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EFFECT OF *BHAVANA* WITH AMLA JUICE ON *IN-VITRO* ANTIDIABETIC AND ANTIOXIDANT ACTIVITY OF AMALAKI (*Emblica officinalis* Gaertn.)

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Abstract
Objective: *Amalaki (Emblica officinalis Gaertn., Fam: Euphorbiaceae) is a well known and potent rasayanas drug and increases our defense mechanism. The drug is included in best antidiabetic drugs mentioned in Ayurvedic text. According to Acharya charaka mentioned bhavana (trituration) with fresh juice of itself increases drugs potency by many fold so that the dose of medicine reduces. The aim of present study was to explore the effects bhavana on in-vitro hypoglycemic activity and in-vitro antioxidant activity.*
Methods: *500g of powder E. officinalis was triturated with fresh fruit juice of E. officinalis 21 times. 20g of the triturated material were collected after 5^{th}, 10^{th}, 15^{th} and 21^{st} trituration and hydroalcoholic extract (methanol:water::80:20) of each sample was used for experimental purpose.*
Results: *The result shows that the raw and processed possess α-glucosidase and α-amylase inhibition activity which was increases with the number of trituration and dose. The IC_{50} value of 21^{th} triturated amalaki was found to be 32.74 µg/mL and 48.22 µg/mL respectively and was comparable with standard drug acarbose. The in-vitro antioxidant potential of E. officinalis and effect of processing (bhawana) was investigated three in-vitro methods viz. DPPH free radical scavenging, scavenging of hydroxyl radical by deoxyribose method and nitric oxide scavenging. The results demonstrate that the free radicals were scavenged by the drug in dose dependent manner. Moreover the potency increases as number of trituration increases. The IC_{50} values for E. officinalis were found to be 38.68 µg/mL, 43.04 µg/mL and 55.85 µg/mL respectively.*
Conclusion: *These findings reveal that bhavana increses in-vitro antidiabetes as well as in-vitro antioxidant activity of E. officinalis.*
Keywords: *Emblica officinalis, Bhavana, Hypoglycemic, Antioxidant, Free Radical.*

Introduction: World's most serious health concerns diabetes mellitus (DM) is a chronic disease the prevalence of which is rapidly increasing in the current scenario with the increase in obesity and advancing age in the general global population. It is estimated that the number of persons in the world suffering from Type 2 DM will reach at least 380 million in 2025 [1]. Type 2 DM is primarily caused by defective glucose absorption, insufficient insulin production and its resistance. It is considered to be a preventable disease. In DM, the postprandial phase is characterized by a rapid increase in plasma glucose levels [2] and this postprandial "hyperglycemic spikes" play an important role in the progress of type 2 DM and leads to several micro- and macro-vascular, complications such as retinopathy, nephropathy, and neuropathy[3]. Moreover, postprandial state also contributes in the development of atherosclerosis and cardiovascular disease [2]. Controlling postprandial hyperglycemia plays an important role in delaying or preventing Type 2 DM and its micro- as well as macro-vascular complications [4]. Dietary control is the best way to control the postprandial hyperglycemia and it has synergistic effect with oral hypoglycemic agents (OHA)[5]. However, it depends upon depend on types and quantity of food consumed and such type of dieatery control is not seem to be possible in the present life style. Another possible therapeutic approach for decreasing postprandial hyperglycemia involves the retardation of fast uptake of glucose in the intestine [6]which is

possible by the inhibition of carbohydrate-hydrolyzing enzymes (especially pancreatic α-glucosidase and α-amylase) in the gastrointestinal tract[7]. Several synthetic α-glucosidase and α-amylase inhibitors, like acarbose are in clinical practices for reducing the sudden rise of blood sugar levels after taking food [8]. However, the continuous use of OHA may cause side effects such as flatulence, abdominal distention, vomiting, possibly diarrhea, renal tumors, serious hepatic injury and acute hepatitis[9,10]. Moreover, excessive inhibition of α-amylase may leads to abnormal bacterial fermentation of undigested carbohydrates in the colon [11].Hence, in the search of effective α-glucosidase and α-amylase inhibitors with lesser side effect, numerous in vivo as well as *in-vitro* studies were carried out and still going on [12].

Emblica officinalis Gaertn. (Euphorbiaceae) is a well known and potent rasayanas drug which reputed to promote health and longevity by increasing defense against diseases [13].The fruits, fresh, dried or stewed act as a tonic, a diuretic and a laxative. The fruits are useful in treating diabetes, cough, asthma, bronchitis, intermittent fevers and cardiac disorders [14]. *E. officinalis* has been reported to possess free radicals scavenging effect and is considered as a rich source of vitamin C. It consists of large amount of Vitamin C (ascorbic acid), tannins 30%, phyllemblic acid, phyllembin, gallic acid, ellagic acid in natural form and cytokine like substances identified as Zeatin, Zriboside, Z nucleotide [15]. Acharya charaka mentioned that if a powdered drug was triturated with its fresh juice, the potency of drugs increases [16]. Through the *bhavana* (trituration) one can reduced the dose of drug and enhance its effect. *Bhavana* is a special process describe in Ayurveda, commonly used in *shodhana* (detoxification with therapeutic enhancement) and *marana* (incineration) process of *rasa* (mineral origin) drugs. It was also described for many plant origin dugs too [17]. This study was designed to evaluate the effect of trituration on *in-vitro* antidiabetic and antioxidant activities of *E. officinalis*.

Materials and Methods

Sample Preparation: Dry fruit of *E. officinalis* were homogenised to a fine powder by using mechanical grinder and passed through mesh sieve (85#). The powder was stored in opaque screw-top jars at room temperature (20±2°C) until use. 500g of powder was then triturated with fresh fruit juice of *E. officinalis* in motor pestle until dry fine powder obtained. The process was repeated 21 times. 20g of the triturated material were collected after 5^{th}, 10^{th}, 15^{th} and 21^{st} trituration and stored in opaque screw-top jars at room temperature until use. The drug samples (20g), were extracted with hydroalcoholic solvent (methanol:water::80:20) (100 mL) using cold maceration process for 10 day. After 10 days the content was filtered and the filtrate obtained was concentrated under reduced pressure in rotary evaporator (Perfit India, Pvt. Ltd., India) below 60°C. The extracts were store at room temperature in air tied container.

Drug and Chemical: α-glucosidase (EC 3.2.1.20), pancreatic-amylase (EC 3.2.1.1), 4-nitrophenyl-a-D-glucopyranoside (pNPG) and DPPH were purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO). While acarbose tablet was purchased from local market. All reagents used in the experiment are of analytical grade.

***In-vitro* anti-diabetic Activity**

α-glucosidase Inhibition Assay: Determination of α-glucosidase inhibitory activity has been done as per the method of Ranilla et al., 2010 [18]. 50µl of extract has been added at different concentrations (5, 10, 20, 40 and 80 µg/mL in DMSO) with 1mL of 0.1M potassium phosphate buffer (pH 6.9) containing α-glucosidase solution. The mixture was then incubated at 25 °C. After incubation, 500µl of 5mM pNPG solution in 0.1M potassium phosphate buffer was then added to the mixture. It was then reincubated at 25 °C for 5min. The absorbance was then measured using a U.V. –Visible spectrophotometer (Varian-carry-100Bio), before and after the incubation period. The absorbance was compared to that of control, containing 500µL of buffer solution instead of extract. The percentage enzyme inhibition was calculated using the following expression:

Inhibition (%) = $[1-(\Delta A_{samp}/\Delta A_{std})]$ x100

Where,

A_{sample} =Absorbance of sample drug

A_{std} = absorbance of the standard drug

α-amylase Inhibition Assay: Determination of α-amylase inhibitory activity was done as per the method of Ranilla et al., 2010 [18]. 200µL of extract has been mixed at different concentrations (5, 10, 20, 40 and 80 µg/mL in DMSO) with 1mL of sodium phosphate buffer (pH6.8) containing 400µL of α- amylase solution. The mixture was then incubated at 37

°C for 10min , after which 300 µ L of starch solution (1%) in 0.05 M sodium phosphate buffer (pH6.8) containing 1m M CaCl2 was added. The mixture was then reincubated at 37 °C for 20min. The reaction was stopped by the addition of 0.2mL of di-nitro-salicylic acid solution which was then boiled in water both for 10min. The total volume was make up 8.0mL by using distill water. The absorbance was compared with that of control containing buffer solution in place of extract at 540nm. Acarbose has been used as positive control. The percentage enzyme inhibition was calculated as above.

***In-vitro* antioxidant Activity**

DPPH Free Radical Scavenging Activity: DPPH scavenging atctivity has been assessed by spectrophotometric analysis [19].A stock solution containing 25mg DPPH (150 µ M) & 100mL of Ethanol was prepared. 3.8mL of DPPH was added to 0.2 mL of extract of different concentrations. Control was prepared in the same manner without test sample. DPPH was replaced by ethanol in case of blank. The reaction mixture was kept in dark for 20minutes to complete the reaction. The absorbance was then measured at 517nm by using V.U- Visible spectrpophotometer (Varian-Cary-100Bio). Vitamin C was taken as standard.

%DPPH inhibition was calculated as follows:

Inhibition (%) = $[1-(\Delta A_{samp}/\Delta A_{std})] \times 100$

Where,

A_{sample} = Absorbance of sample drug

A_{std} = absorbance of standard drug

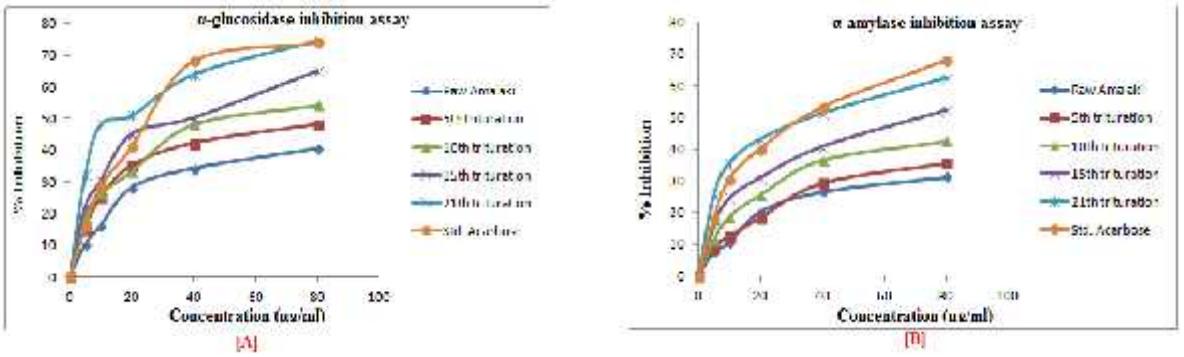
Scavenging of Hydroxyl Radical by Deoxyribose Method: 0.2mL of various concentrations of extracts or standard was added to the reaction mixture containing deoxyribose (3mM, 0.2mL), Ethylenediamine tetra acetic acid sodium salt (EDTA)(0.1mM ,0.2mL), ferric chloride (0.1mM, 0.2mL) and hydrogen peroxide (2mM, 0.2mL) in phosphate buffer (pH7.4, 20m M). The mixtures were then incubated at 37°C for 30min. Thiobarbituric acid (0.2mL, 15%) in 0.25N HCl were added to the reaction mixture after incubation. The reaction mixtures were then boiled in a water bath for 30min, cooled and absorbance was measured at 532nm [20].The % inhibition was calculated as above.

Nitric Oxide Scavenging Activity: Method of Singh et al., 2014[19] was followed for the determination of nitric oxide scavenging activity. At physiological pH sodium nitro prusside (aqueous solution) spontaneously produce nitric ions when interacts with oxygen, which was determined colorimetrically. 1mL of different concentration of extracts were mixed with 2mL of sodium nitro prusside (10m M) in phosphate buffer saline and the reaction mixture was incubated ay 37 °C for 4h. The same procedure was followed with control without test sample. Gries reagent (0.5mL) was then added after incubation. The absorbance was measured at 546nm. Percentage inhibition of nitric oxide generation was calculated by comparing absorbance of control and test. Vitamin C was taken as standard. Percentage nitric oxide inhibition was as above.

Results

***In-vitro* α-glucosidase and α-amylase Inhibition Activity:** Figure 1 illustrates inhibitory property of the raw and processed amalaki as well as standard acarbose on α-glucosidase and α-amylase. The results confirm α-glucosidase and α-amylase inhibition activity of amalaki. Moreover, the inhibitory potential was increases with the number of trituration and dose. At the highest concentration of 80 µg/mL, the % inhibition of α-glucosidase was increases from 40.54 to 74.78% after 21th trituration, while % inhibition of α-amylase was increases from 31.25 to 62.45%. The IC_{50} value of 21th triturated amalaki was comparable with standard drug acarbose. Therefore we can conclude that this fruit extract have moderate α-amylase inhibitory activity.

Figure 1. *in-vitro* antidiabetic activity of the raw and processed *E. officinalis*.

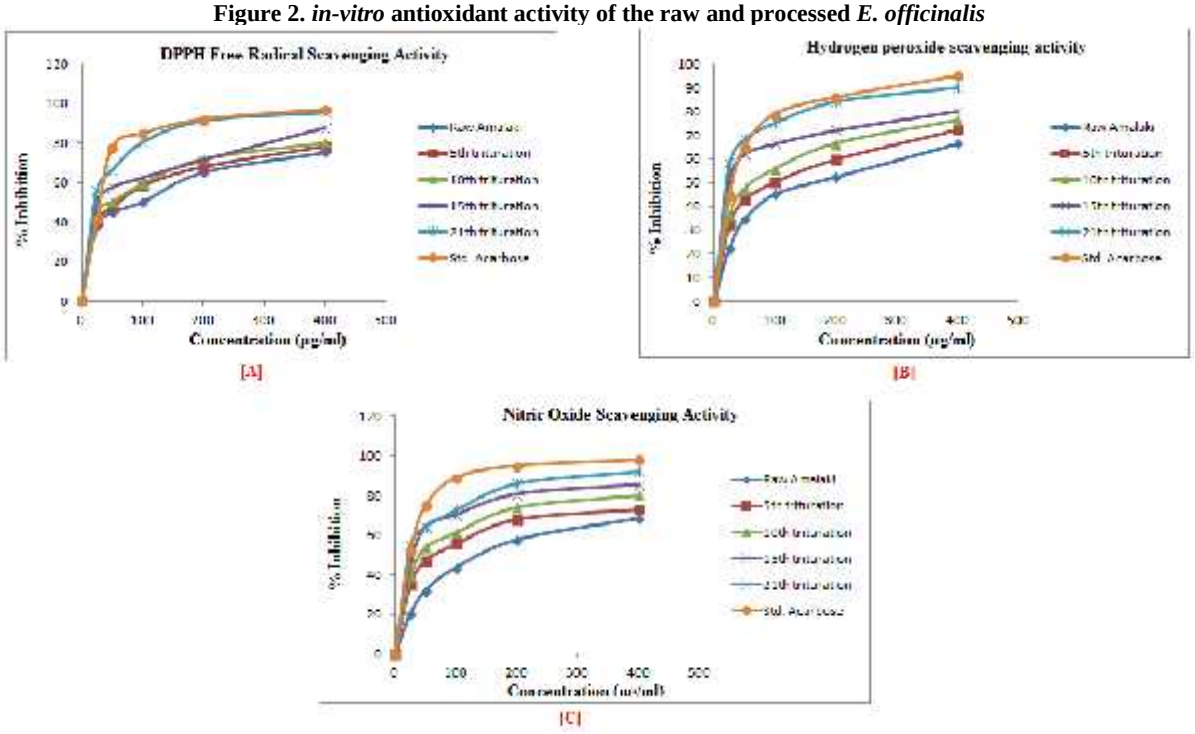


***In-vitro* Antioxidant Activity:** The *in-vitro* antioxidant potential of *E. officinalis* and effect of processing (*bhawana*) was investigated three *in-vitro* methods. The results demonstrate that the free radicals were scavenged by the drug in dose dependent manner. Moreover the potency increases as number of trituration increases. Results of *in-vitro* antioxidant activity were demonstrated in figure 2. Fig 2A shows that the *E. officinalis* possess DPPH radical scavenging ability. Maximum activity (95.68 %) was observed at 400 µg/mL concentration and the IC_{50} value for *E. officinalis* decreases as number of trituration increases. The IC value of 21^{th} triturated amalaki and ascorbic acid were found to be 38.68 µg/mL and 32.36 µg/mL respectively (table. 1). Fig. 2B reveals that the drug also possesses hydroxyl radical scavenging activity in which increases with trituration. Maximum scavenging activity (90.25%) was observed at 400 µg/mL concentration and the IC_{50} value of 21^{th} triturated amalaki and ascorbic acid were found to be 43.04 µg/mL and 62.16 µg/mL respectively (table 1). The nitric acid scavenging power measurements were shown in Fig 2C. In this assay, the hydro-methanolic extract of 21^{th} triturated amalaki at dose 400 µg/mL had maximum reductive potential (92.36%) with IC_{50} of 55.85 µg/mL.

Table 1. IC_{50} values of standard and different processed samples of *E. officinalis*

Activity	IC_{50} values (µg/mL)						
	Raw Amalaki	5^{th} trituration	10^{th} trituration	15^{th} trituration	21^{th} trituration	Std. Acarbose	Ascorbic Acid
α-glucosidase inhibition	88.62	69.78	60.02	47.43	32.74	39.56	----
α-amylase inhibition	120.25	104.98	83.89	66.35	48.22	46.10	----
Free Radical Scavenging by DPPH Method	159.21	136.63	120.20	97.29	38.68	---	32.36
Nitric Oxide Scavenging Activity	226.84	180.83	149.77	86.45	43.04	----	62.16
Scavenging of hydroxyl radical by deoxyribose method	216.73	154.14	118.75	77.73	55.85	-----	22.90

Figure 2. *in-vitro* antioxidant activity of the raw and processed *E. officinalis*



Discussion: The conventional pharmaco agents aimed to reduce the blood sugar level toward normal. After meal a sudden rise in postprandial blood glucose occurs due to digestion of carbohydrates by α-amylase and absorption of glucose by α-glycosidase [21].The clinically used OHA are having insufficient hypoglycemic effect on postprandial spike of glucose level [22].Agents which have inhibitory effect on α-glucosidases and α-amylase, may be added in clinical practice along with other OHA such as metformin, glimepride. α-Glucosidase enzyme is present on the brush-border surface of intestinal cell membrane [23]. It is a exo-type carbohydrase enzyme [24] which catalyze the hydrolysis of the a- (1, 4) -glucosidic linkage of starch and

disaccharides by providing hydrogen [24]. So hydrogen scavengers may act as α-glucosidase inhibitors like acarbose [23].Pancreas and salivary glands secrets α-amylase which catalyses the hydrolysis of α-1, 4-glucosidic linkages of starch, glycogen, and oligosaccharides [25].α-amylase inhibitors acts through two mechanisms. They either form a complex with enzyme and limit its activity [26] or reduce the diffusion rate of glucose from the active site [27, 28].Therefore, scientific communities are still investigating natural origin drugs for their possible role in the inhibition of these enzymes [12] .

Generation of oxidative stress in diabetes mellitus is well known. Use of antioxidants with OHA increases at present. Free radicals are produced in different oxidation reactions in the body, which participates in progress of diseases. Antioxidants terminate the oxidative chain reactions through free radicals scavenging activity and act as reducing agents like ascorbic acid and polyphenols [29].Production of reactive oxygen species (ROS) is a continuous process and different intracellular enzymatic and nonenzymatic antioxidants play important role in protection of cells from these ROS [30]. Hence, Antioxidants possess reducing power, free radical scavenging power, metal chelating power, and may activate antioxidative defense enzyme system of body [29]. Therefore; *in-vitro* antioxidant activity evaluation of herbal drugs may prove their therapeutic significance. Previously a variety of plants and phyto-molecules were investigated for their protective role in oxidative stress [31] .

However, *E. officinalis* has been used in Ayurveda for a number of disorders such as liver diseases, atherosclerosis and diabetes. It is also consider as one of the best rasayana (immunomodulatory) drug. Many researchers were reported its efficacy in diabetes mellitus *in vivo* as well as *in-vitro* [32].The present article deals with effect of trituration of *E. officinalis* with its juice on its antidiabetic and antioxidant activity *in-vitro.* The results of study reveal that the *in-vitro* anti-diabetic as well as antioxidant potency of *E. officinalis* was increases after successive trituration with its juice. The tannoid principles from fruits of *E. officinalis* were reported to be responsible for its antioxidant activity [33]. Because of the antioxidant activity of *E. officinalis* extract and quercetin, they are also found to possess cytoprotective effects. [34]. *E. officinalis* significantly scavenges superoxide as well as inhibits its generation and aqueous *E. officinalis* has been found to be potent antioxidants *in-vitro*.

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