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DESGINING, METHOD DEVELOPMENT AND STANDARDIZATION OF PROCESS FOR EXTRACTION OF MARKER COMPOUND FROM *TERMINALIA CHEBULA* FRUIT

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ABSTRACT: The present study is done to study and optimize the process of enzyme-assisted extraction of Chebulinic acid from the dried fruit of *Terminalia chebula*. A selective extraction process after the treatment with enzymes is proposed by using 30% (v/v) methanol which releases increased yield of the Chebulinic acid, present in the dried fruit compare to aqueous extract. The optimal conditions were as follows: pH value was 4.5, concentration of cellulase solution was 2.5 mg/mL, incubation time was 8 h, and incubation temperature was 50 º C and solid: solvent ratio was 1:8. Enzymes have the ability to degrade or disrupt cell walls and membranes, thus enabling better release and more efficient extraction of bioactives. Enzyme-assisted extraction methods are gaining more attention because of the need for eco-friendly extraction technologies. Enzyme-assisted extraction was proven to be highly efficient in the designing, process development and standardization of extraction of marker compound from herbal drugs and could be used in making enriched phytopharmaceuticals which may be translated into herbal drug for treating a medical condition.

INTRODUCTION: *Terminalia chebula* popularly known as Harde, belongs to the family Combretaceae. It is used commonly in many Ayurvedic preparations as laxative, diuretic, cardiotonic. *Terminalia chebula* Retz is reported to be anticarries, antioxidant, antimicrobial, anticancer, anti-urolithiasis and radioprotective properties [1-7].

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It is extensively used in Unani, Ayurveda and Homeopathic medicine. This is used in traditional medicine due to the wide spectrum of pharmacological activities associated with the biologically active chemicals present in this plant. It is used for the treatment of number of diseases like cancer, paralysis, cardio vascular diseases, ulcers, leprosy, arthritis, gout, epilepsy etc.

T. chebula is rich in tannins which on hydrolysis give chebulinic acid and D-galloyl glucose [8]. The chief constituents of tannin are chebulinic acid **(Figure 1),** chebulagic acid, ellagic acid, gallic acid, terchebin, terchebulin, and syrigic acid [8-11]. The dried fruits of *Terminalia chebula* is used to produced the dye.

The appearance of dye powder is brown and the main colouring component is chebulinic acid.

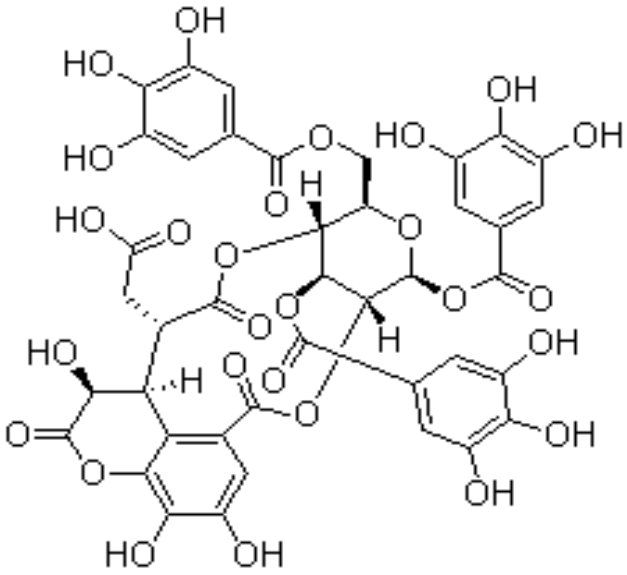

FIGURE 1: CHEMICAL STRUCTURE OF CHEBULINIC ACID

Although many reports about extracting Chebulinic acid by using different extraction methods have been published [12-13], some disadvantages still exist such as large organic solvent consumption, and low efficiency. Therefore, high efficient extraction method of Chebulinic acid represents a hot spot in *Terminalia chebula* research. Enzyme-assisted extraction is a method applied to the study of secondary metabolites releasing from biogenic materials. It possess the advantages of environmental- friendship, high efficiency and easy operation process. It has been represented as an alternative way for extracting marker compound from the herbal drug. Hydrolytic enzymes including cellulase, beta-glucosidase and pectinase, which are commonly used in extraction [14-16], can interact on cell wall; break down its structural integrity so as to increase the releasing of Chebulinic acid notably.

The main aim of the present study is to examine and optimize the process of enzyme-assisted extraction of Chebulinic acid from the Fruits of *Terminalia chebula*. For this purpose, the selection of enzyme type, pH and the concertration of enzyme solution, incubation time and temperature were studied, in order to obtain high yields of above natural products economically and environmental friendly.

MATERIAL AND METHODS:

Plant material: The dry Hartike was collected and ground into fine powder using a high-speed blender. The dry, ground Hartike was packed in a plastic bag, sealed and kept in the refrigerator (5°C) until used.

Chemicals and reagents: Chebulinic acid, and Cellulase were provided by Radiant Research Pvt. Ltd as gift sample. Methanol of analytical grade was purchased from Rankem Ltd. and double-distilled water was used in all experiments.

Enzyme-assisted extraction and pretreatment: Cellulase was quantified accurately and dispersed in deionized water to obtain enzyme solutions of certain concentrations (0.25-4 mg/mL). 100 g dry powder was added to the enzymatic solution and adjusted to certain pH (3.5-7.0) with 0.1 M HCl solution and shaken on a flat-bed orbital shaker for a period of time (1-10 hr) at certain temperature (30-55ºC). After the treatment fulfilled, the extract was filtered through Whatmann filter paper no 1. Filtrate collected was concentrated in vacuo (55ºC) in a rotary evaporator and analyzed by spectrophotometer. All the experiments were performed in triplicate.

Quantification of Chebulinic acid: Quantitative determination of total Chebulinic acid content in each sample of *Hartike* was performed by the described method [17]. Chebulinic acid content was calculated using an area under standard curve. Analysis of each sample was done in triplicate. For preparation of standard solution, standard Chebulinic acid (100.00 mg) was accurately weighed and transferred to a 5-ml volumetric flask. Distilled water was added and adjusted to a final concentration of 1.0 mg/ml.

From this solution, concentrations of 0.8, 1.6, 2.0, 2.4 and 3.2 µg/ml were prepared and used for preparation of the calibration curve. For preparation of sample solution from Hartike, the extract (500 mg) add 50 ml of water, reflux for 15 minutes, cool and filter and dilute to 100.0 ml. Dilute 10.0 ml of the solution to 25 ml with water.

RESULTS AND DISCUSSION: Cellulase catalyzes the breakdown of cellulose into glucose, cellobiose and higher glucose polymers; therefore, it is used to extract Chebulinic acid from Hartike Fruit.

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Quantification of Chebulinic acid: Standard and test solutions were injected into column of HPLC. The percentage of Chebulinic acid present in *Terminalia* extract was calculated by comparison of the areas measured for the sample and standard solution as per IP, 2007.

Effect of pH value of enzyme solution: It has been reported [18] that the activity of cellulase can be influenced by pH very much, and it is believed that it works better with pH<7. The effect of pH was studied in this experiment in order to pick out the proper pH value which would make the cellulase work best. **Figure 2** shows the effect of pH on the extraction yields of the Chebulinic acid. It can be observed that the yields of Chebulinic acid varied unregularly with different pH value. The yields of Chebulinic acid achieved the maximum at pH 4.5.

Effect of enzyme concentration: The effect of concentration of cellulase on the extraction yields of Chebulinic acid was studied and the results are shown in **Figure 3.** According to the results, it is obvious that with the increasing of cellulase concentration, the yields of these Chebulinic acids increased gradually until 2.5 mg/mL. Comparing with the yields of Chebulinic acid at the concentration of 2.5 mg/mL, 4.0 mg/mL did not show distinct advantage. Considering the economic influence, 2.5 mg/mL was selected for the pretreatment of the extraction process.

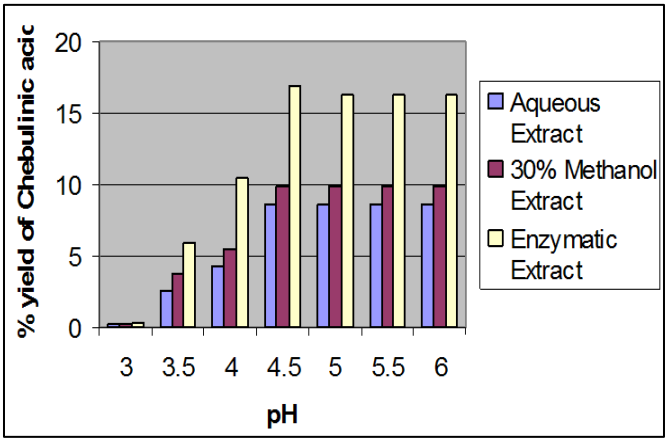

FIGURE 2: EFFECT OF PH ON THE YIELD OF CHEBULINIC ACID

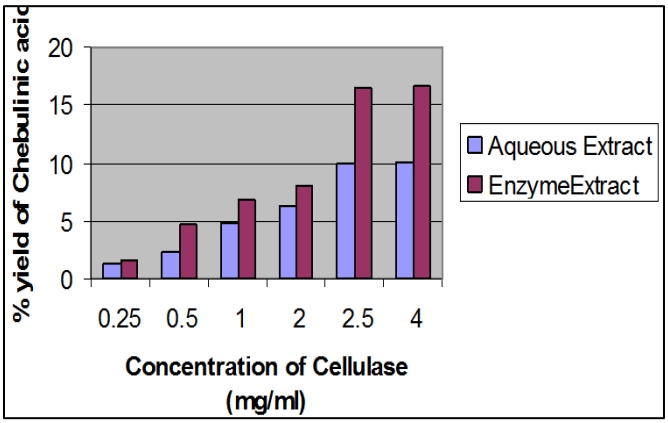

FIGURE 3: EFFECT OF CONC. OF CELLULASE ON YIELD OF CHEBULINIC ACID

Effect of incubation time: Figure 4 showed the results of the effect of cellulase incubation time on the extraction yields of Chebulinic acid. The yields of Chebulinic acid increased notably along with the extending of incubation time. The yields of Chebulinic acid reached the peak at 8 h and the yields began to decrease in additional time. Thus, 8 h was considered to be enough for cellulase to catalyze the hydrolysis of cell wall of *Terminalia* fruit.

Solid Solvent Ratio: Different solid: solvent ratios ranging from 1:2 to 1: 12 were studied and the optimum ratio for the extraction of Chebulinic acid was found to be 1:8 g/ml **(Figure 5).**

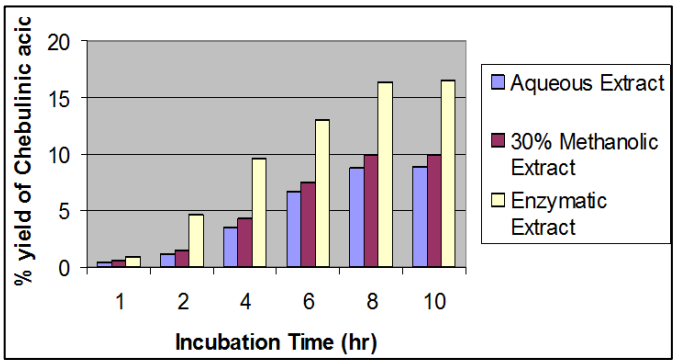

FIGURE 4: EFFECT OF INCUBATION TIME ON THE YIELD OF CHEBULINIC ACID

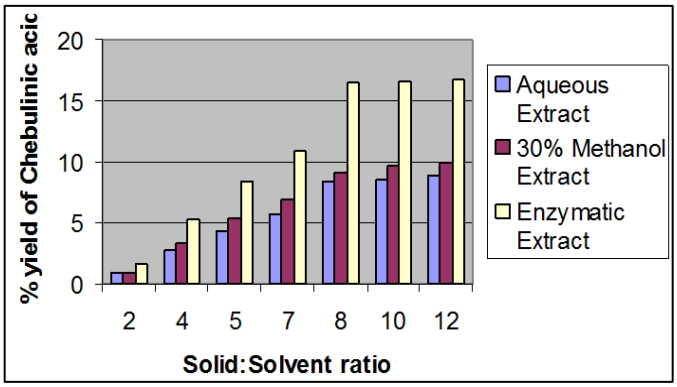

FIGURE 5: EFFECT OF SOLID: SOLVENT RATIO ON THE YIELD OF CHEBULINIC ACID

Effect of temperature on enzyme activity: The study of the thermal effect on the extraction yields was also carried out in this work. The results are presented in **Figure 6.** The yields of Chebulinic acid varied with the change in temperature. With the increase in temperature, the yields of Chebulinic acid increased gradually until 50ºC. The yields of Chebulinic acid increased upto 1.9 fold. Therefore, 50ºC was chosen for cellulase incubation temperature in this assay.

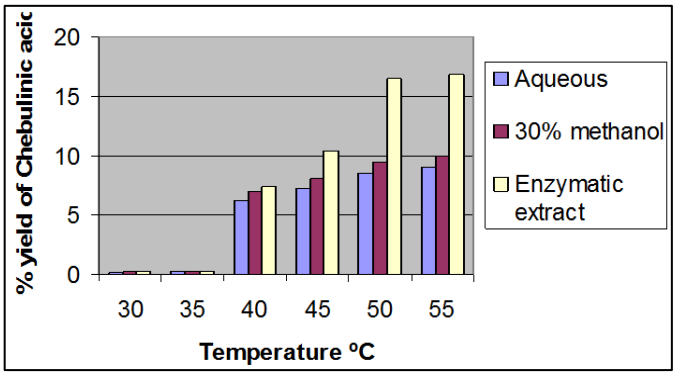

FIGURE 6: EFFECT OF TEMPERATURE ON THE YIELD OF CHEBULINIC ACID

Statistical Analysis: All results were subjected to statistical analyses. Mean values of all data were obtained from triplicate experiment and significance of differences was evaluated.

CONCLUSION: Enzyme assisted extraction Chebulinic acid, from Fruits of *T. Chebula* was carried out in present study. The effect of hydrolytic enzyme was studied and it was proved that cellulose at a concentration of 2.5 mg/ml to be most effective for extracting Chebulinic acid from Fruit of *Terminalia chebula*. As per the economic effect, cellulase was chosen for the treatment of the Fruit. The extraction conditions including pH and the concentration of cellulase solution, solid: solvent ratio, incubation time and incubation temperature were optimized. Results showed that all these factors were important for the extraction of Chebulinic acid.

The optimal conditions were as following: pH value was 4.5, concentration of cellulase solution was 2.5 mg/mL, incubation time was 8 h, and incubation temperature was 50ºC and solid: solvent ratio was 1:8. Pass through the treatment by cellulase, the contents of Chebulinic acid were 1.9-fold, of those in the control which showed that cellulase destroy the structures of plant cells and results in higher extraction yields of Chebulinic acid. Enzyme-assisted extraction may provide a feasible way for the extraction of Chebulinic acid from *T. Chebula* and other species of Terminalia, it has the advantages of environment friendship, lower cost, easy operation and higher efficiency, and it is promising for industry application broadly.

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