



RESEARCH ARTICLE

ISOLATION & EVALUATION OF ANTIFUNGAL ACTIVITY OF THE SEMI SYNTHETIC DERIVATIVES OF LYCOPENE

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Abstract

The aim of the study was to evaluate the antifungal activity of lycopene tomato-extracts and their derivative used in fungal infections against the *Candida albicans* fungus. Lycopene is red carotenoid pigment having scientific values because of its potential biological function. It has a natural source of antioxidant. This project highlights the scientific value and significance of lycopene as therapeutic agent. Now a day's human fungal infection and fungal resistance is increasing due to increased Tuberculosis, cancer, HIV and various infections. So is makes necessary to discover or modify the new class of antifungal compounds to treat and cure the fungal infections. As we know that plants contains rich source of many bioactive secondary metabolite such as carotenoids, terpenoids, flavonoids, saponins, alkaloids and other constituents were reported to have in vitro antimicrobial properties. Therefore the research on natural products and isolated contents of natural products has been increases in the recent years due to their easy availability and their importance in drug discovery. The use of herbal antimicrobial such as lycopene and lycopene semi synthetic derivatives have been an alternate and effective way of treating candidiasis fungal infections because of the large scale resistance and adverse activities of the commercially available azole group of synthetic antifungal drugs for e.g. ketoconazole, fluconazole, miconazole, itraconazole etc because azoles antifungal are the most frequent class to treat candida infections. Approximately 1.5 million people affected due to various fungal infections globally.^[9] Candidiasis is common and serious fungal infection caused by the *candida albicans*. However; candida normally lives on the skin surface and inside the body like; gut, vagina, mouth and throat without affecting and causing any problem. It can infect the individual cause infection grows out of control or if is enters deep inside the body for e.g. in brain, kidney and blood stream.

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Introduction:-

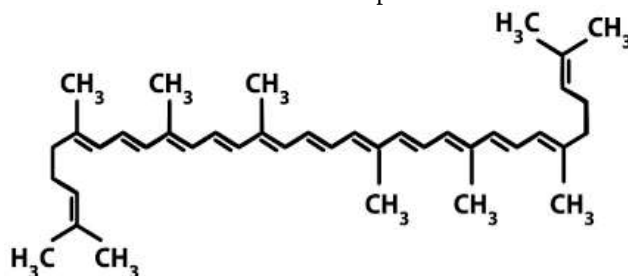
Lycopene is dominant acyclic carotenoid pigment (chemically) found in tomato, watermelon, papaya, and red pepper. It is powerful antioxidant that may help protect cells from damage.^[10] The most important sources of lycopene are tomatoes (*Lycopersicon esculentum* L.) although it can also be synthesized by the fermentation of fungi or by using bacteria's.^[2]

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The major coloring pigments of lycopene extract from tomato are mainly trans-lycopene. Lycopene in tomatoes consists predominantly of all-trans-lycopene (35-96% of the total content) however trans form is poorly absorb by the body and low levels of cis-lycopenes (1-22% of the total content).^[8] The reactivity of lycopene carotenoid biologically; depends on the physical and molecular structure, location or site of action within the cells, interaction with other contents, concentration and partial pressure of oxygen.^[11]

Lycopene for food use is also manufactured by chemical synthesis or produced by fermentation of *Blakeslea trispora*. The lycopene content of tomato extract ranges from 5% to 15%, depending on the nature of the fruit from which it was extracted, and the amount of tomato seed oil that is included in the extract.^[8] The intended use of lycopene extract from tomato is to provide color shades from yellow to red to various foods and other products. Lycopene in the extract can be **stable** when stored at room temperature and at 4°C for up to 37 months.^[8]



Previously, in-vitro and in-vivo studies were performed on lycopene that exhibited beneficial role in treatment of chronic diseases such as; cancer, atherosclerosis, cardiovascular diseases and neurodegenerative disorders.^[11]

Physical properties of lycopene – Lycopene has molecular formula $C_{40}H_{56}$ and molecular weight 536.85 Da. The melting point of lycopene were reported around 172-175 °C. In crystal form Lycopene are long needles separate form a mixture of carbon disulfide and ethanol though in powder form appeared as dark reddish brown color. It is soluble in chloroform, hexane, benzene, carbon disulfide, acetone, petroleum ether and vegetable oil but insoluble in water, ethanol, and methanol. Lycopene is sensitive to exposure of light, oxygen, high temperature, acids, catalyst and metal ions.^[11]

Isolation Of Lycopene From Tomato:

Prepared 100 gram fresh tomato paste & mixed with 50 ml acetone vigorously stirred for 5 minutes to remove all water soluble substances. Decant the extra liquid from the mixture and removed total moisture from the content by using filter or soaking paper. Take out drained tomato paste separately in beaker and mixed with petroleum ether and methylene dichloride (1:1) followed by small amount anhydrous magnesium sulphate powder to remove any fine or traces of water. Filtered the solution, collected the filtrate and evaporated the content on hot plate to get dry powdered as residue.^[3]

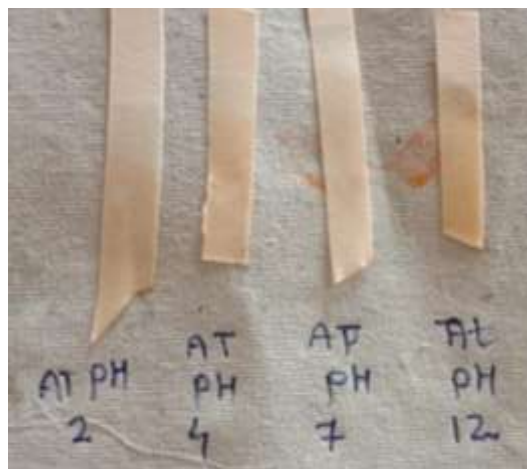


Ph And Titrametric Analysis Of Lycopene Extract:

First we had prepared the pH paper with the use of Watts's man filter paper by soaking it in the extracted solution of lycopene in the petri plate and allowed for drying. Dried filter papers cut in to 4 strips. Different buffer and chemical solutions of varying pH were prepared. The color change of strips was checked with prepared various pH ranges of solutions.

Result:-

There is no color change of lycopene was found to be at pH 2, 4, 7, 12. Hence it can be concluded that the color of lycopene is not varying with the pH. It has no any significant indicator property in acid-base titrations performed on 1M HCL against 1M NaOH with respect to phenolphthalein. Hence, lycopene is stable, non-toxic or quite inert compound with reference to reactivity & pH therefore it can be used in enteric coating drug delivery system.



Identification Test For Lycopene:-

Identification test were performed using color chemical reactions.

1. In order to identify the lycopene, a few crystal of extracted Lycopene was dissolved in concentrated sulfuric acid, imparting an indigo blue color to the solution.
2. In another test, by adding a solution of antimony trichloride in chloroform to a solution of lycopene in chloroform, an intense unstable blue color appeared. These tests proved the presence of lycopene in the extract.^[3]
3. Identification test of lycopene from tomato: - TLC: Retardation factor: Mobile phase – methanol: chloroform – 9.5: 0.5 Formula: retardation factor= distance travelled by solute / distance travelled by solvent = 2.5 / 6.1 = 0.4098.^[7]

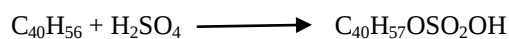
Reactivity & Semi Synthetic Derivative Of Lycopene:

It can undergo Reduction reaction with platinum forms perhydrolycopene, Ozonolysis reaction gives acetone and levulonic acid, Oxidation reaction yields acetic acid.

Sulphated Derivative Of Lycopene:

Sulfur has antifungal, antibacterial, and keratolytic activity. In the past, its use was widespread in dermatological disorders such as acne vulgaris, rosacea, seborrheic dermatitis, dandruff, pityriasis versicolor, scabies, and warts. Adverse events associated with topically applied sulfur are rare and mainly involve mild application site reactions. Sulfur, used alone or in combination with agents such as sodium sulfacetamide or salicylic acid, has demonstrated efficacy in the treatment of many dermatological conditions.^[31]

The alkenes react with concentrated Sulfuric acid in the cold to produce alkyl hydrogen sulphates. Therefore lycopene react to give lycopene hydrogen sulphate.^[25]



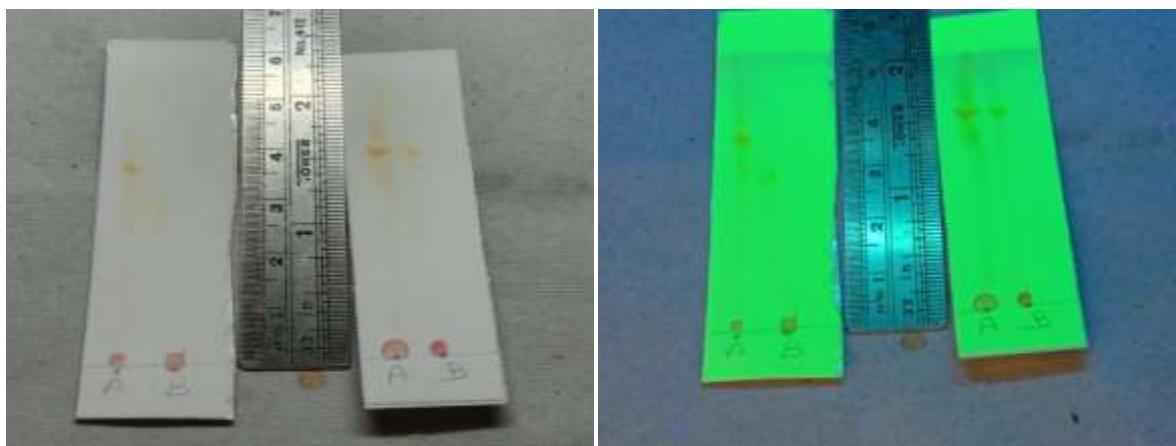
This reaction is Electrophilic addition reaction. The **mechanism** of the reaction between alkenes (lycopene) and sulfuric acid: The hydrogen atoms are attached to very electronegative oxygen atoms which mean that the hydrogen will be slightly negative. In the mechanism, we just focus on one of the hydrogen to oxygen bonds, because the other one is too far from the carbon-carbon double bond to be involved in any way. ^[26]

Procedure:

1. Weighted 0.5 gram lycopene and dispersed in 15 ml of distilled water.
2. Added conc. H_2SO_4 1-1 ml with an interval of 2 minutes until the color turns in to reddish blue. This is highly exothermic reaction; the temperature of flask will be around 60°C . During the addition of acid, appears as dark blue color but disappears after well stirring.
3. Flask kept a side until its temperature cools up to room temperature & then immediately poured in to 100 ml crushed ice water.
4. After melting of crushed ice pieces added NaOH to neutralize the excess acidity.
5. Filtered the product under suction pump, recrystallized, and dried in oven at 60°C .
And Melting point of this product was reported to be more than 300°C .

TLC analysis of Sulphated derivative of lycopene:

Sr.	Solvents	Ratio	Detecting reagent	Component	Rf value
1	Methanol : Acetone ^{[21][22]} (Test 1)	8 : 2	Uv Chamber	A- Pure lycopene	0.673
				B- Sulphate derivative	-
2	Methanol : Acetone (Test 2)	8 : 2	Uv Chamber	A	0.686
				B	0.686



Azole Derivative Of Lycopene:

Azole is the five member heterocyclic compound containing nitrogen atom. ^[12] These compounds are the broad spectrum and largest class of antifungal agents as compare to other antifungals. These azoles compounds are approved by the FDA for the treatment of candidiasis, chromomycosis etc. It inhibits the fungal infection by inhibiting the ergosterol biosynthesis in fungal cell which is absent in human and in animals. ^[13] Some marketed azole derivatives are ketoconazole, clotrimazole, oxiconazole, miconazole.

Procedure:

1. In the 100 ml RBF, Placed 1.25 gram of OPD and 1.25 gram lycopene & added 4 ml of 90% formic acid.
2. Attached reflux condenser for 1.5 hours by direct or electrical heating.
3. Cool the solution & poured in beaker.
4. Added 10% aqueous NaOH solution with continues stirring till it becomes alkaline to litmus.
5. Filtered the product at suction pump & washed with cold water approximately 20 to 30 ml.
6. Recrystallized the product by using water, drained the content
7. Reported yield (0.75 gram) and melting point.

**Bromine Derivative Of Lycopene:**

The reaction of bromine with diene can produce regioisomers; the outcomes can be altered depending on the conditions used. ^[14] Bromide is good allylic system like SN_1 can take place in which both the nucleophile and the electrophile are bromide. Bromide just adds across one of the double bond to give a 1,2 – dibromide. This is the kinetic product. ^[15]

Procedure:

1. Weighted 2.5 gram lycopene and dissolved in 11.25 ml of GAA in 250 ml in conical flask.
2. Cooled to below 5°C .
3. 1.05 ml bromine in added drop wise in 6.25 ml cold acetic acid with stirring.
4. Bromine solution is transferred to lycopene solution slowly with stirring & flask is placed in cold water.
5. Color changed to orange color.
6. Allowed to stand at room temperature.
7. Poured the content in to beaker having 50 ml ice cold water, reaction turned in to dark orange red color indicates the reaction is completed.
8. Solution is colored so added the sodium metabisulfite to decolorize the product.
9. Filtered the product and washed with cold water.
10. For purification- dissolved the product in cold water and filtered it, dried in oven.



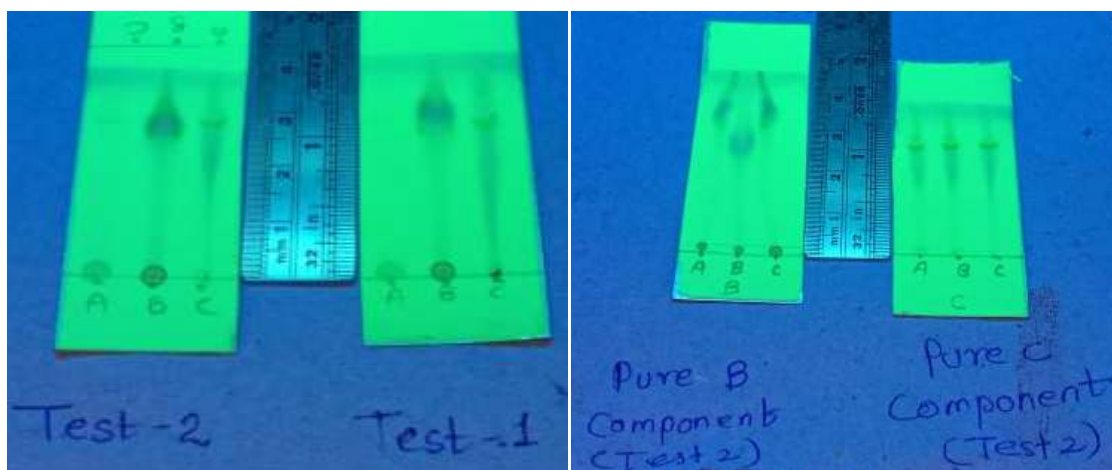
[Lycopene does not show any significant reactivity with the chlorine water (at high or low temperature) and conc. HCl (with water in presence of HCl).]

TLC Analysis of Bromine and Imidazole derivative of lycopene:

TLC Analysis of Lycopene as standard, azole derivative and bromine derivative of derivative were dissolved separately in methanol and ethyl-acetate in proportions of 1:2 V/V. Prepared mobile phase of methanol and chloroform (9.8: 0.2) run on to the stationary silica gel phase and observed under the UV chamber. Result – Not separated and identified the spots.^[16]

Now due to solubility issue of lycopene, it must be dissolve in that solvent in which extract dissolved completely. In an article it is mentioned that lycopene is freely soluble in chloroform, ether and vegetable oil.^[17] Imidazole & bromine derivative of lycopene also dissolved in chloroform because it is less polar solvent.^{[18][19][20]}

Sr.	Solvents	Ratio	Detecting reagent	Component	Rf value
1	Methanol : Chloroform ^{[21][22]} (Test 1)	9.8 : 0.2	Uv Chamber & Vanillin	A	0.717
				B	-
				C	0.731
2	Methanol : Acetone (Test 2)	8 : 2	Uv Chamber & vanillin	A	0.707
				B	-
				C	0.743



To get précised RF value, we have also performed the TLC of individual component B & C.

Sr.	Solvents	Detection Method	Component	Rf value
1	Methanol : Acetone (8 : 2)	UV Chamber	Component-B (Imidazole derivative of Lycopene)	0.70
				0.56
				0.70
2	Methanol : Acetone (8 : 2)	UV Chamber	Component-C (Bromine derivative of Lycopene)	0.68
				0.68
				0.68

Other detecting reagents: ^{[21]-[24]}

1. Iodine- Stain the TLC plate with iodine vapor because it has high affinity for both unsaturated and aromatic compounds and organic compounds.
2. Vanillin- Vanillin is very good staining reagent. (Prepare solution of 15 gram of vanillin in 250 ml ethanol and add 2.5 ml conc. H₂SO₄).
3. KMnO₄- It is excellent reagent for the detection of the functional group which is sensitive to oxidation. (Alkenes and alkynes will appear readily on TLC, stain will appear as bright yellow spot on a bright purple Background).

Antifungal Activity Against Candida Albicans: ^{[32][33]}

Fungal strain of *Candida albicans* were obtained from the Department of the Microbiology “Institute of Science, Fort- Mumbai”. Fungal strain was maintained on Peptone Dextrose (PD) agar. The inoculum of isolate was prepared by first growing the fungus on peptone dextrose (PD) agar slants for seven days at 35°C. The slant was rubbed carefully with a sterile cotton swab and transferred to a sterile tube with fresh PD broth (20 ml).

The medium most commonly employed for the isolation and growth of fungi is glucose peptone agar also called as Sabouraud’s agar. The components of the Peptone Dextrose Agar (PDA) are Peptone – 1 gram, Dextrose – 4 gram, Agar – 1.5 gram and Distilled water – 100 ml. ^[34] various derivatives of lycopene and solvents are used in following concentrations to find out antifungal activity.

Sr.		Drug & Contents	Role	Qty.	Dilution up to	Concentration
1	A	Distilled water	Negative control	-	10ml	-
2	B	Chloroform	Solvent used	-	10ml	-
3	C	Etraconazole (Standard)	Positive control	20mg	10ml	2mg\ml
4	D	Lycopene	Pure drug	20mg	10ml	2mg\ml
5	E	Sulfate derivative	Derivative	10mg	5ml	2mg\ml
6	F	Bromine derivative	Derivative	10mg	5ml	2mg\ml
7	G	Imidazole derivative	Derivative	10mg	5ml	2mg\ml

Preparation of Candida subculture (broth):

Washed and sterilized the all glass wares in hot air oven and broth media in autoclave for 30 minutes at around 120°C. Allowed to cool at room temperature and added the *Candida* slant by the sterilized loop in aseptic medium. Incubated in incubator at 36°C for 24 hours or until get turbidity or growth of *Candida albicans* in media.

Screening For Antifungal Activity Was Screened By:

Agar well diffusion method:

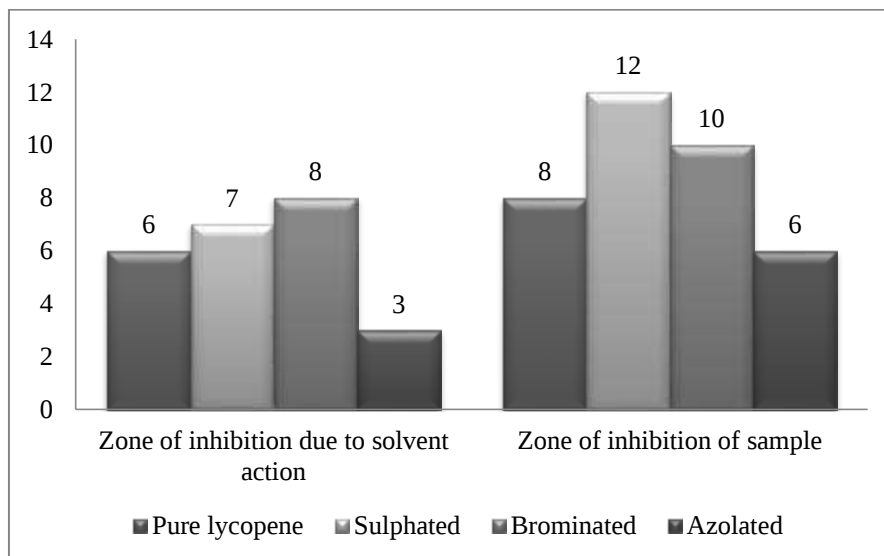


Prepared different samples of lycopene derivatives in chloroform solvent in 10 ml volumetric flask. ^{[36][37]} The different derivatives of lycopene were tested against pathogen *Candida albicans*. The PDA medium (50°C) was mixed with sub culture then poured in to the sterile petriplates and allowed to solidify. Then wells (6 mm) were made in the medium using sterile cork borer. The derivatives were transferred into the separate wells. The plates were incubated at 37°C for 24 – 48 hrs. After the incubation, confluent fungal growth was observed. Inhibition of the bacterial growth was measured in mm. ^{[34][35]}

Samples	Petri dish 1	Petri dish 2	Petri dish 3	Petri dish 4
Negative control	0 mm	0 mm	0 mm	0 mm
Positive control	13 mm	-	15 mm	-
Solvent	6 mm	7 mm	8 mm	3 mm

Drug & derivatives	8 mm	12 mm	10 mm	6 mm
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The zone of inhibition of positive control that is itraconazole was found to be more among all because it is synthetic marketed antifungal drug however; others are the natural or semi synthetic derivatives, moreover the concentrations of all samples are taken to be same 2mg/ml.



Agar diffusion Paper- disc method:

Natural products were dissolved and diluted with solvent as mentioned in table. 7 mm filter paper disc (Whatman paper) was impregnated with 5 ml of each of the different samples. The discs were allowed to remain at room temperature until complete diluents evaporation. Discs loaded with samples were placed on to the surface of the solidified media. The all plates were incubated at 37°C for 24 – 48 hrs. After the incubation the plates were observed for formation of clear incubation zone around the paper discs, indicated the presence of antifungal activity. The zone of inhibition was recorded. ^{[38][39]}



Result:-

The antifungal activity of lycopene and lycopene derivative against *Candida albicans* was not showing any significant actions by paper disc method because disc couldn't absorb enough samples to inhibit growth in the media.

Lycopene Absorption, Transportation And Distribution In Human:**Absorption:**

Lycopene, as a fat soluble compound, in the stomach and intestine; it will separate from the food matrix and dissolved in the lipid phase. Lipid vesicles will absorb in to small intestine via passive diffusion process. The release of lycopene from the diet is higher in large intestine around 57% than the small intestine (40%), but potential for the lycopene to be absorbed in the large intestine is negligible. However cis-isomer has higher bioavailability than the all trans-isomers. ^{[40]- [43]}

Transportation:

After the absorption by intestinal mucosa, it parceled in to chylomicrons and secreted in to lymph transport system, and then transferred to the liver. It is transported by the lipoproteinoic plasma and the distribution depends on its chemical structure. As hydrophobic compound, it is found at the lipophilic part of lipoproteins; therefore lycopene is mostly transported by low density lipoprotein. ^[44]

Distribution:

The distribution of lycopene in organs and plasma was found to be within the range of 0.2-21.4 nmol/g tissue. In an article, it is reported that lycopene concentration was highest in human tests, followed by adrenal gland > liver > prostate gland > breast > pancreatic gland > skin > colon > ovary > lung > stomach > kidney > fat tissue > cervix. Higher concentration of lycopene was found in liver, adrenal and reproductive tissue that is 10 times higher than other tissue. ^[45]

Metabolism:

The lycopene metabolites are formed by reaction with carotenoid monooxygenase enzyme; mainly cleavagic and oxidative products. ^[11]

Excretion:

The excretion of lycopene through feces and urine was also reported. ^[46]

Role & Application Of Lycopene:

As lycopene is an anti oxidizing agent, used as quench reactive oxygen species, in anti-lipid peroxidation and anti DNA oxidative damage. It is also follow the non oxidative mechanism, to perform the gap junction communication (to decrease proliferation of tumors cells), green function regulation, hormone and immune modulation, anti-proliferation and pro-differentiation. It has found to improve mouth opening and reduce burning sensation. ^{[27]- [30]} The lycopene shows preventive effect towards diseases like; Oxidative stress, Cardiovascular disease, Cancers, Diabetes etc. ^[11]

Discussion:-

Sulphated derivative of lycopene is synthesized by accidentally unique process which shows antifungal activity more than other derivatives of lycopene against *Candida albicans*. The synthesis of sulphated derivative of lycopene is still under study for more chemical, analytical and biological evaluation. In pH stability study we found that lycopene is almost stable at all pH so, this can be utilized in novel drug delivery system and for enteric coating of such formulations.

Conclusion:-

In the human health, many articles as evidence shows that consumption of lycopene and lycopene rich foods can help in preventing many degenerative diseases, microbial infections and in novel drug delivery system but very limited studies have found a beneficial role of the consumption of lycopene alone. On the other hand, some beneficial effects may due to the lycopene isomers or its metabolites but information about this is scarce.

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