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A COMPARATIVE STUDY ON ANTI-OXIDATIVE PROPERTIES OF DIFFERENT CULTIVARS OF *PIPER BETLE* COLLECTED FROM HOWRAH DISTRICT (WEST BENGAL)

Medicinal Plants

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ABSTRACT

The incidences of inflammatory diseases are increasing in Asia with maximum incidence in India among the south-east Asian countries. There is a need for development of novel approaches to treat or prevent the diseases using eco-compatible drugs. Paan or *Piper betle* has been traditionally known as a recreational food. However due to its properties it is a functional food as well. Ayurveda illustrates the use of Paan as a nutraceutical. Paan is known to possess antioxidant, anti-inflammatory, anti-apoptotic, anti-cancer and anti-microbial properties. The essential oil (1%–3%) in leaves are used in making ayurvedic medicines and herbal products. Exploration of its anti-oxidative potential may reveal some bioactive or higher phenolic and flavonoid compound to enhance its nutritive value and help in combating various inflammatory and degenerative diseases. With this aim, we have planned to screen a number of varieties of paan collected from different districts of West Bengal, by a panel of different cell free anti-oxidative biochemical assays, to compare four different anti-oxidative potentials in four different cultivars of a particular geographical location and in a particular season (summer) of collection from Howrah district of West Bengal. The study revealed the best variety of Howrah district which has the potential to combat the inflammatory and degenerative diseases.

KEYWORDS

Paan, geographical location, anti-oxidative potential, phenolic and flavonoid compound, comparative study

INTRODUCTION

Inflammation is the key driving force for most disorders and it is the breakdown of the body's homeostasis through regulatory disruption that leads to the clinical manifestation of diseases such as asthma, rheumatoid arthritis, psoriasis, multiple sclerosis, obesity and inflammatory bowel disease. It is a complex response to stimulus in which the body repairs tissue damage and defends itself from foreign materials.

Oxidative damage is another important effect of ionizing radiation on biological membrane. Free radicals generated from the radiolytic decomposition of water can attack fatty acid chains of membrane lipid. A free radical that has sufficient energy to abstract allelic hydrogen from the methylene carbon of polyunsaturated fatty acid can initiate per oxidative process (Verma *et al.* 2010).

Piper betle Linn. (Local name paan) of piperaceae, is a widely distributed, dioecious, annual creeper plant in tropical and subtropical regions. Its leaves are largely consumed as a mouth freshener, mild stimulator and chewable. It climbs by many small adventitious roots, growing to a height of about 1 meter, and is generally grown in hotter and damper parts of the country. It is glorified as an evergreen, perennial plant with a strong pungent aromatic flavour, and the heart-shaped leaves are used as a mild stimulant. It has some traditional as well as modern medicinal uses. The nutritive leaves, which hold a considerable quantity of vitamins and minerals, are credited with digestive, detoxification, anti-carcinogenic, hepato-protective and anti-mutagenic properties. Previous studies have reported that extracts of *Piper betle* leaves contain many bioactive molecules but there have been no systematic studies to examine different varieties of leaves and the effects of different extraction methods on the composition of different bioactive compounds present in the extract. Due to difference in culture in different geographical locations with different soil constitution, water, air quality and agronomical parameters, there are subtle differences in the *P. betle* activity.

The nutritional and pharmacological uses of different varieties of *P. betle* have been well elucidated in which its anti-oxidative and anti-inflammatory properties have been studied, suggesting possible therapeutic effects in inflammation (Das *et al.*2015, Shah *et al.*2016). The ethanolic extract of *P. betle* showed down regulation of nitric oxide (Ganguly *et al.* 2007). The use of *P. betle* for gastro-protective effect has been studied which showed the therapeutic effect of hot aqueous extract and cold ethanolic extract of *P. betle* in gastric ulcers (Arambewela *et al.*2014). Apart from the antioxidant, anti-inflammatory, anti-apoptotic, anti-cancer and anti-microbial properties, the essential oil (1%–3%) component of the leaves has been used in making ayurvedic medicines and herbal products (Das *et al.* 2016). The *P. betle* leaves also contains vitamins such as Vitamin-C, vitamin-A, nicotinic acid, thiamine and riboflavin, and studies showed the production of enzymes from the leaves which helps in digestion along with anti-microbial activity which acts against a broad spectrum of microbes (Hossain *et al.*2017).

The presence of hydroxyl group-containing polyphenolic compounds like hydroxyclavicol, chatecol, allylpyrocatecol, eugenol and hydroquinone may directly contribute to the anti-oxidant, anti-microbial, analgesic activities in *Piper betle* leaf extract, which inhibits free radical damage and radiation-induced lipid per-oxidation very effectively. It reduces most of the ferric (Fe^{3+}) ion and possesses strong reductive ability (Manigauha *et al*. 2009). Bio active compounds isolated from the extracts have demonstrated different beneficial effects. Due to low cost, easy accessibility and fewer side effects ethno-medicines are extensively used in India.

The consumption of food and dietary anti-oxidants has been associated with the reduction of cognitive dysfunction in aged human, as well as inflammatory and metabolic disorders. Dietary anti-oxidants have been shown to be effective scavengers of harmful free radicals, preventing the oxidation of biomolecules (such as DNA, LDL) (Halliwell 2000).

Generally, plants that have important therapeutic properties are found to be wealthy in phenolic compounds, with high antioxidant properties. This correlation has been verified with the antioxidant effect being detected in the extract of betel splint. The consumption of antioxidant-rich foods helps neutralize the free radicals in the body, therefore precluding or delaying the oxidative damage of lipids, proteins and nucleic acids. This correlation has been confirmed with the antioxidant activity being detected in the extract of betel leaf. It has been shown that antioxidants could reduce mortality rate of cardiovascular disease, and protect against cancer and other chronic diseases (Sarma *et al.*2018).

High content of various phenolic and non-phenolic compounds, high flavonoid content, total antioxidant capacity and ferric reducing power potential may contribute to the use of paan, not only as a highly nutritive, edible plant part, but also as a nutraceutical substance, to be used therapeutically in oxidative inflammatory diseases (Shahidi *et al.* 2015). With this in mind, the aim of this investigation was to detect good antioxidant activities of 4 cultivars of *Piper betle* with two separate leaf extracts. The best variety would then be analysed to

determine which components of the extract are responsible for the anti-oxidant and/or anti-inflammatory activity. Further, the extract would be tested in a pre-clinical mouse model of an inflammatory disease, for ultimate translational use. However, these are beyond the scope of this paper, which is only concerned with the *in vitro* assessment of the activities, as mentioned above.

MATERIALS AND METHODS

Sample collection, storage and extraction:

Healthy and well grown young greenish *Piper betle* leaves were collected in sterile polythene bags, directly from the cultivated fields of **Howrah district, Ulu Beria block II**, and transferred direct to the lab. The leaves were then washed thoroughly with tap water and distilled water alternatively, then surface sterilization was done with 10% concentrated sodium hypochlorite solution to prevent the growth of microbes, then rinsed again with sterile distilled water. The materials were then placed in zip lock bags, containing silica gel, and further shade dried at room temperature.

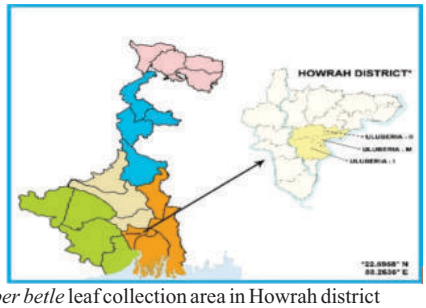
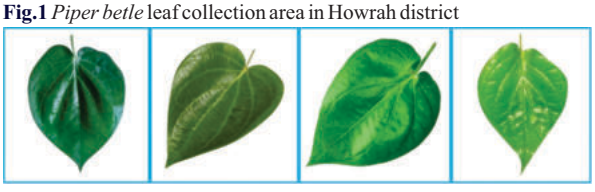


Fig.1 *Piper betle* leaf collection area in Howrah district



A **B** **C** **D**

Fig.2 Four varieties of *Piper betle* leaf of Howrah district. **A**- Kalibangla, **B**- Bagerhati, **C**- Ghanagete and **D**- Meetha paata

Preparation of plant extract:

Fresh betel leaves were washed thoroughly, shade dried for 15 days and then powdered. Dried, powdered leaves were extracted with a choice of solvents, like extreme non-polar such as petroleum ether and polar as water (Lei *et al*. 2003) with the help of normal maceration, at room temperature for about 48-72 hours and frequent shaking.

The extract was filtered victimisation Whatman paper no.1, and also the solvent was allowed to evaporate completely by rotary vacuum evaporator to obtain the concentrated extract. The extract was stored in -20°C for freezing. The frozen extracts were then run through a lyophilizer to complete evaporation of the solvent. Then the dried extracts were stored in 4°C for future use (Shrunkala *et al*. 2020).

Assessment of Antioxidant Activity:

Four different anti-oxidative assays were performed. Each assay was performed twice in triplicates.

I. Determination of total phenolic content (TPC)

Folin-Ciocalteau reagent was used to determine total phenolic content of the non-polar and polar *P. betle* leaf extracts. Gallic acid was used as positive control and the results were expressed as Gallic acid equivalent (GAE), which is number of milligrams of Gallic acid/ gram of the extract (Lamuela-Raventos *et al*. 2018).

II. Determination of total flavonoid content (TFC)

Aluminium chloride was used to determine total flavonoid content of the non-polar and polar *P. betle* leaf extracts. Fisetin was used as a positive control. Total content of flavonoid in the plant extracts were expressed as Fisetin equivalent (milligram of fisetin /gram of extract) (John *et al*. 2014).

III. Determination of ferric reducing power

Various concentrations of the petroleum ether and water extracts were added to phosphate buffer and 1% potassium ferricyanide (Himedia India) to determine ferric reducing power of the sample (Spiegel *et al*. 2020).

IV. Determination of total antioxidant capacity

Total antioxidant activity of the extracts, expressed as the number of equivalents of ascorbic acid, was measured by the phospho-molybdenum method, which is based on the reduction of Mo(VI) to Mo(V) (Mukherjee *et al*. 2014).

Statistical analysis

All results were recorded in triplicates. All data have been expressed as mean ± standard deviation (SD), and p values less than 0.05 were accepted as significantly different at 95% confidence interval.

RESULTS AND DISCUSSION:

Natural merchandise square measure in nice demand due to their intensive biological properties and bioactive parts that have tested to be helpful against an outsized variety of diseases. it's been tested that gift extracts of *Piper betle* leaves show a large array of anti-oxidant activities.

Various reactive chemical element species (ROS) may be shaped in cells by reactions mediate by transition metals, particularly Fe(II), at the side of chemical element metabolism, and radiation exposure, resulting in hurtful effects on membrane lipids and DNA. within the gift study, the inhibitor activity of the petroleum ether and water extracts of four varieties of *P. betle* leaves were screened, using cell-free *in vitro* assays to determine the total phenolic content by the Folin-Ciocalteu method, total flavonoid content by aluminium chloride, ferric reducing power potential by P-FRAP assay, and total anti-oxidant capacity by phospho-molybdenum method.

I. Determination of total phenolic content

Phenols belong to the biggest cluster of secondary metabolites of plants. Plant phenolic resin compounds have numerous biological effects, including radical-scavenging and anti-oxidant activities. Consequently, the inhibitor activities of plant/herb extracts can be correlated to their TPC.

Total phenolic content of the *Piper betle* leaves was assessed using the Folin-Ciocalteau method, using Gallic acid as a positive control, and results were expressed as GAE. It was seen that TPC of the polar extract of Meetha paata had the highest phenolic content (**40.613 GAE**) and polar extract of Kalibangla had the lowest (**9.867 GAE**) (Fig.3A). All polar extracts results were found to be significantly higher with respect to petroleum ether viz. 5.2 fold ($p<0.05$) higher TPC in polar extract of Kalibangla, 2.2 fold ($p<0.05$) higher TPC in polar extract of Ghanagete, 3.3 fold ($p<0.05$) higher TPC in polar extract of Bagerhati, and 4.4 fold ($p<0.05$) higher TPC in polar extract of Meetha paata (Fig. 3A). The phenol content of the samples decreased in the order:

Meetha paata>Ghanagete>Bagerhati>Kalibangla (in both type of extracts).

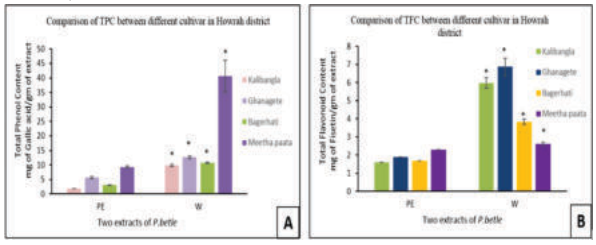


Fig. 3 (A) Graphical representation of total phenolic content in different cultivars of *P. betle* (Kalibangla, Ghanagete, Bagerhati, Meetha paata) of Howrah district, showing high phenolic content in polar extracts in all the cultivars, with maximum TPC in Meetha paata cultivar. **(B)** Graphical representation of total flavonoid content in different cultivars of *P. betle* (Kalibangla, Ghanagete, Bagerhati, Meetha paata) of Howrah district, showing high flavonoid content in polar extracts in all the cultivars, with maximum TFC in Ghanagete cultivar. (*: $p<0.05$ of polar water extract, with respect to non-polar petroleum ether extract).

II. Determination of total flavonoid content

Total flavonoid content of the *P. betle* leaves was assessed using the aluminium chloride ($AlCl_3$) method, using fisetin as a positive control, results were expressed as fisetin equivalents. The TFC of polar extract of Ghanagete had the highest flavonoid content (**6.866 fisetin equivalent**) and the polar extract of Meetha paata had the lowest (**2.623 fisetin equivalent**) (Fig.3B). All polar extracts results were

found to be significantly higher with respect to petroleum ether i.e. Kalibangla showing 3.7 fold ($p<0.05$) higher TFC, the polar extract of Ghanagete showing 3.6 fold ($p<0.05$) higher TFC, the polar extract of Bagerhati showing 2.3 fold ($p<0.05$) higher TFC, and the polar extract of Meetha paata showing 1.1 fold ($p<0.05$) higher TFC, compared to the non-polar extracts (Fig. 3B). The flavonoid content of the samples decreased in the order:
Ghanagete>Kalibangla>Bagerhati>Meetha paata (according to polar extract value)

III. Determination of ferric reducing power

Ferric reducing power of *P. betle* leaves was assessed using the potassium ferricyanide method, using ascorbic acid as a positive control. Maximum ferric reducing power, proportional to the absorbance at 700 nm, was seen at a concentration of **10 mg/ml** (Fig.4).

Among the polar extracts, Meetha paata (Fig.4D) showed the highest ferric reducing power (**0.774±0.012**) and Bagerhati (Fig.4C) showed the least (**0.200±0.005**). On the other hand, among the non-polar extracts, Meetha paata (Fig.4D) showed the highest ferric reducing power (**0.309±0.003**) and Kalibangla (Fig.4A) showed the least (**0.032±0.001**).

The polar extracts of Meetha paata, Kalibangla, Ghanagete and Bagerhati showed 2.5 fold ($p<0.05$), 8.1 fold ($p<0.05$),1.3 fold ($p<0.05$), and 2.3 fold ($p<0.05$) higher ferric reducing power respectively, compared to the non-polar extracts (Fig. 4).

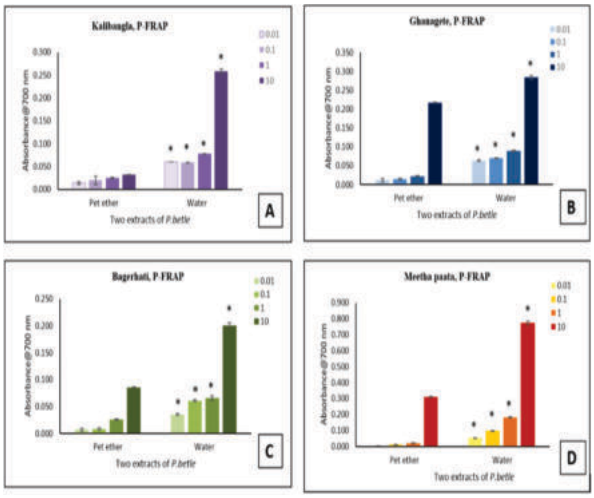


Fig.4 Graphical representation of ferric reducing power potential (P-FRAP) of four different cultivars of Howrah district- **(A)** Kalibangla, **(B)** Ghanagete, **(C)** Bagerhati and **(D)** Meetha paata, in both extracts (non-polar petroleum ether and polar water). Four concentrations of extracts were taken (10 mg/ml, 1 mg/ml, 0.1 mg/ml and 0.01 mg/ml), and in all cases the 10 mg/ml concentration showed maximum potential.

Maximum reducing power among the varieties was seen in both extracts of Meetha paata. (*: $p<0.05$ of polar water extract, with respect to non-polar petroleum ether extract).

IV. Determination of total antioxidant capacity

Total antioxidant capacity of paan was assessed using the phospho-molybdenum reagent, using ascorbic acid as standard, and results were expressed as ascorbic acid equivalent.

The petroleum ether extracts of Kalibangla (Fig. 5A) and Ghanagete (Fig. 5B) showed higher antioxidant capacity **(0.477±0.035 and 0.770±0.005 respectively)** than their polar counterparts. However, the other two cultivars, i.e. Bagerhati (Fig. 5C) and Meetha paata (Fig. 5D) showed higher total anti-oxidative capacity **(1.073±0.011 and 0.797±0.015 respectively)** in their polar extracts, than the non-polar extracts.

Unlike the above assays, here we found that the water extracts of Bagerhati showed 2.2 fold and Meetha paata showed 1.4 fold more antioxidant capacity than the non-polar extracts, while the non-polar extracts of Kalibangla and Ghanagete showed greater anti-oxidant capacity than the polar extracts (Fig. 5)

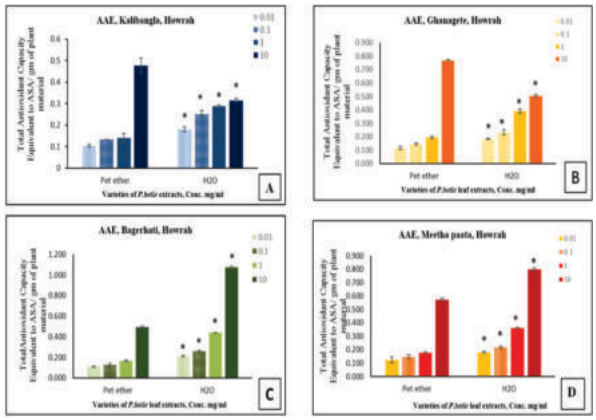


Fig.5 Graphical representation of total antioxidant capacity of different cultivars of *P. betle*-(A) Kalibangla (B) Ghanagete, (C) Bagerhati, and (D) Meetha paata of Howrah district showing the maximum activity in polar extract of Bagerhati cultivar (1.073±0.011 ASA equivalent). Four concentrations of extracts were taken (10 mg/ml, 1 mg/ml, 0.1 mg/ml and 0.01 mg/ml), and in all cases the 10 mg/ml concentration showed maximum antioxidant capacity. (*: $p<0.05$ of polar water extract, with respect to non-polar petroleum ether extract).

The results of the above assays have been summarized in the table below (Table 1), where we have attempted to rank the varieties according to their activities.

Table.1 Comparison among four different cultivars of *Piper betle* of Howrah District showing maximum anti-oxidative activities in the polar extracts in comparison to non-polar extracts.

Sl No	Samples	Extracts	TPC	TFC	P-FRAP	AAE
1	Kalibangla	Pet ether	+	+	+	++
		Water	++	++	++	+
2	Ghanagete	Pet ether	+	+	+	++
		Water	++	++	++	+
3	Bagerhati	Pet ether	+	+	+	+
		Water	++	++	++	++
4	Meetha paata	Pet ether	+	+	+	+
		Water	++	++	++	++

Wellness and therapeutic properties are required for those people requiring high energy, who are fatigued. They need a pick-me-up drink but without free glucose-induced high oxygen demand, in which a sudden burst of glucose-induced oxidation leads to a number of undesirable side effects, including but not limited to metabolic overdrive, tooth decay and a short term feeling of energy burst that does not sustain itself physically and mentally. This study was aimed to characterize the mesophyll tissue for bio-prospecting of anti-oxidative bioactive compounds, as well as to explore its use as a functional food for providing non-nutritional health benefits, such as repair of damaged tissue.

We had selected Howrah district for the collection of different varieties of *P. betle* for its highly fertile soil quality, and because the environmental conditions during the summer season is suitable for the extensive cultivation of paan. Four cultivars (Kalibangla, Ghanagete, Bagerhati and Meetha paata) were selected for a comparative analysis of possible health benefits wellness and/or therapy.

This study indicates that the polar fractions have better anti-oxidative potential (Table1). From these results, specific cultivars of paan could be positioned to assess quality and yield of extraction of water insoluble fractions, which a user has no access to in the present (chewable) form.

The goal of this study was to optimize the carefully in-bred, cross-bred, intra-bred varieties developed indigenously for its maximum economic potential.

From our data using the crop yield in summer season it may be inferred that while the activity of the polar extracts is important, extracts in non-polar solvents, which are inaccessible to users, also containing bio-active compounds, are now significantly open to further investigation. Detailed chromatogram of the medicinal chemical compound will be

investigated further and based on taste, flavour, aroma and texture, paan leaves can be classified for its value-added market value as functional food.

CONCLUSION

This study for the first time revealed that the anti-oxidative potential and the functional profile of *Piper betle* leaves expressed markedly in the polar (water) extracts than the non-polar (petroleum ether) extracts. Among the four cultivars of Howrah district, Meetha paata showed the better anti-oxidative reducing power potential with its high phenol content, high total anti-oxidative capacity, high flavonoid content and highest ferric reducing power than the other three varieties in the polar extracts. Therefore, it is evident that the same geographical location gives rise to differential functional profile among the varieties and can help ensure the best cultivar of the Howrah district to be cultivated in large scale. Further work is going on to investigate its bioactive compounds which will lower the inflammation and degeneration and save human health.

Statement of Declaration:

Competing Interest:

The authors have declared that no conflict of interest exists.

Abbreviations:

P. betle*: *Piper betle, **Pet ether:** Petroleum ether, **GAE:** Gallic acid equivalent, **TPC:** Total phenol content, **TFC:** Total flavonoid content, **P-FRAP:** Potassium ferricyanide ferric reducing power potential, **AAE:** Ascorbic acid equivalent, **DMSO:** Di methyl sulphoxide, **TCA:** Tri chloro acetic acid

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Supplementary File:

MATERIALS AND METHODS

I. Determination of total phenolic content (TPC)

Folin-Ciocalteau reagent was used to determine total phenolic content of the non-polar and polar *P. betle* leaf extracts. The reaction mixture, containing diluted extract and freshly prepared diluted Folin-Ciocalteau reagent (SRL) and 7.5 % sodium carbonate (SRL). The mixtures were incubated at 45˚C for 15 minutes, followed by the incubation in room temperature for 30 minutes to complete the reaction. The absorbance and development of blue colour was appeared at 765 nm. Gallic acid was used as positive control and the results were expressed as Gallic acid equivalent (GAE), which is number of milligrams of Gallic acid/ gram of the extract (Lamuela-Raventos *et al*. 2018), i.e. T = (CxV)/M

Where,

T = total content of phenol compounds (mg of GA/gm of extract)
C = the concentration of Gallic acid established from the calibration curve (mg/mL)
V = volume of extract (mL)
M = weight of petroleum or water plant extract (gm)

II. Determination of total flavonoid content (TFC)

Fisetin was used as a positive control. Both non-polar and polar leaf extracts were added to 75% methanol, followed by the addition of 1M potassium acetate (CH_3COOK; E. Merck), 10% $AlCl_3$ and distilled water. Then the reaction mixture was vortexed well and incubated for 30 minutes at room temperature, allowing the colour of the solution to change from yellow to orange (John *et al*. 2014). Finally, the absorbance was measured at 435nm. Total content of flavonoid in the plant extracts were expressed as Fisetin equivalent (milligram of fisetin /gram of extract) and calculated by the formula: T = (CxV)/M

Where,

T = total content of flavonoid compounds (mg of fisetin/gm of extract)
C = the concentration of fisetin established from the calibration curve (mg/mL)
V = volume of extract (mL)
M = weight of petroleum or water plant extract (gm)

III. Determination of ferric reducing power

Various concentrations of the petroleum ether and water extracts were added to phosphate buffer and 1% potassium ferricyanide (Himedia India), mixed well and incubated at 50˚C for 20 minutes. After incubation, 10% TCA (SRL) was added, followed by centrifugation at 3000 rpm for 10 minutes. The reaction mixture was diluted with deionised water and 0.1% ferric chloride (Himedia India) was added, for the development of bluish green colour. Absorbance was measured at 700nm using UV spectrophotometer (Spiegel *et al*. 2020).

IV. Determination of total antioxidant capacity

Total antioxidant activity of the extracts, expressed as the number of equivalents of ascorbic acid, was measured by the phospho-molybdenum method, which is based on the reduction of Mo(VI) to Mo(V). The extract and the reagent mixture, containing 0.6 M sulfuric acid (Emparta), 28 mM sodium phosphate (Himedia India) and 4 mM ammonium molybdate (SRL) were incubated at 95°C for 90 mins. The mixture was then cooled to room temperature, after which the absorbance was measured at 695 nm against a blank (Mukherjee *et al*. 2014). Results were expressed as ascorbic acid equivalent (ASA)/ gm of plant material.

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