

*Running title: Antidiabetic potential of aqueous leaf extracts of Aegle marmelos, Azadirachta indica and its herbal formulation.*

## COMPARATIVE EVALUATION OF ANTIDIABETIC POTENTIAL OF AQUEOUS LEAF EXTRACT OF AZADIRACHTA INDICA, AEGLE MARMELOS AND ITS FORMULATION

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### ABSTRACT

#### Introduction

Phytochemicals are secondary metabolites which act as antioxidants and possess various protective roles. Alpha Amylase and Alpha Glucosidase enzymes catalyse hydrolysis of starch to simple sugars. Aegle marmelos belong to rutaceae, they may grow to a height of 6-8 meters with pleasant smelling leaves. Azadirachta indica belongs to the meliaceae family which have been used by generations in folk medicine due to its health promoting activity and its major role in scavenging free radicals.

#### Materials and method

The herbs were collected from a local herb store and the extraction of the herbs was done. Phytochemicals screening test was done followed by the evaluation of antioxidant and antidiabetic potential. The data were analysed statistically by a one-way analysis of variance (ANOVA) followed by Duncan's multiple range test was used to see the statistical significance among the groups. The results with the  $p < 0.05$  level.

#### Results

When compared the formulation of Azadirachta indica and Aegle marmelos a significant antioxidant ( $IC_{50}=270$   $\mu\text{g/ml}$ ) and antidiabetic potential ( $IC_{50}$  of Alpha Amylase and Alpha Glucosidase inhibitory potential at 250 and 300  $\mu\text{g/ml}$  respectively), than the individual extract.

#### Conclusion

From the study, it was evident that the formulation exhibited an increased antioxidant and antidiabetic potential when compared to the individual extract thus proving the synergistic action. Further studies need to be focussed to validate the health benefits of herbal formulations to enrich its potential for the benefits of the society.

**Keywords:** Azadirachta indica, Aegle marmelos, Antidiabetic activity, Innovative technology, Novel method.

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#### INTRODUCTION:

Herbalism makes use of medicinal plants. Nature has equipped mankind with a large array of

medicines to treat any ailment. In rural and tribal areas, they are the most readily accessible source of healthcare (1). *Aegle marmelous* belong to the family of rutaceae. They may grow up to a height of

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6 to 8 meters with pleasant smelling leaves. Its flowers are sweet, scented nearly 2cm wide and shape is like oblong pyriform (2). Its leaf extract has potential to manage diabetes mellitus also taking more than a level may affect kidney, liver etc... Its found that methanolic extract of aegle marmelos may reduce blood sugar level which was tested on alloxan diabetic rats (3). Extensive laboratory and clinical studies show that Aegle marmelos has antidiarrheal, antimicrobial, and antifungal properties. *Azadirachta indica* which is neem belong to the meliaceae family and has been used by many generations due to its health promoting activity and its derivatives play a major role in scavenging free radicals and its biochemical parameters play a major role in controlling diabetes. It's been used in ayurveda for treating many diseases like diabetes mellitus (4,5). Neem has a therapeutic simplification in pathogenesis, antioxidant, nimbin, nimbin, salarin and antimicrobial effects neem. Its main function is scavenging microbes and free radicals present in the body (6,7). It also has cancer managing activity and is anti-inflammatory (8). Therapeutic role of *Azadirachta indica* is due to its richness in nimbin, nimbol. Its property in activating antioxidative enzymes resulting in cure of diseases (9). Cell membranes and other structures, such as cellular proteins, lipids, and DNA, are damaged by the oxidation process in the human body (10). As oxygen is metabolised, it produces unstable molecules called "free radicals," which steal electrons from other molecules, causing DNA and other cells to be damaged (11). Some free radicals can be tolerated by the body, and it requires them to act properly. However, over time, the damage caused by an excess of free radicals can become irreversible, leading to diseases such as heart disease and liver disease, as well as some cancers (such as oral, oesophageal, stomach and bowel cancers) free radicals.

These free radicals are triggered by smoking, alcohol, sunlight etc. Many research are done to test the importance of ayurvedic medicine in curing various ailments. Also it has reduced the side effects and costs very low and very easily available. Natural products or their derivatives are becoming increasingly popular in the treatment and prevention of diseases due to their lack of side effects (12). Neem and its constituents have therapeutic properties and have been used in traditional medicine around the world, especially in the Indian Subcontinent, since ancient times. (13), (14), (15). Clinical studies have shown that neem is effective in preventing a number of diseases (16), (17). Hence the aim of the study is to evaluate the antidiabetic potential of aqueous leaf extract of *Azadirachta indica*, *Aegle marmelos* and its herbal formulation (18).

## MATERIALS AND METHOD:

### CHEMICALS:

All chemicals and reagents used for this research work were purchased from Sigma Chemical Company St. Louis, MO, USA; Invitrogen, USA; Eurofins Genomics India Pvt Ltd, Bangalore, India; New England Biolabs (NEB), USA.

### COLLECTION OF PLANT MATERIAL:

Leaves of *Azadirachta indica* and *Aegle marmelos* were collected from a farm in Chennai district, Tamil Nadu, India.

### PREPARATION OF PLANT EXTRACT:

The plants collected were washed, crushed and made into powder. Leaf powder was utilized to prepare an 80% aqueous extract. Equal volume of the extract was mixed and a formulation was prepared. Antioxidant and Antidiabetic potential of *Aegle marmelos* and *Azadirachta indica* and its herbal formulation were calculated.

### Phytochemical Screening test

#### Test for phlobatannin

1ml of the extract was treated with 1ml of 1% HCl and boiled for 10 mins. The formation of red color precipitate indicates the presence of phlobatannin.

#### Test for Carbohydrates

Three to five drops of Molisch reagent was added with 1 mL of the extract and then 1 mL of concentrated sulphuric acid was added carefully through the side of the test tube. The mixture was then allowed to stand for two minutes and diluted with 5 mL of distilled water. The development of a red or dull violet ring at the junction of the liquids showed the presence of carbohydrates (19).

#### Test for Flavonoids

Few drops of 1% liquid ammonia were taken in a test tube and along with it 1ml of the extract was added resulting in the formation of yellow color thereby indicating the presence of flavonoids.

#### Test for Alkaloids

2ml of sample was mixed with 2ml of HCl. Then 6 drops of HCN was added and further 2 drops of picric acid was added that resulted in a creamish pale yellow ppt indicating the presence of alkaloids.

#### Test for Terpenoids

2 ml of sample along with 2ml of chloroform and 3ml of con. H<sub>2</sub>SO<sub>4</sub> was added. Red color ppt obtained indicates the presence of terpenoids.

#### Test for proteins

One milliliter of ninhydrin was dissolved in 1 mL of acetone and then a small amount of extract was added with ninhydrin. The formation of purple colour revealed the presence of protein.

#### Detection of saponins

Foam test: A fraction of the extract was vigorously shaken with water and observed for persistent foam.

#### Test for steroids

One milliliter of chloroform was mixed with 1 mL of extract and then ten drops of acetic anhydride and five drops of concentrated sulphuric acid were added

and mixed. The formation of dark red colour or dark pink colour indicates the presence of steroids

**Antioxidant activity:**

**DPPH free radical scavenging activity**

Scavenging of 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) radical was assessed by the method of Hatano et al, (1989). DPPH solution (1.0 ml) was added to 1.0 ml of extract at different concentrations (0.1 to 0.5mg/ml). The mixture was kept at room temperature for 50 minutes and the activity was measured at 517 nm. Ascorbic acid at the same concentrations was used as standard. The capability to scavenge the DPPH radical was calculated and expressed in percentage (%) using following formula:

$$\text{DPPH radical scavenged (\%)} = \frac{\text{Control OD} - \text{Sample OD}}{\text{OD}} \times 100$$

**Alpha amylase inhibitory activity of aqueous leaf extract of *Azadirachta indica* and *Aegle marmelos* and its herbal formulation:**

Alpha amylase inhibitory activity of extract was carried out according to the standard method of Ademiluyi et al[2013](20), In a test tube a reaction mixture containing 500 mu/l phosphate buffer (100mM ; pH=6.8). 100 mu alpha amylase (2 mu/l) and varying concentration of extract (0.1 - 0.5 mg/ml) was Incubated at 37degree Celsius for 20 minutes. Then the 200 mu/l of 1% soluble starch(100 MM phosphate buffer 6.8) was added as a substrate and incubated further at 37 degree Celsius for 30 minutes, 1000 mu/l of the 3,5 Dinitrosalicylic acid[DNS], DNS colour reagent was then added and boiled for 10 minutes. The absorbance of the resulting mixture was measured at 540 nm using a multi plate reader. Acarbose at various concentrations (0.1-0.5 mg/ml) was used as a standard.

$$\text{Inhibitory activity [\%]} = (1 - \text{AS}/\text{AC}) \times 100$$

AS= absorbance in the presence of test substance ; AC=absorbance of control.

**Alpha glucosidase inhibitory activity of aqueous leaf extract of *Azadirachta indica* and *Aegle marmelos* and its herbal formulation:**

Alpha glucosidase inhibitory activity of extract was carried out according to the method of Ademiluyi et al. Reaction mixture containing 500 mu/l phosphate buffer(100mM pH 6.8), 100mu/l glucosidase (10 ml) and varying concentration of extract (0.1 to 0.5 mg /ml) was pre incubated at 37 degree Celsius for 15 minutes. Then 200 mu/l p-NPG(5mM) was added as a substrate and incubated further at 37degree Celsius for 30 minutes. The reaction was stopped by adding 50 mu/l sodium carbonate (0.1M). The absorbance of the released p- nitrophenol was measured at 405 nm using multiple readers. Acarbose at various concentrations (0.1-0.5mg/ml)was used as a standard.

$$\text{Inhibitory activity [\%]} = (1 - \text{AS}/\text{AC}) \times 100$$

AS=absorbance of test substance; AC= absorbance of control.

**Statistical Analysis**

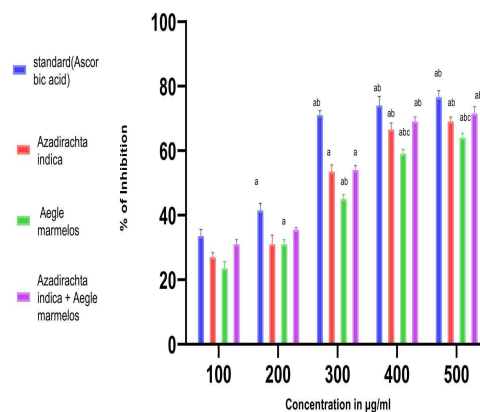
The data were subjected to statistical analysis using Two-way analysis of variance (ANOVA) and Tukey’s multiple range test to assess the significance of individual variations between the groups. In Tukey’s test, significance was considered at the level of  $p < 0.05$ .

**Results:**

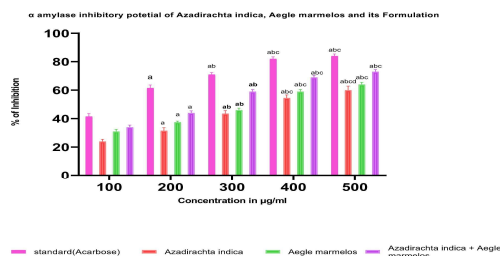
**Table 1: Phytochemical analysis of aqueous leaf extract of *Azadirachta indica*, *Aegle marmelos* and its formulation**

s. no	phytochemical	Azadirachta indica	Aegle marmelos	formulation
1.	carbohydrate	+	-	-
2.	flavonoids	+	+	+
3.	alkaloids	+	++	+
4.	terpenoids	++	+	++
5.	proteins	-	-	-
6.	saponins	+++	+	+
7.	steroids	-	+	-

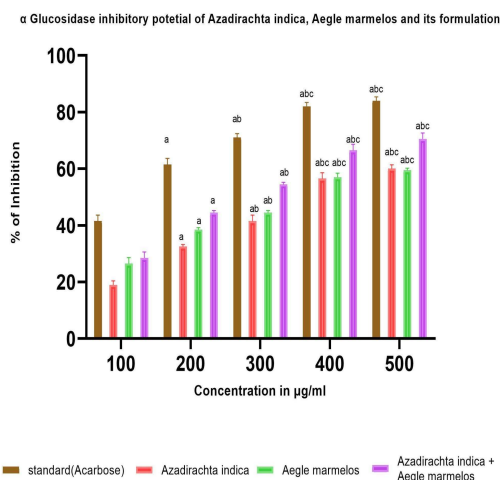
Antioxidant potential of *Azadirachta indica*, *Aegle marmelos* and its formulation



**Graph 1** Represents Antioxidant potential of aqueous leaf extracts of *Azadirachta indica*, *Aegle marmelos* and its herbal formulation compared with the standard (Vitamin C)- DPPH Assay. “X” axis represents various concentrations of the extracts of *Azadirachta indica*, *Aegle marmelos* and its herbal formulation. “Y” axis represents the percentage of inhibition. Blue colour denotes standard drug (vitamin C), orange colour represents *Azadirachta indica*, green colour represents *Aegle marmelos* and purple colour represents formulation. Each line represents Mean  $\pm$  SEM of 3 independent observations. Significance at  $p < 0.05$ .



**Graph 2** represents Alpha amylase inhibitory potential of aqueous leaf extracts of *Azadirachta indica* and *Aegle marmelos* and its herbal formulation compared with the standard (Acarbose). “X” axis represents concentration of the extracts of *Azadirachta indica* and *Aegle marmelos* and its herbal formulation. “Y” axis represents the percentage of inhibition. Pink colour denotes standard drug (Acarbose), orange colour represents *Azadirachta indica*, green colour represents *Aegle marmelos* and purple colour represents formulation. Each line represents Mean  $\pm$  SEM of 3 independent observations. Significance at  $p < 0.05$ .



**Graph 3** represents Alpha Glucosidase inhibitory potential of aqueous leaf extracts of *Azadirachta indica* and *Aegle marmelos* and its herbal formulation compared with the standard (Acarbose)

“X” axis represents concentration of the extracts of *Azadirachta indica* and *Aegle marmelos* and its herbal formulation. “Y” axis represents the percentage of inhibition. Brown colour denotes standard drug (Acarbose), orange colour represents *Azadirachta indica*, green colour represents *Aegle marmelos* and purple colour represents formulation. Each line represents Mean  $\pm$  SEM of 3 independent observations. Significance at  $p < 0.05$ .

**Phytochemical analysis:**

From the study, it was evident that the aqueous leaf extract of *Azadirachta indica*, *Aegle marmelos* and its formulation was found to be rich in phytochemicals such as Alkaloids, flavonoids, terpenoids, saponins and steroids. (Table 1) The presence of these phytochemicals is responsible for the medicinal value of the individual extract and its formulation.

**Antioxidant analysis:**

Antioxidant analysis of the aqueous leaf extract of *Azadirachta indica*, *Aegle marmelos* and its formulation was analysed and compared with the standard vitamin- C.  $I_{c50}$

was found to be 300,350,270µg/ ml for aqueous leaf extract of *Azadirachta indica*, *Aegle marmelos* and its formulation respectively. ( Graph 1)Antioxidant potential of the extract increased in a dose dependent manner as compared to the standard (Vitamin C).

**In vitro antidiabetic activity:**

Antidiabetic potential of the extract was analysed by estimating the extract’s  $\alpha$ - Amylase and  $\alpha$ - Glucosidase inhibitory potential and compared with the standard Acarbose. The enzymes amylase and glucosidase act on starch and release free glucose molecules. If the extract has significant inhibition of these enzymes it is proportional for its antidiabetic potential.

The extract exhibited a significant  $\alpha$ - Amylase inhibitory potential with an  $I_{c50}$  of 320,290,250µg/ ml for aqueous leaf extract of *Azadirachta indica*, *Aegle marmelos* and its formulation respectively. (Graph 2) and  $\alpha$ - Glucosidase inhibitory potential with an  $I_{c50}$  of 410,390,300µg/ ml for aqueous leaf extract of *Azadirachta indica*, *Aegle marmelos* and its formulation respectively. (Graph 3) Antidiabetic potential of the extract increased in a dose dependent manner as compared to the standard- Acarbose.

**DISCUSSION:**

*Azadirachta indica* and *Aegle marmelos*, commonly known as neem and vilvam are very common trees found in Asian countries, especially in India. Owing to its enriched medicinal properties these trees are considered more devotional and thus usually associated with temples. These trees are a part of indigenous folk medicine. Many research has been done on every part of both the trees neem and vilvam, but there is only a little research done with its formulation. Folk medicine usually comes as a

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formulation of different herbal extracts in different concentrations. The aim of any formulation is to improve the medicinal property of all the extracts as a formulation. Synergistic role of individual extracts is usually found in a formulation.

When phytochemical constituents were analyzed for the individual extracts and its formulation, both the extracts and its formulation was found to be rich in aromatic phytochemicals such as alkaloids, flavonoids etc. In the presence of phytonutrients like flavonoids, alkaloids indicate that the extract can be a potential antioxidant. Scavenging free radicals becomes a basis for any substance to possess medicinal properties like antidiabetic potential or anti cancer agent.

The extracts were analysed for antioxidant activity by DPPH free radical scavenging assay. Free radicals are molecules that possess unpaired electrons emerging in oxidative stress. The results obtained in this activity showed that all the extracts exhibited significant antioxidant potential, but comparatively the formulation of *Azadirachta indica* and *Aegle marmelos* exhibited significantly higher antioxidant potential than its individual extract.

In this study in vitro  $\alpha$ -amylase and alpha glucosidase inhibition of extract studied to explore the antidiabetic potential of the extracts. Acarbose is a standard drug used for study. The extracts revealed a significant inhibitory activity on  $\alpha$ -amylase and  $\alpha$ -glucosidase enzymes, but comparatively formulation was found to have significantly more antidiabetic activity than the individual extract.

The enzyme inhibitory activity ( $\alpha$ -amylase and  $\alpha$ -glucosidase) would delay degradation of carbohydrate which would in turn cause decrease in glucose absorption as a result reduce blood glucose level.

Popularity of natural products or their derivatives role in disease cure and prevention is increasing worldwide due to less side effects exhibited. As neem and vilvam are good antioxidants, their formulation can act as excellent medicine for diabetic treatment.

Previous research was conducted to determine the efficacy of neem mouth rinse in terms of its antigingivitis effect, and the results revealed that *Azadirachta indica* mouth rinse is just as effective as chlorhexidine in reducing periodontal indices. A research was conducted to test the 70 percent alcoholic neem root bark extract (NRE) in diabetes, and the findings revealed that in an 800 mg/kg dosage, neem root bark extract showed statistically significant results. The antioxidant activity of the flowers and seed oil of the neem plant *Azadirachta indica* A. Juss. was tested, and the results showed that the ethanolic extract of the flowers and seed oil at 200 g/mL provided the highest free radical scavenging activity, with 64.17 0.02 percent and 66.34 0.06 percent, respectively(21).

In a previous study, the regeneration of beta cells of experimental animals treated with alloxan was studied, where *Aegle marmelos* was used as a treatment drug and compared with glibenclamide-treated groups was demonstrated histopathologically. In alloxan-induced diabetic rats, the ethanolic extract of *Aegle marmelos* leaves showed promising antidiabetic activity.

Further research has to be done to exhibit the medical property of indigenous herbal drug formulations with the recorded concentrations to explore the synergistic role of these herbal constituents.

### Conclusion

Usage of natural herbs is always recommended over allopathic medicine which have side effects. Clinical studies prove that natural herbs possess antibacterial, antifungal and wound healing properties. From this study, it was evident that the formulation exhibited an increased antioxidant and antidiabetic potential when compared to the individual extract thus proving the synergistic action. More and more research has to be done to explore and create an awareness in replacing the synthetic drugs with indigenous herbal formulations which are easily available and cost effective. Research should also be focussed to validate the health benefits of herbal formulations to enrich its potential for the benefits of the society.

### Conflict of Interest :

The authors hereby declare that is no conflict of interest in this study

### Author Contribution :

S.Jeswin Immanuel - contributed in designing the study, execution of the project, statistical analysis, manuscript drafting.

R.Gayathri - contributed in study design, guiding the research work, manuscript correction.

V.Vishnu Priya, S.Kavitha - study design, statistical analysis, manuscript proofreading and correction.

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