

Estimation of Antioxidant Activity and Total Phenol, Flavonoid Content among Natural Populations of Caper (*Capparis moonii*, Wight) from Western Ghats Region

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ABSTRACT

Antioxidant activity (%) of *Capparis moonii*, Wight leaves and stem collected from Amboli, Western Ghat (India) was measured using DPPH, Phosphomolybdenum reduction, FRAP, Reducing power assay and H₂O₂ radical scavenging assay along with its total phenol and flavonoid content. Current research focuses on exploring antioxidants of plant origin. **Method:** In this study, the antioxidant activity of hexane, chloroform, ethylacetate, methanol and aqueous crude extracts of leaves and stem of *Capparis moonii* were evaluated by above mentioned various antioxidant assays. The various antioxidant activities were compared to standard antioxidants such as butylated hydroxytoluene (BHT) and Ascorbic acid. Phenolic and Flavanoid content was correlated with antioxidant activity. **Results:** Crude extracts were found to be more effective as compared to standard antioxidants like ascorbic acid and BHT. No correlation was observed between the antioxidant activities of various extracts of leaves and stem of *Capparis moonii* and total phenol, flavonoid content. **Conclusion:** The antioxidant activities of the plant extracts largely depend on the composition, concentration of the extracts, conditions of the test system and synergistic action of secondary metabolites.

Key words: Phenols, Flavanoids, Antioxidant activity, *Capparis moonii*, Wight.

INTRODUCTION

Antioxidant is a compound that protects biological systems against the harmful effects of processes or reactions that can cause excessive oxidation.¹ Oxidative damage caused by free radicals and ROS is the reason for aging, cancer and occurrence of other diseases.² Antioxidants of natural origin are preferred over synthetic due to synthetic antioxidants having toxicological effects. Therefore, current research focuses on exploring antioxidants of plant origin. Since nature is storehouse of infinite molecules having therapeutic value, bioprospecting of natural resources holds prospect in identifying lead molecules or consortia of molecules with potent therapeutic properties.

Genus *Capparis* is well known for ethno medicinal importance. In Arab traditional system of medicines, *C. spinosa* is used for the treatment of various human disorders like gastro-intestinal problems, hypertension, strangury, inflammation, emmenagogue, anemia, liver dysfunction, rheumatism, dropsy, diabetes.^{3,4} *Capparis zeylanica* is ethno medicinally used in ailments like spasmolyte, blisters, boils, pneumonia, cholera, dysentery.^{5,6} *Capparis* species are reported to be utilized as contemporary ethno medicines. Species like *sepiaria*, *flexulosa*, *decidua*, *zeylanica*, *baducca*, *tomentosa*, *horrida*, *acuminata*, *pyrifolia*, *cordata*, *ovalifolia* are used in treating numerous diseases.⁶

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Capparis moonii, Wight belongs to the family Capparidaceae frequently found in the Konkan regions and grows vigorously in hot semi-arid conditions.⁶ Its worldwide distribution is restricted to only Indian Subcontinent i.e. southern India, Sri Lanka exhibiting its endemism.⁷ Capparidaceae family comprising different species of *Capparis* is distributed in tropical and sub-tropical India, whose medicinal usage has been reported in the traditional systems of medicine. Plants belonging to the Capparidaceae family have been described as a rasayana herb and have been used extensively as an adaptogen to increase the non-specific resistance of antioxidant and immunostimulant effects.⁸ *Capparis moonii*, Wight commonly known as Waghati in Marathi and Rudanti in Sanskrit. Therapeutic Uses of Rudanti are described in Ayurveda. Rudanti nourishes each and every cell of the body (Rasayani). It is useful in under nutrition and emaciating conditions (Shoshghani). Signs of aging (Jara Vinashnam) are delayed by use of rudanti and is also useful in diseases which are having devastating effects on all the systems of the body (Rajyakshma Shasyate). Rudanti was widely used to get relief from asthma and cough.⁹ Due to significant ethno medicinal importance of genus capparis, *Capparis moonii*, Wight emerges as an extremely valuable plant for bioprospecting.

In the present study, the antioxidant activity of hexane, chloroform, ethyl acetate, methanol and aqueous crude extracts of leaves and stem of *Capparis moonii* were evaluated by above mentioned various antioxidant assays like DPPH, Phosphomolybdenum reduction, FRAP, Reducing power assay and H₂O₂ radical scavenging assay. The various antioxidant activities were compared with standard antioxidants such as BHT and Ascorbic acid. Correlation analysis was studied between the antioxidant activities of various extracts of leaves and stem of *Capparis moonii* and total phenol, flavanoid content.

MATERIALS

The stem and leaves of *Capparis moonii* was collected freshly from Amboli, district Sindhudurg, Maharashtra (India) in the month of April, 2012 and was authenticated from Botanical Survey of India, Western Circle, Pune (Registration no BSI/WRC/Tech./2012/ and Voucher no PAYCAM 1).

Chemicals

2,2-Diphenyl-2-picrylhydrazyl (DPPH), 2,4,6-tripyridyl-s-triazine (TPTZ), potassium ferricyanide, potassium persulfate, trichloroacetic acid, tannic acid, rutin, FeCl₃, ascorbic acid, and butylated hydroxyl toluene (BHT) were purchased from HIMEDIA Laboratories Pvt. Ltd.

(Germany); Folin-Ciocalteau phenol reagent, anhydrous sodium carbonate (Na₂CO₃), hydrochloric acid (HCl), glacial acetic acid, potassium chloride, sodium acetate trihydrate and solvent methanol were obtained from Merck Chemical Supplies (Damstadt, Germany).

METHOD

Extract preparation

The collected plant material was cleaned, dried under shade at room temperature and powdered. The powdered material was subjected to sequential extraction along with sonication. Solvents used for sequential extraction are Hexane, Chloroform, Ethyl acetate, Methanol and Water. 10 g of powdered material was soaked in 100 ml of solvent and sonicated for 20 min followed by filtration using Whatmans filter paper. The extract was concentrated using rotavapor and further stored at -4°C until use. The residue obtained after filtration was subjected to extraction exhaustively.

Phytochemical analysis

The extracts of *Capparis moonii* were subjected to different chemical tests for the detection of phytoconstituents such as carbohydrates, glycosides, alkaloids, proteins, tannins, phenolics, saponins, steroids.^{10,11}

Tests for carbohydrates

The carbohydrates were tested by using Benedict's test, Fehling's test and Molisch test.

Tests for glycosides

Keller Kiliani Test was used for the analysis of glycosides.

Tests for alkaloids

The alkaloids have been tested by using Dragendorff's test and Wagner's test.

Test for proteins

Various extracts were dissolved in few ml of water and treated with Millon's reagent.

Tests for tannins

Test for tannins was performed by adding 2-3 drops of ferric chloride to 1 ml of extract for the formation of a dark blue or greenish black colour product which shows the presence of tannins.

Test for saponins

The procedure adopted for the identification of saponins was to take 1 ml of extract which is diluted with 20 ml distilled water and then shaken in a graduated cylinder

for 15 minutes. A 1 cm layer of foam indicates the presence of saponins.

Tests for steroids

The steroids were tested by using Libermann-Burchard test.

Determination of total Phenolics

Total phenol content in the extracts was determined by using modified Folin-Ciocalteau method.¹² Absorbance of the solution was measured at 765 nm. Total phenolic content was expressed as mg/g tannic acid equivalent using the following equation based on the calibration curve: $y=0.1216 \times s$ ($r^2=0.9365$), where x was the absorbance and y was the Tannic acid equivalent (mg/g).

Determination of total Flavonoids

Estimation of the total flavonoids in the plant extracts was carried out using the method of Ordon et al, 2006.¹³ The absorbance of the solution was measured at 420 nm and yellow color indicated the presence of flavonoids. Total flavonoid content was calculated as quercetin (mg/g) using the following equation based on the calibration curve: $y=0.0255x$ ($r^2=0.9812$), where x was the absorbance and y was the rutin equivalent (mg/g).

DPPH radical scavenging assay

The effect of the extracts on DPPH radical was estimated using the method of Miliauskas et al, 2004.¹⁴ The absorbance of the mixture was measured spectrophotometrically at 517 nm using Ascorbic acid as reference. The ability to scavenge DPPH radical was calculated by the following equation: DPPH radical scavenging activity (%) = $[(\text{Abs Control} - \text{Abs Sample})]/(\text{Abs Control}) \times 100$ where; Abs Control is the absorbance of DPPH radical methanol; Abs Sample is the absorbance of DPPH radical+sample extract/standard.

Phosphomolybdenum assay

The total antioxidant capacity of extracts was evaluated by method of Prieto et al, 1999 and expressed as equivalents of ascorbic acid ($\mu\text{mol/g}$ of extract).¹⁵ An aliquot of 0.1 ml of sample solution was combined in an Eppendorf tube with 1 ml of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate). The tubes were capped and incubated in a thermal block at 95°C for 90 min. After the samples had cooled to room temperature, the absorbance of the aqueous solution of each was measured at 695 nm against a blank. A typical blank solution contained 1 ml of reagent solution and the appropriate volume of the same solvent used for the sample, and it

was incubated under the same conditions as the rest of the samples.

Ferric reducing antioxidant power (FRAP) assay

The FRAP assay was conducted using method of Wong et al, 2006.¹⁶ The increase in absorbance was measured using spectrophotometer at 593 nm. The percent of antioxidant was calculated using the formula, percent of antioxidant (%) = $[(\text{Abs Sample} - \text{Abs Control})/\text{Abs Sample}] \times 100$.

Reducing Power Assay

The reducing power was determined according to a described procedure Ferreira et al, 2007.¹⁷ Various concentrations of sample extracts (2.5 mL) were mixed with 2.5 mL of 200 mmol/L sodium phosphate buffer (pH 6.6) and 2.5 mL of 1% potassium ferricyanide. The mixture was incubated at 50°C for 20 min. After incubation 2.5 mL of 10% trichloroacetic acid (w/v) were added and then the mixture was centrifuged at 1000 rpm in a refrigerated centrifuge for 8 min. The upper layer (5 mL) was mixed with 5 mL of deionised water and 1 mL of 0.1% of ferric chloride, and the absorbance was measured spectrophotometrically at 700 nm.

H_2O_2 radical scavenging assay

The ability of the extract to scavenge hydrogen peroxide was determined according to the method given by Ruch et al.¹⁸ A solution of hydrogen peroxide (2 mmol/l) was prepared in phosphate buffer (pH 7.4). Extracts (50 $\mu\text{g/ml}$) were added to hydrogen peroxide solution (0.6 ml). Absorbance of hydrogen peroxide at 230 nm was determined after 10 min against a blank solution containing phosphate buffer without hydrogen peroxide and compared with ascorbic acid, the reference compound.

Statistical analysis

The data were subjected to a one-way analysis of variance (ANOVA) and the significance of the difference between means was determined by Duncan's multiple range test ($P<0.05$) using SPSS 16.0. Values are expressed as mean \pm standard deviation.

RESULTS

Hexane, Chloroform, Ethyl acetate extracts of stem and leaves showed presence of steroids and alkaloids in preliminary phytochemical investigations. Proteins and Tannins were present in methanolic and aqueous extracts. Saponins were present only in aqueous extracts. Carbohydrates were present in chloroform, methanol

and aqueous extracts. Chloroform, Ethyl acetate and Methanol showed presence of glycosides.

Analysis of phenolic content was done by Folin-Cio-calteau method and expressed in terms of tannic acid equivalents. Table 1 denotes presence of highest phenolic content in hexane stem extract (16.19 mg TAE/g dry wt) followed by chloroform leaves extract (15.57 mg TAE/g dry wt). Flavanoid content was determined by Aluminium chloride method spectrophotometrically. Chloroform leaves extract showed maximum flavanoid content (2.52 mg rutin equivalents/g dry wt) as shown in Table 1.

The proton radical scavenging action is known to be one of the various mechanisms for measuring antioxidant activity. Figure 1 shows 50% inhibitory concentration of extracts (IC_{50}). The radical scavenging activity was calculated and accordingly extract concentration for 50% Inhibitory activity was determined. Methanolic stem extract showed least IC_{50} value (31.83) thereby determining potent radical scavenging ability almost equivalent to standard Ascorbic acid activity.

The phosphomolybdenum assay method is based on the reduction of Mo (VI) to Mo (V) by the antioxidant compounds and the formation of a green Mo (V) complex, which has a maximal absorption at 695 nm. Figure 2 exhibits methanolic leaves extract with highest antioxidant capacity (41.57%).

The ferric reducing ability of methanolic stem extract of *Capparis moonii* was highest as demonstrated in Figure 3. Similarly methanolic leaves extract proved to have strong capacity to reduce ferric ions as compared to other extracts of leaves. Among all the tested extracts for reducing power assay hexane leaves extract showed maximum activity amongst the tested plant extracts of varied polarity as shown in Figure 4. Standard antioxidant BHT showed highest activity than any other tested extracts for reducing power assay.

The scavenging ability of different extracts of *C. moonii* on hydrogen peroxide is shown in Figure 5 and compared with BHT as standard. Ethyl acetate leaves and stem extract possessed 22% and 21% antioxidant potential which was highest among other tested extracts.

DISCUSSION

Our findings suggest that crude extracts are more potent than antioxidant standards like ascorbic acid and BHT. Methanolic extracts showed better activity as compared to other tested extracts. These results are in agreement with previous studies done by Fejes et al where Methanolic extracts from parsley leaves found to exhibit considerable antioxidant activity against lipid peroxidation.

¹⁹ Study of crude extracts is significant as synergistic action of metabolites is very important attribute of crude extracts. Strong nematocidal activity of turmeric was attributed to synergistic action of curcuminooids whereas pure curcuminooids proved to be ineffective against nematocides.²⁰ Synergism is very interesting property from drug development point of view.²¹ Efficacy of biologically active compounds depends upon synergistic interactions with other components.²² Multicomponent therapeutics is a new strategy to overcome one disease-one target- one drug strategy which often fails as pathogenesis of diseases involves multiple factors.^{23,24} Multicomponent therapeutics modulates biological networks modestly and thereby overcome complex diseases.²⁵ Moreover many compounds are powerful in combination than alone. For example the combination of *Realgar*, *Indigo naturalis*, *Radix sahvia miltiorrhizae* and *Radix pseudostellariae* constitute a formula in TCM that has proven effective against human promyelocytic leukemia.²⁶ The radical scavenging and antioxidant properties of isolated metabolites demonstrate that the antioxidant properties of isolated metabolites cannot be ascribed exclusively to one class of secondary metabolites. The synergy of natural products renders antioxidant activity.²⁷ Also several studies report that plant polyphenols alongwith other

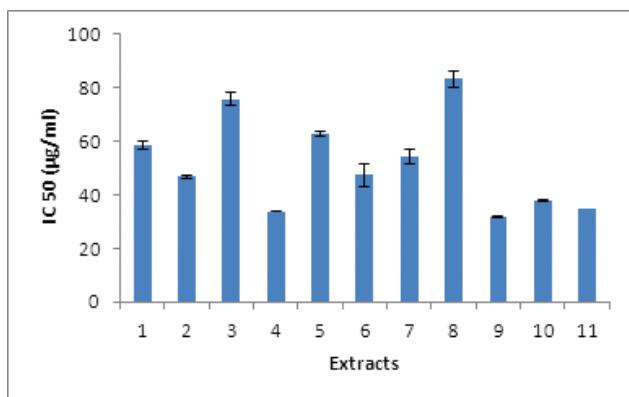
Table 1: Total Phenol and Flavanoid content

| Extract | Total Phenolic Content (TPC) Tannic acid Equivalents | Total Flavanoid Content (TFC) Rutin Equivalents |
|---------------|---------------------------------------------------------|----------------------------------------------------|
| Leaves | | |
| Hexane | 13.08±0.44 ^e | 0.61±0.009 ^f |
| Chloroform | 15.02±0.53 ^b | 2.5±0.012 ^a |
| Ethyl acetate | 14.6±0.33 ^{bcd} | 1.6±0.016 ^b |
| Methanol | 8.18±0.60 ^{gh} | 1.5±0.028 ^c |
| Water | 8.14±0.20 ^{ghi} | 0.3±0.018 ^l |
| Stem | | |
| Hexane | 16.45±0.61 ^a | 0.49±0.021 ^{gh} |
| Chloroform | 10.82±0.12 ^{ef} | 0.84±0.026 ^d |
| Ethyl acetate | 10.88±0.004 ^f | 0.81±0.04 ^{de} |
| Methanol | 14.6±0.36 ^{bc} | 0.5±0.036 ^g |
| Water | 8.66±0.44 ^g | 0.4±0.09 ⁱ |

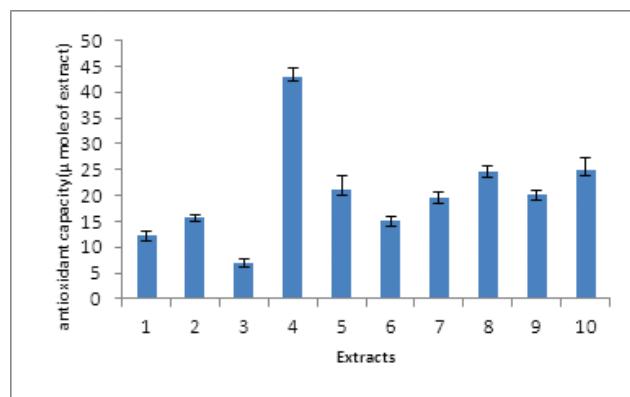
Means with different letters are significantly different according to Duncan's test ($p < 0.05$).

Table 2: Correlation analysis

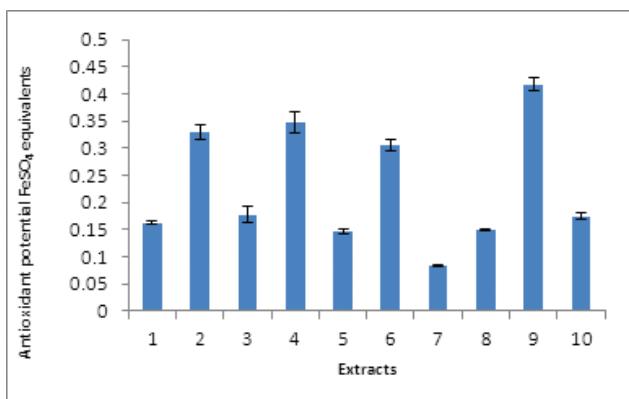
| Antioxidant activity | Phenolics | Flavanoids |
|-------------------------|-----------|------------|
| DPPH | 0.06 | 0.21 |
| Phosphomolybdenum assay | -0.58 | 0.009 |
| FRAP | 0.419 | 0.306 |
| H_2O_2 scavenging | 0.093 | 0.135 |
| Reducing power assay | 0.400 | 0.035 |

**Figure 1: DPPH radical scavenging assay**

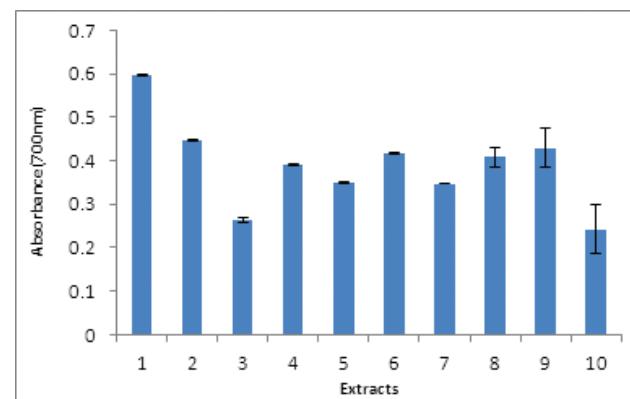
1- Leaves hexane, 2- Leaves chloroform, 3-Leaves ethyl acetate, 4- Leaves methanol, 5- Leaves water, 6- Stem hexane, 7- Stem chloroform, 8 - Stem ethyl acetate, 9 - Stem methanol, 10- Stem water, 11- Ascorbic acid.

**Figure 2: Phosphomolybdenum assay**

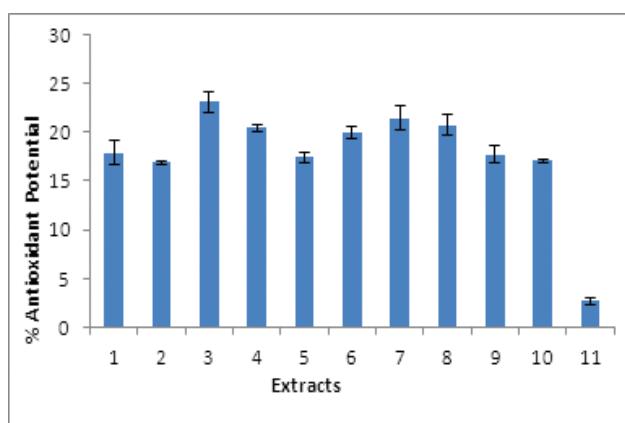
1- Leaves hexane, 2- Leaves chloroform, 3-Leaves ethyl acetate, 4- Leaves methanol, 5- Leaves water, 6- Stem hexane, 7- Stem chloroform, 8 - Stem ethyl acetate, 9 - Stem methanol, 10- Stem water.

**Figure 3: FRAP Assay**

1- Leaves hexane, 2- Leaves chloroform, 3-Leaves ethyl acetate, 4- Leaves methanol, 5- Leaves water, 6- Stem hexane, 7- Stem chloroform, 8 - Stem ethyl acetate, 9 - Stem methanol, 10- Stem water.

**Figure 4: Reducing Power assay**

1- Leaves hexane, 2- Leaves chloroform, 3-Leaves ethyl acetate, 4- Leaves methanol, 5- Leaves water, 6- Stem hexane, 7- Stem chloroform, 8 - Stem ethyl acetate, 9 - Stem methanol, 10- Stem water.

**Figure 5: H₂O₂ radical scavenging assay**

1- Leaves hexane, 2- Leaves chloroform, 3-Leaves ethyl acetate, 4- Leaves methanol, 5- Leaves water, 6- Stem hexane, 7- Stem chloroform, 8 - Stem ethyl acetate, 9 - Stem methanol, 10- Stem water, 11- ButylatedHydroxytoluene.

metabolites have synergistic effect and thereby cause elevated antioxidant activity.²⁸

As represented in Table 2 Correlation analysis between total phenol and flavonoid content showed no relation with the antioxidant activity. These results are in agreement with previous studies conducted in plant species of lamiaceae where no relation was exhibited between antioxidant activity and the antioxidant activity of the polar and non-polar extracts.²⁹ The ambiguous relation between the antioxidant activity and the total phenolics is due to numerous reasons. The total phenolic content does not feature all the antioxidants.³⁰ Antioxidant properties of single compounds within a group can vary remarkably so that the same levels of phenolics do not necessarily correspond to the same antioxidant responses. Secondary metabolites other than phenols and flavanoids are also responsible for antioxidant activity.³¹ The activity may also come from the other antioxidant secondary metabolites, such as volatile oils, carotenoids and vitamins. Structure and the interaction between the antioxidants is also major factor contributing to antioxidant activity. The antioxidant activity of phenolics mainly depends on the number and the position of hydrogen donating hydroxyl groups on the aromatic cycles of the phenolic molecules. Also it should be mentioned that Folin-Ciocalteau reagent gives a crude estimate of the total phenolic compounds present in an extract specific for polyphenols, but many interfering compounds such as ascorbic acid and sugars may react with the reagent to give an elevated phenolic concentration.^{32,33} The response of phenolics in the Folin-Ciocalteu assay also depends on their chemical structure. Thus, the radical scavenging activity of an extract cannot be predicted on the basis of its total phenolic content.³⁴

CONCLUSION

The antioxidant activities of the plant extracts largely depend on the composition and concentration of the extracts as well as on the conditions of the test system. The antioxidant activities are influenced by many factors, which cannot be described with one single method. Therefore, it is necessary in this study to perform more than one type of antioxidant activity measurement to take into account the various mechanisms of the antioxidant action. Synergistic action of secondary metabolites is very important property of crude extracts. Presence of various types of secondary metabolites like alkaloids, steroids, tannins, glycosides is responsible for antioxidant activity. Further research focuses on

elucidating mechanism of action involved in synergistic action of crude extracts.

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CONFLICT OF INTEREST

Authors declare no conflict of interest.

ABBREVIATION USED

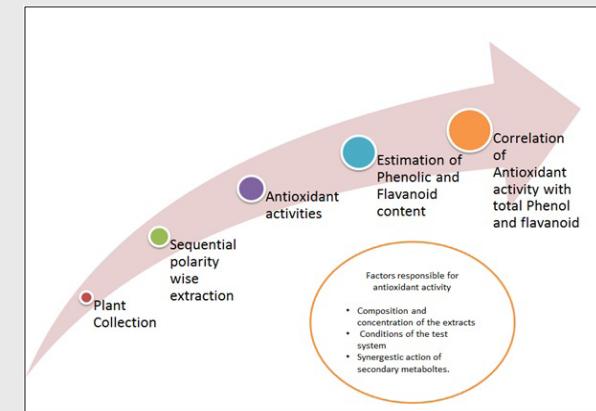
BHT: Butylated hydroxytoluene; **TPC:** Total Phenol Content; **TFC:** Total Flavanoid Content; **IC50:** Half maximal concentration; **Mo:** Molybdenum.

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PICTORIAL ABSTRACT



SUMMARY

- *Capparis moonii* Wight is an endemic plant belonging to Family Capparidaceae is endemic to Indian subcontinent.
- This plant is commonly known as Waghati in Marathi and Rudanti in sanskrit. Rudanti possess several therapeutic properties a mentioned in Ayurveda
- Our findings reveal potency of crude extracts as compared to standard antioxidants. Therefore synergistic action of compounds is the mechanism postulated for the presence of antioxidant activity.