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Study on antibacterial and antioxidant property from methanol extract of *Vitis vinifera* fruit and its application in hand sanitizer

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Abstract

The goal of this study is to assess the antibacterial and antioxidant activity of a methanol extract of *Vitis vinifera*, as well as to find possible natural sources for the development of novel medications to combat rising antimicrobial resistance. Its major application is to make hand sanitizer. The bactericidal activity of a crude methanol extract of *Vitis vinifera* was tested using the agar well diffusion method, which revealed an estimated potential zone of inhibition of 10.62mm for *Escherichia coli*. The antioxidant activity of the extracts was then measured, with test sample 2 showing 0.728 ug percent antioxidant activity, followed by test sample 1 and test sample 3, which had antioxidant activity of 0.372 ug percent and 0.156 ug percent, respectively. After that, the thesis moves on to the application section, where hand sanitizer compositions were created and evaluated based on colour, odour, clarity, and skin irritation. All created formulations were tested for antibacterial activity, with formulation 3 (made mainly of dried black grapes) showing the most significant findings. Spot inoculation (contact time test) and Spread plate count were performed using just one or two test organisms, due to the method's limitations. Both *Escherichia coli* and *Staphylococcus aureus* grew in a contact time test. SPC was measured before and after hand sanitizer treatment to see if there was a decrease in culture density. Log reduction was observed which helped in determining that the formulations prepared have the capacity to decrease the bacterial load. Antibacterial assay was performed for all prepared formulations, in which formulation 3 (made up of dried black grapes) showed significant results. Keeping in considerations the limitation of this method, spot inoculation (contact time test) and Spread plate count were taken using only one or two test organism. Contact time test showed growth for both *Escherichia coli.*

Keywords: *Vitis vinifera*; Antimicrobial; Antioxidant; Methanol Extract; Hand Sanitizer.

1 Introduction

Vitis vinifera (common grape vine) is a species of *Vitis* that is primarily grown in Iran's east and north. The shrub grows to a height of 32 metres and has flaking bark [11] There are around 5000 to 10,000 species of *Vitis vinifera* in the world [12] The fruit is commonly referred to as a berry, but it is best known as a grape, and it comes in a wide variety of colours and sizes. The fruit of grown plants can grow to be fairly large, up to 3 cm long, and can be red, green, or purple (black) in colour. The grape has been used as a fresh fruit since ancient times, and it is also processed to make wine or juice or dried to produce raisins. Usage of natural antioxidants including fresh fruits and vegetables has curative effects against various diseases. This effect is mostly related to the presence of several components such as vitamins, flavonoids, anthocyanins and other phenolic compounds [11] *Vitis vinifera* is found extensively in Mediterranean region, Central Asia and southwestern Asia from Morocco and Portugal north to southern Germany and east to northern Iran [12] Grape is commonly regarded as one of the primary sources of phenolic compounds, such as resveratrol, the flavanol quercetin, catechins, procyanidins, and anthocyanins, which have been shown to have health advantages as natural

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antioxidants. Polyphenols found in this fruit have been demonstrated to have health benefits, including lowering the risk of cardiovascular disease, improving cognitive and brain function with ageing and neurodegenerative illnesses, and acting as an anticancer, anti-inflammation, and antimicrobe. Furthermore, phenolic chemicals could be absorbed by the human digestive system and successfully injected into the bloodstream with minimal risk of harm. Wine, as well as the seeds and skins of grapes, contain high levels of phenolic compounds, making it a suitable source of grapes. The nutritional and therapeutic properties of *Vitis vinifera* fruits have been known for thousands of years. Grapes are a good source of nourishment and include numerous chemicals that have antibacterial, antioxidant, and anti-inflammatory properties. Grapes, in particular, are high in polyphenols, a class of chemicals produced by plants' secondary metabolism. Secondary metabolites play an important role in the flowering of various plant lineages because they satisfy particular physiology and morphological needs. Because their ability to create these chemicals has varied over evolutionary time in order to resist environmental risks, various plants synthesis different quantities of multiple polyphenols [6]. The skin of grapes, especially those of the red and black varieties, is extremely rich in resveratrol which is a derivative of stilben. Studies have shown that resveratrol is one of the strongest known natural antioxidants [3].

Plant extracts' key functional qualities, such as antibacterial and antioxidant activity, are the foundation of their use in food preservation, medicines, cosmetics, alternative medicine, and natural therapies [3]. As a result, investigations on the extraction of natural chemicals capable of preventing the development of germs have piqued attention in recent years, particularly in relation to microbes that cause food spoilage and are responsible for a wide range of diseases [6]. To get high-value goods, special emphasis has been paid to the utilization of grape pomace, a winemaking sector byproduct made up of skins, seeds, and stems that is produced in vast quantities around the world.

We can design novel disinfectants and drugs/antibiotics against some diseases if we can prove that plant extracts have antibacterial action. There is a need to develop new medications or antibiotics since microbes are becoming resistant to older drugs due to the development of a resistance mechanism that cannot be broken. The WHO has traditionally stated both Gram-positive and Gram-negative bacteria spread illnesses. Many studies have shown that plant extracts have antibacterial properties.

According to research, alcohols are germicidal immediately when applied to the skin, but have little obvious long-term residual activity. However, it has been demonstrated that bacteria regrowth is modest following use. This could be owing to the alcohol's sublethal effect on the remaining bacteria. Alcohol-based hand rubs containing chlorhexidine, octenidine, or triclosan may also provide some long-term protection. 4 percent chlorhexidine has bactericidal activity against methicillin-resistant Staphylococcus aureus for up to 4 hours after application. Ethanol, the most prevalent alcohol constituent, appears to be the most effective against viruses, but propanol is regarded to be a more effective antibacterial alcohol. When two or more alcoholic beverages are combined, they may have a synergistic effect. The amount of alcohol in hand sanitizers has an effect on their effectiveness, according to one study. ABHS frequently include humectants, such as glycerin, which assist avoid skin dryness, as well as emollients or moisturizers, such as aloe vera, which help replenish some of the water lost during use. None of the aforementioned alcohols have been demonstrated to cause acquired bacterial resistance, hence they are considered extremely effective for usage in medical contexts.

Disease-causing bacteria, viruses, and other microorganisms are transmitted mostly through the hands. As a result, hand cleanliness is the most critical step for preventing the transmission of hazardous microorganisms and thereby diseases. Sanitizing hands with sanitizer is the simplest and least expensive way to maintain hand hygiene. Although alcohol-based hand sanitizers claim to destroy 99.99 percent of microorganisms and are quite effective, they do have significant drawbacks, as stated above. Any plant or fruit extract that has antibacterial properties and keeps hands germ-free can be used as herbal component in the formulation. There are a few natural ones available on the market that have a similar effect to the synthetic ones. The most common fruits or plant plant used are Neem (*Azadirachta indica)*, Aloevera *(Aloe barbadensis miller)*, Tulsi (*Ocimum tenuiflorum*), Orange (*Citrus x Sinensis*) etc. But looking at future prospective there might be chances that these herbal also become resistant or might change their property. Thus a study should be carried out to screen more such natural products that show beneficial effects and can be used in disinfectants. In this research we will study if *Vitis vinifera* shows antibacterial effect against some test organism that are usually present on skin especially hands.

2 Material and methods

2.1 Collection and storage of fruit sample

The grape fruit, *Vitis vinifera*, samples were taken from a local market in India in February 2022. The collected material was properly washed in running water, rinsed in distilled water, and then crushed with a mortar and pestle before being stored in an airtight plastic container under refrigeration until extraction process is carried out.

2.2 Sample Preparation

2.2.1 Preparation of Extracts using Alade and Irobi's extraction Method:

Alade and Irobi's cold extraction Method

50gm of crushed sample (*Vitis vinifera* fruit) was taken individually in 50 ml methanol and kept for 72 hours (3 days) at room temperature and agitate with the help of a glass rod in every 24 hours' time interval. After 3 days, the mixture was filtered via the Whatman's filter paper no:1, the filtrate was air dried to eliminate the solvent contents and concentrate it. Then the concentrated extract was stored in the refrigerator (4°C) for further use.

*All the flask top was wrapped with aluminium foil paper in order to resists methanol from evaporating.

- Sample .1- Dried grapes + methanol.
- Sample.2- Wet grapes + methanol.
- Sample.3- Red Wine grapes + methanol.

2.3 Reference Bacterial strains

The strains were maintained on Nutrient agar slant at 4°C.

Table 1 Reference bacterial strains

Sr.no	Test Microorganism	
1	Normal flora / human pathogens.	*Escherichia coli*
2		*Staphylococcus aureus*
3		*Pseudomonas aeruginosa*
4		*Klebshiella pnemonia*

2.4 Determination of antibacterial Activity of Extracts

The antibacterial susceptibility test (AST) was developed to determine the efficacy of prospective antimicrobials derived from biological extracts against a variety of human infections. The antibacterial activity of crude fruit extracts was tested using the Agar well diffusion method. [9]. Determination of antioxidant activity of extract

Samples- Methanol extract of all 3 grapes samples.

2.4.1 Phosphomolybdenum method

This spectroscopic method is very useful in quantitative determination of antioxidant capacity using Total antioxidant capacity assay, through the formation of phosphomolybdenum complex. Antioxidant capacity for unknown can be expressed as equivalents of Ascorbic acid. (Abdullah Ebtesam Saleh, 2011; Biglari F, 2008; Fahad Mohammed Al- Jasass et al; Karthikeyan Ravichandran, 2015; M.A Ibrahim, 2012)

2.5 Preparation of hand sanitizer using extracts and testing it for antibacterial activity

2.5.1 Making 4 formulations of hand sanitizer. (Reference: as per WHO Formulations)

Table 2 Synthetic formulation

Ingredients	Volume (100ml)	Role of ingredient
Isopropyl alcohol 99.8%	75.15 ml	Surface disinfectant
Glycerol 1.45%	1.45 ml	Humectant is used for skin care and to increase acceptability of the product.
Hydrogen peroxide 3%	4.17 ml	Antiseptic, Kills virus, germs, bacteria. Low concentrations of H2O2 help to eliminate contaminating spores.
Distilled water	Add to make 100ml	Vehicle

Table 3 Herbal formulation (For all 3 extracts)

Ingredients	Volume (100ml)	Role of ingredient.
Freshly prepared methanol grape extract	75.15 ml	Testing for antibacterial activity
Glycerol 1.45%	1.45 ml	Humectant is used for skin care and to increase acceptability of the product.
Hydrogen peroxide 3%	4.17 ml	Antiseptic, Kills virus, germs, bacteria. Low concentrations of H2O2 help to eliminate contaminating spores.
Distilled water	Add to make 100ml	Vehicle

** Formulation 1, 2, 3&4 can be noted as F1, F2, F3 & F4 also.

2.5.2 Evaluation test for formulations of hand sanitizer have following test

- Appearance
  - Odour: It can be determined manually
  - Colour: It can be determined visually.
- Clarity

Clarity test was determined to evaluate presence of particulate matter visually.

- Skin Irritation Test

Skin Irritancy of hand sanitizer was evaluated by taking small amount of formulation on palm. Checked for local irritation or any inflammatory reactions (if present or not).

Testing for efficacy of hand sanitizer in terms of its antibacterial effect

The prepared formulations of hand sanitizer extract were scrutinized against various human pathogens using Agar well diffusion method. 20ml of sterile and molten Mueller Hinton Agar, inoculated with 0.5ml of test cultures (OD 530nm= 0.1), mixed gently and poured into sterile Petri dishes to achieve a uniform/ even inoculums. Wells were made using 6mm cup borer and then filled with crude extract until it fills the well. Methanol (99.5%) was used as solvent control. The plates were incubated at 37°C for 24 hours for bacteria. Zones of inhibition were measured in milimetre (mm) and the investigations was executed in duplets[10] The antibacterial activity was inferred from the size of the diameter of zone of inhibition measured in millimetre using a transparent ruler[10].

Formulation 1 (F1) - complete synthetic one.

- Formulation 2 (F2) - made using wet grape extract.
- Formulation 3 (F3) - made using dry grape extract.
- Formulation 4 (F4) - made using wine grape extract.

2.5.3 Contact time test between sanitizer and test organism.

2ml of hand sanitizer + 0.2ml of test organism→ mix gently and spot inoculate on Nutrient agar plate→ check for growth/ no growth. This test helps to determine whether growth is been promoted even after treating the culture with sanitizer, accordingly measures should be taken. (Kwandong Univ. Coll. Med. Hyukmin Lee)

2.5.4 Plate count (SPC) of standard test organism before and after treating with hand sanitizer

This phase will involve a proper enumeration of the organism before and after it has been treated with hand sanitizer, which will allow us to see if there has been any drop in cell count. (Erin R. Sanders)

3 Results

Samples used were 1. Wet black grapes 2. Dried black grapes 3. Wine red grapes

3.1 Study of Antibacterial activity of Methanol extract of *Vitis vinifera* by agar well diffusion method

- The crude extract of plant sample was subjected to antibacterial assay using Meuller Hinton Agar.
- Test organism used were: *Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa, Klebsiella pneumoniae*.
- Diameter of Zones of inhibition were measured in mm.
- *All the values in table below are as per zone of organism- extract combination.

Table 4 Antibacterial activity of methanol extract against test cultures

Test organism	S1	S2	S3	S4	S5	S6	S7	S8
Diameter of Zones of inhibition in mm								
Escherichia coli	11	15	9	16	8	9	11	6
Pseudomonas aeruginosa	10	10	9	10	8	9	9	6.5
Staphylococcus aureus	8	9	6	7	6	6	6	6
Klebsiella pneumoniae	10	12	7	8	7	8	8	6.5

Key* S1- Wet grapes methanol extract,S2- Dried grapes methanol extract, S3- Wine grapes methanol extract, S4- Wet+ dried grapes methanol extract ,S5-Dried+ wine grapes methanol extract, S6- Wet+ wine grapes methanol extract ,S7- Wet+ Dried+ wine grapes methanol extract ,S8- Solvent control (99.5% methanol)

His diameters of the areas of growth inhibition by the methanol extracts from skin of the grapes varieties in terms of high to low inhibitions are as follows:

Comparision on basis of average zones of inhibition:

Escherichia coli (10.62 mm) > Pseudomonas aeruginosa (8.94 mm) > Klebsiella pneumoniae (8.31 mm) > Staphylococcus aureus (6.75 mm).

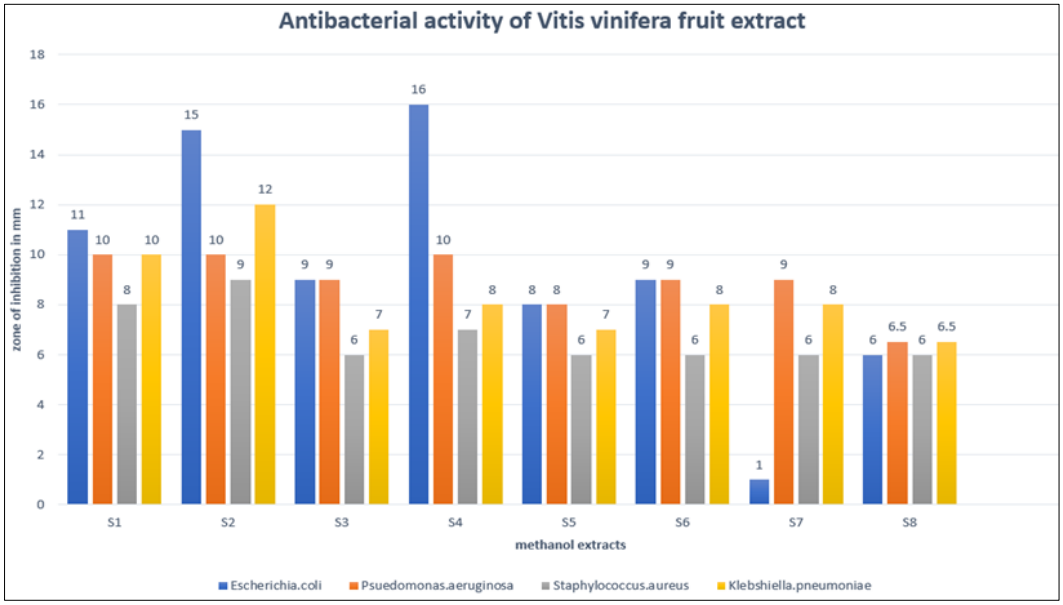

Figure 1 Graphical representation of antibacterial activity of *Vitis vinifera* fruit extract

3.2 Determining Antioxidant activity by Phosphomolybdenum Method

Standard used is Ascorbic acid (60ug/ml), O.D was measured at 670 nm.

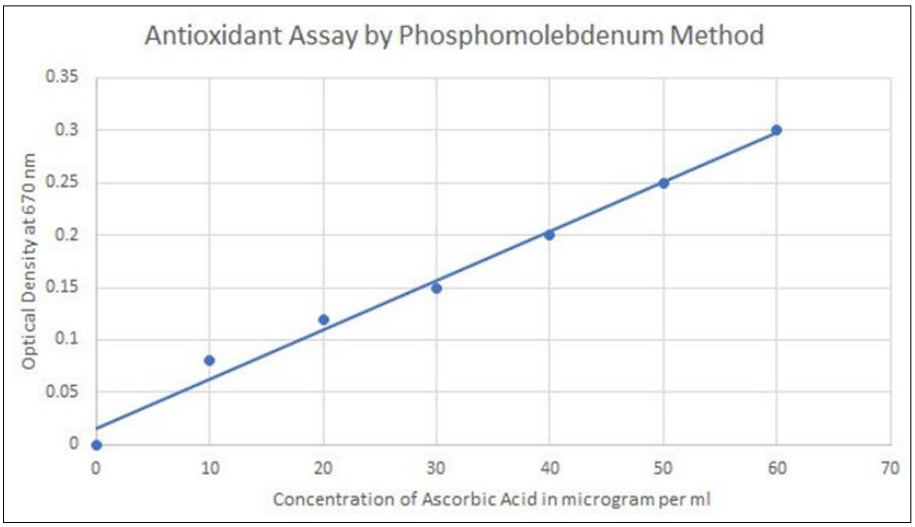

Figure 2 Graphical representation of antioxidant assay by phosphomolybdenum method

Since, the value of O.D of test samples TS 1, TS 2, TS 3 do not fall in range of standard graph their values of concentration (ug/ml) were found by calculation method.

TS 1- wet grapes extract, TS 2- dried grapes extract, TS 3- wine grapes extract

Table 5 Antioxidant activity in ug % of test samples

Test samples	Concentration(ug/ml)	(ug %)
TS 1	3.72	0.372
TS 2	7.28	0.728
TS 3	1.56	0.156

This table denotes that test sample S2 > test sample S1 > test sample S3 in terms of antioxidant activity.

3.3 On preparation of formulations of hand sanitizer they are to be tested for its evaluation

Figure 3 4 formulations of hand sanitizer

3.3.1 Evaluation test for formulations of hand sanitizer have following test

Table 6 Results for evaluation test of hand sanitizers

Properties	**F1**	**F2**	**F3**	**F4**
COLOUR	Colourless	Light voilet	Brown	Light brown
ODOUR	Alcoholic	Fruity	Pungent	Fruity
CLARITY	No solid precipitates	No solid precipitates	No solid precipitates	No solid precipitates
SKIN IRRITATION	Absent	Absent	Absent	Absent

Formulation 1 (F1)- complete synthetic one; Formulation 2 (F2)- made using wet grape extract; Formulation 3 (F3)- made using dry grape extract; Formulation 4 (F4)- made using wine grape extract.

3.3.2 Testing for efficacy of hand sanitizer in terms of its antibacterial effect:

Formulations of laboratory prepared hand sanitizers were subjected to antibacterial assay using Mueller Hinton agar.

- Formulation 1 (F1) - complete synthetic one.
- Formulation 2 (F2) - made using wet grape extract.
- Formulation 3 (F3) - made using dry grape extract.
- Formulation 4 (F4)- made using wine grape extra

Formulation 3 compared to other formulations showed fair results.

One limitation of this method is that at time there might not be proper diffusion of test sample and hence no proper zones of inhibition seen.

Hence, spot inoculation and spread plate count was taken of all these formulations using standard cultures.

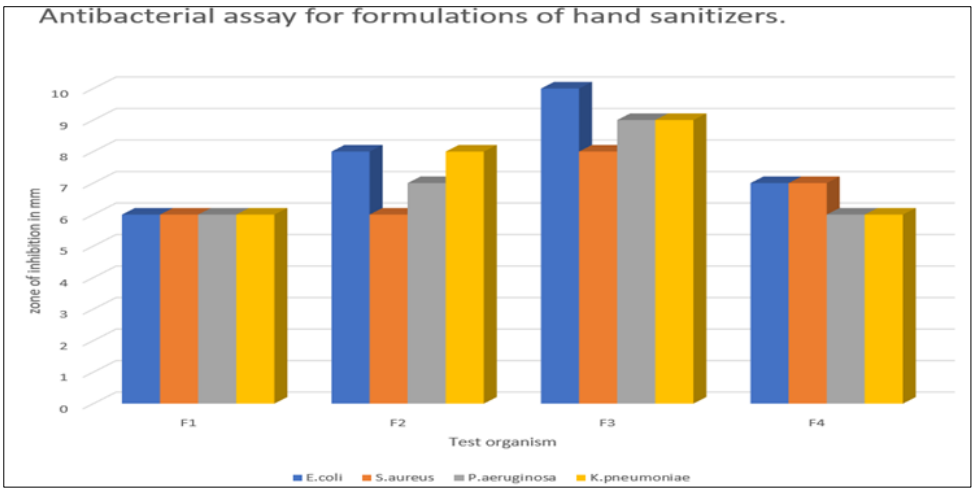

Figure 4 Graphical representation of Antibacterial assay for formulations of hand sanitizers

Table 7 Results for 10 seconds exposure to different formulations of hand sanitizer

Test organism	**F1**	**F2**	**F3**	**F4**
Escherichia coli	-	+	+	+
Staphylococcus.aureus	-	+	+	+

Key: + → growth - → no growth

This table indicates that after a certain contact period between culture and hand sanitizer formulation F1 inhibited growth of both test organism where as other formulations 2,3 &4 could not inhibit its growth.

3.3.3 Plate count of standard test organism before and after treating with hand sanitizer

In this step there will be proper enumeration of organism before treating with hand sanitizer and after treating it with sanitizer, which would help us to understand if at all there are decrease in cell count.

This step is also a confirmatory step to the initial one in which on spot inoculation growth was observed for formulations 2,3 &4. Here test organism used was *Escherichia.coli.* Initial plate count- 3.95 x 10^6 cfu/ml.

Table 8 Results for 10 seconds exposure to different formulations of hand sanitizer

Formulations	Before exposure (cfu/ml)	After 10 sec exposure (cfu/ml)	Log reduction	% reduction
F1	3.95 x 10^6	1.3 x 10^3	4.483	99.997
F2		9.36 x 10^3	3.625	99.976
F3		2.25 x 10^3	4.244	99.994
F4		4.3 x 10^3	3.963	99.99

4 Discussion

4.1 Antibacterial activity of methanol extracts

The present study aims the evaluation of antimicrobial potential of methanol extract of *Vitis vinifera* fruit and to identify potential natural sources for the synthesis of new pharmaceutical products to address the growing antimicrobial resistance. Initially after preparing methanol grape extract it was tested for its antibacterial activity. Three types of grape fruit were uses namely- wet black grapes, dried black grapes & wine red grapes. Antibacterial property of the crude methanol grapes extract was evaluated against 4 test organism using agar cup method which demonstrated the inhibition on basis of methanol extract added to the wells. On comparing the extracts in different wells, original solvent S2 i.e of dried black grapes was more effective in terms of getting zones of inhibition against the 3 out of 4 test cultures. Dried grapes probably have more antioxidant and phytochemical content due to less water concentration which would be one of the interfering agent. Whereas extract of wine red grapes i.e S3 showed minimum inhibition. To have more number of test extracts there was mixing done and accordingly extracts were made. Out of which S4 i.e mixture of wet and dried grapes showed maximum inhibition, followed by S7, S6 and S5 at the last showing minimum or no inhibition. This study tells us that mixture of 2 or 3 different samples can be made in order to get better zones. In phytomedicine research, synergy assessment between plants can be made [1] [10]. In terms of cultures being inhibited *Escherichia.coli >Klebshiella.pneumoniae >Psuedomonas.aeruginosa > Staphylococcus.aureus*.

4.2 Antioxidant activity by Phosphomolybdenum Method

Phytochemical analysis (Chemical Tests by Tube Method) revealed the presence of phytoconstituents mainly antioxidants. Phosphomolybdenum method was used to calculate and demonstrate the total antioxidant capacity. Antioxidant capacity in terms of % it was found that test sample 2(dried grapes) > test sample 1 (wet grapes) >test sample 3(wine grapes) with antioxidant capacity of 0.728%, 0.372% and 0.156% respectively.

This table indicates antioxidant capacity of both dried matter and juice extract, since our sample was in form of methanol juice extract values will be correlated on that basis. On comparing with standard values for grapes 3.9% to the test sample 0.7%, indicating differences in antioxidant levels.

The total antioxidant capacity varies considerably from one kind of fruit to another. For example, on the basis of the wet weight of a fresh fruit, the total antioxidant capacity of strawberry was 2 times the capacity measured in oranges or red grapes, 7 times the capacity measured in apple and banana, 11 times the capacity measured in pears, and 16 times the capacity measured in honeydew melon (Hong Wang, 1996).

Therefore, on basis of examples of fruits given in reference table, strawberry is the fruit with maximum antioxidants.

4.3 Evaluating the prepared formulations of hand sanitizer

All the prepares formulations of hand sanitizers were evaluated on the basis of its colour, odour, clarity and skin irritation. All the 4 formulations showed fair results.

Test were performed to correlate the efficacy between the prepared hand sanitizers. On performing antibacterial assay by agar cup method, it was found that formulation 3 showed fair zones of inhibition as compared to other formulation (table no 6). This method may not be that accurate enough that only on this basis the efficacy of sanitizer can be determined. Efficacy may alter dure to agar composition or depth of zones such that not maximum amount of extract is been diffused into the agar medium. Thus, contact time test and standard plate count was taken for all formulations of prepared sanitizers. In contact time test, table 8, after spot inoculating on Nutrient agar plate growth was promoted for all 3 formulations except formulation 1. This signifies that even after contacting the test culture with hand sanitizer for few seconds, growth was promoted, indicating that more contact time then 10 sec might be needed to inhibit growth or decrease the number of organism. To check if there is decrease in density of culture, plate count was taken before and after treating it with hand sanitizer formulations. This test method is designed to measure the reduction in bacterial flora on the skin. It is intended for determining immediate and persistent microbial reduction (World health Organization; 2009). Spread plate count shows that formulations 1, 2, 3&4 shows 99.997, 99.976, 99.994 &99.99% reduction

This indicates that synthetic formulation 1 can be replaced by formulation 3 which nearly shows same log reduction i.e their efficacy to kill and reduce bacterial growth is almost the same.

Hand sanitizers containing alcohol enhance the risk of fire. Accidental poisoning via ingesting, fire hazard, organ toxicity, and other dangers are all possible. Antimicrobial properties, antioxidant activity, and a high microbial load are all found in herbal sanitizers. Herbal hand sanitizers are superior to alcohol-based hand sanitizers in that they are more efficient against microorganisms, nontoxic, soften the hands, and keep them clean. Plants have antibacterial capabilities due to their abundance of secondary metabolites such as tannins, terpenoids, alkaloids, and flavonoids. Many traditional healers have employed plants to prevent or cure infectious diseases.

5 Conclusion

The purpose of this thesis was to investigate the antibacterial and antioxidant properties of Methanol extract of *Vitis vinifera* fruit and their use in hand sanitizers. Many research, identification, and quantification of diverse classes of phytochemicals with a high potential application in the burgeoning businesses of functional foods and nutraceuticals have been conducted in recent years due to an explosion of interest in the multiple health advantages of grapes. Researchers have discovered that phytochemicals have the potential to stimulate the immune system, prevent toxic substances in the diet from becoming carcinogenic, reduce oxidative damage to cells, slow cancer cell growth, trigger damaged cells to self-destruct (apoptosis) before they can reproduce, help regulate intracellular signaling of hormones and gene expression, and activate insulin receptors. Tannins, Saponins, Flavonoids, Glycosides, Alkaloids, and Terpenoids were identified as phytochemical constituents in the study. The presence of large levels of Terpenoids chemicals suggests that these compounds have antibacterial, antiviral, and antioxidant activities. Both normal flora and human pathogenic diseases are moderately resistant to the grape fruit's antibacterial properties. To avoid the resistance that synthetic disinfectants demonstrate, herbal disinfectants should be utilized instead of synthetic disinfectants. We must be prepared for any similar emergency that may arise in the near future, given the current pandemic issue. If all synthetic items are used at the same time, resistance will develop at a faster rate. The main reason for avoiding excessive synthetic use is to minimize skin irritation, allergies, and other negative side effects. Such discoveries like those presented in this study are expected to be useful in a variety of sectors such as pharmaceuticals, food processing, nutraceuticals, Ayurveda, cosmetics, biotechnology, fisheries, nanomedicine, agriculture, bio pesticide, green chemistry, phytomedicinal research etc. We certainly hope that this study will contribute in a small but significant way to the ever expanding realm of knowledge and research in the field of Microbiology and Phytomedicinal research.

Compliance with ethical standards

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Disclosure of conflict of interest

No conflict of interest.

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