

Research Article

Effect of Enzymes on Extraction of Phytoconstituents From *Holarrhena Antidysenterica*

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ABSTRACT

Holarrhena antidysenterica (L) (Kutaja-Wall) Apocynaceae, is a common plant widely cited in literature for its medicinal value due to the presence of a large number of alkaloids. Various parts of the tree viz. bark, root, stem and seeds are known to have various medicinal properties including acute and chronic diarrhea and dysentery activity. The alkaloids are present in the polymeric matrix network of proteins and polysaccharide. There are specific enzymes that can break down this proteinaceous and polysaccharide matrix leading to an increase in the release of the drug, which will then facilitate the extraction. The overall effect will be an increase in the overall extraction of the constituents of interest. It was observed that cellulase was most effective in the release of alkaloids.

Keywords: *Holarrhena antidysenterica*, Extraction, Enzymes, Alkaloids, Phytoconstituents

INTRODUCTION

Herbal medicines remain the major source of health care for the world's population. We have yet to explore fully the vast storehouse of indigenous, tribal or folklore and traditional system of medicine of our country. In spite of advances in modern system of medicine, there are various areas like tropical diseases, herpes, AIDS, cancer and bronchial asthma etc, which will remain a formidable challenge to present day drug therapy. There is a possibility of finding a cure for them from the drugs of herbal origin.

Plants are a rich reservoir of potential leads for drug discovery. Almost half of the useful drugs today are derived from natural sources¹. Recently there has been a tremendous increase in research of medicinal plants. With emphasis being on scientific validation, a large number of plants are being biologically and chemically evaluated for their acclaimed properties. *Holarrhena antidysenterica* (L) (Kutaja-Wall) Apocynaceae, is a common plant known as kurchi, is widely cited in literature for its medicinal value due to the presence of a large number of alkaloids present². Various parts of the tree viz. bark, root, stem and seeds are known to have various medicinal properties like bitter, carminative, astringent, aphrodisiac, tonic, given in the infections of chest, asthma, colic, diuresis, used as a febrifuge in dysentery, diarrhea, intestinal worms, supposed to favor conception and used after delivery, galactogogue, aphrodisiac, are said to be good in chronic bronchitis, fever, piles, leprosy, skin diseases etc³.

Holarrhena antidysenterica is a source of steroidal alkaloids of conanine and aminopregnane types. The total alkaloid content of Indian kurchi is 0.22 – 4.2 % (av. 2.2 %). The bark of the plant contains about 30 alkaloids, the principal being Conessine, a stenol with a structure resembling 7-ergosten-3-ol and -stigmastanol. The other

important alkaloids that are present in the plant in smaller quantities are nor-conessine, conessimine, isoconessimine, kurchimine, kurchinine, kurchine, holarrhimine, conaine, conarrhimine, conkurchine^{4,5}. These alkaloids are present in the polymeric matrix network of proteins and polysaccharide and if this matrix can be broken down by some method, it may help in releasing the alkaloids. Chemical methods like acid hydrolysis can be used but they may bring about degradation of active constituents. Hence enzymatic method of extraction is beneficial due to its non requirement of heat, less energy requirement, milder conditions and specificity of enzymes as compared to the Conventional method and it can easily break down this polymeric matrix to release the phytoconstituents. The overall effect of these enzymes is an increase in the overall extraction of the constituent of interest⁶.

MATERIALS AND METHODS

Quantification of total alkaloids, carbohydrates, proteins and lipids present in Kurchi: The content of primary metabolites like carbohydrates, proteins and lipids alongwith total alkaloids present in the drug need to be determined which will help in utilizing the right enzyme in appropriate concentration for further studies.

*Determination of Total Alkaloids in Kurchi bark*⁷: It is very essential to find out the total alkaloid content in the kurchi bark sample used in the study so as to compare the effects of various enzymes used. The following experimental method was used.

Procedure: 10 of 80# kurchi bark powder was moistened with 50% ammonia. The ammonia was then removed and the powder is refluxed at 60°C for 4 hours. The extract is then filtered off and the filtrate is collected and the volume made to 100 ml with methanol. From this 2 ml of aliquot is withdrawn and the method for the determination of total

Release of sugar, protein, lipid and drug release without enzyme pretreatment:

Time (Hrs)	% S	% P	% L	% DF	% DP
0	0	0	0	0	0
1	2	1.5978	1.6914	0.0429	0.5464
2	2.053	1.9049	1.7814	0.1888	0.5288
4	2.1159	2.3233	1.9433	0.2056	0.4707
8	2.1507	2.5842	2.1052	0.2599	0.4113
12	2.195	2.6758	2.2672	0.3419	0.3563
24	2.2438	2.7676	2.4292	0.3606	0.3454

Table1: Effect of cellulase on release of sugar, protein, lipid and drug release:

Time (Hrs)	% S	% P	% L	% DF	% DP
0	0	0	0	0	0
1	2.1652	2.3608	2.1052	0.2311	0.4914
2	2.2695	2.7050	2.2672	0.3623	0.4510
4	2.4408	2.7528	2.4292	0.3670	0.4293
8	2.4856	2.9930	2.7530	0.5280	0.3530
12	3.0470	3.1396	3.0770	0.5494	0.3248
24	2.7571	3.2587	3.2388	0.5857	0.2474

Table2: Effect of amylase on release of sugar, protein, lipid and drug release:

Time (Hrs)	% S	% P	% L	% DF	% DP
0	0	0	0	0	0
1	2.3602	2.9238	2.1052	0.1104	0.6363
2	2.5662	3.1725	2.1052	0.2545	0.5330
4	2.7620	3.1854	2.2672	0.2908	0.5249
8	2.9786	3.4142	2.4292	0.2964	0.4360
12	3.2169	3.4647	2.5911	0.3283	0.3989
24	3.2408	3.6667	2.7530	0.3607	0.3269

alkaloids is followed. The yellow complex obtained was measured at 435 nm using thiourea reagent as the color reagent.

The total alkaloid content was found to be 0.8858%

Carbohydrates⁸: The total carbohydrates were determined in terms of glucose by DNS method.

Procedure: 2 g of the sample was accurately weighed and transferred completely to 250 ml round bottom flask. 100 ml of distilled water and 20 ml of 25 % HCl was added. The content of the flask was heated for three hours. The flask was cooled and the excess acid was neutralized with 40% NaOH. Volume of the solution was made up to 250 ml and sugar was estimated by DNS. 0.5 ml of the solution was taken, volume made up to 1 ml with distilled water. 1 ml of DNS reagent was added. It was heated on water bath for 10 minutes, cooled and volume made up to 10 ml with distilled water. The absorbance was measured at 540 nm. The amount of total carbohydrate present in the plant was found out in terms of glucose content from standard graph plotted.

Proteins⁹: The proteins were estimated by Folin Lowry method.

Procedure: 1 g of sample was weighed. 30 ml of acetate buffer was added and heated for 20 min. It was filtered into a 50 ml volumetric flask and volume made up to 50 ml with acetate buffer. From this 0.2 ml was taken and the volume made up to 1 ml with water. 5 ml of alkaline copper sulphate solution was added and allowed to stand for 10 min. After 10 min, 0.5 ml Folin-Ciocalteu reagent (50%) was added and was kept at room temperature for 30 min.

The absorbance was measured at 750 nm. The protein content was calculated from the standard graph.

Lipids¹⁰

Procedure: 2 g drug sample was accurately weighed and placed in a Soxhlet apparatus. The drug was extracted with 100 ml of petroleum ether (40-60 °C). Extraction was carried out for 12 hr. The petroleum ether extract thus obtained was evaporated on rotary vacuum evaporator. The dried residue was weighed and the lipid content was determined in terms of petroleum ether extract

The content of primary metabolites of kurchi are as follows:

Proteins: 4.55 %

Carbohydrates: 15.9 %

Lipids: 5.20 %

Standardization and Dose Optimization of Enzymes:

Amylase¹¹: The required amount of the amylase was determined by the ability of the enzyme to hydrolyze the starch.

Test solution of amylase: 100 mg of given amylase sample was accurately weighed and dissolved in sufficient acetate buffer pH 5 and volume was made to 100 ml.

Standard substrate: 100 mg corn starch was accurately weighed, added in sufficient distilled water and warmed gently, cooled and 5 g sodium chloride was added in the starch solution and finally volume was made to 100 ml with water.

Method: Into each of the 10 test tubes (numbered from 1 to 10), 1 ml (i.e. 1 mg) of standard substrate solution was added and test tubes were placed in water bath maintained

Table3: Effect of papain on release of sugar, protein, lipid and drug release:

Time (Hrs)	% S	% P	% L	% DF	% DP
0	0	0	0	0	0
1	2.0279	3.1017	1.9433	0.1411	0.4967
2	2.1119	3.2317	2.1052	0.2410	0.4787
4	2.2300	3.4114	2.2672	0.3744	0.3882
8	2.4348	3.6775	2.4292	0.4887	0.2986
12	2.5249	3.7527	2.5911	0.5508	0.2375
24	3.1259	4.5838	2.7530	0.5587	0.2175

Table4: Effect of lipase on release of sugar, protein, lipid and drug release:

Time (Hrs)	% S	% P	% L	% DF	% DP
0	0	0	0	0	0
1	2.1881	2.6392	2.2672	0.1730	0.6146
2	2.2533	2.9147	2.5911	0.2096	0.5031
4	2.3553	2.9455	2.7530	0.3312	0.4492
8	3.1694	3.1733	2.9150	0.3618	0.3986
12	2.5602	3.4583	3.0770	0.5234	0.3501
24	2.3940	3.7902	3.5628	0.5074	0.3454

S: Average % sugar release.

P: Average % protein release.

L: Average % lipid release

DF: Average % drug release in filtrate

DP: Average % drug release in powder

at 40 °C. When temperature of solution in the tubes had reached 40 °C, blank, 0.2, 0.4, 0.6, 0.8, 1, 2, 3, 4, 5 of test solution was added in no. 1 to 10 test tubes respectively. The tubes were mixed thoroughly and kept in the water bath at 40°C. After exactly an hour the tubes were removed and in each tube 0.05 ml of 0.02M iodine was added, mixed and observed for the colour change. A minimum 1.0 mg of given enzyme is required for the complete digestion of 1.0 mg substrate. Thus the quantity of enzyme required was **100%** of the substrate quantity.

Cellulase^{12,13}: The quantity determination is based on the principal that cellulase hydrolyses carboxymethylcellulose and produces carboxymethyl oligosaccharides; which on reacting with 3, 5-dinitrosalicylic acid (3, 5-DNSA) produces red color which is measured at 540 nm.

Preparation of sodium carboxymethylcellulose: 500 mg was dissolved in 100 ml acetate buffer. **Enzyme solution:** 200 mg of cellulase was weighed and dissolved in acetate buffer and the final volume was made to 100 ml with buffer.

Method: 2 ml of sodium carboxymethylcellulose was added to 15 test tubes (Numbered 1-15). In these test tubes blank, 0.2, 0.4, 0.6, 0.8, 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 ml of enzyme solution was added respectively. The tubes were kept in water bath at 50⁰ ± 1⁰ C for 2 h. The test tubes were cooled and 1 ml DNS reagent was added. The tubes were kept in boiling water bath again for 10 mins. They were cooled at room temperature. Volume was made up to 15 ml with distilled water. Absorbance was measured at 540 nm. Minimum 3 mg of dose of enzyme is required for complete digestion of 10 mg substrate.

Papain¹⁴: The proteolytic activity of the papain was measured in terms of amount of casein breakdown. Casein

that is not hydrolyzed is coagulated and filtered out. The hydrolyzed casein is measured in terms of amino acids.

Casein substrate: 1 g of casein was dispersed in 50 ml of 0.05 M dibasic sodium phosphate and placed in a boiling water bath for 30 min. with occasional stirring. Then it was cooled to room temperature and pH was adjusted to 6.0 ± 0.1 with 0.05 M citric acid. The solution was stirred rapidly and continuously, while addition of citric acid to prevent precipitation. Then it was diluted with water to make 100 ml.

Standard enzyme solution: 100 mg of standard papain was dissolved in 100 ml buffer solution and mixed well. 2 ml of this stock was dissolved in 50 ml of buffer solution. The final concentration of standard enzyme solution was 0.04 mg / ml.

Method: 14 test tubes (numbered from 1 to 14), each containing 2 ml (i.e. 20 mg) of standard substrate solution, were kept in water bath at 40⁰ for 10 min. Test tubes were taken out and into each test tube, blank, 0.2, 0.4, 0.6, 0.8, 1, 2, 4, 4.5, 5, 5.5, 6, 7 and 8 ml of test solution of papain was added respectively and the final volume was made up to 10 ml by buffer solution. The solution was mixed properly by swirling, time was noted and tubes were replaced in 40⁰ C water bath for 60 min. After 60 min, test tubes were taken out and into all; 3 ml of trichloroacetic acid solution was added and again replaced in water bath for 30-40 min to allow coagulation. Then the test tubes were taken out and the solution was filtered, first 5 ml of the filtrate was discarded and the remaining was read at 280 nm. 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.

Fig 1. Release of sugar, protein, lipid without enzyme

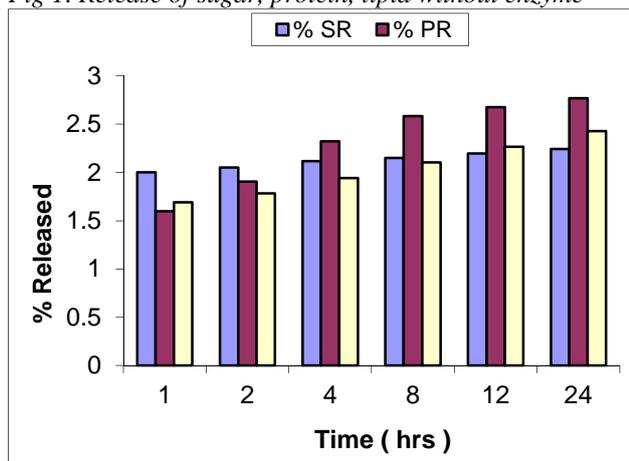


Fig 2. Effect of cellulase on sugar, protein, lipid release

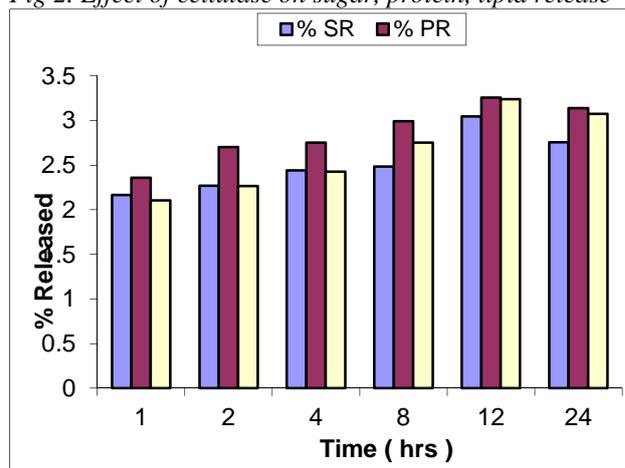


Fig 3. Effect of amylase on sugar, protein, lipid release

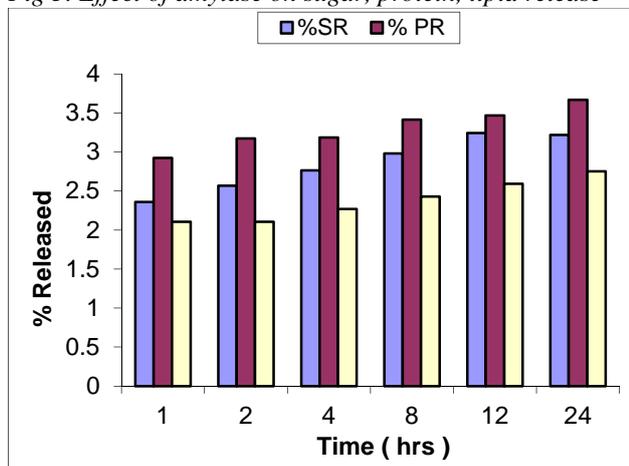


Fig 4. Effect of papain on sugar, protein, lipid release.

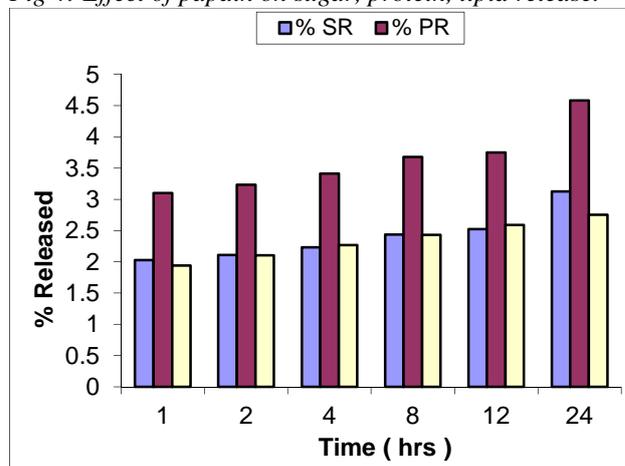


Fig 5. Effect of lipase on sugar, protein, lipid release

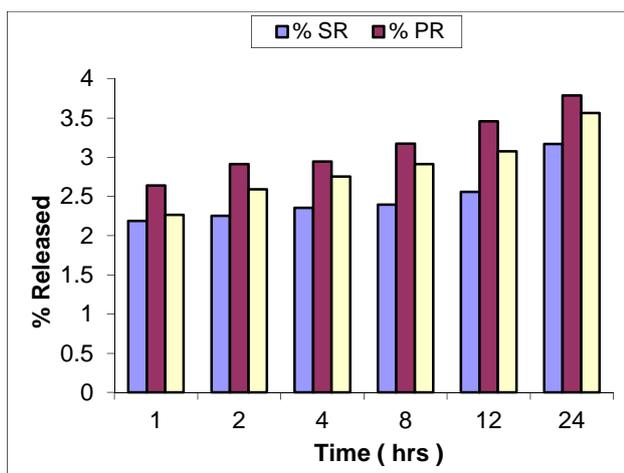
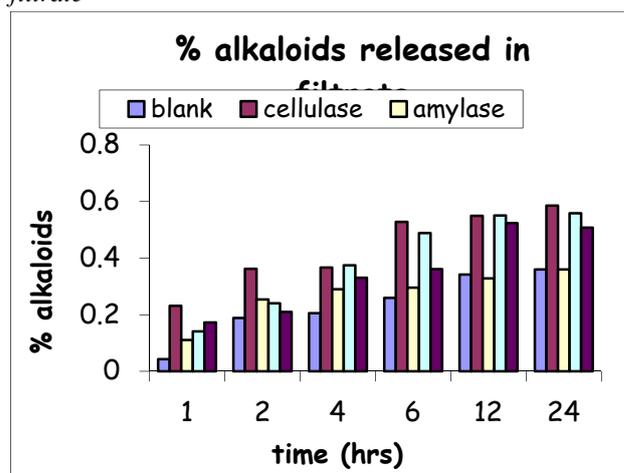


Fig 6. Effect of different enzymes on release of alkaloids in filtrate

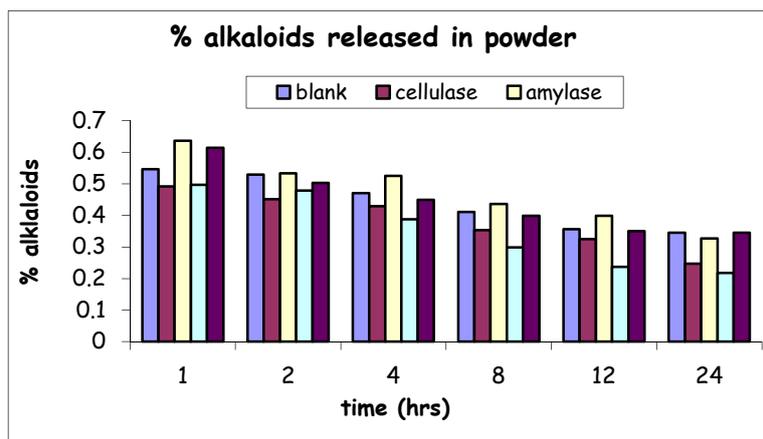


Lipase¹⁵: The quantity of lipase was decided on the basis of the quantity required to digest standard substrate (olive oil) completely in 24 h at pH 5 and at 37⁰ C.

Test solution of lipase: It was prepared by triturating accurately weighed 200 mg lipase in 60 ml of acetate buffer. The volume was made to 100 ml with buffer

Standard substrate solution: Olive oil is used as standard substrate. 1 g olive oil was weighed and dissolved in methanol and diethyl ether (1:1) and diluted to 50 ml
 Procedure: In 15 test tubes (numbered from 1 to 15), each containing 1.0ml (20 mg) of standard substrate solution, blank, 0.2,0.4,0.6,0.8,1,2,3,4,5,6,7,8,9 and 10 ml of test

Fig 7: Effect of different enzymes on the amount of alkaloids in powder



solution of lipase was added respectively. All test tubes were placed in a beaker and which in turn kept for shaking for 24 h at 37 °C. After 24 hr, the solution of each test tube was titrated with 0.1M KOH solution using phenolphthalein as indicator. 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.

From the above experiments; the amount of each enzyme to be used was calculated as follows on the basis of composition of drug:

Screening of Individual Enzymes: Screening of individual enzymes at 37 °C was processed in the following steps:

Pretreatment of drug with enzyme: 2.5 g crude drug (80 mesh) was used each time dispersed in 50 ml of buffer medium. Through this study, the quantity of the drug was kept constant. The enzyme mediated phytoconstituents release with respect to time was carried out at 37 °C in a shaker kept at 135 rpm. Time interval of 1, 2, 4, 8, 12 and 24 hr were fixed.

MATERIALS AND METHOD

Preparation of Enzyme solutions: The solutions of various enzymes were prepared in the acetic acid buffer I.P (pH 5). 2.385 mg dose of amylase, 0.1193 mg dose of cellulase, 1.0242 mg dose of papain and 0.624 g dose of lipase were dissolved separately in sufficient acetate buffer and the volume of each solution was made to 50 ml.

Procedure: 2.5 g powdered crude drug passed through 80 mesh was suspended in a 50 ml of enzyme containing buffer solution (containing required dose). 6 conical flasks were prepared as such and kept for shaking on orbital shaker incubator at 135 rpm and 37 °C for intervals of 1, 2, 4, 8, 12 and 24 h. After the period of respective time

RESULTS AND DISCUSSION

The experiment brought out some interesting facts. Though all the enzymes i.e. amylase, papain, cellulase and lipase are generally effective in enhancing the release of phytoconstituents by breakdown of the cell wall, these generally did not show significant increase in the release of the carbohydrates, lipids, proteins and very particularly alkaloids. But a small rise was seen in the release of these phytoconstituents than the normal extraction methods

interval, the conical flasks were removed and filtered. The filtrate was analyzed for sugar and protein release, while the residue was dried in incubator and used for analysis of lipid and drug release. At the same time, blank was carried out under identical conditions replacing enzyme solution by 50 ml buffer to compare the effect of enzymes.

Determination of Reducing sugars⁸: Determination of sugars was done by 3, 5-dinitrosalicylic acid (DNS) method.

Determination of Proteins⁹: Protein release was analysed by Folin-Ciocalteu reagent.

Fat determination¹⁰: Fat release was determined using titrimetric method with 0.05 M KOH solution using phenolphthalein indicator

Determination of Drug release⁷: To study the effect of enzymes on the facilitation of total alkaloid release, pretreated crude drug residue was analyzed by colorimetric method. For this purpose every time, drug released from fixed quantity of pretreated dried residue within fixed time was analyzed.

Screening of enzyme for drug release in the residue: 2 g of accurately weighed dried pretreated crude drug residue was moistened with 50 % ammonia, and was extracted for 10 minutes with methanol. After complete removal of ammonia the extract was filtered and final volume made to 10 ml with methanol. Appropriate volume of aliquot was taken and further analyzed for the total alkaloids by colorimetric method using thiourea (3 %) and the absorbance was measured at 435 nm.

Screening of enzyme for drug release into the filtrate: 5 ml of the filtrate after enzymatic treatment was used and further analyzed for drug release by colorimetric method using thiourea (3%) and the absorbance was measured at 435 nm

without these enzymes. It was observed that the enzymes, which caused more release of sugars and proteins, also release more amounts of alkaloids.

Cellulase was most effective in the release of alkaloids. From this it can be inferred 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 of the cell wall thus releasing them.

1. The ease in alkaloid release was in the following order: cellulase > papain > lipase > amylase > blank
2. The ease in sugar release was in the following order: amylase > papain > lipase > cellulase > blank
3. The ease in protein release was in the following order: papain > lipase > amylase > cellulase > blank
4. The ease in lipid release was in the following order: lipase > cellulase > papain , amylase > blank

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