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Research article

The Quality Evaluation of In-Vitro Antimicrobial, Antioxidant Effect of different Pharmaceutical Dosage Forms of Vitamin C Compounds

Marwa Riyadh Chalati[1], Mohamed S Ali[1,2]*, Fatma R Khalaf [2]

[1]*Department of Pharmacy, College of Pharmacy, Nursing and Medical Sciences, Riyadh Elm University, Riyadh, Saudi Arabia.*
[2]*Al Azhar university, Faculty of pharmacy, Microbiology and immunology Department Cairo, Egypt.*
[3]*Pimary health care department, Faculty of Nursing. Al-Ahliyya Amman University, Amman, 19111 Jordan, Family and Community health Nursing, Assiut University, Asyut 71515, Egypt.*

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***Corresponding Author: Mohamed S Ali**, Department of Pharmacy, College of Pharmacy, Nursing and Medical Sciences, Riyadh Elm University, Riyadh, Saudi Arabia. Al Azhar university, Faculty of pharmacy, Microbiology and immunology Department Cairo, Egypt.
Email id: mohamedsalah@riyadh.edu.sa

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Abstract

Background: The quality of commercially available ascorbic acid preparations varies widely, raising concerns about substandard vitamin C products in the market. Since vitamin C exhibits both antimicrobial and antioxidant properties, evaluating these characteristics across different brands is essential for ensuring consumer safety and therapeutic effectiveness. **Objective:** This in vitro study aimed to assess the quality of various vitamin C formulations available in the market and to identify the type with superior antimicrobial and antioxidant activity. **Materials and Methods:** Six vitamin C products in tablet and effervescent dosage forms were purchased and coded as S2–S7, while pure ascorbic acid powder (S1) served as the standard. Ascorbic acid content was quantified using iodine redox titration and compared with S1. Antibacterial activity was evaluated using the agar diffusion method against standard Gram-positive strains (Bacillus subtilis ATCC 6633, Staphylococcus aureus ATCC 25923) and Gram-negative strains (Klebsiella pneumoniae ATCC 13883, Pseudomonas aeruginosa ATCC 27853, Escherichia coli ATCC 25922, Proteus mirabilis ATCC 29906). Minimum inhibitory concentrations (MICs) were determined for each sample. Antioxidant activity was assessed using DPPH radical scavenging measured by UV–VIS spectrophotometry. Data were analyzed using one-way ANOVA with significance set at $p < 0.05$. **Results:** ANOVA revealed significant differences among the tested products ($p < 0.05$). Sample S4 (Zein Pharma® Vitamin C 1000 mg with 10 mg zinc) showed the closest ascorbic acid content to the standard and demonstrated the largest inhibition zones against S. aureus, B. subtilis, P. aeruginosa, and E. coli at 100 mg/mL. S4 also exhibited the lowest MIC values across all tested strains. Additionally, S4 displayed synergistic enhancement of antioxidant activity compared with pure ascorbic acid. **Conclusion:** Vitamin C combined with zinc demonstrated superior product quality, stronger antimicrobial activity, and enhanced antioxidant potential compared with other tested formulations.

1. Introduction

Vitamin C or L-ascorbate is a water-soluble vitamin that plays an important role in human nutrition. It is a highly effective antioxidant and reduces oxidative stress [1]. However, several studies have shown that vitamin C, in the form of ascorbic acid, exerts significant antibacterial effects on pathogenic organisms like *Staphylococcus aureus, Enterococcus faecalis, Bacillus subtillis, Corynebacterium diphtheria, and Escherichia coli* [2]. Vitamin C is produced only in non-humans as primate species, guinea pigs, fishes, and birds. Although most animals can synthesize their needs of vitamin C, humans suffer from a mutation in the DNA coding of gulonolactone oxidase, which is the primary enzyme responsible for ascorbic acid synthesis. Due to this mutation, the external supplement of vitamin C becomes a must. The main source of vitamin C for human beings is mainly found in fruits and vegetables. Citrus fruits and other types are wealthy sources of vitamin C as cantaloupe, watermelon, berries, pineapple, strawberries, cherries, kiwi fruits, mangoes, and tomatoes [3].

Also, there are two critical biological forms of vitamin C, the reduced form of ascorbic acid and the oxidized form, DHA (dehydroascorbic acid). Vitamin C has a vital role in maintaining a healthy immune system, and its deficiency causes immune insufficiency and multiple infections. The ascorbic acid level is lowered in various body fluids during bacterial infections. Thus, it is commonly used as adjunctive treatment in many infectious diseases such as hepatitis, HIV, influenza, and periodontal diseases. Vitamin C administration modifies and enhances both innate and adaptive immune responses. It neutralizes the bacterial toxins, especially endotoxins, by blocking the essential signal for lipopolysaccharides (LPS) formation. On the other hand, LPS blocks the passage of ascorbic acid through the blood-brain barrier and inhibits its uptake by various cells. Ascorbic acid improves the phagocytic properties and activity of various immune cells, including neutrophils, natural killer cells, macrophages, and lymphocytes. In addition, vitamin C increases lymphocytes proliferation and antibody production [4]. Various prospective studies on the relation between vitamin C and cardiovascular disorders were reported. For example, the National Health and Nutrition Examination Surveys (II and III) and the Eastern Finland Study supported a protective role for vitamin C in risk reduction for several endpoints representing cardiovascular diseases and coronary artery disease in separate models [5]. The present study was designed to evaluate the quality of pharmaceutical dosage form contains vitamin C components.

2. Material and Methods

2.1 Determination of Vitamin C Concentration

As a quality evaluation, first determined the vitamin C concentration in each pharmaceutical dosage form of vitamin c samples using simple titration. This titrimetric method was described by Outreach College of Science, University of Canterbury, New Zealand and Abeysuriya HI *et al.*, [6]. As the iodine is added during the titration, the ascorbic acid is oxidized to dehydroascorbic acid, while the iodine is reduced to iodide ions.

Ascorbic acid + $I_2 \rightarrow 2\ I^-$ + dehydroascorbic acid

Due to this reaction, the iodine formed is immediately reduced to iodide as long as there is any ascorbic acid present. Once all the ascorbic acid has been oxidized, the excess iodine is free to react with the starch indicator, forming the blue-black starch-iodine complex. This is the endpoint of the titration. The method is suitable for vitamin C tablets, fresh or packaged fruit juices, and solid fruits and vegetables [7, 8]. Equipment Burette and stand, 100 mL or 200 mL volumetric flask, 20 mL pipette, 10 mL and 100 mL measuring cylinders and 250 mL conical flasks.

2.1.1 Sample Preparation

A single tablet of each vitamin C sample S2, S3, S4, S5, S6, and S7 was dissolved in 200 mL of distilled water in a volumetric flask. 1000 mg of pure ascorbic acid powder S1 as a stander was also taken in 200 ml of distilled water.

2.1.2 Solutions and Reagents

Iodine solution: (0.005 mol L^{-1})

Weigh 2 g of potassium iodide (CAS # 7681-11-0) into a 100 mL beaker, 1.3 g of iodine (CAS # 7553-56-2) from Fisher® was added, then a few mL of distilled water and swirl for a few minutes until iodine is dissolved. Iodine solution was transferred to a 1 L volumetric flask, filled up to the 1 L mark with distilled water.

Starch indicator solution: (0.5%)

0.25 g of soluble starch (CAS # 9005-84-9) was dissolved by adding 50 ml of boiling water in a 100 ml conical flask. Redox titration Standardization of iodine solution 50 mL of the ascorbic acid standard solution with concentration o.5 mg/ml, 20 drops (~1 mL) of 0.2 M acetic acid (CAS # 64-19-7), and ten drops of 2% starch solution was added and titrated against 0.005 mol/L iodine solution 10 mL of each sample was titrated against 0.005 mol/L iodine solution until endpoint shown in figure 4. The volume of iodine solution consumed was recorded and three times repeated for each sample.

2.2 Microbiology Agar Diffusion Method

The agar diffusion method, also known as Kirby–Bauer test, disc-diffusion antibiotic susceptibility test, disc-diffusion antibiotic sensitivity test, and KB test, is widely used to evaluate the antimicrobial activity of compounds in vitro.

2.2.1 Sample Preparation

All vitamin C samples were prepared by directly dissolving one effervescent/crouched tablet or 1000 mg of L-ascorbic acid powder with 10 ml of water for injection to be uniform in concentration100mg/1ml. Each sample was given S1, S2, S3, S4, S5, S6, and S7 Bacterial strains and culture media preparation Six types of bacteria were used: Gram-positive *Bacillus subtilis* ATCC® 6633 ™*, Staphylococcus aureus* ATCC® BAA-977™, and Gram-negative bacterial strains *Klebsiella pneumonia* ATCC® 700603 ™*, Pseudomonas aeruginosa* ATCC® 27853 ™*, Escherichia coli* ATCC® 25922 ™*, and Proteus mirabilis* ATCC® 12453 ™ Microbiologics®, MediMark® Europe. The strains were obtained from AL-Hammadi Hospital, Medical Laboratories, Microbiology Department, Riyadh-KSA.

Mueller-Hinton agar CM0001, OXOID LTD., (Sigma-Aldrich Company) was used as a culture medium to grow bacteria. MHA medium prepared according to manufacturer's instructions and dissolved by distilled water. Sterilize in an autoclave (LabTechat®) 121°C for15 minutes. The agar was poured into a sterile petri dish to a depth of 4mm in laminar airflow. After solidifying, the petri dishes were incubated for 24 h at 37°C.

Antibacterial test for each type of organism, bacterial suspension was prepared from pure bacterial cultures; four colonies were taken by an inoculation loop and transferred to 5 ml of 0.9% saline solution. Then 50 µl of suspension containing test organism was added into plates through a micropipette and spread over the whole petri plates with a sterile cotton swab. Petri plates were dried under sterile conditions for 5 minutes, forming wells (5 mm diameter). In the remaining wells, 50 µl of each test solution with various types of vitamin C was added via micropipette. One well filled with water as a negative control and selective antibiotic disk for each type of bacteria placed on plates by using sterile forceps as a positive reference standard. After 24 h of incubation (labTech®) incubator at 37°C, the zones of inhibition around the samples were calculated with a graduated scale in millimeters (mm). A parallel analysis study with commercial antimicrobial agents for most susceptible antimicrobial discs was used based on CLSI interpretive criteria: B. subtilis ATCC® 6633 was used Amikacine 30 ug (Thermo Scientific ™ Oxoid ™), Staph. aureus ATCC® BAA-977™ was used Ampicillin 10ug (Thermo Scientific ™ Oxoid ™) , K. pneumonia ATCC® 700603 ™ was used Amikacine 30 ug (Thermo Scientific ™ Oxoid ™) , Pseudomonas aeruginosa ATCC® 27853 ™ was used Gentamicin 10 ug (Thermo Scientific ™ Oxoid ™), E. coli ATCC® 25922 ™ was used Gentamicin 10 ug (Thermo Scientific ™ Oxoid ™), and P. mirabilis ATCC® 12453 ™ was used Amikacine 30 ug (Thermo Scientific ™ Oxoid ™) according to CLSI M100 ED31:2021-Performance Standards for Antimicrobial Susceptibility Testing, 31st Edition. All samples have been studied three times and recorded. A vitamin C solution with various types was tested to check the antibacterial effect against Gram-negative and Gram-positive pathogens.

2.2.2 The Minimum Inhibitory Concentration (MIC)

The MIC, or minimum inhibitory concentration, is the lowest concentration in mg/mL of a test solution that inhibits the growth of a given strain of bacteria. The Minimum Inhibitory Concentration (MIC) of the different types of vitamin C solution tested was determined using the method of serial micro dilutions. A Series of 10 tubes containing 1 mL of sterile Nutrient Broth (N.B) (Oxoid, BTL) were prepared. In total, 1 ml of the test solution with a 100mg/ml concentration was added to the first tube from each series, thus obtaining 2 mL of solution in the first tube. The tube contents were mixed thoroughly and then transferred 1 mL of the solution to the next tube in the series and mixed again. The same procedure was followed until the end of the series. For the last tube, 1 mL was discarded to equalize the volume of the remaining tubes in series. For each type of organism, bacterial suspension inoculum 50 µl was added to the prepared ranks, and the clear-control sample containing only liquid medium 1 mL, and + control containing 1 ml of a liquid medium with a different type of bacterial suspension was performed. Series incubations were carried out at 37 ◦C for 24 h. The MIC value was defined as the lowest concentration of the tested sample, in which no visual growth of bacteria was noted.

2.2.3 Determination of Antioxidant Activity

Vitamin C is a potent reducing agent and scavenger of free radicals in biological systems. It is involved in the first line of antioxidant defense, protecting lipid membranes and proteins from oxidative damage. As a water-soluble molecule, vitamin C can work both inside and outside the cells, neutralize free radicals, and prevent free radical damage. Vitamin C is an excellent source of electrons for free radicals seeking out an electron to regain stability. Vitamin C can donate electrons to free radicals and quench their reactivity [9].

2.2.4 2, 2-Diphenyl-1-picrylhydrazyl Assay

The percentage of antioxidant activity (AA %) of each ascorbic acid type was assessed by 2,2-Diphenyl-1-picrylhydrazyl DPPH from SIGMA-ALORICH, MF:$C_{18}H_{12}N_5O_5$, MW:394.32, MP:135 °C, green color, free radical assay. The DPPH radical scavenging activity was measured according to the methodology described by Brand [10]. One tablet 1000 mg of different vitamin c form from each sample was completely dissolved in 10 ml D.W to make a stock solution of 100 mg/ml. The samples were reacted with the stable DPPH radical in an ethanol absolute 99.9 for analysis MF: CH_3CH_2OH, MW: 46.07g/mol, PanReac-AppliChem. The reaction mixture of samples is prepared by adding 0.5 mL of sample stock solution, 3 mL of absolute ethanol, and 0.3 mL of DPPH radical solution 0.5 mM in ethanol (prepared using 10 mg of DPPH in 100 ml of ethanol). When DPPH reacts with an antioxidant compound, which can donate hydrogen, it is reduced. The changes in color from deep violet to light yellow and disappear were visible in all samples and were read [Absorbance (Abs)] at 517 nm after 100 min of reaction using a UV/VIS double beam spectrophotometer model Helios zeta Thermo (England). The mixture of ethanol (3.3 mL) and sample (0.5 mL) serve as blank was prepared for each sample. The control solution was prepared by mixing ethanol (3.5 mL) and DPPH radical solution (0.3 mL) [10]. The scavenging activity percentage (AA %) was determined by method described by Chandra *et al.* [11]

$$\%\ \mathrm{AA} = \left\{1 - \frac{(\mathrm{Abs\ sample} - \mathrm{Abs\ blank})}{(\mathrm{Abs\ control} - \mathrm{Abs\ blank})}\right\} \times 100\%$$

The experiment was done in triplicate for each substance.

2.3 Statistical Analysis

The results were expressed as a percentage decrease concerning control values and compared by one-way ANOVA. P value <0.05 is set as significant.

3. Results and Discussion

3.1 Determination of Vitamin C Concentration Determination

The obtained results have been described in table 1; ascorbic acid content for each sample was calculated according to the following equation of the titration, which determines the number of moles of ascorbic acid was reacting. Ascorbic acid + $I_2 \rightarrow 2 I^- +$ dehydroascorbic acid means the 1 mole of ascorbic acid will react with 2 moles of iodine. The iodometric determination method of ascorbic acid content in different six samples of pharmaceuticals dosage form compared with a pure ascorbic acid powder S1, all samples it was around 1g –/+ 0.1 or 0.2 of pure ascorbic acid present in one tablet and this matches what was written on the tablet content in the recipe except S2.

The differences of ascorbic acid content between the pharmaceuticals samples showing clear in figure 1, and comparing with slandered ascorbic acid S1, the highest content of one tablet and nearest to stander was for S4:Zein Pharma ®, vitamin C 1000mg with 10mg zinc per one effervescent tablet, made in Germany then S5: Vitamin C Forte 1000®, L-ascorbic acid gained from plants biotechnologically with the bioflavonoid, followed by S3:Cal-C-Vita®,1000mg of vitamin C combined with calcium 250mg, vitamin D 300 I.U, S6: NOW®, Ascorbic acid 1000mg with rose hips fruit 25mg, S7: Ester-C®, Calcium Ascorbate 1000mg with vitamin D3 cholecalciferol 125mcg (5000 IU), calcium as calcium ascorbate 111mg, tablet, Ester-C®, and Better Vitamin C® company, manufactured by American Health, INC. the lowest content of ascorbic acid sample tablet was S2: Redoxon®, 1000mg vitamin C effervescent tablet.

Table 1. Ascorbic Acid Content for Each Sample.

Samples	Mean (ml)	Std. Error	AA. tit g/10 ml	AA. ta g/200 ml	Mean ± S.D.
S1	30.06 ±0.32	0.2333	0.05	1.05	1.05±0.32
S2	21.5 ± 0.24	0.2887	0.037	0.75	0.75±0.24
S3	28.46 ± 0.40	0.2906	0.049	0.98	0.98±0.40
S4	29.53 ± 0.40	0.2906	0.052	1.04	1.04±0.40
S5	28.96 ± 0.22	0.2603	0.055	0.99	0.99±0.22
S6	26.2 ± 0.24	0.4163	0.045	0.91	0.91±0.24
S7	24.83 ± 0.19	0.441	0.043	0.87	0.87±0.19

*Data are expressed as Mean ± Standard deviation (n = 3).

**AA. tit: Ascorbic acid titrated in g calculated by $2 \times M_1 \times V_1 = M_2 \times V_2$ » 2 x 0.005 x (mean volume ml) = M_2 x 10 ml of each sample was used » Mass (g) =moles x M. Wt. of ascorbic acid=176.12 g/mol x volume of sample in liter.

***AA. ta: Ascorbic acid content in one tablet.

**Df= Degrees of Freedom, SS= Sum of Squares, MS= Mean Square.

**Data represent mean ± SD, $n = 3$, the F-ratio value is 90.1911. The p-value is 0.000. The result is highly significant at $p < 0.05$.

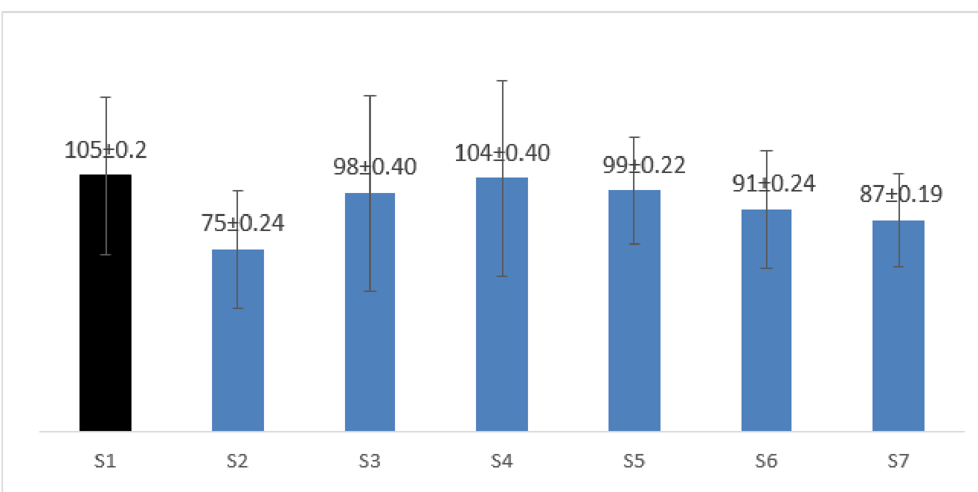

Figure 1. % yield of ascorbic acid present in one tablet for different pharmaceuticals samples.

One study in Romania proposed dosing method was described by volumetric quantitative analysis of pure ascorbic acid in two brands of pharmaceutical tablets has confirmed the following deviations from the declared active substance content: if content up to 10 mg, the maximum allowed deviation ± 10 and only ± 5 for 100 mg and over the content. According to Romanian Pharmacopoeia, 10-th Edition according to European and International in force rules [8], Moreover, if we applied with our deviations results, for all samples, the content accepted was SD ≤ ± 0.4. For all bacterial strains, outcomes of ANOVA test revealed that there was a significant variance ($p<0.05$) and rejected null hypothesis in the antibacterial activity at constant concentration 100 mg/ml of different types of vitamin c samples. Compared to other bacteria they were tested for Gram-positive bacteria *Staph. aureus* BAA-977, *B. subtilis* ATCC 6633, the largest inhibition zone diameters of 33 and 29 mm treated with S4 (vitamin C 1000mg with 10 mg zinc), respectively, were demonstrated. In addition, S4 showed suitable antibacterial activities against *K. pneumoniae* ATCC 700603 with 23 mm and largest zones of inhibitions diameters29, 22, and 29mm for *Ps. aeruginosa* ATCC 27853*, E. coli* ATCC 25922*, and P. mirabilis* ATCC 12453 respectively figure 2.

3.2 Minimum Inhibitory Concentrations

In figure 3, the MIC against *Bacillus subtilis* ATCC 6633 was lowest concentration 0.5 mg/ml in S4: vitamin C 1000mg with 10 mg zinc as the best combination to inhibit *Bacillus subtilis* compared with other samples. For other gram-positive *Staphylococcus aureus* ATCC BAA-977 the minimum concentration was S5: Vitamin C Forte 1000®, L-ascorbic acid gained from plants biotechnologically with bioflavonoid and S7: Ester-C®, Calcium Ascorbate 1000mg with vitamin D3 cholecalciferol 125mcg (5000 IU), calcium as calcium ascorbate 111mg. The gram-negative *Klebsiella pneumonia* ATCC 700603**,** the minimum concentration was 0.07mg /ml for S1: ascorbic acid pure powder followed by S7: Ester-C®, Calcium Ascorbate 1000mg with vitamin D3 cholecalciferol 125mcg (5000 IU), calcium as calcium ascorbate 111mg. And for *Pseudomonas aeruginosa* ATCC 27853*,* the lowest concentration is 0.29mg/ml for S4: vitamin C 1000mg with 10 mg zinc per one effervescent tablet. The minimum inhibitory concentration against *Escherichia coli* ATCC 25922 was 0.29 for S1; ascorbic acid pure powder followed by S6: NOW®, Ascorbic acid 1000 mg with rose hips fruit 25mg in each tablet. The Last bacteria strain, *Proteus mirabilis* ATCC 12453, was inhibited by lower concentration for almost all samples.

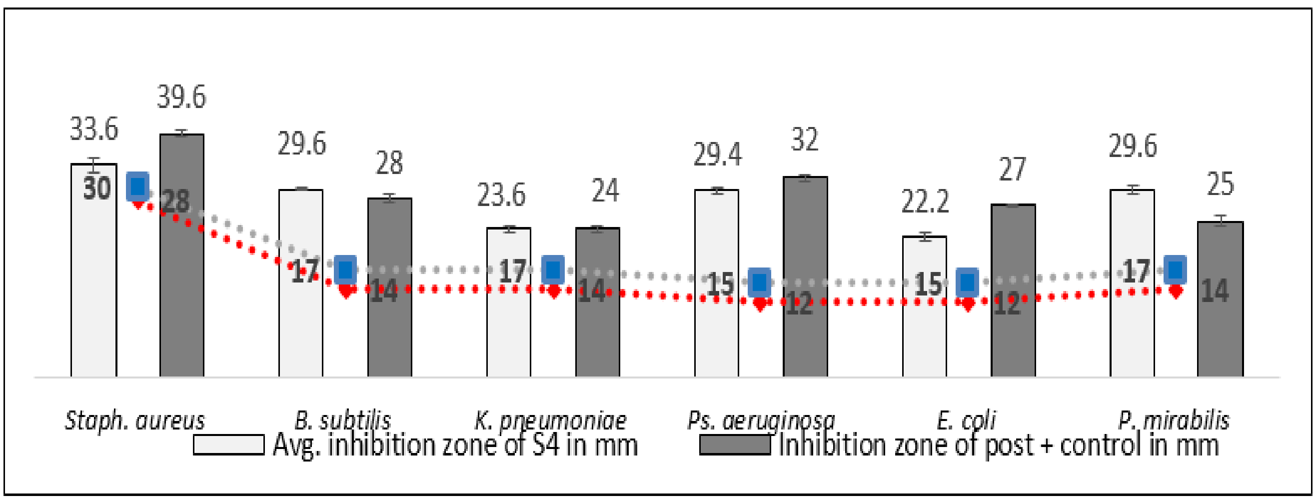

Figure 2. Antibacterial activity of S4 against different types of bacteria.

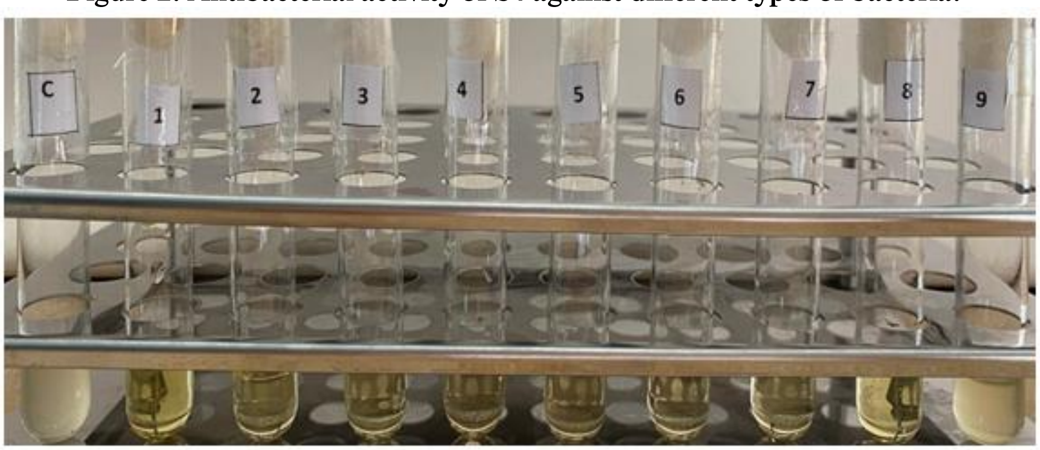

Figure 3. MIC of S3 against E. coli (the last test tube clear with concentration 0.39mg/ml + first test tube turbid with concentration 0.19mg/ml divided by 2 equal to 0.292 mg/ml).

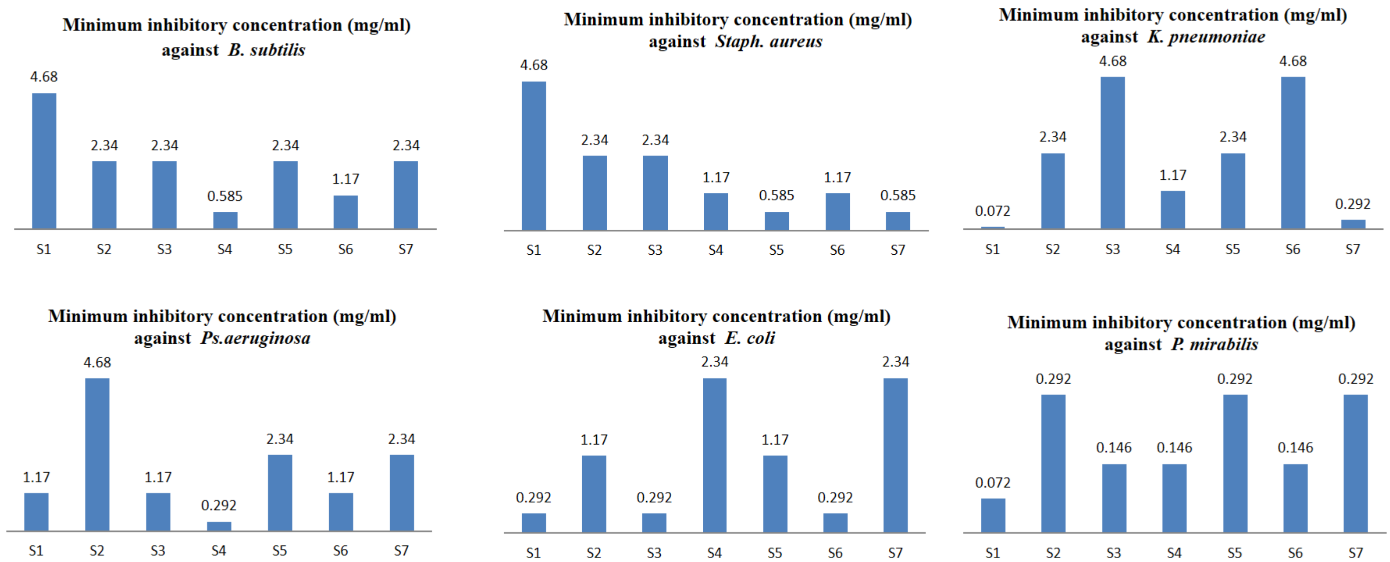

Figure 4. Minimum inhibitory concentration of vitamin C against various microorganism.

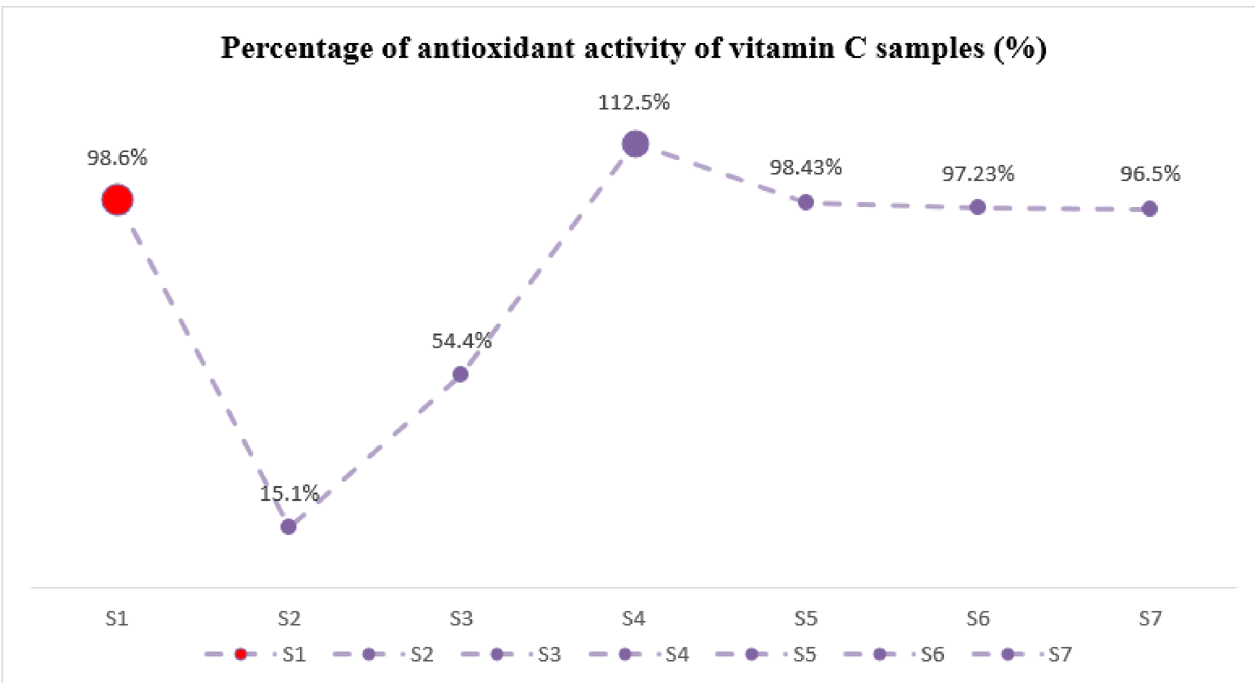

Figure 5. Antioxidant activity of vitamin C samples at 100mg/mL by DPPH assay.

3.3 Antioxidant Activity

This study determines and compares the antioxidant activity of different vitamin C forms in different pharmaceuticals dosage. The combination between ascorbic acid and zinc or bioflavonoid rose hips fruit, and Ester-C® Calcium ascorbate has been shown better antioxidant potential by DPPH radical scavenging method when compared to standard ascorbic acid powder S1 and the value found to be highest 112% then 98 and 96% equal to a near ascorbic acid standard which was 98 %. So, we can say these combinations and forms of vitamin C have maximum antioxidant activity instead of S3, Cal-C-Vita®, 1000 mg of vitamin C combined with calcium 250mg, vitamin D 300 I.U. vitamin B_6 15mg for Bayer Company. The lowest antioxidant activity for S2, Redoxon®, 1000 mg vitamin C. Bayer Company, is only 15%.

4. Conclusion

The present investigation reveals that six pharmaceuticals, as mentioned above forms of vitamins c have been a concentration of ascorbic acid 1g +/- 0.1,0.2 as mentioned in the product value of each sample, except S2, Redoxon®, 1000 mg vitamin C effervescent tablet with orange flavor. Bayer Company. The potent antimicrobial activity of all samples showed significant inhibition against Gram-positive *Bacillus subtilis* ATCC 6633*, Staphylococcus aureus* BAA-

977*,* and Gram-negative bacterial strains *Klebsiella pneumonia* ATCC 700603*, Pseudomonas aeruginosa* ATCC 27853*, Escherichia coli* ATCC 25922*, and Proteus mirabilis* ATCC 12453**.** However, the highest zone of inhibition was found in S4, vitamin C 1000 mg with 10 mg zinc, at a constant concentration (100 mg/ ml) of vitamin C in all Gram-positive and Gram-negative bacterial strains that have been tested. Also, in these studies, highlights of each sample's minimum concentrations have been inhibiting the previous bacteria strains. On the other hand, the study shows that all vitamin c samples have antioxidant properties compared to stander ascorbic acid. Therefore, the highest product was S4, vitamin C 1000 mg with 10 mg zinc, recommended as the best combination antimicrobial and antioxidant dosage form.

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Conflict of interest
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Ethics Approval and Consent to Participate
Not applicable.

Author Contribution
All authors are contributed equally in the research.

Declaration of Generative AI
This manuscript has utilized OpenAI's ChatGPT (version 4) to enhance the clarity and coherence of the language. The tool was employed exclusively for language improvement purposes with ethical and academic standards. The authors take full responsibility for the content and integrity of the manuscript.

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