In vitro synergistic antibacterial activity of Cassia Auriculata and Allium sativum

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Abstract:

The present study carried out to evaluate the In vitro Antibacterial activity of crude extract of locally available plants like Cassia Auriculata and Allium sativum. The extract was subjected to well diffusion technique with different concentrations. Both the drugs may show antibacterial activity against Escherichia coli and Staphylococcus aureus. According to the Checkerboard analysis formula Cassia Auriculata and Allium sativum may show synergistic antibacterial activity.

1. Introduction:

Herbal medicine is still used from ancient years because of better compatibility with the human body and having fewer side effects. However from the last few years there have increased use in developed countries also. Medicinal herbs are easily available, non toxic, easily affordable and play major role in research and development. The Rig-Veda (3700B.C.) mentioned the use of medicinal plant. Indian traditional medicines like Ayurveda, siddha, Homeopathy and Unani utilized healing potential of many plants.

The 20th century, even now bacterial resistance to antibiotics was recognized shortly after their introduction to clinical use, the spectrum of these is miracle drugs that led some to conclude that the war on infectious diseases had been won. The rapid onset of bacterial resistance was recognized early on as a biological phenomenon. However it was prevented from becoming a medical problem by the regular introduction of newer and more efficacious agent.

Antimicrobial activity: Ability to destroys bacteria or suppresses their growth or their ability to reproduce.

Antibacterial agents: Those agents destroy or suppress their growth of microorganism.

Antibiotics: A medicine (such as penicillin or its derivatives) that inhibits the growth of or destroys microorganism.

Cassia Auriculata consists of fresh leaves, flower, seed of plant Cassia Auriculata commonly called as Tanners senna, Avaram tree. Cassia auriculata is known as Tanner's Cassia belonging to family Caesalpiniaceae. Leaves are alternate, stipulate, paripinnate compound, very numerous, closely placed, 8.8-cm long, narrowly furrowed, slender, pubescent, with an erect linear gland between the leaflets of each pair, leaflets 16-24,very shortly stocked 2-2.5 cm long1-1.3 cm broad. Flowers are irregular, bisexual, bright yellow and large (nearly 5 cm across), the pedicles glabrous and 2.5 cm long. Fruits are a short legume, 7.5-11 cm long,1.5 cm broad, oblong, obtus, tipped with long style base, flat, thin, papery, undulately crimpled, pilose, pale brown.12-20 seeds per frit are carried each in its separate cavity. It consists of nonucosane and

nonacosan-6-one, chrysophanol, emodin, rubiadin, β sitosterol, polysaccharides, flavonoids, antracene derivatives and has some diametric procyanidins, saponins and tannins. Cassia Auriculata may used in diabetes, pink eye, liver disease, urinary track diseases, skin health, male infertility, as an aphrodisiac.

Allium sativum, also known as clove Garlic is part of the lilliaceae plant family. Garlic is a member of the onion family and is one of the nature's most versatile medicinal plants. Allium Sativum is a perennial flowering plant growing from a bulb. It has a tall, erect flowering stem that grow up to 1 m (3 ft). The leaf blade is flat, linear, solid, and approximately 1.25-2.5 cm (0.5-1.0 in) wide, with an acute apex. Alliin is an odorless sulfur containing chemical derived from the amino acid cysteine. When garlic bulbs are crushed, Alliin is converted into another compound called Allicin. Allicin is further broken down to a compound called Ajoene, which may be the substance that inhibits blockage in vessels from clots and atherosclerosis. Germanium, magnesium, selenium, vitamin A, vit c, volatile oil, sulphur containing compound, zinc etc. It also contains 65% water, 28% carbohydrate, 2.3 % organosulphur compound, 2% protein. 1.2% free amino acid (mainly arginine), 1.5% fiber, 0.15 % lipid, 0.08% phytic acid, 0.07 % saponins. Allium Sativum used in atherosclerosis, heart health, diabetes, hyperlipidemia, cancer (Prostate, Stomach, Breast, Colon, Rectal, Esophagus, Lung, Blood), GIT infections.

2. Preliminary phytochemical analysis:

Qualitative phytochemical screening tests were carried out based on the method used by Guruprasad C. Nille, K. R. Reddy (2015) to determine the presence or absence of alkaloids,tannins,flavonoids,saponines,phenols,terpinoids,steroids,glycosides and antraquinones in methanol extract of C.auriculata. The C.auriculata flower extract were analyzed by the following procedures for the presence of steroid, terpenoids, phenols, tannins, flavonoids, phytosterol, Quinones etc. The flower part of Cassia auriculata is reported to have significant amount of flavonoids (rutin, vicenin, and auricuoflavonoids) and phenols which are present in plant that showing antibacterial activity. Antibacterial activity of garlic is widely attributed to allicin.

I. Detection of steroids:

The extract were treated with 5 ml of chloroform and equal volume of con sulphuric acid were added along the side of test tubes, yellowish green color formation indicates the presence of steroid.

II. Detection of phenols:

The extracts were treated with three to four drops of Ferric chloride solution. The bluish black color indicates the presence of phenol.

III. **Detection of tannins**:

The extracts were treated with equal volume of Ferric chloride solution. Brownish green color formation indicated presence of tannins.

IV. Detection of flavonids:

To the extracts few drops of 20%Sodium Hydroxide solution were added, which results in yellow color formation that disappear while adding dilute acid indicate the presence of flavonoid.

V. Detection of terpenoids:

The extract were treated with chloroform, filtered and treated with few drops of concentrated sulphuric acid, shaken and allowed to stand. Change of reddish brown color revealed the presence of terpenoids.

VI. Detection of phytosterol:

The extract were treated with chloroform and then filtered. A few drops of concentrated sulphuric acid were added, shaken and kept. The golden yellow color formation indicates the presence of triterpine.

VII. **Detection of Quinones**:

1 ml of extract was mixed with 1 ml of 2% NaOH. The formation of blue green or red color indicates presence of quinones.

3. Materials and Methods:

3.1 Collection of plant material:

Cassia auriculata flowers were collected from the local area of Kolhar, during Jan-Feb2021. Allium sativum bulbs were collected from local market of Kolhar. The both plant material was botanically identified and confirmed from the expert of department of botany P.V.P. Arts Commerce and science college Loni. The flowers of Cassia auriculata were examined carefully. The flower of Cassia auriculata were cleaned from extraneous matter and old, infected, and damaged flowers of were removed. Extract were prepared from dried flowers. Healthy flowers were spread out and dried at room temperature for about ten days and ground into fine powder using electric blender.

3.2 Preparation of plant extract:

Cassia auriculata: Cassia auriculata flowers were dried in oven below 60ºc for 2 hrs. They were finely powdered and extracted with 80% methanol using Soxhlet apparatus at 55ºc. The soluble part was concentrated over water bath maintained below 60ºc and dried in a vacuum oven to obtain free flowing reddish brown powder. The extract obtained was termed as methanolic extract of cassia auriculata.

Allium sativum: The bulb of garlic 50gm of Garlic bulbs were crushed and sock in 100 ml of 95% ethanol in a sterile sample bottle and kept at room temperature for 4 days. The socked garlic was then filtered using filter paper (Whatman no.1).Different concentration of garlic extract was made by diluting the crude extract with ethanol

3.3 Test micro organism:

Escherichia coli and Staphylococcus aureus were selected for the present experiment from microbiology lab of padmashri vithalrao vikhe patil medical college, Viladghat.

3.4 Preparation of inoculums:

Active cultures for experiment were prepared by transferring loopful of cells from stock cultures to test tubes of nutrient broth and the tubes were incubated at 37ͦc for 24 hrs that are used as inoculums.

3.5 Antimicrobial screening:

Qualitative assay of antibacterial activity of methanol extract of C.auriculata and A. sativum and combination of both extract was performed by standard methodology by well diffusion technique (Saeed et al., 2005).The Mueller Hinton Agar plates were seeded with 0.1 ml of standardized inoculums of bacteria. The inoculums were spread evenly over the plate with sterile glass spreader. The seeded plates were allowed to dry in the incubator at 37ºc for 20 minutes. A standard crack border of 6 mm diameter was seed to cut uniform wells on the surface of plates and 0.1 ml of each concentration was introduced in the well. The inoculated plates were incubated at 37ºc for 24 hrs and zone of inhibition diameter was measured to using nearest millimeter (mm).

3.6 Minimum Inhibitory Concentration:

Minimum inhibitory concentration (MIC) of each plant extracts and antibiotics alone was determined by the micro-dilution method according to the NCCLS Serial double dilutions of the tested plant extracts, as well as the antibiotics, were prepared in Mueller-Hinton broth and transferred into a 96-well microtiter plate over the final concentration range of 0.125–128.0 µg/ml and 0.78–300 mg/ml for antibiotics and plant extracts respectively. Ten µl of working inoculum suspension was added to the wells. The final volume was 100 µl and the final bacterial concentration was 5×10^5 CFU/ml in each well. Two controls were included, a medium with no inoculum for control of sterility, a medium with no plant extracts or antibiotics for control of inoculum viability. The plates were then incubated for 24 hr at 37 °C. After incubation, ten µl of an aqueous 0.5% triphenyl tetrazoliumchloride TTC) solution (Sigma-Aldrich) was added to each well and further incubated for 1 hr. The plates were then examined to determine a color change. Viable microorganisms interact with the TTC solution to cause a color change from no color to red. MIC was defined as the lowest concentration showing no color change which exhibited complete inhibition of growth .The tests were performed in triplicate.

3.7 Synergistic antibacterial effect:

Synergy measurement by Checkerboard analysis is used to determine the impact on potency of the combination of antibiotics in comparison to their individual activities. This comparison is then represented as the fractional Inhibitory Concentration (FIC) index value. The FIC index value takes into account the combination of antibiotics that produces the greatest change from the individual antibiotics MIC.

The checkerboard broth micro dilution method was used for the determination of synergy between the antibiotics and plant extracts. Two fold serial dilutions of the antibiotic and two fold serial dilutions of the plant extracts were prepared for every combination tested and 50 µl aliquots of each component was placed into the wells of the sterile 96-well microtiter plate. The using inoculums concentration was as in MIC determination method. After incubation of microtiter plates at 37 °C for 24 hr, 10 µl of an aqueous 0.5%

TTC solution was added to each well and further incubated for 1 hr. MIC was defined as the lowest concentration showing no color change which exhibited complete inhibition of growth.

The checkerboard method is often combined with calculation of fractional inhibitory concentration (FIC) index (FICI). The FIC was derived from the lowest concentration of antibiotic and plant extracts combination showing no color change of TTC. FIC value for each agent was calculated using the formula:

FICI = ΣFIC = FIC (antibiotic) + FIC (plant extract)

Where: FIC (antibiotic) = MIC of antibiotic in combination/ MIC of antibiotic alone

FIC (extract) = MIC of extract in combination/ MIC of extract alone

The interactions were classified as being synergistic for ΣFIC values of ≤0.5, additive (≥ 0.5–1.0), indifferent (≥1.0–≤4.0) or antagonistic (ΣFIC > 4.0)

To quantify the interactions between the antibiotics being tested (the FIC index), the following equations is used-

$$\frac{A}{MIC_A} + \frac{B}{MIC_B} = FIC_A + FIC_B = \text{FIC Index}$$

Where A and B are the MIC of each drug in combination, and MIC $_a$ and MIC $_B$ are the MIC of each drugs individually. The FIC index value is then used to categorize the interaction of the two drugs tested.

Table 1. FIC value by Checkerboard analysis method

	FIC value
Synergy	<0.5
Antagonism	>4
Additive or indifference	0.5-4

Synergy: When the combination of compounds result in an FIC value of <0.5, then the combination of compounds increases the inhibitory activity (decrease in MIC) of one or both compounds than the compounds alone.

Additive or indifference: When the combination of compounds result in an FIC value of 0.5-4, then the combination of compounds has no increases the inhibitory activity or slight increase in the inhibitory activity from the additive effect both compounds combined.

Antagonism: When the combination of compounds result in an FIC value of >4, then the combination of compounds increases the MIC, or lowers the activity of compounds.

3. Results and discussion:

3.1 Ethno botanical data of plant:

In present study checked synergistic antibacterial activity of herbal extracts like Cassia auriculata belonging to family Caesalpiniaceae and Allium Sativum belonging to family Lilliceae. Ethno botanical data was shown in table -2.

Table.2. Ethno botanical data for C.auriculata flower and A. sativum bulb

Botanical name	Family	English name	Local name	Part of plant used	Area of collection
Cassia auriculata	Caesalpiniaceae	Tanners cassia	Aavarai	Flower	Local area of Kolhar
Allium Sativum	Lilliceae	Garlic	Lahsuna	Bulb	Local market of Kolhar

3.2 Preliminary phytochemical present in the extract:

MECA has shown presence of Carbohydrate, Steroids, Terpenoids, Flavonoids, Phenol, and Amino acid while EEAS has shown presence of Anthraquinone, Saponins, Tannins, and Alkaloids etc as reported in table no-3

Table.3. Phytochemical data for C.auriculata flower and A. sativum bulb

Test	Ethanol extract of Allium Sativum	Methanol extract of Cassia auriculata
Carbohydrate	-	+
Anthraquinone	+	-
Saponins	+	-
Steroids	-	+
Terpenoids	-	+
Flavonoids	-	+
Tannins	+	-
Alkaloids	+	-
Phenol	-	+
Protein	-	+
Amino acid	-	+

3.3 Invitro antibacterial activity of extracts:

MECA was found to have maximum activity against all organisms. The present investigation confirmed the antimicrobial activity of flower of cassia auriculata and allium sativum bulb against S aureus and E. coli. Leaf extract of CA exhibited significant broad spectrum activity against S aureus Antibacterial activity of CA

flower has been reported previously. The extract of CA was found to have potent microbicidal activity against E coli in poultry. In this study methanolic extract of flowers was found to have higher inhibitory activity against S aureus and E. coli. Invitro antibacterial activity of methanol extract of flower of Cassia auriculata was shown in table 4-

Table 4.Invitro antibacterial activity of methanol extract of flower of Cassia auriculata

(Values are mean of three replicate)

Concentration	Zone of inhibition (mm in diameter)	
	Methanol extract of CA(2mg)	Tetracycline (30µg)
S aureus	20	24
E. Coli	18	22

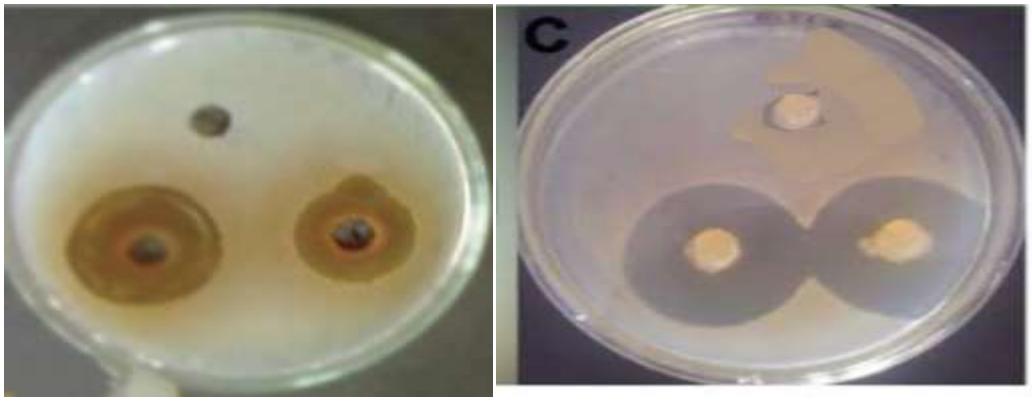

Fig 1. Antibacterial activity of CA extract in E coli and S aureus

The result obtained by study showed that garlic possessed anti –bacterial effect against gram positive (S aureus) and gram negative (E. coli) bacteria just as it was described by Louis Pasteur that onion and garlic juices posses antibacterial activities against both gram positive and gram negative bacteria (Whitemore and Naidu, 2000). .In vitro antibacterial activity of ethanol extract of allium sativum bulb was shown in table 5-

Table 5.Invitro antibacterial activity of ethanol extract of bulb of Allium Sativum

(Values are mean of three replicate)

Micro organism	Zone of inhibition (mm in diameter)	
	ethanol extract of AS(2mg)	Tetracycline (30µg)
S aureus	18	26
E. coli	16	20

Fig 2. Antibacterial activity of AS extract in S aureus and E coli

3.4 Minimum inhibitory concentration:

The present studies suggested that flower of cassia auriculata extract have significant antibacterial potential. The Minimum inhibitory concentration of methanol extract of cassia auriculata ranged between 12.5 mg/ml to 75 mg/ ml depending on micro organism shown in table 6- The MIC for ethanol extract of AS in S. aureus and E. coli at 200 mg/ml respectively was shown in table 7-

Table6. Minimum inhibitory concentration of cassia auriculata flowers against micro organism

Concentration (mg/ml)	Minimum Inhibitory Concentration	
	S. aureus	E. coli
300	-	-
200	-	-
100	-	-
75	+	-
50	+	-
25	+	-
12.5	+	+
6	+	+
3	+	+
1	+	+
0.78	+	+
0	+	+

+no inhibition, -there is inhibition

The result for MIC for methanol extract of CA in S. aureus and E. coli at 75 mg/ml and 12.5 mg/ml respectively.

Table7: Minimum inhibitory concentration of Allium Sativum against micro organism

Concentration (mg/ml)	Minimum Inhibitory Concentration	
	S. aureus	E. coli
300	-	-
200	-	-
100	+	+
75	+	+
50	+	+
25	+	+
12.5	+	+
6	+	+
3	+	+
1	+	+
0.78	+	+
0	+	+

+no inhibition, -there is inhibition

The result for MIC for ethanol extract of AS in S. aureus and E. coli at 200 mg/ml respectively.

3.5 Synergistic antibacterial effect of extracts:

Table 8: Synergistic antibacterial effect of methanol extract of flower of Cassia auriculata and Tetracycline in E coli and S aureus.

FIC	**S aureus.**	**E coli**
FIC (Tet)	0.06	0.06
FIC (C.A)	0.06	1.13
Σ FIC	0.12	0.18

Where Tet- Tetracycline, C.A- Cassia auriculata extract, FIC-Fractional inhibitory concentration

Σ FIC value is 0.12 and 0.18 <0.5, hence both drugs show synergistic effect with each other

Table 9: Synergistic antibacterial effect of ethanol extract of Allium Sativum and Tetracycline in E coli and S aureus.

FIC	**S aureus.**	**E coli**
FIC (Tet)	0.03	0.06
FIC (A.S)	0.13	0.13
Σ FIC	0.16	0.19

Where Tet- Tetracycline, A.S- Allium Sativum extract, FIC-Fractional inhibitory concentration

FIC value is 0.16, 0.19<0.5, hence both drugs show synergistic effect with each other.

Table 10: Synergistic antibacterial effect of methanol extract of Cassia auriculata and ethanol extract of Allium Sativum in E coli and S aureus.

FIC	**S aureus.**	**E coli**
FIC (C.A)	0.03	0.25
FIC (A.S)	0.25	0.13
Σ FIC	0.28	0.38

Where C.A - Cassia auriculata extract, A.S- Allium Sativum extract, FIC-Fractional inhibitory concentration FIC value is 0.28, 0.38<0.5, hence both drugs show synergistic effect with each other

4. Conclusion:

In this study the results suggested that extract of drugs may show antibacterial activity. Antibacterial action of cassia auriculata and Allium sativum may indicate their potential as antibacterial herbal remedies. The present study justified that Cassia auriculata and Allium sativum both drugs may show synergistic antimicrobial activity.

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