3 mins[17]. Table3: explains the experimental design of PEF pretrearment combination given to the Basil leaves.

Table3: Pretreatment combination on basil leaves.

Electric field force (E)	T_1 (1 min)	T_2 (2 mins)	T_3 (3 mins)
E_1 (2kV cm^{-1})	E_1T_1	E_1T_2	E_1T_3
E_2 (3kV cm^{-1})	E_2T_1	E_2T_2	E_2T_3
E_3 (4kV cm^{-1})	E_3T_1	E_3T_2	E_3T_3

Effect of pretreatment

TPC and TFC were found to be increased in Pulsed electric field pre-treatment combination of 3kV cm^{-1} for 2 mins i.e E_2T_2. Table4: explains the influence of PEF on basil leaves comparing to sample extracted without any pretreatment. With pulsed electric field pre-treatment TPC was found to be 115.203±10115 mg GA E/g and total flavonoid content was found to be 75.516±0.723 mg GA E/g.

Table4: Total phenolic and flavonoid content with and without PEF pretreatment.

	With PEF E_2T_2	Without PEF
Phenolic content	115.203±1.115 mg GA E/g	16.680±2.653 mg Q E/g
Flavonoid content	75.816±0.723 mg GA E/g	12.270±1.746 mg Q E/g

4. Enzymatic pre-treatment

Holarrhena antidysenterica i.e Khurchi is a source of conanine and amino pregnane type of steroid alalkaloids[18]. Khurchi is used in the treatment of several serious diseased conditions such as amoebic dysentery, diarrhea, hemorrhoids, chest affections, dyspepsia,diuresis, dropsy, and toothache[19]. The TAC of Indian khurchi is about 0.22 – 4.2 % (av. 2.2%)[20]. Polymeric matrix network of proteins and polysaccharide in khurchi contains alkaloids like conessimine, kurchinine, kurchine, holarrhimine, isoconessimine, kurchimine, conaine, conarrhimine, conkurchine. These can be extracted by various methods but efficiently by enzymatic pretreatment methods. There are chances of degradation of active constituents when other conventional methods were used. Enzymatic pre-treatment can easily degrade this polymeric matrix and facilitate the release of phytoconstituents. The complete effect of this enzymatic pre-treatment is an increase in the overall extraction of the constituent of interest. According to a study conducted on *Holarrhena antidysenterica*, the following enzymes were used.

Amylase: The ability of the enzyme to hydrolyze thestarch helped to determine the required amount of amylase and 1mg of the given enzyme was required for the complete digestion of 1 mg substrate. Thus the quantity of enzyme required was 100% or equal of the substrate quantity.

Cellulase**:** The quantity determination was based on the principle that cellulase hydrolyzes carboxymethylcellulose and produces carboxymethyl oligosaccharides; which on reacting with 3, 5-dinitrosalysilic acid (3, 5-DNSA) produces red color which is measured at 540 nm. Minimum 3 mg of a dose of the enzyme was required for complete digestion of 10 mg substrate quantity.

Papain: The proteolytic activity of the papain was measured in terms of the amount of casein breakdown. Free casein that is not hydrolyzed is coagulated and filtered out. The hydrolyzed casein is measured in terms of amino acids. Minimum 0.18 mg of the given enzyme was required for the complete digestion of 20 mg of the substrate. Thus the quantity of enzyme required was 0.9 %of the substrate quantity.

Lipase: The quantity of lipase were finalized based on the quantity required to digest standard substrate (oliveoil) completely. Minimum 16 mg of the given enzyme was required for the complete hydrolysis of 20 mg substrate. Thus the quantity of enzyme required was 80% of the substrate quantity.

Procedure:
The sample was treated with four different enzymes i.e cellulase, papain, amylase, lipase. 2.5g crude sample (80mesh) was used for each and dispersed in 50 ml of buffer medium. Throughout the study, the quantity of the sample was kept constant. The enzyme-mediated Phytoconstituents release concerning time was carried out at 37˚C in a shaker kept at speed of 135 rpm. For this experiment, time intervals of 1, 2, 4, 8, 12, and 24 hr were fixed.

Effect of pretreatment:
Cellulase was the enzyme found to be most effective and suitable for the release of alkaloids.
From this, it can be expected that the plant cell contents are enclosed in a complex polymeric network made of proteins, polysaccharides, and fatty material and the enzyme cellulase brings about a breakdown and degradation of the cell wall thus releasing them.
The ease in alkaloid release was in the following order:
cellulase > papain > lipase > amylase > blank[21].

III. Total content determination

Determination of total phenolic content (TPC)[22-24]

Spectrophotometric methods arecommonly used for the quantification of phenolic content. Estimation of TPC in the selected plant extract was measured spectrophotometrically by Folin–Ciocalteu method, using Gallic acid as the standard and expressing results as gallic acid equivalent (GAE) per gram of sample. Different concentrations (0.01-0.1 mg/ml) of gallic acid were prepared in methanol. Aliquots of 0.5 ml of the test sample and each sample of the standard solution were taken, mixed with 2 ml of Folin–Ciocalteu reagent (1:10 in deionized water) and 4 ml of a saturated solution of sodium carbonate (7.5% w/v). The tubes were covered with the help of silver foils and incubated at room temperature for 30 minutes with occasional shaking. The absorbance was taken at 765 nm using methanol as blank. All the samples were analyzed in three replications. The total phenol was determined with the help of the standard curve prepared from pure phenolic standard (gallic acid). Folin–Ciocalteu is a very sensitive reagent that contains phosphomolybdate and phosphotungstate that form the blue coloured complex in alkaline solution by the reduction of phenols.

Determination of total flavonoid content (TFC)[25-27]

The TFC of the seed extract was determined by aluminum chloride colorimetric assay. Briefly, 0.5 ml aliquots of the extract and standard solution (0.01-1.0 mg/ml) of rutin were added with 2 ml of distilled water and subsequently with 0.15 ml of sodium nitrite (5% $NaNO_2$, w/v) solution and mixed. After 6 minutes add 0.15 ml of (10% $AlCl_3$, w/v) solution to above mixture. Allow the solution to stand for further 6 min and then 2 ml of sodium hydroxide (4% NaOH, w/v) solution was added to the mixture. The final volume was adjusted to 5 ml with immediate addition of distilled water, mixed thoroughly and allowed to stand for next 15 min. The absorbance of each mixture was determined at 510 nm against the same mixture but without seed extract as a blank. TFC was determined as mg rutin equivalent per gram of sample with the help of the calibration curve of rutin. All determinations were performed in triplicate.

Determination of total alkaloid content (TAC)[28-30]

TAC was determined by the spectrophotometric method. This method is based on the reaction between alkaloid and bromocresol green (BCG). The plant extract (1 mg/ml) was dissolved in 2 N HCl and then filtered. Add 0.1N NaOH to the phosphate buffer solution to make pH neutral. 1 ml of this solution was transferred to a separating funnel, and then 5 ml of BCG solution along with 5 ml of phosphate buffer were added. The mixture was shaken and the complex formed was extracted with chloroform by vigorous shaking. The extract was collected in a 10 ml volumetric flask and diluted to volume with chloroform. Take absorbance of complex in chloroform at 470 nm. The whole experiment was conducted in three replicates for better results.

IV.Conclusion

The presented review explains the effect of different pretreatment exerts a quantitative increase in total alkaloid, flavonoid, and phenolic contents. The main conclusions were: a) The TPC in black currant fruit with different ultrasonic pre-treatment for 10 mins at high amplitude (70%), was found to be increased by approximately 4% in

all three cases of sample preparation i.e (frozen, freeze dried, oven air dried).b) The TFC in leaves of *Dryopteris erythrosora* with varying temperature drying pre-treatment for leaves shade dried for one day then oven-dried at 75°C for two days was found to be highest i.e 7.6%. c) The TPC and TFC, in the Basil leaves giving PEF pre-treatment with varying electric field strength and time of exposure, was found to be increased in a combination of 3kV cm^{-1} for 2 mins. With pulsed electric field pre-treatment TPC was found to be 115.203±10115 mg GA E/g and TFC was found to be 75.516±0.723 mg GA E/g. d) The TAC, in *Holarrhena antidysenterica*,when exposed to different enzymatic pretreatment, was found to be maximum by enzyme cellulose a followed by papain, lipase, amylase, and blank.

Conflict of interest

NA

Acknowledgment

Authors are thankful tothe Principal, management, and professors of Gahlot Institute of Pharmacy, koparkhairane, Navi-Mumbai, for their kind help and suggestion. The authors are also thankful to the informants for sharing valuable information.

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