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Evaluation of Anti Cholesterol and Antioxidant Potentiality of Aqueous Extracts of *Citrus aurantifolia, Zingiber officinale* and its Formulation - a Comparative *In vitro* Study

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Background: Several plant extracts and herbs have been used for treating and prevention of cardiovascular diseases hypertension, angina pectoris, congestive heart failure, atherosclerosis, cerebral and venous insufficiency and arrhythmia. Similarly, aqueous extracts of *Citrus aurantifolia* and *Zingiber officinale* which is commonly called as lemon and ginger respectively and its formulation were analysed for its antioxidant and anti cholesterol activity.
Objective: This research has been performed in order to evaluate the anti cholesterol and antioxidant potentiality of aqueous extracts of *Citrus aurantifolia*, *Zingiber officinale* and its formulation respectively.
Methods: The study setting carried out for this research was in vitro, hence the work was performed outside the living organism. *Citrus aurantifolia* and *Zingiber officinale* were purchased from a farm in chennai. The experiment began starting from the preparation of aqueous extract of lemon and ginger. A formulation was made combining equal amounts of the two extracts followed by this, a phytochemical screening test was conducted. Antioxidant and anti cholesterol potential of the extracts and its formulation were also analysed. The data was statistically examined by one-

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way analysis of variance (ANOVA) continued by Duncan's multiple range test, it was compiled to see the statistical significance among the groups present. The results carrying $p<0.05$ level were contemplated to be statistically significant.

Result: From the study, though both the extracts possessed a good antioxidant and anti cholesterol potential, comparatively the formulation exhibited an increased antioxidant and anti cholesterol potential respectively (IC_{50} = 250 µg/ml) and (IC_{50}= 375µg per ml). Here, the formulation exhibited significantly more activity than the individual extracts.

Conclusion: Even though there is sufficient knowledge among citizens about the nutritional value present in herbal formulations, there isn't enough in-depth study conducted on the formulation of these two extracts based on their cholesterol inhibitory activity. From this particular study it was proven that the formulation showed synergism. Hence the formulation of these extracts could be preferred over other synthetic drugs since it is natural, cost effective and easily accessible.

Keywords: Citrus aurantifolia; zingiber officinale; lemon; ginge;, antioxidant; anti cholesterol; innovative technology; novel method.

1. INTRODUCTION

Citrus aurantifolia and *Zingiber officinale* are among the world's most common fruits and stems. Citrus which is commonly called lemon is a species of miniature evergreen tree belonging to the flowering plant family called 'Rutaceae'. This tree's yellow fruit is popularly being used for culinary and non culinary purposes around the world, specifically for its juice and the nutritional value it contains [1]. The juice extracted from the fruit is composed of 5% to 6% citric acid. It stands in the pH range of around 2.2, hence providing it with a very sour taste on its own. It's a very rich source of vitamin-C, pertaining 64% of the daily value in 100g of it's essential nutrient amount. Lemons also contain numerous phytochemicals, including steroids, saponins, terpenoids, alkaloids, flavonoids and protein (amino acid) [2]. Lemon juice is known to have more citric acid content as compared to lime juice, it is nearly 5 times the amount of citric acid found in orange juice and twice the citric acid of grapefruit juice [3]. The second plant extract involved in this research is *Zingiber officinale commonly called ginger* that belongs to the flowering plant family 'Zingiberaceae'. The health promoting perspective of *Zingiber offcinale* i.e ginger is imputed to its abundant phytochemical properties by its nature like, protein (amino acid), flavonoids, alkaloids, terpenoids, saponins, steroids and carbohydrates [4]. Besides the extract of ginger, the rhizome of the stem is also widely used in traditional herbal medicatio [5,6]. Ginger has great deal of potential for treating numerous degenerative, digestive, cardiovascular disorders along with vomiting, diabetes mellitus and cancer. Furthermore it contains antimicrobial potential which helps in treating infectious disease [7].

In this analysis we look upon the antioxidant potential of *Citrus aurantifolia* and *Zingiber offcinale*. Generating free radicals or reactive oxygen species during the ongoing metabolism further to the antioxidant capacitance in a biological system leads to oxidative stress. It plays a crucial role in case of cancer, neurodegenerative disease, heart problems etc.

The reactive oxygen species are chemically derived from oxygen like that of hydrogen peroxide, superoxide anion and hydroxyl radicals within living organisms by metabolic pathways, while the antioxidant system is able to defend against it to maintain balance [8]. However modern lifestyle involves a number of factors that may raise the level of oxygen reactive species which has a critical part in the pathogenesis of several diseases [8,9]. Cholesterol is generally involved with fatty foods but the majority of the wax substance present in the cholesterol is produced by the body on its own. At standard levels, cholesterol plays a crucial role in assisting the cells to do the job. High cholesterol levels are also commonly found high in the population due to unhealthy and carefree lifestyles in relation to one's diet. Low-density lipoproteins, which are frequently referred to as bad cholesterol, are associated with increased risk of heart disease. LDL constitutes more cholesterol that makes it lighter in weight than protein. When oxidation takes place, LDL promotes inflammation and forceful accumulation of lipids on the walls of vessels in the heart and rest of the body, leading to plaque formation. These plaques could thicken over time and limit or completely block blood and nutrient supply to the affected tissues or organs. Hence, consuming natural plant extracts like that of lemon and ginger would help maintain the body's cholesterol level, along with its antioxidant

property [10]. There is always a good correlation existing between the anti cholesterol and antioxidant activities of *Citrus aurantifolia* and *Zingiber officinale* or between any other plant species [10,11]. Our department has substantial knowledge and research encounters that have led to immense quality publications. [12-31]. This research aimed to evaluate the anti cholesterol and antioxidant potential of aqueous extract of *Citrus aurantifolia, Zingiber offcinale* and its formulation through *in vitro* analysis.

2. MATERIALS AND METHODS

2.1 Preparation of Aqueous Extract of *Citrus aurantifolia, Zingiber officinale* and its Formulation

Citrus aurantifolia and *Zingiber officinale* were purchased from a farm in Chennai. *Citrus aurantifolia* was crushed and juice was extracted. *Zingiber officinale* was peeled and crushed with water to get an 80% extract . Equal volume of *Citrus aurantifolia* and *Zingiber officinale* extract was mixed to prepare a formulation.

2.2 Phytochemical Screening Test

2.2.1 Test for phlobatannin

1ml of the extract was taken and added to 1ml of 1% HCl. This mixture was boiled for 10 mins. There was a formation of red colour precipitate. This indicates the presence of phlobatannin [32].

2.2.2 Test for carbohydrates

Around three to five drops of Molisch reagent was added to 1 mL of the extract. Followed by, 1 mL of concentrated sulphuric acid added carefully through the side of the test tube. The mixture was kept aside for two minutes on stand. After 2 minutes, it was diluted by adding 5 mL of distilled water to it. The presence of a red or dull violet ring at the junction of the liquids indicates the presence of carbohydrates.

2.2.3 Test for flavonoids

Few drops of 1% liquid ammonia is taken in a test tube. 1ml of the extract was added which resulted in the formation of yellow colour. This colour indicates the presence of flavonoids.

2.3.4 Test for alkaloids

2ml of HCl is mixed with 2ml of sample. 6 drops of HCN was added to the mixture followed by 2 drops of picric acid. This resulted in a creamish pale yellow precipitate. This precipitate indicates the presence of alkaloids.

2.3.5 Test for terpenoids

2 ml of chloroform along with 2ml of sample and 3ml of con. H2SO4 was added in a test tube. The presence of Red colour ppt indicates the presence of terpenoids.

2.3.6 Test for proteins

One millilitre of ninhydrin was dissolved in 1 mL of acetone. A small amount of extract was added with ninhydrin which led to the formation of purple colour. This purple colour tested the presence of protein.

2.3.7 Detection of saponins

Foam test: A fraction of the extract and some water was taken and was vigorously shaken. It was then observed for the presence of persistent foam.

Test for steroids: One millilitre of extract was mixed with 1 mL of chloroform. Ten drops of acetic anhydride and five drops of concentrated sulphuric acid were added to this mixture and mixed well. There would be a formation of dark red colour or dark pink colour that indicates the presence of steroids.

2.4 Antioxidant Activity

2.4.1 DPPH free radical scavenging activity

Hatano et al. [33] method was used for the assessment of scavenging of 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) 1 ml of the DPPH solution was added to 1 ml of the extract in a test tube. It was added at different concentrations varying from 0.1 to 0. 5mg/ml. The mixture was set aside at room temperature for about 50 minutes. After the duration the activity was measured at 517 nm. The standard used was Ascorbic acid. It was used at the same concentrations to carry out the step [34]. The capacity of DPPH radical scavenging activity was calculated. It was expressed in the form of percentage (%). The formula used was as following:-

DPPH radical scavenging (%) = Control OD - Sample OD X 100/ Control OD.

2.4.2 In vitro anti-cholesterol activity

The anti-cholesterol assay carried out was as per the kit method description (Spinreact, S.A.U-Ctra Santa Coloma, Spain and Girona). The Cholesterol was then dissolved in chloroform. The dissolution was at the concentration of 2.5 mg mL/ml. Ten microliter of the extract was pipetted out into a microtiter plate. Which was followed by the addition of 2000 µL and 10 µL of R1 reagent and cholesterol as samples respectively. Twenty microliter of distilled water were used as blank to carry out this step along with 2000 µL of R1 reagent [35]. The negative control consisted of 20 µL and 2 ml of cholesterol and R1 agent respectively. On the other hand, the standard consisted of 20 µL simvastatin and 2000 mL of R1 reagent. The contents were incubated at room temperature for a duration of 0-30 minutes. The absorbance was read against reagent blank in a UV-Vis spectrophotometer at 500 nm.

Anti-cholesterol assay of the extract was calculated using the following equation for calculation:-

$$\text{Inhibition } (\%) = \frac{\text{Negative control} - \text{Sample.} \times 100}{\text{Negative control}}$$

2.5 Statistical Analysis

The data were subjected to statistical analysis by the means of two-way analysis of variance (ANOVA) along with Tukey's multiple range test. They were used to assess the significance of individual variations between the groups. In Tukey's test, when the value is $p<0.05$, it was considered to be at the level of significance.

3. RESULTS AND DISCUSSION

The concentration of Ninhydrin reagent is found to be present in the extract of *Zingiber officinale* but absent in *Citrus aurantifolia* extract. The phytochemicals, Protein (Amino acid), Flavonoids, Alkaloids, Terpenoids and steroids are less in concentration in *Zingiber officinale* extract in comparison with *Citrus aurantifolia* extract. which in. Saponin is present in equal concentration in the extracts of *Zingiber officinale* and *Citrus Aurantifolia*. The Carbohydrate concentration is present in the extracts of *Zingiber officinale* but is not present in the extract of *Citrus aurantifolia.* This was identified by the phytochemical analysis of aqueous extract of *Zingiber officinale* and *Citrus Aurantifolia* (Table-1).

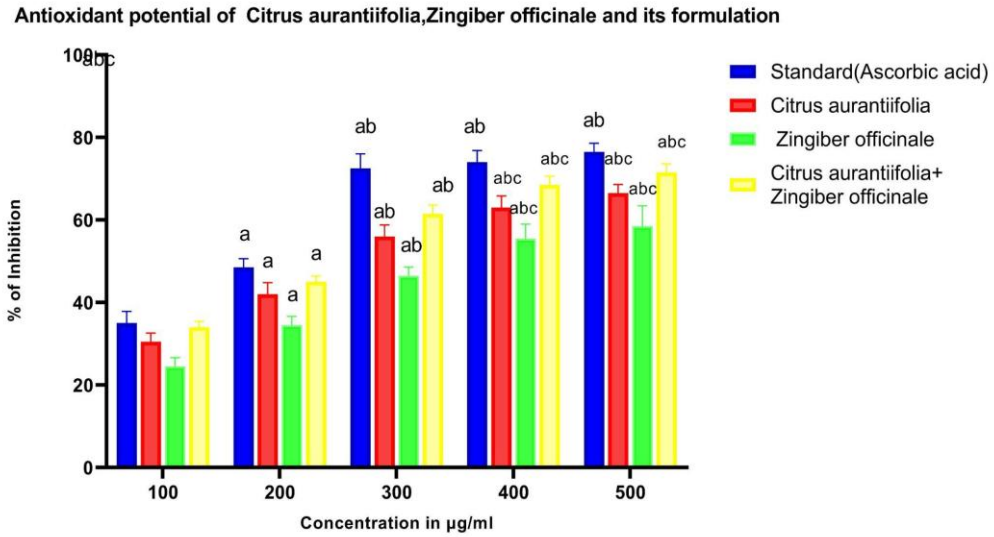
From the study, it was evident that both the plant extracts were rich in phytochemicals such as alkaloids, flavonoids, terpenoids, saponins (Table 1) etc. When compared, Citrus *aurantifolia* extract showed a stronger presence of these phytonutrients than *Zingiber officinale* extract. Phytochemicals are secondary metabolites which are present only in plants. The medicinal value of a plant extract depends on its rich source of various phytonutrients.

Antioxidant activity of aqueous extract of *Citrus aurantifolia* and *Zingiber officinale* and its formulation was determined. The methodology used for its determination was DPPH free radical scavenging assay [36]. Molecules possessing unpaired electron / free radicals leads to oxidative stress. Phenolic compounds have great deal of importance in the activity of free radical scavengers [37]. Similar findings by various research suggest a significant total antioxidant activity possessed by both the aqueous plant extracts . Haoua KB et al. [2,38] has analysed the total antioxidant activity. The methodology used for the analysis was DPPH radical scavenging activity. The effects of the antioxidants on DPPH free radical scavenging was due to their high hydrogen donating ability. The results derived from the study showed that aqueous extract of Momordica charantia , seed kernel extract of Mangifera indica and its herbal formulation exhibit significant antioxidant activity with IC_{50} of 275µg/ml, 350µg/ml, 250 µg/ml respectively as compared with the standard Vitamin C (Graph 1). Formulation was found to exhibit a significantly more antioxidant potential than the individual extracts. However further studies may be required to identity the potential health benefits of the extracts in prevention and scavenging of free radicals.

In this study, the in vitro anti cholesterol activity of *Citrus aurantifolia* and *Zingiber officinale* aqueous extracts and its formulation were duly examined. Results revealed a dose dependent increase in the inhibitory activity percentage. The extracts were identified with potent anti cholesterol activity in a dose dependent manner as compared to the standard drug used. The extracts were analysed compared to standard simvastatin for its anti-cholesterol activity. IC_{50} for anti cholesterol activity was found to be 390µg/ml, 360µg/ml, 340µg/ml respectively (Graph 2).

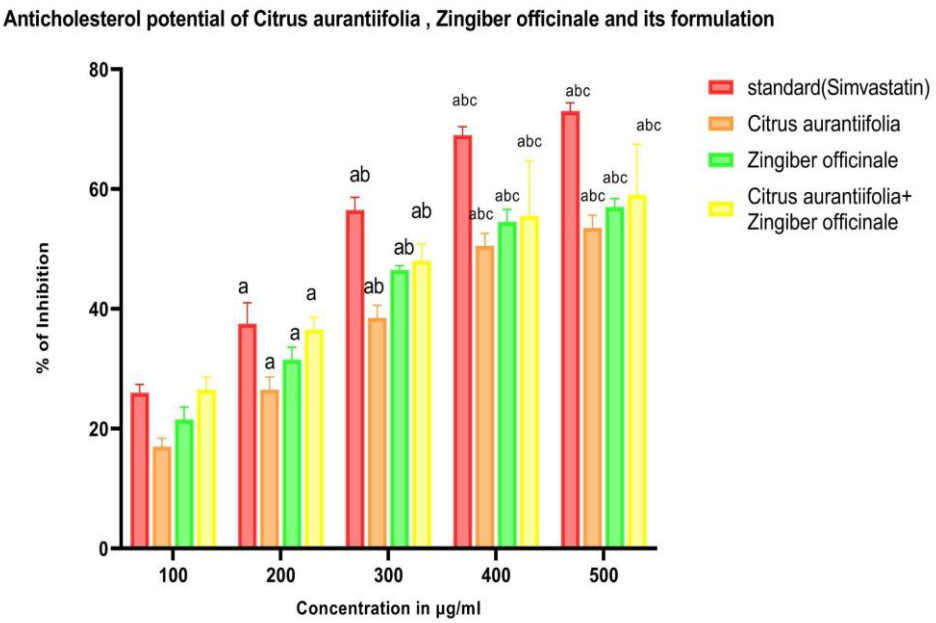
Table 1. Phytochemical Analysis of aqueous extract of *Zingiber officinale* and *Citrus aurantifolia*

S.No	Phytochemicals	Presence of *Zingiber officinale*	Presence of *Citrus aurantifolia*
1.	Ninhydrin reagent	+	-
2.	Protein (amino acid)	+	+ +
3.	Flavonoids	+	+ +
4.	Alkaloids	+	+ +
5.	Terpenoids	+	+ +
6.	Saponins	+	+
7.	Steroids	+	+ +
8.	Carbohydrate	+	-



Graph 1. Represents antioxidant potential of aqueous extract of *Citrus aurantifolia, Zingiber offcinale* and its formulation- DPPH assay against the standard Ascorbic acid. The X axis represents the concentration in the µg/ml unit. The Y axis represents the inhibitory potential of the extracts used. The blue bar represents the standard (vitamin C). The red bar represents the aqueous extract of *Citrus aurantifolia* i.e lemon. The green bar represents the aqueous extract of *Zingiber officinale* i.e ginger. The yellow bar represents the formulation of these two extracts. Each line represents a Mean of ± SEM. The mean is of 3 independent observations in the graph. The Significance was observed to be at p < 0.05

Lemons, *Citrus aurantifolia* has been proved for several health benefits such as cancer prevention, maintaining a healthy complexion and blood pressure, preventing asthma, increasing iron absorption etc [39]. For *Zingiber offcinale,* it's known for its anti arthritis, anti-informatory, anti diabetic, anti bacterial, anti fungal and anti cancer properties [40,41]. However there are not sufficient and many in depth studies conducted on the antioxidant and anti cholesterol properties of the *Citrus aurantifolia* and the *Zingiber officinale* and its formulation. This research was intended to detect the properties that natural and herbal extracts possess which could be used as an alternate source since it is more cost effective, natural and easily accessible [36]. Also since there is no cure for medical conditions which result due to oxidative stress, prevention using natural ingredients should be promoted. There are several synthetic drugs such as Atorvastatin, Fluvastatin, Lovastatin etc. Thus there are also several synthetic antioxidants such as propygallate (Ph) and butylated hydroxyanisole (BHA) [42,43]. But all these synthetic drugs taken over a longer period of time lead to various side effects and other complications.This particular research would fulfil the deficiency of having a natural source as an antioxidant and anti cholesterol agent. Since it is natural, cost effective and a familiar source that is preferred over synthetic chemicals [42].



Graph 2. Represents anti-cholesterol potential of aqueous extract of *Citrus aurantifolia, Zingiber offcinale* and its formulation against simvastatin which is the standard drug used. The X axis represents the concentration in µg/ml unit. The Y axis represents the inhibitory potential of the two extracts used. The red bar represents the standard drug that is Simvastatin. The orange bar represents the aqueous extract of *Citrus aurantifolia* i.e lemon. The green bar represents the aqueous extract of *Zingiber officinale* i.e ginger. The yellow bar represents the formulation of these two extracts used. Each line represents a Mean of ± SEM. The mean is of 3 independent observations in the graph. The Significance was observed to be at p < 0.05

From the study, it was evident that both the plant extracts and its formulation exhibited a significant antioxidant and anti cholesterol activity, but comparatively the formulation showed a better potential as to the individual extracts. Thus this study opens up a new avenue of research to study the potential of various formulations.

In countries like India, there are treasures of ayurvedic and siddha formulations based on herbal extracts. All these formulations are traditionally used and are indegenous medicines [44]. More studies have to be made to explore the synergistic role of these herbal formulations. Synergism helps in alleviating the potential of herbal activity, which cannot be done even with an external catalyst. Research on various herbal formulations can create awareness and help mankind from various disorders [44,45]. Further in vitro studies need to be carried out in the future to prove the potential health benefits involved in the generation and prevention of cholesterol, free radicals and reactive oxygen species associated disorders [44-46]. The rich phytochemical constituents of the extracts indicates the ability of the extract to act as a potential anti-cholesterol agent.

4. CONCLUSION

It is well known that lemon (*Citrus aurantifolia*), and ginger (*Zingiber offcinale*), contains numerous health benefits and is commonly used as a herbal medicine in day to day life. Even though there is sufficient knowledge among citizens about the nutritional value present in them, there isn't enough in-depth study conducted on the formulation of these two extracts based on their cholesterol inhibitory activity. From this study it was made to the understanding that the formulation showed synergism. Hence the formulation of these extracts could be preferred over other synthetic drugs since it is natural, cost effective and easily accessible.

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CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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