

Evaluation of the polyherbal extract for antioxidant, anticancer and antidiabetic activity

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Abstract

In the present investigation, methanolic and hydro alcoholic polyherbal extracts having *Terminalia arjuna* (Roxb.) Wight & Arn, *Piper nigrum* Linn. and *Cuminum cyminum* Linn. were evaluated for their *in vitro* antioxidant activity by 1,1-diphenyl-2-picryl hydrazyl (DPPH) and superoxide radical scavenging method; *in vitro* antidiabetic activity by α -glucosidase and α -amylase inhibitory method; and anticancer activity by 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay against A549 human lung cell carcinoma. Antioxidant activity of methanolic extract of *Terminalia arjuna* (S_1) was found to be more potent compared to other polyherbal extractions. S_1 also inhibited α -glucosidase enzyme and hydroalcoholic extract inhibited α -amylase enzyme. Few polyherbal combinations are found to possess significant anticancer activity against A549 human lung cell carcinoma.

Key words: A549 human lung cell carcinoma, Anticancer activity, *Terminalia arjuna*, *Piper nigrum*, *Cuminum cyminum*.

Introduction

In recent years, more interest has been paid to protect human beings against oxidative damage caused by free radicals which leads to ageing and human diseases like diabetes and cancer. One possible solution is to explore the potential antioxidant and anticancer properties of herbal or polyherbal extracts (Namiki, 1990). Antioxidants, which can scavenge free radicals against damage and decay, have an important role in biological system and may be helpful in the prevention of cancer, diabetes mellitus and heart diseases (Tiwari, 2004).

There are several traditional medicinal plants which possess rich antioxidant principles and strong antioxidant activities. It has been argued that major antidiabetic activities from these plants might originate from their antioxidant principles (Scartezzini and Speroni, 2000 and Cune and Johns, 2002). There is an immediate requirement to search herbal extracts which possess antioxidant activity including antidiabetic and anticancer activity.

The medicinal plant, *Terminalia arjuna* which belongs to *Combretaceae* family, is an important plant described in the Ayurveda, having many therapeutic activities (Kokate, 1994). Piperine, an alkaloid amide isolated from pepper has been reported to show cytotoxic activity towards several tumor cell lines (Bezzera *et al.*, 2006). The volatile oils in cumin are found to increase the bioavailability of valuable phytochemicals present in other species (Amal *et al.*, 2009).

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So far polyherbal extracts having these herbs are not investigated for antioxidant activity. Hence, the polyherbal extract of these three plants were evaluated for their *in vitro* pharmacological activities. However, in addition to this, the origin and distribution, description, healing properties and the chemical composition of three plants are given below.

i. *Terminalia arjuna* (Roxb.) Wight & Arn

Origin and distribution

Arjuna tree is indigenous to India. It is found throughout the sub-Himalayan tracts, the Deccan regions, Myanmar and Sri Lanka. It grows chiefly along water channels or marshy belts.

Description

Arjuna is the large size deciduous tree. The height of the Arjuna tree reaches up to 60 -85 feet. It is the evergreen tree with the yellow flowers and conical leaves. It has a smooth gray bark. It has a buttressed trunk and a vast spreading crown from which the branches drop downwards. Mainly, the bark of the tree is used in medicines. This tree has been named nadisarjja in the Sanskrit, wherein its bark has been describes as a cardiac tonic. India was the first to discover the bark of this tree for heart diseases.

Healing properties

The bark of the Arjuna tree contains calcium salts, magnesium salts, and glucosides have been used in traditional Ayurvedic herbalism. Juice of its leaf is used to cure dysentery and earache. Arjuna helps in maintaining the cholesterol level at the normal rate, as it contains the antioxidant properties similar to the Vitamin E. It strengthens the heart muscles and maintains the heart functioning properly. It also improves functioning of cardiac muscle. Arjuna is used for the treatment of coronary artery disease, heart failure, edema, angina and hypercholesterolemia. Its bark power possesses diuretic, prostaglandin enhancing and coronary risk factor modulating properties. It is also considered as beneficial in the treatment of asthma.

Arjuna can help to lower cholesterol as much as 64% - people taking Arjuna preparation saw their LDL levels plummet by an average of 25.6%.

Used for Cardiomyopathy like Myocardial infraction, angina, coronary artery disease, heart failure, hypercholesterolemia, hypertension. In case of heart attack though it can not act against, like streptokinase or eurokinase, but regular use of it after just recovering from heart attack, reduces the chance of further attack to a great level.

Arjuna reduces angina episodes much better than nitroglycerin. In one study, angina episodes were cut in half by the Arjuna, with none of the nasty side effects. Plus, it can be used as long as we like, because no toxicity or side effects has so far be found and it can be advocated to use in regular basis for a strong and well functioning heart.

Chemical composition

Arjuna contains specific medically active constituents namely triterpine glycosides like arjunetosides I, II, III, IV, arjunine, arjunetein, arjunic acid, arjunolic acid, arjungenin, arjunglycosides, phytosterols (beta- sitosterol). The bark is rich in saponnins, natural anti-oxidants (flavonoids- arjunone, arjunolone, leteilin) gallic acid, ellagic acid, oligomeric proanthocyanidins, phytosterols, rich in minerals like calcium, magnesium, zinc and copper.

ii. *Piper nigrum* Linn.

Origin and distribution

Black pepper is native to Southeast Asia and China, and is extensively cultivated there and elsewhere in tropical regions. Currently Vietnam is the world's largest producer and exporter of pepper, producing 34% of the world's *Piper nigrum* crop as of 2008.

Description

The pepper plant is a perennial woody vine growing up to 4 meters in height on supporting trees, poles, or trellises. It is a spreading vine, rooting readily where trailing stems touch the ground. The leaves are alternate, entire, 5 to 10 cm long and 3 to 6 cm across. The flowers are small, produced on pendulous spikes 4 to 8 cm long at the leaf nodes, the spikes lengthening up to 7 to 15 cm as the fruit matures. The fruit of the black pepper is called a drupe and when dried it is a peppercorn.

Medicinal uses

Black pepper (or perhaps long pepper) was believed to cure illness such as constipation, diarrhea, earache, gangrene, heart disease, hernia, hoarseness, indigestion, insect bites, insomnia, joint pain, liver problems, lung disease, oral abscesses, sunburn, tooth decay, and toothaches. Nevertheless, black pepper, either powdered or its decoction, is widely used in traditional Indian medicine and as a home remedy for relief from sore throat, throat congestion, cough *etc.*

Piperine present in black pepper acts as a thermogenic compound. Piperine enhances the thermogenesis of lipid and accelerates energy metabolism in the body and also increases the serotonin and beta-endorphin production in the brain. So piperine can dramatically increase absorption of selenium, vitamin B, beta-carotene and curcumin as well as other nutrients.

Pepper contains small amounts of safrole, a mildly carcinogenic compound. Also, it is eliminated from the diet of patients having abdominal surgery and ulcers because of its irritating effect upon the intestines, being replaced by what is referred to as a bland diet. However, extracts from black pepper have been found to have antioxidant properties and anti-carcinogenic effects, especially when compared to chili.

iii. *Cuminum cyminum* Linn.

Origin and distribution

Cumin sometimes spelled cummin, *Cuminum cyminum* Linn. is a flowering plant in the family Apiaceae, native from the east Mediterranean to India.

Description

Cumin is the dried seed of the herb *Cuminum cyminum* Linn., a member of the parsley family. The cumin plant grows to 30–50 cm tall and is harvested by hand. It is an herbaceous annual plant, with a slender branched stem 20–30 cm tall. The leaves are 5–10 cm long, pinnate or bipinnate, thread-like leaflets. The flowers are small, white or pink, and borne in umbels. The fruit is a lateral fusiform or ovoid achene 4–5 mm long, containing a single seed. Cumin seeds resemble caraway seeds, being oblong in shape, longitudinally ridged, and yellow-brown in color, like other members of the Umbelliferae family such as caraway, parsley and dill.

Medicinal uses

Cumin is useful as warming oil and helps relieve muscular pains and osteoarthritis. In the digestive system, it is a stimulant that helps with colic, dyspepsia, flatulence, bloating and indigestion. For the nervous system, it is a tonic and has a beneficial effect on headaches, migraine and nervous exhaustion. It is found to increase the bioavailability of the species with which it is given.

Materials and Methods

Plant material

The bark of *Terminalia arjuna* (Roxb.) Wight & Arn. was collected from Gubbalamangamma range, west Godavari district in December 2009. The *Piper nigrum* Linn. and the *Cuminum cyminum* Linn. seeds are collected from the fields near Godavari district. Drying of *Terminalia arjuna*, *Piper nigrum* and *Cuminum cyminum* was carried out at Department of Taxonomy, Laila Impex, Vijayawada. Plants were completely dried to eliminate moisture content. Dried plants are stored in shade with proper labels and used as and when required.

Extraction

The dried bark of *Terminalia arjuna* is pulverized and the material was taken in round bottomed flask and refluxed with methanol. The extract is collected four times at every 1 h interval with the addition of fresh solvent. The resulting extract is methalic extract of *Terminalia arjuna* (S₁). Similarly the three plants, *Terminalia arjuna*, *Piper nigrum*, *Cuminum cyminum* were extracted with methanol to give mixed methonalic extract (S₂). *Terminalia arjuna* bark and the

mixture of equivalent weight of black pepper seeds, cumin seeds and dried bark of *Terminalia arjuna* is refluxed with 60 % methanol and 40 % water (hydroalcohol) in the similar manner to obtain hydro alcoholic extract of *Terminalia arjuna* (S₃) and mixed hydro alcoholic extract (S₄).

Extraction of volatile oil from Piper nigrum Linn. and Cuminum cyminum Linn.

The pulverized material of black pepper and cumin were taken in a separate round bottomed flask which is attached to Clevenger's apparatus. Volatile oil present in the drug gets separated at 100°C and deposited as a layer on top of the water layer. During hydrodistillation, 5-10 % of glycerin is added to give better yield of volatile oil due to elevation in boiling point of water. Total volatile oil collected from pepper (P₁) and from cumin (C₁) is measured in the graduated receiver.

Extraction of piperine from Piper nigrum Linn.

Black pepper is ground to fine powder and extracted with ethanol in a soxhlet extractor for 2 h. The solution is filtered and concentrated. Alcoholic potassium hydroxide is added to filtrate and after a while decanted from insoluble residue. The alcoholic solution is left overnight where upon yellow needles of piperine (P₂) are deposited. The crystals formed are collected and dried. The melting point of the crystals was found to be 136-138°C.

Determination of total phenolic content

Total phenolic content of each extract obtained by the above methods was determined by the method of Singleton and Rossi (1965) and then expressed as microgram/gram gallic acid equivalents. In brief, a 100 µl-aliquot of the samples was added to 2 ml of 0.2 % (w/v) Na₂CO₃ solution. After 2 minutes of incubation, 100 µl of 500 ml/l Folin-Ciocalteu reagent was added and the mixture was then allowed to stand for 30 minutes at 25°C. The absorbance was measured at 750 nm using a spectrophotometer (UV1800, Shimadzu). The blank was also run to nullify the effects of reagents and solvents. The total phenolic content was determined using the standard gallic acid calibration curve.

In vitro studies: Antioxidant activity

DPPH radical scavenging activity

DPPH radical scavenging activities of all the fractions were determined by the method of Blois (1958). Initially, 0.2 ml of the fractions at a concentration of 25 µg/ml, 50 µg/ml, 75 µg/ml and 100 µg/ml was mixed with 1 ml of 0.2 mM DPPH which is dissolved in methanol. The reaction mixture was incubated for 20 minutes at 28 °C under dark condition. The control contained all reagents except the extract fraction while methanol was used as blank. The DPPH radical scavenging

activity was determined using vitamin C as standard. Values are determined by measuring the absorbance at 517 nm using a spectrophotometer (Table 1). IC₅₀ values were calculated using linear regression analysis. The DPPH radical scavenging activity (%) of the sample was calculated as:

$$\begin{aligned} & \% \text{DPPH scavenging activity} \\ & = 1 - \frac{\text{absorbance of sample}}{\text{absorbance of control}} \times 100 \end{aligned}$$

Superoxide anion radical scavenging activity

Superoxide radical scavenging activity of methanolic and hydroalcoholic extracts of *Terminalia arjuna* was determined by Nitro Blue Tetrazolium (NBT) riboflavin photo reduction method of McCord and Fridovich (1969). The assay mixture contained EDTA solution (6.6 mM) containing NaCN (3 µg), riboflavin (2 µM), NBT (50 µM), test substances and phosphate buffer (67 mM, pH 7.8) in a final volume of 3 ml. The absorbance at 560 nm were measured before and 15 min after illumination (Table 1). All tests were run in triplicate and mean values were used to calculate percentage scavenging ability and IC₅₀ values were calculated using linear regression analysis using gallic acid as the standard.

The superoxide anion radical scavenging activity (%) was calculated as:

$$\begin{aligned} & \% \text{superoxide scavenging activity} \\ & = 1 - \frac{\text{absorbance of sample}}{\text{absorbance of control}} \times 100 \end{aligned}$$

Antidiabetic activity

α-Glucosidase inhibitory activity

The enzyme α- glucosidase inhibitory activity was determined by incubating solution (0.1 ml) of an enzyme preparation with 0.2 M Tris buffer, pH 8.0 (1.0 ml) containing various concentrations of extract at 37 °C for 60 minutes by using glucose as working standard. The reaction mixture is heated for two minutes in boiling water bath to stop the reaction. The amount of liberated glucose is measured by glucose oxidation method (Prashanth *et al.*, 2001). (Assay condition 37 °C±0.1 °C, pH-8.0; O.D at 540 nm. Results are given in Table 1). IC₅₀ values were calculated using linear regression analysis and acarbose as the standard. The percentage inhibition is calculated as:

$$\begin{aligned} & \% \text{ inhibition} = \\ & \frac{\text{Enzyme activity of the control} - \text{Enzyme activity of the extract}}{\text{Enzyme activity of the control}} \times 100 \end{aligned}$$

α-amylase inhibitory activity

A starch solution (0.1 % w/v) was obtained by stirring 0.1 g of potato starch in 100 ml of 16 mM of sodium acetate buffer. The enzyme solution was prepared by mixing 27.5 mg of

α-amylase in 100 ml of distilled water (Anrew *et al.*, 1969). The colorimetric reagent is prepared by mixing sodium potassium tartarate solution and 3, 5- dinitrosalicylic acid solution 96 mM. Both standard (Acarbose) and plant extracts were added with starch solution and left to react with α-amylase solution under alkaline conditions at 25 °C. The reaction was measured over 3 minutes. The generation of maltose was quantified by the reduction of 3, 5-dinitro salicylic acid to 3-amino-5- nitro salicylic acid. This reaction is detectable at 540 nm (Table 1). IC₅₀ values were calculated using linear regression analysis. (Temperature 25 °C ± 0.1 °C, pH- 4.8; O.D at 540 nm).

% inhibition =

$$\frac{\text{Enzyme activity of the control} - \text{Enzyme activity of the extract}}{\text{Enzyme activity of the control}} \times 100$$

Anticancer activity

The samples for anticancer activity were prepared by individually extracting piperine from pepper (P₂) and volatile oil from black pepper (P₁) and volatile oil from cumin (C₁) and evaluating them in combination with methanolic extract of *Arjuna* (S₁) against A549 human lung carcinoma using MTT assay.

Anticancer assay

The cytotoxicity potential of the test compounds was evaluated on A549 human lung carcinoma cells using MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a yellow tetrazole) based cell proliferation assay (Mosmann, 1983). The carcinoma cell lines were obtained from National Centre for Cell Science (NCCS), Pune and cultivated in Dulbecco's modified Eagle's red medium (DMEM) (Sigma Life Science, USA) containing 10 % fetal bovine serum (FBS). Equal number of cells were seeded in each well of 96 well micro plates and incubated at 37 °C with 5 % CO₂. The cells are treated with different concentrations of test compounds (0-100 µg/ml) for 72 h. control wells were supplemented with 0.5 % DMSO.

After 72 h treatment, 5 µl of MTT reagent along with 45 µl of phenol red and FBS free DMEM was added to each well and plates are incubated at 37 °C with 5 % CO₂ for 4 h. Thereafter, 50 µl of solubilization buffer was added to each well to solubilize the colored formazan crystals produced by the reduction of MTT. After 24 h, the optical density was measured at 550 nm using micro plate reader. Cisplatin, the conventional drug is used as standard.

Results

The total phenolic content of methanolic extract of *Terminalia arjuna* is 0.74% as compared to gallic acid as a standard and is prominent compared to the other extracts.

DPPH antioxidant activity

A comparison of various antioxidant activities of extracts is shown in Table 1 and Figure 1. The DPPH radical scavenging activity of all the fractions increased with increase in fraction concentration. The IC₅₀ values of the extracts are in the order of S₁ > S₃ > S₂ > S₄. So methanolic extract of *Terminalia arjuna* S₁ is potent compared to other extracts.

Superoxide scavenging antioxidant activity

In this study, the superoxide anion scavenging effects of various fractions were analyzed and the results are given in Figure 1. Among the fractions tested, S₁ exhibited the highest superoxide anion scavenging activity with IC₅₀ values in increasing order S₁ > S₃ > S₄ > S₂.

α-glucosidase inhibitory antidiabetic activity

The *in vitro* α-glucosidase inhibitory studies demonstrated that S₁ had significant α-glucosidase inhibitory activity. The IC₅₀ value of methanolic extract of *Terminalia arjuna* was found to be 0.70 μg/ml which is compared with the standard acarbose having IC₅₀ value 0.1 μg/ml Figure 2. The IC₅₀ values of α -glucosidase inhibitory activity in increasing order was S₁ > S₃ > S₂ > S₄.

α-amylase inhibitory antidiabetic activity

The *in vitro* α-amylase inhibitory studies demonstrated that S₃ of *Terminalia arjuna* had potent α-amylase inhibitory activity. The IC₅₀ of S₃ was found to be 34.24 μg/ml which is compared with standard acarbose having IC₅₀ value 38 μg/ml Table 1 Figure 2. The IC₅₀ values of α - amylase inhibitory activity in increasing order was S₃ > S₄ > S₂ > S₁.

Cytotoxic activity

The cytotoxicity of *Terminalia arjuna*, *Piper nigrum* and *Cuminum cyminum* and cisplatin were investigated using an 3-(4, 5-dimethylthiazole 2yl)-2,5-diphenyl tetrazolium bromide assay on human cancer cell line, A-549 Figure 3. The anticancer activity of different extracts and cisplatin are presented in Table 2. Individually the methanolic extract of *Terminalia arjuna* and piperine isolated from black pepper are not active against A549 cell line. But the extracts when given in combination (S₁ + P₂) have showed potent antitumor property than the standard Cisplatin. Also the combination of all the four extracts (S₁ + C₁ + P₁ + P₂) showed good anticancer activity.

Discussion

The methanolic extract of *Terminalia arjuna* S₁ was found to possess both antioxidant and antidiabetic properties. The current hypothesis is that total phenolics and flavonoids reduce the DPPH radicals by their hydrogen donating ability. The superoxide anion scavenging activity might be due to the action of polyphenolic compounds. Among the evaluated six polyherbal extracts for anticancer activity, the combination of methanolic extract of *Terminalia arjuna* and piperine (S₁ + P₂) treatment have showed potent antitumor property than the standard Cisplatin. The increased anticancer activity of (S₁ + P₂) and (S₁ + C₁ + P₁ + P₂) may be due to the enhanced bioavailability of the species when given in combination. Further investigation is being carried out to identify the potency of these polyherbal extracts against various other human cancer cell lines.

Table 1. Dose, % inhibition and IC₅₀ values of methanolic and hydro alcoholic polyherbal extract for various *in vitro* antioxidant and antidiabetic activities

S. No.	Sample	Antioxidant activity IC ₅₀ (μg/ml)		Antidiabetic activity IC ₅₀ (μg/ml)	
		DPPH	Superoxide	α-glucosidase	α-amylase
1	S ₁	4.28	2.65	0.70	55.83
2	S ₂	5.94	6.79	1.63	54.67
3	S ₃	4.59	3.06	1.51	34.24
4	S ₄	6.94	5.88	1.57	41.7
5	Std*	3.53	0.63	0.009	37.99

*Standard: For DPPH, Vitamin C is the standard. For Superoxide scavenging activity, Gallic acid is used as standard. For α-glucosidase and α-amylase, acarbose is used as standard.

Table 2. IC₅₀ values of different combinations of substances against A549 human lung carcinoma

S. No.	Test substance*	IC50 values
1	S ₁	NA
2	P ₂	NA
3	S ₁ + C ₁	68.6
4	S ₁ + P ₁	92.4
5	S ₁ + P ₂	39
6	S ₁ + C ₁ + P ₁ + P ₂	56.3
7	Cisplatin (Standard)	49.5

* S₁ (methanolic extract of *Terminalia arjuna*), P₁ (volatile oil from pepper), P₂ (piperine from pepper), C₁ (volatile oil from cumin) NA= not active.

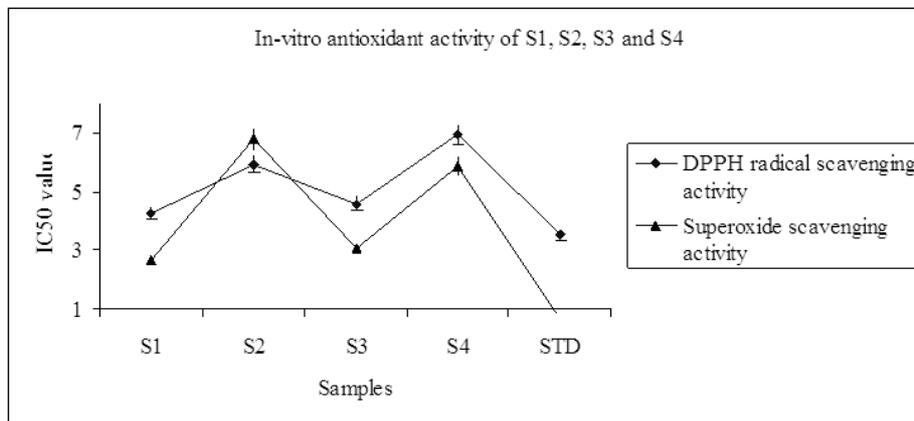


Figure 1. DPPH radical scavenging and superoxide radical scavenging activity of S₁ (methanolic extract of *Terminalia arjuna*), S₂ (methanolic extract of *Terminalia arjuna*, *Piper nigrum*, *Cuminum cyminum*), S₃ (hydroalcoholic extract of *Terminalia arjuna*) and S₄ (hydroalcoholic extract of *Terminalia arjuna*, *Piper nigrum*, *Cuminum cyminum*).

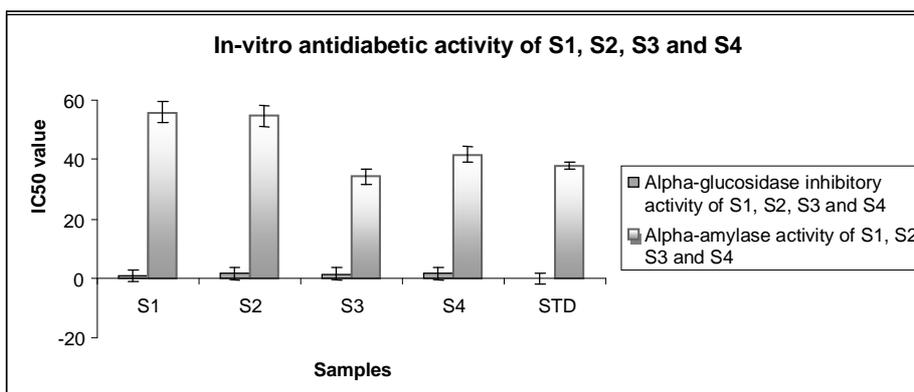


Figure 2. α -Glucosidase enzyme inhibitory activity and α -Amylase enzyme inhibitory activity of S₁ (methanolic extract of *Terminalia arjuna*), S₂ (methanolic extract of *Terminalia arjuna*, *Piper nigrum*, *Cuminum cyminum*), S₃ (hydroalcoholic extract of *Terminalia arjuna*) and S₄ (hydroalcoholic extract of *Terminalia arjuna*, *Piper nigrum*, *Cuminum cyminum*).

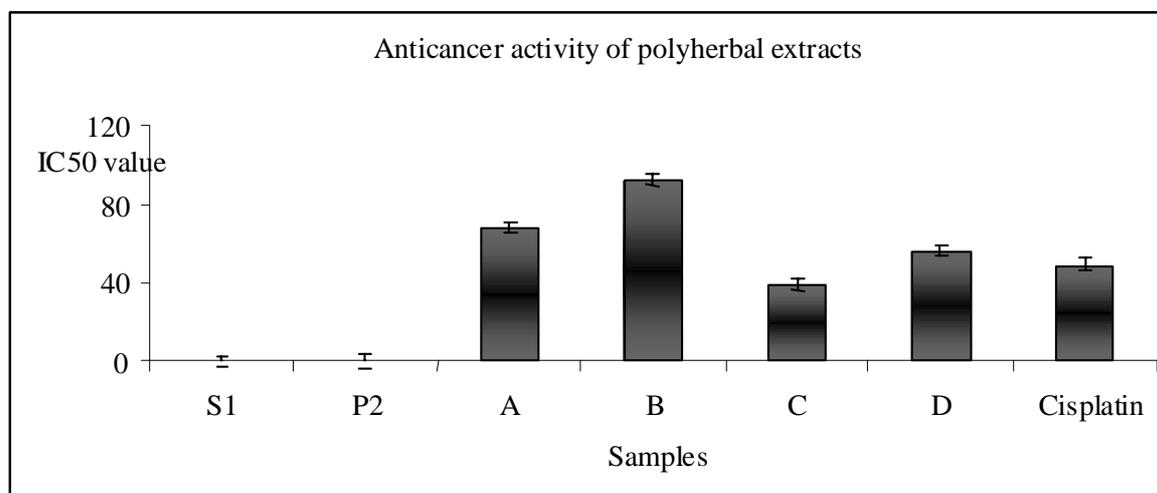


Figure 3. IC₅₀ values of various combinations of the polyherbal extract for their cytotoxic activity against A549 human lung carcinoma; A= S₁+ C₁, B= S₁+P₁, C= S₁+ P₂, D= S₁+ C₁+ P₁+ P₂.

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