EVALUATION OF ANTIOXIDANT AND ANTI CHOLESTEROL POTENTIAL OF AQUEOUS EXTRACT OF *CITRUS AURANTIFOLIA ZINGIBER OFFICINALE* AND ITS FORMULATION - A COMPARATIVE *IN VITRO* STUDY

Running title:- Evaluation o*f Citrus aurantifolia, Zingiber officinale* extracts and its formulation.

ABSTRACT

Background: Several plant extracts and herbs have been used for treating and prevention of cardiovascular diseases congestive heart failure, hypertension, angina pectoris, atherosclerosis, arrhythmia, cerebral and venous insufficiency. Similarly the 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 conducted to evaluate the antioxidant and anti cholesterol potential of aqueous extract 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~~Chennai (Mention the known country?). 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 analysed by one-way analysis of variance (ANOVA) followed by Duncan's multiple range test, it was used to see the statistical significance among the groups. The results with $p<0.05$ level were considered 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 potential (IC_{50} = 250 µg/ml) and anti cholesterol potential (IC_{50}= 375µg/ml). The formulation exhibited significantly more activity than the individual extracts .

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Conclusion: Even though there is sufficient knowledge among citizens about the nutritional value present in ~~herbal formulations~~herbal formulations, there isn't enough in-depth study conducted on the formulation of these two extracts based on their anti-cholesterol activity. From this study it was evident 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.

Key words: *Citrus aurantifolia; Zingiber officinale;* Lemon; Ginger;, Antioxidant; Anti cholesterol, Innovative technology, Novel method

INTRODUCTION

Citrus aurantifolia and *Zingiber officinale* are among the world's most common fruits and stems. Citrus lemon is a species of small evergreen tree in the flowering plant family known as Rutaceae. The tree's yellow fruit is being used for culinary and non culinary purposes around the world, mainly for its juice (1). The juice of the lemon is composed of 5% to 6% citric acid. It contains a pH of around 2.2, giving it a very sour taste. It is a rich source of vitamin-C, providing 64% of the daily value in a 100g of essential nutrient amount. Lemons also contain numerous phytochemicals, including steroids, saponins, terpenoids, alkaloids, flavonoids and protein (amino acid) (2). Lemon juice contains slightly more citric acid than lime juice, nearly twice the citric acid of grapefruit juice and about 5 times the amount of citric acid found in orange juice (3). The second plant extract involved in this research is *Zingiber officinale*, ginger, it belongs to the family Zingiberaceae. The health promoting perspective of ginger is attributed to its rich phytochemical properties such as , protein (amino acid), flavonoids, alkaloids, terpenoids, saponins, steroids and carbohydrates.(4). Besides this the rhizome of ginger also is being used in traditional herbal medicine (4,5). Ginger has starting potential for treating a number of degenerative disorders, digestive disorders, cardiovascular disorders, vomiting, diabetes mellitus and cancer. Furthermore it has antimicrobial potential which helps in treating infectious disease (6).

In this study we also look upon the antioxidant properties of *Citrus aurantifolia* and *Zingiber offcinale*. Generation of free radicals or reactive oxygen species during metabolism beyond the antioxidant capacity in a biological system results in oxidative stress (7). It plays an essential role in heart diseases, neurodegenerative disease, cancer ~~etc~~. The reactive oxygen species are chemically derived from oxygen such as superoxide anion, hydrogen peroxide

and hydroxyl radicals in 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 reactive oxygen species which play a critical role in the pathogenesis of various diseases (8,9). Cholesterol is usually associated with fatty foods but most of the waxy substance present in it is produced by the body itself. At normal levels, cholesterol actually plays an important role in helping cells do the job but culture actually plays an important role in helping cells do their job. But cholesterol levels are high in the population due to unhealthy lifestyles. Low-density lipoproteins, also called "bad cholesterol," has a bad reputation from the fact that high levels of it are associated with increasing the risk of heart disease. LDL contains more cholesterol than protein hence, making it lighter in weight. When it gets oxidized, LDL promotes inflammation and forces lipids to accumulate on the walls of vessels in the heart and rest of the body, leading to formation of plaques. These plaques can thicken and limit or completely block blood and nutrients 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 always exists a good correlation between the antioxidant and anti cholesterol activities of *Citrus aurantifolia* and *Zingiber officinale* or between any other plant species (10,11). Our team has extensive knowledge and research experience that has translate into high quality publications (12),(13),(14),(15),(16),(17),(18),(19),(20),(21),(22),(23),(24),(25),(26),(27),(28),(29),(30),(31)??This study aimed to evaluate the antioxidant and anti cholesterol potential of aqueous extract of *Citrus aurantifolia, Zingiber offcinale* and its formulation through *in vitro* analysis.

MATERIALS AND METHODS :

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.

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.(32)

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Comment [u4]: What about the remaining 20% extract??

Comment [u5]: What is then the exact CONCENTRATION??

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Test for Carbohydrates

Three to five drops of Molisch reagent was added along with 1 mL of the extract. After that, 1 mL of concentrated sulphuric acid was added carefully through the side of the test tube (REFERENCES??). The mixture was then kept to stand for about two minutes. After 2 minutes, it was 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.

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 (Ref??).

Test for Alkaloids

2ml of sample was mixed with 2ml of HCl, after which 6 drops of HCN was added and further 2 drops of picric acid was added. This resulted in a creamish pale yellow precipitate indicating the presence of alkaloids.

Test for Terpenoids

2 ml of sample along with 2ml of chloroform and 3ml of con. H2SO4 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. This purple colour revealed the presence of protein.

Detection of saponins

Foam test: A fraction of the extract taken and was vigorously shaken along with some water. It was then 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. There was a formation of dark red colour or dark pink colour. This indicated the presence of steroids.

Antioxidant activity

DPPH free radical scavenging activity

Scavenging of 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) radicals was assessed by the method of (33) Hatano et al. (1989). The DPPH solution (1.0 ml) was added to 1 ml of the extract. It was added at different concentrations (0.1 to 0. 5mg/ml). The mixture was kept at room temperature for 50 minutes. After that the activity was measured at 517 nm. Ascorbic acid at the same concentrations was used as standard to carry out the step (34). The capability to scavenge the DPPH radical was calculated. It was expressed in percentage (%). The following formula was used:

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

***In vitro* anti-cholesterol activity :**

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

Anti-cholesterol assay of the extract was calculated. The following equation was used for the calculation:

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

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, the significance was considered to be at the level of $p<0.05$.

RESULTS AND DISCUSSIONS:

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 acids), Flavonoids, Alkaloids, Terpenoids and Ssteroids are less in concentration in *Zingiber officinale* extract in comparison with *Citrus aurantifolia* extract. ~~which in.~~ Saponin is present

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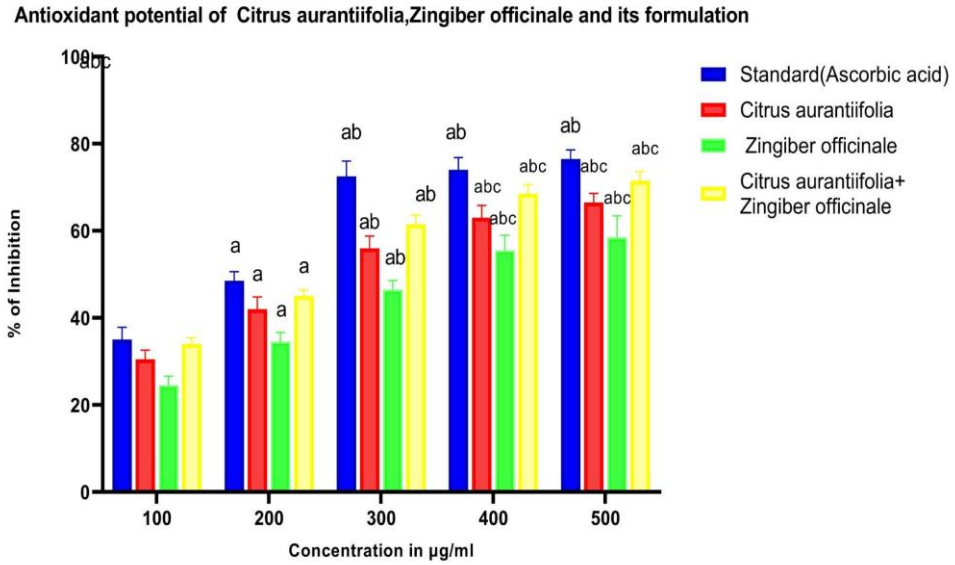
in equal concentration in the extracts of *Zingiber officinale* and *Citrus Aurantifolia*. The Carbohydrate concentration is present in the extracts of *Zingiber officinale* but is not present in the extract of *Citrus aurantifolia.* This was identified by the phytochemical analysis of aqueous extract of *Zingiber officinale* and *Citrus Aurantifolia*. **(Table-1).**

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	+	-

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

Note the meanings of each of the sign +, ++, and - ???



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

Note what a, ab, abc are stands for ? How is the significance determined with respect to these letters??

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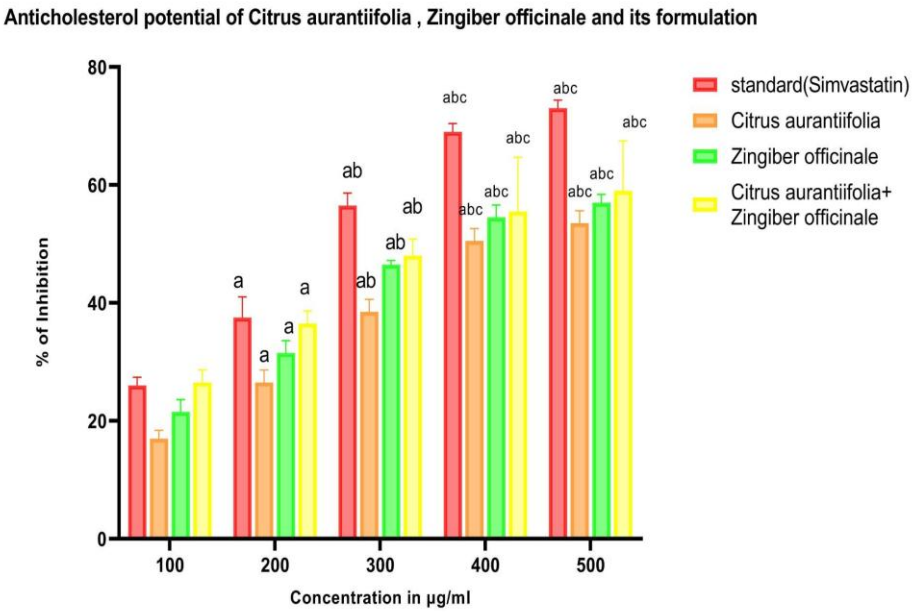

Graph-2 :- Represents anti-cholesterol potential of aqueous extract of *Citrus aurantifolia, Zingiber offcinale* and its formulation against the standard drug simvastatin..X axis represents the concentration in µg/ml and Y axis represents the inhibitory potential of the extractsThe red bar represents the standard (Simvastatin), the orange bar represents the aqueous extract of *Citrus aurantifolia*, the green bar represents the aqueous extract of *Zingiber officinale* and the yellow bar represents the formulation of these extracts. Significance at $p < 0.05$..Each line represents Mean ± SEM of 3 independent observations

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 by DPPH free radical scavenging assay (36). Free radicals / molecules possessing an unpaired electron leads to oxidative stress. Phenolic compounds

have great importance in free radical scavenging activity(37). Similar findings by various research suggest a significant total antioxidant activity possessed by both the aqueous plant extracts . Haoua KB et al , 2018, (2,38) has analysed the total antioxidant activity by DPPH radical scavenging activity. The effects of the antioxidants on DPPH free radical scavenging was considered to be due to their hydrogen donating ability. The results obtained in the study show 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.Further studies may be needed to find out the potential health benefits of the extracts in prevention and scavenging of free radicals.

In the study, in vitro anti cholesterol activity of aqueous extracts of *Citrus aurantifolia* and *Zingiber officinale* and its formulation were studied. Results revealed a dose dependent increase in % of inhibitory activity. The extracts showed potent anti cholesterol activity in a dose dependent manner as compared to the standard.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).**

Lemons, *Citrus aurantifolia* has been proved for several health benefits such as maintaining blood pressure, cancer prevention, maintaining a healthy complexion, 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 butylated hydroxyanisole (BHA) and propygallate(Ph)(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)

From the study, it was evident that both the extracts and its formulation exhibited a significant antioxidant and anti cholesterol activity, but when compared the formulation showed a better potential compared 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 may be needed to prove the potential health benefits in the prevention and generation of free radicals, reactive oxygen species and cholesterol 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.

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 anti-cholesterol activity. From this study it was evident 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.

NOTE:

The study highlights the efficacy of "HERBAL" which is an ancient tradition, used in some parts of India. This ancient concept should be carefully evaluated in the light of modern medical science and can be utilized partially if found suitable.

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