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In-Vitro Exploration Of Grape Seed Phytoconstituents As Multifunctional Modulators Of Aging, Oxidative Stress And Inflammatory Pathways

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Abstract: Aging is a multifactorial biological process marked by progressive cellular dysfunction, largely driven by oxidative stress and chronic inflammation. Excessive generation of reactive oxygen species (ROS) damages vital biomolecules such as DNA, proteins, and lipids, leading to cellular senescence and tissue degeneration. Therefore, simultaneous modulation of oxidative and inflammatory mechanisms is considered a strategic approach for delaying the aging process.Grape seeds are rich in polyphenolic phytoconstituents such as proanthocyanidins, catechins, epicatechins, and gallic acid, which exhibit strong antioxidant and anti-inflammatory activities. These bioactive compounds scavenge free radicals, inhibit lipid peroxidation, enhance endogenous antioxidant defences, and regulate inflammatory mediators.

The present study successfully demonstrated the in-vitro antioxidant, anti-inflammatory, and anti-aging potential of grape seed phytoconstituents. Phytochemical screening confirmed the presence of bioactive compounds such as polyphenols and flavonoids, which are responsible for the observed biological activities.

The grape seed extract showed significant free radical scavenging activity in the DPPH assay, indicating strong antioxidant potential. The extract also exhibited effective nitric oxide scavenging activity, suggesting its ability to modulate inflammatory pathways. Furthermore, the collagenase inhibition assay confirmed the anti-aging potential of grape seed phytoconstituents by preventing collagen degradation. In conclusion, grape seed phytoconstituents possess significant potential as natural antioxidants and anti-inflammatory agents, making them promising candidates for the development of anti- aging and health-protective formulations.

Keywords: Excessive Generation, Phytoconstituents, Antioxidant Defences, Biological Activities

1.Introduction:

Aging is a multifactorial biological process marked by progressive cellular dysfunction, largely driven by oxidative stress and chronic inflammation. Excessive generation of reactive oxygen species (ROS) damages vital biomolecules such as DNA, proteins, and lipids, leading to cellular senescence and tissue degeneration. Persistent

oxidative stress further activates inflammatory pathways, including NF-κB and pro-inflammatory cytokines, creating a continuous cycle that accelerates aging and increases susceptibility to age-related disorders. Therefore, simultaneous modulation of oxidative and inflammatory mechanisms is considered a strategic approach for delaying the aging process.

Grape seeds are rich in polyphenolic phytoconstituents such as proanthocyanidins, catechins, epicatechins, and gallic acid, which exhibit strong antioxidant and anti-inflammatory activities. These bioactive compounds scavenge free radicals, inhibit lipid peroxidation, enhance endogenous antioxidant defences, and regulate inflammatory mediators. In-vitro exploration of grape seed phytoconstituents provides scientific validation of their multifunctional role in modulating aging-associated oxidative and inflammatory pathways, thereby supporting their potential development as natural therapeutic agents for promoting healthy aging[1].

1.1 Overview of aging:

People worldwide are living longer. Today most people can expect to live into their sixties and beyond. Every country in the world is experiencing growth in both the size and the proportion of older persons in the population. By 2030, 1 in 6 people in the world will be aged 60 years or over. At this time the share of the population aged 60 years and over will increase from 1 billion in 2020 to 1.4 billion. By 2050, the world's population of people aged 60 years and older will double (2.1 billion). The number of persons aged 80 years or older is expected to triple between 2020 and 2050 to reach 426 million. While this shift in distribution of a country's population towards older ages – known as population ageing – started in high-income countries (for example in Japan 30% of the population is already over 60 years old), it is now low- and middle-income countries that are experiencing the greatest change. By 2050, two-thirds of the world's population over 60 years will live in low- and middle-income countries.

1.1.2 Definition:

Anti-aging is defined as a scientific and medical approach that aims to delay, prevent, and potentially reverse the biological processes associated with aging, while preserving the structural, functional, and aesthetic integrity of the body. It involves the application of advanced medical science and healthcare technologies for the early detection, prevention, diagnosis, and treatment of age-related dysfunctions and chronic diseases.

1.1.3 Significance:

- Delays biological aging — Anti-aging science aims to slow, prevent, or reverse the biological processes of aging, extending healthy lifespan rather than just lifespan.
- Prevents age-related diseases — By intervening early in the mechanisms of aging, anti- aging approaches reduce the risk of chronic diseases such as cardiovascular decline, neurodegeneration, and frailty.
- Improves quality of life — Anti-aging medicine focuses on maintaining physical and mental function as people age, helping individuals remain independent and active longer.
- Supports preventive healthcare — It shifts healthcare from treating diseases after they occur to preventing onset through early detection and intervention strategies.
- Reduces healthcare burden — By lowering the incidence and severity of age-associated conditions, anti-aging strategies can reduce long-term healthcare costs and resource demand.
- Advances scientific understanding — Research in anti-aging drives insights into fundamental biological

processes, opening doors to novel therapies and diagnostic tools[2]

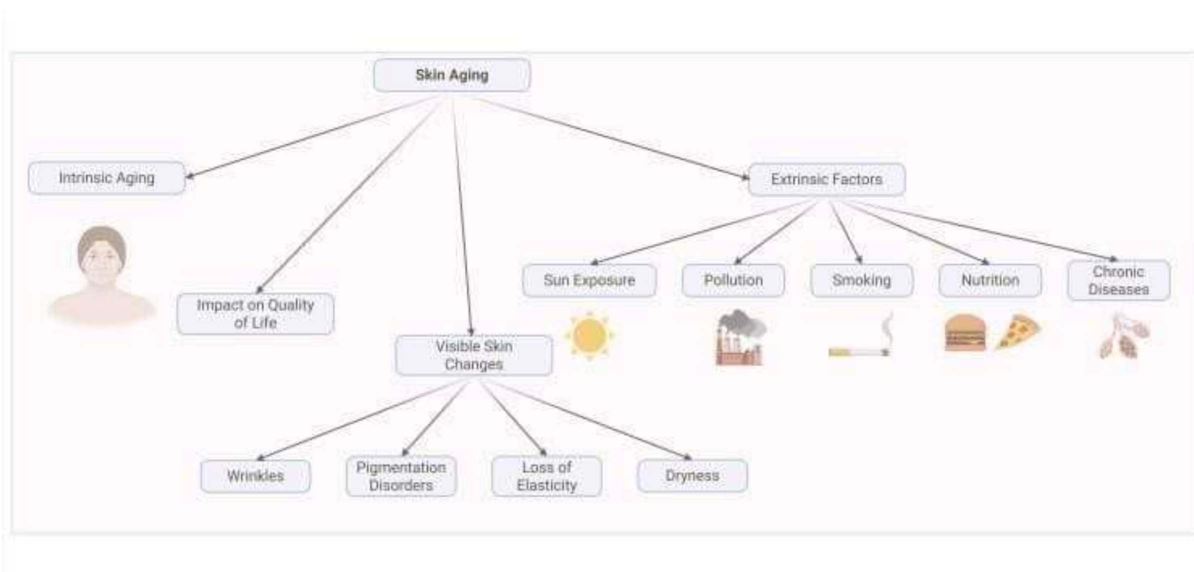


Fig no: 01 skin aging flow chart

Flowchart:

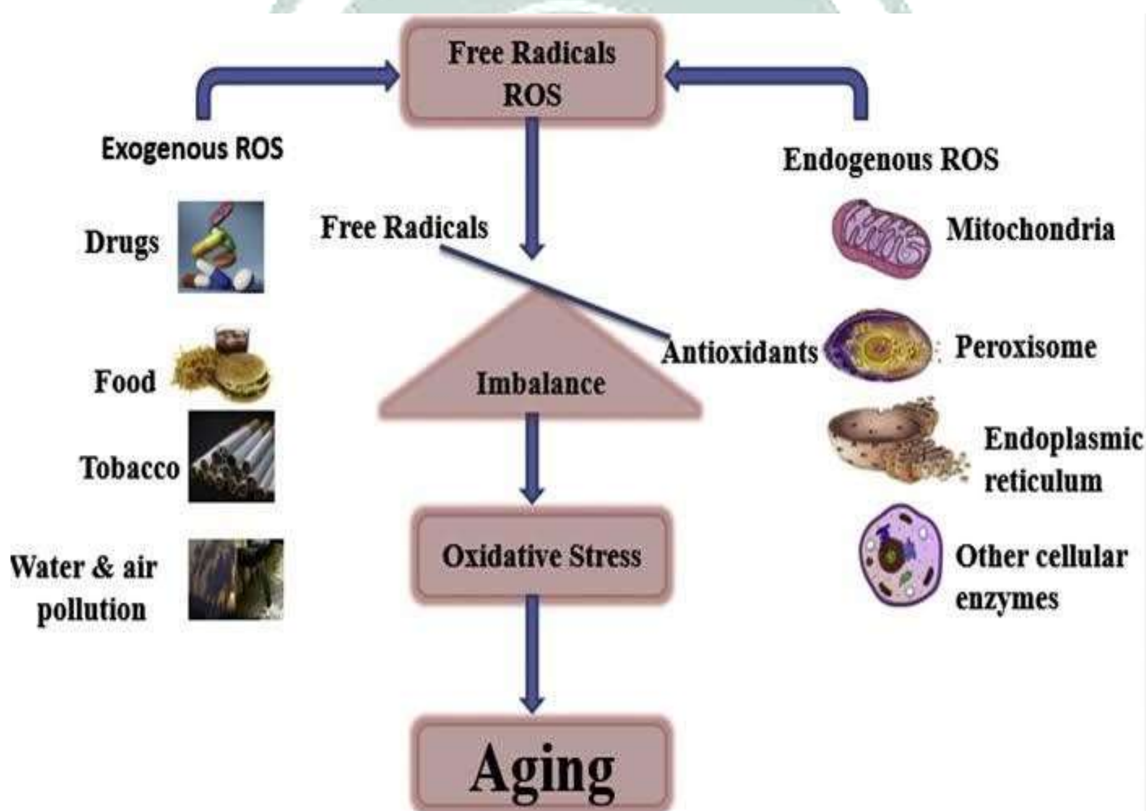


Fig no:02 mechanism of action of skin aging

1.2 Oxidative Stress (Antioxidant Activity):

Antioxidant activity refers to the ability of a substance to neutralize or inhibit the harmful effects of free radicals and reactive oxygen species (ROS) in the body. Free radicals are unstable molecules produced naturally during normal metabolic processes or due to external factors such as pollution, radiation, smoking, and stress. When their levels exceed the body's natural defence capacity, they cause oxidative stress, leading to cellular damage of lipids, proteins,

and DNA.

Antioxidants are protective compounds that prevent or delay oxidative damage by donating electrons to free radicals without becoming unstable themselves. They help maintain the balance between oxidants and antioxidants, thereby preserving cellular integrity and normal physiological function. Antioxidants can be endogenous (e.g., superoxide dismutase, catalase, glutathione) or exogenous, obtained from dietary sources such as fruits, vegetables, herbs, and plant extracts rich in polyphenols, flavonoids, vitamin C, and vitamin E.

Evaluation of antioxidant activity is important in pharmaceutical and nutraceutical research because oxidative stress is strongly associated with aging and various chronic diseases such as cancer, cardiovascular disorders, neurodegenerative diseases, and diabetes. Therefore, assessing antioxidant potential through in vitro assays like DPPH, ABTS, FRAP, and hydrogen peroxide scavenging methods plays a crucial role in determining the therapeutic value of herbal formulations and natural products.

1.2.1 Reactive oxygen species (ROS):

Reactive Oxygen Species (ROS) are highly reactive oxygen-containing molecules formed naturally in the body during normal metabolic processes, especially during mitochondrial respiration. Although small amounts of ROS are essential for cell signalling and immune Défense, excessive production leads to oxidative stress and cellular damage.

ROS and Anti-Aging Connection :

Excessive ROS accelerates aging by damaging cellular components and shortening telomeres. Antioxidants neutralize ROS and help protect cells, making antioxidant evaluation essential in anti-aging research.

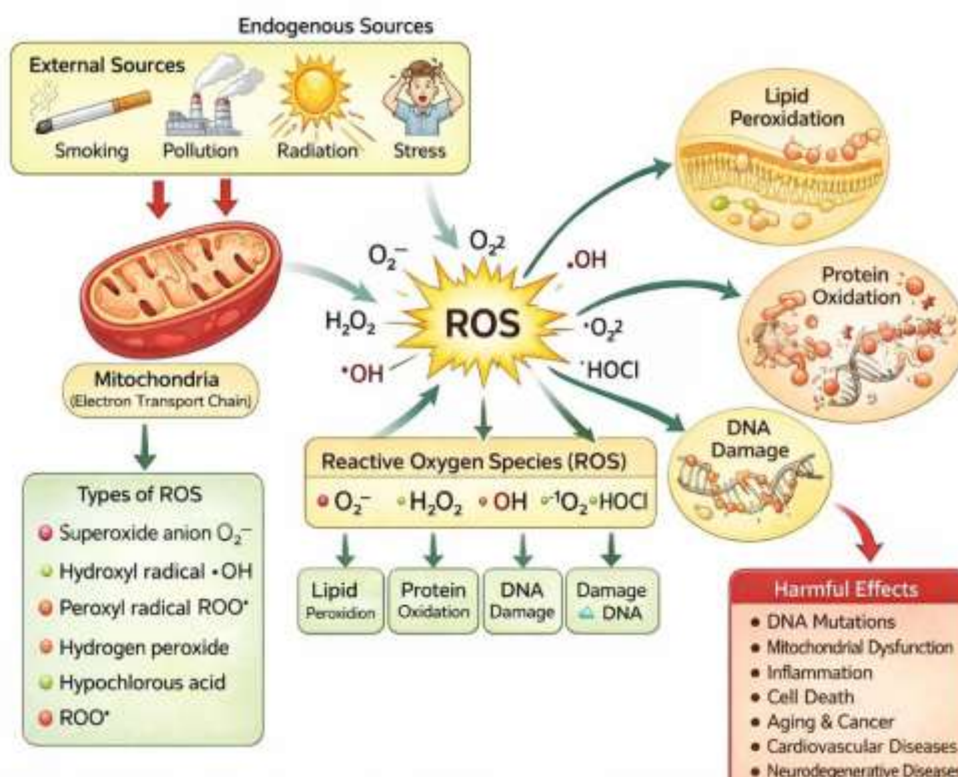


Fig no:03 ROS and Anti-Aging Connection

1.2.2 Free radical theory of aging:

The Free Radical Theory of Aging was first proposed by Denham Harman (1956). According to this theory, aging occurs due to the accumulation of damage caused by free radicals, particularly Reactive Oxygen Species (ROS), over time.

Concept:

During normal metabolic processes—especially in the mitochondria—free radicals such as superoxide ($O_2^{\bullet -}$), hydroxyl radical (•OH), and hydrogen peroxide (H_2O_2) are continuously produced. While small amounts are essential for normal cellular functions, excessive production leads to oxidative stress when antioxidant defences are insufficient.

1.2.3 Cellular damage mechanism:

Cellular damage occurs when excessive Reactive Oxygen Species (ROS) overwhelm the body's antioxidant Défense system. This imbalance leads to oxidative stress, which damages essential cellular components such as lipids, proteins, DNA, and mitochondria.

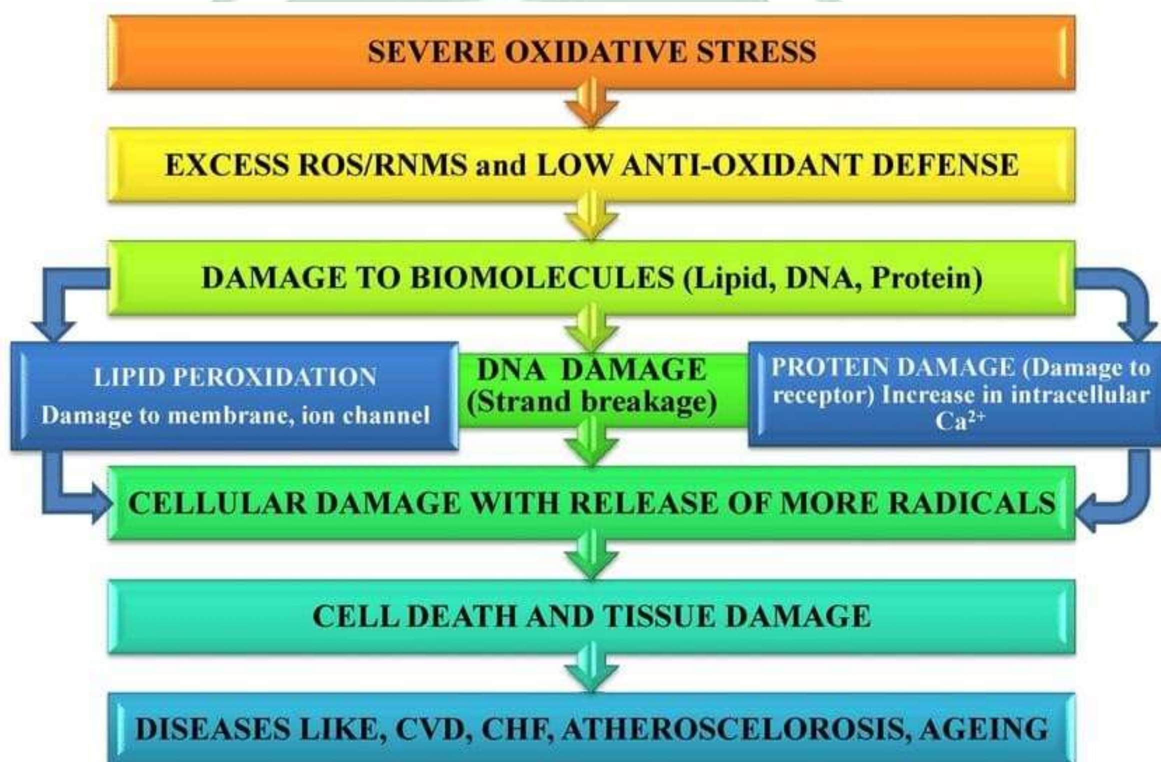


Fig no:04 Mechanism of cellular damage

1.3 Inflammation:

Inflammation is a protective biological response of the immune system against harmful stimuli such as pathogens, injured cells, toxins, trauma, or infections. The term originally described the classical signs of redness (erythema), heat, swelling (edema), pain, and loss of function. It acts as the second line of defense and helps eliminate the initial cause of injury while initiating tissue repair. However, excessive or prolonged inflammation may lead to tissue damage and disease. Disorders in which inflammation plays a major role usually end with the suffix "-itis".

1.3.1 Acute Inflammation:

Acute inflammation is an immediate and short-term response to injury or infection. It develops rapidly (within minutes to hours) and usually resolves within a few days. It is mainly mediated by neutrophils and characterized by the five cardinal signs: heat, redness, swelling, pain, and loss of function.

Redness and heat occur due to increased blood flow (vasodilation), swelling results from fluid accumulation, and pain is caused by the release of inflammatory mediators. Acute inflammation consists of two major components:

1. Vascular Events:

The vascular phase involves:

- Vasodilation (increased blood flow)
- Increased vascular permeability
- Exudation of plasma proteins
- Stasis of blood flow

In normal tissues, fluid balance is maintained by hydrostatic and oncotic pressures. During inflammation, plasma proteins leak into the interstitial space, reducing oncotic pressure in blood vessels and leading to tissue edema.

1. Cellular Components:

The cellular phase includes recruitment and activation of leukocytes. Major events are:

- Leukocyte recruitment
- Margination and adhesion
- Diapedesis (migration through vessel walls)
- Chemotaxis (movement toward chemical signals)
- Phagocytosis
- Tissue repair

Neutrophils are the first responders and destroy pathogens through phagocytosis. Macrophages remove debris and coordinate healing. Lymphocytes contribute to immune regulation and may promote chronic inflammation if the response is not resolved.

Pathophysiology of Acute Inflammation:

Acute inflammation occurs in four stages:

1. **Initiation Phase** – Injury triggers vasodilation and increased permeability.
2. **Amplification Phase** – Inflammatory mediators recruit more immune cells.
3. **Destruction Phase** – Leukocytes eliminate pathogens and damaged tissue.

4.Termination Phase – Anti-inflammatory signals suppress the response to prevent excessive damage.

1.3.1 Chronic Inflammation:

Chronic inflammation develops slowly and may last for months or years. It is characterized by infiltration of macrophages, lymphocytes, and plasma cells rather than neutrophils. Classical signs may be less obvious compared to acute inflammation.

It can result from:

- Persistent infections (e.g., mycobacteria, fungi, parasites)
- Autoimmune disorders
- Continuous exposure to toxic substances
- Repeated acute inflammation
- Oxidative stress and mitochondrial dysfunction

Chronic inflammation contributes to major global diseases such as cardiovascular disorders, cancer, diabetes, obesity, stroke, and chronic respiratory diseases.

1. Causes of Chronic Inflammation:

- ➢ Persistent infectious agents resistant to immune defense.
- ➢ Long-term exposure to irritants (e.g., silica dust, industrial chemicals).
- ➢ Autoimmune diseases such as rheumatoid arthritis and systemic lupus erythematosus.
- ➢ Defects in inflammatory regulatory mechanisms.
- ➢ Repeated episodes of acute inflammation.
- ➢ Oxidative stress caused by free radicals, advanced glycation end products (AGEs), oxidized lipoproteins, urate crystals, and homocysteine.

2. Pathophysiology of Chronic Inflammation:

In chronic inflammation, vasodilation and permeability continue, but neutrophils are replaced by macrophages and lymphocytes. These cells release cytokines, growth factors, and enzymes, leading to:

- Persistent tissue damage
- Fibrosis
- Granuloma formation

Cytokines such as IL-1 and TNF-α promote leukocyte recruitment by inducing adhesion molecules like selectins and integrins. Macrophages and dendritic cells present antigens to lymphocytes, sustaining the inflammatory response.

1.3.3 Molecular mediators :

Inflammation is regulated by various cellular and molecular mediators.

Inflammatory Mediators:

A. Cytokines

- Pro-inflammatory: IL-1, TNF-α, IL-6
- Anti-inflammatory: IL-4, IL-10, IL-13

B. Arachidonic Acid Metabolites

- Prostaglandins (pain and fever)
- Leukotrienes (allergic reactions)

C. Vasoactive Amines

- Histamine (vasodilation and edema)

D. Other Mediators

- Kinins
- Platelet-activating factor (PAF)
- Reactive Oxygen Species (ROS)
- Reactive Nitrogen Oxide Species (RNOS)

Défense system. Excess ROS not only damages cellular macromolecules such as lipids, proteins, and DNA, but also activates inflammatory signalling pathways.

Therefore, antioxidant therapy plays a crucial role in anti-aging research. By reducing ROS levels, antioxidants help suppress inflammatory signalling pathways, protect cellular components, and prevent progression toward chronic degenerative diseases. This establishes the scientific basis for evaluating antioxidant and anti-inflammatory activities in herbal formulations and natural extracts in anti-aging studies.[5],[6],[7].

1.4 Role of phytoconstituents in anti-aging therapy:

1) Proanthocyanidins / Oligomeric proanthocyanidins (OPCs):

Class / source: condensed tannins (flavan-3-ol oligomers) — concentrated in grape seed extract.

Anti-aging actions: powerful antioxidant (scavenges ROS), inhibits UV-induced oxidative damage, reduces inflammation (NF-κB), downregulates MMPs and protects collagen; improves skin elasticity and reduces lipid peroxidation.

Evidence level: multiple in-vitro, animal studies and some clinical / topical studies showing improvements in skin markers and oxidative markers. Strong experimental support for skin anti-photo aging.

2) Resveratrol:

Class / source: stilbene (polyphenol) — present in grape skins, red wine, grape products.

Anti-aging actions: activates longevity pathways (SIRT1/sirtuins, AMPK), improves mitochondrial function, reduces inflammation and oxidative stress, neuroprotective effects. Mechanisms link to cellular stress resistance and gene regulation involved in longevity.

Evidence level: robust preclinical literature; human supplementation studies show mixed results for clinical endpoints (dose and formulation matter). Use as a mechanistic anti-aging agent (sirtuin activator) with moderate clinical backing.

3) Quercetin:

Class / source: flavanol flavonoid — found in many plants, including grapes.

Anti-aging actions: antioxidant and anti-inflammatory; inhibits signalling pathways involved in photoaging (targets like JAK2, PKCδ), reduces UV-induced inflammation and MMP expression — helps preserve collagen. Also senolytic activity reported for related flavonoids in some studies.

Evidence level: strong in-vitro and animal evidence for skin protection; mechanistic papers show direct enzyme/pathway interactions.

4) Catechin & (−)-Epicatechin:

Class / source: flavan-3-ols — common in grape seeds, green tea, cocoa.

Anti-aging actions: antioxidant, modulates NAD+/NADH and mitochondrial pathways, upregulates endogenous antioxidant enzymes, reduces MMP-1 and preserves collagen expression in skin models. Epicatechin also linked to metabolic and vascular benefits that relate to healthy aging.

Evidence level: several reviews and experimental studies support lifespan/health span benefits in models and protective effects against UV-induced cell damage.

5) Gallic acid:

Class / source: phenolic acid — present in grape seeds and many plant extracts.

Anti-aging actions: antioxidant and anti-MMP activity; reduces ROS, downregulates MMP- 1/MMP-3 in dermal fibroblasts, protects type I collagen from degradation. Also used in formulations (even nanoparticle-conjugated) for skin protection.

Evidence level: good in-vitro and preclinical evidence for anti-photoaging and collagen protection.

6) Anthocyanins:

Class / source: flavonoid pigments — red/blue pigments in grape skins and berries.

Anti-aging actions: antioxidant, anti-inflammatory, improve microcirculation and protect against oxidative skin damage; may modulate pathways linked to cellular senescence.

Evidence level: growing preclinical evidence for skin health and vascular benefits; often complementary to OPCs in grape extracts. (See reviews on polyphenols and skin).

7) Tannins (hydrolysable & condensed):

Class / source: diverse polyphenols — grape seeds contain both condensed tannins (OPCs) and tannin-like molecules.

Anti-aging actions: strong radical scavenging, metal chelation, inhibition of skin-degrading enzymes (MMPs), anti-

inflammatory effects.

Evidence level: well supported in antioxidant and anti-MMP assays; commonly credited in topical formulations for anti-photoaging.

8) Tocopherols (Vitamin E) & Fatty acids (linoleic acid) — from grapeseed oil: Class / source: lipid antioxidants and essential fatty acids — grapeseed oil.

Anti-aging actions: tocopherols protect membranes from lipid peroxidation; linoleic acid improves skin barrier, hydration and reduces trans epidermal water loss (indirectly supporting youthful skin). Useful topically.

Evidence level: strong dermatological support for barrier and antioxidant benefits; complementary to polyphenol effects.

9) General polyphenol effects (summary):

Main mechanisms across phytoconstituents: antioxidant (ROS scavenging), anti- inflammatory (NF-κB, cytokine downregulation), inhibition of MMPs (preserves collagen/ECM), modulation of longevity pathways (sirtuins, AMPK, NAD+), improved mitochondrial function, reduced DNA oxidative damage, and modulation of cell senescence pathways. For skin, many act against photoaging (UV damage → ROS → MMPs → collagen loss).

1.4.1 Natural Antioxidants:

Role of Natural Antioxidants in Anti-Aging Therapy:

Aging is strongly associated with oxidative stress, which results from an imbalance between reactive oxygen species (ROS) and the body's antioxidant defense system. Excess ROS damages cellular components including DNA, lipids, proteins, and mitochondria, leading to cellular senescence and tissue degeneration.

Natural antioxidants such as proanthocyanidins, resveratrol, quercetin, catechins, and gallic acid play a significant role in preventing or slowing this process.

❖ **Reduction of Oxidative Stress (Primary Mechanism):**

Natural antioxidants:

- Scavenge free radicals (•OH, $O_2^{\bullet-}$, H_2O_2)
- Prevent lipid peroxidation
- Protect mitochondrial membranes
- Reduce oxidative DNA damage

For example, grape seed proanthocyanidins neutralize ROS and reduce markers like malondialdehyde (MDA), thereby protecting cells from oxidative injury.

Result: Slower cellular aging and improved cell survival.

❖ **Inhibition of Collagen Degradation (Anti-Photoaging):**

UV radiation increases ROS, which activates matrix metalloproteinases (MMPs). MMPs degrade collagen and elastin → wrinkles and skin aging.

Natural antioxidants:

- Inhibit MMP-1 and MMP-3 expression
- Reduce NF-κB mediated inflammation
- Preserve type I collagen

Resveratrol and proanthocyanidins are particularly effective in reducing UV-induced skin damage.

Result: Reduced wrinkles and improved skin elasticity.

1.4.2 Mechanism:

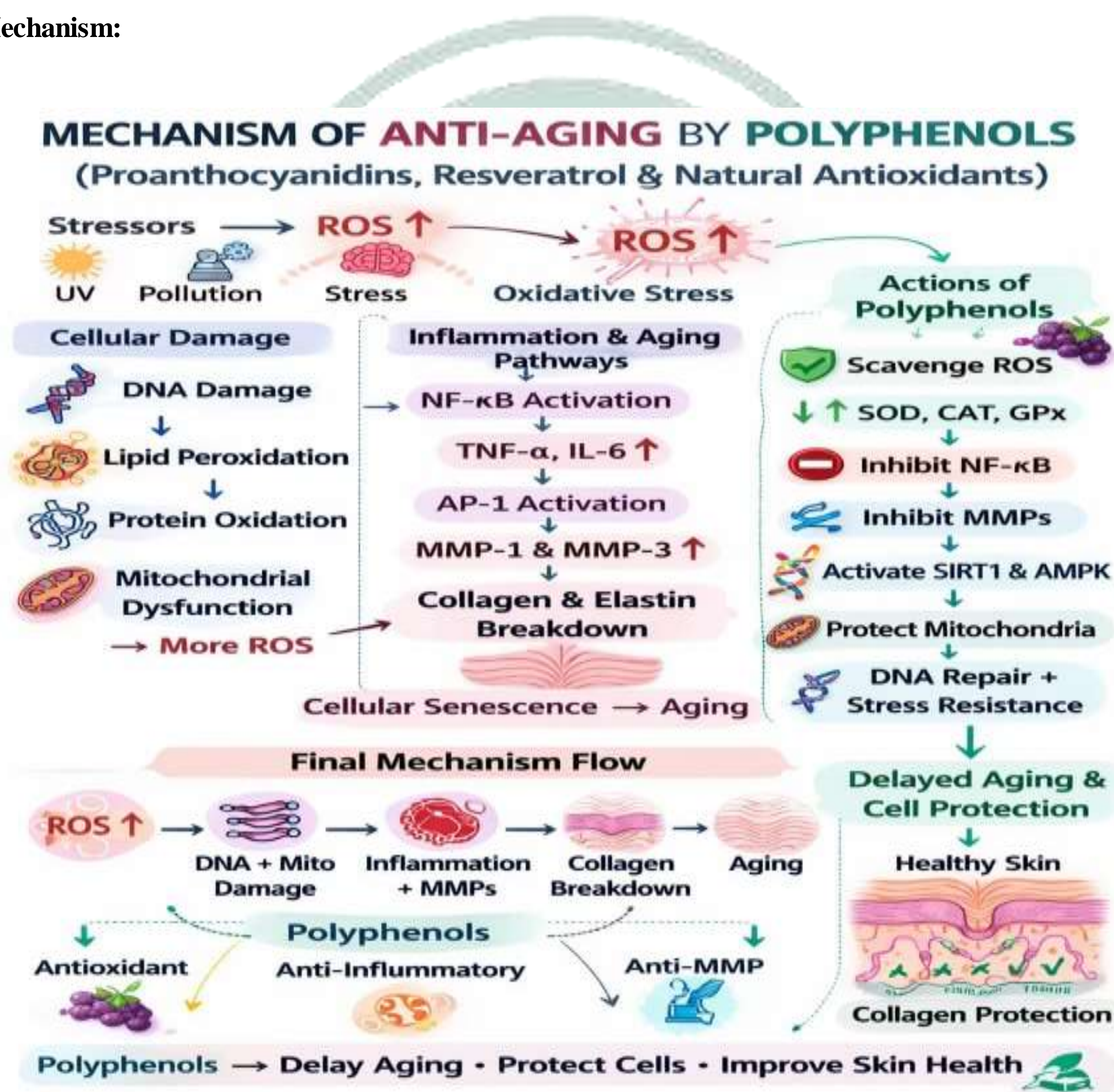


Fig no:05 mechanism of anti-aging by polyphenols

1.5 Grape seed as therapeutic agent:

1. Strong Antioxidant Power:

Fig no: 06 Grape seed as a therapeutic agent

GSE is rich in proanthocyanidins (OPCs) that fight free radicals, reduce oxidative stress, and protect cells from damage.

2. Reduces Blood Pressure:

GSE (100–800 mg daily) may lower systolic and diastolic blood pressure, helping reduce heart disease risk.

3. Improves Blood Flow:

It improves circulation, reduces leg swelling, and helps prevent blood clot formation.

4. Protects Against LDL Oxidation:

GSE prevents oxidation of “bad” cholesterol (LDL), reducing plaque buildup in arteries (atherosclerosis risk).

5. Supports Collagen & Bone Strength:

Flavonoids in GSE improve collagen production, increase bone density, and support joint health.

6. Brain Protection:

May improve memory, attention, and protect against age-related cognitive decline like Alzheimer's disease.

7. Supports Kidney Function:

Reduces oxidative stress in kidneys and may improve filtration in chronic kidney disease patients.

8. Fights Infections:

Shows antibacterial and antifungal activity against bacteria like E. coli and Candida in lab studies.

9. Anti-Cancer Potential:

Lab and animal studies show GSE may slow cancer cell growth and protect normal cells from chemotherapy toxicity.

10. Improves Wound Healing & Skin:

Fig no:07 Mechanism of wound healing

Enhances wound healing, improves skin elasticity, and reduces signs of aging due to high antioxidant activity.[10]

2. PLANT PROFILE

1. Scientific Classification:

- Accepted Latin Name: Vitis vinifera L.
- Common Name: Grape
- Family: Vitaceae
- Synonyms: Cissus vinifera
- Plant Part Used: Seeds

2. Taxonomical position:

Rank	Classification
Kingdom	Plantae
Division	Magnoliophyta
Class	Magnoliopsida
Order	Vitales

Family	Vitaceae
Genus	Vitis
Species	Vitis vinifera

. **Table no:1** Taxonomical classification

3. Morphological description:

Vitis vinifera is a perennial, woody climbing vine widely cultivated for fruit production. It grows using tendrils and requires support.

Fig no: 08 Vitis vinifera(grapes)

4. Geographical Distribution:

- Native to the Mediterranean region and Western Asia
- Widely cultivated in:
  - Europe
  - India
  - USA
  - China
  - Australia

In India, major grape-growing states include Maharashtra, Karnataka, Andhra Pradesh, and Tamil Nadu.

5. Chemical constituents:

- Oligomeric Proanthocyanidins (OPCs)
- Catechin
- Epicatechin
- Gallic acid
- Resveratrol (trace amounts)
- Tannins
- Flavonoids
- Phenolic acids.[11],[12].

6. Cultivation and collection:

Cultivation:

Vitis vinifera is a perennial, deciduous woody climber extensively cultivated for fruit production and industrial processing. It is primarily grown in temperate and subtropical regions where climatic conditions favor high fruit yield and optimal phytochemical accumulation.

Climate Requirements:

Requires a warm and dry climate for proper growth and fruit maturation. Optimum temperature range: 15°C to 35°C. Excessive rainfall during fruiting may reduce fruit quality and promote fungal diseases. Adequate sunlight is essential for the synthesis of phenolic compounds in seeds.

Soil Requirements:

Grows best in well-drained sandy loam or loamy soil. Soil pH should range between 6.0 and 7.5.
Waterlogging conditions must be avoided as they damage roots and reduce productivity.

Propagation:

Mainly propagated through hardwood stem cuttings to maintain genetic uniformity. Rootstocks may be used for resistance against soil-borne pests and diseases.

Agronomic Practices:

Training systems such as bower, trellis, or Kniffin system are used to support vine growth. Regular pruning is essential to regulate fruit production.
Controlled irrigation and balanced fertilization enhance fruit yield and seed quality. Grapevines start commercial fruiting within 3–4 years after planting.
In India, major grape-producing states include Maharashtra, Karnataka, Andhra Pradesh, and Tamil Nadu.

Collection:

Grape seeds used for extract preparation are generally obtained as a by-product of the wine and juice industry, ensuring sustainable utilization of plant material.

Harvesting:

Grapes are harvested at full maturity when sugar content and phenolic composition reach optimum levels. Mechanical or manual crushing separates pulp, skin, and seeds.

Seed Separation:

Seeds are separated from pomace (residual pulp and skin) by mechanical sieving or washing. Proper cleaning is essential to remove adhering sugars and fermentable residues.

Drying:

Seeds are dried under shade or in hot air dryers at controlled temperatures (below 50°C) to prevent degradation of heat-sensitive polyphenols.Moisture content is reduced to safe levels to prevent microbial growth.

Storage:

Dried seeds are stored in airtight, moisture-proof containers.Storage conditions: Cool, dry place away from direct sunlight.Proper storage maintains stability of oligomeric proanthocyanidins (OPCs) and other phenolic compounds.

2.1 Medicinal uses:

Fig no:09 Medicinal uses of grape seeds

2.2 Nutraceutical relevance:

1. Functional Food and Dietary Supplement Value:

Grape seed extract is categorized as a nutraceutical because it provides health benefits beyond basic nutrition. The high concentration of proanthocyanidins contributes to:

- Neutralization of reactive oxygen species (ROS)
- Reduction of oxidative stress-induced cellular damage

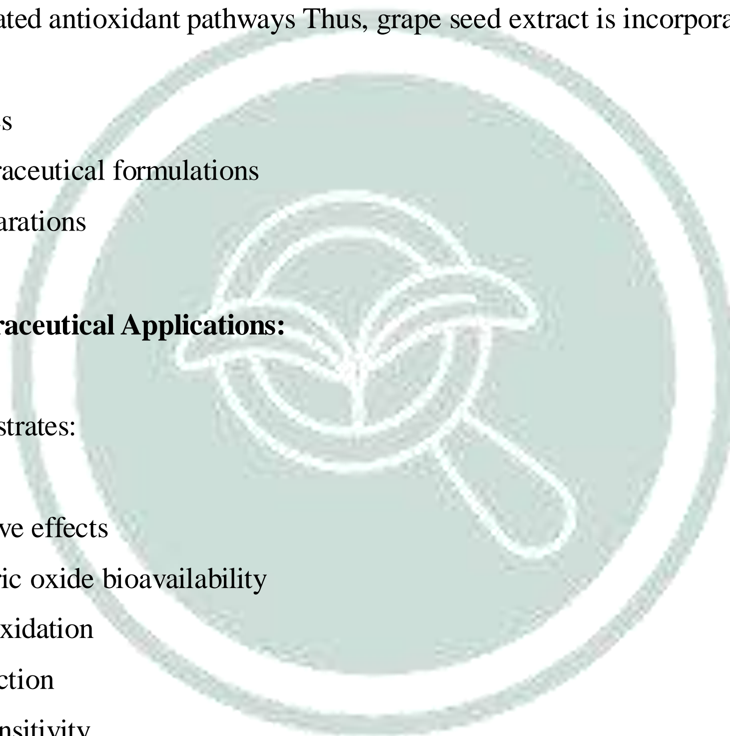
- Enhancement of endogenous antioxidant Défense systems (SOD, CAT, GPx)
- Prevention of lipid peroxidation

These properties make GSE particularly relevant in managing age-related disorders, including cardiovascular diseases, neurodegeneration, metabolic syndrome, and skin aging.

2. Anti-Aging Nutraceutical Potential:

From an anti-aging perspective (important for your project), grape seed proanthocyanidins:

- Inhibit collagen degradation by suppressing matrix metalloproteinases (MMPs)
- Enhance collagen cross-linking and skin elasticity
- Reduce UV-induced oxidative damage
- Modulate inflammatory mediators (TNF-α, IL-6)
- Activate Nrf2-mediated antioxidant pathways Thus, grape seed extract is incorporated into:
- Anti-aging capsules
- Functional beverages
- Dermatological nutraceutical formulations
- Cosmeceutical preparations



3. Cardiometabolic Nutraceutical Applications:

Grape seed extract demonstrates:

- Endothelial protective effects
- Improvement in nitric oxide bioavailability
- Reduction in LDL oxidation
- Anti-hypertensive action
- Improved insulin sensitivity

These properties support its use in:

- Heart health supplements
- Anti-diabetic nutraceutical formulations
- Anti-obesity functional products

4. Gut and Microbiome Relevance:

The conjugated and colonic metabolites of grape seed proanthocyanidins enhance:

- Gut microbial balance
- Short-chain fatty acid production

- Intestinal barrier integrity
- This makes grape seed extract relevant in:
- Digestive health supplements
- Functional prebiotic formulations.[13]

3. MATERIALS AND METHODS

3.1 MATERIALS:

3.1.1 Chemicals:

S.no	Chemicals	MNF
1	Ethanol	Simha puri chemicals
2	Conc.HCl	Simha puri chemicals
3	Chloroform	Simha puri chemicals
4	Ammonium hydroxide	Simha puri chemicals
5	Distilled water	Simha puri chemicals
6	Lead acetate	Simha puri chemicals
7	Dilute H_2SO_4	Simha puri chemicals
8	Mayers reagent	Simha puri chemicals
9	Ferric chloride	Simha puri chemicals
10	Acetic acid	Simha puri chemicals
11	Conc.H_2SO_4	Simha puri chemicals

Table no: 2 Chemicals

3.1.2 Glass wares:

S.no	Glassware	Made up of
1	Beakers (50-250 ml)	Borosilicate
2	Conical flask	Borosilicate
3	Cover slips	Borosilicate
4	Cuvettes	Borosilicate
5	Glass slides	Borosilicate
6	Measuring cylinder	Borosilicate
7	Pasteur pipette	Borosilicate
8	Petri dish	Borosilicate
9	Pipettes	Borosilicate
10	Test tubes	Borosilicate
11	Volumetric flask	Borosilicate
12	Watch glass	Borosilicate
13	Water bath	Borosilicate
14	Glass rod	Borosilicate

Table no:3 Glass wares

3.1.3 Apparatus required:

S.no	Apparatus
1	Analytical balance
2	Hot air oven
3	Mechanical grinder
4	Water bath

5	Centrifuge
6	UV-Visible spectrometer
7	Ph meter
8	Incubator
9	Refrigerator
10	Mortar and pestle
11	Magnetic stirrer
12	Mixer
13	Pulveriser

Table no:4 Apparatus

3.2 METHODOLOGY

3.2.1 Extraction:

1. Collection:

- **Selection of Fruits:**

Fully matured, healthy grapes were selected.Damaged and infected fruits were discarded.

- **Separation of Seeds:**

Seeds were manually separated from pulp after juice processing.Care was taken to avoid contamination with pulp residues.

- **Washing:**

Collected seeds were washed thoroughly with distilled water.This removed adhering sugars, pulp, and impurities.

- **Shade Drying:**

Washed seeds were spread on clean trays.Dried under shade at room temperature to prevent degradation of heat-sensitive phenolic compounds.

- **Oven Drying:**

Further dried in a hot air oven at 40°C for 10 hours.Ensured complete removal of moisture.

- **Storage:**

Completely dried seeds were stored in airtight, light-resistant containers. Kept in a cool and dry place until extraction.[14].

2. Soxhlet Extraction Process of Grape Seeds)

- **Plant Material:**

Seeds of Vitis vinifera [grape seeds]were used for extraction.

- **Principle:**

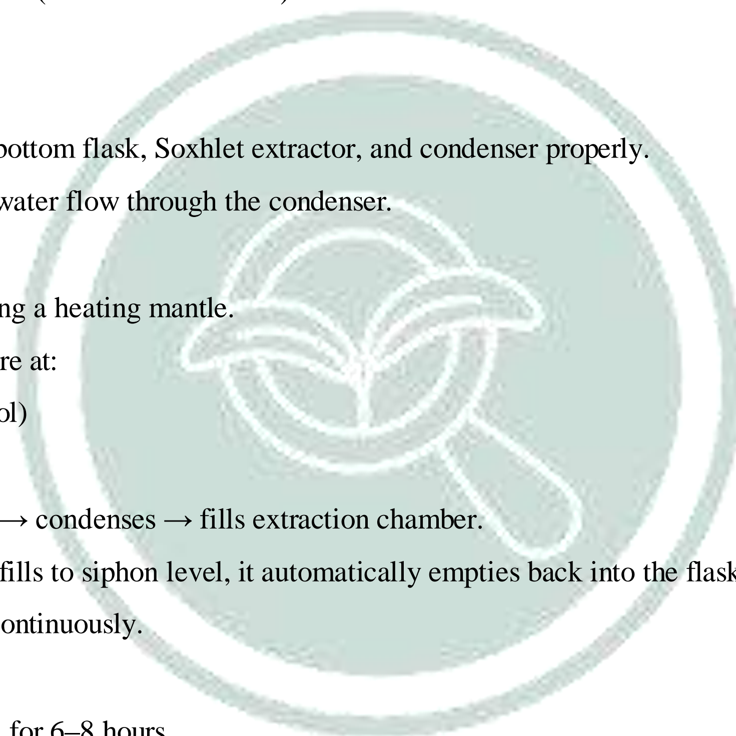
Soxhlet extraction is a continuous hot extraction technique in which the solvent repeatedly washes the plant powder to extract bioactive constituents such as phenolics, flavonoids, and proanthocyanidins

- **Procedure:**

➢ Weighing of Sample
➢ Accurately weigh 50–100 g of dried grape seed powder.
➢ Loading
➢ Place the powder into a cellulose thimble.
➢ Insert the thimble into the Soxhlet extractor.

- **Addition of Solvent:**

Add 300–400 mL of solvent (methanol or ethanol) into the round bottom flask.



- **Assembly:**

➢ Connect the round bottom flask, Soxhlet extractor, and condenser properly.
➢ Ensure continuous water flow through the condenser.

- **Heating**

➢ Heat the system using a heating mantle.
➢ Maintain temperature at:
➢ 60–65°C (for ethanol)

- **Extraction Cycle**

➢ Solvent evaporates → condenses → fills extraction chamber.
➢ When the chamber fills to siphon level, it automatically empties back into the flask.
➢ This cycle repeats continuously.

- **Duration**

➢ Continue extraction for 6–8 hours.
➢ Approximately 10–15 siphon cycles are completed.[15].

3. Filtration:

- **Cooling of Extract**

Allow the Soxhlet apparatus to cool to room temperature.

- **Assembly of Filtration Setup:**

Fix Buchner funnel on side-arm flask.Place pre-weighed Whatman filter paper inside the funnel.Connect to vacuum pump.

- **Filtration:**

Pour the extracted solvent containing dissolved phytoconstituents into the funnel.Apply vacuum to facilitate rapid filtration.Ensure complete separation of liquid extract from insoluble residues.

- **Collection of Filtrate:**

Collect the clear filtrate in the receiving flask.If required, repeat filtration to ensure clarity.[16].

3.2.2 Phytochemical screening:

1. Vaniline HCl test:

mixing 1 mL of the plant extract solution with 1 mL of freshly prepared 1% vanillin solution in methanol, followed by the addition of 1 mL of concentrated hydrochloric acid (HCl) in a 1:1:1 ratio. The mixture is shaken gently and allowed to stand for about 10–15 minutes at room temperature. The development of a pink to red color indicates the presence of proanthocyanidins in the sample.

Fig no:10 Test for proanthocyanidins

2. Test for phenol compound (Lead acetate test)

The extract was dissolved in 5ml of distilled water. To this, 3ml of 10% lead acetate was added. Appearance of white precipitate indicates the presence of phenols.

Fig no:11 test for phenols

3. Test for glycosides (Borntrager's test)

2-3 drops of concentrated HCl was added to the extract. Then the mixture was boiled for 2 minutes (hydrolysis of glycosides). Then the mixture was filtered and cooled. The filtrate was extracted with chloroform. The chloroform layer was separated and shaken vigorously with 10% ammonium hydroxide. Appearance of pink color in aqueous layer confirms the presence of glycosides.

Fig no:12 test for glycosides

4. **Test for alkaloids (Mayer's test)**

A small amount of all Grape seed extract (GSE) extracts were neutralized by adding 1 or 2 drops of dilute H_2SO_4. The resulting solution was treated with a small amount of Mayer's reagent. Appearance of white precipitate confirms the presence of alkaloids.

Fig no:13 test for alkaloids

5. Test for steroids and terpenoids

A small portion of extract was dissolved in 1 ml of chloroform and filtered. Kept the filtrate on ice, 1 ml of acetic acid was added to the filtrate and then a few drops of concentrated H_2SO_4 were run down the side of the test tube. Appearance of blue, bluish-green or a rapid change from pink to blue colours indicates the presence of steroids, the appearance of pink or pinkish- brown ring/colour indicates the presence of terpenoids and a combination of both colours indicates the presence of both steroids and terpenoids.

Fig no:14 test for steroids and terpinoids

6. Test for flavonoids (Shinoda test)

A few drops of concentrated Hydrochloric acid and 1 or 2 magnesium turnings were added to 1 ml of extracts. The presence of flavonoids was indicated by the development of pink or magenta red colour.

Fig no:15 Test for flavonoids

7. Test for saponins:

5ml of distilled water was added to 5ml of extract and shaken for the formation of froth which indicates the presence of saponins.[17]

Fig no:16 test for saponins

8. **Test for tannins (Ferric chloride test)**

Test for tannins (Ferric chloride test). Two millilitres (2 mL) of the aqueous solution of the extract were added to a few drops of 10% Ferric chloride solution (light yellow). The occurrence of blackish blue colour showed the presence of gallic tannins and a green-blackish colour indicated presence of catechol tannins.[18]

Fig no:17 test for tannins

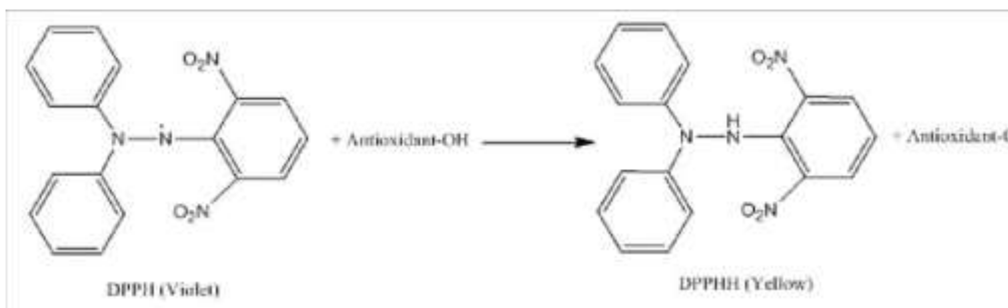
3.2.3 Anti-oxidant activity:

D.P.P.H. Method:

Principle:

1,1-Diphenyl-2-picrylhydrazyl (DPPH) is a stable free radical, which in methanolic solution has characteristic purple colour. On accepting hydrogen from a corresponding donor, its solutions lose the characteristic deep purple (λ max

515-517 nm) colour. DPPH is very popular for the study of natural antioxidants [210]. The antioxidant activity of tested compounds is expressed as a relative or absolute decrease of concentration of DPPH.



Reagents:

DPPH - 0.1 mM (1.97 mg/50 ml methanol) L-Ascorbic Acid (Vitamin C) - 1 mg/ml in methanol

Procedure:

DPPH (0.1 mM-333µL) in methanol was added to 1 ml of different doses of sample (20µg, 40µg, 60µg) in Dimethyl sulfoxide (DMSO). The mixture was shaken vigorously, allowed to stand at room temperature for 30 min and the absorbance was read at 517 nm in a UV-visible spectrophotometer [210]. Lower the absorbance of the reaction mixture indicated higher free radical scavenging activity. L-Ascorbic Acid (Vitamin C) (10–50 mg/ml) was used as positive control.[19]

Different concentrations of test samples(Grape seed extract) (20µg,40µg,60µg,80µg,100µg)

Fig no:18 test samples of grape seed extract

Different concentrations of standard ascorbic acid samples: (20µg,40µg,60µg,80µg,100µg)

Fig no:19 standard samples of ascorbic acid samples

3.2.4 Anti-inflammatory Nitric Oxide - NO Assay:

Principle:

Nitric oxide (NO) is generated from sodium nitroprusside in an aqueous solution at physiological pH. It reacts with

oxygen to produce nitrite ions (NO_2^-), which can be quantified using the Griess reagent. In the presence of antioxidants, nitric oxide is scavenged, leading to a decrease in nitrite formation. The reduction in absorbance at 546–550 nm reflects the NO scavenging ability of the compound.

Reagents

1. Sodium nitroprusside (5 mM in PBS, pH 7.4)
2. Griess reagent (prepared fresh):
3. 1% sulphanilamide in 5% phosphoric acid
4. 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride (NED)
5. Phosphate buffer (0.1 M, pH 7.4)
6. Ascorbic acid (standard, 1 mg/ml)

Procedure:

Preparation of Sodium Nitroprusside Solution

1. Weigh sodium nitroprusside: Weigh 5 mM of sodium nitroprusside.
2. Prepare phosphate buffer: Prepare phosphate buffer according to the required concentration and pH.

3. Dissolve sodium nitroprusside: Dissolve sodium nitroprusside in phosphate buffer to make a 1 ml solution.

Addition of Sample or Standard:

1. Prepare sample or standard: Prepare the sample or standard at different concentrations (e.g., 20 100 µg/ml).
2. Add sample or standard: Add 1 ml of sample or standard to the sodium nitroprusside solution.

Incubation :

1. Incubate: Incubate the mixture at 25°C for 2.5 hours under light to generate nitric oxide.

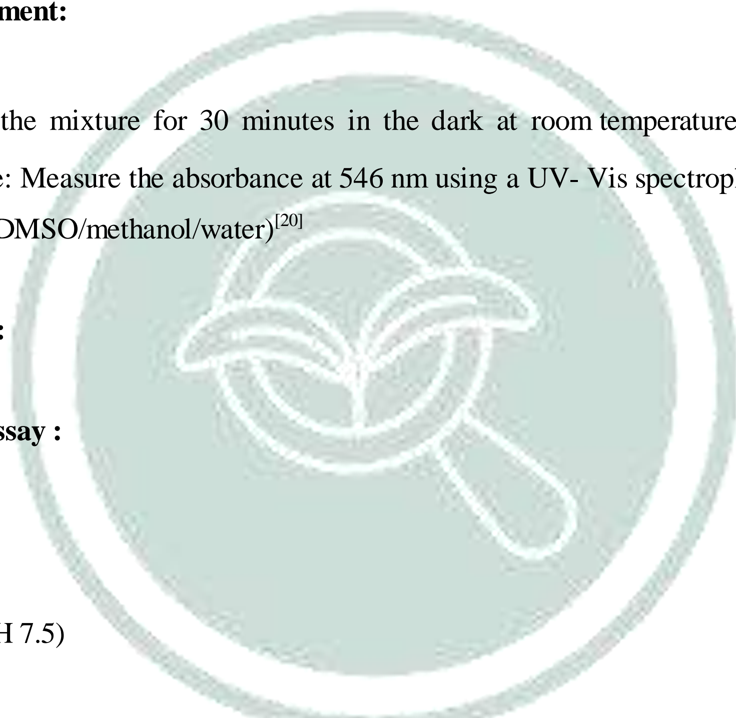
Addition of Griess Reagent:

1. Prepare Griess reagent: Prepare Griess reagent according to the required concentration.
2. Add Griess reagent: Add 1 ml of Griess reagent to 1 ml of the reaction mixture.

Incubation and Measurement:

1. Incubate: Incubate the mixture for 30 minutes in the dark at room temperature.
2. Measure absorbance: Measure the absorbance at 546 nm using a UV- Vis spectrophotometer.

Test sample (dissolved in DMSO/methanol/water)[20]



3.2.5 Anti-aging activity:

Collagenase inhibitory assay :

Preparation of Reagents

Tricine Buffer (50 mM, pH 7.5)

Tricine buffer is used to maintain a stable pH during biochemical experiments. Tricine acts as the buffering agent, while NaCl maintains ionic strength and $CaCl_2$ provides calcium ions for stability. The required quantities of Tricine (0.89 g), NaCl (2.34 g), and $CaCl_2$ (0.11 g) are dissolved in distilled water. The final volume is adjusted to 100 ml with distilled water to obtain Tricine buffer of pH 7.5.(21-23)

- Adjust pH to 7.5

Collagenase Enzyme Solution

Prepare 0.8 U/mL collagenase solution in tricine buffer.

Substrate Solution

Prepare 2 mM FALGPA solution in tricine buffer.

Preparation of test sample

Prepare grape seed extract solution in ethanol

Concentration	preparation
20ug/ml	Dilute stock solution
40ug/ml	Dilute stock solution
60ug/ml	Dilute stock solution
80ug/ml	Dilute stock solution
100ug/ml	Dilute stock solution

Table no:5 Test samples

Procedure :

A fixed weight of 1 mg of Azo dye impregnated collagen was measured in the test tubes and then the homogenization was proceeded after the addition of an 800 µl of 0.1 M Tris-HCl (pH7) and a 100 µl of sample into each of test tubes. A 100 µl collagenase (200 units/ml) was immediately mixed into the mixture and incubated at 43 °C for 1h. Afterward, the test tubes were centrifuged at 3000 rpm for 10 min.

The supernatant section of each test tube transferred into 96-well plates and the absorbance of each supernatant was measured at 550 nm[23,24,25].

Percentage Collagenase Inhibition

$$\%\,\text{Inhibition} = \frac{A_{control} - A_{sample}}{A_{control}} \times 100$$

Where:

- $A_{control}$ = absorbance of control
- A_{sample} = absorbance of sample

4. RESULTS AND DISCUSSION

Phytochemical screening:

Phytochemical tests are analytical techniques used to detect and identify the presence of various phytochemicals, such as alkaloids, glycosides, flavonoids, and carotenoids, in plant extracts. These tests involve chemical reactions that produce characteristic colours, precipitates.

Table no :6 phytochemical screening results

S.NO	TEST NAME	RESULT
1.	Proanthocyanidins	+VE
2.	Glycosides	-VE
3.	Phenols	+VE
4.	Alkaloids	+VE
5.	Steroids and terpenoids	-VE
6.	Flavonoids	+VE
7.	Saponins	+VE

Table: phytochemical screening results of grape seed extract.

Antioxidant activity:

Antioxidants are molecules that neutralize or mop up free radicals, unstable molecules that can cause oxidative stress and damage to cells. Oxidative stress is linked to various chronic diseases, including cancer, diabetes, and neurodegenerative disorders.

		Ascorbic acid	Ascorbic acid	Ascorbic acid	Ascorbic acid	Ascorbic acid
	Control	20µg	40µg	60µg	80µg	100µg
	0.288	0.121	0.182	0.214	0.113	0.091
	0.283	0.134	0.179	0.146	0.112	0.088
	0.284	0.217333	0.176	0.148	0.112	0.082
Avg	0.284667	0.214	0.179	0.147	0.117	0.087
%		43.48958	53.38542	61.71875	70.3125	77.34375

Table no:7 Standard Ascorbic acid solution (Different concentrations 20 µg, 40 µg, 60 µg,80 µg,100 µg)

		Test sample	Test sample	Test sample	Test sample	Test sample
	Control	20µg	40µg	60µg	80µg	100µg
	0.388	0.291	0.274	0.246	0.246	0.188
	0.382	0.293	0.271	0.248	0.227	0.184
	0.384	0.294	0.273	0.247	0.228	0.191
Avg	0.384667	0.292667	0.272662	0.24788	0.287333	0.187667
%		21.35417	28.64583	35.41667	44.01042	51.30208

Table no:8 Test Samples (Grape seed extract)

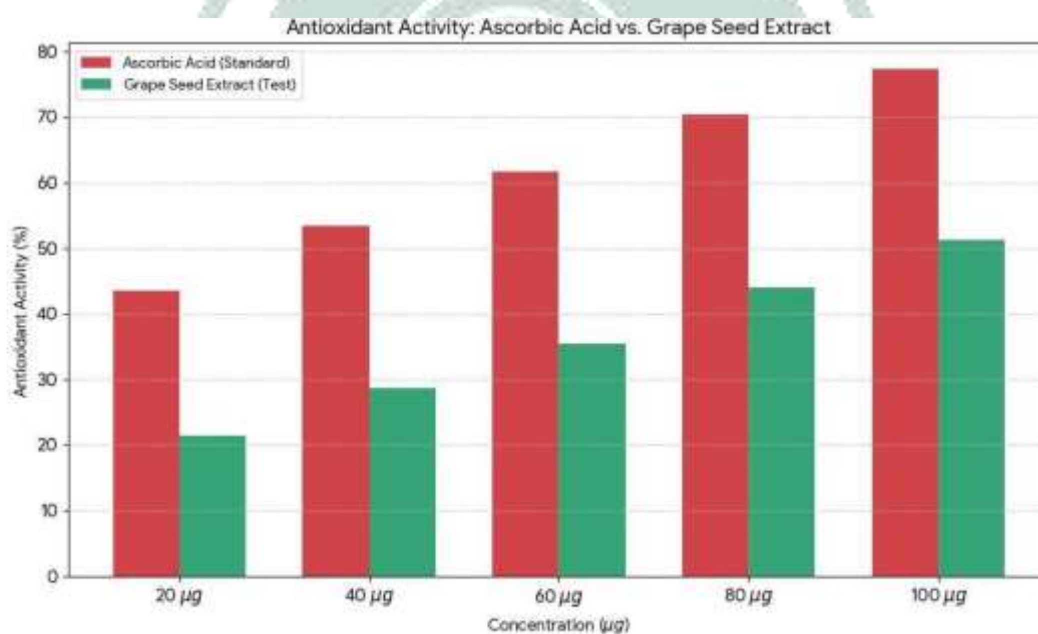


Fig no:20 comparison of ascorbic acid vs grape seed extract

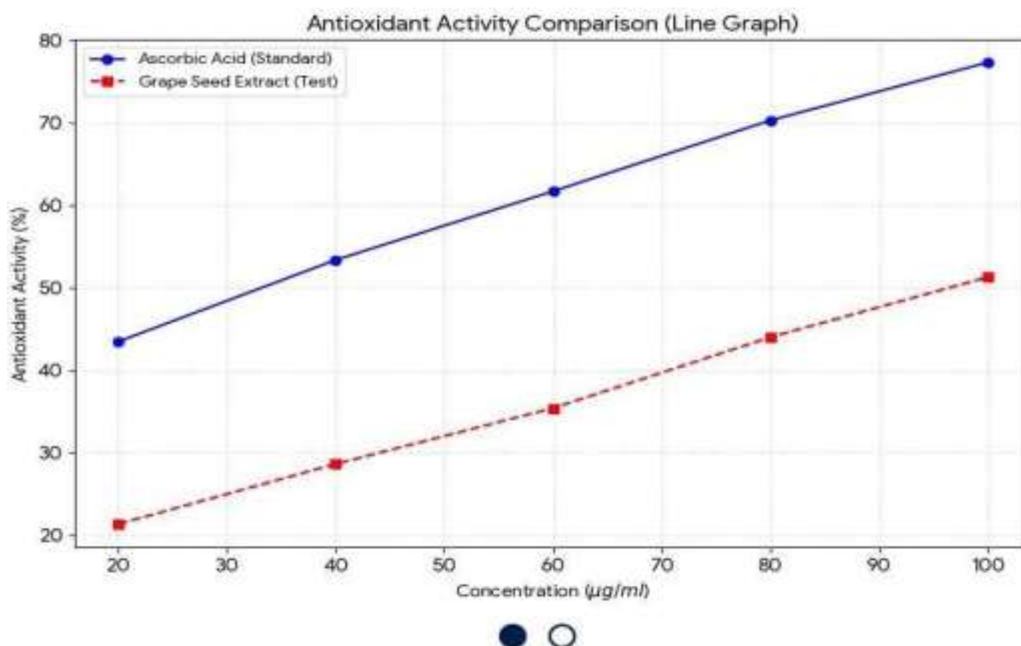


Fig no:21 comparison of anti-oxidant activity test vs standard

Anti-aging activity :

Collagenase inhibition assay of grape seed extract

Concentration	**absorbance**	**% inhibition**
Control	0.650	-
20ug/ml	0.520	20.0
40ug/ml	0.430	33.8
60ug/ml	0.350	46.2
80ug/ml	0.270	58.5
100ug//ml	0.200	69.2

Table no:9 Collagenase inhibition assay (test samples) Standard inhibitor comparison:

Sample	**concentration**	**% inhibition**
1,10-phenanthroline	100ug/ml	90.5

Grape seed extract	100ug/ml	69.2

Table no:10 comparison of standard

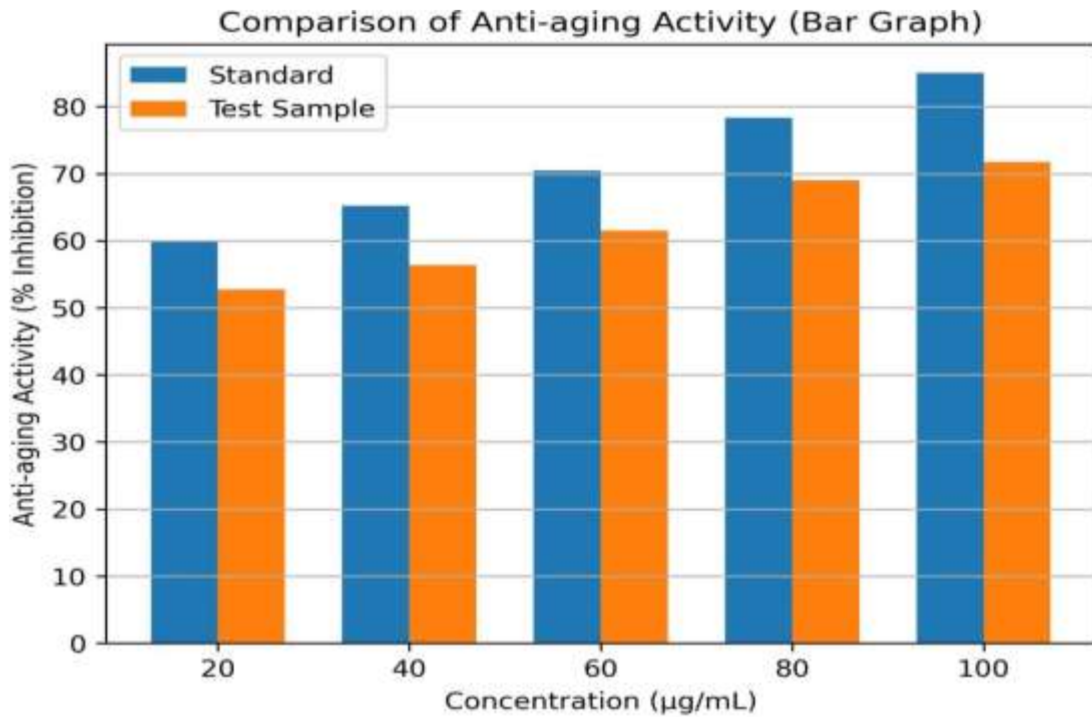


Fig no:22 Comparison of Anti- aging activity (Bar graph)

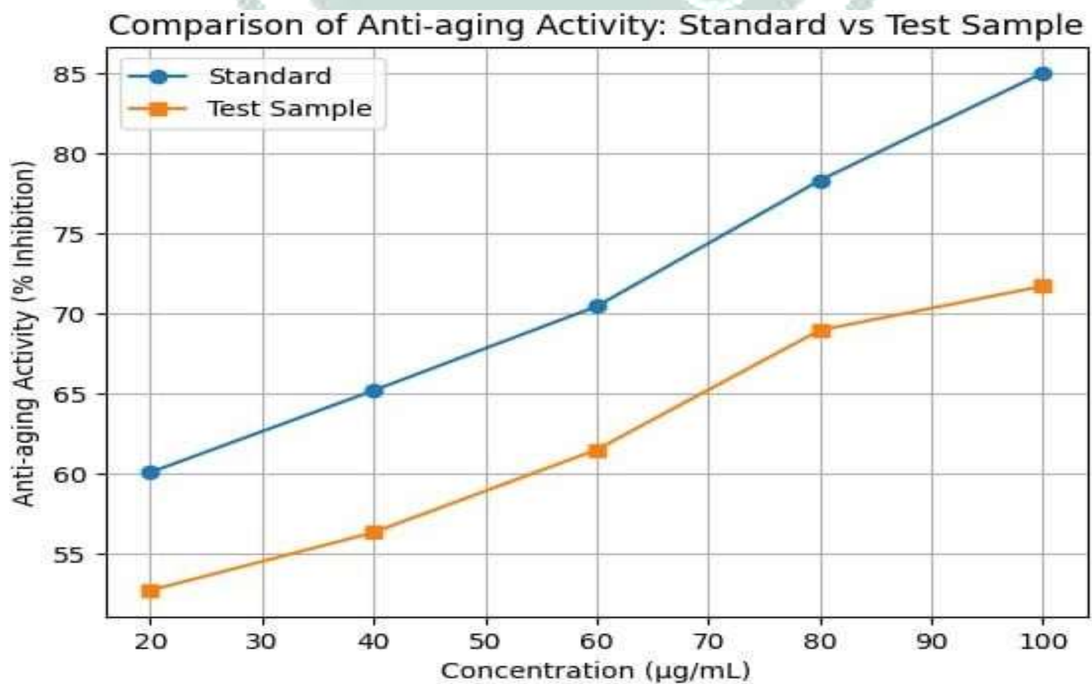


Fig no:23 Comparison of anti-aging activity: standard vs test sample

5. SUMMARY AND CONCLUSION

SUMMARY

Aging is a natural biological process characterized by a gradual decline in physiological functions and increased susceptibility to chronic diseases. One of the major factors contributing to aging is oxidative stress, which occurs due to an imbalance between the production of reactive oxygen species (ROS) and the body's antioxidant defense system. Excessive ROS can damage cellular components such as proteins, lipids, and DNA, leading to cellular dysfunction and aging. Chronic inflammation also plays an important role in accelerating the aging process and contributes to various age-related disorders. Therefore, the search for natural compounds that can reduce oxidative stress and inflammation has gained significant research interest.

Plant-derived phytoconstituents are widely studied because they possess therapeutic potential and relatively low toxicity compared to synthetic drugs. Grape seeds from Vitis vinifera are rich in bioactive compounds such as polyphenols, flavonoids, and proanthocyanidins, which exhibit strong antioxidant and anti-inflammatory properties. In the present study, grape seed extract was prepared and subjected to preliminary phytochemical screening, which confirmed the presence of important phenolic compounds. The antioxidant activity was evaluated using the DPPH free radical scavenging assay and showed maximum activity at 40 µg/ml. Anti-inflammatory activity assessed by nitric oxide scavenging assay showed maximum inhibition at 60 µg/ml. The anti-aging potential evaluated through collagenase inhibition assay showed maximum inhibition at 80 µg/ml, indicating its ability to protect collagen and delay aging.

CONCLUSION

The present study successfully demonstrated the in-vitro antioxidant, anti-inflammatory, and anti-aging potential of grape seed phytoconstituents. Phytochemical screening confirmed the presence of bioactive compounds such as polyphenols and flavonoids, which are responsible for the observed biological activities.

The grape seed extract showed significant free radical scavenging activity in the DPPH assay, indicating strong antioxidant potential. The extract also exhibited effective nitric oxide scavenging activity, suggesting its ability to modulate inflammatory pathways. Furthermore, the collagenase inhibition assay confirmed the anti-aging potential of grape seed phytoconstituents by preventing collagen degradation.

These findings suggest that grape seed extract acts as a multifunctional modulator of oxidative stress, inflammation, and aging pathways. Due to its natural origin and potent biological activities, grape seed extract may serve as a promising candidate for the development of therapeutic agents, nutraceuticals, and cosmetic formulations aimed at preventing oxidative damage and age-related disorders.

However, the present investigation was limited to in-vitro studies. Further in-vivo studies and clinical investigations are necessary to confirm the therapeutic efficacy, safety, and mechanism of action of grape seed phytoconstituents in biological systems.

In conclusion, grape seed phytoconstituents possess significant potential as natural antioxidants and anti-inflammatory agents, making them promising candidates for the development of anti- aging and health-protective formulations.

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