

Anti-inflammatory activity of *in vitro* regenerated calli and *in vivo* plant of *Stevia rebaudiana* (Bert.) Bertoni

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Abstract- Anti-inflammatory activities of methanolic extract of callus and intact plant part of *Stevia rebaudiana*(Bert.) Bertoni was studied for the first time. Acute inflammation model, carrageenan induced rat hind paw edema model was employed to investigate the anti-inflammatory activity. The methanolic extract from fresh callus cultures of *Stevia rebaudiana* cultured on Murashige and Skoog (MS) medium fortified with BAP (2.0 μ M) and α -NAA(1.0 μ M) for 2 - 12 weeks was studied and compared with intact plant extract. Results from the anti-inflammatory activity testing indicated that the callus extract showed a less significant inhibition in the edema as compared to extract of intact plant. It was also revealed that there is an increase in the accumulation of secondary metabolites in callus cultures of sixth week of incubation. The methanolic extract of callus culture and intact plant part exhibited significant, dose-dependent activity on the tested experimental animal models and produced a significant inhibition of carrageenan- induced paw edema in rat. The study suggests that the plant *Stevia rebaudiana* is also a potent source for phytochemistry development in future.

Index Terms- Callus culture, Carrageenan, Paw edema, Inflammation, *Stevia rebaudiana* Bert., *in vitro*, *in vivo*. Medicinal plant

Abbreviation- α -NAA (α -Naphthaleneacetic acid), MS (Murashige and Skoog), CMC (Carboxymethylcellulose), BAP (6-Benzylaminopurine), SPL (*Stevia rebaudiana* Bert. leaves), SPC (*Stevia rebaudiana* Bert. calli), SP(P) (*Stevia rebaudiana* plant intact/parts), 2,4,5-T (Trichlorophenoxy acetic acid), ROS (Reactive oxygen species).

I. INTRODUCTION

Inflammation is a very complex, multifactorial and dynamic process involving many systems which is closely associated with the process of repair. Inflammation is defined as local response of living mammalian tissue to injury due to any agent and manifests usually in form of painful swelling associated with some changes in skin covering the site. Inflammation can be classified either as acute or chronic. Acute inflammation is the initial response of the body to harmful stimuli and is due to the increased movement of plasma and leukocytes from the blood into the injured tissues. Chronic inflammation is due to a progressive shift in the type of cells which are present at the site of inflammation which is characterized by simultaneous destruction and healing of the tissue from the inflammatory process. Carrageenan-induced paw edema model is widely used for determining the acute phase of the inflammation. Histamine, 5-hydroxytryptamine and bradykinin are the first detectable mediators in the early phase of carrageenan-induced inflammation whereas prostaglandins are detectable in the late phase of inflammation [1,2].

Plant based traditional medicine system continues to play an essential role in health care with about 80% of world's inhabitants relying on it for their primary health care [3,4]. *Stevia rebaudiana*(Bert.)Bertoni 'the sweet herb of Paraguay' is an herbaceous perennial belongs to the Asteraceae family. *Stevia* contains a number of diterpenesteviol glycosides which are about 300 times sweeter than sucrose at their concentration of 4% (w/v) [5]. Besides its sweetening property *Stevia* is also known for its medicinal properties [6,7] which comes from a variety of constituents besides the steviosides and rebaudiosides. This includes sterols, triterpenes, flavonoids, tannins, and an extremely rich volatile oil comprising rich proportions of aromatics, aldehyde, monoterpenes and sesquiterpenes.

In India and in other countries scientists have developed micropropagation protocol for the regeneration of *Stevia* through different *in vitro* regeneration pathways viz. bud induction [8], somatic embryogenesis [9,10] and organogenesis from callus culture [8,11]. Callus initiation in *Stevia rebaudiana* is reported from different explants viz. leaves shoot tip and shoots discs [9,10,12]. Fewer reports are available on the regeneration of *Stevia* from unorganized callus tissues derived from different explants by dedifferentiation induced by exogenous growth regulators [11,13]. Another popular aspect of callus culture is the production of secondary metabolite as it cannot be synthesized economically on commercial basis [14].

Since micropropagation of *Stevia rebaudiana* is one way to increase the biomass of this medicinal plant, Therefore, the primary aim of present investigation was to develop a well standardize micropropagation protocol for *Stevia* through callus culture technique and to investigate the anti – inflammatory activity of plant and callus extracts of *Stevia*.

The investigation of plants for their anti-inflammatory activities can play an important role in drug development program in the pharmaceutical industries.

The aim of present investigation was to develop callus induction and to investigate

II. MATERIALS AND METHODS

Plant Materials

Young leaves and shoots of *Stevia rebaudiana*(Bert.) Bertoni were used as explant to initiate callus culture and the leaves were used for the extract preparation.

Drugs and Chemicals

The drugs and chemicals used were Carrageenan (Sd Fine Chemicals Limited, Mumbai), Gum acacia, Indomethacin (Indocap, Jagsonpal Pharmaceuticals Ltd., Faridabad) and Methanol (BDH, Mumbai). Murashige and Skoog medium (Himedia Laboratories Pvt. Ltd., Mumbai), Agar Type 1 (Central Drug House Pvt. Ltd. New Delhi), Sucrose (RFCL Ltd., New Delhi), α -Naphthalene acetic acid (Central Drug House Pvt. Ltd., New Delhi) and 6-Benzylaminopurine (Sigma, China).

Animals

A permission from Institutional Animal Ethics Committee was granted and after wards male Wistar rats (150-250 g) were maintained under standard husbandry conditions and acclimatized to the laboratory environment for a period of one week prior to the experimental session. All the animals were divided into different groups each consists of six animals. The experimental animal models were made to fast overnight prior to the experiments.

Callus Culture Conditions

Young leaves and stem of *Stevia rebaudiana*(Bert.) Bertoni rinsed with distilled water and were sterilized in 70% ethanol for 1 min and then followed by five times washing with sterile distilled water and explants were aseptically cut in segments of 0.5 cm length. These explants were cultured in sterile polystyrene flasks containing 40 ml of MS medium. The medium was fortified with a mixture of BAP (2.0 μ M) and α -NAA(1.0 μ M) prior to autoclaving at 120°C (1.5 Kg/cm²) for 30 min, the pH of all media was adjusted to 5.8 with 0.1N NaOH. All cultures were incubated at 25°C with photoperiod for 16 h and darkness for 8 h in a culture room. The initiated calli were routinely sub-cultured onto a fresh (multiplication) medium fortified with same concentration of growth regulators as in initiation medium to proliferate the calli at faster rate.

Measurements

Fresh weights of the calli were recorded immediately after the cultivation time and dried weights were determined by drying to a constant weight at 60°C in an oven for 24 h.

Preparation of Extracts

The plant leaves were shade dried, powdered and extracted with methanol while calli were dried in oven at 35°C and extracted with methanol. The extracts were filtered and concentrated and kept in a vacuum desiccator for complete removal of the solvent. Methanol extract of leaves and calli were obtained in the yield of 10.04% and 52.01% w/w respectively. The extracts were stored at 4°C in glass vials for use in anti-inflammatory activities.

Phytochemical Screening

The methanolic extract was examined for the presence of various phyto-constituents like alkaloids, tannins, flavonoids, phenolics and glycosides by employing standard phytochemical tests[15]

Anti-inflammatory Activity

The animals were divided into six groups. Group I (n=6) served as Control, received the vehicle only (1% CMC, 10 ml/kg oral dose). Group II (n=6) served as Standard, received Indomethacin at dose of 1 mg/kg body weight. Group III (18 animals in subgroups of 6), Group IV (18 animals in subgroups of 6) served as test, received methanolic extract of *Stevia rebaudiana*(Bert.) Bertoni leaves (SPL) and *Stevia rebaudiana*(Bert.) Bertonicalli (SPC) at oral doses of 100, 200 and 400 mg/kg body weight respectively.

Paw Edema Induction

Carrageenan induced paw edema was used to determine the anti-inflammatory activity of the extracts [16]. The animals pretreated with extract or Indomethacin one hour before were injected with 0.1 ml of 1% Carrageenan (in 1% CMC) solution into the sub-plantar region of right hind paw. Paw volume was measured by dislocation of the water column in a Plethysmometer (UgoBasile, Italy) immediately after Carrageenan application at 0, 1, 2, 3, 4 and 6 h after the stimulus. Reduction in the paw volume compared to the vehicle-treated control animals was considered as anti-inflammatory response.

The percentage inhibition of edema was calculated as follows:

$$\text{Percentage of Inhibition} = \frac{(V_T - V_O) \text{ Control} - (V_T - V_O) \text{ Treated group}}{(V_T - V_O) \text{ Control}} \times 100$$

V_O= paw volume of the rat before administration of Carrageenan.

V_T= paw volume of the rat after the administration of Carrageenan at different time intervals.

Percentage inhibition of paw edema was found to be proportional to anti-inflammatory activity.

III. RESULTS AND DISCUSSION

MS medium fortified with BAP (2.0 μM) and α -NAA(1.0 μM) was found best for initiation and multiplication of callus from leaf and shoot explant. The callus cultures were maintained in dark conditions in a culture room. For further multiplication the callus were transferred on to the multiplication medium. The physical appearance of all calli was friable and ranging from yellow to brown. **Figure 1** shows that the growth of different callus cultures (2, 4, 6, 8, 10 and 12 weeks) expressed as the increase or decrease in fresh and dry weights. The weights of fresh callus extracts significantly increased with the duration of cultivation and then decreases. The dried callus weight was maximum of sixth week of callus culture. The maximum secondary metabolites were also seen in the sixth week incubation of callus culture. After extraction, the extracts were subjected to qualitative phytochemical tests (

Table 1).

The anti-inflammatory effect of the extract and the reference drug in carrageenan induced paw edema model in rats is shown in **Table 2**. Carrageenan induced paw edema in rats reached to a peak value at 4 h and graded doses of methanolic extract of plant and callus produced a significant inhibition in the edema volume ($P < 0.001$). The leaf extract at the test doses 100, 200 and 400 mg/kg body weight reduced the edema induced by carrageenan by 28, 37 and 42% respectively at 4 h and callus extract reduced the edema by 25, 28 and 34% respectively at 4h, whereas the standard drug showed 85% of inhibition as compared to the control group. All the doses of methanolic extract of SP(P) are significantly inhibit the paw edema in rats than all the doses of SP(C). The study suggests the methanolic extracts of intact plant part of *Stevia rebaudiana* has more potent inhibition than callus culture for carrageenan induced paw edema in rat.

Carrageenan induced paw edema is widely used for determining the acute phase of the inflammation [1] and is characterized by biphasic event with involvement of different inflammatory mediators. In the first phase (during the first 2 h after carrageenan injection), chemical mediators such as histamine, dextran and serotonin play role, while in second phase (3-4 h after carrageenan injection) Kinin and prostaglandins are involved [17,18]. The results of present study revealed that administration of methanolic extract inhibited the edema starting from the first hour and during all phases of inflammation, which probably leads to inhibition of different aspects and chemical mediators of inflammation.

It is well known that in chronic and sub-acute inflammation ROS play an important role in modulating the extent of inflammatory response and consequent tissue and cell injury[19]and antioxidants are considered as possible protective agents reducing oxidative damage of human body from ROS and retard the progress of many diseases [20]. The natural phenolic, alkaloids, tannins, glycosides and flavonoids compounds function as antioxidants by different mechanisms and according to present study, the high contents of these phytochemicals in both the extracts can explain its anti-inflammatory activity[21] Thus, it can be concluded that the methanolic extract of *in vivo* plant possesses higher anti-inflammatory activity than leaf and shoot callus. Further studies involving the purification of the chemical constituents of the plant and the investigations in the biochemical pathways may result in the development of a potent anti-inflammatory agent with low toxicity and better therapeutic index.

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RESULTS

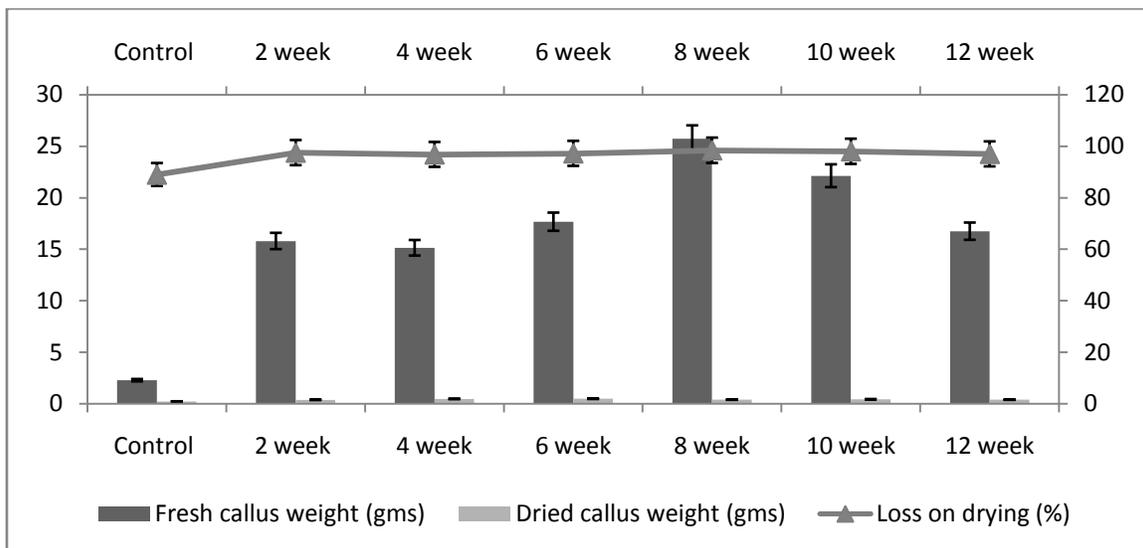


Figure 1: Weights (gms) of callus cultures after 2, 4, 6, 8, 10 and 12 weeks in MS medium fortified with BAP (2.0 μM) and α-NAA(1.0 μM).

Note: callus texture was yellow to brown up to 12 weeks.

Table 1: Qualitative Phytochemical test of Extracts of SP(P) and SP(C)

| S.No | Test/Reagent used | Sample SP(P) | Sample SP(C) |
|--|--------------------------|--------------|--------------|
| Test for alkaloids | | | |
| 1 | Mayer's reagent | + ve | +ve |
| 2 | Dragendorff's reagent | + ve | + ve |
| Test for Carbohydrates and Glycosides | | | |
| 5 | Molish's reagent | + ve | + ve |
| 6 | Fehling's solution | + ve | - ve |
| Test for Tannins and phenolic compounds | | | |
| 7 | Ferric chloride solution | + ve | + ve |
| 8 | Geletin solution | + ve | - ve |

| | | | |
|----------------------------|---|------|------|
| 9 | Lead acetate solution | + ve | + ve |
| Test for lignin | | | |
| 10 | Extract + Alcoholic solutions of phloroglucinol and hydrochloric acid | + ve | - ve |
| Test for Terpenes | | | |
| 11 | Extract + chloroform+ acetic anhydride + Sulfuric acid | + ve | + ve |
| Test for Flavonoids | | | |
| 12 | Methanolicextract + Hydrochloric acid + Magnesium ribbon | + ve | + ve |

Table 2: Anti-inflammatory effect of the extract and reference drug in carrageenan induced paw edema in rats

| Groups | Dose mg/kg b.w | Paw Edema Volume | | | | | | | |
|----------|----------------|------------------------|-------|------------------------|-------|------------------------|------|------------------------|------|
| | | 1hr | | 2hr | | 3hr | | 4hr | |
| | | Mean ± S.E.M | %PEI | Mean ± S.E.M | %PEI | Mean ± S.E.M | %PEI | Mean ± S.E.M | %PEI |
| Control | ---- | 0.28±0.01 | ----- | 0.85±0.02 | ----- | 0.89±0.01 | ---- | 0.46±0.01 | ---- |
| Standard | 10 | 0.22±0.03 | 30 | 0.27±0.02 ^c | 72 | 0.27±0.01 ^c | 78 | 0.18±0.01 ^c | 84 |
| SP(P) | 100 | 0.19±0.04 | 12 | 0.60±0.05 ^b | 21 | 0.61±0.04 ^c | 23 | 0.31±0.03 ^b | 22 |
| | 200 | 0.15±0.02 ^c | 18 | 0.52±0.04 ^c | 24 | 0.52±0.04 ^c | 30 | 0.25±0.03 ^c | 34 |
| | 400 | 0.23±0.03 | 21 | 0.60±0.03 ^c | 28 | 0.61±0.02 ^c | 33 | 0.33±0.03 ^b | 37 |
| SP(C) | 100 | 0.14±0.02 ^b | 10 | 0.61±0.03 ^c | 16 | 0.60±0.02 ^c | 15 | 0.29±0.04 ^b | 21 |
| | 200 | 0.25±0.02 | 18 | 0.61±0.06 ^a | 21 | 0.64±0.06 ^a | 21 | 0.38±0.04 | 25 |
| | 400 | 0.14±0.03 ^a | 23 | 0.56±0.04 ^c | 25 | 0.56±0.03 ^c | 30 | 0.31±0.04 ^b | 30 |

Values are expressed as mean ± S.E.M; n=6; significance at p< 0.05^a, 0.01^b and 0.001^cas compared to the control. PEI – Paw Edema Inhibition

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