

Phytochemical Investigation and Antioxidant activity of *Annona squamosa*, *Glycyrrhiza glabra* and *Zingiber officinale* extracts

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Abstract

Medicinal plant is a promising source of bioactive metabolites for management of diseases related to oxidative stress. Among such common botanicals used for ages in day-to-day staple food are *Annona squamosa*, *Glycyrrhiza glabra* and *Zingiber officinale* that have antioxidant, anti-inflammatory as well as antimicrobial action. The research was also carried out for analyzing the phytochemical constituents and their antioxidant activity in single and polyherbal form of these plants. The leaves of *Annona squamosa*, roots of *Glycyrrhiza glabra* and rhizomes of *Zingiber officinale* were collected, identified by botanist and extracted with petroleum ether, chloroform and ethanol successively. TLC fingerprint with quick detection of multicomponents was developed based upon these spectroscopic data revealed the presence of various compounds. Phytoconstituents were characterized on Thin Layer Chromatography (TLC) and bioactive markers were established as rutin, quercetin, glycyrrhizic acid, 18 β -glycyrrhetic acid, 6-gingerol and zingerone by Reverse Phase high-performance liquid chromatography (RP-HPLC). Antioxidant activity was determined using the DPPH radical scavenging assay. Concentration of phytochemicals was found to be highest for ethanol extracts. RP-HPLC confirmed the presence of all markers in polyherbal product. Concentration dependent antioxidant activity of the polyherbal formulation was revealed by the DPPH assay

and 89.45 % inhibition was registered at dose concentration of 100 µg/mL which was similar to ascorbic acid (90.67%). In summary, the study validates antioxidant property of the tested botanicals and also supports standardised polyherbal formulated products. The further development for therapeutic use of in vivo confirming and formulation optimization.

Keywords: *Annona squamosa*, *Glycyrrhiza glabra*, *Zingiber officinale*, phytochemical profile, RP-HPLC DPPH assay, antioxidant activity, Polyherbal formulation

Introduction

Plants have been used as sources and admixtures of traditional medicinal therapies worldwide, presenting an abundant source for bioactive compounds that exert therapeutic effects [1]. Phytochemicals and antioxidant activity Phytochemicals as bioactive molecules have drawn the attention of the scientific community during the last decades and are under systematic investigation not only for their intrinsic properties but also for their established pharmacological efficacy especially in the domain of antioxidant capacity that is recognized to have a pivotal role in oxidative stress alleviation (suppression) which constitutes a major contributing factor in aging and occurrence pathogenesis especially chronic diseases including cancer, diabetes, neurodegenerative diseases and cardiovascular ailments[2]. Among the plethora of botanicals investigated for their antioxidant potential, *Annona squamosa*, *Glycyrrhiza glabra*, and *Zingiber officinale* are more favored choice because of there ethnomedicinal uses, phytochemical diversity and broad spectrum bioactivity [3].

Annona squamosa Linn., also known as custard apple or sugar apple, is a member of the family Annonaceae and is grown in tropical and subtropical regions[3]. Different plant parts such as leaves, bark seeds and fruit are traditionally used for the management of fever, ulcers, dysentery and skin disorders. Several recent phytopharmacological studies have demonstrated that *A. squamosa* abounds in flavonoids, alkaloids, tannins and phenols, thereby giving it the antioxidant [4], antidiabetic [5] hepatoprotective and antimicrobial effects. Rutin and quercetin, two phytochemicals present in its leaves, are well reported for their strong free radical scavenging activity and regulation of inflammatory pathways [5].

Glycyrrhiza glabra, also known as licorice root, is a commonly used medicinal plant from the Fabaceae family. It possesses demulcent, antiinflammatory and expectorant properties [6] has been widely used in Ayurveda, Unani and traditional Chinese system of medicines. The root consists of a complex combination of triterpenoid saponins, flavonoids as well as

polysaccharides and its major bioactive marks are glycyrrhizic acid and 18 β -glycyrrhetic acid. These compounds have shown strong antioxidative, hepatoprotective, and antiviral activities in several cell lines and animal models. In particular, glycyrrhizic acid has been reported to exhibit inhibitory effect on lipid peroxidation and glutathione levels in such a way to participate in cellular protection against oxidative stress [4]. The pharmacological freedom of *G. glabra* highlights its potential as an inextended drug for polyherbal preparations intended to be used in countering oxidative stress and inflammation.

Ginger The botanical name for ginger is *Zingiber officinale* Roscoe and it belongs to the family Zingiberaceae, ginger It is a rhizomatous plant which fruit are spice together with haematinic purposes [8]. Its rhizome is abundant in volatile oils, phenolic compounds, and terpenoids; the most studied are 6-gingerol and zingerone. These compounds possess potent antioxidant, anti-inflammatory and anticancer activity partly due to their ability to scavenge ROSs (reactive oxygen species), inhibit pro-inflammatory cytokines as well as regulate cell signals like NF- κ B or MAPK [9]. The antioxidant potential of ginger has been demonstrated through several methods such as DPPH, ABTS and FRAP (25) with ethanolic extracts invariably exhibiting potent radical-scavenging effects. Its use as an ingredient in polyherbal combination adds to the therapeutic profile by providing antioxidant and digestive effects [8].

The justification for selecting these three plants *Annona squamosa*, *Glycyrrhiza glabra* and *Zingiber officinale* includes their similar pharmacognostical features and compatible phytochemical constitution along with their synergistic antioxidative mechanisms. Whereas *A. squamosa* provides flavonoids and phenolics, *G. glabra* gives saponins and glycosides, *Z. officinale* supports volatile oils and terpenoids in the formula [10]. Such polyherbal combinations are becoming more popular in phytomedicine for their efficacy due to the action on several biochemical pathways, reduction of side effects and additive/ synergistic activities. Nevertheless, the efficacy of these formulations will rely on strict standardization and phytochemical profiling as well as validation of bioactivity using well-known analytical tools [11]. In this study, we analyze the phytochemical profile and antioxidant potential of mono extracts as well as a polyherbal formulation containing leaves of *Annona squamosa* L., roots or rhizomes of *Glycyrrhiza glabra* L. and *Zingiber officinale* Roscoe [12].

Material and method

Chemicals and reagents

All chemicals and reagents utilized were analytical grade from reliable manufacturers. Methanol, ethanol, n-hexane, ethyl acetate and distilled water were used for extraction as

solvents. Reactants The following reagents were used, Phytochemical (test) screening and Biuret (protein), Millon's (tyrosine) Ninhydrin (amino acid) test; DPPH for radical scavenging activity [13].

Plant Collection and Authentication

The plant materials were collected from genuine botanical sources (leaves of *Annona squamosa*, roots of *Glycyrrhiza glabra* and rhizomes of *Zingiber officinale*) [14]. Morphological comparison with herbarium specimens and consultation with an expert were employed for the authentication of the plant. Voucher specimens (V. No. 9234) were preserved for reference material.

Physiochemical properties of collected plants

Loss on Drying (LOD)

A known weight of the sample was heated at a particular temperature until a constant weight was obtained. This gave the percentage weight loss and was calculated in percentage form for establishing loss on drying.

Water Soluble Extractive Value

An appropriate amount of powder sample was blended with distilled water for a fixed time. This was filtered and the resulting clear liquid was evaporated to dryness. The residue was weighed to determine the value of the water-soluble extractive samples.

Ethanol Soluble Extractive Value

The sample was treated at maceration with ethanol at a determined environmental condition. The extract was dried to a constant weight after filtration, and the weight was used to calculate the ethanol soluble extractive value.

Petroleum Ether Soluble Extractive Value

The powdered substance was subjected to petroleum ether extraction in a Soxhlet apparatus. The petroleum ether soluble extractive value was measured by weighing the residue remaining after evaporation of the solvent.

Chloroform Soluble Extractive Value

The sample was extracted with chloroform according to conventional method [6]. Extract was concentrated, dried and weighed to determine the value of chloroform soluble extractives.

Total Ash Value

The sample was burned at 500 °C for 2 h in a muffle furnace to yield white ash. Either the ash was cooled and weighed to measure total ash content.

Acid Insoluble Ash Value

Dilute hydrochloric acid was used to boil the total ash, and the insoluble part was filtered out. The remained ash after washed, dried and burned was weighted and correctly expressed in terms of the acid insoluble ash value.

Preliminary Pharmacognostical Evaluation:**Macroscopic Evaluation**

The morphological and macroscopic characters are important tools for the preliminary identification of plant materials along with its organoleptic characters, which have been described for leaves (*Annona squamosa*), roots (*Glycyrrhiza glabra*) and rhizomes (*Zingiber officinale*). Its characteristic colour, an important character of distinction that could be an indication of some phytoconstituents or deterioration²⁴ was also noted and recorded from all parts observed under day light. As per perception the smell and taste were checked, that could serve as some qualitative clues towards presence of volatile oils, alkaloids or any other secondary metabolite. FT-IR ²² Calibration of calipers and conformation to standard pharmacognostic parameters²⁶ were used for the determination of size and shape of plant material. Texture (the appearance/trichome type and cuticle) was also visual- and hands-evaluated [16]. Such features are useful in distinguishing plant materials from adulterants and help in establishing the botanical identity of pharmacognostical studies.

Microscopic Evaluation

The transverse sections and powder of the plant materials were studied microscopically to reveal their internal cellular structure and diagnostic characters. Stained either safranin or iodine solution, the samples will then compared on a compound microscope to provide an excellent contrast and ease of observation [17]. Madular cells: Cell tissue was consist of parenchyma; storage and metabolic function, thin walled cell with large air space, collenchyma,

rough thick wall cell providing mechanic support; sclerenchyma(lignified) for rigidity. The type and distribution of stomata was studied, because these characters are species specific and also taxonomically important. Trichomes (glandular and non-glandular protective) were observed on the epidermal surfaces reflecting plant defense system as well as storage site of phytochemical[18]. To determine the xylem-phloem pattern, vascular bundles were analyzed and secretory cells (often associated with essential oil or resin production) were observed in terms of number and shape. This microscopic observation provided important information for the botanical identity and quality of plant material [19].

Extraction of collected plants

The plant fractions (dried & powdered) such as leaves of *Annona squamosa*, roots of *Glycyrrhiza glabra* and rhizomes of *Zingiber officinale* were subjected to successive extraction with solvents by Soxhlet apparatus [20]. A cellulose thimble was filled with 100 g of powder material from each plant and the solvent extraction was carried out using polar solvents stepwise: petroleum ether (60–80°C), chloroform followed by ethanol. Extraction was carried out with petroleum ether (500 mL) for 6–8 h to remove fatty materials, waxes and lipophilic terpenoides. The marc, after completed extraction, was allowed to air-dry and then extracted with 500 mL chloroform for 6–8 hrs (to extract themoderately polar constituent viz., alkaloids, flavonoid s [21]. The defatted residue was ultimately acted upon by 500 mL ethanol for 8–10 h in order to get the polar class of phytoconstituents such as phenolics, glycosides and saponins. The solvent extracts obtained were filtered with Whatman No. 1 filter paper and evaporated in vacuo giving semi-solid crude extracts at a temperature of 40–45°C, separately. The dried extracts were weighed, labeled and stored in airtight containers at refrigeration temperature (4°C) for further phytochemical screening and chromatographic analysis [22].

Phytochemical Screening:

Qualitative Tests:

Test for Alkaloids

Alkaloids were detected from 2 mL of the extract sample by acidifying with dilute hydrochloric acid and filtering. The filtrate was also tested separately with Mayer's reagent, Wagner's reagent and Dragendorff's reagent. The occurrence of cream precipitate (Mayer's), reddish brown precipitate (Wagner's) and orange-red precipitate (Dragendorff's) were serve as the presence of alkaloids [23].

Test for Flavonoids

To 2 mL of each extract was added some drops of a dilute solution of sodium hydroxide. The development of a bright yellow color which faded on addition of diluted HCl was testimony for the presence of flavonoids [24].

Test for Tannins

To 2 mL of each extract, a drop or two of 1% ferric chloride was added. The formation of a blue-black or greenishblack color showed the presence of tannins [25].

Test for Saponins

A 2-mL of each extract was diluted in 5 mL of distilled water and then stirred for 2 min. A stable and persistent froth indicated the presence of saponins [26].

Test for Terpenoids

2 mL of extract was added with 1.5 mL down to pyrogallol and ascorbic acid/acetone and then mixed with 2 mL chloroform prior to adding 3 mL concentrated sulfuric acid. The formation of reddish brown interface indicated the presence of terpenoids [25].

Test for Steroids

Each extract (2 mL) was added to 2 mL of chloroform and 2 mL of concentrated sulfuric acid. A red UV-absorbing upper layer and yellow-green fluorescence in the lower layer indicated steroids [27].

Test for Glycosides

To 2 mL of extracts, 1 mL of glacial acetic acid and a few drops of ferric chloride solution were mixed to which concentrated sulfuric acid was added. The formation of a brown ring at the surface indicated the presence of cardiac glycosides [28].

Test for Phenols

Two milliliters of all the extracts were added to 2 mL 2% ferric chloride solution. The presence of phenolic compound was identified by deep bluish-green colour [25].

Test for Proteins

To 2 mL of each extract, the Biuret test was applied using 1 mL of 1% copper sulfate solution and 1 mL of 10% sodium hydroxide. Violet or pink colour indicated proteins [29].

Quantitative Methods

Thin Layer Chromatography (TLC) Procedure

TLC analysis was conducted to get phytochemical leads of *Annona squamosa*, *Glycyrrhiza glabra* and *Zingiber officinale* extracts [30]. To a concentration of 10 mg/mL reconstituted in methanol were dried extracts. Silica gel 60 F₂₅₄ TLC plates (Merck) were employed as the stationary phase. Each sample solution (5 µL) was spotted 1 cm above the bottom of the plate using a capillary tube. The plates were then developed in a saturated chamber using some solvents depending on the extract, chloroform:methanol (9:1) for *Annona squamosa*, ethyl acetate:formic acid:acetic acid:H₂O (100:11:11:26) for *Glycyrrhiza glabra* and toluene:ethyl acetate [7:3] for *Zingiber officinale*. The plates were air-dried and examined under UV light (at 254 and 366 nm) after development. Additional TLC detection was carried out by spraying with specific reagents, including Dragendorff's (for alkaloids), ferric chloride (for phenolics) and vanillin-sulfuric acid (terpenoids) and heating at 105°C for 5 min. R_f values of each visible spot were determined to help assess the identity of the compound [30].

Bioactive Markers Analysis Using RP-HPLC Profile

For our study, RP-HPLC was used by Shimadzu I-Series with a photodiode array (PDA) detector. A reverse-phase C18 column (4.6 mm × 250 mm, 5µm particle size) was used to perform chromatographic separation, which was run in gradient mode for fast separation of the bio-active markers [31]. It was operated using LC LabSolution software for accurate instrument method control, real-time data capture and easy analytical flow integration.

Preparation of Test solutions for RP-HPLC

The extraction of each plant ingredient (*Annona squamosa* L. leaves, *Glycyrrhiza glabra* L. roots and *Zingiber officinale* Rosc rhizomes) and polyherbal formulation (AS-GG-ZO blend) was performed by using the sonication-assisted extraction (SAE) technique [32]. Two grams of each plant part coarse powder was precisely weighed and extracted in 10 mL methanol by sonication (50 Hz) for 15 min at controlled temperature (40°C). The filtrates were sequentially passed through Whatman No.41 filter paper to remove coarse particles followed by filtration through 0.22µm PTFE syringe filter to form clear and particle free solution. All single

extracts were re-solved to achieve a final concentration of 10mg/mL with methanol as the dilution solvent [33]. For the polyherbal formulation 2 g of the mixed powder (1:1:1 ratio) of *Annona squamosa*, *Glycyrrhiza glabra* and *Zingiber officinale* were also extracted with 10mL of methanol under same SAE conditions. The filtrate was diluted to produce two working solutions (10mg/mL and 50mg/mL, respectively) [34]. The samples were kept in amber vials at 4°C until analysis. The dried extracts were analyzed by a RP-HPLC for the qualitative and quantitative estimation of bioactive markers *Rutin*, *Quercetin*, *Glycyrrhizic acid (GGA)*, *18β-Glycyrrhetic acid (18β-GA)* and *6-Gingerol and Zingerone*, using validated chromatographic conditions [35].

Preparation of stock solution

Similarly, in order to determine the content quantitatively, a separate stock solution of reference standards—rutin, quercetin for *Annona squamosa*; *glycyrrhizic acid*, *18β-glycyrrhetic acid* for *Glycyrrhiza glabra* and *6-gingerol and zingerone* for *Zingiber officinale* were prepared at a concentration of 1mg/mL by dissolving an accurately weighed quantity in HPLC grade methanol. These standardized marker solutions were used as calibration standards during the RP- HPLC analysis, to ensure accuracy and reproducibility in the identification and quantification of studied phytoconstituent[36].

DPPH radical scavenging activity

The antioxidant potential of the plant extracts was determined by DPPH (2, 2-diphenyl-1-picrylhydrazyl) radical scavenging assay. 1 mL of each extract (at different concentration levels-10, 25, 50, 75 and 100 µg/mL) were mixed with 2 mL of freshly prepared DPPH solution (0.1 mM in methanol), in this approach [37]. The mixtures were vortexed for a few seconds then kept in the dark at room temperature for 30 minutes to provide DPPH radicals and antioxidant compounds ample time for interaction. The absorbances of the solutions were determined at 517 nm after incubation with a UV-Visible spectrophotometer. Blank was methanol and positive control ascorbic acid [38]. The DPPH radical scavenging activity percent was calculated by using the following equation:

$$\text{Antioxidant scavenging activity (\%)} = (A_{\text{control}} - A_{\text{sample}} / A_{\text{control}}) \times 100$$

where A " control" is the absorbance of DPPH solution without extract, and A " sample" is with plant extract. The response was plotted as percentage inhibition versus concentration and the IC₅₀ values were calculated (the concentration of extract required to scavenge 50% DPPH radicals).

Results

Physicochemical analysis

The comparative analysis using physicochemical properties showed different profiles of extractives and ash content in the three samples. Loss on drying (LOD) In between 4.73% to 6.5% (moderate moisture content) *Annona squamosa* had the highest water and ethanol soluble extractives (51.43% and 49.2%, respectively) indicating this material is rich in polar and semi polar constituents, whereas *Zingiber officinale* had lowest values (Table 1). There were significant higher extractives of petroleum ether and chloroform of *Annona squamosa* which reflects higher content of non-polar and semi polar compounds. The total ash values ranged from 5.04%–8.35% and *Zingiber officinale* contained the lowest inorganic residue, thus lowest Effectiveness of inorganic elements. Among spices, *Zingiber officinale* (1.8%) showed higher acid insoluble ash value suggesting more siliceous matter.

Table 1 Physicochemical Analysis of selected plants

S.N.	Parameters	<i>Annona squamosa</i>	<i>Glycyrrhiza glabra</i>	<i>Zingiber officinale</i>
1	Loss on drying (LOD)	6.37%	4.73%	6.5%
2	Water soluble extractive value	51.43%	41.50%	29.45%
3	Ethanol soluble extractive value	49.2%	39.7%	19.95%
4	Petroleum ether soluble extractive value	14.50%	1.75%	7.17%
5	Chloroform soluble extractive value	15.25%	6.97%	8.37%
6	Total ash value	8.35%	5.92%	5.04%
7	Acid insoluble ash value	1.23%	0.39%	1.8%

Macroscopic examination

Annona squamosa leaves were green in color, ovate and having entire margins. They were of a slightly leathery consistency and with soft non-glandular trichomes on the abaxial face. The smell was slightly fragrant, and a little bit taste was bitter which could be an indication for *flavonoids and alkaloids*. The roots of *Glycyrrhiza glabra* were cylindrical in shape and externally brownish yellow in color, fibrous in nature with longitudinal striations. They had a

characteristic sweet flavor, which is considered to be due to the *glycyrrhizin* content. It was rough with some rootlets and no trichomes (Table 2). The rhizomes of *Zingiber officinale* were pale to light brown in color, branched and irregular in shape and knobby. They smelled pungent and spicy taste, acrid could be attributed to volatile oil and gingerol derivatives. It was nodes, had a smooth surface and revealed fibrous roots at the base. These macroscopic characters were in accordance with traditional pharmacognostic parameters and established the botanical identity and quality of the plant material, thereby justifying their utilization for phytochemical as well as pharmacological investigations.

Table 2 Macroscopic Evaluation of *Annona squamosa* Leaves, *Glycyrrhiza glabra* Roots, and *Zingiber officinale* Rhizomes

Parameter	<i>Annona squamosa</i> (Leaves)	<i>Glycyrrhiza glabra</i> (Roots)	<i>Zingiber officinale</i> (Rhizomes)
Color	Green	Brownish-yellow	Pale yellow to light brown
Shape	Ovate with entire margins	Cylindrical with tapering ends	Irregular, branched, knobby
Size	5–15 cm long, 3–7 cm wide	10–20 cm long, 1–2 cm thick	4–10 cm long, 1.5–3 cm thick
Surface Texture	Slightly leathery, soft	Fibrous, longitudinal striations	Smooth with visible nodes and root scars
Trichomes	Non-glandular, present on lower surface	Absent	Absent
Odor	Faintly aromatic	Sweet, characteristic	Pungent, spicy
Taste	Mildly bitter	Distinctly sweet	Sharp, acrid
Cuticle Integrity	Intact, smooth	Rough, with occasional rootlets	Intact, fibrous at base

Microscopic examination

The three plant materials exhibited specific anatomical characteristics under microscopic examination. The leaves of *Annona squamosa* exhibited spongy mesophyll parenchyma types,

anisocytic stomata, non-glandular trichomes and collateral vascular bundle (Table 3 and Figure 1). Parenchyma in the cortex, scanty collenchyma, lignified *sclerenchymatous* cells around xylem and radial arrangement of vascular elements with secretory cells related to its sweetness are found in the roots of *Glycyrrhiza glabra* (Figure 2). *Rhizomes of Zingiber officinale* had conspicuous starch-storing parenchyma, numerous secretory cells containing essential oils, a few sclerenchymatic fibers and anomocytic stomata (Figure 3). Examination of the powder made it possible to identify the trichomes, starch grains and oil cells which are diagnostic features that assisted in the botanical characterization.

Table 3 Microscopic features of *Annona squamosa*, *Glycyrrhiza glabra*, and *Zingiber officinale*

Diagnostic Feature	<i>Annona squamosa</i> (Leaves)	<i>Glycyrrhiza glabra</i> (Roots)	<i>Zingiber officinale</i> (Rhizomes)
Parenchyma Cells	Thin-walled, abundant in mesophyll	Present in cortex and central region	Prominent in ground tissue, storing starch
Collenchyma Cells	Found below epidermis, unevenly thickened	Sparse, mainly near vascular region	Absent or poorly developed
Sclerenchyma Cells	Present around vascular bundles	Lignified fibers near xylem	Scattered fibers, supporting rhizome structure
Stomatal Type	Anisocytic, mostly on lower epidermis	Paracytic, rare	Anomocytic, distributed irregularly
Trichomes	Non-glandular, unicellular on abaxial surface	Absent	Glandular and non-glandular types present
Vascular Bundles	Collateral, closed, surrounded by bundle sheath	Radial arrangement of xylem and phloem	Scattered, closed collateral bundles

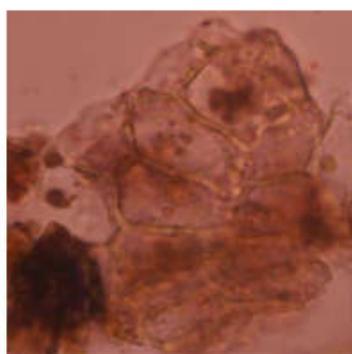
Secretory Cells	Rare, near veins	Present, associated with sweet compounds	Abundant, linked to essential oil production
Powder Microscopy	Shows fragments of epidermis, trichomes, and veins	Displays starch grains, fibers, and vessel elements	Reveals oil cells, starch grains, and parenchyma clusters



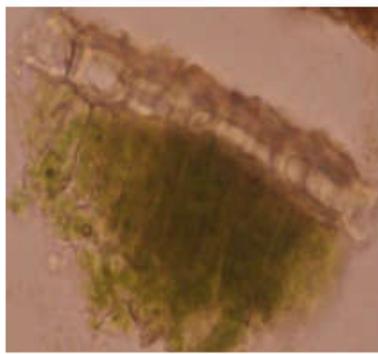
Thick walled uni and multicellular Trichomes



Fibres



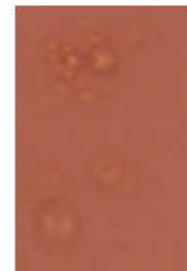
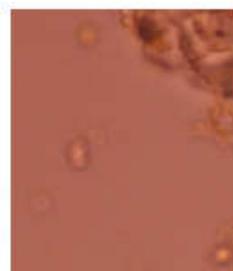
Thick walled parenchymatous cells filled with starch grains



Upper epidermis with Palisade cells

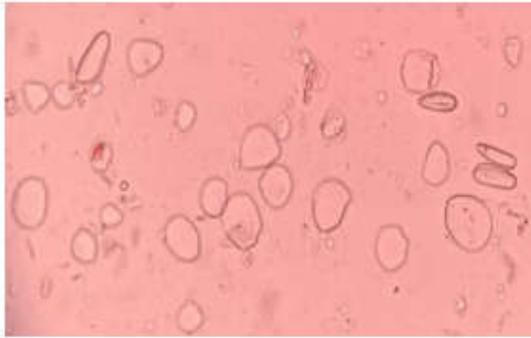


Stomata surrounded with parenchyma

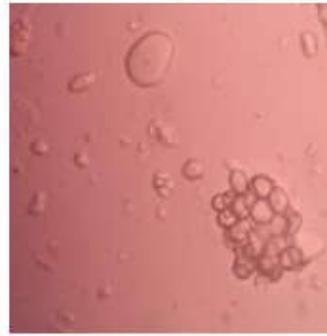


Starch grains

Figure 1 Microscopy of *Annona squamosa*



Single & compound starch grains



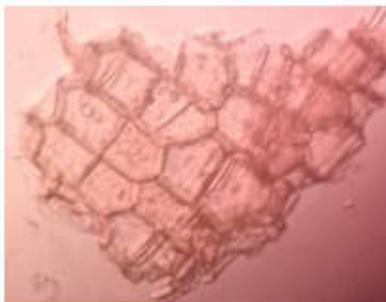
Reticulate thickening



Fibres with prismatic crystals



Fibre



Parenchymatous cells filled with starch grains



Thick walled cork cells

Figure 2 Microscopy of *Glycyrrhiza glabra*

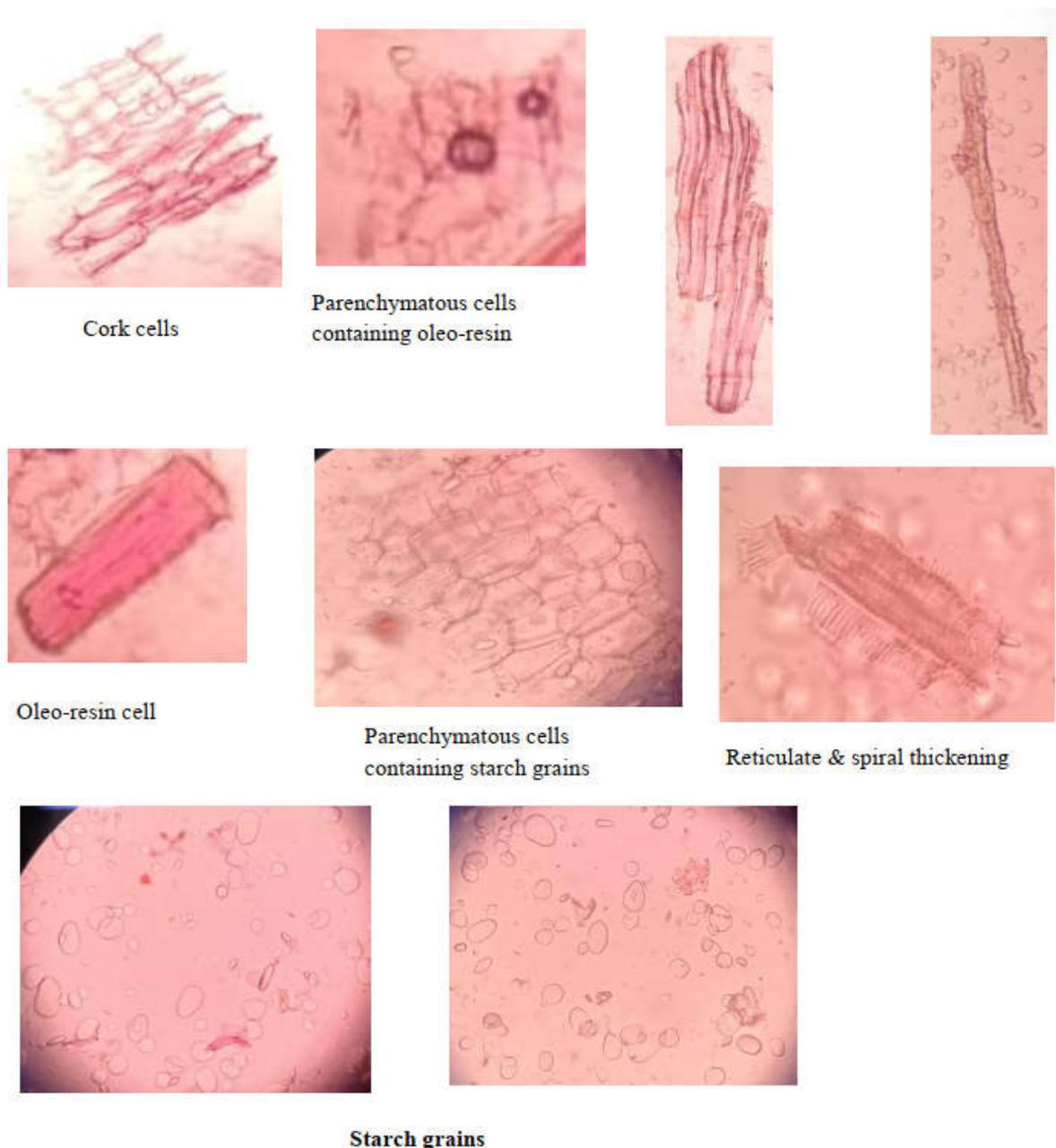


Figure 3 Microscopy of *Zingiber officinale*

Phytochemical screening

The phytochemical screening (Table 4) showed the existence and absence of major secondary metabolites in ethanol, chloroform and petroleum ether extracts of *Annona squamosa* leaves, *Glycyrrhiza glabra* roots and *Zingiber officinale* rhizomes. The spread of phytochemicals in the ethanol extracts from the three plants was very broad, which means that it had higher polarity and optimal extraction of various bioactive compounds. Both ethanol and chloroform extracts in *Annona squamosa* were positive for alkaloids and flavonoids, however no oil (petroleum ether) was found to contain such constituents. Tannins, saponins, glycosides,

phenols, and proteins were observed in only the ethanolic extract, while terpenoids and steroids were common to all three extracts indicating that they possess a broad spectrum of solubility. Proteins were remarkably found in the petroleum ether extract, and this rarely happens but it may be due to any lipophilic proteins fraction. Namely, for *Glycyrrhiza glabra* ethanol extracts contained all examined phytochemicals, with the exception of steroids and terpenoids which could be detected in each of three tested extracts. Chloroform extract was found to constitute of alkaloids, glycoside and steroids; it also disclosed the presence of tannins, saponins, glycosides and proteins. Notably, the petroleum ether extract of *Glycyrrhiza glabra* has been reported to contain saponins, terpenoids and steroids indicating that some of its components are lipophilic. For *Zingiber officinale*, the ethanol extract tested positive to all phytochemicals assayed except glycosides and steroids that were also present in chloroform extract. As the other two plants, terpenoids and steroids were found in all three solvent extracts confirming their high solubility. The petroleum ether extract of *Zingiber officinale*, did not contain most of the phytochemicals except for terpenoids and steroids which indicated that it could extract only a polar substances.

Table 4 Phytochemical screening results for plant extracts

Phytochemical Test	<i>Annona squamosa</i> (Ethanol)	<i>Annona squamosa</i> (Chloroform)	<i>Annona squamosa</i> (Petroleum Ether)	<i>Glycyrrhiza glabra</i> (Ethanol)	<i>Glycyrrhiza glabra</i> (Chloroform)	<i>Glycyrrhiza glabra</i> (Petroleum Ether)	<i>Zingiber officinale</i> (Ethanol)	<i>Zingiber officinale</i> (Chloroform)	<i>Zingiber officinale</i> (Petroleum Ether)
Alkaloids	+	+	–	+	+	–	+	+	–
Flavonoids	+	+	–	+	+	–	+	+	–
Tannins	+	–	–	+	–	–	+	–	–
Saponins	+	–	–	+	–	+	+	–	–
Terpenoids	+	+	+	+	+	+	+	+	+
Steroids	+	+	+	+	+	+	+	+	+
Glycosides	+	–	–	+	–	–	+	+	–
Phenols	+	+	–	+	+	–	+	+	–
Proteins	+	–	+	+	–	–	+	–	–

+ = Present; – = Absent

TLC estimation of plant extracts

Plate A (*Annona squamosa*) which has a weak blue-green spot near the top and a yellowish band that run vertically, it could be due presence of classic macroporous or semi-polar compounds. The blue-green colour in UV or post-spray may be due to the presence of flavonoid or alkaloids and the yellow streak is associated with terpenoids or glycosides. The position of the spot indicates an Rf value approximately 0.90. Plate B (*Glycyrrhiza glabra*) with a distinct central band of darker yellow streak suggest the presence of a band like zone of compounds e.g glycyrrhizin or similar saponin (Figure 4). The position indicates an Rf value of 0.85. The presence of more than one well-defined spots might reveal the dominance of a finite component or overlapping bands. Plate C (*Zingiber officinale*) exhibiting a very light purple spot at the top and less distinct streak indicate some volatile constituent or terpenoids. The purple color could be due to vanillin-sulfuric acid spray, which is often used for detection of essential oils. The Rf seems at 0.95. The TLC profile of all the three ethanolic extracts was found to be varying as *Annona squamosa* and *Zingiber officinale* had several phytoconstituents, whereas in *Glycyrrhiza glabra* a prominent band was visible. These findings suggest the occurrence of flavonoids, alkaloids, terpenoids, and glycosides and justify the TLC as a preliminary phytochemical discrimination method.

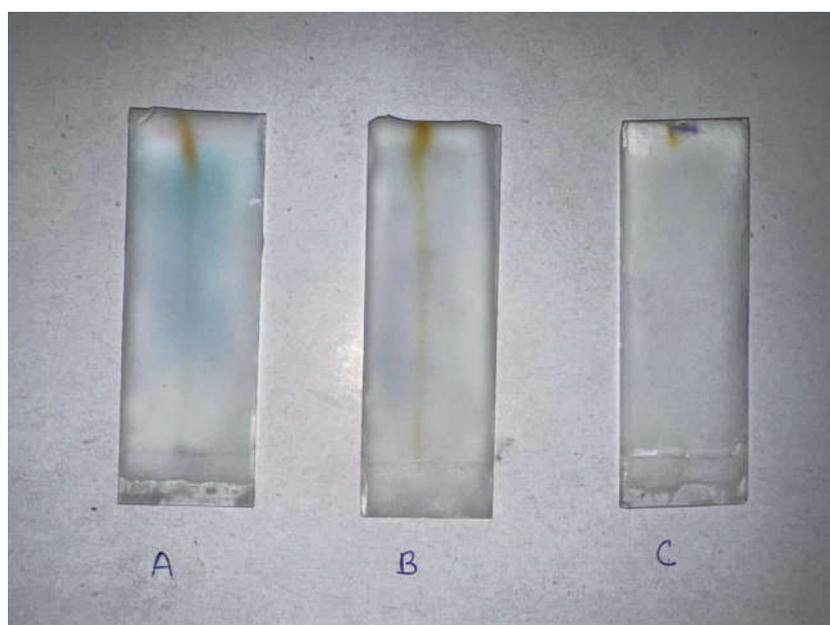


Figure 4 TLC image for selected plants ethanolic extract

Plate A (*Annona squamosa*); Plate B (*Glycyrrhiza glabra*); Plate C (*Zingiber officinale*)

HPLC estimation of biomarkers in polyherbal formulations

The HPLC profile comparison chromatogram of the standard mixture (Polyherbal formulation *Annona squamosa*, *Glycyrrhiza glabra* and *Zingiber officinale*) with a UV detection at 254nm has been shown in Figure 5. Chromatogram of mixed standard solutions The chromatogram shows six well separated peaks, corresponding to the known biologically active components such as rutin (A), quercetin (B), glycyrrhizic acid (C), 18 β -glycyrrhetic acid (D), 6-gingerol(E) and zingerone(F) with their respective characteristic retention time. The polyherbal chromatogram also reveals several peaks in close proximity with marker compounds, which signifying the existence of these phytoconstituents in formulation. The good overlap of the retention time and comparable intensity (peak) indicate that successful extraction and coexistence of isolated phytocompound derivatives in the mixture has occurred. This chromatographic profile confirms the phytochemical integrity of the formulation and substantiates its standardization on essential bioactive markers.

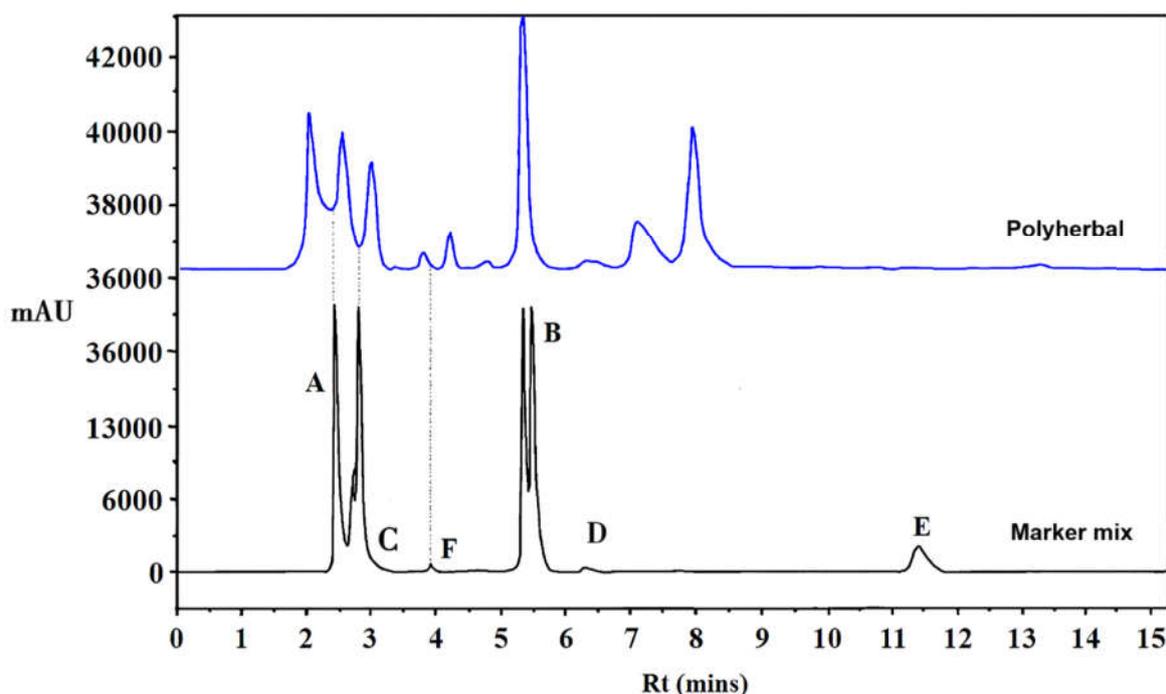


Figure 5 HPLC chromatogram of polyherbal formulation and marker compounds mixture

(A: Rutin, B: Quercetin, C: Glycyrrhizic acid, D: 18 β -Glycyrrhetic acid, E: 6-Gingerol, F: Zingerone @ 254 nm)

Antioxidant activity of polyherbal formulation

As presented in the DPPH radical scavenging activity Table 5, a dose-dependent enhancement of antioxidant capacity was observed on all samples examined. Ascorbic acid was always the most active (considered as control) at all the concentrations used. Of the single plant extracts, *Glycyrrhiza glabra* had a more powerful antioxidant role than *Zingiber officinale* and *Annona squamosa*. The polyherbal formulation also showed better activity in comparison to the extracts alone at 100 µg/mL with the ability to inhibit up to 89.450% and was well comparable to ascorbic acid (90.67%). This indicates a synergistic improvement of antioxidant ability following the combination of those three botanicals.

Table 5 DPPH radical scavenging activity of polyherbal formulation

Concentration ($\mu\text{g/ml}$)	Control	Ascorbic acid	<i>Annona squamosa</i>	<i>Glycyrrhiza glabra</i>	<i>Zingiber officinale</i>	Polyherbal formulation
20	0.976	53.68 \pm 0.24	44.27 \pm 0.34	51.58 \pm 0.66	49.93 \pm 0.45	50.45 \pm 0.78
40		65.36 \pm 0.77	49.67 \pm 0.54	59.87 \pm 0.70	55.37 \pm 1.34	60.67 \pm 1.05
60		75.30 \pm 0.64*	59.21 \pm 0.55	62.54 \pm 1.04	62.47 \pm 0.68	72.45 \pm 1.21*
80		83.19 \pm 1.04*	71.04 \pm 0.69*	72.89 \pm 1.03*	68.37 \pm 0.55	78.44 \pm 0.45*
100		90.67 \pm 0.79*	76.07 \pm 1.20*	79.74 \pm 0.94*	73.32 \pm 1.33*	89.45 \pm 1.01*

Data are represented as mean \pm SD (n=3), significantly different at *p<0.05 in comparison to the control group

Discussion

The phytochemical profile and antioxidant capacity of the leaves of *Annona squamosa*, roots of *Glycyrrhiza glabra* and rhizomes of *Zingiber officinale* were systematically studied by successive solvent extraction, thin layer chromatography (TLC) profiling, and reversed phase high performance liquid chromatography (RP-HPLC)-based determination. The results correlate and add more information to the published work confirming the pharmacognostical and phytochemical importance of these botanicals in traditional system of medicine as well as modern scientific principles.

The flavonoids, alkaloids, tannins and phenolic compounds were higher in chemical content in the ethanolic extract of *Annona squamosa* L., a plant commonly known as custard apple while greatest anti-microbial activity was observed in acetone extracts of the plants with some exceptions. This supports the findings of Kumari N et al. who recorded higher amount of total phenolics and flavonoids in methanolic leaf extracts of *A. squamosa* and they contributed antioxidant and antidiabetic properties, respectively in these plants [39]. The healing potential of this plant may be substantiated by the presence of rutin and quercetin already confirmed in our study using RP-HPLC. Rutin a well established vasoprotective flavonoid, quercetin a powerful antioxidant have been earlier reported in *A. squamosa* by Mandal AK et al., who thus highlighted their antioxidants potential ability to reduce oxidative stress and inflammation. Our TLC analysis showed a high R_f value (0.90) for flavonoid-rich bands, which is in accordance with the chromatographic behavior found in other studies under UV and vanillin-sulfuric acid visualization [40].

Glycyrrhiza glabra (licorice) root constituents were characteristically rich in glycyrrhizic acid and 18 β -glycyrrhetic acid, which were verified by RP-HPLC. This triterpenoid saponin is known for its anti-inflammatory, hepatoprotective and antiviral properties. Sharma et al. reported that the consecutive solvent extraction of *G. glabra* bark gave best phytochemical recovery in methanol and glycyrrhizin as major bioactive marker [41]. Correspondingly in our study, ethanol proved to be an effective solvent in releasing a diversity of phytoconstituents such as alkaloid, phenol and flavonoids. Notably, the petroleum ether extract of *G. glabra* revealed, through thin layer chromatography for saponins and steroids, the membrane partitioning of some potentially active compounds, which corroborates with previous work related to solubility of glycyrrhizin derivatives in non-polar solvents [42].

Zingiber officinale, (known to be used medicinally as well as culinary) also depicted a strong terpenoids, steroid and phenolic presence in all the solvent extractions. RP- HPLC analysis of 6-gingerol and zingerone, the main bioactive markers which contribute to its antioxidative and

anti-inflammatory activities was positive [43]. These results are in agreement with Vagare RD et al., who found the high antioxidant potential of methanolic extracts of *Z. officinale*, due to higher content of gingerol-related molecules [13]. Our TLC result demonstrated an appearance of a light purple spot at Rf 0.95 for volatile oil fraction, consistent to that chromatograph in study using vanillin-sulfuric acid spray for terpenoid screening [36].

The results of comparative phytochemical screening indicated that ethanol was the best solvent for extraction of diverse secondary metabolites from all three plants. This phenomenon is sustained by initiation procedures showing ethanol with higher polarity and much efficiency to solubilize variety phytoconstituents such as glycosides, flavonoids and phenolics [44]. The universal presence of terpenoids and steroids in all solvent extracts confirms their wide solubility and structural resistance, which supports them as robust standardization markers.

Anatomical characterization and quality control microscopic features were depicted. The presence of hypocytic stomata and non-glandular trichomes in leaves of *Annona squamosa*, radial vascular bundles in roots of *Glycyrrhiza glabra*, as well as abundant secretory cells on the rhizome of *Zingiber officinale* are indeed diagnostic characteristics that correspond to previously documented pharmacognostic standards [45]. These anatomical characters not only help in affirming species identities, but are related to the location of phytochemical reserves, such as essential oils and glycosides.

The RP-HPLC chromatogram of the polyherbal formulation showed separate peaks for all six marker compounds: rutin, quercetin, glycyrrhizic acid, 18 β -glycyrrhetic acid, 6-gingerol and zingerone—indicating successful co-extraction as well as compatibility between the mixed botanicals [46]. This justifies the use of polyherbal formulations to potentiate combined activities of different compounds. The retention time overlap and peak height analogy with reference standard fellow proves the robustness & repeatability, which is a pre-requisite in herbal drug quality control [47].

In comparison, our study provides an even more holistic approach that includes both macroscopic and microscopic microscopic on organoleptic characters with (chromatographic), biological (phytochemical), micronutrient characterization as well as microscopically quantification using thin layer chromatography other studies have focused on pieces of this as antioxidant tests or single marker(quantification). Gempo N et al. highlighted the antidiabetic potential on *A. squamosa* bark by pharmacological activity assay with solvent extraction but is without TLC as well as RP-HPLC profiling [48]. Similarly, in another study but based solely on the total phenolic content and the antioxidant capacity without the anatomical or chromatographic confirmation. Integrating various analytical dimensions, the full roadmap

represents a general guide to characterise botanical extracts and can expand their application in the field of pharmacological as well nutraceutical formulations.

The results of the DPPH assay in this study indicated that the activities of antioxidants increased with increasing concentrations of all extracts tested (extracts from *Annona squamosa*, *Glycyrrhiza glabra*, *Zingiber officinale*) as well as their polyherbal formulation. At 100µg/mL concentration the polyherbal mix showed 89.45% inhibition, which was comparable to standard antioxidant ascorbic acid (90.67%), indicating potent free radical scavenging capacity [49].

Glycyrrhiza glabra extract was consistently more active than the other extracts: *Zingiber officinale* and *Annona squamosa*, with exception of middle to highest concentration. It is in confirmation with the study of Joseph J N et al., who noted appreciable antioxidant potential in the methanolic extracts of *G. glabra* and had attributed it to the presence of glycyrrhizic acid and flavonoids. RP-HPLC analysis of our preparations also showed the presence glycyrrhizic acid and 18β-glycyrrhetic acid in accordance with their respective biological activities [50]. The combination was better than individual extract in higher concentration suggesting the synergistic activity among the phytoconstituents. Such synergism is further indicated by the simultaneous retention times and peak intensities in the RP-HPLC chromatogram, which provides evidences for the co-presence of rutin, quercetin, glycyrrhizic acid, 18β-glycyrrhetic acid, 6-gingerol as well as zingerone. These combinations might result in synergy on radical scavenging as their mechanisms of action complement each other such as hydrogen donation, metal chelation and through inhibition of lipid peroxidation [51].

Dwivedi S et al. also investigated DPPH free radical scavenging of *Annona squamosa* leaf extracts and ethanolic fractions were found to be more antioxidant active than the hexane and the ethyl acetate extracts. This is consistent with the conclusion that ethanol is the best solvent for extracting various kinds of antioxidant ingredients [52].

Apparently this detection of protein in the petroleum ether extracts of *A. squamosa* is an uncommon occurrence since it may imply either the existence of lipophilic-protein fractions or protein-bound- secondary products. This deserves to be probed further, since proteins are extracted by water or polar solvents. These anomalies illustrate the need for deliberative solvent selection and analytical cross-validation in phytochemical research [53]. The comparative HPTLC, DSC, and FT-IR results support the suitability of ethanol as a solubilizing agent for extraction of broad-spectrum phytochemicals and RP-HPLC for marker-based standardization. The polyherbal also showed higher activity, which may be attributed to synergy interactions of bioactive molecules [54]. These results are in agreement with the literature and confirm the

therapeutic interest of these plants for diseases related to oxidative stress. These results add to the increasing library of evidence for beneficial properties and therapeutic potential use of these botanical drugs, and warrant further research in formulation development and clinical trials

Conclusion

The results of the present investigation provide further evidence for the potentially beneficial antioxidant effects of *Annona squamosa*, *Glycyrrhiza glabra*, and *Zingiber officinale* alone and in combination (polyherbal formulation). This enabled us to verify by means of detailed pharmacognostic examination, phytochemical screening and chromatographic profiling that major active compounds -rutin, quercetin glycyrrhizic acid 18 β -glycyrrhetic acid 6-gingerol/zingiberone-would be present in the mixture, demonstrating a biomedical significance. The synergistic activity among the tested constituent of polyherbal formulation led to its improved DPPH radical scavenging potential (comparable with ascorbic acid). These findings not only support the ethnobotanical use of these botanicals for diseases involving oxidative stress, but also form groundwork for further research and product development. Include in vivo validation of antioxidant effects, formulation optimization for enhanced bioavailability and mechanistic investigation of their molecular pathways will need to be addressed by prospective studies. Moreover, incorporation of such extracts in functional foods, nutraceuticals or standardized herbal medicines may provide safe and effective preventive healthcare products. The integrative methodology in the study establishes a solid working platform for applications to transference from ethnopharmacology to modern phytomedicine.

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Authors credit

PST – writing original draft, VKS – protocol design and supervision, VDS – editing and proofreading

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