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CHARACTERIZATION & IMMUNO-MODULATORY ACTIVITY OF *CAPSICUM ANNUUM* L. VAR. *GROSSUM* SENDT. BY USING LABORATORY ANIMALS

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ABSTRACT:-

The present study was aimed to investigate Immuno-modulatory activity of *Capsicum annuum* L. var. *grossum* Sendt.The assessment of immuno-modulatory activity was carried out by using Neutrophil Adhesion Test (In-Vivo Method) & T-Cell Population (In-Vitro Method). Oral administration of extract of *Capsicum annuum* L. var. *grossum* Sendt. Significantly showed Immuno-modulatory activity increases percentage neutrophil adhesion, increases lymphocytes & No. of rosette count when result compared with control group. Separation was done by Thin Layer Chromatography (TLC) & then isolation was done by column chromatography technique & confirmed by spectroscopic techniques. In conclusion, ethanolic & aqueous extract of *Capsicum annuum* L. var. *grossum* Sendt. showed presence of flavonoids, phenolic compound & vitamin C. as well as alkaloids, glycosides. But aqueous extract showed maximum immunomodulatory activity by both In-vivo & In-vitro methods. So, further first performed Thin Layer chromatography & then flavonoid quercetin was isolated from aqueous extract of *Capsicum annuum* L. var. *grossum* Sendt. by column chromatography technique. Its presence was confirmed by spectroscopic analysis (UV, IR, NMR & MS) techniques.

Keywords: - Immuno-Modulatory, *Capsicum annuum* L. var. *grossum* Sendt, Neutrophil Adhesion Test, T-Cell Population, Immunostimulant's.

1. Introduction:-

Botanical herbs / plants are found in the form of natural medicines considered as a gift from God to human beings. Traditional and ethno-veterinary practices have been used for centuries, transferring knowledge from one generation to the next and are accessible, easy to prepare and administer, with little or no charges. Although modern developments in the therapeutic field caused rapid declines in traditional medicine, herbal remedies continue to play a crucial role as a potential source of therapeutic aids in health systems around the world, for both humans and animals [1].

The World Health Organization (WHO) was included among the 21,000 medicinal plants, 2,500 species are native to India, which ranks first in the production of medicinal herbs. This numberless treasure of medicinal herbs/plants gives India the distinction of "botanical garden of the world". Today, immune treatments are obtaining more importance's than monovalent approaches that have limited benefits. In addition to actions such as treating disease, controlling ecto-parasites and endo-parasites, enhancing fertility, bone formation, and mismanagement of childbearing, a variety of herbal medications have been reported to have effects immunomodulators such as modulation of cytokines secretion/production, histamine release, immuno-globulin secretion, cells co receptor expression, lymphocyte expression and phagocytosis, etc [1].

The modulation of the immune response through the use of Ayurvedic herbal medicines as possible therapeutic measures has become a subject of scientific research. The idea in modern scientific comprehension would mean the enhancement of the immune response capacity of organisms against a pathogen by non-specific activation of the immune-system using immuno-modulatory agents obtained from plant origin. It is now recognized that modulation of the immune responses could provide an alternative to conventional chemotherapies for a variety of diseases with impaired immune response or when selective immunosuppression must be induced in situations such as autoimmune disorders and organ transplants. The basic concepts of immuno-modulation not only lived in Ayurveda, but have been practiced by Ayurvedists for centuries. Actually, one of the therapeutic/curative strategies in Ayurvedic medications is instead of directly affecting disease causing agents it enhances body's total immune power [2].

Herbal drugs are easily affordable and less potent than synthetic prescription immuno-modulators but are also less likely to cause side effects. Therefore, there is a needed to search for plants with immuno-modulatory activity to offer novel strategies for the treatment of infectious disease.

❖ Immunomodulation:

Immunomodulation is defined as the alteration of the immune response that can increase or decrease the immune response. Immunomodulators are generally two types based on their effects immunostimulant's & immunosuppressant's. Increases the immune responsiveness is called immunostimulation. Decreases the immune responsiveness is called immunosuppression [3]. Potential uses of immuno-modulators in clinical drugs include reconstitution of immunodeficiency (e.g., treatment of AIDS) & suppression of extreme immune function (e.g., treatment of graft's rejection or auto-immune disease) [4].

The immune system is designed to prevent the hosts from invading pathogens and to eliminate disease. The human body is occurring two types of immune response:

I) Innate immune response: The innate immune response is the first line of the defense mechanism against physical, biochemical and cellular components.

II) Adaptive immune response:

a) Humoral immunity - Antibody production - killing extracellular organisms.

b) Cell-mediated immunity - cytotoxic / killer T cells - that kill viruses and tumor cells [5].

In Covid-19 situation immunity is important criteria for human being because weak immunity is causes corona virus infection. So enhancement of immune responsive is more important to prevent corona virus infection.

❖ Future Scope:

As immune system is known to be involves in the etiology as well as pathologic mechanisms of many diseases it has tremendously increased the need of drugs which are effective on immune system. Synthetic immunomodulators have drawbacks, so there is a need of development of herbal immunomodulators. This study will help the researchers to find out the new plants with immunomodulatory activity based on the parts used and their chemical constituents [6].

Plant Information

1. *Capsicum annuum* L. var. *grossum* Sendt:

Botanical Name: - *Capsicum annuum* L. var. *grossum* Sendt.
Family : - *Solanaceae*
Synonym : - Red Bell Pepper
Common Name: - Sweet pepper, Simla Mirchi.

Fig. No: 1. *Capsicum annuum* L. var *grossum* Sendt

❖ **Vernacular Names in India:-**

Table No: 1. Vernacular Names

English	Red Bell Pepper
Marathi	Bhopli Mirchi
Hindi	Simla Mirchi/ Mota Marcha
Sanskrit	Mahamarichika
Kannada	Donne Menasinakai
Gujarati	Simla Marchan/ Mota Marcha

❖ **Occurrence and Description of Plant:-**

➢ **Geographical Distribution:**

Cultivation & collection of *Capsicum* in almost all the tropical countries. East Africa, West Africa and India are the regions producing the drug on commercial scale. In India it is grown in Andhra Pradesh, Uttar Pradesh, Gujarat, Maharashtra, Assam & Tamil Nadu [7].

➢ **Plant Description:**

Species name *annuum* means "annual" (from Latin annus "year"), the plant is not an annual but is frost tender [8]. In general, the *Solanaceae* have a female part of the flower & it formed two carpel's. This is known as gynoecium. However, *Melananthus* has a monocarpelar gynoecium; there are 3or 4 carpel's in *Capsicum* [9].

It grows up to 0.75-1.8 m in cultivated varieties with many angular branches. The leaves are simple of varied and alternate forms, elliptical to lanceolate, with smooth margins (whole) generally wrinkled. The small flowers (about 1.5 cm or 1 inch in diameter) are white or purple, in clusters of two or more [7].

Green fruits are generally green or, less often, pale yellow or purple. One variety, Permagreen, maintains its green color even when fully ripe. Red, yellow & orange bell peppers come from different

seeds and are different cultivars of bell pepper. Red bell peppers are just ripe green bell peppers. Green bell peppers are slightly bitter & less sweet than yellow bell peppers or orange bell peppers, with red bell peppers being the sweetest [10].

❖ **Chemical composition:**

The Capsicum annuum fruit contains capsaicin (8-methyl-N-vanillyl-6-nonenamide) and several related chemicals that contain a series of homologous straight and branched chain alkyl vanillylamides, collectively referred to as capsaicinoids as their main chemical entity. The main capsaicinoids present are capsaicin (48.6%) followed quantitatively by 6, 7-dihydrocapsaicin, the minor capsaicinoids that are present nor-dihydrocapsaicin (7.4%), homo-dihydrocapsaicin (2%) and homocapsaicin (2%). Other part of the plant contains steroidal alkaloid glycosides (solanines, solanidines & solasodines). The seeds contain the steroidal glycosides capsicoside A to D, all furostanol. *Capsicum annuum* is rich in carotenoid pigments, including capsanthin, capsorubrine, carotene, lutein, zeaxanthin, and cucurbitaxanthin A [7].

All types of Bell peppers are good resources of antioxidants, such as vitamin C, provitamin- A, vitamin E, and flavonoids, the nutritional contents and phyto-constituents are also varied among different coloured Bell peppers [11].

2. MATERIAL & METHODS:-

i) Drugs, chemicals & solvents: (Analytical grade drugs & chemicals are used.

All the drugs chemicals were analytical grade. Drugs & chemicals used in this experimentation as follows chloroform**,** ethanol, pet ether (40-60), ethyl acetate, methanol, diethyl ether, benzene, silica for TLC, silica for column, Levamisole, EDTA, Alsever's solution.

ii) Collection of plant material:

The fresh fruits of *Capsicum annuum* L. var. *grossum* Sendt. was collected in the month of November 2020 from the area of ganeshwadi (Kolhapur district), Maharashtra.

iii) Authentification of plant:

The plant was authenticated by Mr. M. D. Wadmare, H. O. D. of Botany Department, Smt. K. W. C. Sangli.

iv) Drying of plant material:

In the current study the collected ripe fruit was sorted carefully & washed thoroughly to remove dirt & debris. The plant material was cutting & spread out in thin layer on drying trays, kept in shade for 30 days. The drying trays were placed at a sufficient height above the ground to ensure proper air circulation and consistent drying of plant material. & avoid mould formation. After complete drying of flowering stalk was powdered by mixer grinder to obtain coarse powder.

v) Extraction of plant material:

➢ **Aqueous Extraction:**

Chloroform water was prepared as 10% chloroform & 90% water (10:90). Then take 150-200gm of dry powder of *Capsicum annuum* L. var. *grossum* Sendt. was added in one liter of chloroform water I.P. (10%) contained in a round bottom flask. The flask was plugged with muslin cloth & kept at room temperature. It was shaken periodically up to seven days. Then it was filtered, mark was pressed & filtrate was collected. The extract was stored in a bottle in refrigerator at 4^{o}C.

➢ **Organic Solvent Extraction:**

Organic solvents extraction is carried out by using Soxhlet extraction. Soxhlet extraction performed in PG Chemistry lab.

Fig. No: 2. Soxhlet Extraction Apparatus

Extraction was performed with organic solvents chloroform & ethanol (70%). About 100-150 gm of dry fruit powder of *Capsicum annuum* L. var. *grossum* Sendt.was extracted by chloroform by continuous Soxhlet extraction. The extraction was continued till the solvent became colorless. The chloroform extract was filtered & powder in the extraction apparatus is removed from extractor, dried & then it was used for extraction with ethanol. These chloroform & ethanol extract was stored in separate bottles & labeled.

vi) Phytochemical Investigation

Capsicum annuum L. var. *grossum* Sendt. three ethanolic & aqueous extracts was prepared, optimized & then to check phytochemical test [12, 13].

❖ **Animals:-**

The protocol used in this study for the use of rat as animal model for immunomodulatory activity was approved by Institutional Animal Ethical Committee (IAEC) of ABCP, Sangli. (Protocol No:-IAEC/ABCP/11/2020-21).

Swiss albino rat eight weeks old, either male/female, weight 150-200 gm were used for study. The animal care & handling was carried out according to CPCSEA guidelines. The animal study was performed in pharmacology research laboratory, ABCP, Sangli.

❖ **Acute Toxicity:-**

➢ The acute toxicity of *Capsicum annuum* L. var. *grossum* Sendt. was reported. LD_{50} of aqueous & ethanol extracts of fruit of *Capsicum annuum* L. var. *grossum* Sendt. was above 5000mg/kg [14].

❖ **Immunomodulatory Activity Methods:-**

A) IN-VIVO STUDY:-

1) Neutrophil Adhesion Test:-

➢ **Procedure:-**

- In this test animals are divided into 6 groups comprising 5 animals in each group.
- Group I was kept as a control & received vehicle only water (10 ml/kg).
- Group II was kept as a standard & received standard drug levamisole (50 mg/kg).
- Group III was kept as a test-III & received the ECA sample no-I.
- Group IV was kept as a test-IV & received the ACA sample no-II.

Table No. 2. Group & Treatment Schedule for Neutrophil Adhesion Test

Sr. No.	Groups	Treatment	Dose
1	Group I	Control (water)	10ml/kg
2	Group II	Standard (Levamisole)	50mg/kg
3	Group III	Ethanolic *Capsicum annuum* L. var. *grossum* Sendt.(ECA)	0.16ml
4	Group IV	Aqueous *Capsicum annuum* L. var. *grossum* Sendt.(ACA)	0.16ml

- On 16th day of the treatment blood sample from the entire group was collected by puncturing retro-orbital plexus under mild anesthesia.
- Blood was collected in vials pretreated by disodium EDTA & analyzed for Total Leukocyte Count (TLC) & Differential Leukocyte Count (DLC).
- After initial count blood sample was collected with nylon fiber (80mg/ml, previously sterilized by alcohol) for 15 min at 37° C & the incubated drug sample was analyzed for TLC & DLC.
- The product of TLC & % neutrophils adhesion was calculated as follows [15].

$$\% \text{ Neutrophil adhesion} = \frac{NI_U - NI_T}{NI_U} \times 100$$

Where,
NI_U - Neutrophil index before incubation with nylon fiber.
NI_T - Neutrophil index after incubation with nylon fiber.

B) IN-VITRO STUDY:-

1) T-Cell Population:-

➢ **Procedure:-**

- On 0th day, all groups were sensitized with 0.1 ml of SRBC containing 1×108 cells, i.p.
- Animals were divided into different groups each containing 5 animals.
- Group I was kept as a control & received vehicle only saline (10 ml/kg).
- Group II was kept as a standard & received standard drug levamisole (50 mg/kg).
- Group III was kept as a test-I & received the ECA sample no-I.
- Group VI was kept as a test-II & received the ACA sample no-II.

Table No. 3. Group & Treatment Schedule for T-Cell Population Test

Sr. No.	Groups	Treatment	Dose
1	Group I	Control (water)	10ml/kg
2	Group II	Standard (Levamisole)	50mg/kg
3.	Group III	Ethanolic *Capsicum annuum* L. var. *grossum* Sendt.(ECA)	0.16ml
4.	Group IV	Aqueous *Capsicum annuum* L. var. *grossum* Sendt.(ACA)	0.16ml

- On 11th day, blood was collected from the retro-orbital plexus & anticoagulanted with Alsever's solution in separate test tube.

- Test tube containing blood was kept in sloping position (45º) at 37º c for 1 hour. RBC were allowed to settle at bottom & supernatant was collected from each test tube by using micro-pipette contains lymphocytes.
- 50 µl of lymphocyte suspension & 50 µl SRBC were mixed in test tube & incubated.
- Resultant suspension was centrifuged at 200 rpms for 5 min & kept in a refrigerator at 4º c for 2 hrs.
- The supernatant fluid was removed & one drop of cell suspension was placed on a glass slide.
- Total lymphocytes were counted & a lymphocyte binding with three or more erythrocytes was considered as rosette & no. of rosette was counted [16].

Table no. 4. Composition of Alsever's Solution

Chemicals	Quantity (g/L)
Sodium chloride	4.2 gm
Sodium citrate	8.0 gm
Citric acid anhydrous	0.55 gm
Glucose	20.5gm
Distilled water q.s.	1000ml

❖ **Isolation process by chromatographic Techniques:**

➢ **Thin Layer Chromatography:-**

Fig. No: 3. Thin Layer Chromatography Plate

Different constituents of extracts were separated on thin layer chromatographic plates size (5.2×10.2) containing silica gel. Different solvent systems were tried but best resolution of constituents was obtained in the **Pet ether: Ethyl acetate: Methanol (5:1:1)** solvent system. Retention Factor (R_f) value was calculated by using following formula.

$$R_f \text{ value} = \frac{\text{Distance travelled by solute front (cm)}}{\text{Distance travelled by solvent front (cm)}}$$

➢ **Isolation of active constituents by column chromatography:**

Fig. No: 4. Column Chromatography

➢ **Experimental Procedure:-**

➢ **Selection of stationary phase & column:**

150-200 gm of silica gel (100-200 mesh) was used as stationary phase. It was activated in hot air oven at 110^{o} C for one hour. A glass column, three times longer than the total volume of adsorbent was used for separation. Slurry of activated silica gel was prepared in benzene & it was introduced into previously dried column. Small quantity of benzene was maintained on top of the prepared column to prevent its drying. After setting of the column, the enriched aqueous extract of *capsicum annuum* L. var. *grossum* sendt dissolved in water was introduced into it without disturbing the column.

➢ **Elution of mobile phase:**

The mobile phase was selected from TLC **pet ether: ethyl acetate: methanol, 5:1:1 v/v/v**. was saturated & then introduced into column. In large quantity of mobile Phase was prepared for column chromatography according to TLC mobile Phase. That mobile phase was used for column chromatography. The active constituents were separated into different fractions. Flow rate was controlled by adjusting the outlet valve. Flow rate is maintained till active constituent got separated into individual fractions. Then the collected in separate tubes & test for flavonoids is performed.

Table No. 5. Details of column chromatography *capsicum annuum* L. var. *grossum* sendt

Adsorbant	Silica gel –100-200 mesh size (activated at 110^{o}C)
Length of column	41 cm
Length of adsorbant	20 cm
Diameter of column	Outer 3 cm & inner diameter 2.9 cm
Rate of elution	4-5 drops per minute
Volume of each fraction collected	7-15 ml each
Mobile phase used	pet ether: ethyl acetate: methanol (5:1:1 v/v/v)

3. RESULTS & DISCUSSION:-

Phytochemical Investigation

Table No. 6. Results of Phytochemical Investigation

A. Test for Flavonoid:

Sr.No.	Chemical Test	Observation	Inferences	
			Capsicum annuum L. var. *grossum* Sendt.	
			Ethanol	Aqueous
1.	**Alkaline Test:** Plant extract + 10 % ammonium hydroxide solution	Yellow Fluorescence	**+ ve**	**+ ve**
2.	**Lead Acetate Test:** Plant extract + 10% lead acetate sol^{n} (few drops)	Yellow Precipitate	**+ ve**	**+ ve**
3.	**Conc. Sulphuric Acid Test:** Plant extract + Conc. H2SO4	Orange Colour	**+ ve**	**+ ve**
4.	**Shibata's Test:** Plant extract + dissolve in 1-2 ml 50% methanol by heating + metal magnesium + 5-6 drops of conc. HCl.	Red colour	**- ve**	**+ ve**

B. Test for Phenolic Compound:

1.	**Iodine test:** 1mL extract + few drops of dil. Iodine sol.	A transient red colour	**+ ve**	**+ ve**
2.	**Gelatin test:** Plant extract is dissolved in 5mL distilled water + 1% gelatin solution + 10% NaCl	A white precipitate	**+ ve**	**+ ve**
3.	**Lead acetate test:** Plant extract is dissolved in 5mL distilled water + 3mL of 10% lead acetate sol^{n}.	A white precipitate	**+ ve**	**+ ve**
4.	**Potassium dichromate test:** Plant extract + few drops of potassium dichromate solution	A dark colour	**+ ve**	**- ve**

C. Test for Alkaloids:

1.	**Dragendroff's test:** Few ml filtrate + 1-2 ml *Dragendroff's reagents*	A reddish-brown precipitate	**+ ve**	**+ ve**
2.	**Wagner's test :** Few ml filtrate + 1-2 drops of *Wagner's reagent* (Along the sides of test tube)	A brown/reddish precipitate	**- ve**	**+ ve**

D. Test for Cardiac Glycosides:

1.	**Keller-Killani test :** 1mL filtrate + 1.5mL glacial acetic acid + 1 drop of 5% ferric chloride + conc. H2SO4 (along the side of test tube)	A blue coloured solution (in acetic acid layer)	**+ve**	**-ve**
2.	**Test for Cardenolides :** Extract + pyridine + Sodium nitroprusside + 20% NaOH	A red colour, fades to brownish yellow	**+ve**	**+ve**
3.	**Baljet test :** 2mL extract + a drop of *Baljet's reagent*	A yellow-orange colour	**+ve**	**-ve**

E. Test for Vitamin C:

1.	Add 2ml of a 2% w/v solution + few ml of 2,6-dichlorophenolindophenol solution	Solution is decolorized	**+ ve**	**+ ve**
2.	Add 2 ml of 2% w/v solution + 2ml of water + 0.1 gm of sodium bicarbonate + 20 mg of ferrous sulphate shake & allow to stand. After that add 5ml of 1M H_2SO_4.	Deep violet colour is produced after add H_2SO_4 colour will disappears.	**+ ve**	**+ ve**

Discussion:-

The presence of flavonoids, phenolic compounds, vitamin C, alkaloids & Cardiac glycosides major constituents in ethanolic & aqueous extract of fruit of *Capsicum annuum* L. var. *grossum* sendt.

Pharmacological Screening

In-Vivo Method

I] Result of Neutrophil Adhesion Test:

❖ **Effect of *Capsicum annuum* L. var. *grossum* Sendt. on Neutrophil Adhesion Test:-**

Table no. 7. Neutrophil Adhesion Test Observation

Animals	% Neutrophil Adhesion			
	Control	**Standard**	**ECA**	**ACA**
1.	26.48%	66%	59.53%	62.22%
2.	31.42%	70.48%	57.30%	65.36%
3.	24.78%	65.17%	61.79%	59.83%
4.	26.47%	66.29%	59.13%	64.93%
5.	27.12%	68%	58.09%	62.22%

➢ ***Capsicum annuum* L. var. *grossum* Sendt:**

Values are expressed as (Mean ± S.E.M) n=4**** P<0.0001 statistically significant when compared with control group by ANOVA followed by Dunnett test. The result is as follows:

Table No. 8. Results of % Neutrophil Adhesion

Sr.No.	Group	% Neutrophil Adhesion
1.	Control	27.25 ±1.111
2.	Standard	67.19 ±0.943****
3.	ECA	59.17 ±0.7635****
4.	ACA	62.91±1.013****

Effect of ECA & ACA extract on neutrophils activation by the neutrophil adhesion test is shown in [Table: 9].

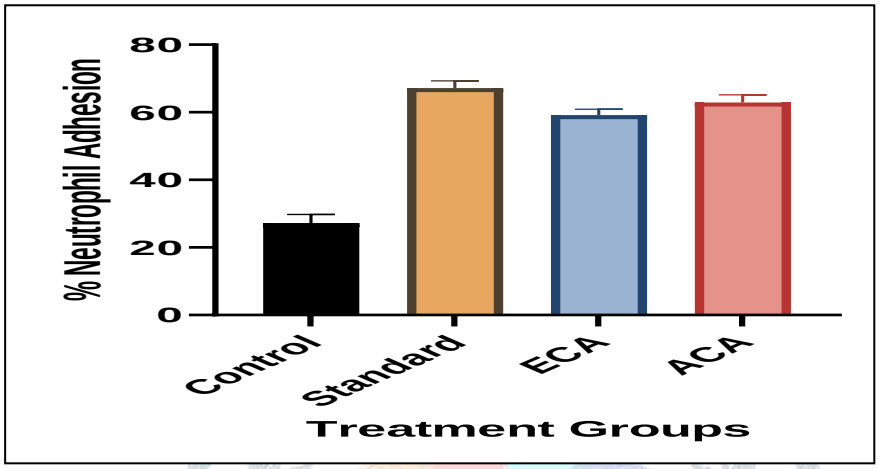


Fig: 5.Graphical Representation of Neutrophil Adhesion Test

Discussion:-

Above graphical representation observed that ACA showed highest % neutrophil adhesion (62.91±1.013) than ECA (59.17±0.7635). When compare with control group showed possible Immunostimulant effect.

In-Vitro Method

I] Results of T-Cell Population Test:

❖ **Effect of *Capsicum annuum* L. var. *grossum* Sendt. T-Cell Population Test:-**

Table No. 9. T-Cell Population Test Observation

Animals	% Absolute Lymphocyte Count				No. of Rosettes			
	Control	Standard	ECA	ACA	Control	Standard	ECA	ACA
1.	11%	19%	15%	19%	12	27	12	18
2.	9%	22%	17%	15%	8	24	15	20
3.	14%	21%	13%	20%	14	29	11	24
4.	14%	23%	16%	18%	13	22	14	17
5.	11%	18%	18%	17%	7	32	16	19

➢ ***Capsicum annuum* L. var. *grossum* Sendt:**

Values are expressed as (Mean ± S.E.M) n=4**** P<0.0001 statistically significant when compared with control group by ANOVA followed by Dunnett test. The result as follows:

Table No. 10. Results of % Lymphocyte

Sr.No.	Group	% Absolute Lymphocyte
1.	Control	11.8 ±0.9695
2.	Standard	20.6 ±0.9274****
3.	ECA	15.8 ±0.8602*
4.	ACA	17.8 ±0.8602***

Effect of ECA & ACA extract on lymphocyte activation by the t-cell population test is shown in [Table no. 13].

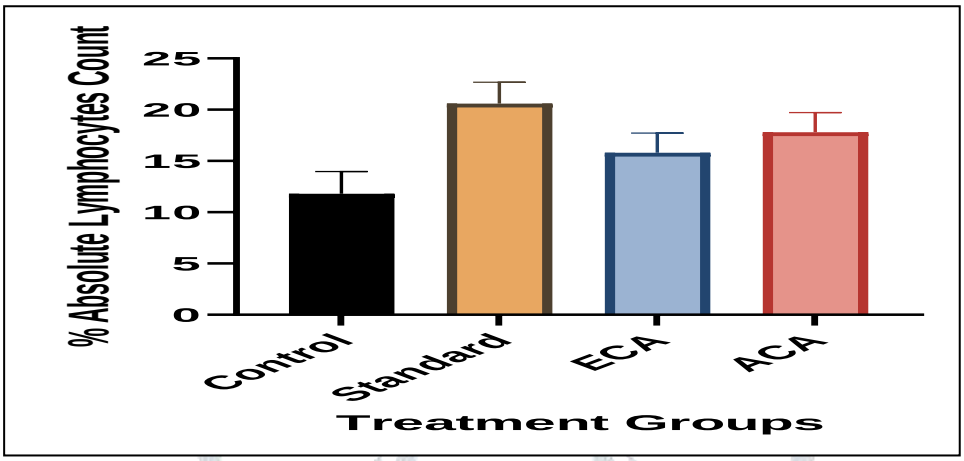


Fig: 6. Graphical Representation of T-Cell Population Test

Discussion:-

Above graphical representation observed that ACA showed highest % absolute lymphocyte (17.8 ±0.8602) than ECA (15.8 ±0.8602). When compare with control group showed possible Immunostimulant effect.

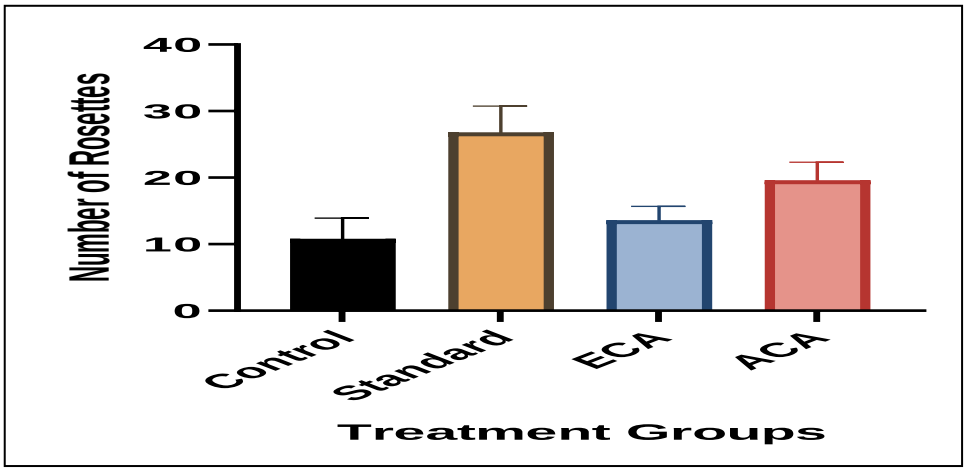
- ***Capsicum annuum* L. var. *grossum* Sendt:**

Values are expressed as (Mean ± S.E.M) n=4**** P<0.0001 statistically significant when compared with control group by ANOVA followed by Dunnett test. The results as follows:

Table No. 11. Results of No. of Rosette Count

Sr.No.	Group	No. of Rosette Count
1.	Control	10.8 ±1.393
2.	Standard	26.8 ±1.772****
3.	ECA	13.6 ±0.9274
4.	ACA	19.6 ±1.208***

Effect of ECA & ACA extract on no. of rosette by the t-cell population test is shown in [Table: 14].



Discussion:-

Above graphical representation observed that ACA showed highest no. of rosette count (19.6 ±1.208) than ECA (13.6 ±0.9274). When compare with control group showed possible Immunostimulant effect.

❖ **Thin Layer Chromatography:**

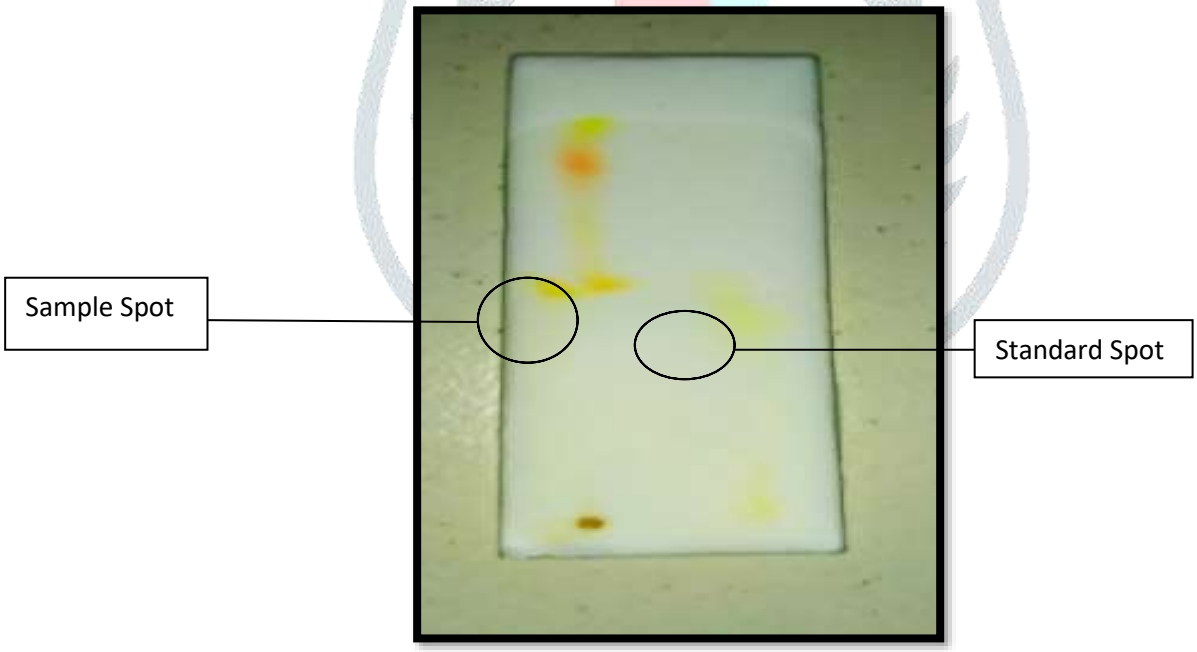


Fig. No: 8. Thin Layer Chromatography

➢ Observation:-

i) Distance travelled by solvent= **5.5 cm**
ii) Distance travelled by solute (std)= **1.9 cm**
iii) Distance travelled by solute (test)= **2.1 cm**

- **Calculation:-**

i) Rf Value of Standard:-

R_f value= Distance travelled by solute front (cm) / Distance travelled by solvent front (cm)

= 1.9cm / 5.5cm

= **0.34**

ii) Rf Value of Test:-

R_f value = Distance travelled by solute front (cm) / Distance travelled by solvent front (cm)

= 2.1cm / 5.5cm

= **0.38**

The R_f value of standard (quercetin) is 0.34 & R_f value of test (ACA) is 0.38 are approximately matching with each other.

Structural Elucidation

- **Characterization of isolated component obtained from ACA:**

A) UV:-

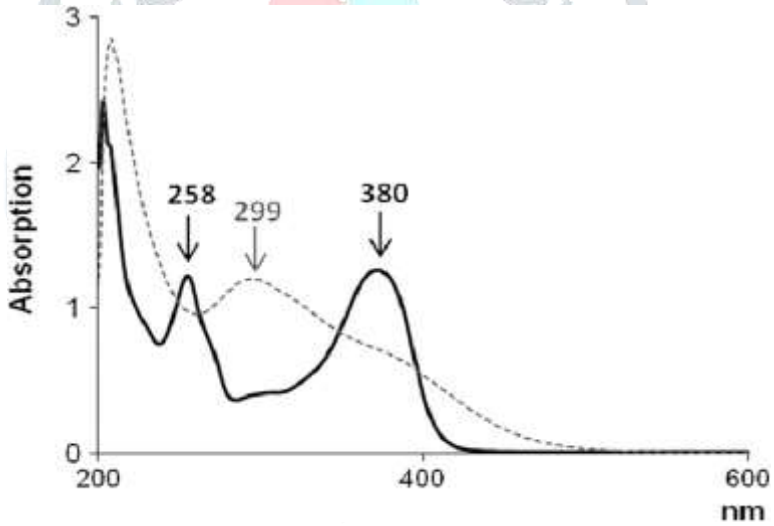


Fig. No: 9. λmax of Standard for comparison

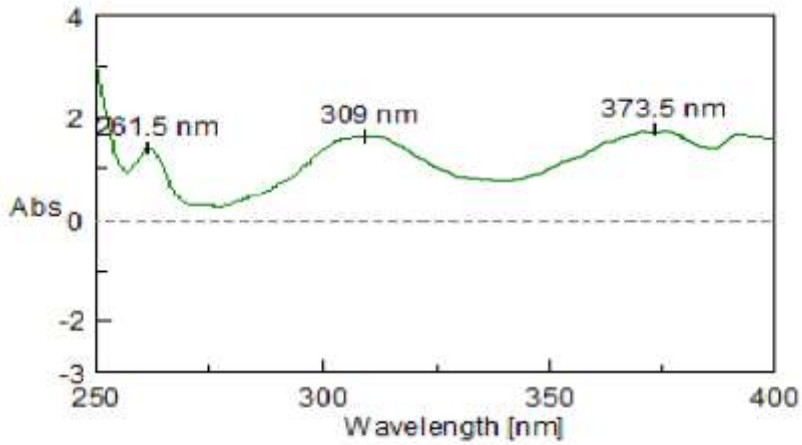


Fig. No: 10. UV of isolated component (quercetin) obtained from ACA.

Discussion:-

Fig. No. 9 & 10 - indicated presence of quercetin in the sample because λmax of standard quercetin is 258nm & 380nm are approximate matching with λmax of sample is 261.5 nm & 373.5nm of isolated component obtained from *Capsicum annuum* L. var. *grossum* Sendt [17].

B) IR:-

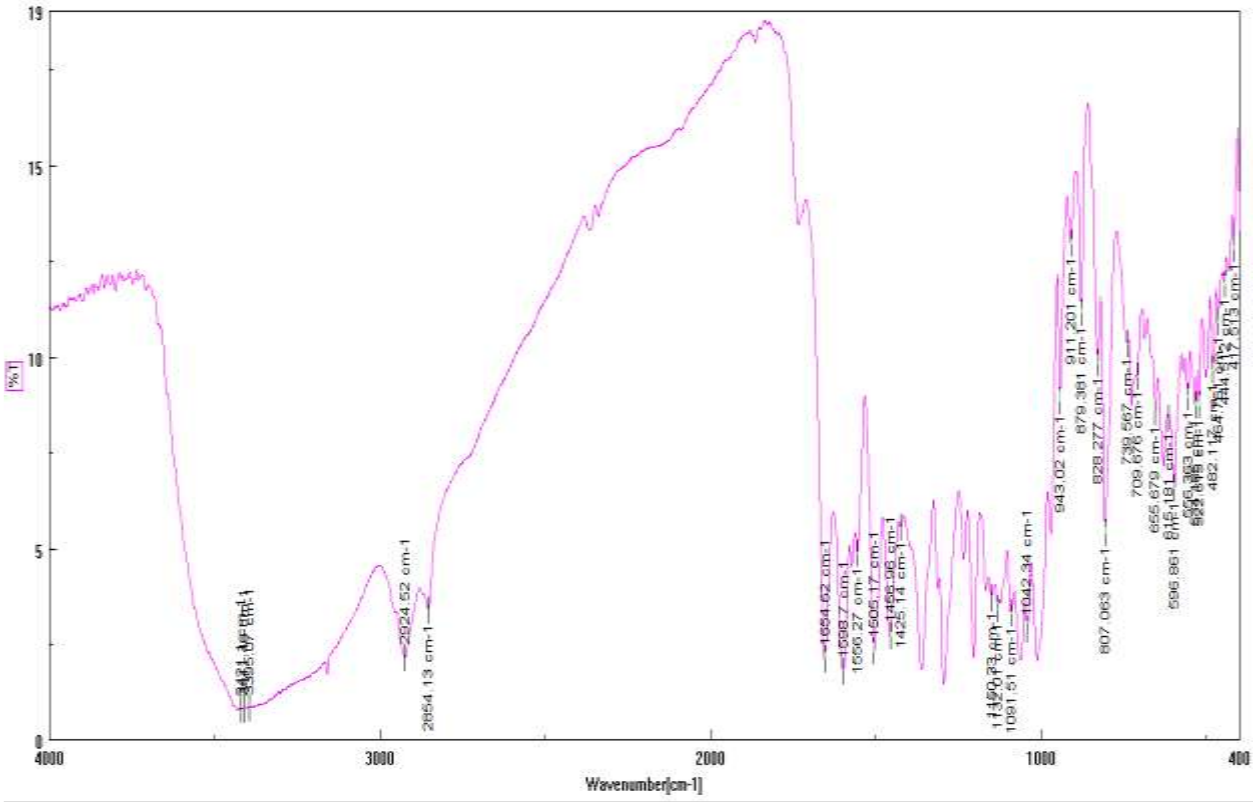


Fig. No: 11. FTIR of isolated component (quercetin) obtained from ACA.

Table No. 12. FTIR data of isolated component from ACA.

Sr.No.	Peak values(cm^{-1})	Functional group	Assignment
1.	**3395**	**Tertiary alcohol (Ar-OH bonded)**	**O-H stretch**
2.	**2924**	**Alkanes**	**C-H stretch**
3.	**2854**	**Alkanes**	**C-H stretch**
4.	**1654**	**Alkenes**	**C=C stretch**
5.	**1556**	**Carboxylic acid**	**R-C-O-H**
6.	**1456 & 1425**	**Aromatic carbon (C-C in ring)**	**Aromatic C=C stretch**
7.	**943**	**Tertiary alcohol (Ar-OH bonded)**	**O-H bending**
8.	**828**	**Aromatic C-H**	**Out of plane bending**

Discussion:-

The above FTIR spectrum indicates that functional group of standard quercetin according to reference & isolated compound is approximately same. So according to IR spectrum quercetin may present [18].

C) Nuclear Magnetic Resonance:-

i) NMR (^{1}H):-

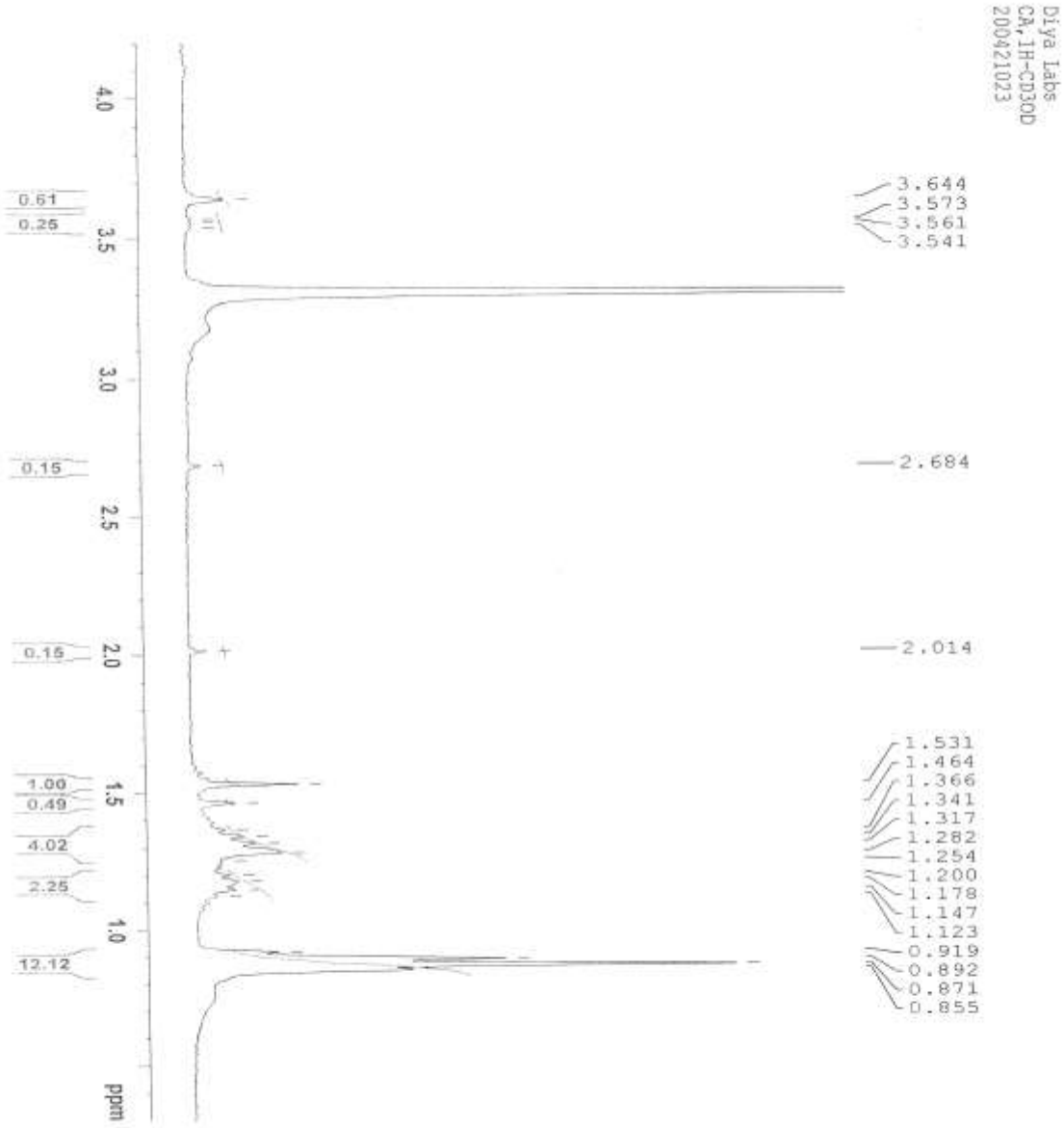


Fig. No: 12. ^{1}H NMR of isolated component (quercetin) obtained from ACA.

Table No. 13. Assignment of ^{1}H NMR peaks for quercetin moiety

Sr.No.	Functional group	Type of peak	δ value in ppm
1.	One proton of alkyl group	triplet	0.8
2.	One Proton of alkyl group	singlet	1.25
3.	One proton of alkene group	singlet	2.01
4.	One proton of aromatic ring	singlet	2.68
5.	One proton of –OH group	singlet	3.64
6.	One proton of –OH group	singlet	3.57
7.	One proton of –OH group	singlet	3.56
8.	One proton of –OH group	singlet	3.54

Discussion:-

The above ^{1}H NMR spectrum indicates that no. of protons of standard (quercetin) & isolated compound is approximately same. So according to this ^{1}H NMR spectrum quercetin may present [18]

ii) NMR (^{13}C):-

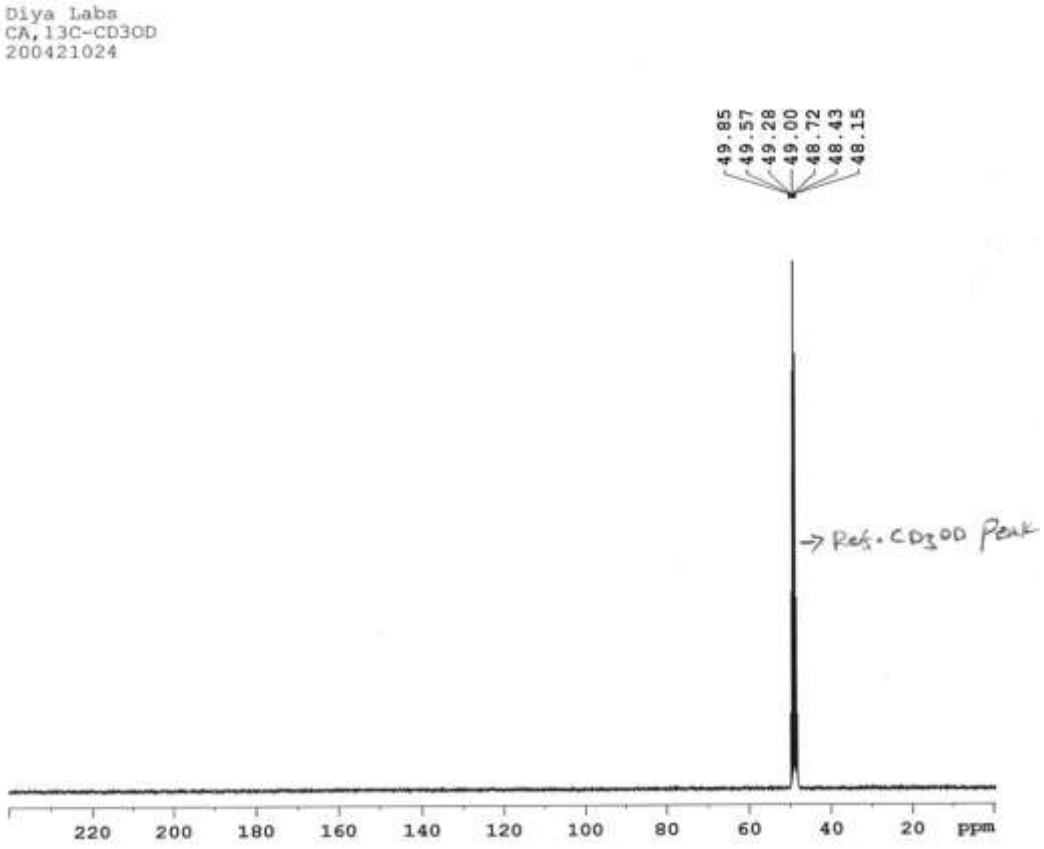


Fig. No: 13. ^{13}C NMR of isolated component (quercetin) obtained from ACA.

Table No. 14. Assignment of ^{13}C NMR peaks for quercetin moiety

Sr.No.	Functional group	Type of peak	δ value in ppm
1.	One carbon of alcoholic group	Singlet	48.15
2.	One carbon of alcoholic group	Singlet	48.43
3.	One carbon of alcoholic group	Singlet	48.72
4.	One carbon of alcoholic group	Singlet	49.00
5.	One carbon of ketone group	Singlet	49.28
6.	One carbon of phenyl group	Singlet	49.57
7.	One carbon of carbonyl group	Singlet	49.85

Discussion:-

The above ^{13}C NMR spectrum indicates that no. of carbons of standard (quercetin) & isolated compound is approximately same. So according to this ^{13}C NMR spectrum quercetin may present [18].

D) Mass Spectroscopy:-

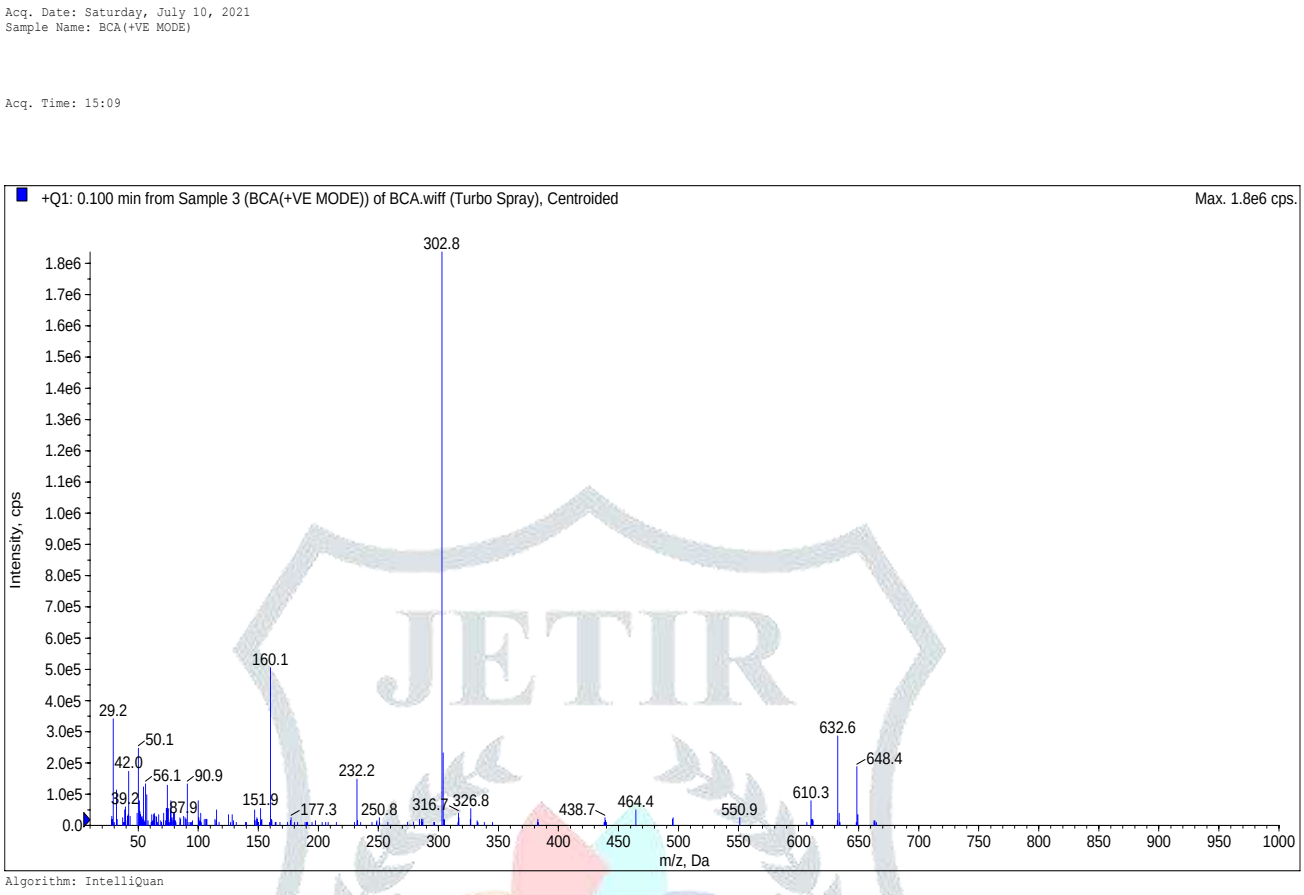


Fig. No: 14. Mass Spectrum of isolated component (quercetin) from ACA.

Discussion:-

According to above mass spectrum we can say that they may of quercetin, because molecular weight of standard (quercetin) & isolated compound molecular weight is approximately same [18].

4. Conclusion:-

Ethanolic & aqueous extract of Capsicum annuum L. var. grossum Sendt. showed presence of flavonoids, phenolic compound & vitamin c. But aqueous extract showed maximum immuno-modulatory activity by both In-vivo & In-vitro methods.

First performed Thin Layer chromatography & then flavonoid quercetin was isolated from aqueous extract of *Capsicum annuum* L. var. *grossum* Sendt. by column chromatography technique. Its presence was confirmed by spectroscopic analysis (UV, IR, NMR & MS) techniques.

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