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Comparative Study on Phytochemical Extraction, Antibacterial and Synergistic Activity of *Zingiber rubens* with Tetracyclin on *Homo sapiens* and *Gallus domesticus* Originated Enteropathogenic Bacteria

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Authors' contributions

This work compiled by the contribution of all authors. All authors collected data from different sources. Author VSB collected the sample, this sample processed by author NB. Authors VSB and SK designed the protocol and all contributed in practical work. Static work compiled by authors VSB and SK. All authors read and approved the final manuscript.

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Original Research Article

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ABSTRACT

Introduction: Nature is a rich source of medicinal plants, these medicinal plants used as a therapeutic agent from thousands years. At present medicinal properties of many plants compiled and these plants are source of many drugs, but properties of some medicinal plants is still unknown. The main object of this study is determination of phytochemical of *Zingiber rubens,* and biological activity of these active compounds against enteropathogenic microbes for determination of

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antibacterial activity and synergetic activity between plant extract and Tetracycline.
Methods: Firstly tubers of *Zingiber rubens*are shade dried and then extraction completed from powder form of plant material with different kinds of solvents (water, ethanol, petroleum ether and chloroform). After extraction their phytochemical constituents resolute through biochemical analysis and their antibacterial and synergetic activity examined through disc diffusion method on different strains of human and cock isolated enteropathogenic bacterial species.
Results: In our study we observed that organic solvent extract comprise more active compounds than other extract and the antibacterial activity of these organic extract is also higher than aqueous extract. In aqueous extraction between the temperatures at 30-50 ℃ maximum numbers of phytochemicals are screened. Chloroform extract shows maximum inhibitory activity along and with Tetracycline against *E. coli* (12 mm/25 mm), cold water extract show minimum inhibitory activity along and with Tetracycline against *S. aureus* (6 mm/13.5 mm). Antibacterial activity expressed in terms of zone of inhibition.
Conclusion: from the results we concluded that extraction depends on nature of solvent and extraction temperature. Temperature between 30-50 ℃ appropriate for organic and aqueous extraction. At this temperature maximum number of photochemical screened in warm water and all organic solvent extract. *Zingiber rubens* contained both antibacterial and synergetic activities. Antibacterial activity of Tetracycline increases, due to the synergetic activity between Tetracyclin and *Zingiber rubens* phytochemicals.

Keywords: Zingiber rubens; synergistic activity; antibacterial activity; phytochemical analysis; shoxhlet apparatus.

1. INTRODUCTION

Usage of medicinal plants for curing diseases documented in history of all civilizations. The interest of medicinal plants has been shown all over the world due to their safe and effective principle [1]. India is rich equally in fauna and flora. The Himalayan and sub Himalayan region of India cover many types of medicinal plants. These plants contain enormous medicinal compounds with a lot of medicinal activity. Due to their harmless effect they are used from past for curing many diseases and health problems. Activity of many plants is studied and some are not studied.

For antimicrobial and synergetic study, we select *Zingiber rubens* (common name-forest zinger) belongs to family Zingiberaceae. This plant normally seen in winter time at higher altitude of sub Himalayan region and used as a medicine by many people's from past. Many older literatures, explain information related to medicinal use of this plant. As a medicine, roots, seeds and flower part are used but there is no scientific documentation with proof behind this plant, so we pick out this plant for study to determine the active components of this plant, antibacterial activity for isolated enteropathogenic bacteria and their synergetic activity with Tetracycline against isolated enteropathogenic bacteria.

2. MATERIALS AND METHODS

2.1 Collection and Preparation of Plant Extract

Zingiber rubens (tubers) collected from the forest of IBT Patwadanger Nainital in winter session. Tubers are dried after proper washing and then powdered. Extraction accomplished from this powdered form using distilled water and organic solvents (ethanol, methanol, petroleum ether and chloroform) in Shoxhlet apparatus. Aqueous extraction has done at different temperature and organic solvent extraction done at a fixed temperature below to their boiling point due to lower boiling point.

2.1.1 Aqueous extraction

Maceration method used for aqueous extraction, 50 g powdered form soaked in 150 ml sterile distilled water at different temperature [2]. On the basis of different temperature gradient aqueous extraction divided in following types.

After extraction, this mixture filtered using Whatman filter paper and filtrate is concentrated by vacuum drier. Dried concentrate extract dissolved in DMSO and stored in dark bottle at 4°C.

2.1.2 Organic solvent extraction

50 g plant powder sample soaked in 150 ml ethanol, methanol, chloroform, petroleum ether separately in a Shoxhlet apparatus and then Shoxhlet is heated for 8 hours at 40-50℃. After extraction filtrate concentrated in vacuum drier [3]. After concentration concentrated form is dissolved in DMSO and stored in dark bottle at 4°C.

2.1.3 Test Microbial culture

Test microbes isolated from human (*Homo sapiens*) and cock (*Gallus domesticus*) for confirmation of these isolated microbes, biochemical tests are used (Table 4).

2.1.4 Sample collection

Sample collected aseptically through sterile cotton swab from human nose, mouth and cock mouth. Then these swabs are kept in sterile phosphate buffer and when buffer become turbid, then a loop full buffer added in nutrient agar broth and incubated at 37°C.

2.1.5 Isolation and determination of bacteria

Identification of microbes was done using standard biochemical manuals (Table 4) [4,5].

3. PRELIMINARY PHYTOCHEMICAL ANALYSIS

Preliminary phytochemical analysis of the different extracts carried out by qualitative determination of major phytoconsituents shown in Table 2 [6].

Table 1. Different types aqueous extraction

S no.	Extraction type	Temperature	Duration
1	Cold water extraction	15℃	For 30 min
2	Warm water extraction	40℃	For 30 min
3	Hot water extraction	70℃	For 30 min
4	Boiling water extraction	100℃	For 30 min

Table 2. Specific qualitative tests for phytochemicals

Experiment name	Procedure for test
Benedict's test (for reducing sugar)	Mix equal volumes of benedict's reagent and test solution in test tube. Heat for 5 minutes in boiling water bath. Solution appears green, yellow, and red depending on amount of reducing sugar present.
Iodine test (for non-reducing sugar)	To 3 ml test solution add few drops of dilute iodine solution. Blue colour appears. It disappears on boiling and reappears on cooling.
Biuret test (for protein)	To 3 ml test Solution add 4% NaOH and few drops of 1% $CuSO_4$ solution. Violet or pink colour appears.
Ninhydrin test (for amino acid)	Heat 3 ml Test solution and 3 drops 5% Ninhydrin solution in boiling water bath for 10 min. Purple or bluish colour appears.
Salkowski Reaction (for steroid)	To 2 ml of extract add 2 ml chloroform and 2 ml conc. H_2SO_4. Shake well chloroform layer appears red and acid layer shows greenish yellow florescence.
Borntrager's test (for anthraquinoneglycoides)	To 3 ml extract add dil. H_2SO_4, boil and filter. To cold filtrate add equal volume of benzene or chloroform. Shake well, separate the organic solvent. Add ammonia. Ammoniacal layer turns pink or red.
Wagner's test (for alkaloid)	To 5mg plant extract added dilute HCl, and then filtered. To the filtrate added few drops of wagner's reagent. Red/brown precipitate forms
Erdmann's test (for alkaloid)	To 1ml plant extract added few drops of Erdmann's reagent. Red or violet colour appears.
Keller kiliani test (forCardiac glycosides)	To 2 ml of extract add 1 ml of acetic acid and 3 drop of $FeCl_3$ then add 1 ml H_2SO_4. Pale green colour appears on the surface.
Foam test (for saponins)	To 1 ml extract add 2 ml distilled water shake it. Persistent foam forms.
Shinoda test (for Flavonoids)	To 1 ml extract few drops of conc. HCl and few mg of magnesium Ribbon. Pink colour observed.
Acetic acid solution (for Tannins and phenolic compounds)	To 1 ml test solutions add 1 ml acetic acid drop by drop. Red colour solution observed.

4. DETERMINATION OF ANTIBACTERIAL ACTIVITY OF DIFFERENT PLANT EXTRACT

Anti bacterial activity determined by disc diffusion method according to Bauer et al. [7]. 1-2 $\times 10^{8}$ cfu /ml bacterial culture of *S. aureus, E. coli P. aeruginosa* spreaded on the Mueller Hinton agar plates and incubated for 3 hours at 37°C. Tetracycline used as a standard drug for antibacterial study.

4.1 Turbidity Standard for Inoculum Preparation

To standardize inoculum density for a susceptibility test, 0.5 McFarland standard prepared as described by NCCLS, 1997[8]. One percent v/v sulphuric acid and 1.175% w/v barium chloride prepared in distilled water. Then 0.5 ml barium chloride added to 1% sulphuric acid (99.5 ml) and mixed well and stored in the dark at room temperature.

4.2 Synergetic Effect of Different Plant Extract with an Antibiotic (Tetracycline)

Synergetic activity determined according to *Muroi H*, 1996 by disc diffusion method [9], after few modifications. Antibiotic mixed with plant extract and paper disc dipped on this mixture then these discs applied on bacterial culture plates. Rise in antibacterial activity calculated and percent increase in antibacterial activity calculated as follows:

4.3 Percentage Increase in Antibacterial Activity by Synergetic Effect= inhibition by Combination – Inhibition by Extract/ Inhibition by Combination× 100

4.3.1 Disc preparation

Sterile paper discs (6 mm diameter) dipped in various extracts and slightly dried at 37℃. Then discs are placed on bacterial culture plates which are incubated at 37℃. Plates are incubated in incubator at 37℃ for 24 hour After 24 hour zone of inhibition observed and measurement should be done in mm.

For antibacterial activity triplicate of a sample used. Antibiotic disc (Tetracycline-10 µg/ disc) used as a standard and sterile disc dipped in only PBS (phosphate buffer saline), used as a blank.

4.4 Statistical Analysis

Plant extract antibacterial activity compared with tetracycline, following student t- test used followed by F- test for determination of significance of difference. Data is significant when $p < 0.05$.

5. RESULTS AND DISCUSSION

5.1 Preliminary Phytochemical Analysis

Phytochemical analysis screened by specific biochemical testing for specific phytochemical group. Different extract contain different types of phytochemicals, which are shown in table no 3. Active compounds which are detected in different extracts, shows antimicrobial, antioxidant, analgesic, antiproliferative and many other biological activities. Organic solvent extract contain more active compounds than aqueous extract. This shows that organic solvent (mostly polar) are suitable for extraction. Most of the secondary metabolite components were isolated and identified in the polar plant crude extracts [10].

Some changes comes at the time of drying of plant material, in some older study researcher found that fresh plant extract contain more bioactive compounds and more antibacterial and antioxidant activity [9]. Maximum bioactive compounds derived at 30-50℃.

5.2 Isolated Enteropathogenic Bacterial Determination

Gram staining and standard biochemical testing protocols used for determination of isolated bacterial culture, (Table 4). [4,5] *E. coli, S. aureus, P. aeruginosa* are isolated from human and cock swab samples.

5.3 Antibacterial Activity of Different Extracts

Different extract shows inhibitory action against bacterial growth. A zone of inhibition formed around the disc due to the inhibitory action of plant extract and Tetracycline (Fig. 1). Chloroform extract show maximum zone of inhibition for *E. coli* (12.5 mm), *P. aerugiosa* (12 mm) and *S. aureus* (12 mm), Tetracycline (22 mm).

Table 3. Phytochemical analysis of dry plant sample

S. NO.		Cold water extract	Warm water extract	Hot water extract	Boiling water extract	Organic solvent			
						Ethanol	Methanol	Petroleum ether	Chloroform
1.	Reducing Sugar	+	+	-	+	+	+	+	+
2.	Non-reducing sugar	-	-	+	-	-	-	-	-
3.	Non-reducing sugar Polysaccharides(Starch)	-	+	+	-	+	+	+	+
4.	Proteins	-	-	-	-	-	-	-	-
5.	Amino-acids	+	+	-	-	+	+	+	+
6.	Steroids	-	-	-	-	-	-	-	-
7.	Cardiac glycosides	+	+	++	++	+	+	+	+
8.	Anthraquinone glycosides	-	-	-	-	-	-	-	-
9.	Saponins	++	+++	+++	-	+	+	+	+
10.	Tannins&phenolic Compounds	-	-	-	-	-	-	-	-
11.	Flavonoids	-	-	-	-	-	-	-	-
12.	Alkaloids	-	-	-	-	-	-	-	-

(-) indicates absence, (+) indicates presence at good concentration, (++) indicates presence at high concentration.

Table 4. Specific test for isolated enteropathogenic bacteria

S.NO.	Test	*E. coli*	*S. aureus*	*P. aeruginosa*
1	Colonies on agar media	shiny, mucoid colonies which have entire margins and are slightly raised	Circular, pinhead colonies which are convex with entire margins.	Large, flat, slightly greenish colony
2	Microscopic study by gram staining	Gram negative, rod shaped	Gram positive, Cocci shaped,	Rod shaped gram negative
3	Growth on Mac-conkey agar media	Sugar fermentation	Sugar fermentation	No Sugar fermentation
4	Eosin Methylene Blue Agar (EMB Agar)	Growth	No growth	Growth
5	Catalase	+	+	+
6	Methyl red	+	+	-

Table 5. Antibacterial activity of plant extracts along and with antibiotic in terms of zone of inhibition

S. No.	Bacteria	Aqueous extracts (15 mg)				Organic solvent extracts (15 mg)				
		S-1 (mm)	S-2 (mm)	S-3 (mm)	S-4 (mm)	E (mm)	M (mm)	P.E. (mm)	Chl. (mm)	Tetracycline (mm)
		Zone of inhibition of plant extract								
1.	*E. coli*	7#	7#	10#	7#	7#	9#	7#	12.5*#	12
2.	*S. aureus*	6#	6.5#	9#	7#	7#	9#	8#	12*#	12.5
3.	*P. aeruginosa*	8#	8#	9#	8#	8#	9#	8#	12*#	14
Synergetic effect of Tetracycline and plant extract(5microgram Tetracycline+10 mg plant extract)										
3.	*E. coli*	15*#	14*#	17*#	16#	15#	16#	15.5#	25#	
4.	*S. aureus*	13.5*#	16*#	15*#	15.5#	17#	16#	15#	22#	
5.	*P. aeruginosa*	15*#	14*#	16.5*#	16#	16.5#	15#	16#	17.5#	

S-1 cold water extract, S-2 warm water extract, S-3 hot water extract ,S-4 boiling water extract, (E) ethanolic extract,(M) methanolic extract (P.E.) petroleum ether extract, (Chl.) chloroform extract, 15 mg extract used for antibacterial activity and 10 μg Tetracyclin disc used as a standard. In synergetic activity 5 μg Tetracyclin with 10 mg plant extract used.

#significant variance in F- test

**significant in t-test*

Table 6. Percent increase in antibacterial activity of different extract after synergetic effect

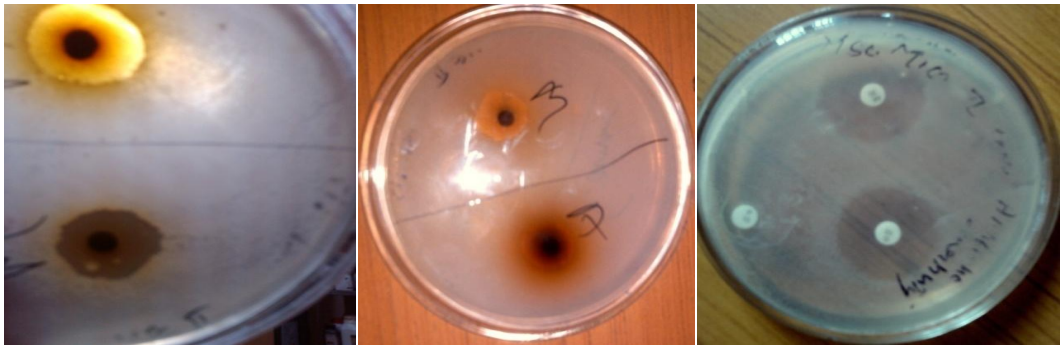
S. No.	Bacteria	Aqueous extracts (15 mg)				Organic solvent extracts (15 mg/140 ml)			
		S-1 (mm)	S-2 (mm)	S-3 (mm)	S-4 (mm)	E (mm)	M (mm)	P.E. (mm)	Chl. (mm)
		Zone of inhibition of plant extract							
1.	*E.coli*	55.55	57.57	40.00	54.83	58.82	43.75	46.66	50.00
2.	*S.aureus*	55.55	57.57	40.00	54.83	58.82	43.75	46.66	45.45
3.	*P.aeruginosa*	46.66	42.85	47.87	50.00	51.51	40.00	50.00	31.42

Organic solvent extract shows maximum antibacterial activity, shown in Table 5. These bacterial species are enteropathogenic and cause illness in humans and animals (*E. coli* most common bacterium of which is virulent strains cause gastroenteritis, urinary tract infections and *Pseudomonas aeruginosa* which infects the pulmonary tract, urinary tract, burns and wounds). Cold water extract show minimum inhibitory action against *S. aureus* (6 mm) in compare to other solvent.

Stastical analysis showed on Table 5.

5.4 Synergetic Antibacterial Activity of Plant Extract with Tetracycline

Increase in antimicrobial activity and zone of inhibition observed in combination of plant extract and Tetracycline. This increase in inhibition occurs due to the synergetic activity of plant extract and Tetracycline. These results illustrated that when antimicrobial agent is applied with plant extract, their antimicrobial activity increases in compare to alone effect of antimicrobial agent. Chloroform extract show maximum synergetic activity against *E. coli.* (25 mm), and cold water extract show minimum



(a) (b) (c)

Fig. 1. Antibacterial activity on bacterial plates by plant extract (a & b) and tetracyclin (c)

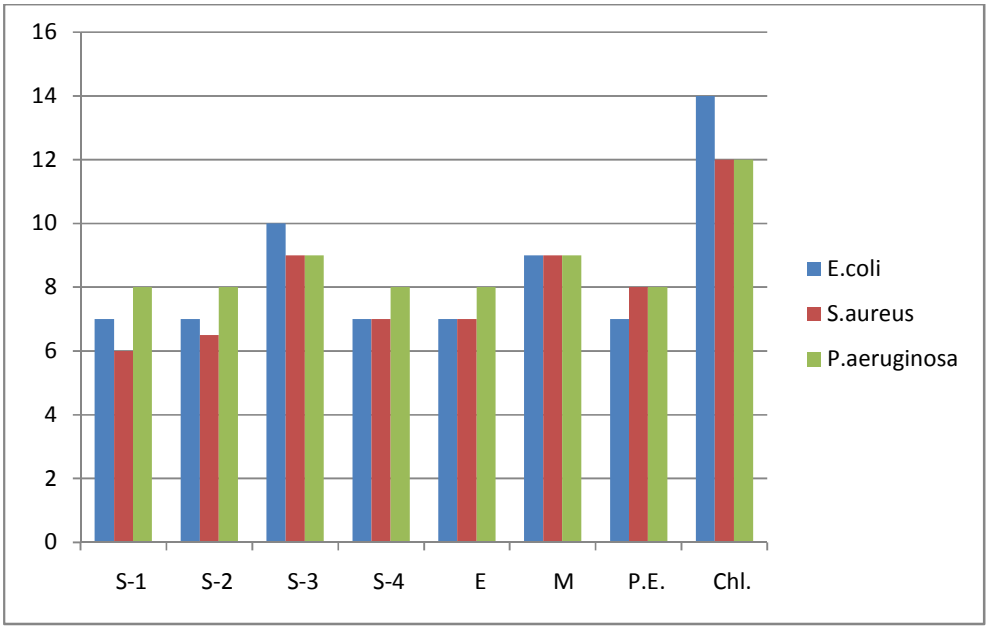


Fig. 2. Antibacterial activity of different plant extract for different enteropathogenic bacteria

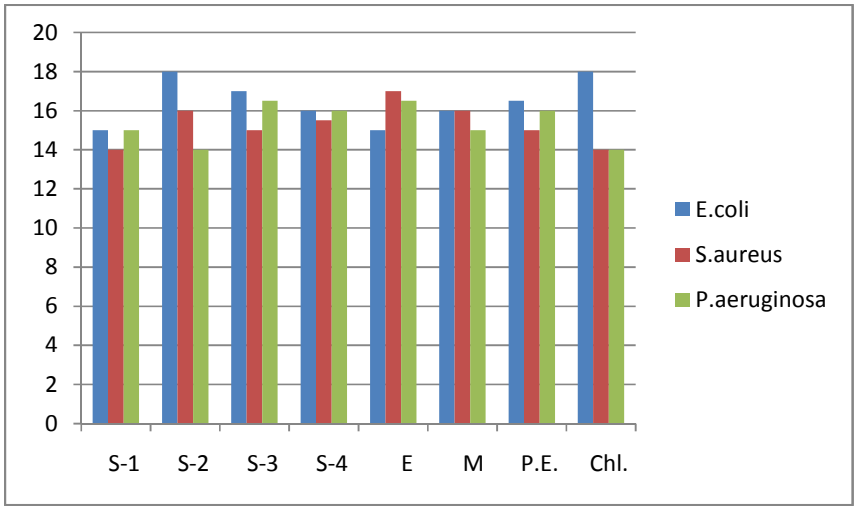


Fig. 3. Synergetic effect of different plant extract with antibiotic (Tetracycline)

synergetic activity (13.5 mm) against *S. aureus* in Table 5. Maximum 55.55% increase in antibacterial activity observed for cold water extract against *E. coli* and *S. aureus* (Table 6).

Many older studies also show synergetic activity of plant extract and antimicrobial agents for different normal and drug resistant bacterial strains [10-14]. Medicinal plants contain antimicrobial activity after this they are not used for clinical use as antibiotics due to the less activity [14]. Different extract contain different types of active compounds, these active compounds interact with antimicrobial agent and show synergetic antibacterial activity, this type of interaction is supported by many researchers [12].

Few studies have found that the efficiency of antimicrobial agents can be improved by combining them with crude plant extracts against different pathogens including *S.aureus, P. aeruginosa, E. coli,* β-lactamases producing multidrug resistant *E. coli* vancomycin-resistant enterococci (*Enterococus fecalis*) [16-19,11,20,13,16,21,22,12,23].

6. CONCLUSION

From this study we concluded that diffrent extract of *Zingiber rubens* conatin diffrent types of phytochemicals. Extraction of these phytochemicals depends on the solvent nature and extraction temperature. Organic solvent is better than the water for extraction and 30-40°C temperature is suitable for extraction. *Zingiber rubens* has antebacterial activity against diffrent enteropathogenic bacteria. Plant extract show synergetic effect with tetracyclin and antibacterial activity of this interaction is higher than their along effects.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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