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Research Paper

Amylase: Its application in the extraction of phytochemicals

***R. Amudan[1], D.V. Kamat[2], S.D. Kamat[3]**
[1]S.I.E.S. College of Arts, Science and Commerce, Sion (West), Mumbai- 400 022, INDIA
[2, 3] Mithibai College, Vile Parle (West), Mumbai 400056, INDIA

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Abstract

Plants are a rich source of drugs used in traditional systems of medicine, modern medicines, nutraceuticals, food supplements, folk medicines and pharmaceutical intermediates. They are also used as chemical entities for synthetic drugs. Efficient methods for maximum extraction of important phytochemicals are the need of the day. The traditional methods of physical and chemical extraction are effective but may affect the structure, quality and yield of the phytochemicals extracted. Amylase is an important enzyme that is generally used in the food and laundry industry. The paper aims at studying the effect of partially purified amylase obtained from *Aspergillus niger* in the extraction of phytochemicals from *Terminalia chebula* and *Syzygium aromaticum.* Amylase assisted extraction of *Terminalia chebula* showed a 13.3% increased release of phenolics in Tannic acid equivalents (TAE) and a two fold increase in Gallic acid equivalents (GAE). In the case of *Syzygium aromaticum,* amylase assisted extraction showed a two fold increase in volume of clove oils extracted and 40% increase in the weight of the oil extracted. The amylase assisted extracts of both the plant sources showed a greater antibacterial activity against *E. coli* and *S. aureus.*

Keywords: Amylase, antibacterial, clove oils, phenolics, *Syzygium aromaticum, Terminalia chebula*.

Introduction

Plants show the presence of a large number of phytochemicals. These phytochemicals are an enormous variety of organic substances that accumulate in herbs and plants and are used for combating diseases since ancient times. A continued search for medicinal plants has resulted in the discovery of new species of plants that are of great use in the treatment of diseases and for promoting health.

Plants contain a wide spectrum of metabolites, as many as 2, 00, 000 different compounds, though not every metabolite occurs in every species [1]. These metabolites represent many different classes of compounds and their derivatives such as amino acids, fatty acids, carbohydrates, and organic acids.
The extraction of active compounds from plants is one of the most critical steps in the development of natural products for medicinal and health benefits. In view of the large number of plant species potentially available for study, it is essential to have systems for the rapid and efficient extraction of phytochemicals. Extraction is one of the most important steps in sample pre treatment. The conventional techniques of solvent extraction of plant materials are usually based on the choice of solvents and the use of heat to increase the solubility of the desired compounds. Usually, conventional techniques require longer extraction time, thus running a risk of thermal degradation of some of the phytoconstituents [2]. The solvents used in the extraction of phytochemicals from plants also increase the risk of environmental pollution. The fact that a single plant can contain up to several thousand secondary metabolites makes the need for the development of high performance and rapid extraction methods an absolute necessity [3]. Many new alternative methods of extraction of nutraceuticals from

plants have been studied. One of them is the use of commercial enzymes with a broad range of activities that rupture the cell wall and cell membrane.

Studies on enzyme action on chilli have shown to give increased yield of capsaicinoids and carotenoids [4].Enzymes are used in many industries like the paper, laundry, food and beverage industry for processing and production. Thermostable α -amylase from *B. licheniformis* is used in the manufacture of high fructose containing syrups [5].

Primary walls isolated from higher plant tissues and cells are composed predominantly of polysaccharides together with lesser amounts of structural glycoproteins (hydroxyproline-rich extensins) , phenolic esters (ferulic and coumaric acids), ionically and covalently bound minerals (e.g. calcium and boron) and enzymes.

Alkaloids, terpenoids, phenolic compounds and volatile oils are known bioactive components. The present study involves the use of amylase in the extraction of bioactive compounds from *Terminalia chebula* fruit and *Syzygium aromaticum* buds. *T. chebula* is used as traditional medicine since antiquity. The fruit shows the presence of phenolics which has been extensively used in Ayurveda, Unani and Homoeopathic medicine. The chief constituents of tannins are chebulic acid, chebulagic acid, corilagin and gallic acid [6]. *T. chebula* has 32% tannin content and besides this, fructose, amino acids, succinic acid, β-sitosterol, resin and purgative principle of anthroquinone and sennoside is also present [7]. Flavonol glycosides, triterpenoids, coumarin conjugated with gallic acids called chebulin as well as other phenolic compounds were also isolated [8]. This plant is known to exhibit potential antibacterial, antifungal and antioxidant activities [9].

In good quality cloves, the content of essential oil may exceed 15%. The oil itself is dominated by eugenol (70 to 85%), eugenol acetate (15%) and β-caryophyllene (5 to 12%) which together makes up 99% of the oil. Cloves contain triterpene oleanolic acid, vanillin, crategolic acid, tannins, gallotannic acid, methyl salicylate, flavonoids triterpenoids and several sesquiterpenes. The clove bud is used as traditional medicine and as a spice that adds flavor to food.

Materials and Methods

All the components of enrichment and nutrient media were obtained from HiMedia Laboratories Pvt. Ltd. Chemicals were obtained from Loba Chemicals Pvt. Ltd.

Plant source

The dried fruit of flowering evergreen plant belonging to the Family: *Combretaceae,* Genus: *Terminalia,* Species: *chebula* also called as Black Myrobalan or Harda was used as the source of phenolics. The aromatic dried flower buds of an evergreen tree**,** Family*: Myrtaceae,* Genus: *Syzygium,* Species: *aromaticum* also called as clove, lavang was used as a source of essential oils. The dried fruits of *T.chebula* and buds of *S. aromaticum* were identified by the department of Botany.

Sample collection

Plant polysaccharides are known to contain different proportions of pectin, starch and cellulose in their cell walls. Thus soil containing decomposed flowers was used as a potential source of amylase producers. 10g of soil was used as inoculums for enrichment of amylase producers.

Enrichment, Isolation and Identification

Sterile Starch broth was used to enrich the soil sample and incubated at room temperature for a period of one week. From the enrichment broths, pure amylase producing fungal culture was obtained after isolation on Starch agar plate. The isolated pure fungal cultures were then identified by using monographs and 16SrRNA sequencing. The use of 16SrRNA gene sequences has been used extensively to study bacterial phylogeny and taxonomy [10].

Enzyme source

The extracellular amylase obtained after 72hr of incubation of the fungal amylase producer in sterile Starch broth was subjected to partial purification with ammonium sulphate at 50% saturation. This partially purified enzyme showing a fold purity of 3.55 and specific activity of 1.5U/mg was used for the enzyme assisted extraction of phenolics from *T. chebula* and oils from *S. aromaticum*.

Extraction procedures for *T. chebula*
10 g% of powdered *T. chebula* extract was subjected to cold water extraction overnight with 2ml of partially purified amylase. Similar extraction with denatured amylase was used as control for the study. The filtrates were then studied for the presence of phenolic content. Each extraction was replicated five times.

Extraction procedures for *S. aromaticum*
100g of *S. aromaticum* bud powder was soaked in 100ml of distilled water. 2ml of the partially purified amylase was added to this and incubated overnight at room temperature (RT). A similar set of pre treatment with 2ml of boiled enzyme was used as denatured enzyme control. The extracts were then hydro distilled using a clevenger apparatus with 100ml of distilled water for 3hr. The volatile distillate was collected over water in a graduated tube in the clevenger apparatus. Each extraction was replicated five times.

Estimation of total Phenolics by Colorimetric assay
Total phenolics were estimated by a modification of Folin Ciocalteau method. 0.5ml of Folin Ciocalteau reagent (1:2 diluted) and 2ml of sodium carbonate (20%) was added to 3ml of extract. It was incubated in boiling water bath for 1 min and absorbance was read at 650 nm in a spectrophotometer [11]. The gallic acid content in the extracts was also estimated by using the Folin Ciocalteau method. The standards used were tannic acid (2-20 µg/ml) and gallic acid (25-150 µg/ml).

Estimation of essential oils
The weight, volume and specific gravity of oils obtained from amylase pre treated *S. aromaticum* and control was ascertained. This was done to quantify the yield of oils obtained by the amylase assisted extraction. The specific gravity of the oil was compared with that of eugenol.

Antimicrobial activity of extracted Phenolics from *T. chebula*:
Sterile Mueller-Hinton agar obtained from Hi Media chemicals Ltd. was used. The method used to study the antimicrobial activity of the amylase assisted extract from *T. chebula against E. coli* and *S. aureus* was the well diffusion method, which is a modified disc diffusion test [12]. The controls used were gallic acid, tannic acid and elagic acid. The plates were incubated overnight at 37°C. The zones of inhibition were then measured in millimeters.

Results and Discussion

Isolation and Identification of Samples
The monograph study that included the study of macro-morphology and micro- morphology of the fungi identified the amylase producer as *Aspergillus niger.* The 16SrRNA sequencing also identified the amylase producer as *Aspergillus niger.* Earlier studies have shown that terrestrial isolates of fungi like *Aspergillus* species and *Penicillium* species are known to produce amylases [13].

Estimation of total Phenolics:
The cold water extract of enzyme pre treated samples of *T. chebula* and control were estimated for the presence of phenolics by the modified Folin Ciocalteau method. The phenolics estimated were higher in amylase assisted extractions as against control. There was a 13.3% increase in phenolics in tannic acid equivalents (TAE) and the amount of phenolics released in gallic acid equivalents (GAE) was twice in amylase assisted extractions in comparison to that of phenolics obtained in control (Table1, Figure 1a, 1b). Studies carried out by other researchers to quantify the amount of phenolics in gallic acid equivalents, have shown that the phenolics of 44.26 ± 0.923 mg/ml GAE was estimated in *T. catappa* followed 12.4 ± 0.4 mg/ml GAE in *T. arjuna* and 1.43 ± 0.621 mg/ml GAE in *T. chebula* [14]. In comparison to studies by other researchers, the yield of phenolics in GAE in the *T. chebula* extracts used in this study was higher in control and amylase assisted extracts. This indicates that amylase is indeed helping in the increased release of phenolics by acting on the outer cell wall and cell membrane of the fruit.

Table 1: Average Phenolics in Tannic Acid and Gallic Acid Equivalents

Samples	Phenolics mg/g (Tannic acid) Mean±S.D	Phenolics mg/ml (Tannic acid) Mean±S.D	Phenolics mg/g (Gallic acid) Mean±S.D	Phenolics mg/ml (Gallic acid) Mean±S.D
Control	101.50 ± 0.216	10.15 ± 0.216	4.7 ± 0.294	4.7 ± 0.294
Amylase	115.00 ±1.914	11.50 ± 1.914	10.8 ±0.311	10.8 ± 0.311

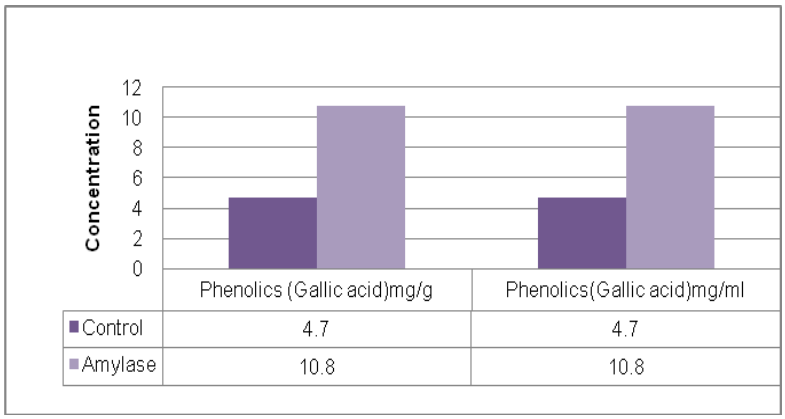

Figure 1a: Average Phenolics in Gallic Acid Equivalents (GAE)

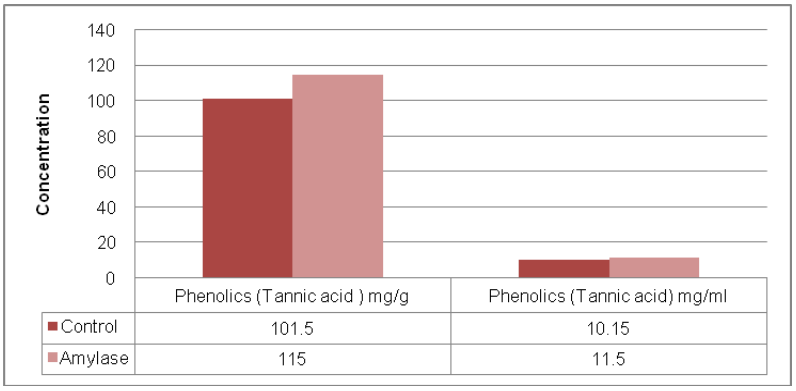

Figure 1b: Average Phenolics in Tannic Acid Equivalents (TAE)

Estimation of essential oils

Dried buds of *S. aromaticum* were subjected to amylase assisted extraction followed by hydro distillation. The specific gravity of clove oil obtained by amylase assisted extracted oil showed a higher value of 1.062 when compared with specific gravity of eugenol and control which were 1.006 and 1.015 respectively (Table 2). The weight and volume of clove oil (in gram percentage) obtained by amylase assisted extractions were higher than that of the control. The volume of essential oil extracted in terms of eugenol was 14.8ml in case of amylase assisted extraction. The volume obtained in control was around 7.2 ml. Similar results were also obtained when weight of the extracted clove oil was measured. The weight of the extracted oil from amylase assisted extraction was 16g/g% respectively. The weight obtained in control conditions was around 11.2g/g% (Table 2, Figure 2). Earlier studies conducted on clove have shown the yield of essential oils obtained on steam distillation of clove buds to be around 10.1% and on hydro distillation as 11.5%. Soxhlet assisted oil extraction has also been reported for clove and the yield obtained was around 41.8%. The oil obtained however was in the form of a brown ointment and not pale yellow oil as desired [15].The results of this study indicate that amylase pre treatment is enhancing the secretion of volatile essential oils from clove buds and the yield obtained is more than some conventional methods used for extraction. The extracted oil obtained was also pale yellow in colour.

Table 2: Characterization of clove oil on amylase extraction

Samples	Average Weight of the extracted clove oil (g %) Mean±S.D	Average Volume of the extracted clove oil (ml) Mean±S.D	Specific gravity
Control	11.2 ±0.216	7.2 ± 1.588	1.015
Amylase	16 ±0.294	14.8 ±0.311	1.062
Eugenol (Standard)	-		1.006

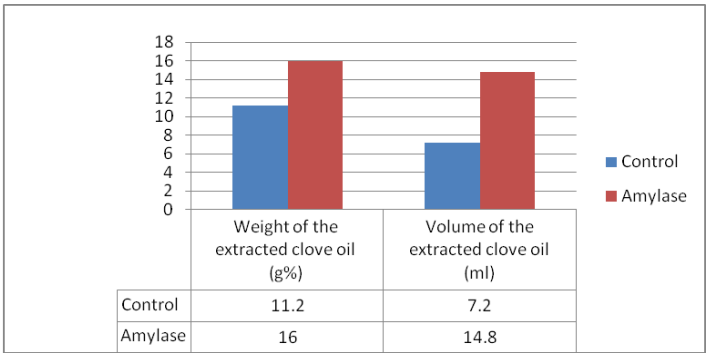

Figure 2: Yield of oil obtained on hydro distillation after amylase pre treatment

Antimicrobial activity of extracted Phenolics from *T. chebula*:
The amylase pre treated extracts of *T. chebula* inhibited both *S. aureus* and *E.coli*. Higher values of zone of inhibition against both *S. aureus* and *E. coli* were seen in the wells having enzyme pre treated extracts as against control and standards like gallic acid, tannic acid and elagic acid (Table 3, Figure 3a, 3b). This indicates the possibility that the enzyme extraction is possibly enhancing the extraction of bioactive compounds that may have the potential of being broad spectrum antibacterial compounds. Work done by other researchers has also indicated that ether, alcoholic and water extracts of black myrobalan (*Teminalia chebula* Retz inhibit growth of *Helicobacter pylori* and that the extracts may contain a heat stable agent with possible therapeutic potential [16].

Table 3: Antimicrobial activity of samples and standards against *E. coli and S. aureus*

Samples	Zone of inhibition against *S. aureus* (mm) Mean ±S.E(mean)	Zone of inhibition against *E. coli* (mm) Mean ±S.E(mean)
Elagic acid (Standard)	18.0 ± 0.33	13.0 ± 0.29
Tannic acid (Standard)	17.0 ± 0.33	17.0 ± 0.29
Gallic acid (Standard)	18.5 ± 0.29	20.5 ± 0.29
Amylase	25.0 ± 0.33	24.0 ± 0.33
Control	20.0 ± 0.17	20.0 ± 0.33

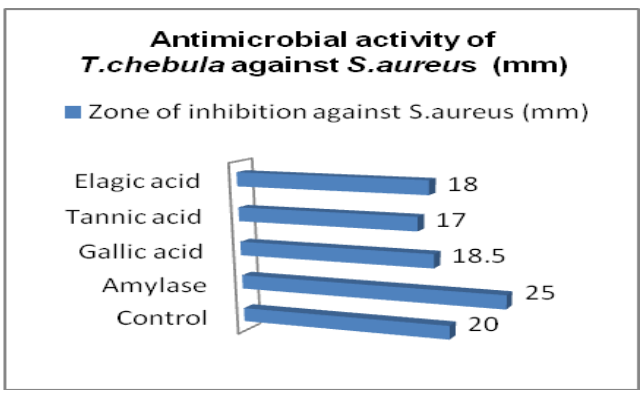

Figure 3a: Antimicrobial activity of *T. chebula* against *S. aureus* (mm)

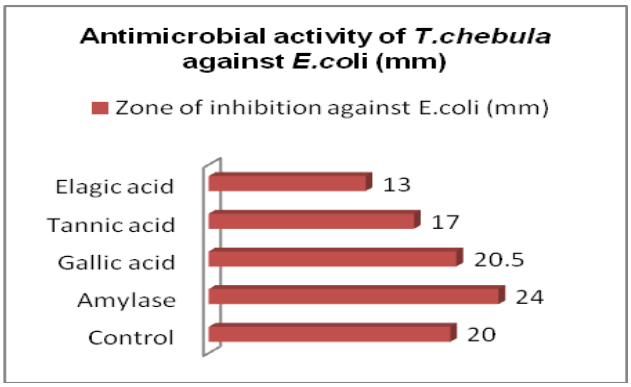

Figure 3b: Antimicrobial activity of *T. chebula* against *E. coli* (mm)

Antimicrobial activity of hydro distilled essential oils from *S. aromaticum*

The antibacterial activity of the vapors and liquid of clove oil extracted was studied. The oil extracted using amylase assisted extraction inhibited *S. aureus and E. coli* giving larger zones of inhibition than control and eugenol (Table4, Fig 4a). This indicates that amylase assisted extraction may be helping in opening up the cell wall and cell membrane of the clove bud thereby releasing compounds in minute amounts that have a greater antibacterial activity. This indicates that clove oil also possess a broad spectrum antibacterial property. Studies by other researchers have indicated that clove bud oil possesses antibacterial, anti insecticidal, anti fungal, antioxidant activity [17].The oil is also known to be effective against *L. monocytogenes* and *S. enteritidis* present in food items like tryptone soy broth and cheese [18].

Table 4: Antibacterial activity of essential oils

Sample	Zone of inhibition against *S. aureus* (well method) (mm)	Zone of inhibition against *S. aureus* (vapor method) (mm)	Zone of inhibition against *E. coli* (well method) (mm)
Control	20 ± 0.17	21 ± 0.32	16 ± 0.32
Amylase	24 ± 0.57	38 ± 0.57	19 ± 0.57
Eugenol	20 ± 0.32	10 ± 0.32	16 ± 0.28

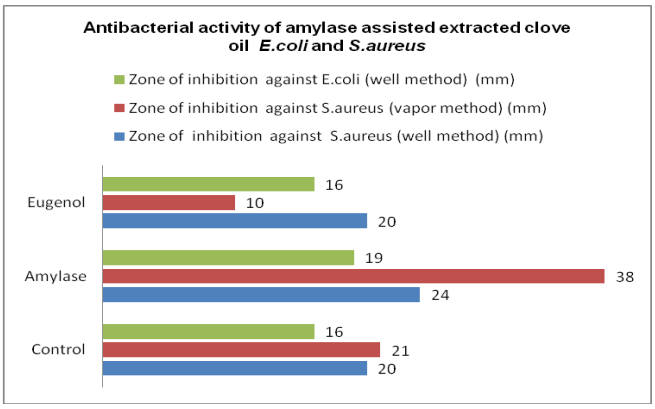

Figure 4: Antibacterial activity of amylase assisted extracted clove oil on *E. coli* and *S. aureus*

Conclusion

The fruits of *T. chebula* were treated with amylase and then subjected to cold water extraction. An increase in phenolics was observed on colorimetric estimation by modified Folin Ciocalteau method as against control conditions. There was a 13.3% increase in phenolics and a twofold increase in terms of tannic acid and gallic acid equivalents respectively by the amylase assisted extractions. This indicates

that the enzyme treatment may have brought about a possible change in the cell wall structure of the fruits of *T. chebula* resulting in the increased release of phenolics.
The results of antibacterial activity of phenolics showed enhanced zones of inhibition in extracts obtained by amylase pre treatment as against standards gallic acid, elagic acid, tannic acid and control extract.

In the study of oils extracted from *S. aromaticum,* the volatile oil obtained using amylase assisted extraction had a specific gravity greater than in control and eugenol. The volume of oil obtained was nearly twice that in control conditions and the weight of oil extracted was nearly 40% greater than that obtained in control. The oil obtained by amylase assisted extraction showed greater inhibition of *S. aureus and E. coli* than oil extracted from control conditions and standard eugenol.
The above results do indicate the possibility of developing a new environmental friendly enzyme assisted extraction along with conventional phytochemical extraction methods. This new method indicates an increase in the yield of extracted phytochemicals with an efficient antibacterial activity.

Of late, there is a rise in the numbers of resistant pathogenic organisms. Hence, there is a need to discover newer drugs to combat these disease causing organisms. The complex organic structure of phytochemical makes it difficult for the pathogen to inactivate it thus making it a potential candidate against various pathogenic organisms. The enzyme assisted extractions may help in increasing the yield of phytochemicals and also some bioactive components that may be active against many resistant organisms. These extractions may also reduce the use of solvents associated with the extractions helping in the onset of novel extraction procedures that are environment friendly. The application of enzymes in the extraction of phytochemicals appears to be a good alternative to the conventional methods used in the industries. An increase in yield and an improvement in quality would really be a milestone in the work done on phytochemicals.

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