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RESEARCH ARTICLE

EVALUATION OF SECONDARY METABOLITES, ANTI OXIDANT AND ANTIMICROBIAL ACTIVITIES OF *Cucurma caesia* and *Kaemferia galanga,* TWO ENDANGERED MEDICINAL PLANTS OF MANIPUR (INDIA)

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ABSTRACT

The comparative studies of secondary metabolites investigation used in pharmacuetical drug research using qualitative test is being performed on *Cucurma caesia* and *Kaemferia galanga Zingerbaracae* family. These medicinal plants are of high economic value due the presence of antioxidant and antimicrobial activity. The samples were extracted using various solvents like methanol, ethanol, ethyl acetate and water (aqueous). Among the various solvent extract methanol gives the highest positive result followed by ethanol, water (aqueous) and ethyl acetate respectively. The phytochemical screening revealed the presence of various compounds like tannins, terpenoids, alkaloids, saponins, cardiac glycosides, amino acids and carbohydrates. The percentage of scavenging of the methanolic extract were almost same at different concentrations, however the total phenolic content of *Cucurma caesia* and *Kaemferia galanga* were 121.13 and 144.8 mgGAE/g . For the antimicrobial activities, the test was screened against gram +ve and gram -ve bacteria and fungi by paper disc method. The studies showed the medicinal plants could play a vital role in health and diseases as they contained pharmacologically useful active principle elements.

INTRODUCTION

The use of various plant parts to cure specific ailments by different ethnic groups around the world is in vogue since time immemorial. The world is endowed with rich wealth of medicinal plants. Of the total 297,000-510,000 plant species in the world, 70,000(10-18 per cent) are estimated to be employed in health care. In India, of the total 17,500 native plant species, 6,000 (34.3 per cent) are well known to have medicinal importance. The Himalayas including North East India harbor about 8,000 plant species of which 2,500 (21.3 per cent) have been reported to have medicinal properties (Trivedi 2002). Medicinal plants play a pivotal role in the health care of ancient and modern cultures. Ayurveda, the Indian system of medicine mainly uses plant based drugs or formulations to treat various human ailments because they contain the components of therapeutic value (WHO, 1993). Medicinal values of medicinal plants lies in some chemical substances that produce definite physiological actions on the human body and these chemical substances are called phytochemicals. These are non nutritive chemicals that have disease preventive property. The most important of these phytochemicals are alkaloids, flavanoids, tannins and phenolic compounds (Hill, 1952). Many of these indigenous plants are used as spices and current research has shown that polyphenols contribute to the prevention of cardiovascular diseases, cancers, and osteoporosis and antioxidant activity with potential health benefits. In addition, plant based drugs remain an important source of therapeutic agents because of the availability, relatively cheaper cost and non-toxic nature when compared to modern medicine (Agbor and Ngogang 2005). Many of these indigenous plants are used as spices and current research has shown that polyphenols contribute to the prevention of cardiovascular diseases, cancers, and osteoporosis and antioxidant activity with potential health benefits. They are known to have beneficial effects on cardio vascular system and have a role in the prevention of neurodegenerative diseases and diabetes mellitus. For long time, plants have been an important source of natural products for human health. The antimicrobial properties of plants have been investigated by a number of studies worldwide and many of them have been used as therapeutic alternatives because of their antimicrobial properties. These properties are due to compounds synthesized as secondary metabolites of the plants, such as phenolic compounds, which are part of the essential oils, as well as tannins (Adams and Addy, 1994). The development of science of phyto pharmaceuticals and the hopes of the remedies in diseases generated enthusiasm in the researchers to develop herbal medicines (Kokate *et al.,* 1999). Plants have a limitless ability to synthesize viz aromatic substances, most of the phenols or their oxygen-substituted derivatives. They are widely used as ingredients in dietary supplements used for health purposes such as attempting to prevent cancer and heart diseases.

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The term antioxidant (also anti-oxygen) originally referred specifically to a chemical that pretended the consumption of molecular oxygen (Mattill, 1947). Antioxidants from plant materials terminate the action of free radicals thereby protecting the body from various diseases (Lai and Chou 2001). There is a growing interest all over the world for discovering the untapped reservoir of medicinal plants. The possible mechanisms of action of antioxidants were first explored thoroughly by Moreau and Dufrasse1926, who recognized that a substance with anti oxidative activity is likely to be one that is itself a target for oxidation (Wolf, 2005). The normal antioxidant defense system in biological system consists of both enzymatic and non enzymatic system. Many herbs contain antioxidant compounds which protects the cells against the damaging effects of reactive oxygen species. Reactive Oxygen Species (ROS) such as superoxide anion, hydroxyl radical and hydrogen peroxide play a crucial role in the development of various ailments such as arthritis, asthma, dementia, mongolism, carcinoma and parkinson's disease. The free radicals in the human body are generated through aerobic respiration or from exogenous sources (Halliwelland Gutteridge 1999). Some of the *in vivo* free radicals play a positive role in phagocytosis, energy production and regulation of cell growth etc. However, free radicals may also be damaging. Free radicals produced in the body react with various biological molecules namely lipids, proteins and deoxyribonucleic acids resulting in the imbalance between oxidants and antioxidants. Even though our body is safeguarded by natural antioxidant defense, there is always a demand for antioxidants from natural sources (Rimbach *et al*., 2005). Phenolic compounds from medicinal plants possess strong antioxidant activity and may help to protect the cells against the oxidative damage caused by free-radicals (Kahkonen *et al*., 1999). They are well known as radical scavengers, metal chelators, reducing agents, hydrogen donors, and singlet oxygen quenchers (Proestos *et al*., 2006). Antioxidants are the substances that are capable of counteracting the damaging but normal, effects of the physiological process of oxidation in animal tissue. Antioxidants are nutrients (vitamins and minerals) as well as enzymes (proteins in our body). They are believed to play a role in preventing the development of such chronic diseases as cancer, heart disease, stroke, Alzheimer's disease, Rheumatoid arthritis and cataracts. Antioxidants block the process of oxidation by neutralizing free radicals. In doing so, the antioxidants themselves become oxidized. That is why there is a constant need to replenish our antioxidant resources. How antioxidants work can be classified in two ways:

(a) Chain breaking: When a free radical releases or steals an electron, a second radical is formed. This molecule, continuing to generate more unstable products. The process continues until termination occurs – either the radical is stabilized by a chain-breaking antioxidant such as beta-carotene and vitamin C and E or it simply decays into a harmless product.

(b) Preventive: Antioxidant enzymes like superoxide dismutase, catalase and glutathione peroxidise prevent oxidation by reducing the rate of chain initiation. That is, by scavenging initiating radicals such antioxidants can thwart an oxidation chain from ever setting in motion. They can also prevent oxidation by stabilizing transition metal radicals such as copper and iron. There are two basic categories of antioxidant namely synthetic and natural-synthetic antioxidant. Natural-synthetic antioxidant are compound with phenolic structures of various degree alkyl substitution whereas natural antioxidant can be phenolic compounds (tocopherols, flavonoids and phenolic acids) nitrogen compounds (alkaloids, chlorophyll derivatives, amino acids and amines) or carotenoid as well as ascorbic acids. Most of these antioxidant compounds in a typical diet are derived from plant sources and belong to various classes of compounds with a wide variety of physical and chemical properties.

About the plant

Zingiberaceae is among the plant families that are widely distributed throughout the tropics, particularly in South East Asia. It is an important natural resource that provides man with many useful products for food, spices, medicines, dyes, perfume and aesthetics (Burkill, 1996).

Figure 1. *Kaemferia galanga* plant and sliced rhizome

Kaempferia galanga (Zingiberaceae), is an acaulescent parenial growing in Southern China, Indochina, Malasia, India and Thailand (Wibool *et al*., 2008).This species is annual and 2-4 plants can be obtained in a year from one rhizome (Chirangini *et al*., 2005). It is found throughout the plains of India and is cultivated for its aromatic rhizomes (Geetha *et al*., 1997). Since the rhizomes of this plant contain volatile oil and other important compounds of enormous medicinal values, they are very demanding to the traditional health care practitioner (Rahman *et al*., 2005) .The rhizome of this plant has been used traditionally for the treatment of many ailment and few biological activities have proven its importance. The rhizome is rich in essential oils and is being used for the treatment of indigestion, cold, pectorial and abdominal pains,

headache, expectorant, diuretic, carminative (Jagadish *et al*., 2010), stomachic, coughs, pectoral affections, stoppage of nasal blocs, asthma and hypertension (Rahman *et al*., 2004). It has reported to have expectorant, carminative, diurectic, coughs, pectorial infectious and inflammatory tumors (Fatima *et al*., 2000), antioxidants (Sataporn *et al*., 2008) and antimicrobial (Parvez*et al*., 2005). The methanolic extract of the rhizome contains ethyl p-methoxy trans-cinnamate which is highly cytotoxic to HELA cells (Vincet *et al*., 1992). The rhizome extract has been potentially active against bacterial infections (Supinya *et al*., 2005).In Thailand, the rhizome has been used as cardiotonic and CNS stimulant (Mokkhasmit *et al*., 1971). The rhizome of *K.galanga* has been used for treatment of fungal derived skin diseases as well as eczema (Tungstronjit, 1978). Which contains essential oils has been used in Chinese medicine as a decoction or powder for treating indigestion, cold, pectoral and abdominal pains, headache and toothache. Due to its increasing demand and overexploitation without ensuring its regeneration, the plant has recently been categorized as an endangered species, the plant is also having some amount of antifungal protein against drug-resistant *Candida albicans* (Mannangatti and Narayanasamy, 2008).

Figure 2. *Curcuma caesia* plant with their fluorescenct and sliced rhizome

Curcuma caesia Roxb.is a member of the family Zingiberaceae and popularly known as Kali haldi. In India it is found in West Bengal, Madhya Pradesh, Orissa, Chhattisgarh, and Uttar Pradesh states. It flourishes well in moist deciduous forest areas (Nadkarni, 1976). Rhizomes of the plant are used for sprains and bruises and also employed in the preparation of cosmetics. The genus *Cucurma* under the family Zingiberaceae comprises of over 80 species of rhizomatous herbs. The effective use of *Curcuma longa* L. is well known since a long time; it is laxative, anthelmintic, and vulnerary, besides this it is used in blood disorders. The genus *Curcuma*, a member of the Zingiberaceae family, comprises of 80 species, some of which have been used in traditional systems of medicine (Ayurveda, Siddha, Unani) for a long time. *Curcuma* plants (rhizomes and leaves) have a camphoraceous aroma and contain many functional compounds such as phenolics, flavonoids and different antioxidant enzymes. Rhizomes of the plant are used for sprains and bruises and also employed in the preparation of cosmetics (Anonymous 1999). Since free radicals are the cause for several major disorders, evaluation of antioxidant compounds activity in plants could result in the discovery of natural antioxidants with pharmacological and food value. The importance of phenolic compounds in plants as natural antioxidants and their use as substitutes to synthetic antioxidants in food additives is well known.

MATERIALS AND METHODS

Plant Collection and Identification

Kaemferia galanga and *Curcuma caesia* were collected from the natural habitat. The plant material was identified and authenticated by botanist from Department of Life Science, Manipur University. From the plants collected, desired parts were separated and cleaned for shade dried in a clean environment to avoid contamination for 10 days and oven dried at 60^0C to remove the moisture content. The oven dried rhizome were ground into fine powder and stored at room temperature for further analysis.

Extract Preparation

The powdered sample was mixed with different solvents, ethyl acetate, methanol, ethanol and water extraction was carried out in a soxhlet extractor. The extract was then concentrated to dryness under reduced pressure and it was preserved in a refrigerator. The soxhelation process was carried out until the solvent was found to be colorless. Then the solvent was filtered and distilled off. Final traces of methanol were removed under pressure by using rotary vacuum flask evaporator. Each extract was re suspended to make 2mg/ml stock solution.

Phytochemical Screening

Phytochemical test were carried out on four different solvent extracts (ethyl acetate, methanol, ethanol and water) on the powdered specimens using standard procedures to identify the constituents as described by Sofowara 1993, Trease and Evans, 989 and Harborne, 1973.

Test for tannins

About 0.5g of the dried powdered sample was boiled in 20 ml of water in a test tube and filtered. A few drops of 0.1% ferric chloride was added and observed for brownish green or a blue black precipitation.

Test for saponin

About 2g of the powdered sample was boiled in 20 ml of distilled water in a water bath and filtered. 10ml of the filtrate

was mixed with ml of distilled water and shaken vigorously, then observed the formation of emulsion.

Test for Steroids

Two ml of acetic anhydride was added to 0.5g of each extract of each sample with 2ml of H_2SO_4. The color changed from violet to blue or green in some samples indicating the presence of steroids.

Test for Terpenoids (Salkowasi Test)

5 ml of each extract was mixed in 2ml of chloroform and concentrated H_2SO_4 was carefully added to form a layer. A reddish brown coloration of the interface was formed to show positive results for the presence of terpenoids.

Test for Cardiac glycoside (Killer-killani test)

5 ml of each extract was treated with 2 ml of glacial acetic acid containing one drop of ferric chloride solution. This was under layered with one ml of concentrated sulphuric acid. A brown ring of the interface indicates deoxysugar characteristic of cardenolides. A violet ring may appear below the brown ring, while in the acetic acid layer, a greenish ring may form just gradually throughout thin layer.

Test for amino acids (Ninhydrin)

A few drops of ninhydrin reagent were added to 2ml of the extract. The mixture was allowed to stand in water bath for 5 minutes and appearance of purple colour indicates the presence of amino acids.

Test for carbohydrates (Molisch Test)

In two test tubes 2ml of extract was added and another second test tube added 2ml of conc. H_2SO_4. In the first test tube 2 drops of molisch reagent was added and mixed well with glass rod and slowly the mixture was poured to the second test tube. Purple colour formation at the interface between the two layers indicated the positive result.

Antioxidant assays

The antioxidant activity of plant material was assayed by employing the following methods

DPPH radical scavenging assay (Mensor, and Meneze 2001**)**.

DPPH (2, 2-diphenyl picryl hydrazyl) is a commercially available stable free radical, which is purple in colour. The antioxidant molecules present in the herbal extracts, when incubated, react with DPPH and convert it into di-phenyl hydrazine, which is yellow in colour. The degree of discoloration of purple to yellow was measured at 520 nm, which is a measure of scavenging potential of plant extracts. 10 µl of plant extract was added to 100 µl of DPPH solution (0.2mM DPPH in methanol) in a microtitre plate. The reaction mixture was incubated at 25^o C for 5 minutes, after that the absorbance was measured at 520 nm. The DPPH with corresponding solvents (without plant material) serves as the control. The methanol with respective plant extracts serves as blank. The percentage of free radical scavenging activity was expressed as percent inhibition from the given formula:

$$\% \text{ inhibition of DPPH} = (AC - AS)/AC \times 100$$

Where Ac is the absorbance of the control and As is the absorbance of the sample.

Determination of total phenolics

Phenols react with phosphomolybdic acid in Folin-ciocalteau reagent in alkaline medium and produce a blue colored complex (molybdenum blue) that can be estimated colorimetrically at 650 nm. Weighed exactly 0.5 to 1.0 g of the plant sample and ground it with a pestle and mortar in 10X volume of 80% ethanol. Centrifuge the homogenate at 10000 rpm for 20 minutes. Save the supernatant. Re-extracted the residue with five times the volume 80% ethanol, centrifuged and pool the supernatants. Then, the supernatant was evaporated upto dryness. Dissolve the residue in a known volume of distilled water. Pipetted out different aliquots (0.2 to 2 ml) into test tubes. Made up the volume in each tube to 3.0 ml with water. Added 0.5 ml of Folin-Ciocalteau reagent. After 3 minutes, added 2.0 ml of 20% sodium carbonate solution to each tube. Mixed thoroughly, placed the tubes in a boiling water bath for exactly 1 minute, cooled and measured the absorbance at 650nm against reagent blank (Mallick ans Singh , 1980).

ANTIMICROBIAL ACTIVITY ASSAY

Micro Organisms

The tested micro organisms used in the study included Gram-positive bacteria *Bacillus subtilis* MTCC 121Gram Negative bacteria- *Escherichia coli* MTCC 25922 and fungi *Rhizoctonia solani* MTCC 4633 and *Candida albicans* ATCC90028.

Media

The medium used in the assay for the antibacterial test was Mueller Hinton agar (MHA, HiMedia) and that for antigungal test Sabouraud Dextrose agar (SDA, HiMedia Laboratories Pvt. limited. Mumbai)

Reference Antibiotics

Norfloxacin (10mcg/disc, HiMedia), Clotrimazole(10mcg/disc, HiMedia), were used as reference antibiotics.

SUSCEPTIBILITY TEST

Disc diffusion susceptibility method (Kirby- Bauer Method) was used to screen the antimicrobial activity. Sterile disc (6mm) were impregnated with at the concentration of 50 µl of crude extract at the concentration of 100µg/ml. For the bacteria the microbes were placed on the surface of MHA, whereas SDA was used for fungi. The methanolic extract was tested in triplicate. The plates were inoculated at 37^{0}C and 30^0 C for 24 hours and 48 hours for the bacteria and fungi respectively. The standard antibiotics consisted of norfloxacin

and clotrimazole10mcg/disc. The results of agar disc diffusion assay were evaluated by measuring the inhibition zone diameter (in mm).

RESULT AND DISCUSSION

The preliminary assessments of phytochemical analysis of C.caesia and K.galanga in different solvent extracts in order to test the potential activity were performed (Table I). Phytochemicals, the non nutrient plant chemicals that contain protective, disease preventing compounds. Plant can produce these chemicals to protect themselves from bacteria and other predatorial invaders, however recent research has discovered that plants with phytochemical abilities may also protect from illness [36 Janaky *et al*., 2010].

Table IV. Antimicrobial activity of *K.galanga*and *C.caesia*with standard antibiotics by agar disc diffusion method

Microbes	Diameter of inhibition zone (mm)		
	Methanolic extract of Plants		Antibiotic used
	K.galanga	*C.caesia*	Norfloxacin
Escherichia coli (ATCC 25922)	8	7	31
Bacillus subtilis (MTCC 121)	14	13	39
Staphylococcus aureus (ATCC 2592)	12	11	23
Candida albicans (ATCC90028)	22	22	Clotrimazole 26
Rhizoctoniasolani (MTCC 4633)	13	12	21

Table I. Phytochemical Analysis of *K.galanga* and *C.caesia*

Medicinal plant used	SOLVENT EXTRACT	Tan	Ste	Alk	Sap	Fla	C.Gl	AA	Car
Curcuma caesia	Methanol	+ve	+ve	+ve	+ve	+ve	+ve	-ve	-ve
	Ethanol	-ve	-ve	-ve	+ve	-ve	+ve	-ve	-ve
	Ethyl acetate	-ve	-ve	-ve	-ve	-ve	+ve	-ve	-ve
	Aqueous	-ve	-ve	+ve	+ve	-ve	+ve	+ve	+ve
Kaempferia galanga	Methanol	+ve	-ve	+ve	+ve	-ve	-ve	+ve	-ve
	Ethanol	-ve	-ve	+ve	-ve	-ve	+ve	-ve	+ve
	Ethyl acetate	-ve	-ve	-ve	-ve	+ve	-ve	+ve	+ve
	Aqueous	-ve	-ve	+ve	+ve	+ve	-ve	+ve	+ve

Abbreviations: Tan-Tannins, **Ste-** Steroids, **Alk-** Alkaloids, **Sap-**Saponins, **Fla-**Flavanoids, **C.Gl-** Cardiac glycoside **AA-** Amino acids, **Car-** Carbohydrates

Table II. Total phenolic content

Medicinal plants	Total phenolic content
Curcuma caesia	121.8
Kaemferia galanga	144.13

Total phenolic content expressed as mg of GAE/100g of dry weight.

Table III. Dpph radical scavenging assay

Conc.of extracts (µg/ml)	ANTIOXIDAT ACTIVITY (%)		
	Ascorbic Acid	*Curuma caesia*	*Kaemferia galanga*
100	64.12	57.75	62.18
200	72.23	63.49	70.51
300	79.05	75.51	78.43
400	89.72	83.17	87.20
500	96.35	90.40	93.68

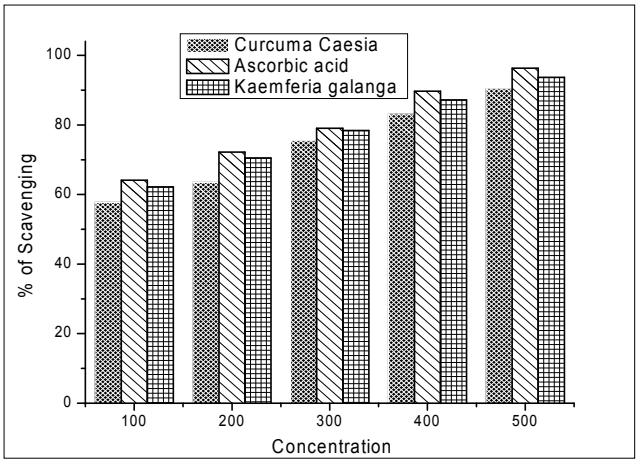

Figure 3. DPPH scavenging activity. Effect of *C.caesia*, *K.galanaga* and standard ascorbic acid on DPPH radical scavenging study

The present studies showed the presence tannins, steroids, alkaloids, saponins, flavanoids, amino acids and carbohydrates. They are well known to show medicinal activity as well as exhibiting physiological activity, Safowara 1993. In this investigation the steroid was found to be absent in all the solvent extracts used in *K.galanga*. Of the four different solvent extracts methanol extract had got the highest positive result than other three extracts, ethyl acetate extract gave the least positive result. Phenolic posses a wide spectrum of biochemical activity such as antioxidant, antimutagenic, anticarcioenic as well as ability to modify the gene expression. Numerous epideomiological studies confirm significant relationship between the high dietary intake of flavanoids and reduction of cardiovascular and carcinogenic risk. The formulation of preventive and healthy nutrition requires information about phenolic and flavanoid composition in plant foods. The total phenolic content and scavenging activity of the two medicinal plants were performed in methanolic extract only, as the in methanolic extract showed the maximum positive results in the phytochemical tests. In our study *Kaemferia galanga* (121.8 mg of GAE/100g of dry weight.) were found more phenolic content than the *Curcuma caesia* (mg of GAE/100g of dry weight.). From this study it can revealed that more phenolic content higher the anti oxidant capacity. The scavenging activities of the two medicinal plants were screened at different concentrations of methanolic extract, ascorbic acid taken as standard. DPPH assay is often used to evaluate the ability of antioxidants to scavenge the free radicals from the supplied samples, whereby the free radicals cause biological damage through oxidative stress and such processes leads to many disorders like neurodegenerative disorders, cancer and AIDS. Therefore, DPPH assay is an effective method to measure their scavenging power. The principle of the DPPH is based on the color changes from purple (DPPH solution) to yellow. The color changes can be measured quantitatively at the absorbance 517nm.

The percentages of free radical scavenging activity at different concentrations were shown in the Table III. Here also *Kaemferia galanga* has the higher percentage of inhibition than the *Curcuma caesia* at different concentrations. Antioxidant activity of the plant extract is often associated with the phenolic compounds present in them. Plant phenols constitute the major group of compounds that act as primary antioxidant (Hatano *et al*., 1989). They can react with active oxygen radicals, such as hydroxyl radicals (Hussain *et al*., 1987), superoxide anion radicals (Afanselv *et al*., 1989) and lipid peroxyl radicals and inhibit the lipid peroxidation at an early stage. This is because of their scavenging ability due to their hydroxyl groups.The antimicrobial activity of two medicinal plants of methanolic extracts were tested against gram positive, negative bacteria and fungi (Table II). The minimal inhibitory concentration study were qualitatively assayed by evaluating the presence of inhibition zones and zone diameter measurements in millimeter. For antimicrobial activity methanolic extract of *K.galanga* exhibited marked activity against the bacteria and both fungi human pathogenic and phytopathogenic organism. The gram negative bacteria *E. coli* shown resistant towards to both the plant extracts used however the gram negative bacteria *B.subtilis* exhibited intermediate inhibitory with the zones between 13-14 mm and 11-13 mm. Among the pathogenic fungi the *C. albicans* shows the higher inhibitory zone than the phytopathogenic fungi *R.solani*. The study revealed that the plant *K.galanga* could be used for treatment of some microbial infections, which also agrees with the traditional use of this plant in treatment of those fungal and bacterial derived skin diseases. Therefore, the present work has been performed to establish the various pharmacognostical and phytochemical parameters, which could serve as a measure of authentication and quality control for commercial samples of crude drug.

Conclusion

In conclusion the present work clearly indicates the two medicinal plants showed the remarkable potentiality for future wealth. The phytochemical screening of four different extracts indicated the presence of major phytocompounds including tannins, steroid, saponin flavanoids, tannins, alkaloids, cardiac glycosides, carbohydrates, amino acids etc. Among the extracts used methanolic extracts contained higher positive results than the other rest three extracts. So, being an anti oxidant the extract can be used as easily accessible source of natural antioxidants and as a possible food supplement or in pharmaceutical industry. Both the *K.galanga* and *C.caesia* showed significant and sensitizing quality against *Escherichia coli*, *Bacillus subtilis*, *Staphylococcus aureus*, *Candida albicans* and *Rhizoctonia solani*. These specific endemic two medicinal plants should be further investigated for isolation of new compounds may be obtained. The constituents molecules obtained from the methanolic extract can be good candidates for structure elucidation and then structural modification for desired antibacterial, antifungal molecules with desired efficacy.

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