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Phytochemical analysis of methanolic extract of leaves of *Gymnema sylvestre R. Br.* from Niyamagiri hill, Kalahandi district, Odisha, India

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Abstract
This study was to carry out phytochemical screening, to determine the total phenolic and flavonoid content as well as to evaluate the antioxidant proper ties of methanolic extract of leaves of Gymnema sylvestre plants. Antioxidant properties were determined by *1, 1- diphenyl, 2-picrylhydrazyl* (DPPH), hydroxyl radical (OH^-), superoxide radical (O_2), hydrogen peroxide (H_2O_2) assay and total antioxidant activity by the phosphomolybdenum assay. The result showed that the powdered leaves was extracted with methanol and the percentage of yield was 29.2%. The total phenolic and flavonoid contents of the extracts were 18.67±0.23mg/g and 2.51±0.22mg/g dry weight respectively. The plant sample possesses high free radical scavenging activity and phytochemical constituents which might be useful for further studies to fight against oxidative stress.

Keywords: *Gymnema sylvestre*, phytochemical screening, antioxidant properties, Kalahandi district, Odisha

Introduction
In present day's human civilization the most used, acceptable and recognized form of medicine is plant derived medicinal products throughout the world. Plant generates several types of secondary metabolites that are biosynthetically produced from primary metabolites and these phytocompounds are the primary metabolites and these phytocompounds are the primary sources of herbal, pharmaceutical and nutraceutical formulations. Natural products, especially those of wild origin, have always been an important source of therapeutic agents. About 25%-30% of drugs available for the treatment of disease are derived from natural products.[1] Natural product research is frequently based on ethnobotanical information, as of now many of the drugs used are developed from medicinal plants employed in indigenous societies.[2] A major part of ethno pharmaceutical research in recent years has been directed towards a better understanding of the pharmacological effects of individual medicinal plants.[3] Many studies carried out in this field show that plants used in traditional medicine have been tested to be effective models for pharmacological studies.[4] Thus, the medicinal plants and natural product extracts have been considered as an alternative therapy against various diseases [5].

Gymnema sylvestre R. Br. is belongs to the class dicotyledonous of the family Asclepiadaceae locally called them as gudamari in Odia. Its medicinal value has been reported in Indian traditional medicine such as Ayurveda, Unani, and Siddha. The herbal medicines are becoming accepted due to better results and safe use as compared to marketed drugs and more effective treatment of health problem [6], The plant is a good quality of an bioactive compounds [7]. It has deep roots in history, being one of the major botanical used in Ayurvedic system of medicine to treat conditions ranging from diabetes, malaria to snakebite [8]. The objective of this study was to perform the phytochemical screening, to determine total phenolic and flavonoid content as well as antioxidant activities of leaves extracts.

Materials and methods
Plant collection and extractions
The plant is collected from Niyamgiri (Kalahandi district of Odisha, India) in December of 2019 (coordinates 20.47° N and 84.23° E). The plant was authenticated by the renowned taxonomist. The sample was placed at the herbarium house of Botany department of Berhampur University, Odisha, India. The leaves of the plant were dried separately in an oven at 80°C. The dried plant material was pulverized to powder with a mechanic grinder. The powder (11 g) of five plant was extracted using solvent methanol (300 mL) through a Soxhlet apparatus.[9,10] After extraction, the filtrate was concentrated by evaporating in a water bath under normal pressure.[11] The dried extracts were weighed to determine the percentage of yield[12] of the soluble constituents using the formula;

$$\% \text{ Yield} = \frac{\text{Weight of dry extract}}{\text{Weight of extraction}} \times 100$$

The dried extracts were stored at 4°C for further investigation of potential *in vitro* free radical scavenging activity.

Qualitative phytochemical screening
Qualitative analysis of methanolic extract was extract carried out to determine the presence of various bioactive compounds using the standard qualitative procedures [13–15].
Quantitative analysis of Phytochemicals

Estimation of total phenol content (TPC) and total flavonoid content (TFC)
Total phenolic content (TPC) was analyzed by the Folin-Ciocalteu method using gallic acid as a standard curve and expressed as mg/g gallic acid equivalent. [16] Total flavonoid content (TFC) was analyzed using rutin as standard and this was expressed as mg/g rutin equivalent. [17]

Determination of antioxidant activity
1, 1 Diphenyl-2-picrylhydrazyl radical (DPPH) scavenging activity
The free radical scavenging activity of the methanolic extracts was determined using DPPH assay.[18] Various concentrations of methanolic extract of the sample (1 mL) were mixed with 1 mL of methanolic solution containing *1, 1 Diphenyl-2-picrylhydrazyl radical* (DPPH) radicals resulting in the final concentration of DPPH being 0.2 mM. The mixture was shaken dynamically and left to stand for 30 mins, and the absorbance was measured at 517 nm. Ascorbic acid was used as a standard. The percentage of DPPH decolorization of the sample was calculated using the following formula:

% decolorization = (Abs: of control - Abs: of sample / Abs: of control) ×100

Hydroxyl radical scavenging activity
The reaction mixture (3 mL) containing 1 mL $FeSO_4$ (1.5 mM), 0.7 mL hydrogen peroxide (6 mM), 10% of 0.3 mL sodium salicylate (20 mM) and varying concentrations of the extracts (10–500 µg/mL) were taken. After incubation for 1 hr at 37°C, the absorbance of the hydroxylatedsalicylated complex was measured at 562 nm. [19] Ascorbic acid was used as the standard. The percentage scavenging effect was calculated as:

% scavenging activity = $[1- (A_1 — A_2) / A_0] \times 100$

Where A_0 was the absorbance of the control (without extract), A_1 was the absorbance in the presence of the extract with sodium salicylate, and A_2 was the absorbance without sodium salicylate.

Superoxide anion radical scavenging activity
This assay was based on the reduction of nitro blue tetrazolium (NBT) in the presence of nicotina- mide adenine dinucleotide (NADH) and phenazinemethosulfate (PMS) under aerobic condition.[20] TrisHCl buffer (3 mL, 16 mM, pH 8.0) containing 1 mL NBT (50 µM) solution, 1 mL NADH (78 µM) solution and a sample solution of extract (10–500 µg/mL) in distilled water mixed. The reaction was started when 1 mL of PMS solution (10 µM) was added to the mixture. The reaction mixture was incubated at 25°C for 5 min, and the absorbance was read at 560 nm against the corresponding blank samples. Ascorbic acid was used as a standard. The decreased absorbance of the reaction mixture indicated increased superoxide anion scavenging activity.

Hydrogen peroxide radical scavenging activity
The capability of the extract to scavenge hydrogen peroxide (H_2O_2) was estimated according to the method of.[21] A solution of hydrogen peroxide (40 mM) was prepared in phosphate buffer, pH 7.4. The concentration of hydrogen peroxide was determined by absorption at 230 nm using a UV- visible spectrophotometer. The extracts (10–500 µg/mL) in distilled water were added to a hydrogen peroxide solution at 230 nm was determined after 10 mins against the blank solution containing phosphate buffer without hydrogen peroxide. Ascorbic acid was used as a standard.

Total antioxidant activity by phosphomolybdenum method
The total antioxidant capacity of the methanol extract was determined by the phosphomolybdenum method.[22] The assay is based on the reduction of Mo (VI) to Mo (V) by the extract and subsequent formation of green phosphate complex at acid P^H. A 0.3 ml extract was combined with 3 ml of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molyb- date). The tubes containing the reaction solution were incubated at 90°C for 90 mins. Then, after cooling the absorbance of the solution was estimated at 695 nm using a spectrophotometer against the blank. Methanol (0.3 mL) in the place of the extract was used as the blank. The total antioxidant activity is expressed as the number of gram equivalent of ascorbic acid. The calibration curve was prepared by mixing ascorbic acid (10–500 µg/mL) with methanol.

Statistical analysis
All the experiments were carried out in triplicate. Experimental results are expressed as mean ± standard deviation (SD) of three parallel measurements. Linear regression analysis was used to calculate the IC_{50} value.

Results
After the plant sample was extracted, the yield percentage of the methanolic extract of leaves extract of *G. sylvestre* is 29.2%.

Phytochemical screening
The analysis of phytochemical screening may be useful in the detection of the bioactive com- pounds and subsequently may lead to the drug discovery and pharmacological formulation. Phytochemical analysis of *G.sylvestre* plant was carried out in methanolic extract and results are shown in Table 1.

Table 1: Phytochemical screening of methanolic extract of G. sylvestre plants.

Compound	Methanolic Plant extract
Alkaloids	+
Glycosides	+
Reducing sugar	-
Proteins	+
Terpenoids	+
Phenol and Tannins	+
Steroid	+
Saponin	-
Coumarin	+
Anthocyanin	+
Leucoanthocyanin	+

Total phenolic and flavonoid content
The total phenolic content of methanolic extract of *G. sylvestre plant* measured by Folin-ciocalteu reagent in terms of gallic acid equivalent (the standard curve equation: $y = 0.0006x + 0.0367$, $R^2 = 0.9317$). The value obtained for the concentration of total phenols of 18.67±0.23mg/g dry weight respectively. The flavonoid content was expressed in terms of rutin equivalent (the standard curve equation: $y = 0.0013\ x + 0.0021$, $R^2 = 0.9877$). The concentration of flavonoid in plant extract of leaves 2.51±0.22mg/g dry weight. (Table 2). It has been recognized that flavonoid shows antioxidant activity and their effects on human nutrition and health are considerable. The result strongly shows that the phenol is important components of this plant and some of the pharmacological effects could be attributed to the presence of this invaluable component.

Table 2: Total phenolic and flavonoid contents of 5 medicinal plant extracts

Plant extract	Total phenolic compounds mg/g plant extract (in GAE)	Total flavonoids mg/g plant extract (in RUE)
G. sylvestre	18.67±0.23	2.51±0.12

Antioxidant activity
DPPH free radical scavenging activity
This assay is based on scavenging of the DPPH radical from the antioxidants, which produces a decrease in absorbance at 517 nm. The antioxidant activities of methanolic extract of *the plants were* 27.12% to 77.97% respectively and the standard ascorbic acid were 45.01% to 85.50%, at concentrations of 10 to 500 µg/mL (Figure 1).

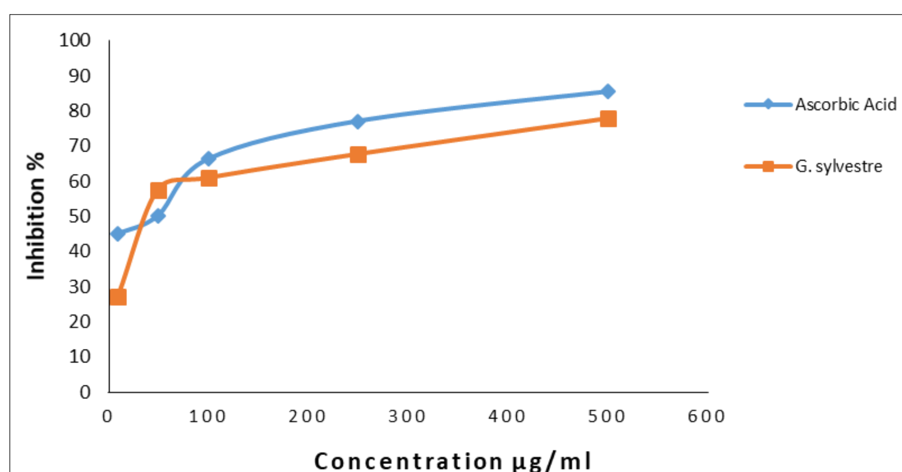

Fig 1: DPPH radical scavenging activity of methanolic extract of the plants and ascorbic acid. Values are expressed as the mean ± standard deviation (n = 3).

Hydroxyl radical scavenging activity

The methanolic extract of this plants showed the potential inhibitory effect of hydroxyl radical scavenging activity. The plant extract exhibited the minimum activity of *G.sylvestre* plants was 46.70% at 10 µg/mL and the maximum activity of 73.10% at 500 µg/mL and the standard ascorbic acid were 24.36% to 98.48%, at concentrations of 10 to 500 µg/m.
(Figure 2).

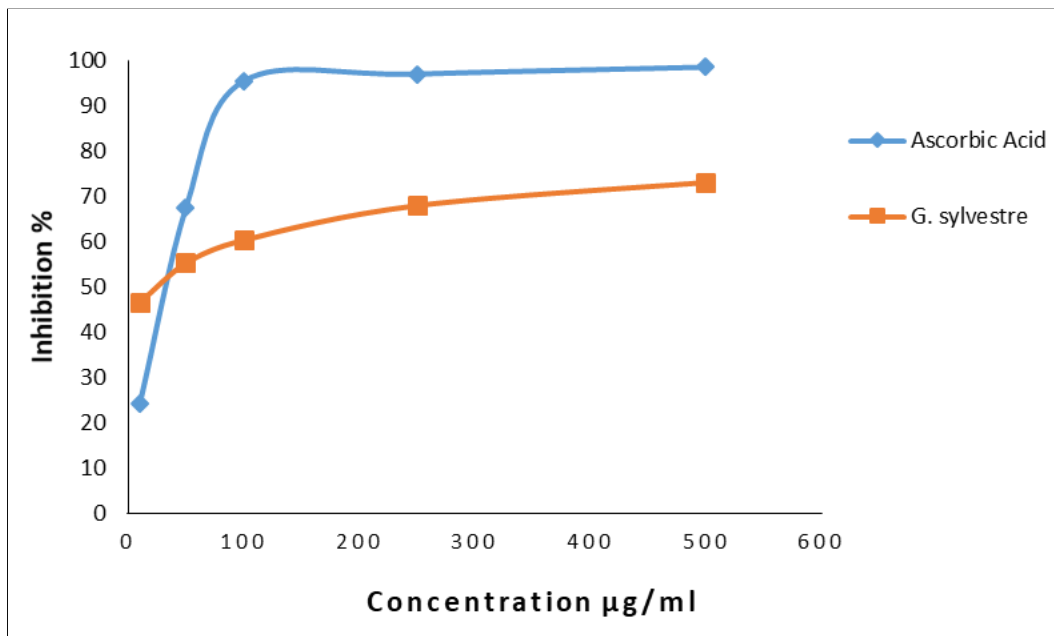

Fig 2: Hydroxyl radical scavenging activity of methanolic extract of the plants and ascorbic acid. Values are expressed as the mean ± standard deviation (n = 3).

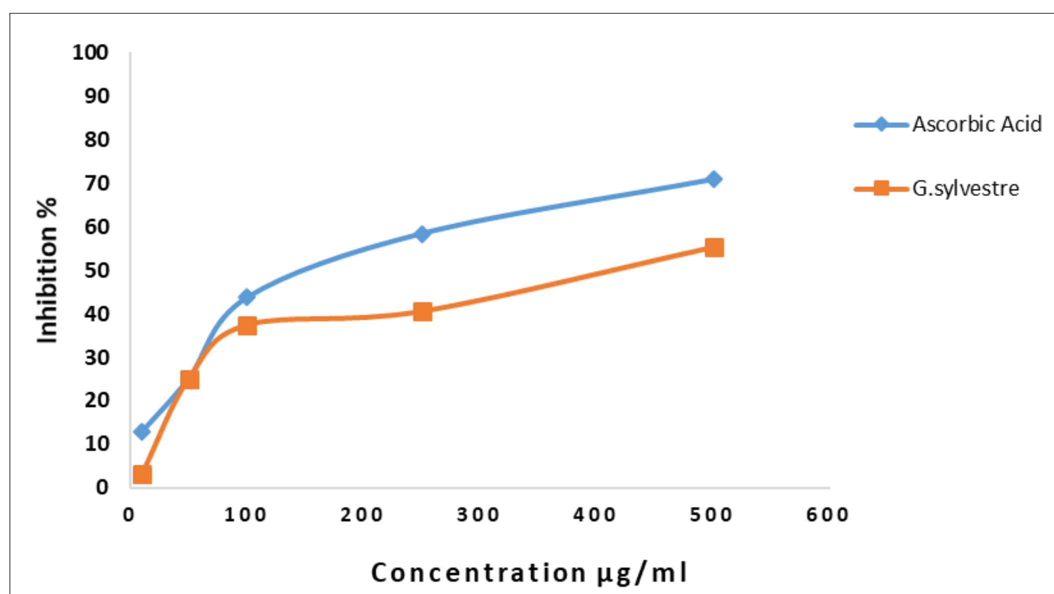

Fig 3: Superoxide radical scavenging activity of methanolic extract of the plants and ascorbic acid. Values are expressed as the mean ± standard deviation (n = 3).

Superoxide radical scavenging activity

The superoxide radical reduced NBT to blue colored formazan that can be measured at 560 nm. At 10–500 µg/mL, the superoxide scavenging activity of methanolic extract of *G.sylvstre* plant was 3.17% to 55.25% and then the standard ascorbic acid value was 12.9% to 70.81%. The result shows the concentration-dependent radical scavenging activity is increased with sample concentration (Figure 3).

Hydrogen peroxide radical scavenging activity

The free radical scavenging activity of *G. sylvestre* was evaluated by hydrogen peroxide (H_2O_2) scavenging method. From the results, the methanolic plant extract showed concentration-dependent activity and the H_2O_2

scavenging effect was 21.74% to 72.17% at concentrations of 10 to 500 µg/mL. This was comparable to the scavenging effect of ascorbic acid (49.02% to 88.54%,) (Figure 4).

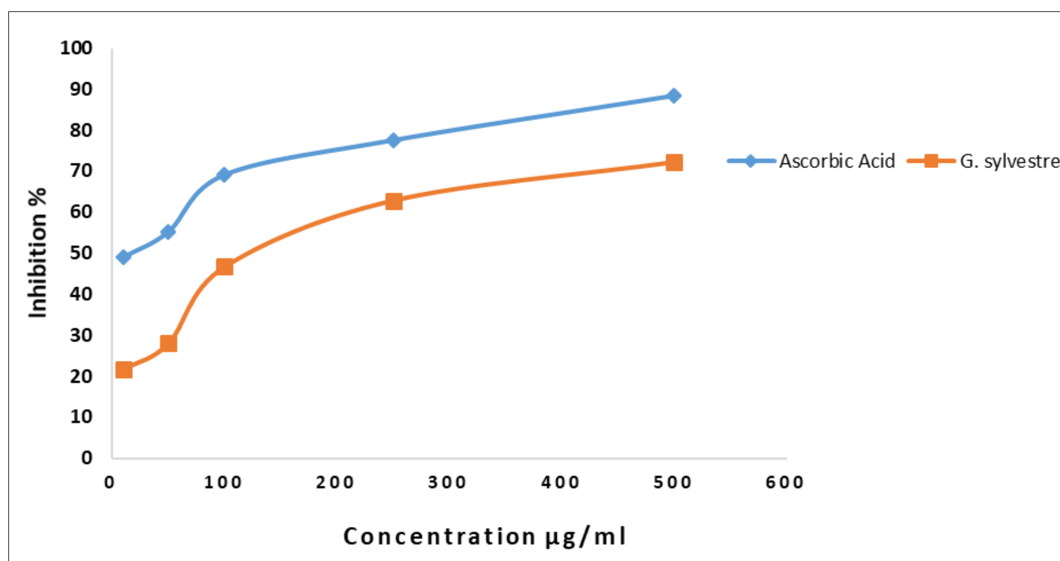

Fig 4: Hydrogen peroxide radical scavenging activity of methanolic extract of the plants and ascorbic acid. Values are expressed as the mean ± standard deviation (n = 3).

Total antioxidant activity by phosphomolybdenum method

The total antioxidant activity of the plants were evaluated by based on the reduction of Mo (VI) to Mo by the extract and subsequent formation of green phosphate complex at acid PH. From the results, the total antioxidant activity of the methanolic plant extract was prepared as ascorbic acid equivalents (AAE) per gram. The plant extract showed high antioxidant capacity (87.38 ± 0.04) (Table 2).

Table 3: IC_{50} values of the free radical scavenging activities of the methanolic extract of G.sylvestre.

	IC50 Value (µg/Ml)	
Parameters	*G.sylvestre*	Ascorbic acid
DPPH scavenging assay	38.81 ± 0.599	4.89 ± 0.380
Hydroxyl Radical scavenging assay	146.29 ± 1.726	61.78 ± 0.100
Hydrogen peroxide Radical scavenging assay	66.8 ± 0.992	55.84 ± 0.210
Super oxide radical scavenging assay	385.81 ± 0.599	248.93 ± 0.340

Table 3 showed the inhibited concentration (IC_{50}) values of the methanolic extract of *G.sylvestre*. It should be noted that the lowest value of IC_{50} indicates the strongest activity against free radicals. The results obtained showed that DPPH was the maximum trapping of the free radical with IC_{50} value was 38.81± 0.599 µg/mL followed by hydrogen peroxide, hydroxyl, and superoxide, with IC_{50} values were 66.8 ± 0.992 µg/mL, 146.29 ± 1.726 µg/mL and 385.81 ± 0.599 µg/mL respectively.

Discussion

G. sylvestre was commonly used to traditionally treat many diseases whose pathogenesis is, among other factors linked to oxidative stress. However, in order to antioxidant potentials of this plant that could be relevant in the treatment of such diseases have not been investigated. In a study, the phytochemical screening of methanolic extracts of *G. sylvestre* found that all the bioactive compounds are detected except saponin in methanolic extract. Phytochemicals are currently receiving the increased attention of interesting new findings regarding their biological activities. These compounds play some metabolic role and control development in a living system [23, 24].

So far as plant phenolics constitute one of the major groups of compounds acting as primary antioxidants or free radical terminators, it was reasonable to determine their total amount in the selected plant extract. Flavonoids as one of the most diverse and widespread groups of natural compounds are probably the most important natural phenolics. These compounds possess a broad spectrum of chemical and biological activities including radical scavenging properties. Such properties are especially distinct for flavonols. [17] Therefore, the content of both groups of phenolics was also determined in the extract (Table 2). In this study, the total phenolic and flavonoid content of methanolic leaf extracts of *G. sylvestre* were 18.67±0.23 mg/100 g and 2.51±0.12mg/100 g respectively.

IC_{50} value is defined as the concentration of substrate that causes 50% loss of the free radicals activity and was calculated by linear regression mentioned of plots of the percentage of antiradical activity against the

concentration of the tested compounds.[25] In this study, methanolic extract displayed the strongest inhibition of DPPH activity (IC_{50} 38.81± 0.599 µg/mL), showing less potency than the standard ascorbic acid (IC_{50} 4.89 ± 0.38 µg/mL). However, hydrogen peroxide (IC_{50}
66.8 ± 0.992µg/mL) showing more potency than the standard (IC_{50} 55.84 ± 0.21 µg/mL). This potency may also be related to the high antioxidant activity of the plant's extract, thereby mopping up free radicals that could be generated under hyperglycaemic condition.[26]

Conclusion

Today, antioxidant properties of this plant have become a vast interest due to their possible uses as natural additives to substitute synthetic ones. Thus, the results obtained in the present study showed that the methanolic extract of leaves of *G. sylvestre* plants contains the maximum antioxidant compound which can scavenge different Reactive oxygen species (ROS) and free radicals under *in vitro* conditions. The present study suggests that it can be used as a good source of natural antioxidants for health benefits and the bioactive compounds are required for identifying the unknown compounds to establish their pharmacological properties.

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Conflict of interest –The authors declare no conflict of interest.

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