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EFFECT OF AGARICUS SUBRUFESCENS AS IMMUNOSTIMULANT/ IMMUNOMODULATORY USING IN VIVO AND INVITRO PROCEDURES EXPERIMENTAL ANIMALS

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ABSTRACT

Agaricus subrufescens possesses immunomodulatory activity. The aqueous and ethanolic extracts was administered at either (400mg/kg, *p.o*) and (200mg/kg, *p.o*). The evaluation of immunoprophylactic effect was also carried out to determined the effect of *Agaricus subrufescens* on specific immunity by challenge with 0.5ml/100gm dose of *Escherichia coli* was administered to determine the survival of the animals against invading pathogens. However, the aqueous and ethanolic extracts of *Agaricus subrufescens* reduced the mortality and decreased the abdominal peritonitis in immunoprophylactic effect. Based on the above observations, it was concluded that Aqueous extract and Ethanolic extracts of (*Agaricus subrufescens*) produced significant immunostimulatory effect in comparison with control and standard. This concludes that the *Agaricus subrufescens* has immunostimulatory effect.

KEYWORDS: *Agaricus subrufescens*, immunostimulatory, Ethanolic extract.

INTRODUCTION

The management of patients with inflammatory disorders, such as atopic dermatitis or psoriasis remains a challenging aspect of clinical practice. Besides genetic and environmental factors, imbalance of the adaptive immune system is thought to play a role in their pathogenesis resulting in infiltration and accumulation of inflammatory cells, mainly T-lymphocytes, in the affected tissue (Cai et al., 2012). T-lymphocytes initiate a cell-mediated immune-inflammation process *in situ* and maintain activation of dendritic cells and macrophages by transforming them into tissue destructive effector cells (Cai et al., 2012). Immunosuppression, the targeted reduction of the activation or efficacy of the immune

system, is an option for the treatment of these conditions. Established pharmaceuticals to treat such inflammatory diseases are (i) locally applied corticosteroids or calcineurin inhibitors, like cyclosporine A, a cyclic non-ribosomal undecapeptide of fungal origin which down regulate the immune system or (ii) systemic immunosuppressant's used for severe conditions. Since inflammatory immune disorders are characterized by an increased proliferation of T-lymphocytes, most immunosuppressive drugs aim to block cell cycle progression of these cells (Macian, 2005). Besides the registered drugs as first-line therapy, which may have many and sometimes severe side effects (De Mattos et al., 2000), there are numerous traditional and alternative herbal treatments with promising but yet not proven efficacy (Reuter et al., 2010).

Heartsease (*Viola tricolor* L.) is a traditional medicinal plant and member of the *Violaceae* family. It has been described and used for centuries in Europe for the therapy of inflammatory lung diseases and for the treatment of inflammatory skin disorders, such as atopic dermatitis (Hoppe, 1951; Hager, 1999) or psoriasis (Amenta et al., 2000). Its traditional use as herbal remedy is documented in several handbooks of phytotherapy (Madaus, 1938; Czygan and Wichtl, 2002), as well as in complementary medine, especially in Anthroposophical Medicine (Pelikan, 1978) and is furthermore registered in the German commission E Monograph (phytotherapy and herbal substances) of the German Federal Institute for Drugs and Medical Devices (Bundesanzeiger (BAnz) 1986), as well as described in the Pharmacopoeia of Europe (European Pharmacopoeia (EP) (2011)). *Viola tricolor* is well-known to contain flavonoids (Vukics et al., 2008a, 2008b), polysaccharides, phenylcarbonic acids, salicylic acid derivatives, catechins and cumarins (Czygan and Wichtl, 2002). In addition, the family *Violaceae* and in particular *Viola tricolor* have been appreciated as rich source of naturally-occurring macrocyclic peptides, so called cyclotides (Schopke et al., 1993; Goransson et al., 2004). Cyclotides are ribosomally-synthesized plant compounds (Gruber et al., 2007) that display the unique structural topology of a head-to-tail cyclized backbone combined with three conserved disulfide bonds arranged in a knotted configuration, which confers them with remarkable stability (Colgrave and Craik, 2004; Colgrave et al., 2005; Clark et al., 2006). Cyclotides were recently reported to act as immunosuppressive peptides which inhibited the proliferation of T-lymphocytes (Grundemann et al., 2012).

Since lymphocytes play an important role in the pathological process of inflammatory diseases, our aim was to investigate the influence of an aqueous extract prepared from *Viola*

tricolor herbs on the cell division and function of activated human lymphocytes *in vitro*. Using a bioactivity-guided fractionation approach and detailed cell-based investigations the 'active' principle of a complex plant crude extract was purified and analytically characterized by reversed-phase chromatography and mass spectrometry. The significance of the identified active compounds for the observed effect was highlighted in this study revealing a potential new source of immunosuppressive natural compounds.

PLANT INTRODUCTION

Agaricus Subrufescens

Agaricus subrufescens (*syn. Agaricus blazei*, *Agaricus brasiliensis* or *Agaricus rufotegulis*) is a species of mushroom, commonly known as almond mushroom, mushroom of the sun, God's mushroom, mushroom of life, royal sun agaricus, *jisongrong* or *himematsutake* (Chinese: 杏仁松茸, Japanese: 姫まつたけ, "princess matsutake") and by a number of other names. *Agaricus subrufescens* is a choice edible, with a somewhat sweet taste and fragrance of almonds. The fungus is also well known as a medicinal mushroom, for its purported medicinal properties, due to research which indicates it may stimulate the immune system.

AGARICUS SUBRUFESCENS

AGARICUS SUBRUFESCENS

Plant Extract

The plant collected and dried on filter paper sheets under shade at room temperature, plant dried for 1 month until the completely dried it was again dried and then powdered. This powdered material was kept for ethanolic extraction in Soxhlet apparatus for 18hours.

The powdered material was weighed about 50mg it was dissolved in 150ml of ethanol(1:3 ratio) and ethanolic extraction was done in Soxhlet apparatus. The extract which was formed in round bottom flask, this ethanolic extract were evaporated under reduced pressure at room

temperature(30^0c) to dryness to yield yellowish brown color extract of *Agaricus subrufescens* stored in an airtight container for further experimental studies. (Dashputre *et al.*, 2010).

Test Compound Formulations

The dilution of aqueous extract of *Agaricus subrufescens* (AEAS) was prepared in distilled water and the aqueous suspension of ethanolic extract of Leaves *Agaricus subrufescens* (EEAI) was prepared in distilled water prior to oral administration to animals. It was used within 7 days and stored at 8^0C while for further use, freshly prepared solution was used. The vehicle alone served as control. (Dashputre *et al.*, 2010; Baizid *et al.*1993).

Fresh Culture Preparation

Nutrient broth was prepared by 1.3gms of agar was dissolved in 100ml of distilled water. Then this solution was sterilized under autoclave for 2hours and cooled. Culture of *Escherichia coli* was transferred in to this sterilized medium under aseptic conditions in laminar air flow chamber. It was taken in to rotary shaker for 18hours to increase the growth of organisms in that sterilized medium. This will shows "log phase" and rapid growth of microorganisms in the culture medium.

100ml of distilled water was taken it was kept in autoclave for 1hour at 15lb pressure. Then 0.5gms of Phosphate buffer solution (PBS) was transferred in to sterilized water under aseptic conditions in laminar air flow chamber by this 0.5% of Phosphate Buffer Solution was prepared. In this solution 0.5ml of Escherichia coli was added a dose of 0.5ml/100kg body weight was used (Yadav *et el.*, 2010).

Acute Toxicity Studies

Acute toxicity studies were performed according to organization for economic cooperation and development (OECD) guidelines, received draft guidelines 425, received from CPCSEA. Rats 120-180g weighing between 120-180gm in groups of five were used (n=6). The animals were fasted for 4 hr. with free access to water only. The both EEAI and AEAI extracts was administered orally in doses of 1000 and 2000 mg/kg to different groups of mice and observed over 14 days for mortality and physical/behavioral changes. The experiments were performed after the experimental protocols had been approved by the Institutional Animal Ethical committee.

Antigenic Material

***Preparation of Sheep RBCs* (SRBCs)**

Induction: Sheep blood was collected in sterile Alsevere's solution in 1:2 proportion of Alsevere's solution (freshly prepared). Blood was kept in the refrigerator and processed, for the preparation of Sheep RBCs batch, by centrifugating at 2000 rpm for 10 minutes and washing with physiological saline 4-5 times and then suspending into buffered saline for further use. (Dashputre *et al.*, 2010).

Preparation of Alsever's Solution

Composition

Dextrose: 2.05 gm

Sodium citrate: 0.8 gm

Sodium chloride: 0.4 gm

Citric acid: 0.05 gm

All the ingredients were weighed and dissolve in 100ml of distilled water. Alsever's solution was used in the proportion of 1:2 (Sheep blood: Alsever's solution) for washing sheep blood. (Vaghasiya *et al.*, 2010).

TREATMENT

***In-vitro* methods**

Neutrophil adhesion test

- Group I - Control for 14 days.
- Group II - Aqueous extract (400 mg/kg/day/per oral) daily for 14 days respectively.
- Group III - Ethanolic extract (200 mg/kg/day/per oral) daily for 14 days respectively.
- Group IV- Levamisole (50 mg/kg/per oral) for 14 days.
- On the 14th day of the treatment, blood samples from all the groups were collected by puncturing retro-orbital plexus under mild ether anesthesia.
- Blood was collected and analyzed for total leukocyte count (TLC) and differential leukocyte count (DLC) by fixing blood smears and staining with Leishman's stain.
- After initial counts, blood samples were incubated with nylon fiber (80 mg/ml of blood sample) for 15 min at 37^0C. The incubated blood samples were again analyzed for TLC and DLC. (Dashputre *et al.*, 2010).
- The product of TLC and % neutrophil gives neutrophil index (NI) of blood sample. Percentage of neutrophil adhesion was calculated as follows,

Neutrophil adhesion= NIU - NIT x 100

NI_U

Where,

NIU: Neutrophil Index before incubation with nylon fiber.

NIT: Neutrophil Index after incubation with nylon fiber.

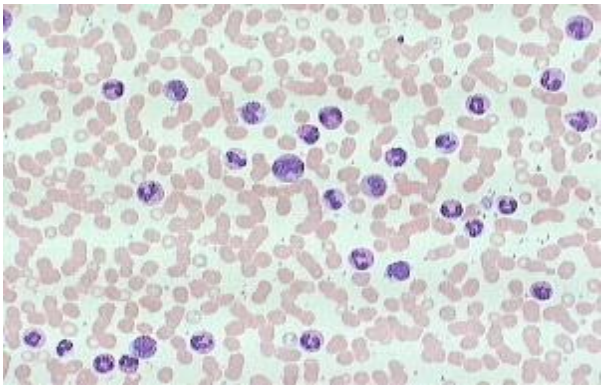


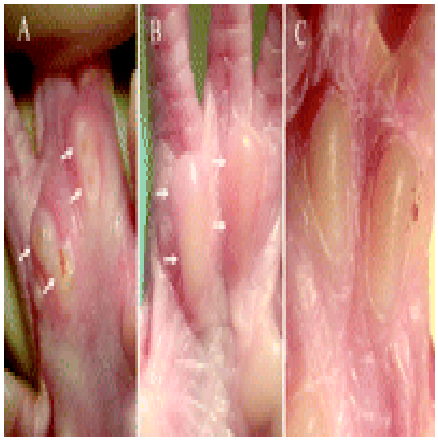
Figure 5: White blood cells.

***In-vivo* methods**

Delayed type hypersensitivity (DTH) response

- Group I: Control for 21 days.
- Group II: Aqueous extract (400 mg/kg/day/ Per oral) daily for 21 days.
- Group III: Ethanolic extract (200mg/kg/day/per oral) daily for 21 days.
- Group IV: Levamisole (50 mg/kg/per oral) for 21 days
- On 14th day of the study, all the groups of I, IV were immunized with Sheep RBCs (0.1ml of 20% Sheep RBCs intra peritoneal) in normal saline.
- On day 21st all animals from all the groups were challenged with 0.03 ml of 20% Sheep RBCs in sub plantar region of right hind paw Foot pad edema in rat was used for detection of cellular immune response.
- On 21st day, injection of 0.1ml of 20% SRBCs in the sub plantar region of right hind paw in the volume of 0.03 ml and normal saline in left hind paw in same volume.
- Foot pad reaction was assessed after 24hours on 22nd day, in terms of increase in the thickness of footpad as a result of hypersensitivity reaction due to edema, the thickness of the right hind footpad was measured using Plethysmometer.
- The footpad reaction was expressed as the difference in the thickness (millimeter) between the right foot pad injected with Sheep RBC and the left footpad injected with normal saline (Dashputre *et al.*, 2010).

Subplantar region of hind paw



Foot paw edema

Figure 6: Measuring of Foot paw edema with Plethysmometer.

Evaluation of Immunoprophylactic Effect

- Group I:- Control for 15 days.
- Group II:- Aqueous extract (500mg/kg/body weight) orally for 15 days.
- Group III:- Ethanolic extract (500mg/kg/body weight) orally for 15 days.
- On 15th day, 3hr.after the last dose of aqueous extract and ethanolic extract *Escherichia coli* (0.5ml/100kg body weight, intra peritoneal) was injected to control, aqueous and ethanolic groups of rats and percentage of mortality was observed after 24hours (Yadav *et al.*, 2010).

RESULTS AND DISCUSSION

Neutrophil Adhesion Test

Table 1: Effect of *Agaricus subrufescens* treatment on neutrophil activation by neutrophil adhesion test.

No. of observations	Control Group	Aqueous Group	Ethanolic Group	Levamisole Group
1	44.44	65.11	68.88	72.72
2	60	72	63.63	69.56
3	55	66.66	54	64.58
4	40	55.76	58.33	60
5	40.62	63.63	69.56	60
6	34.28	72.72	61.7	78.57
Mean	45.72	65.98	62.68	67.57
SD	9.81	6.21	6.031	7.41

All values are Mean ± SD, n= 6, $p<0.001$ when compared to control groups. Statistically analyzed by one- way analysis of variance (ANOVA) followed by Tukey-Kramer multiple comparison test.

Delayed Type Hyper Sensitivity

Table 2: Effect of *Agaricus subrufescens* Treatment on Cell Mediated Immune Response by Delayed Type Hypersensitivity Induced Footpad Edema.

No. of observations	Control Group	Aqueous Group	Ethanolic Group	Levamisole Group
1	0.8	0.4	0.5	0.6
2	0.9	0.5	0.3	0.6
3	0.6	0.6	0.6	0.5
4	0.5	0.5	0.4	0.4
5	0.6	0.5	0.5	0.3
6	0.9	0.3	0.3	0.5
Mean	0.7	0.466	0.433	0.483
SD	0.187	0.103	0.121	0.116

All values are Mean ± SD, n= 6, $p<0.05$ when compared to control groups. Statistically analyzed by one- way analysis of variance (ANOVA) followed by Tukey-Kramer multiple comparison test.

Evaluation of Immunoprophylactic Effect

Table 3: Effect of aqueous and ethanolic extract of *Agaricus subrufescens* on *Escherichia coli* induced in rats.

No. of observations	Control Group	Aqueous Group	Ethanolic Group
1	-	+	-
2	-	+	+
3	-	-	+
4	-	+	-
5	-	+	-
6	-	+	+
Total number of animals died	6/6	1/6	3/6
Protection	0%	84%	50%

+ ve group indicates survival and –ve group indicates death of the animals.

Neutrophil Adhesion Test and Delayed Type Hypersensitivity

Table 4: Effect of *Agaricus subrufescens* treatment on Neutrophil adhesion test and Delayed type hypersensitivity.

Groups	Treatment	Neutrophil adhesion test	Delayed type hyper sensitivity response
1	Control	45.72±9.81	0.7±0.187
2	Aqueous extract (400mg/kg)	65.98±6.21	0.46±0.103
3	Ethanolic extract (200mg/kg)	62.68±6.03	0.43±0.121
4	Levamisole (50mg/kg)	67.57±7.41	0.48+0.116

Neutrophil Adhesion Test

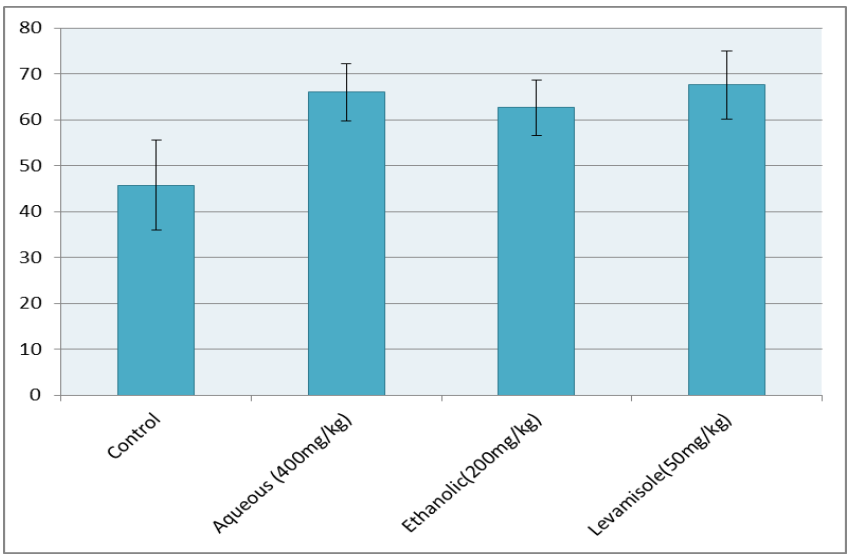


Figure 7: Neutrophil adhesion test.

Incubation of blood samples with nylon fibers produced a reduction in neutrophil count in all the treatment groups. The percentage neutrophil adhesion was significantly ($p<0.001$) increased by Aqueous and Ethanolic extracts of *Agaricus subrufescens* when compared with control groups, showed possible immunostimulant effect(Dashputre *et al.*, 2010).

Delayed type hyper sensitivity response

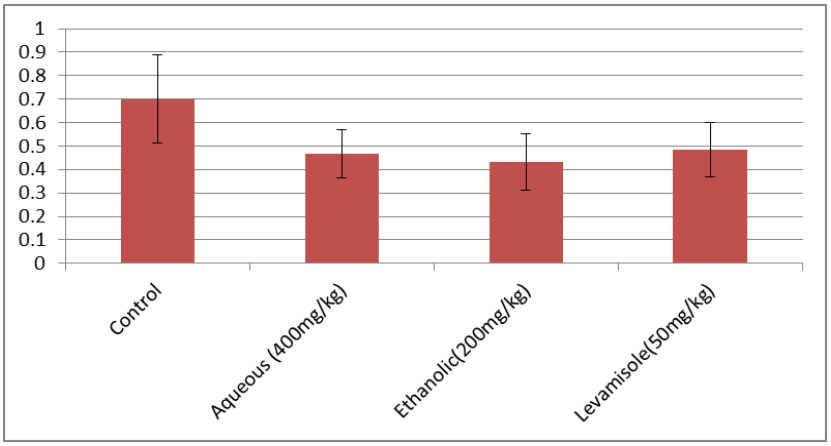


Fig: 8 Delayed type hyper sensitivity response.

In all the groups of mice with normal immune status, of Aqueous and Ethanolic extracts of *Agaricus subrufescens* showed significant ($p< 0.05$) potentiated Delayed Type Hypersensitivity (DTH) response in terms of increase in the mean difference of paw thickness when compared with control group (Dashputre *et al.*,2010)

Evaluation of immunoprophylactic effect

Table 5: Effect of aqueous and ethanolic extracts of *Agaricus subrufescens* on *Escherichia coli* induced in rats.

Serial number	Total number of animals died	Protection
Control	6/6	--
Aqueous extract	1/6	84%
Ethanolic extract	3/6	50%

Immunoprophylactic effect was significantly enhanced in animals treated with the aqueous and ethanolic extracts before administering *Escherichia coli.* Animals treated with the *Agaricus subrufescens* aqueous and ethanolic extracts in rats, showed only 14 to 50% mortality as compared to 100% mortality in control rats (Yadav *et al.*, 2010).

DISCUSSION

The term "immunity" has traditionally referred to the resistance exhibited by the host towards injury caused by microorganisms and their products. Immunomodulation is a procedure which can alter the immune system of an organism by interfering with its functions; if it results in an enhancement of immune reactions it is named as an immunostimulative drug which primarily implies stimulation of specific and non specific system, i.e. granulocytes, macrophages, complement, certain T-lymphocytes and different effector substances (Dashputre *et al.*, 2010). Generally, Cytokines are secreted by activated immune cells in order for the margination and extravasation of the phagocytes mainly polymorphonuclear neutrophils. A Significant increase in the adhesion of neutrophils to nylon fibers correlates to the process of margination of cells in blood vessels. In this present study, *Agaricus subrufescens* showed a significant increase in the neutrophil count, upon treatment with aqueous and ethanolic extracts of the fruit, compared to control. This may be helpful in increasing immunity of body against microbial infections. (Gayatri *et al.*, 2005) In general cell mediated immunity involves Ag entry and this Ag engulfed by macrophages. Proteins in macrophages combines with Ag, Ag with macrophage proteins presents on macrophage surface then TH cells recognizes Ag present on macrophage and binds to it, and gets activated. Activated TH cells release cytokines (gives to macrophages), and gets itself multiply to Tk cells, macrophages with those chemicals activates cytotoxic t cells (kill t cells) cytotoxic cells attacks on target cells this leads to cell lyses. (Mishra, 2004; Miller, 1991) Cell-mediated immunity (CMI) involves effector mechanisms carried out by T lymphocytes and their products (lymphokines). DTH requires the specific recognition of a given antigen by activated T lymphocytes, which subsequently proliferate and release cytokines. These in turn increase vascular permeability, induce vasodilatation, macrophage accumulation, and activation, promoting increased phagocytic activity and increased concentrations of lytic enzymes for more effective killing. When activated TH1 cells encounter certain antigens, with SRBCs. They secrete cytokines that induce a localized inflammatory reaction called delayed type hypersensitivity. (Mishra, 2004; Miller, 1991) DTH comprises of two phases, an initial sensitization phase after the primary contact with Sheep RBCs antigen. During this period TH1 cells are activated and clonally expanded by APC (antigen presenting cells) with class II MHC molecule (example. langerhans cells and macrophages are APC involved in DTH response). A subsequent exposure to the SRBCs antigen induces the effector phase of the DTH response, where TH1 cells secrete a variety of cytokines that recruits and activates macrophages and other nonspecific inflammatory

mediators. From the present study, the results showed that, the ethanolic and aqueous extracts of *Agaricus subrufescens* possesses anti-inflammatory activity, and found decreased paw edema in rats but less decreased when compared with that of standard drug levamisole. (Miller *et al*., 1991).

The total aqueous and ethanolic extract of plant showed a significant immunostimulatory activity *Escherichia coli* treatment caused 100% mortality of the animals, due to abdominal peritonitis in untreated mice and extract treated mice. However, treatment with *Agaricus subrufescens* reduced the mortality to 14 to 50% showing *Agaricus subrufescens* has immunostimulatory activity (Yadav *et al*., 2010).

CONCLUSION

Agaricus subrufescens possesses immunomodulatory activity. The study evaluate the effect of administration of *Agaricus subrufescens* on the immune system in experimental animals. The aqueous and ethanolic extracts was administered at either (400mg/kg, *p.o*) and (200mg/kg, *p.o*).

The effect was evaluated using three different models representing different components of the immune system. The neutrophil adhesion test was carried out in rats to evaluate the effect on levels of neutrophils. The neutrophil adhesion test was done to determine the effect on neutrophil stimulation.

The evaluation of immunoprophylactic effect was also carried out to determined the effect of *Agaricus subrufescens* on specific immunity by challenge with 0.5ml/100gm dose of *Escherichia coli* was administered to determine the survival of the animals against invading pathogens. However, The aqueous and ethanolic extracts of *Agaricus subrufescens* reduced the mortality and decreased the abdominal peritonitis in immunoprophylactic effect.

The results of the present study suggests that that aqueous (400mg/kg, p.o) and ethanolic(200mg/kg, p.o) decreased the cellular and humeral immunity as indicated by an significant decrease in foot paw in delayed type hypersensitivity response and significant increase in adhesion of neutrophil to nylon fibers in the neutrophil adhesion test.

Based on the above observations, it was concluded that Aqueous extract and Ethanolic extracts of (*Agaricus subrufescens*) produced significant immunostimulatory effect in

comparison with control and standard. This concludes that the *Agaricus subrufescens* has immunostimulatory effect.

Further studies has to be conducted as there is need for isolation and elucidation of individual constituents from the extracts to get more precision over studies.

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