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A brief comparative study of the natural sources (lemons) in the basis of protein, vitamin C, their antibacterial, anthelminthic and cell viability on immune cells

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Abstract
Vitamin C is a vital circulating antioxidant with anti-inflammatory and immune-boosting properties that are required for good health throughout the life cycle. The coronavirus disease (SARSCov-2) pandemic is now being done; every doctor has suggested consuming vitamin C as a dietary supplement. Besides an extensive range of significances, we want to focus the vit C-rich natural resources despite costly drugs and supplements. we have taken 6 different types of citrus fruits; *Citrus aurantifolia* or *C. limon*, the aroma king lemon, *Citrus limetta*, *Citrus reticulata*, *Citrus sinensis*, and *Citrus maxima* all of these are easily available in the market and affordable too. For every financial class in our country. This paper has shed light on comparative nutritional analysis, the mechanism of their vitro cell viability, anthelmintic, and antibacterial activity of these fruits and indigenous. Interestingly. *Maxima* contain (0.972±0.002 mg/ounce) 99.89 % vitamin C in comparison to the limcee tab. amount significant amount of protein is present in *C. limetta* 138.49±4.003 mg BSAE/ml. *Citrus limon* shows good antimicrobial activity, *C. reticulata and C. sinensis* show potentiality about death time and paralysis time of helminths. Ascorbic acid helps in the enhanced survival of macrophages which are the body's first line of defence. Using the current level of knowledge this paper is attracting the focus of healthcare practitioners, who will give suggestions for having different kinds of lemons that will boost the immunity of the community.

Keywords: Anthelmintic, antibacterial, lemon, macrophage, trained immunity, vitamin C

1. Introduction
The modern world is entirely dependent on the synthetically available drugs and medicaments. But most of it is unaware of the underlying properties of the plethora of herbs and plants that provide a potent source of nutritional and medicinal traits. Vitamin C which is one of the key circulating antioxidants with anti-inflammatory and immune-supporting effects that is required for good health throughout the life cycle. Due to various kind of diseases, scientific pathology suggests synthetic medications, but we need to aim at our own healing process which mother nature always gives her best to nurture us. The coronavirus disease (SARScov-2) pandemic is now being done; every doctor has suggested to consume vitamin C as dietary supplement. Vitamin C is one of the key nutrients which essentially help to boost up the immune system of humans [1-5].

When we are discussing vitamin C potency, it can refer to two main aspects: Concentration and bioavailability. Concentration of vitamin C present in a given substance, such as a dietary supplement, food item, or skincare product and Bioavailability indicates the extent to which the body can absorb and utilize the vitamin C present in a substance.

Vitamin C, also known as ascorbic acid, is a primary nutrient that plays an intensive role in various physiological functions. It acts as an antioxidant, supports the immune system, aids in collagen production, helps with the absorption of iron, and has other important functions like collagen hydroxylation, tyrosine metabolism, ferric metabolism [4, 5-7] ascorbic acid, are highly bioavailable and easily absorbed by the body, while others, such as certain mineral ascorbates or esterified forms, may have lower bioavailability. Thus, till now there have been many studies in the past that shed light on the mechanism of the anthelmintic, antibacterial activity of these herbs and indigenous [9-10].

Now, we have indulged in the study of *Citrus* fruits for their anthelmintic and antibacterial (*E. coli*) activity and for the exploration we isolated the activity of vitamin-C as a potent anthelmintic, antibacterial activity causative agent against the artificially present vitamin-c sources like vitamin-c tables and ascorbic acids powders. We also aimed to study the comparison of natural and artificial sources of Vitamin-C and their anthelmintic and antibacterial activity.

A growing number of studies suggest that after being given viruses, monocytes/macrophages may have memory capacities similar to those of the adaptive immune system [11]. Initially Ascorbic acid clearly functions as a donor of single atoms of hydrogen, whereas the reactive anion monodehydroascorbate mostly interacts with the radicals which are free. Ascorbic acid's outstanding antioxidant capabilities are due to the combination of these qualities [12]. We have introduced different series of experimented concentrations of ascorbic acid to macrophages cells to observe the presence of viable cells with certain time lapse.

2. Materials and Method

2.1 Samples taken

We have taken 6 different types of citrus fruits; *Citrus aurantifolia* or *C. limon or C. lemon*, the aroma king lemon, *Citrus limetta*, *Citrus reticulata*, *Citrus sinensis*, *Citrus maxima* all of theseare easily available in the market.
[Figure 1].

2.2 Chemicals required

All the chemicals were analytical grade. Starch as indicator from SRL. Iodine solution (KI + KIO_3) Vitamin C chewing tablet from Abbott, Bradford Reagent, Ascorbic acid bought from Merck biological society of India, NA (Nutrient Agar) and NB (nutrient broth), ampicillin, Ascorbic acid bought from Merck biological society of India, standard anthelmintic Albendazole was purchased from a local medical store. The macrophage cell line J774A1 cell line has been purchased from national centre for cell science, research institute in Pune, Maharashtra.

2.3 Vitamin C Estimation by Iodometric Titration

2.3.1 Sample Preparation

Extracts of samples were centrifuged for 5000 rpm for 5-10 mins and the supernatant was taken and the pellet was discarded. The aliquots were kept for further use in the experimentation in air tight conditions within the 4 °C.

2.3.2 Total vitamin-c quantification

The samples were subjected to vitamin-c quantification assay using Potassium iodate, potassium iodine titration method [13].

2.4 Protein Estimation by UV spectrophotometric method: Bradford Assay

Total protein content was measured by Bradford assay [14] A standard curve was made of bovine serum albumin (BSA) 10 mg/10ml (1, 0.5, 0.25, 0.125, 0.0625,0 g/L) and OD was read at 595 nm through UV spectrophotometer. The contents were indicated as g BSA Equivalent /g of Fresh Weight of the sample (mg BSAE/gm. of FW).

2.5 Disc-Diffusion process (*In-Vitro* Antibacterial assay)

Antibacterial assay done by disc diffusion process against Gram negative bacteria: *Escherichia coli* maintained in nutrient broth at 37 ºC; 100 mL of each stock for Overnight incubation [19]. Then the stock of fresh fruit crude extracts was used and the dilution with distilled water and absolute ethanol was also used (100 mg/ml.) then 20 ul. of sample impregnated to autoclaved blank disc (5-6 mm) [15-18].

2.6 Macrophage Cell Culture

Macrophage Cell line J774A1, supplied by NCCS Pune, were cultured in DMEM medium along with 1% pen strep (penicillin and streptomycin), 10% Foetal Bovine Serum at 37 °C, 5% carbon di oxide and 95% of air.

2.6.1 *In-vitro* cell viability assay

Viability of live cell was calculated via (3-(4,5-dimethylthiazol-2-yl)- 2,5-diphenyltetrazolium bromide dye) assay [MTT] on the basis of serial dilutions of synthetic Vitamin C compound i.e., ascorbic acid (1.5,0.75,0.375,0.1875, 0.093,0.046,0.0234,0.017 µg/ml). After growing a full monolayer sheet, 1×10^5 cells concentration per well were seeded in a microtiter plate (96 wells) prior the day of treatment [19] and incubation period 37 °C for 24 hr. then exposure to the indicated amount of Ascorbic acid for 24 hrs. 48 hours previous used medium was discarded and 8-10 µl. of MTT (HIMEDIA) solution were given, then the plate was again placed into the Co_2 incubator for 2 hours. Next, we added 100 µl. solubilizing buffer after that the 96 well plate was placed on a shaker for 30 min at 15 rpm. Lastly OD (optical density) was measured ELIZA reader (at 570 nm) [20].

2.7 *In-Vitro* anthelmintic activity

The in-vitro anthelmintic assay was performed on the worm *Tubifex tubifex* for their simplicity to use, anatomical and morphological similarity with nematodes and Platyhelminthes (average 2-3 cm, 1g of worms were used for every sample, control and concentrates of 10mg/ ml volume in Petri plates adjoin in fig depicts how it was performed. both medicinal grade Vitamin-C tablet and laboratory grade ascorbic acid both of 10mg/ml concentration, the dilutions used for every sample were ranging from 100, 50, 25 %. total duration of death and paralysis time has been compared to using albendazole (common medication) and double distilled water. approximately 10 ml and the laboratory grade ascorbic acid and vitamin-c dilution also of the similar concentration of about 10mg/ml of a total volume of 10 ml i.e., of 100 mg in a total volume of 10ml and the similar aspects of the paralysis, death and recovery tests were performed.

3. Result

3.1 Vitamin C

For the quantification of vit C [table 1, fig 2] for the samples, we used titration method and the above-mentioned graph represents the Vit C content (mg/ounce) of the lemons and the standard limcee tab (mg/ounce) Result shows *Citrus maxima* (pomelo, pummelo), [30 ml of centrifuged extract from 1 lemon] shows 99.89% similarity, *Citrus sinensis* [30 ml of centrifuged extract from 2 lemons] shows 95.37% similarity, *Citrus aurantifolia* [30 ml of centrifuged extract from 3 lemons] shows 69.064 % similarity, *The aroma king lemon* [30 ml of centrifuged extract from 3 lemons] shows 68.03 %, *Citrus reticulata* [30 ml of centrifuged extract from 2 lemons] shows 44.50 % similarity, *Citrus limetta* [30 ml of centrifuged extract from 2 lemons] shows 40.80 % similarity with the amount of vit c content of limcee tablet [1gm in 50 ml distilled water].

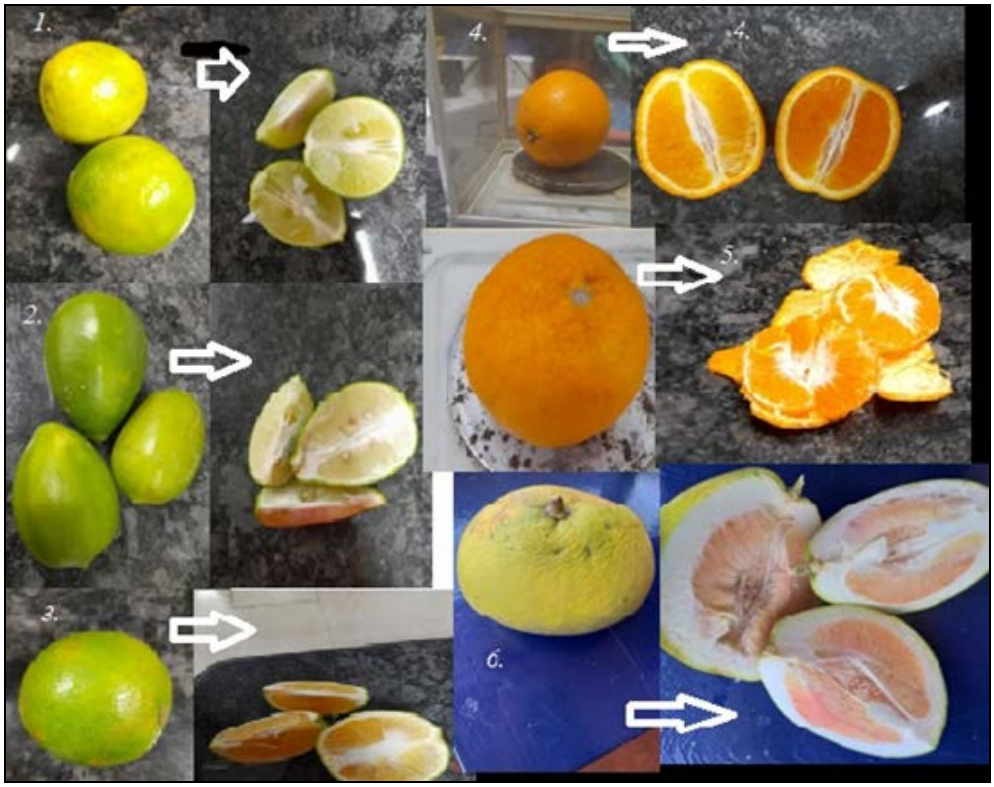

Fig 1: Shows the picture of commonly available lemons which we have taken for the experiments those are *1. Citrus aurantifolia 2. The aroma king lemon, 3 Citrus limetta 4. Citrus reticulata 5. Citrus sinensis, 6. Citrus maxima*

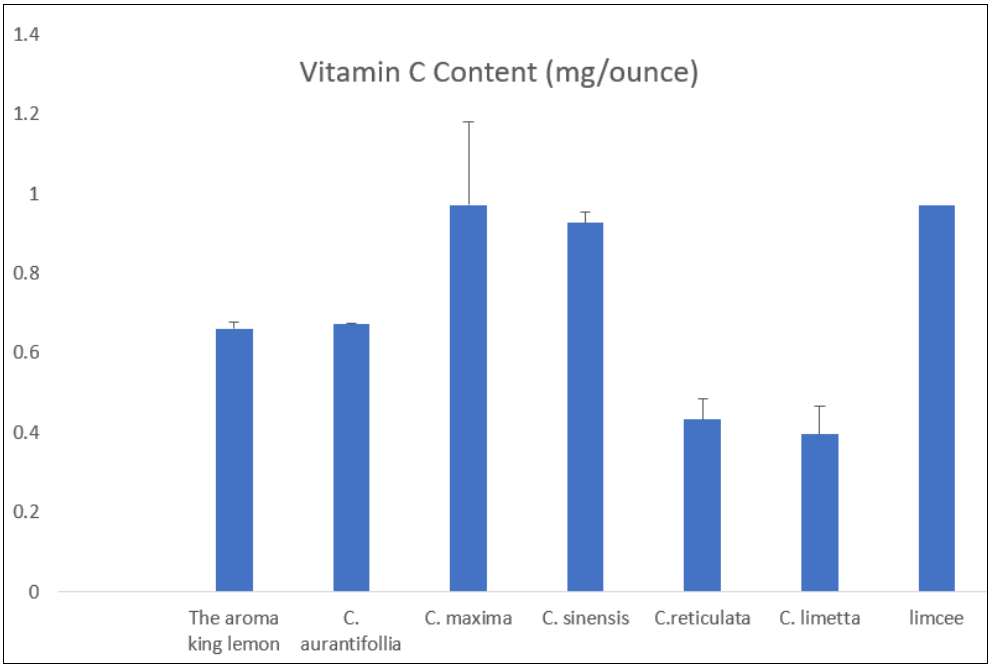

Fig 2: Depicts the vitamin c content of the samples

Table 1: Shows the vitamin c content of the samples.

Sample Name	Vitamin C Content (mg/ounce)
The aroma king lemon	0.662±0.115
C. aurantifollia	0.672±0.015
C. maxima	0.972±0.002
C. sinensis	0.928±0.208
C.reticulata	0.433±0.027
C. limetta	0.397±0.052
limcee	0.973 .069

3.2 Protein

The protein content of the samples is *Citrus aurantiifolia* 111.73±2.18 mg BSAE/ml The Aroma king lemon 127.44±11.9206 mg BSAE/ml, *Citrus sinensis* 121.44±0.719, *Citrus reticulata* 120.44±0.817, *Citrus maxima* 107.85±0.571, *Citrus limetta* 138.49±4.003. *C.limetta* has the highest amount of protein among these samples. [Fig 3]

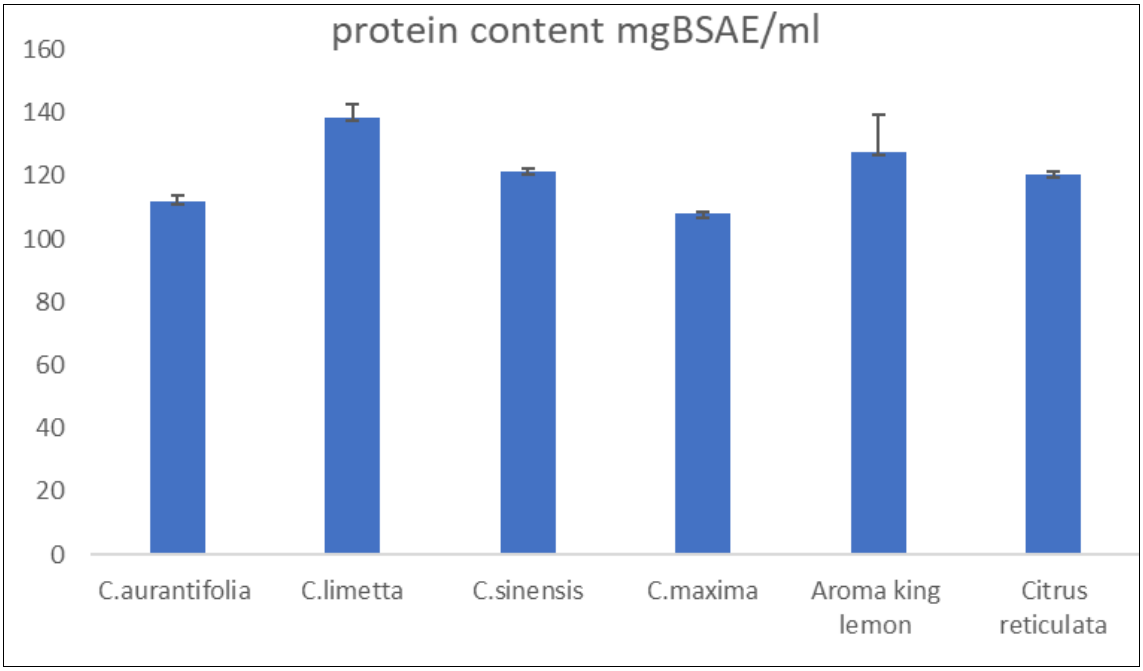

Fig 3: Depicts the protein content of the samples

3.3 Determination of Antibacterial Property and cell viability assay using *E. coli*

Antimicrobial activity of a sample is completely along with the compounds that provincially kill bacteria or decrease the rate of growth, without being highly toxic to nearby tissues. Some common factors, consideration of bacterial strains, inoculum size, antibiotic concentrations, serum effect, and interaction with the microbiota *Escherichia coli* (abbreviated as *E. coli*) large and diverse group of bacteria influencing the antibacterial activity, found in the environment, foods, and intestines of people and animals. The antibacterial activity of the extracts can be determined by using the Disc-diffusion method also known as Kirby-Bauer test developed in 1940 [21]. This is a bactericidal method that does not kill bacteria but inhibits their growth. In this method plates with agar are inoculated with a test organism, here *E. Coli* in standardised inoculum. Filter paper discs of 6 mm diameter are used on the agar surface where the samples are added. The compounds having antibacterial property in the samples diffuse into the agar and inhibit bacterial growth [22]. The sensitivity of *E. coli* to citrus fruits is measured in terms of their zone of inhibition [23]. [Table 2, Fig 4, Fig 5,]

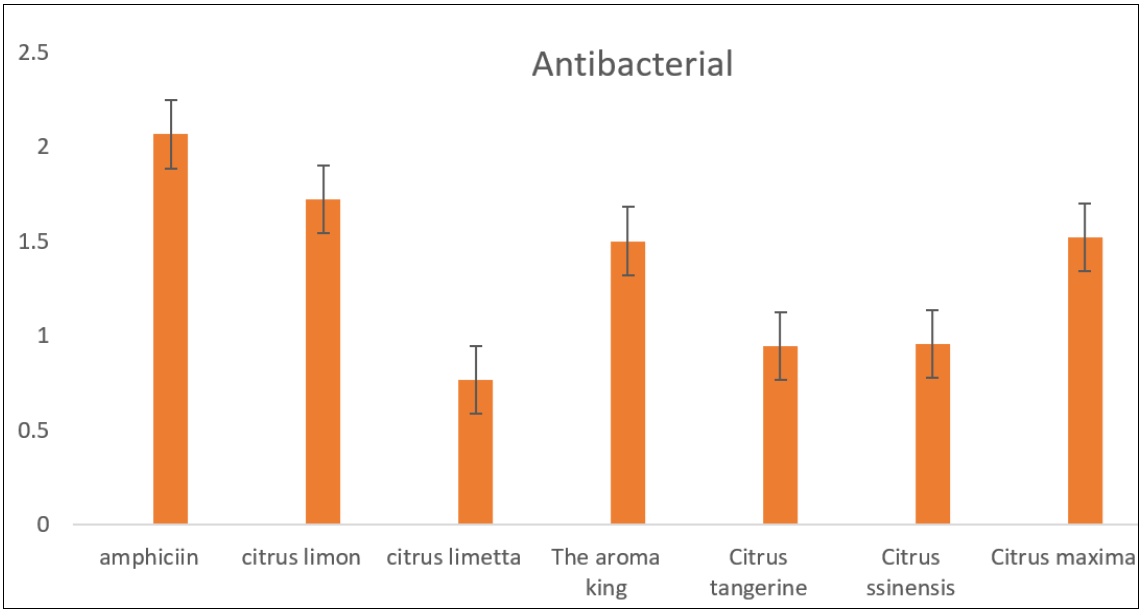

Fig 4: Shows the potentiality of the antimicrobial activity of the samples

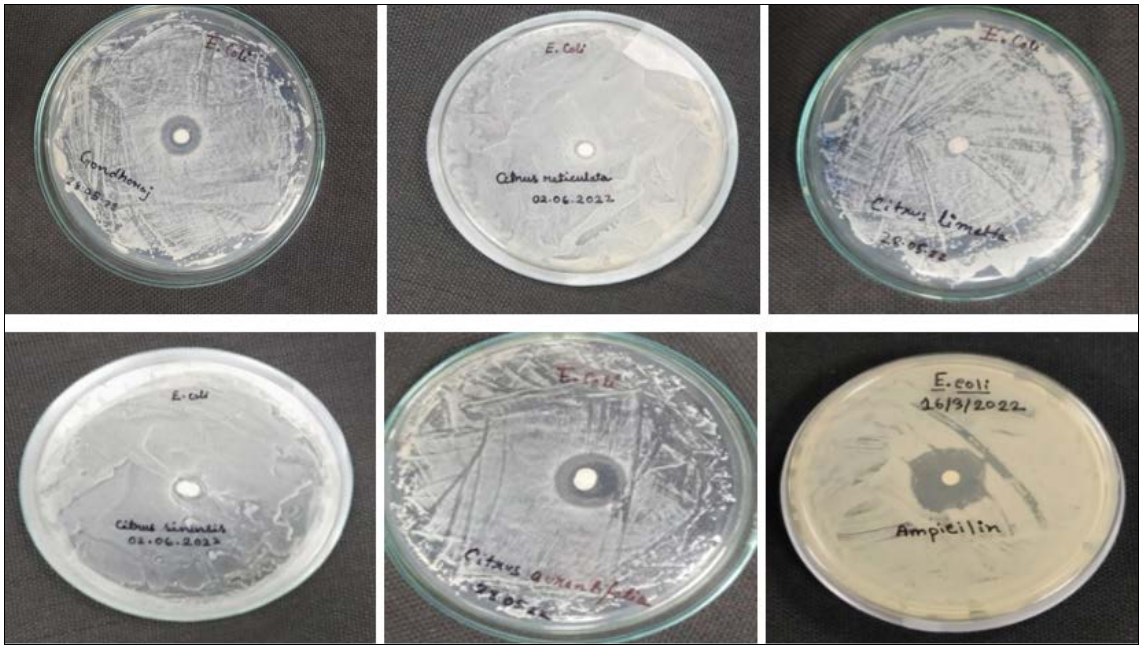

Fig 5: Shows the potency of antimicrobial disc diffusion images using the samples.

3.4 Cell viability assay result

Immunoprotect activity of Ascorbic Acid assessed on J774A1 via MTT assay. The ascorbic acid compound showed significant inhibition and growth of cell proliferation in a dose-dependent manner. J774A1 cells were alive at the concentration of 1.5 µg/ml and very few numbers of cells died at 0.0234 µg/ml, 0.0468 µg/ml, 0.0937 µg/ml. The results reflect the lower toxicity of ascorbic acid upon macrophage cells (incubation time 24 hours and 48 hours). Even low doses of AA show potentiality of survival and at the highest concentration shows a significant number of live cells. [Fig 6]

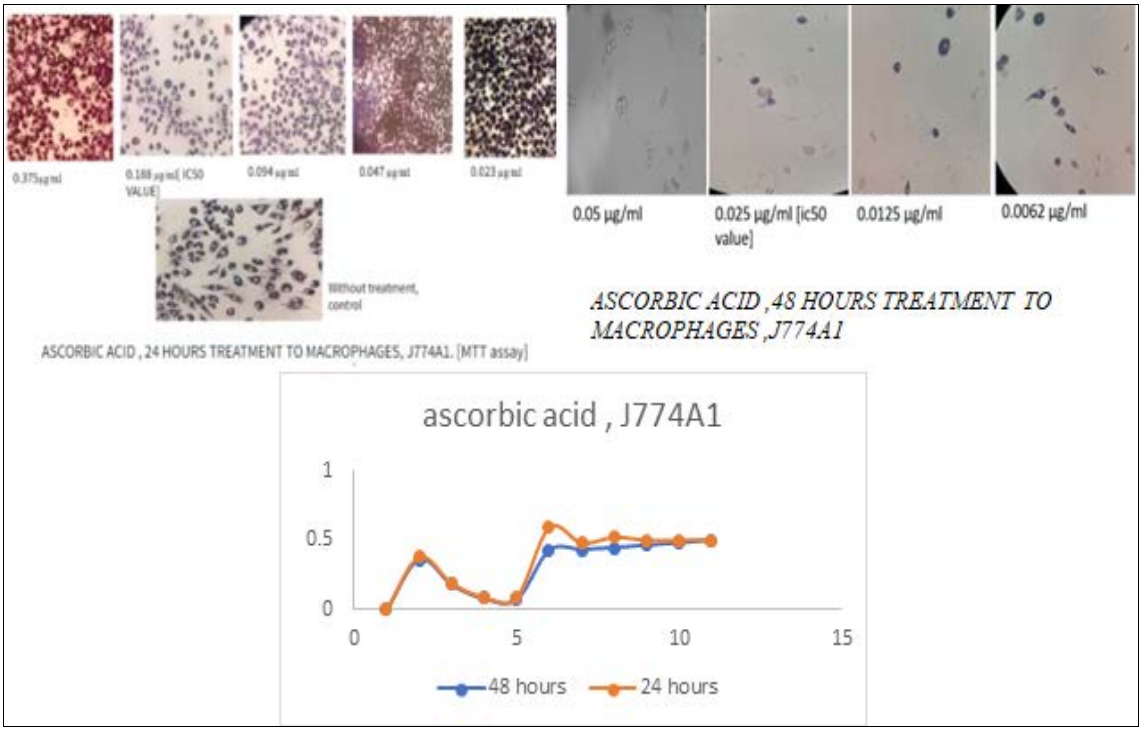

Fig 6: Shows the cytotoxic activity of ascorbic acid on macrophages. We have not used the samples for the cell.

3.5 Anthelminthic property

Generally, the mode of action of any drug used as anthelmintic activity works by causing damaging the cuticles of the worms as they are the most crucial for their development as well survival as these are extremely flexible and resilient exoskeletons that facilitate their locomotion and confer environmental protection thereby helping in their growth. Whereas, the Albendazole works by the inhibitory activity on the tubulin polymerization results in the detriment of cytoplasmic microtubules located in the intestine of the worms causes energy depletion and death of the organism.

In the present study it was found that the citrus fruits of certain varieties had a significant anthelmintic activity with respect to medicinal grade vitamin-C tablets as well as the laboratory grade ascorbic acid solution of similar concentration. However, it was also found that the fruits that specified the most activity were not the ones with the highest Vitamin C concentration. The studies were performed in

contrast to the natural sources of the vitamin C with the artificial source of vitamin C whether it be medical or laboratory grade the natural sources out performed This further needs to be studied for further clarification but nonetheless this makes it clear that the citrus fruits (specifically Aroma King lemon (raw) and *Citrus aurantiifolia* (Pati raw) possess prominent anthelmintic activity and can be potentially developed into useful, economic and safer anthelmintic activity. But this strictly demands more study to clarify detailed insight of the activity as well as to improve the potency. [Fig 7]

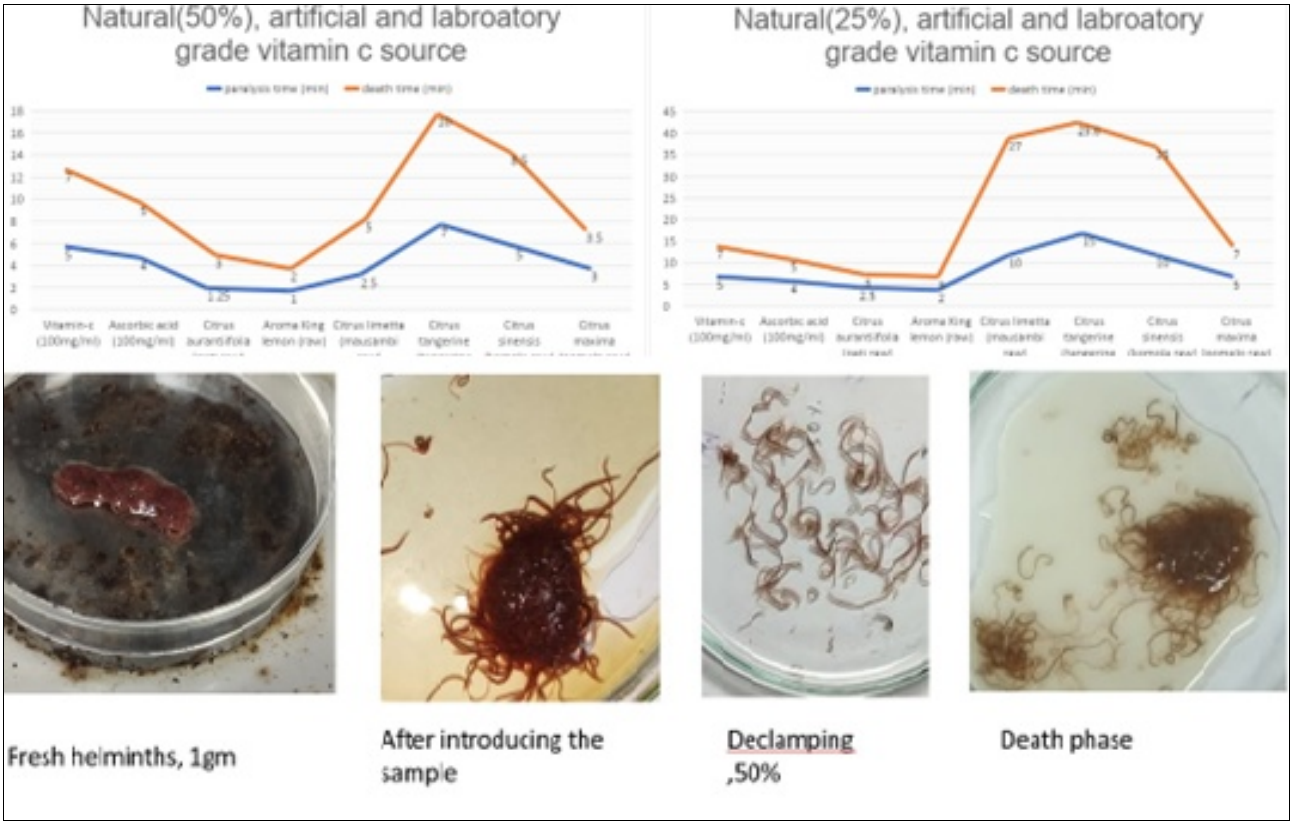

Fig 7: Shows the cytotoxic activity on helminths

4. Discussion

Besides extensive range of significances, we want to focus the vit C rich natural resources despite of costly drugs and supplements, these following mentioned lemons are easily available in the market and affordable too. For every financial class of our country.

Citrus aurantiifolia is thought to be the outcome of a tri-hybrid intergeneric cross between Citrus medica (citron), Citrus grandis (pummelo), and a Micro-citrus species [24]. It produces a spherical fruit that measures 25-50 mm. The lime is typically collected when still green, but when ripe, it turns yellow [25]. Aroma King lemon (Gandhoraj) it is exclusively found in eastern India, specifically Bengal and parts of Assam and Tripura, there is no unique Hindi, English, or even scientific name for this species. This lemon has a peculiar flavour that is somewhat acidic and smells strongly of lily flowers. This is a lime-mandarin orange hybrid [26]. *Citrus limetta* Mosambi (*Citrus limetta*) is a variety of delicious lime. This fruit is gaining popularity around the world due to its abundance of vitamin C and pleasant taste. The extraction, depectinization, pre-treatment, and ultimate clarity of pre-processed juice have all been studied by us [27]. It is tiny and spherical in shape, similar to a common lime [28]. *Citrus reticulata*- A tiny, thin-skinned orange variation of the Rutaceae family's mandarin orange species. Tangerine culture, which was most likely indigenous to Southeast Asia, migrated westward over trade routes as far as the Mediterranean. The soft, juicy, and flavourful pulp is high in vitamin C. Tangerine oil, derived from the fragrant skin of the fruit, is a common ingredient in many flavourings and liqueurs [29]. *Citrus sinensis*- orange, any of numerous kinds of tiny trees or shrubs in the family Rutaceae, genus Citrus with its roughly spherical fruits with leathery and oily rinds and tasty, juicy inside. Also significant was the emerging recognition of the nutritional advantages; oranges are high in vitamin C and also contain some vitamin A [30]. *Citrus maxima* -Pummelo (*Citrus maxima*), usually spelled pomelo, and commonly known as shaddock, is a citrus tree in the Rutaceae family that is grown for its enormous sweet fruits. The fruit shows a long history of usage in traditional and herbal medicine, primarily as an abortifacient and for stimulating menstruation. These medications are unlikely to end a pregnancy; nevertheless, they may cause vomiting, haemorrhages, and liver damage, which can be fatal in severe situations. In addition to being hepatotoxic in high dosages, skin contact with rue can result in severe phytophotodermatitis and burn-like blisters after sun exposure [31] People's interest in herbal remedies has grown in recent years. *C. reticulata* and *C. sinensis* shows potentiality about death time and paralysis time of helminths. Over the last few decades, there has been a noticeable shift in the research and promotion of plant-based medications. This is the primary reason we have chosen three lemons which are easily available in West Bengal. Antibacterial and antibiotic-potentiating activities of lemon is quite noticeable against drug-resistant phenotypes. The aroma king lemon and *Citrus maxima* are rich in antioxidants [32] phytochemicals and biologically active compounds. Recent studies proved that lemon peels, and fresh lemon juice show superior anti-tumor

activity [33]. Regular consumption of citrus fruits (Lemons) is associated with reduced risk for gastric cancer too [34]. The ethyl acetate and petroleum ether extract of *Citrus limon* shows anticancer activity in human cancer cell line [35]. *Citrus limon* (Lemon) relates to Rutaceaea family, has shown an important role in ancient ayurvedic treatments and modern approach in medical science [36]. The concentrated compound, the essential oil Limonene (43.07% in lemon peel) can be found in the Lemon extract, shows anti-angiogenic activity, anti-fungal activity [37] can block VEGF binding with its receptor.

clinical trials on covid 19 patients have been reported to aid in the prevention and support of immunological responses in micronutrient deficient persons at risk of COVID-19 infection, vitamin C supplementation is a viable approach [38].

4.1 Recommended dietary allowances: Over the years, recommended dietary allowances (RDA) for vitamin C have been established in order to maintain suitable body reserves. The RDAs differ per country adult men should take 90 mg per day, while adult women should take 75 mg per day [united states, Canada] whereas 105mg/day for adult men, 85mg/day for adult women in Italy [39-40]. Older research found that supplementing with vitamin C (2-3 g intravenous vitamin C/day) reduced organ dysfunction. Recent modest controlled studies employing pharmaceutical dosages of Vitamin C (6-16 g/day) demonstrate that it reduces vasopressor support and organ dysfunction, and may even reduce mortality [41].Oral vitamin C (2-8 g/day) has been shown to reduce the frequency and length of respiratory infections, whereas the administration of intravenous Vitamin C (6-24 g/day) has been shown to minimise death rates, ICU as well as hospitalisations, and duration on ventilatory support for serious pneumonia [42].

One limcee tablet contains 500mg vitamin c, so we can depict that RDA of vitamin c can be fulfilled through these lemons are in following manner; 30±5 ml of pomelo juice [½ portion of a standard size] (*Citrus maxima*), Citrus *aurantifolia* and the aroma king lemon is 1-2 full lemon or 40±10 ml per day for adults. *Citrus sinensis*1 full lemon or 30±5 ml of juice. and *Citrus reticulata* is 1 full lemon per day or 70±10 ml of juice. *Citrus limetta* 1 full lemon per day or 90±10 ml of juice.

5. Conclusion

The Vitamin C content of the *Citrus* fruits estimated and compared. Ascorbic acid helps in enhanced survival of macrophages which are bodies first line of defence. In general population apart from the specific circumstance's vitamin C can be used for protection or medication. As per the reports considerable spike in vitamin C sales immediately following the declaration of a global pandemic state of emergency. using the current level of knowledge this paper is attracting the focus of health care practitioners, who will give suggestion of having lemons over drugs that will boost immunity of the community and obtain accurate information despite a dearth of data. In future, further scientific evaluations and phytochemical studies will be done for the seeds and peels of these lemons and correct standardisation that must be investigated.

6. Acknowledgement

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7. Conflict of interest

The authors declare that there is no conflict of interest.

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