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INFLUENCE OF STACHYDRINE ON IMMUNOMODULATION IN MICE

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Keywords:

Capparis sepiaria, Stachydrine, Phagocytosis, plaques, Immunostimulation

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ABSTRACT: The Present study was planned to gauge the immune-modulatory activity of the ethanolic extract and the isolated compound Stachydrine from the leaves of *Capparis sepiaria* L. (Capparidaceae). The study was extended to research the chemical compounds within the EECS (Ethanolic Extract of *Capparis sepiaria*). The preliminary phytochemical screening revealed that EECS evidenced the presence of varied phytoconstituents like alkaloids, glycosides, reducing sugars, flavonoids, saponins, starch and terpenoids. The alkaloidal compound Stachydrine was isolated and further evaluated by using UV, IR and NMR. Both *in-vitro* and *in-vivo* studies were administered to gauge the immunomodulatory effect of ethanolic extract of *C. sepairia* and the isolated alkaloid Stachydrine using Phagocytosis and Jerne plaque assay. Both ethanolic extract (25.82% for 70mcg/ml) and the isolated compound (21.28% for 25 mcg/ml) showed the enhancement of phagocytic index compared to regulate (18%) also because the number of plaques proportional to the amount antibody-producing lymphocytes within the cell population after the treatment with extract of *C. sepiaria* (551.25± 4.26 and 620 ± 7.25 for the 100mg/kg and 150 mg/kg) and the isolated Stachydrine (560± 4.32 for 25mg/kg). The result indicated that the extract showed dose-dependent immunostimulant activity and the isolated compound showed no immunostimulant activity.

INTRODUCTION: An increase or decrease in the magnitude of immune response is a process of describing Immunomodulation. The immune-stimulants are the compounds that enhance immune reactions and are involved in nonspecific system stimulation [1]. In contrast, the compounds capable of decreasing the resistance against infections and stress caused by environmental factors or chemotherapy are immunosuppressive [2].

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Medicinal plants with immunomodulatory activity are used for cases of organ transplant rejection or treatment of various autoimmune diseases [3, 14, 15, 16, 17] and are also capable of inhibiting the cellular and humoral responses in the treatment of immunological disorders Capers, comprising of species of the genus Capparis Linn. (family-Capparaceae) [4] reduce flatulence and have anti-rheumatic effects in ayurvedic medicine; capers are recorded as hepatic stimulants and protectors, improving liver function. They have been used to treat arteriosclerosis, as diuretics, disinfectants, vermifuges, and tonics. Traditionally the root bark of the caper has been used for anaemia, arthritis, and gout. Capers contain considerable amounts of the antioxidant bioflavonoid, rutin.

Capparis sepiaria is an astringent and used in cardiac troubles and biliousness. *Capparis sepiaria* a closely related species, is a dense armed handsome shrub, distributed throughout India and hence known as the Indian Caper. It has coriaceous leaves, white flowers, and ovoid to ellipsoid berries [5].

Hence, the present paper deals with the immunomodulatory activity of ethanolic extract of leaves of *Capparis sepiaria* and the isolated alkaloid Stachydrine. We decided to examine its effects on macrophage activation, which was determined by assays phagocytic index and antigen-specific plasma cells by plaques formation.

MATERIALS AND METHODS:

Plant Material: The healthy leaves with petiole of C. *sepiaria* were collected from Sirumalai, Kodai road, Tamilnadu. The taxonomist of the American College, Madurai, Tamilnadu, India, authenticated the plants.

Preparation of Plant Extract: The leaves with petiole of *Capparis sepiaria* were washed thoroughly in tap water, shade-dried and powdered. The powder (1.5 kg) was successively extracted with double quantity of ethanol by maceration for 24, 48 and 72 hours through occasional shaking. A rotary flash evaporator then concentrated the filtrate. The yield of the extract was found to be 5.12 % (W/V). This crude extract was referred to as *Capparis sepiaria* (EECS).

Phytochemical Screening: The Preliminary phytochemical screening was carried out for the EECS by following the methods of Harborne[6] Trease and Evans [7] **Table 4.**

Isolation of Stachydrine Alkaloid: The brown-colored dried extract was mixed with 1:1 acidulated water (Hydrochloric acid: water) and set aside for 12 hrs, then it was filtered. To the filtrate 250 ml of strong ammonia was added and set aside for 6 hours.

Then the precipitate was collected using Whatman No.1 filter paper and dried in a desiccator. The dried substance was recrystallized using hot chloroform and then evaporated. The white colour residue was further dried tested using Dragendorff's reagent, Mayer's reagent and confirmed by TLC, UV, IR and ^{13}C-NMR.

Immunomodulatory Studies:

Animals: Female mice of CD1 strain weighing 20–25 g were obtained and kept under strict ethical conditions according to international guidelines for the care and use of laboratory animals. All experiments involving animals comply with the ethical standards of animal handling and are approved by the Institutional Animal Ethics Committee (IAEC/XXXIV/SRU/284/2013), Sri Ramachandra Institute of Higher Education and Research, Chennai, Tamil Nadu.

Evaluation of Phagocytosis *In-vitro* [8, 9]:

Materials: *Candida albicans* suspension, Hank's balanced salt solution (HBSS), Giemsa stain, ethanolic extract, Stachydrine alkaloid.

Preparation of *Candida albicans* Suspension: *C. albicans* was incubated overnight in sabouraud's agar broth and then centrifuged to form a cell button. The suspension was discarded. The cell button was washed with sterile HBSS for 4-5 hrs. The last cell button was mixed with sterile HBSS and human serum in a proportion of 4:1 and cells were properly mixed in vortex. The cell suspension of a concentration of $1x10^8$ was used for the experiment.

Slide Preparation: Human blood (0.2ml) was obtained by finger prick method in a sterile glass slide. Slides in triplicates were incubated at 37° C for 25 minutes to permit clotting. The grume was gently removed of the slide and drained carefully with normal saline taking care not to wash the adhered neutrophils.

A monolayer of polymorph nuclear leucocytes (PMN) was flooded with a predetermined concentration of the test extract (50 mcg/ ml, 60 mcg/ ml, 70 mcg/ ml) and the Stachydrine (25 mcg/ ml) for quarter-hour at 37° C. The PMN were covered with *C. albicans* for 1 h. The slide was drained, fixed with methanol and stained with Giemsa stain.

Phagocytosis Evaluation: The mean number of Candida cells phagocytosed by PMN on the slide decided microscopically for 100 granulocytes using morphological criteria. This number was taken as phagocytic index (PI) and was compared with PI of control (plate 2). Immunostimulation (%) was calculated by using the following equation.

% immunostimulation = PI (test) – PI (control) / PI (control) × 100

The results were tabulated- **Table 1**

TABLE 1: PHAGOCYTOSIS EVALUATION

S. no.	Concentration of drug	Phagocytic index	% stimulation
1.	Control	33.33 ± 2.1	
2.	50 mcg/ml	37.56 ± 2.3	19.89
3.	60mcg/ml	38.33± 3.19	23.62
4.	70mcg/ml	39.52± 2.6	25.82
5.	Stachydrine 25mg/ml	37.32± 3.2	22.32

Results are expressed as mean ± S.D. P < 0.001vs control.

Enumeration of Antigen-Specific Plasma Cells by Jerne Plaque Assay [10, 11]:

Preparation Beforehand: Eight groups of mice were housed under standard environmental conditions. They were grouped as

G1= Control

G2 = SRBC (antigen) alone treated

G3 = SRBC + extract treated (100 mg/kg)

G4 = SRBC + extract treated (150 mg/kg)

G5= SRBC + Stachydrine treated (25 mg/kg)

On day 15 all animals of G2 to G5 were immunized with 0.25 ml of 2% SRBC. After 3,5 days of immunization animals were sacrificed, blood was collected and the spleen were isolated.

Agar Underlay: Makeup 1.4% DifcoBacto agar in Hank's saline. Agar was melted in a microwave oven and then directly over a Bunsen flame to remove all the lumps if necessary. Care was taken to swirl the agar gently to avoid charring.

Enough agar solution was added to a 5 cm plastic petri dish to just cover its base. An underlay was wont to confirm that the lowest of the assay dish was reasonably flat, so pouring must be done on a levelled surface.

Agar Overlay: 7% agar solution in Hank's saline was prepared to contain 0.5mg/ml of diethylamino ethyl (DEAE) -Dextran (final concentration). DEAE – dextran was wont to prevent anti-complementary activity of the agar.

Materials Required: SRBC – immunized mice Sheep blood in Alsevier's solution, Agar overlay, Petri dishes containing agar underlay, Hank's saline, without phenol red indicator dye, Water bath, 45°, Guinea-pig serum, as a complement source

Method:

1. SRBC was washed 3 times in Hank's saline by centrifugation (300 g for 10 minutes at room temperature).
2. Spleens were prepared into one suspension by teasing them apart with ice-cold saline forceps. The fibrous connective tissues were discarded.
3. Suspension was sucked in and out of a Pasteur pipette to disperse the cells; it is not usually necessary to let the cells stand to settle out the tiny aggregates.
4. Volume adjusted to a whole of 2.5 ml in Hank's saline.
5. 1 ml of spleen-cell suspension was diluted 1:10 with hank's saline.
6. 0.8 ml was pipetted out of overlay into small test tubes in a 45° C water bath.
7. 0.25 ml of the first or diluted spleen-cell suspensions were added to every assay dish
8. Dish was placed on levelled surface.
9. 0.15 ml of SRBC suspension was added to every overlay tube just before use. Mixed well by flicking the end of the tube.
10. Overlay was added to dish and mixed thoroughly with spleen cells.
11. Agar was allowed to line and 1.0 ml of a 1:10 dilution of guinea pig serum as a source of complement was added.

12. Dishes were incubated at 37° C for 1-1.5 hrs. If the plaques were not clear when the dishes were far away from the incubator, allow them to face at temperature for about half-hour before counting. The results were calculated by using the following formula and tabulated in **Table 2.**

TABLE 2: JERNE PLAQUE ASSAY

Days	Control	SRBC (Antigen control)	100 mg/kg + antigen	150 mg/kg + antigen	Stachydrine + antigen
3	450.25±0.945	463± 1.75	520 ± 7.35	559.25± 3.25	454± 1.825
5	450.25±0.945	465±1.85	551.25± 4.25	620 ± 7.25	458.75±1.492

p< 0.001 vs antigen control all the others are non-significant.

Number of Plaques per 10^6 lymphocytes = Number of plaques /spleen x $10^{6\,/}$ Number of cells /spleen

Haematological Profile and Body Weight Determination: The next haematological parameters and weight were determined in Swiss male albino mice in 4 groups treated orally for 10 days with extract (100 mg/kg, 150 mg/ kg and 200 mg/kg / day of ethanolic extract and 25 mg/ kg.

1. Haemoglobin content

2. Haematocrit value

3. Total WBC count

4. Total RBC count

These parameters were determined on the day 10 of drug administration.

The results were tabulated **Table 3.**

TABLE 3: HAEMATOLOGICAL PROFILE OF ECS AND STACHYDRINE TREATED MALE ALBINO MICE

Parameter	Extract treated				
	Control – 5% tween 80	100 mg/kg	150 mg/ kg	200 mg/kg	Stachydrine (25 mg/ kg)
Body weight (gm)	20.15 ± 0.34	20.42 ± 0.38	20.05 ± 0.62	20.20 ± 0.25	20.1± 0.2
Haemoglobin (gm%)	13.2 ± 0.52	14.42 ± 0.85	16.53 ± 0.72	17.8 ± 0.64	19.1 ± 0.12
Haematocrit value (%)	46.6 ± 0.82	48.16 ± 0.65	52.4 ± 0.38	55.18 ± 0.42	46.32 ± 0.62
Total WBC (10^3/ mm^3)	3.9 ± 0.1	5.2 ± 0.28	5.6 ± 0.25	6.5 ± 0.57	3.8 ± 0.1
Total RBC (10^6/ mm^3)	8.3 ± 0.42	9.6 ± 0.25	10.2 ± 0.28	13.2 ± 0.8	8.2 ± 0.2

S. D. P < 0.001

RESULTS & DISCUSSION:

Phytochemical Studies: The Preliminary phytochemical screening revealed the presence of alkaloid (Stachydrine), Carbohydrates, Phyto-sterols, Proteins and free amino acid, Saponins, flavonoids, terpenoids and cyanogenetic glycosides and the absence of other glycosides, gums and mucilage, volatile oils and fixed oil.

*+ indicates -Positive reaction, - Indicates - Negative reaction. An attempt had been made to isolate and characterize the alkaloid (Stachydrine) which is water soluble. The melting point of the isolated compound was 235° C. Chemical tests, alkaloids, TLC, UV, FTIR and ^{13}C NMR spectral studies were also carried out to characterize the compound. It was identified that the compound may be Stachydrine, a 2-carboxylic acid substituted pyrrolidine derivative.

1. UV - ƛ max at 208 nm & 228 nm

2. ^{13}C NMR Spectroscopy

129.522 ppm – Aromatic

169.212 ppm – COOH

TABLE 4: PRELIMINARY PHYTOCHEMICAL SCREENING

S. no.	Phytochemical test	Result
1.	Alkaloids	
	(a) Mayer's Reagent	+
	(b) Dragendorff Reagent	+
	(c) Hager's Reagent	+
	(d) Wagner's Reagent	+
2.	Carbohydrates	
	(a) Molisch's Reagent	+

	(b) Fehling's Reagent	+
	(c) Benedict's Reagent	+
3.	Glycosides	
	(a) Anthraquinone	-
	(b) Cardiac	-
	(c) Cyanogenetic	+
4.	Phytosterols	
	(a) Salkowski test	+
	(b) Liebermann Burchard test	+
5.	Saponins	+
6.	Tannins	-
7.	Proteins and free amino acids	
	(a) Millon's test	+
	(b) Biuret test	+
8.	Gums and Mucilage	
	Ruthenium red solution test	-
9.	Flavonoids	
	Shinoda teat	+
10.	Terpenoids	+
11.	Volatile oil	-

TABLE 5: IR RESULTS

Molecular Structure	Spectral peaks (cm^{-1})
C-H bending and four adjacent hydrogen atoms and two substituted pyrrole type	748.6
C- O stretching	1104.1
C-N stretching	1636
Carboxylate anion	1558

Pharmacological Studies: Immunomodulatory activity of ECS and Stachydrine of the leaves were studied. Plant products are becoming increasingly popular for a variety of diseases in recent times.

A factor that influences the recovery from such an infective process is the host defence mechanism. Immunomodulatory agents of plant origin enhance the immune responsiveness of an organism against a pathogen by non-specifically activating immune system. In the present study *in-vitro* and *in-vivo* immunomodulatory studies were performed.

Evaluation of Phagocytes:

***In-vitro* Study:** Phagocytosis categorised under innate immunity that largely involves granulocytes (PMN) and Macrophages.

ECS (50,60,70mcg/ml) enhances phagocytic index 19.89, 23.62 and 25.82 % of stimulation when compared to control but Stachydrine (18.5% only) near to control and an increase in the percentage stimulation was shown in **Table 6, Fig. 1.** The preliminary observation showed that probably the ethanolic extract possesses immunostimulation.

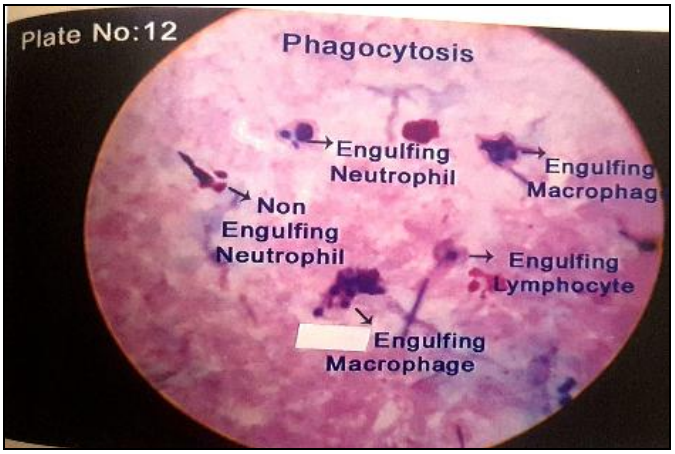

FIG. 1: PHAGOCYTOSIS

TABLE 6: EFFECT OF ECS AND STACHYDRINE ON NEUTROPHIL PHAGOCYTOSIS BY SLIDE METHOD OF BORNE

S. no.	Concentration of drug (mcg/ml)	Phagocytic index	% Stimulation
1.	Control	33.33 ± 2.1	
2.	50mcg/ml	37.56 ± 2.3	19.89
3.	60mcg/ml	38.33 ± 2.19	23.62
4.	70 mcg/ml	39.52 ± 2.43	25.82
5.	Stachydrine	34.22 ± 2.1	18.50

Results are expressed as mean ± SD.

TABLE 7: JERNE PLAQUE ASSAY

Days	Control	SRBC (antigen control)	100 mg/kg+ Antigen	150mg/kg + Antigen	Stachydrine + Antigen
3	450 ± 0.945	463± 1.75	520 ± 7.35	559.25± 3.25	454 ± 1.825
5	450 ± 0.945	465 ± 1.85	551.25± 4.25	620 ± 7.25	458.75 ±1.492

Values are mean ±SD.

The Enumeration of Antigen-Specific Plasma Cells in Jerne Plaque Assay: It was revealed that the increase in the number of antibody-producing cells in the spleen and it will potentiate the humoral responses as evidenced by increasing antibody-secreting cells.

On 3rd Day: It was observed that the antigen-alone treated group and Stachydrine treated group produced less number of plaques (463 ± 1.75 plaques / million cells and 454± 1.825 plaques / million cells respectively) while the extract treated (100 mg/Kg, 150 mg/Kg) showed satisfactorily significant (P<0.001 *vs* antigen control) enhancement of number of plaques formed (*i.e.* 520±7.35, 559.25±b3.25 plaques / million cells respectively). The extract alone treated showed number of plaques (452.25±0.79) almost same as that of control.

On 5th Day: Again there was a gradual increase in the plaque-forming cells in the spleen for extract treated (100 mg/Kg & 150mg/Kg – 55.25 ± 4.26 and 620 ± 7.25) while the antigen control and Stachydrine group still showed no changes.

The results showed an immunostimulation since the number of plaques obtained is proportional to the number of antibody-producing lymphocytes in the cell population **Fig. 2** [12].

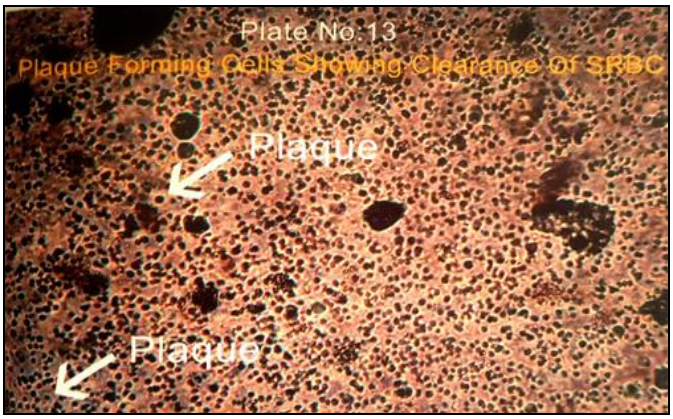

FIG. 2: JERNE PLAQUE ASSAY

The effect of ethanolic extract (ECS) and Stachydrine of the leaves of *C. sepiaria* on haemoglobin and Haematocrit value, total WBC counts and RBC counts were evaluated.

It was observed that significant increase in total WBC and RBC counts, haemoglobin percentage, Haematocrit values in the ECS treated and no longer in the Stachydrine-treated group. No change in body weight was observed during the treatment period **Table 3.**

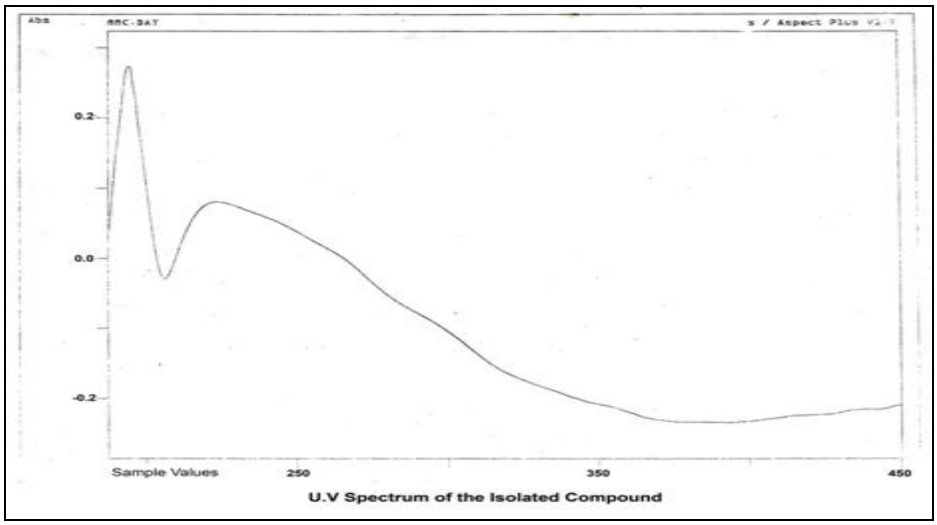

FIG. 3: UV SPECTRA FOR THE ISOLATED STACHYDRINE

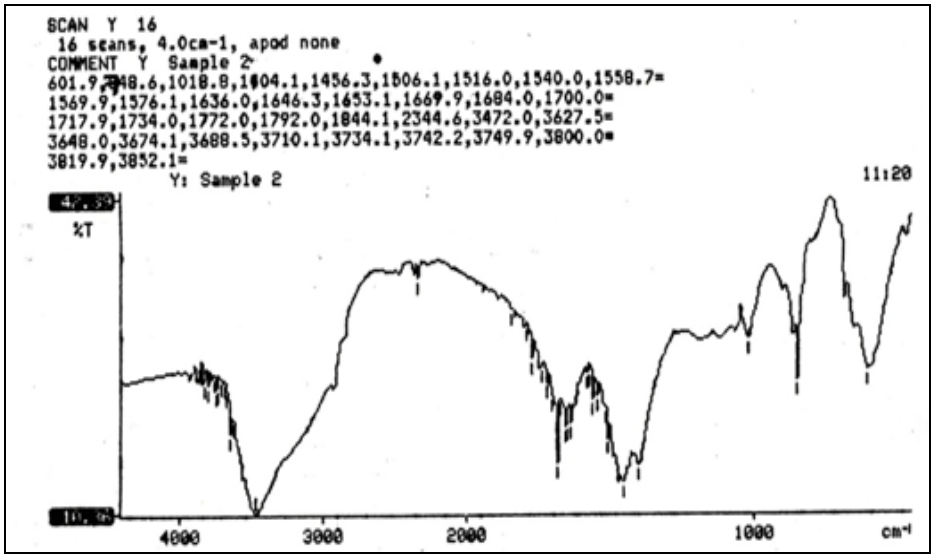

FIG. 4: IR SPECTRUM OF THE ISOLATED COMPOUND

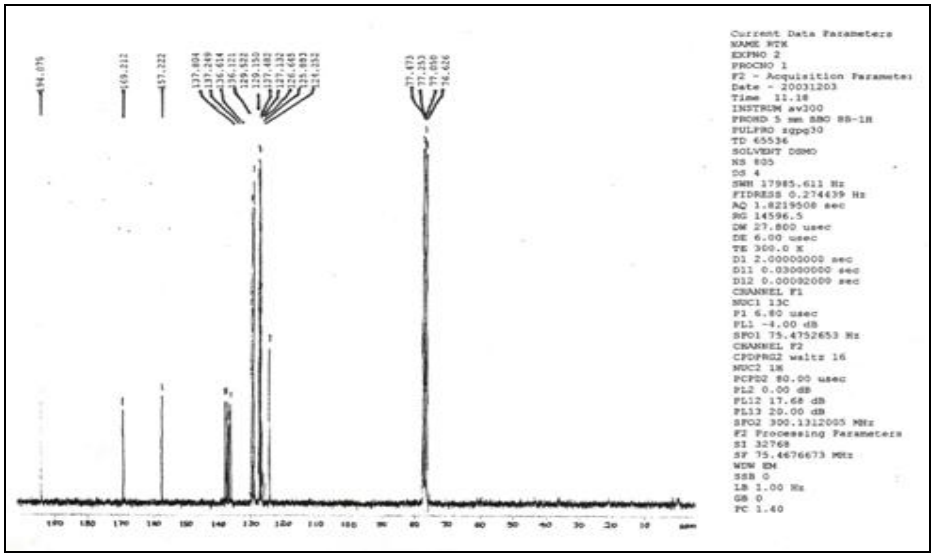

FIG. 5: C NMR SPECTRUM OF THE ISOLATED COMPOUND

CONCLUSION: Human immune system is a tangled interaction of many impulsive cellular and organic chemistry portions [18]. Pathophysiological conditions are caused by alternation in exogenous and endogenous in dynamic immune system. The immune response acts as capsaicin, andro-grapholide, genistein, flavopirido immune-suppressants or enhance the efficacy of vaccines using immunoadjuvants by the immunomodulators which could could either induce stimulation or amplification of the immune response [19].

Most synthetic immunomodulators pose serious toxicity or other side effects than the plant-derived immunomodulators and considered safe [13]. Several commonly known plants like *Acorus calamus, Actinidia macrosperma, Allium sativum, Alpinia galangal, Alternanthera tenella, Andro-graphis paniculata, Angelica sinensis, Argyreia speciosa, Averrhoa bilimbi, Azadirachta indica, Boerhaavia diffusa, Catharanthus roseus, Centella asiatica, Codonopsi spilosula, Cynodon dactylon, Curcuma longa, Ficus benghalensis, Ganoderma tsugae, Morus alba, Murraya koenigii, Ocimum sanctum, Panax ginseng, Pelargonium graveolens, Phyllanthusemblica, Picrorhiza scrophulariiflora, Senegalia catechu, Terminalia arjuna, Tinospora cordifolia, Tridax procumbens, Withania somnifera, Zingiber officinale, etc.* are currently being investigated for their immunomodulatory potentials. On the other hand, many plant-derived bioactive compounds like flavonoids, iridoid glycosides, polysaccharides, saponins, terpenes, cordifolioside A, syringing, curcumin, resveratrol, epigallocatechol – 3 - gallate, quercetin,

colchicinel, combretastatin, lycopene, dendroside, dendronobilosides, *etc*. play an immunomodulatory role. Medicinal plants induce a nonspecific widespread modulation of the immune system, affecting various immune cells, namely, macrophages, natural killer cells, and granulocytes.

The above result showed that the ethanolic extract of *C. sepiaria* showed dose-dependent immunostimulant activity, while the Stachydrine alkaloid showed no activity.

Hence, a better understanding of the immune-modulatory roles of plants, their mode of action, and phytoconstituents would enable us to identify lead compounds from natural sources to develop novel and safe immunomodulators that can fortify current therapies.

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CONFLICTS OF INTEREST: The authors declare no conflicts of interest.

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