Phytochemical Screening and *In vitro* Anti-inflammatory Activity of the Stem of *Coleus forskohlii*.

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ABSTRACT

The extracts of *Coleus forskohlii* prepared by using hexane, chloroform, methanol, 80% methanol and water as solvents were screened for secondary metabolites and for its *invitro* anti-inflammatory activity. The extracts revealed the presence of alkaloids, phenols, tannins, proteins, carbohydrates, saponins, glycosides and cardiac glycosides and most of these compounds were observed in the aqueous, 80% methanol and methanolic extracts. DPPH antioxidant assay and the *in vitro* anti-inflammatory activity assays *viz.*, BSA anti-denaturation and HRBC membrane stabilization assay indicated that the aqueous and methanolic extracts of *Coleus forskohlii* possess constituents with anti-inflammatory properties. TLC profile of the methanolic extract confirmed the presence of forskolin, which is a major bioactive compound isolated from the roots of *Coleus forskohlii*. Of all the extracts that were tested for their *in vitro* anti-inflammatory activity, methanolic and aqueous extracts showed maximum activity.

Key words: *Coleus forskohlii*, anti-inflammatory, DPPH and TLC.

INTRODUCTION

In Ayurveda, *Coleus species* have been used to treat spasmodic pain, digestive problems, heart disease, convulsions, and painful urination.[1] Since the 1970s, research work was predominantly carried out on forskohlin, that was extracted from the tuberous roots of *Coleus forskohlii*. Although most studies recently have used the isolated forskohlin, it is believed that the whole of *Coleus forskohlii* plant may be more effective, due to the presence of multiple compounds that can act synergistically. *Coleus forskohlii* is a perennial member of the mint (Lamiaceae) family and grows in the subtropical temperature climates in India, Nepal, Sri Lanka and Thailand.[2] Inflammation covers a sequence of reparative and defensive reactions to tissue injury, caused either due to infection, autoimmune stimuli or mechanical injury. It involves several types of compounds such as plasma proteins, vasoactive amines, tissue digestive enzymes, biologically derived oxidants and eicosanoids.

As a result of the undesirable side effects, like gastric lesions, caused by NSAIDs and tolerance and dependence induced by opiates, the use of these drugs as anti-inflammatory and analgesic agents have not been successful in all the cases. Therefore, new anti-inflammatory and analgesic drugs lacking those effects are being searched all over the world as alternatives to NSAIDs and opiates.[3] The herbs used in Ayurveda and other traditional methods of treatment can be used as good source of molecules having anti-inflammatory activity and which have lower adverse effects compared to the NSAIDs presently being used. There is a renewed interest in identifying plant constituents that can suppress the inflammatory responses, which is a complex array of enzyme activation, mediator release, cell migration, tissue breakdown and repair.

In the present study, the phytochemical screening of *Coleus forskohlii* shoot extract in various solvents was performed and the anti-inflammatory activity of the extracts was tested using BSA anti-denaturation assay and HRBC membrane stabilization assays. TLC profiling of the methanolic extract was also performed along with the root extract of *Coleus forskohlii*, and an attempt was made to evaluate the anti-inflammatory activity of the shoot extract of *Coleus forskohlii*.

MATERIALS AND METHODS

The dried shoot part of the plant (*Coleus forskohlii*) collected was pulverized and 10 g was refluxed with hexane,

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chloroform, methanol, 80% methanol and water in the ratio 1:10(w/v). The crude extracts were collected in amber coloured sample bottles and stored. All chemicals and reagents used including the solvents were of analytical grade.

Phytochemical screening

Chemical analysis was carried out in the hexane, chloroform, methanolic and water extracts of the shoot of *Coleus forskohlii* using standard procedures to identify constituents, as described by Harborne (1984), Trease and Evans (1979), and Sofowara (1993).[4-6]

DPPH Antioxidant assay

The DPPH antioxidant assay was carried out by adopting the method of Blois (1984).[7] The extracts (5 mL) were dried in vacuum oven and redissolved in 50% methanol. This was used for the DPPH assay. 50 µL (400µg/mL) and 100 µL (800µg/mL) of each fraction was taken and made up to 1 mL with 50% methanol. To this, 1 mL of DPPH (1 mM) was added. The mixture was left for 20 minutes in dark at room temperature. Absorbance was measured after 20 minutes at 517 nm. Control was taken without the extracts.

Anti-inflammatory assays

BSA anti denaturation assay:[8] 5 mL of each extract was dried in vacuum oven and redissolved in 5 mL of isosaline. Then, 1 mg/mL of all extracts were made from the above-mentioned stock solution. To 1.8 mL of 1% of BSA solution, 0.2 mL of extract solution in isosaline was added. The pH was adjusted to 6.5 using 1N HCl. This solution was incubated at 37 ºC for 20 minutes and then heated to 57 ºC for 10 to 15 minutes. After cooling, turbidity was measured at 660 nm. Control was taken without the extracts.

HRBC membrane stabilization assay:[9] Blood was collected freshly and mixed with equal volume of Alsever solution. It was then centrifuged at 3000 rpm for 15 minutes. The packed cells were washed with isosaline and a 10% suspension was made with isosaline. To 0.5 mL of extract, 1 mL phosphate buffer, 2 mL hyposaline and 0.5 mL HRBC suspension were added. This was incubated for 30 minutes at 37 ºC and then centrifuged at 3000 rpm for 20 minutes. Absorbance was measured at 560 nm. Control was taken without the extract.

TLC: Thin layer chromatography was performed with Toluene: Ethyl acetate: Methanol as solvent system and Anisaldehyde-H_2SO_4 as spraying reagent and the Rf values were compared with standard forskolin.

RESULTS AND DISCUSSION

Phytochemical analysis

Coleus forskohlii is one of the most significant potential medicinal crops of the future with its therapeutic properties being scientifically authenticated recently. The roots have been established as the source of forskolin, which is popularly used in medicinal preparations, against asthma, cardiac disorders, eye diseases, hypertension, cancer, and gastric disorders. Forskolin is reported to be responsible for virtually all pharmacological activities attributed to *Coleus forskohlii* and the extracts of this constituent has been used in nearly all existing studies though other plant constituents, such as volatile oils, diterpenoids and coleonols, are also reported to contribute to the pharmacological activity and adsorption of forskolin.[10] Forecasts of the requirements of forskolin for drug development indicate the need for a sustained supply of root material in quantities that could threaten the survival of the species in nature. Concern for species conservation and for a sustained supply of the root material led to the consideration of developing *C. forskohlii* as a medicinal crop. The focus on the development studies, led to an increased yield of root tubers, due to its cultivation as a source for forskolin. In the present study, the possibility of the utilization of the shoot was considered and thus evaluated for the levels of forskolin and for its *in vitro* anti-inflammatory properties.

Phytochemical-screening results (Table 1) of the powdered sample extracted in water, hexane, chloroform, methanol and 80% methanol showed the presence of all the constituents in aqueous and methanol, whereas the chloroform extract

Table 1: Phytochemical analysis of extracts

Chemical constituents	Water	80% Methanol	Methanol	Chloroform	Hexane
Flavonoids	+	+	+	–	–
Alkaloids	+	+	+	–	–
Phenols	+	+	+	–	–
Tannins	+	+	+	–	–
Proteins	+	+	+	+	–
Carbohydrates	+	+	+	+	–
Saponins	+	+	+	–	–
Glycosides	+	+	+	–	–
Cardiac glycosides	+	+	+	+	+

showed the presence of proteins, carbohydrates and cardiac glycosides and the hexane extracts was positive for cardiac glycosides, indicating that water and methanol were able to extract most of the phytochemicals present in the shoot. The TLC chromatogram of the methanolic extract of *Coleus forskohlii* shoot showed similar profile, when compared with the chromatogram of the methanolic extract of *Coleus forskohlii* root (Figure 1), which demonstrates that the shoot parts of *Coleus forskohlii* contained similar chemical constituents as the root portion. The Rf value of 0.589 (red band) (Table 3) corresponded with the Rf of standard forskolin, specified in the literature, confirming the presence of forskolin in both the extracts. Further isolation and identification of the other constituents separated on TLC, is needed by bioassay guided fractionation.

DPPH Assay

The results of the free radical scavenging potential of different extracts tested by DPPH method is given in Table 2. Reduction of the DPPH radicals were observed by a decrease in absorbance and change in the color to yellow that denotes the quenching of the free radical was higher by the compounds in the methanolic extract compared to other extracts. These observations clearly indicate a close linkage between active compounds and antioxidant activity. The high activity of the water and methanolic extracts are generally attributed to the presence of flavonoids and polyphenolics, as the majority of the active antioxidant compounds are flavonoids, isoflavones, flavones, anthocyanins, coumarins, lignans, catechins, and isocatechins. In addition, to the above compounds found in natural foods, vitamins C and E, b-carotene and alpha-tocopherol are known to possess antioxidant potential.

The extracts prepared with water, 80% methanol and methanol showed maximum activity. The concentration and percentage of scavenging the DPPH radical by the solvent extracts are as follows: Aqueous extract 400 µg/mL (78.2%) and 800 µg/mL (100%), 80% methanol extract 400 µg/mL (97.5%) and 800 µg/mL (101.1%) and methanolic extract 400 µg/mL (86.1%) and 800 µg/mL (87.5%), whereas the chloroform and hexane fractions showed low activity. The standard, BHA, is reported to show an IC_{50} value of 53.27 mg/mL,[11] whereas the aqueous and methanolic extracts had an IC_{50} value less than 400 µg/mL.

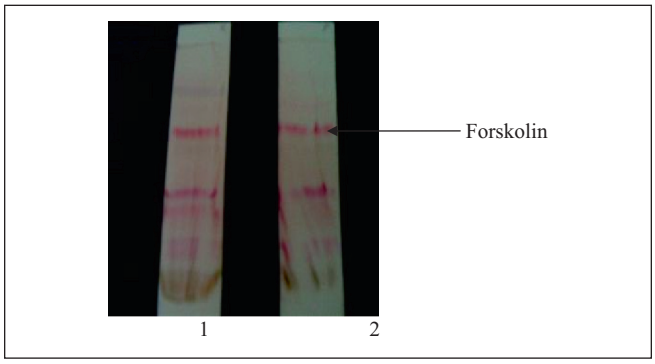

Figure 1: TLC chromatograms of methanolic extracts of *Coleus forskohlii* root (1) and shoot (2) respectively.

Table 2: HRBC membrane stabilization of extracts, standard Forskolin and drug

Concentration	% stabilization by methanolic extract		% stabilization by Diclofenac
	Met Extract	Std Forskolin	
50 µg/mL	77.6	24.32	68.09
100 µg/mL	79.1	30.78	80.48
250µg/mL	80.4	37.73	82.74
500 µg/mL	69.2	28.65	88.21

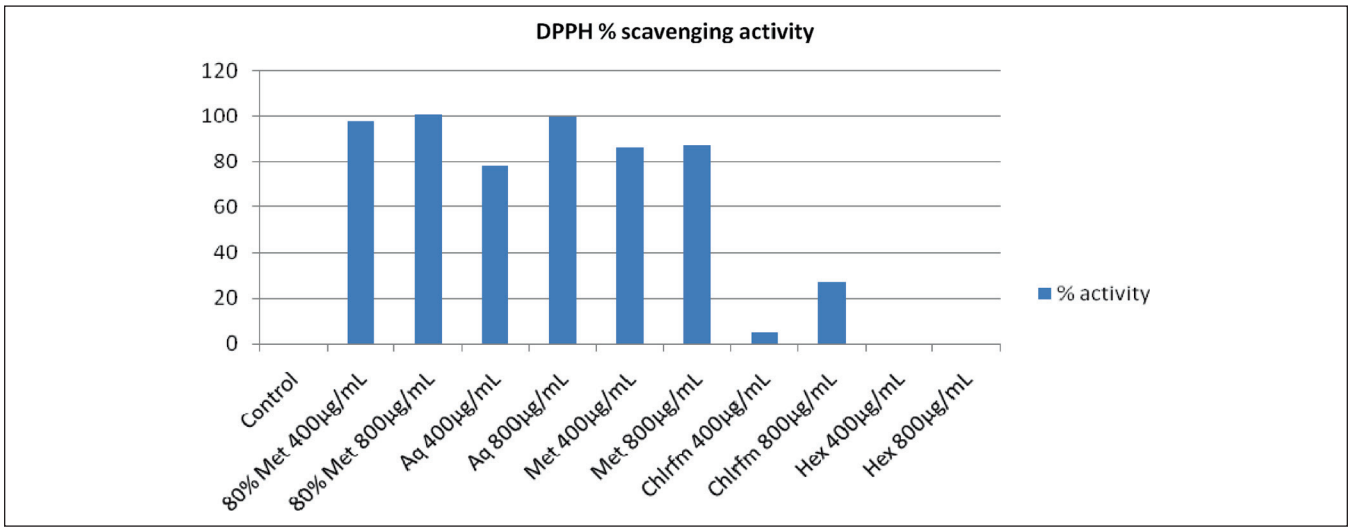

Figure 2: DPPH % scavenging activity of all the extracts.

BSA anti denaturation assay

The inhibitory effect on protein (BSA) denaturation by the extracts is shown in Table 3. All the extracts were tested at 200 µg/mL concentration. The aqueous, methanolic and hexane fractions showed good activity, whereas the chloroform extract showed comparatively lower activity. At 200 µg/mL concentration, aqueous extract showed 69.67% inhibition of denaturation followed by methanol (66.80%); 80% methanol (65.84%), hexane (65.16%) and chloroform (34.69%). Denaturation of proteins is well-documented cause of inflammation and rheumatoid arthritis. Several anti-inflammatory drugs have shown dose dependent ability to inhibit thermally induced protein denaturation.[12] When BSA is heated, it undergoes denaturation and expresses antigens associated with type III hypersensitive reaction and which are related to diseases such as serum sickness, glomerulonephritis, rheumatoid arthritis and systemic lupus erythematosus. Thus, this assay was applied for the detecting compounds, which can stabilize the protein from denaturation process. Several nonsteroidal anti-inflammatory drugs such as Indomethacin, Ibufenac, Diclofenac sodium, salicylic acid and flufenamic acid prevent denaturation of BSA at pathological pH (6.2-6.5).[13]

Table 3: Rf values from chromatogram

Rf value	Color of the band
0.465	Pink
0.589	Red (Forskolin)
0.643	Light red
0.684	Light brown
0.821	Dark pink
0.890	Brown

HRBC membrane stabilization assay

After the initial screening, it was found that the methanolic extract showed activity similar to Diclofenac, the standard anti-inflammatory drug used, for treating inflammation. Various concentrations of the methanolic extracts in iso-saline were tested and it was observed that at 250 µg/mL, both Diclofenac and the extract showed similar effects (Table 2). The analogous activity makes the extract a potential candidate for further studies.

Among all the solvent extracts, methanolic and aqueous extract showed significant anti-inflammatory activity in a concentration dependent manner. Methanolic extract at a concentration of 250 µg/mL showed 80.4% protection of HRBC in hypotonic solution. All the results were compared with standard diclofenac. According to Chou, the erythrocyte membrane can be considered to be related to lysosomal membranes and thus stabilization of the membrane is a significant criterion for assessing the effect of any extract/drug.[14] The methanolic extracts exhibited relatively higher stabilization effect by inhibiting the hypotonicity-induced lyses of erythrocyte membrane. The inflammatory responses get limited by preventing the release of lysosomal constituents of activated neutrophils thereby the damage to the tissue is reduced. Some of the non-steroidal anti-inflammatory drugs are known to possess membrane stabilization properties, which may contribute to the potency of their anti-inflammatory effect. The exact mechanism of stabilization of the membrane by the extract is not known, but it can be observed that the osmotic loss of intracellular electrolytes and fluid components was inhibited under induced hemolysis. According to Iwueke, plant extracts have the potential to stimulate or enhance the efflux of these intracellular

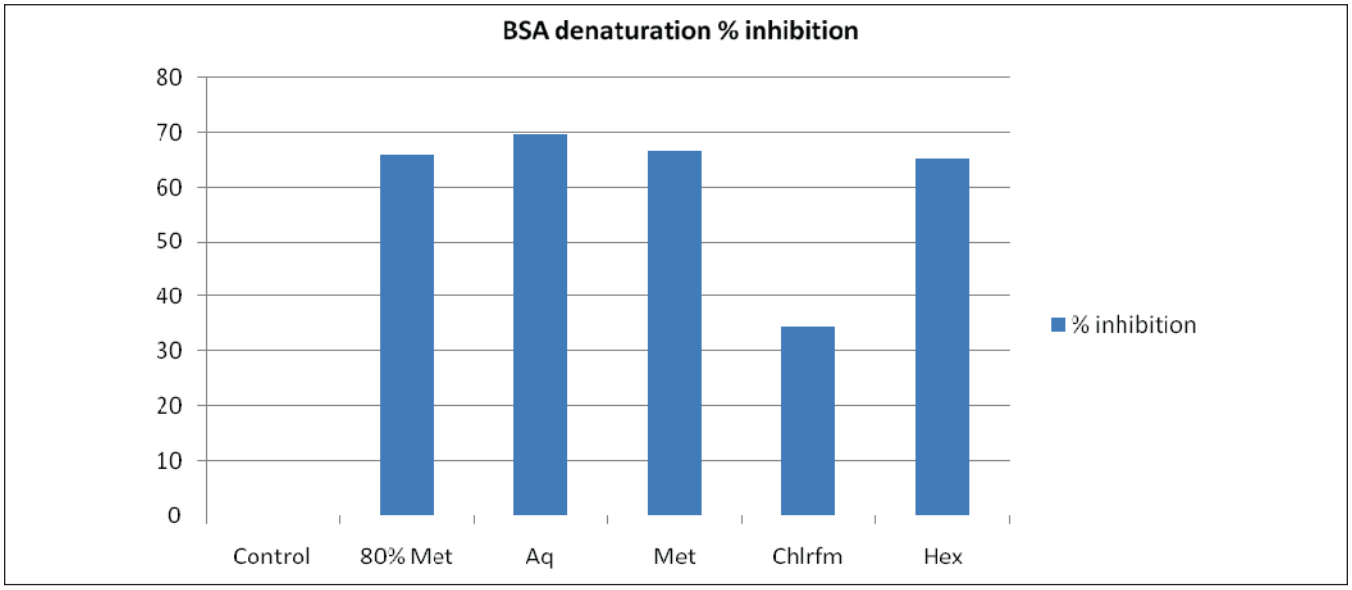

Figure 3: BSA denaturation % inhibition.

components.[15] Since inflammation responses amplify any disease conditions, it can be suggested that the shoot of *Coleus forskohlii* can also be used to reduce the inflammatory responses that is activated in most disease condition, in addition, to the root of *Coleus forskohlii* that is generally used.

The anti-inflammatory effect of the test samples were assessed by *in vitro* assay using the modified assay based on the method of Mizushima and Kobayashi.[16] The data obtained is summarized in Table 4. The analysis revealed that the methanolic extract of the shoot of *Coleus forskohlii* was more effective than 80% methanol, water, chloroform and hexane extracts, and similar to the levels of standard forskolin. Engprasert *et al.,* reported that forskolin is synthesized from Isopentenyl-diphosphate; a common biosynthetic precursor *via* a non-mevalonate pathway by geranyl-geranyl pyrophosphate synthase and is primarily synthesized in the leaves and subsequently accumulated in the stems and roots.[17]

The need for biologically active compounds with low profiles of adverse reactions compared to pharmacological drugs has triggered an extensive investigation of herbal phytochemicals and their mechanisms of action. The net biological activity is determined by the outcome of the multiple cellular effects exerted by a phytochemical and a better understanding of these cellular effects is vital to properly utilize the phytochemicals, as promising agents for promoting health and preventing disease.

CONCLUSION

The shoot extract of *Coleus forskohlii* showed potent antioxidant and anti-inflammatory activities, and TLC confirms the presence of forskolin and other constituents that is similar to that present in the root extracts. Further studies related to the active constituents on lipid-derived eicosanoids, enzyme expression (COX-2, lipoxygenase) and cytokines are necessary to understand the mechanism of action in relation to the observed anti-inflammatory activity.

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