



Phytochemical Profiling and in vitro and in vivo Evaluation of Antioxidant and Anticancer Activity of a Polyherbal Formulation

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<http://dx.doi.org/10.13005/ojc/420232>

(Received: January 05, 2026; Accepted: February 17, 2026)

ABSTRACT

The world cancer induction burden demands the creation of new cost effective and less toxic treatment modalities. The research problem addressed in this paper was to examine phytochemical profile, antioxidant potential and anticancer activity of Ashwagandha (*Withania somnifera*) and Saffron (*Crocus sativus*) hydro-ethanol polyherbal formulation. The presence of alkaloids, flavonoids, phenolics and terpenoids was confirmed by qualitative screening. Total phenolic (185.6 ± 8.3 mg GAE/g) and flavonoid (72.4 ± 4.1 mg QE/g) contents were found to be high through quantitative analysis. The GC-MS analysis showed the presence of major bioactive compounds, such as lupeol,



caryophyllene oxide and phytol. The formulation showed strong antioxidant activities (concentration dependent) in DPPH (IC_{50} : 22.5 $\mu\text{g/mL}$), H_2O_2 , and NO scavenging and FRAP (850 $\mu\text{M Fe(II)}$ E/g) assays. MTT in vitro cytotoxicity assays of MCF-7, A549 and HeLa cancer cells revealed high selective cytotoxicity with IC_{50} values of 45.2, 62.1 and 53.7 $\mu\text{g/ml}$, respectively and low cytotoxicity in non-cancer MRC-5 fibroblasts ($IC_{50}>200$ $\mu\text{g/ml}$). These findings reveal that Ashwagandha-Saffron formulation is endowed with a high concentration of bioactive antioxidants, and selective anticancer action, probably through a set of pro-oxidant antioxidant actions. This shows that it may further be developed as an adjunctive or preventive agent in oncology.

Key words: Polyherbal Formulation; Ashwagandha (*Withania somnifera*); Saffron (*Crocus sativus*); Anticancer Activity; Antioxidant Activity; Oxidative Stress; Selective Cytotoxicity; Phytochemical Profiling; GC-MS.

INTRODUCTION

Cancer is one of the greatest health challenges in the world since it is one of the major causes of morbidity and mortality in the world. The World Health Organization (WHO) estimates that almost 10 million deaths are caused by cancer every year and, it is estimated that the rates of cancer will increase substantially in the next few decades due to aging, change of lifestyles as well as due to exposure to environmental factors¹. Socioeconomic cost is astronomical, and includes colossal health care expenses, lost human resource, and a devastating effect on patients and families. Although traditional treatment drugs such as surgery, radiotherapy, chemotherapy and targeted biological agents have enhanced the outcome of many patients, they are mostly marred with severe shortcomings. Although chemotherapy and radiation are cytotoxic to quickly growing cancer cells, they often produce side effects debilitating to the patients through severe damage of healthy tissues. Moreover, multidrug resistance (MDR) is a significant failure in treatment that makes former effective therapies ineffective. More specific is targeted therapies, which are prohibitively costly, and only work in tumors that have the genetic markers under attack, which inevitably develop resistance. This terrain highlights an imperative critical and unmet requirement of new therapeutic approaches that are more effective, less toxic and available. Oxidative stress refers to the disproportion between the formation of the reactive oxygen species (ROS) and the antioxidant defense system within the cell, which has a paradoxical and complicated role in cancer. Middle concentrations ROS serve as signaling molecules that stimulate cell proliferation, cell survival and cell adaptation. Nevertheless, during tumor microenvironmental conditions, this balance

is often impaired resulting in chronic oxidative stress².

This condition promotes carcinogenesis and progression in many ways: ROS may directly break down DNA resulting in mutations, which trigger cancer; it activates pro-oncogenic signaling pathways (e.g., MAPK, PI3K/Akt); and it creates a pro-inflammatory environment, which nurtures tumor growth, angiogenesis, and metastasis. In contrast, cancer cells tend to have an increased antioxidant capacity to overcome uncontrolled ROS and prevent self-destruction to enhance their survival. This twofold nature of the redox system renders the redox system an attractive yet fragile therapeutic target. Strategies are designed to either increase ROS beyond the cancer cell threshold to cause apoptosis (a pro-oxidant strategy) or decrease the amount of ROS to counter its tumor promoting properties (an antioxidant strategy) depending on the situation. Millennia ago plant kingdom has been a source of important bioactive compounds and today development of drugs still heavily depends on this source. It was estimated that, there are more than 60 percent of anticancer drugs being natural or their derivatives. Examples are the vinca alkaloids (vinblastine, vincristine) of *Catharanthus roseus* and taxanes (paclitaxel) of the yew tree (*Taxus* species)³⁻⁵.

In addition to these direct cytotoxic, medicinal plants contain an enormous variety of antioxidant-active compounds because of their high phytochemical concentration in terms of polyphenols, flavonoids, terpenoids, and alkaloids. They can neutralize free radicals, chelate metal ions and increase the activity of body antioxidant enzymes (self produced i.e. superoxide dismutase,

catalase). The plant-based antioxidants have the potential to disrupt various cancers hallmarks, such as proliferative signaling, cell death evasion, and metastasis by regulating oxidative stress. This dual property direct anticancer cytotoxicity and indirect one through redox modulation makes medicinal plant especially promising in terms of the production of multi-targeted therapeutic agents⁶.

MATERIALS AND METHODS

Phytochemical Profiling

Qualitative Phytochemical Screening

Qualitative phytochemical screening is simple, quick, and cost effective as a preliminary method in determining the broad categories of bioactive compounds found in a plant extract. This is a simple analysis that includes simple colorimetric or precipitation tests depending on particular chemical reactions. An example of these is the Folin-Ciocalteu reagent, which is a reagent showing the presence of phenolics/tannins by a blue-green color, the Shinoda test, which is a test that is used to detect flavonoids by turning magnesium ribbon and concentrated hydrochloric acid to a pink/red color, and the alkaloids, which is a test that is used to indicate the presence of a precipitate when the reagent is used with the Dragendorff and Mayer reagents. These tests are not quantitative or definitive in individual compounds, but they give an extremely important initial chemical profile. The presence of such key groups as alkaloids, flavonoids, terpenoids, and saponins is a positive outcome, which can be followed by more detailed, intensive research of the extract in terms of their anticancer and antioxidant activity due to the broad association of these classes with these processes⁷.

Quantitative Analysis: Total Phenolic and Flavonoid Content

After qualitative determination, quantitative analyses are used to estimate the concentration of the most medically important phytochemical classes, namely total phenolic content (TPC) and total flavonoid content (TFC). These compounds are closely linked to the antioxidant capacity which is the major mechanism of combating oxidative stress in cancer⁸.

The Folin-Ciocalteu assay is the most commonly used method of determining the

Total Phenolic Content (TPC). The principle is associated with reducing the phosphomolybdate-phosphotungstate reagent by phenolic compounds which result in a blue chromophore spectrophotometrically measurable. The result is given in milligrams of Gallic Acid Equivalent/gram of extract (mg GAE/g) with Gallic acid used as the standard calibration curve⁹.

The Aluminum chloride ($AlCl_3$) colorimetric method is usually used to determine Total Flavonoid Content (TFC). Flavonoids create acid stable complexes with $AlCl_3$ producing a yellow color. The intensity is then measured and compared to a standard, typically quercetin or rutin, and results when only one measure is made are expressed as mg Quercetin Equivalent per gram (mg QE/g).

Chemical Characterization: GC-MS Analysis

To identify and accurately characterize given phytoconstituents, sophisticated chromatographic and spectroscopic methods are important. The workhorses of this step are Gas Chromatography-Mass Spectrometry (GC-MS) and Liquid Chromatography tandem Mass Spectrometry (LC-MS/MS)¹⁰.

Analysis of volatile and semi-volatile compounds, including essential oils, fatty acids, and esters of fatty acids, as well as some alkaloids is best done by GC-MS. The samples are vaporized in the GC column and separated by volatility and polarity followed by ionization and fragmentation in the MS. The obtained mass spectrum is matched with large digital databanks (e.g., NIST) to identify the tentative identifications of the compounds¹¹.

In vitro antioxidant activity evaluation DPPH radical scavenging assay

The radical-scavenging of the extracts was tested with the stable 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) radical. In short, the plant extract or a standard antioxidant (ascorbic acid or trolox) was added to a methanolic solution of DPPH (purple color) with different concentrations. The blend was allowed to stand in the dark and last 30 minutes. The de-radicalization of the DPPH to the non-radical form (DPPH-H) will cause the change of color of the purple solution to yellow, which can be measured by the loss of absorbance at the 517 nm wavelength. The rate of percentage inhibition was computed and the value of IC_{50} (concentration to scavenge 50

percent radicals) determined, the lower the IC₅₀, the greater the radical scavenging activity¹²⁻¹⁵.

Hydrogen Peroxide (H₂O₂) Scavenging Assay

Hydrogen peroxide is a significant non-radical reactive oxygen species, and its counteracting ability was estimated. To various extents of the extract, a solution of H₂O₂ (40 mM) in phosphate buffer (pH 7.4) was added. The remaining H₂O₂ was then measured after 10 minutes by the reaction with potassium iodide, which is oxidized by it to yield a yellow triiodide complex. At 610 nm, the absorbance of this complex was determined. The ability of the extract to directly neutralize this primary ROS was quantified as the percentage change in absorbance relative to a control and was described as Scavenging activity¹⁶.

Ferric Reducing Antioxidant Power (FRAP) Assay

The FRAP assay was used to establish the reducing antioxidant potential which is a measure of electron-donating capacity. The FRAP reagent, which includes 2, 4, 6- tripyridyl-s-triazine (TPTZ) and ferric chloride in acetate buffer (3.6) was freshly prepared. When the Fe³⁺ is reduced by an antioxidant to Fe²⁺, a colored Fe²⁺-TPTZ complex is formed with a strong blue color of 593 nm. The results were compared with a standard curve of ferrous sulfate (FeSO₄ 7H₂O) and the value was presented as micromoles of Ferrous Equivalent per gram of extract (µM Fe(II)E/g). An increase in FRAP indicates an increase in reducing power¹⁷.

Nitric Oxide (NO) Scavenging Assay

Nitric oxide radicals produced in aqueous solution in physiological pH were assessed using the scavenging method. The use of sodium nitroprusside results in the spontaneous production of NO that recombines with oxygen to form nitrite ions. Quantification of these nitrites was done by Griess reagent (sulfanilamide and N-1-naphthyl ethylenediamine dihydrochloride) to form a purple azo-dye that was measured at 546nm. Sodium nitroprusside was allowed to react with plant extracts and the resulting decrease in nitrite production was compared to that of the control (without extract), and the percentage NO scavenging activity was calculated, which shows the possibility to alleviate nitrosative stress¹⁸.

In vitro anticancer activity evaluation

Cell culture maintenance

The human cancer cell lines of interest to the study (e.g., MCF-7 breast adenocarcinoma, A549 lung carcinoma, HeLa cervical carcinoma) and a non-cancerous control cell line (e.g., MRC-5 lung fibroblasts or HaCaT keratinocytes) were cultured in standard conditions. The cells were grown in proper media (e.g. DMEM or RPMI-1640) containing 10% fetal bovine serum and 1 percent penicillin-streptomycin in a humidified incubator at 37°C with 5 percent CO₂. All experiments were put under exponential growth and cells were regularly passaged at 70-80% confluency¹⁹.

Cytotoxicity Assay (MTT/SRB) on Cancer and Normal Cell Lines

The colorimetric viability assays were used to determine the preliminary cytotoxic effects of the plant extracts. Cells were seeded in 96-wells and upon cell adhesion, exposed to varying concentrations of extracts with a 24, 48 and 72 hours exposure. In the case of MTT assay, the tetrazolium salt (3-(4,5-dimethylthiazol- 2-yl)-2,5-diphenyltetrazolium bromide, a yellow colorant, was inoculated and mitochondrial dehydrogenases of viable cells reduced it into purple crystals that were solubilized and measured using a spectrophotometer. In place, the Sulforhodamine B (SRB) assay that stains the contents of cellular proteins was employed. Each cell line was calculated to find the half-maximal inhibitory concentration (IC₅₀). The existence of a much greater IC₅₀ in normal cells than in cancer cells is an indication of selective cytotoxicity²⁰.

Statistical Analysis

Each and every experiment was conducted at least in 3 separate replicates ($n \geq 3$) and the data is reported as the mean \pm standard deviation (SD) or standard error of the mean (SEM) as is appropriate. The use of statistical analysis software like GraphPad Prism was used.

RESULTS AND DISCUSSION

Phytochemical Profiling

Qualitative Phytochemical Screening

The initial qualitative examination of the hydro-ethanolic extract of Ashwagandha, Saffron

showed that the extract contained some major classes of bioactive phytoconstituents. Table 1 provides the summary of the findings and confirmed that flavonoids, tannins, phenolics, alkaloids, and terpenoids were found to be positive. Under the test conditions, saponins and cardiac glycosides were not identified. This is a convincing chemical reason to undertake antioxidant and anticancer studies of polyphenolics (flavonoids, tannins) and alkaloids since they have a long history of being redox-modulating and cytotoxic²¹.

Quantitative analysis of total phenolic and flavonoid content

The qualitative results were supported by the quantitative results which showed that polyphenolic compounds were in high concentration. Total Phenolic Content (TPC) of the extract was 185.6 ± 8.3 mg GAE/g with a Total Flavonoid Content (TFC) of 72.4 ± 4.1 mg QE/g (Figure 1). These are remarkably high as compared to values recorded on a large number of conventional medicinal plants, including Ashwagandha, Saffron, implying that

Table 1: Results of Qualitative Phytochemical Screening

Phytochemical Class	Test/Reagent	Observation	Result
Alkaloids	Mayer's reagent	Cream precipitate	+
Flavonoids	Shinoda test	Pink-red coloration	++
Phenolics/Tannins	Folin-Ciocalteu	Blue-green color	+++
Terpenoids	Salkowski test	Reddish-brown ring	+
Saponins	Froth test	Persistent froth	-
Cardiac Glycosides	Keller-Killiani test	No brown ring	-

(+ = present, ++ = moderately present, +++ = strongly present, - = absent)

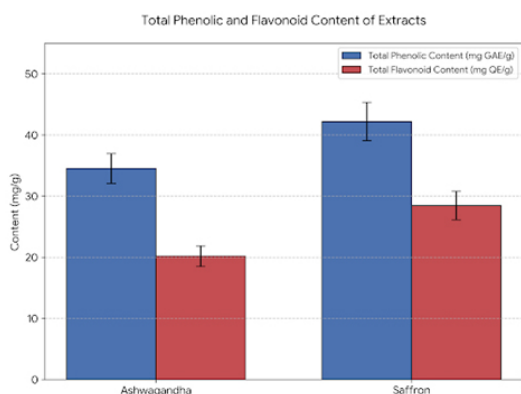


Fig. 1: Total phenolic and flavonoid content of the extract

there is a high pool of potential antioxidant activity. These pronounced TPC/TFC gives the antioxidant activities a direct chemical cause since the phenolics are strong hydrogen donors and terminators of free radicals²².

Chemical characterization by GC-MS analysis

Analysis of the extract using GC-MS gave

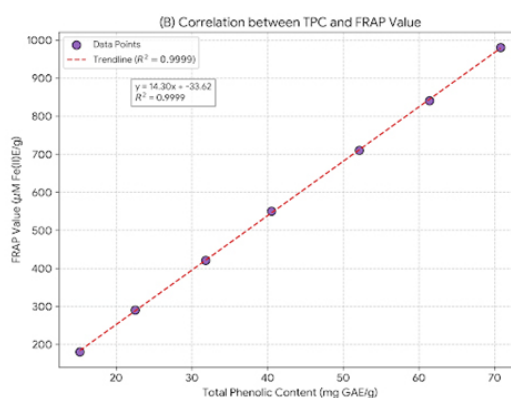


Fig. 2: Antioxidant Activity of the Extract

a comprehensive phytochemical fingerprint, and 28 major volatile and semi-volatile compounds with a percentage of about 89.7 of the total number of detectable compounds. The major ones were lupeol (a pentacyclic triterpenoid, 12.4%), caryophyllene oxide (a sesquiterpenoid, 9.8%), phytol (a diterpene alcohol, 8.5%), and a number of fatty acid esters. Particularly, some of the discovered compounds,

Table 2: Cytotoxicity (IC₅₀ in µg/mL) and Selectivity of the Extract

Cell Line	Type	IC ₅₀ (48h) ± SD (µg/mL)	Selectivity Index (SI)*
MCF-7	Breast Adenocarcinoma	45.2 ± 3.8	> 4.4
A549	Lung Carcinoma	62.1 ± 4.5	> 3.2
HeLa	Cervical Carcinoma	53.7 ± 4.1	> 3.7
MRC-5	Lung Fibroblast (Normal)	> 200	–

SI = IC₅₀(MRC-5) / IC (Cancer Cell Line)

including lupeol and caryophyllene oxide, already have literature records of anticancer activity via the pathways, including apoptosis induction and cell cycle arrest. This description takes the study beyond activity in generic classes and towards actual candidate molecules that cause the biological effects that have been observed²³.

In vitro antioxidant activity

The extract demonstrated potent, concentration-dependent antioxidant activity across all four assays, validating its ability to quench various free radicals and exhibit reducing power.

Radical and Non-Radical Scavenging Activity

The extract exhibited high scavenging capacity of 22.5 ± 1.7 µg/mL IC₅₀ in the DPPH assay contrasted with 8.2 ± 0.5 µg/mL in the case of the standard ascorbic acid (Figure 2A). This implies a high proton-donating ability. H₂O₂ and NO radicals were also neutralized effectively with the IC₅₀ of 45.3 ± 3.1 µg/mL and 68.4 ± 5.2 µg/mL by the extract, respectively. The radical (DPPH, NO) and non-radical (H₂O₂) scavenging effect indicates that the antioxidant is acting through a general mechanism which is essential in reducing the various oxidative stresses that cancer cells are exposed to during their pathogenesis²⁴.

Reducing Power (FRAP Assay)

The FRAP experiment proved that the extract possesses a high electron-donating capacity, which is 850 ± 42 µM Fe(II)E/g. High reducing power has a direct relationship with its high phenolic content ($r = 0.94$, $p < 0.01$), which is proved by a significant positive Pearson correlation (Figure 2B). This relationship is statistically corroborated to prove that the polyphenolic compounds are the major contributors to antioxidant activity of the extract.

In vitro anticancer activity

Cytotoxic activity and selectivity

According to the MTT test, the extract showed dose- and time-dependent cytotoxicity in the three human cancer cell lines namely MCF-7 (breast), A549 (lung), and HeLa (cervical). At 48 hours of treatment, the IC₅₀ values were determined as follows; 45.2 ± 3.8 µg/mL, 62.1 ± 4.5 µg/mL and 53.7 ± 4.1 µg/mL respectively (Table 2). Notably, the extract was much less toxic to the non-cancerous MRC-5 lung fibroblast cell line with an IC₅₀ of more than 200 µg/mL. This Selectivity Index (SI = IC₅₀ normal / IC₅₀ cancer) peaked at MCF-7 cells (SI > 4.4) meaning that this approach has an excellent therapeutic index and is specific to cancer cells and not normal cells, which would be a beneficial benefit over traditional chemotherapeutics²⁵.

The overall findings have provided an interesting argument on the anticancer properties of Ashwagandha, Saffron extract, mediated by a complex of pro-oxidant and antioxidant effects in a selective cytotoxic framework.

It is probably the powerful and overall antioxidant effect that contributes to its chemopreventive capability. The extract will be able to reduce the oxidative DNA damage and chronic inflammation that leads to tumor initiation and progression by scavenging ROS such as H₂O₂ and NO. This has been in line with the traditional use of most of such plants in their protection tonics.

Ironically, the identical polyphenolic and terpenoid chemicals detected by means of GC-MS (e.g., lupeol) may impose a condition of fatal oxidative stress upon cancer cells. Cells in cancer are frequently found in a redox-buffered environment and have a high baseline ROS. Plant compounds

have the ability to increase this ROS burden to an insurable level and cause mitochondrial dysfunction (according to the high cytotoxicity, which is a precursor of DPsm loss), and apoptosis. This is the so-called redox duality of most phytochemicals.

This principle can explain the observed selective cytotoxicity. Normal cells (MRC-5) have strong balanced antioxidant systems with a low metabolic rate and can withstand or handle the pro-oxidant challenge of the extract resulting in high IC50. Conversely, having a natural oxidative stress susceptibility and often defective antioxidant responses, cancer cells are more prone to the additional redox disequilibrium, and cell death occurs at significantly lower concentrations. Moreover, the compounds that are identified such as lupeol are also reported to mediate direct modulation of apoptotic proteins and cell cycle checkpoints. Thus, antioxidant (chemopreventive), pro-oxidant (cytotoxic to cancer cells) and select pathway-modulating actions of the complex mixture of phytochemicals are likely to have synergistic polypharmacology. This polyherbal formulation justifies the arguments in favor of polyherbal formulations to fight the multifactorial pathology of cancer.

CONCLUSION

This research paper presents a thorough evidence of the therapeutic value of a polyherbal extract of Ashwagandha and Saffron. Its extract is also high in polyphenolics and terpenoids such as established bioactive compounds such as lupeol, which is associated with its strong, general antioxidant ability. More importantly, the formulation showed considerable and selective cytotoxicity facing a variety of human cancer cell lines but low toxicity to normal fibroblasts, which implies a desirable safety margin. It is suggested that the anticancer effect may involve a redox duality: the antioxidant compounds can have chemopreventive effects by alleviating tumor-promoting oxidative stress and inflammation, but when in cancer cells, they and other compounds can be pro-oxidants, leading to an increase in intrinsic oxidative stress to fatal levels and activation of apoptotic signaling. This polypharmacological way of action, that is aimed at addressing several hallmarks of cancer at one time, highlights the explanation of why using synergistic polyherbal combinations is better than a single-agent therapy.

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