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Physicochemical, qualitative and quantitative determination of secondary metabolites and antioxidant potential of *Alocasia macrorrhizos* leaf extracts

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Abstract
Plants have served human beings as a natural source for treatments and therapies from ancient times, amongst them medicinal herbs have gain attention because of its wide use and less side effects. In the recent years plant research has increased throughout the world and a huge amount of evidences have been collected to show immense potential of medicinal plants used in various traditional systems. The aim of the present study was to evaluate physicochemical, qualitative and quantitative phytochemical analysis and *in vitro* antioxidant activities of leaf of *Alocasia macrorrhizos* collected from Bhopal region of Madhya Pradesh. The physicochemical evaluations carried out in terms of loss on drying, ash value, extractive values and acid insoluble ash value etc. Qualitative analysis of various phytochemical constituents and quantitative analysis of total phenolics and flavonoids were determined by the well-known test protocol available in the literature. Quantitative analysis of phenolic and flavonoids was carried out by Folins Ciocalteau reagent method and aluminum chloride method respectively. The *In vitro* antioxidant activity of ethanolic extract of the leaf was assessed against DPPH assay method using standard protocols. Phytochemical analysis revealed the presence of phenols, flavonoids, tannins, saponins, alkaloids. The total phenolics content of leaves ethanolic extract was (9.18mg/100mg), followed by flavonoids (2.30mg/100mg). The activities of ethanolic leaves extract against DPPH assay method were concentration dependent with IC 50 values of ascorbic acid and extracts 24.44 and 48.58 µg/ml respectively. These studies provided information for standardization and correct identification of this plant material. The diverse array of phytochemicals present in the plant thus suggests its therapeutic potentials which may be explored in drug manufacturing industry as well as in traditional medicine.

Keywords: *Alocasia macrorrhizos,* physicochemical, qualitative, quantitative phytochemical, antioxidant

Introduction
About 75 to 80% of the world population was comprised of medicinal plants and especially in developing countries, the herbal drugs play a central role in many health care programs. The broad definition of medicinal plants has been incorporated in an ancient Indian literature which portrays that all plant parts to be potential sources of medicinal substances [1]. The lack of citations and inflexible quality control has hindered the acceptance of the alternative medicines in the developed countries by serving a lead obstacle. Hence, documentation is very essential part of research work to be carried out on traditional medicines [2]. It becomes extremely important to make an attempt towards consistency of the plant material to be used as medicine in this scenario. WHO has also recommended the evaluation of physicochemical and phytochemical parameters of medicinal plants for its efficacy, due to lack of confined synthetic drugs [3]. These evaluation parameters help in identification and authentication of the plant material. The safety and efficacy of herbal medicine depends mainly upon the exact identification and quality assurance of the starting materials. Molecular oxygen is required to maintain life, but it can be toxic through the formation of reactive oxygen species (ROS). ROS includes superoxide radical, hydroxyl radical, singlet oxygen and H_2O_2 which have been found to play an important role in the initiation and progression of various diseases such as atherosclerosis, inflammatory injury, cancer and cardiovascular disease [4]. Oxidative stress, initiated by these free radicals, seek stability through electron pairing with biological macromolecules such as proteins, lipids and DNA in healthy human cells and cause protein and DNA damage along with lipid peroxidation. But organisms have multiple mechanisms to protect cellular molecules (DNA, RNA and proteins) against ROS induced damage. These include repair enzymes (DNA glycosylases, AP endonucleases etc.), antioxidant enzymes (SOD, catalase, and glutathione peroxidase) and intra as well as extracellular antioxidants

(glutathione, uric acid, ergothioneine, vitamin E, vitamin C and phenolic compounds [5]. However, this natural antioxidant mechanism can be inefficient for severe and/or continued oxidative stress. Based on this idea, there has been a strong demand of therapeutic and chemo preventive antioxidant agents with limited cytotoxicity to enhance the antioxidant capacity of the body and help attenuate the damage induced by ROS. Antioxidants are a loosely defined group of compounds characterized by their ability to be oxidised in place of other compounds present [6]. *Alocasia macrorrhizos* commonly known as elephant ear taro or giant taro is an indigenous herb belonging to the family Araceae. They are naturally grown on marshy land of tropical area in India. Bangladesh, China. It plant were used traditionally in inflammation [7]. The juice of leaves of the plant is used as a digestive, laxative, diuretic, astringent, antifungal agent and for the treatment of rheumatoid arthritis. The leaves of this plant are used to prevent iron deficiency, to enhance eye sight and as a good source of protein. Also the whole plant is used for jaundice and constipation [8]. Leaf extract of Alocasia macrorrhizos is proven to have anti oxidant [9], antinociceptive, anti-inflammatory [10], laxative, diuretic [11] andhepato protective [12] properties. Rhizome extract has shown antihyperglycemic, antioxidant and cytotoxic activity in various studies [13].

The aim of this work was to determine the quality (types), quantity (amount) of bioactive compounds and *in vitro* antioxidant activity of leaf of *Alocasia macrorrhizos*.

Materials and Methods

Plant Material

The leaves of plant *Alocasia macrorrhizos* was collected from rural area of Bhopal (M.P), India. Chemical reagents

All the chemicals used in this study were obtained from HiMedia Laboratories Pvt. Ltd. (Mumbai, India), Sigma Aldrich Chemical Co. (Milwaukee, WI, USA), SD Fine-Chem Chem. Ltd. (Mumbai, India) and SRL Pvt. Ltd. (Mumbai, India).All the chemicals used in this study were of analytical grade.

Organoleptic Characters

Plant material (leaves) selected for the study were washed thoroughly under running tap water and then were rinsed in distilled water; they were allowed to dry for some time at room temperature. Then the plant material was shade dried without any contamination for about 3 to 4 weeks. Dried plant material was grinded using electronic grinder to obtain a powdered form and then subsequently used for organoleptic characterization. A small amount of powdered plant part was spread on a white tile and physically examined for general appearance i.e. color, taste, texture etc. Dried plant material was packed in air tight container and stored for Phytochemical and biological studies [14].

Physicochemical study [15-17].

Determination of loss on drying

Two grams of crude powder was taken in an evaporating dish and then dried in an oven at 105°C till constant weight was obtained. The weight after drying was noted and loss on drying was calculated. The percentage was calculated on the basis of sample taken initially.

Total ash

3 g of powdered drug was accurately weighed and taken in a tarred silica crucible which was previously ignited and weighed. The powdered drug was spread as a fine even layer on the bottom of the crucible. The crucible was incinerated gradually by increasing the temperature to make it dull red hot until free from carbon. The crucible was cooled and weighed. The ash was weighed and the total ash content was calculated with reference to the air dried drug.

Acid insoluble ash

The ash obtained as described in total ash was boiled with 25ml of dilute hydrochloric acid for 5 minutes. The insoluble ash was collected on an ashless filter paper and washed with hot water. This insoluble ash was transferred into a silica crucible and it was ignited, cooled and weighed. The process was repeated to get constant weight. The percentage of acid insoluble ash was calculated with reference to the quantity of air dried crude drug.

Water soluble ash

Total ash obtained was boiled for 5 minutes with 25ml of water. The insoluble matter was collected in ashless filter paper and washed with hot water. The insoluble ash was transferred into silica crucible and was ignited, cooled and weighed. The process was repeated to get constant weight. The weight of insoluble matter was subtracted from the weight of the total ash. The difference of the weight was considered as the water soluble ash. The percentage of water soluble ash was calculated with reference to the air dried drug.

Solvent ether soluble extractive values

Accurately weighed 5gm of powdered air dried leaves was taken with 100 ml of solvent ether in a stopper flask and kept for 24 hours. The flask was shaken frequently (Maceration) then the solvent ether extract was filtered rapidly through filter paper to prevent excessive loss of solvent ether. 25 ml of solvent ether extract was evaporated to dryness on a water bath and complete the drying in an oven at 100^0C.Then the residue was cooled weighed and kept in desiccators. Then the percentage w/w of solvent ether soluble extractive with reference to the air-dried drug was calculated.

Alcohol soluble extractive values

Accurately weighed 5 gm of powdered air dried leaves was taken with 100ml of alcohol (90 % v/v) in a stopper flask and kept for 24 hours. The flask was shaken frequently (Maceration).Then the alcohol extract was filtered rapidly through filter paper to prevent excessive loss of alcohol. 25ml of alcoholic extract was evaporated to dryness on a water bath and complete the drying in an oven 100^0C. Then the residue was cooled, weighed and kept in desiccators. Then the percentage w/w of alcohol soluble extractive with reference to the air-dried drug was calculated.

Water soluble extractive values

Accurately weighed 5gm of powdered air-dried leaves was taken with 100 ml of water in a stopper flask and kept for 24 hours. The flask was shaken frequently (Maceration).Then the aqueous extract was filtered rapidly through filter paper. 25 ml of aqueous extract was evaporated to dryness on a water bath and complete the drying in an oven at 100^oC. Then the residue was cooled, weighed and kept in desiccators. Then percentage w/w of soluble extractive with reference to the air dried drug was calculated.

Extraction procedure

Defatting of plant material

Powdered plant material (leaves) of *Alocasia macrorrhizos* was shade dried at room temperature. The shade dried flower was coarsely powdered and subjected to extraction with petroleum ether (60-80°C) in a soxhlet apparatus. The extraction was continued till the defatting of the material had taken place.

Extraction

50 g. of *Alocasia macrorrhizos* dried leaves were successive extracted with various solvent (chloroform, ethyl acetate, ethanol and aqueous) and using different drug: solvent ratios using hot continuous percolation for different time (soxhlet apparatus). The extracts were evaporated above their boiling points and stored in an air tight container free from any contamination until it was used. Finally the percentage yields were calculated of the dried extracts [18].

Qualitative phytochemical analysis of plant extract

The *Alocasia macrorrhizos* leaves extract obtained was subjected to the preliminary phytochemical analysis following standard methods by Khandelwal and Kokate [19, 20]. The extract was screened to identify the presence or absence of various active principles like phenolic compounds, carbohydrates, flavonoids, glycosides, saponins, alkaloids, fats or fixed oils, protein and amino acid and tannins.

Quantification of secondary metabolites

Quantitative analysis is an important tool for the determination of quantity of phytoconstituents present in plant extracts. For this TPC and TFC are determined. Extracts obtained from leaves of *Alocasia macrorrhizos* plant material of subjected to estimate the presence of TPC and TFC by standard procedure.

Total phenol determination

The total phenolic content was determined using the method of Olufunmiso *et al.* [21]. A volume of 2 ml of *Alocasia macrorrhizos* leaves extracts or standard was mixed with 5 ml of Folin Ciocalteau reagent (previously diluted with distilled water 1:10 v/v) and 4 ml (75g/l) of sodium carbonate. The mixture was allowed to stand for 15 min under room temperature. The blue colour developed was read at 765 nm using UV/visible spectrophotometer. The total phenolic content was calculated from the standard graph of gallic acid and the results were expressed as gallic acid equivalent (mg/g).

Total flavonoids determination

The total flavonoid content was determined using the method of Olufunmiso *et al.* [21]. 1 ml of 2% AlCl3 methanolic solution was added to 1 ml of extract or standard and allowed to stand for 60 min at room temperature; the absorbance of the reaction mixture was measured at 420 nm using UV/visible spectrophotometer The content of flavonoids was calculated using standard graph of quercetin and the results were expressed as quercetin equivalent (mg/g).

DPPH free radical scavenging assay

DPPH scavenging activity was measured by modified method [21]. DPPH scavenging activity was measured by the spectrophotometer. Stock solution (6 mg in 100ml methanol) was prepared such that 1.5 ml of it in 1.5 ml of methanol gave an initial absorbance. Decrease in the absorbance in presence of sample extract at different concentration (10-100 µg/ml) was noted after 15 minutes. 1.5 ml of DPPH solution was taken and volume made till 3 ml with methanol, absorbance was taken immediately at 517 nm for control reading. 1.5 ml of DPPH and 1.5 ml of the test sample of different concentration were put in a series of volumetric flasks and final volume was adjusted to 3 ml with methanol. Three test samples were taken and each processed similarly. Finally the mean was taken. Absorbance at zero time was taken for each concentration. Final decrease in absorbance was noted of DPPH with the sample at different concentration after 15 minutes at 517 nm. The percentage inhibition of free radical DPPH was calculated from the following equation: % inhibition = [(absorbance of control - absorbance of sample)/absorbance of control] × 100%. Though the activity is expressed as 50% inhibitory concentration (IC50), IC50 was calculated based on the percentage of DPPH radicals scavenged. The lower the IC50 value, the higher is the antioxidant activity.

Results and Discussions

Organoleptic evaluation represents those properties of materials that can be done by using the sense organs. It thereby defines some specific characteristics of the material which can be considered as a first step towards establishing the identity and degree of purity of the material**.** The organoleptic parameters were evaluated represent in Table 1.The crude extracts so obtained after each of the successive soxhlet extraction process were concentrated on water bath by evaporation the solvents completely to obtain the actual yield of extraction. The percentage yield of extraction is very important in phytochemical extraction in order to evaluate the standard extraction efficiency for a particular plant, different parts of same plant or different solvents used. The yield of extracts obtained from the leaves of the plants using petroleum ether, chloroform, ethyl acetate, ethanol and water as solvents are depicted in the Table 2.The physical constituent's estimation of the drugs is an essential parameter to determine adulteration or inappropriate handling of drugs. The physicochemical characters of powder drug of leaves of *Alocasia macrorrhizos* such as total alcohol soluble extractive, water soluble extractive, ash value, acid insoluble ash, and water soluble ash, loss after drying and foreign substances are given in Table3. The leaves showed less moisture content; it was 6.39%%. Moisture content of drugs could be at minimal level to discourage the growth of bacteria, yeast or fungi during storage. These can serve as a valuable basis of information and provide suitable standards to establish the quality of this plant material as future prospects. An ash values are used to decide quality and purity of crude drug; it indicates presence of various impurities like, silicate, oxalate and carbonate. The water soluble ash is used to determine the quantity of inorganic compounds present in drugs. The acid insoluble ash helps to estimate the amount of silica present in the material. The total water soluble portion of the ash is considered as water soluble ash. Less amount of these three parameters indicate that the inorganic matter and silica were less in *Alocasia macrorrhizos* leaf. The results of qualitative phytochemical analysis of the crude powder of leaves of *Alocasia macrorrhizos* are shown in Table 4. Ethanolic and aqueous extracts of leaves sample of *Alocasia macrorrhizos* showed the presence of flavonoids, phenols, tannins, carbohydrate, glycosides and proteins but in chloroform and ethyl acetate extracts all phytoconstituents was absents.

Table 1: Organoleptic character of *Alocasia macrorrhizos*

Plant parts	Color	Odour	Taste	Texture
Leaves	Green colour	Characteristic	NA	Broad, Peltate and petiolated

Table 2: Results of percentage yield of leaf extracts

Plant Name	Percentage yield (%)				
	Pet. Ether	Chloroform	Ethyl acetate	Ethanol	Water
Alocasia macrorrhizos	3.2	4.7	5.9	6.6	7.1

Table 3: Results of physicochemical parameters of leaf

S. No	Parameters (% w/w)	*Alocasia macrorrhizos*
1.	Total ash	2.6%
2.	Acid Insoluble Ash	0.8%
3.	Water soluble Ash	1.8%
4.	Alcohol soluble extractive value	16.1%
5.	Water soluble extractive value	31.6%
6.	Ether soluble extractive value	3.9%
7.	Moisture content	6.39%

Table 4: Phytochemical evaluation of *Alocasia macrorrhizos* leaves

Chemical Tests	Pet. ether	Chloroform	Ethyl acetate	Ethanol	Aqueous
Alkaloids					
Mayer's reagent	-	-	-	-	-
Hager's reagent	-	-	-	+	-
Wagner's reagent	-	-	-	-	-
Dragendorff's reagent	-	-	-	-	+
Glycosides (+Ve)					
Baljet test	-	-	-	-	-
Legal's test	-	-	-	-	-
Keller-Kiliani	-	-	-	-	-
Phenols/Tannins					
Ferric chloride	-	-	+	+	+
Gelatin Solution	-	-	+	+	+
Lead acetate test	-	-	+	+	+
Flavonoids					
FeCl3 test	-	-	+	+	+
Alkaline reagent test	-	+	+	+	+
Shinoda test	-	-	+	+	-
Saponins					
Foam test	-	+	-	-	+
Hemolytic test	-	+	-	-	+
Lead acetate	-	+	-	-	+
Fixed oil/Fats					
Spot	+	-	-	-	-
Saponification	+	-	-	-	-
Gums & Mucilage					
Water	-	-	-	-	+
Carbohydrates					
Molish test	+	-	-	-	-
Fehling's solution	-	-	-	-	-
Benedict's test	-	-	-	-	+
Amino acids					
Ninhydrin Test	-	-	-	-	+
Millons Test	-	-	-	-	+
Xantoprotein Test	-	-	-	-	+
Terpenoids					
L B Test	-	-	-	-	-
Salkowski test	-	-	-	-	-
Steroids					
Lieberman Test	-	-	-	-	-
Protein					
Biuret test	-	-	-	+	-

(+) Indicates 'Presence'; (-) Indicates 'Absence'

The determination of the total phenolic content, expressed as mg gallic acid equivalents and per 100 mg dry weight of sample. TPC of ethanolic, aqueous and ethyl acetate extract of *Alocasia macrorrhizos* leaves showed the content values of

9.1818, 7.3636and 3.7454respectively. But chloroform extracts of *Alocasia macrorrhizos* leaves have no phenolic content. The total flavonoids content of the extracts was expressed as percentage of quercetin equivalent per 100 mg dry weight of sample. The total flavonoids estimation of ethanolic, aqueous and ethyl acetate extracts of leaves of *Alocasia macrorrhizos* showed the content values of 2.300, 1.090 and 0.8375respectively. The above results showed that aqueous extract contain less phenolic and flavonoids content than the alcoholic extract. It may due to the solubility of principle contents presence be higher in case of alcoholic solvent, thus it has been accepted that it is a universal solvent for the extraction of plant constituents. Results are provided in (Table 5 and Fig. 1, 2).

Table 5: Estimation of total phenolics and total flavonoids content in *Alocasia macrorrhizos*

S. No	*Alocasia macrorrhizos* Extracts	Total phenolic content (mg/100mg of dried extract)	Total flavonoids content (mg/ 100 mg of dried extract)
1.	Chloroform	-	0.0222
2.	Ethyl acetate	3.7454	0.8375
3.	Ethanol	9.1818	2.3000
4.	Aqueous	7.3636	1.0900

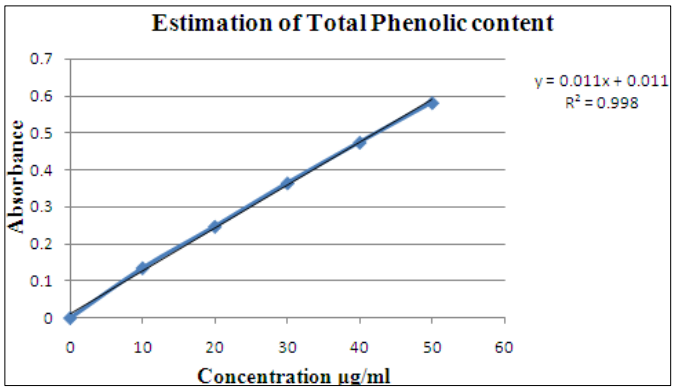

Fig 1: Graph of estimation of total phenolic content

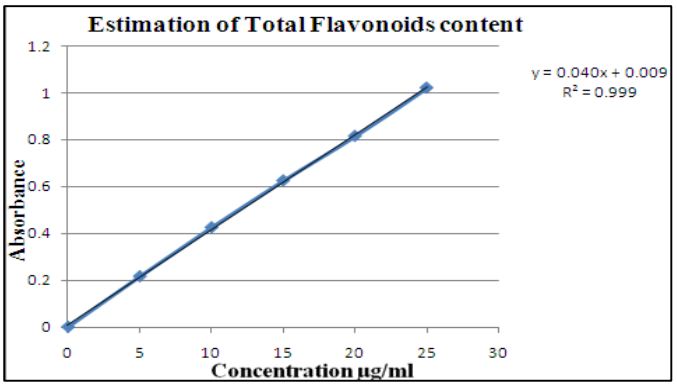

Fig 2: Graph of estimation of total flavonoids content

Antioxidant activity of the samples was calculated through DPPH assay. % inhibition was calculated as an indicative of antioxidant potency. The higher the % inhibition the better the activity. Ascorbic acid was taken as standard and the values were comparable with concentration ranging from 20 µg/ml to 100µg/ml. A dose dependent activity with respect to concentration was observed Table 6.

Table 6: DPPH assay of ascorbic acid and ethanolic extract

S. No.	Conc. (µg/ml)	% Inhibition	
		Ascorbic acid	Ethanolic extract
1	20	47.66	31.56
2	40	58.93	48.30
3	60	68.11	58.77
4	80	81.48	80.03
5	100	90.98	84.21
IC 50		24.44	48.58

Conclusion

It can be concluded that from present investigation the physicochemical and preliminary phytochemical investigation study of *Alocasia macrorrhizos* leaves yielded a set of standards that can serve as an essential basis of evidence to determine the identity and to determine the quality and purity of the plant material as per its future perspectives. The phytochemical investigation gave valuable information about the different phytoconstituents present in the plant, which helps the future investigators concerning the selection of the particular extract for further investigation of isolating the active principle and also gave idea about different phytochemical have been found to possess a wide range of activities. the total phenolic and flavonoid content in ethanolic leaves extract was found to be higher than all the extracts which is further proved by *in vitro* antioxidant studies. Potential antioxidant activity has good correlations with the therapeutic use in the treatment of cardiovascular disorders. Further research to isolate individual compounds, there *in vivo* antioxidant activities with different mechanism is needed.

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