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Phytochemical screening and biological studies of *Cynodon dactylon* (L.) Pers. extracts

Dikshita Lama[a]*, Dharma Prasad Khanal[a], Bishwo Raj Dhungana[b] and Bibek Sigdel[a]

[a,]Manmohan Memorial Institute of Health Sciences, Soalteemode, Kathmandu.
[b]Janamatri Foundation, Hattiban, Lalitpur Nepal

*For correspondence: dikshulama.dl@gmail.com

Abstract: A traditionally well-known perennial grass *Cynodon dactylon* (L.) Pers. has a variety of medicinal use such as a folk remedy in treatment of many symptoms and diseases like cramps, measles, tumors, wounds, warts, fever, and rheumatic affections. The objective of the present study was to evaluate antiulcer activity of the aerial extracts of *Cynodon dactylon* (L.) Pers in experimentally induced ulcers in rats. *Cynodon dactylon* (L.) Pers grass was collected from the Dhankuta district, Nepal, washed with water to, shed dried for a few days, cut into small pieces, and again shed dried for 3 weeks. After that it was pulverized in a grinder. Extraction was carried out using Methanol. The qualitative and quantitative Phytochemical screening was carried out using standard protocols. GC-MS analysis of the extract was done. The reducing power activity of the extracts of Cynodon dactylon (L.) Pers. was determined by the slight modification of the method of Oyaizu, 1986 (Bhalodia et al., 2013). In-vivo antiulcer activity was carried out taking Wistar Albino Rats in indomethacin induced ulcer. The methanolic extract of grass powder of *Cynodon dactylon* (L.) Pers. showed the presence of alkaloids, flavonoids, and cardiac glycosides in phytochemical screening. Similarly, the GC-MS analysis of methanolic extract of grass of *Cynodon dactylon* (L.) Pers. showed the presence of 6 compounds. The extract showed indicative amount of TPC and TFC. The antioxidant activity by RPA and DPPH was also indicative. Similarly, the methanolic extract of the grass of *Cynodon dactylon* (L.) Pers significantly ($p<0.05$) inhibited ulcer index, free acidity and total acidity in indomethacin induced ulcer models as compared to positive control group. The methanolic extract of grass of *Cynodon dactylon* (L.) Pers. can be used as a new source of antiulcer agent. The methanolic extract of grass of *Cynodon dactylon* (L.) showed the impressive ulcer protection in indomethacin induced ulcer in experimental rats, so it may be used for the search of novel antiulcer agents.

Keywords: Peptic ulcer, ranitidine, *Cynodon dactylon* (L.) Pers., ulcer index, indomethacin, methanolic-extract

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INTRODUCTION

Plants have been employed consistently over a long period of time to treat many ailments before their possibilities to use as medicine were being recognized by researchers. (Chong et al., 2012; Gupta et al. 2008). Approximately 80% of the population in the world trusts on traditional medicines for primary health care, utmost of which comprise the usage of plant extracts. (Linga Rao et al., 2008; Luitel et al., 2014). Peptic ulcer disease (PUD) refers to painful sores or ulcers in the lining of the stomach or first part of the small intestine, called the duodenum which impairs the quality of life and is associated with increased morbidity and mortality. (Gopinathan et al., 2014) There are lots of medicines available for the treatment of peptic ulcers but has shown significant adverse effects like impotence, headache, skin rash, atrophic gastritis, stomach distention, belching, constipation, dry mouth, urinary retention, blurred vision, xerostomia and precipitation of glaucoma (Reilly, 1999), edema and hypophosphatemia (Akhtar et al., 1992). Therefore, herbal drugs having better compatibility with human body has been used for the treatment of several ailments. They show less adverse effects and are easily available and cost-effective (Kaur et. al., 2012) *Cynodon dactylon* is a long-lived (perennial) grass, forming thick mats by means of stolons and rhizomes (Lusweti et al., 2011). Progressive studies on this plant have been stated that it has antiulcer, antidiabetic, anti-inflammatory, antibacterial, chemoprotective and hepatoprotective activities (Parekh et al., 2004; Garg et al., 2011). An aim was made to provide scientific basis to the traditional claims. We therefore evaluated the antiulcer activity of *Cynodon dactylon.*

MATERIAL AND METHODS

Plant material: Cynodon dactylon (L.) Pers grass was collected from Patle in the Dhankuta district of the Koshi zone, Nepal. The plant was identified by National Herbarium and Plant Laboratories, Godawari, Lalitpur, Nepal and the voucher number were 076/077/40.

Chemicals: Various chemicals used during the entire experiments are buffer solution pH 4 and pH 7, gallic acid, quercetin Himedia Laboratories Pvt. Ltd., Ranitidine: Lomus Pharmaceuticals Pvt. Ltd., Indomethacin: Omnica Laboratories Pvt. Ltd., Sodium carbonate, Folin-ciocalteu reagent, Methanol, $AlCl_3$, $NaNO_2$, NaOH, Trichloroacetic acid, Potassium ferricyanide, Ferric chloride, Ascorbic acid, and DPPH. These chemicals are purchased and manufactured from Merck and Qualigens Fine Chemicals.

Instruments: UV visible spectrophotometer (EI- 2372, India), Computer set with spectrophotometer software (Sync master 5915, Samsung), Digital pH meter (Labtronics, India), Digital centrifuge (Remi-R-8C, India), Digital hot air oven (Accumax- UG37, India), Digital incubator (Memmert, Germany), Digital weighing balance (RADWAG - AS 220 R2, USA), Rotary vacuum evaporator - RC5100 (Accumax India) and all glassware were from Borosil, Religlas & Aarosil.

Preparation of extracts: The grass *Cynodon dactylon* (L.) Pers was washed with water and shade dried for 2 weeks, cut into small pieces and again shed dried for 3 weeks. After that it was pulverized in a grinder. 100gm of the crushed powder was taken and soaked in 500 ml of

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methanol and macerated for 48 hours. It was then filtered primarily using muslin cloth followed by filtration through Whatman's filter paper No. 1. Subsequently, filtration was carried out for further 2 times and all the filtrate were mixed. It was then subjected to evaporation in rotating evaporator at the temperature of 55-60-degree Celsius and 32-35 revolutions per minute. The extracts were stored in a vial that was covered with parafilm. It was stored further in a desiccator (Chetia et al., 2014).

$$\text{Yield (\%)} = \frac{\text{Dry weight of extract}}{\text{Dry weight of plant powder}} \times 100$$ (Chong et al., 2012)

Qualitative Phytochemical analysis:
Qualitative preliminary phytochemical screening for the identification of alkaloids, tannins, saponins, glycosides and terpenoids were carried out for the extracts (Dahanayake et al., 2019). Tannins: Extract was taken and five drops of $FeCl_3$ was added to it and mixed. It was then observed for the black precipitate formation. Saponins: 5 ml of extract was taken to which 2.5 ml of water was added and the solution was shaken vigorously and observed for a stable persistent froth. The frothing was mixed with few drops of olive oil and shaken vigorously after which it was observed for the formation of an emulsion.Test for alkaloids: Picric acid test: 5 ml of extract was taken and few drops of picric acid was added to it. It was then observed for the formation of yellow color crystalline precipitate. Wagner reagent test: 5 ml of extract was taken and 2 drops of Wagner reagent was added to it. The reddish color confirms the presence of alkaloids.Test for flavonoids: to the test solutions, few magnesium turnings and conc. hydrochloric acid were added. It was then observed for red-orange color. To the test solutions, few drops of dilute ammonia solution followed by conc. H_2SO_4 was added. Intense yellow color was formed that indicates the presence of flavonoids.Test for Cardiac glycosides: 3 ml of extract was taken and 1 ml of glacial acetic acid was added to it. Again, H_2SO_4 was added to it gently. A violet brown or reddish-brown ring between the two liquids was observed. GC-MS analysis: GC-MS technique was used in this study to identify the components present in the extracts. The GC-MS analysis of the extract was conducted in Department of Food Technology and Quality Control, Babarmahal, Kathmandu Nepal for the analysis of compound present in the extract. The GC-MS model used for the analysis is GCMS-QP2010 Ultra Gas Chromatography Mass Spectrometer by Shimadzu, Japan.

Quantitative Phytochemical analysis: Determination of total Phenolic contents: Procedure-The amount of total phenolics in extracts was determined with the Folin-Ciocalteu reagent. (Sultana et al., 2012)Gallic acid was used as a standard and the total phenolics were expressed as mg/g gallic acid equivalents (GAE). Concentration of 0.01, 0.025, 0.05, 0.1 and 0.2 mg/ml of gallic acid were prepared in methanol. Concentration of 1mg/ml of plant extract were also prepared in methanol and 0.5ml of each sample were introduced into test tubes and mixed with 2.5ml of a 10-fold dilute Folin- Ciocalteu reagent and 2ml of 7.5% sodium carbonate. The tubes were covered with parafilm and allowed to stand for 30 minutes at room temperature before the absorbance was read at 760 nm spectrophotometrically. All determinations were performed in triplicate. The Folin-Ciocalteu reagent being sensitive to reducing compounds including polyphenols is producing a blue color upon reaction which is measured spectrophotometrically. Determination of total flavonoids content:
The total flavonoids content of each plant extract was estimated by method described by Zhishen et al. Based on this method, each sample (1.0ml) was mixed with 4ml of distilled

water and subsequently with 0.30ml of a $NaNO_2$ solution (10%). After 5 min, 0.30ml $AlCl_3$ solution (10%) was added fallowed by 2.0ml of NaOH solution (1%) to the mixture. Immediately, the mixture was thoroughly mixed and absorbance was then determined at 510 nm versus the blank. Standard curve of quercetin was prepared (0.01, 0.025, 0.05, 0.1 and 0.2 mg/ml) and the results were expressed as quercetin equivalents (mg quercetin/gm dried extract).

Biological activity- Antioxidant activity: Total reducing power activity: Procedure: The reducing power activity of the extracts of *Cynodon dactylon*(L.) Pers. was determined by the slight modification of the method of Oyaizu, 1986. (Bhalodia et al., 2013). Different concentrations of extract were mixed with 2.5ml of phosphate buffer (200 mM, pH 6.6) and 2.5ml of 1% potassium ferricyanide. The mixtures were incubated for 20 min at 50°C. After addition of 10 % trichloroacetic acid centrifuged at 650 rpm for 10 min. The upper layer (5ml) was mixed with 5ml of distilled water and 1ml of 0.1% ferric chloride and the absorbance of the resultant solutions were measured at 700 nm. The activity of the drug was compared with that of control. Here, ascorbic acid was used as a reference standard.DPPH radical scavenging activity: Procedure: DPPH in methanol (0.1 mM) was prepared and 3.0 ml of this solution was added to 1.0 ml of extract solution in methanol at different concentrations. Thirty minutes later, the absorbance was measured at 517 nm. A blank was prepared without adding extract. Ascorbic acid at various concentrations (10, 20, 30, 40, 50 µg/ml) was used as standard. Lower absorbance of the reaction mixture indicates higher free radical scavenging activity (Bhalodia et al., 2013). The capability to scavenge the DPPH radical was calculated using the following equation:

Calculation
DPPH Inhibition % was calculated using the following formula:

$$\text{Inhibition } \% = \frac{[\text{A(control)} - \text{A(test sample)}] \times 100}{\text{A(control)}}$$

IC_{50} value was determined from the plotted graph of inhibition % against the different concentrations of the sample, which is defined as the total antioxidant necessary to decrease the initial DPPH radical concentration by 50%.

In-vivo antiulcer activity: Experimental Animals: Wistar Albino Rats weighing 150-200 g were housed at 25° ± 5°C in a well-ventilated animal house under 12:12 h light dark cycle. Committee of Ethical guidelines approved the experimental protocol. The animals were maintained under standard conditions in an animal house as per the guidelines of NHRC Ethical Handing of Animals. Animals were grouped as follows: Group 1: Negative Control (Animals were fed with normal water and food *ad libitum*)Group 2: Positive Control (Ulcer was induced by Indomethacin 30 mg/kg b.w, *p.o*) (Sabiu et al., 2015)Group 3: Standard Control (38 mg/kg b.w,p.o- Ranitidine) (Swathi et al., 2015)Group 4: MLD (250 mg/kg b.w, *p.o*– Methanolic Low Dose extract of *Cynodon dactylon*(L.) Pers. Group 5: MHD (500 mg/kg b.w, *p.o*- Methanolic High Dose extract of *Cynodon dactylon*(L.) Pers. Indomethacin induced ulcer model: After 24 hours of fasting, animals were orally administered with indomethacin at a dose of 30 mg/kg. After 4 hours, animals were euthanized by cervical dislocation and parameters like gastric content, gastric pH, free acidity, total acidity, ulcer index and percent inhibition of ulcer are examined (Palle et al., 2018) .Collection of Gastric juice: Gastric juice was collected from stomach of Indomethacin induced rats. Stomachs were removed and

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opened along the greater curvature, gastric juice was collected, after that washed with distilled water and the ulcer was scored. Gastric juice collected was centrifuged for 3000 rpm for 10 minutes and was used for the estimation of pH, free acidity and total acidity (Sabiu et al., 2015).Determination of free acidity and total acidity: 1ml of gastric juice was diluted to 10 ml of distilled water and titrated with 0.01N NaOH using methyl orange indicator till end-point which is colorless. The volume of NaOH consumed is the free acidity. Again, the titration was carried out using phenolphthalein indicator till the appearance of red tinge. The volume of NaOH consumed corresponds to the total acidity. Gastric pH was determined using Digital pH meter. (Katary et al., 2017)

Acidity was calculated by using the formula: (Gopinathan et al., 2014)

$$\text{Acidity} = \frac{\text{Volume of NaOH x Normality of NaOH x 100 mEq/L}}{0.1}$$

Ulcer index (Soma et al., 2017)

$$\text{Ulcer Index} = \frac{\text{Number of ulcer} + \text{ulcer score} + \%\text{incidence}}{\text{Number of Animals}}$$

The number of ulcers/stomachs were noted and severity of the ulcers were scored as below (Muthukumar A et. al., 2016):

0 = Normal colored stomach
0.5 = Red coloration
1 = Spot ulcers
1.5 = Hemorrhagic streaks
2 = Ulcers > 3 but < 5
3 = Ulcers > 5

Mean ulcer score of each animal is expressed as ulcer index. (Soma et al., 2017)

$$(\text{Percent inhibition})\%\ \text{I} = \frac{\text{Ulcer index of control} - \text{ulcer index of test}}{\text{Ulcer index of control}} \times 100$$

RESULTS

Extractive value: The extractive value of methanolic extracts of *Cynodon dactylon*(L.) Pers. extracts was found to be 3.28 %. In the previous research performed by Muhtasim Kader Mukit et. Al, the extractive value for methanolic extraction was found to be 4.55 % (Mukit et al., 2017). This variation may be due to the different geographical conditions, time of collection, solvents used during the experiments and laboratory experimental conditions.

Preliminary Phyto-chemical investigation: The Preliminary Phyto-chemical investigation of methanolic extracts of *Cynodon dactylon* (L.) Pers. showed the presence of terpenes, saponins, alkaloids, flavonoids, cardiac glycosides, while the tannin was absent. In the previous research performed by Bagewadi et al. shows the presence of alkaloids, flavonoids,

tannins and glycosides in methanolic extracts that supports our results while test for anthraquinone and saponin showed the negative results (Bagewdi et al., 2014)
GC-MS Analysis: The GC-MS analysis of methanolic Extract of *Cynodon dactylon* (L.) Pers. showed the presence of main five compounds. Their retention times (RT), name, molecular formula, molecular weights (MW), similarity index (SI), area % and reported bioactivity are shown below:

Table no.: 1 - GC-MS analysis of methanolic Extract

S.N.	RT	Name	Molecular formula	MW	SI	Area %	**Activity
1.	26.236	9,12-Octadecadienoic acid	$C_{18}H_{32}O_2$	280	76	24.48	Anti-inflammatory (Yadav et al., 2017)
2.	26.535	Octadecadienoic acid	$C_{18}H_{36}O_2$	284	92	18.51	Surfactant as Conjugated linoleic acids
3.	25.80	Phytol	$C_{20}H_{40}O$	296	80	15.14	Anti-microbial, Anti-cancer, Diuretic, Anti-inflammatory, joint dislocation, Headache, Hernia (Chandel et al., 2015)
4.	23.451	n-Hexadecenoic acid	$C_{16}H_{32}O_2$	256	91	11.64	Antioxidant, Hypocholesterolemia, Hemolytic, Anti-androgenic (Chandel et al., 2015)
5.	25.625	9,12,15-Octadecatrienoic acid, methyl ester	$C_{19}H_{32}O_2$	292	73	10.51	Anti-microbial, Anti-cancer, Hepato-protective, Diuretic, Anti-arthritic, Anti-asthma (Chandel et al., 2015)

Determination of TPC and TFC: The total phenolic content of the extract was estimated taking gallic acid as standard. Methanolic extract of *Cynodon dactylon* (L.) Pers shows 161 mg/g dry material TPC in terms of gallic acid equivalent. The total flavonoids content of the extract was estimated taking quercetin as standard. Methanolic extract of *Cynodon dactylon* (L.) Pers shows 89.411 mg/g dry material TFC in terms of quercetin equivalent.In the previous study of total phenolic content conducted by Soumen Roy et al. showed 244.6µg/mg GAE in methanolic extract and 4.5µg/mg dry material TFC in terms of quercetin equivalent. (Roy et al., 2016)

The standard plot is shown in figure:

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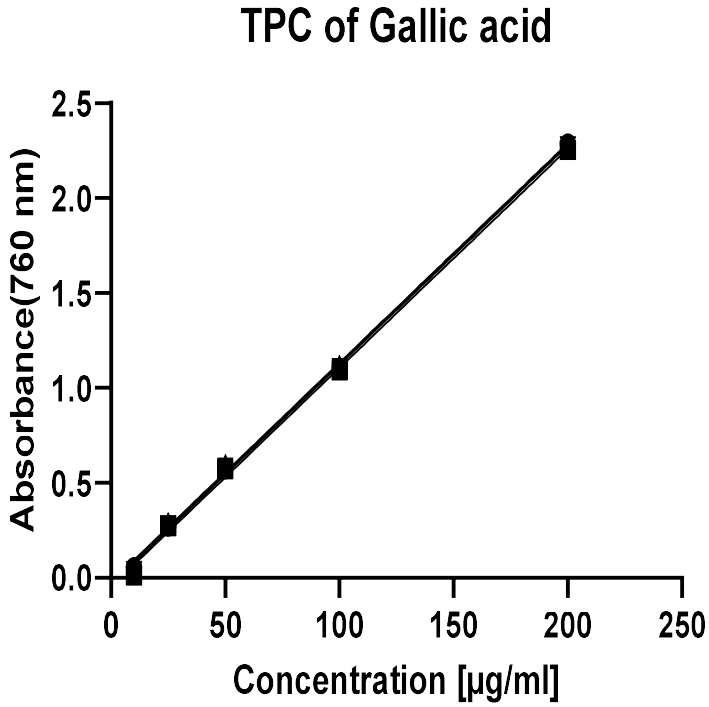

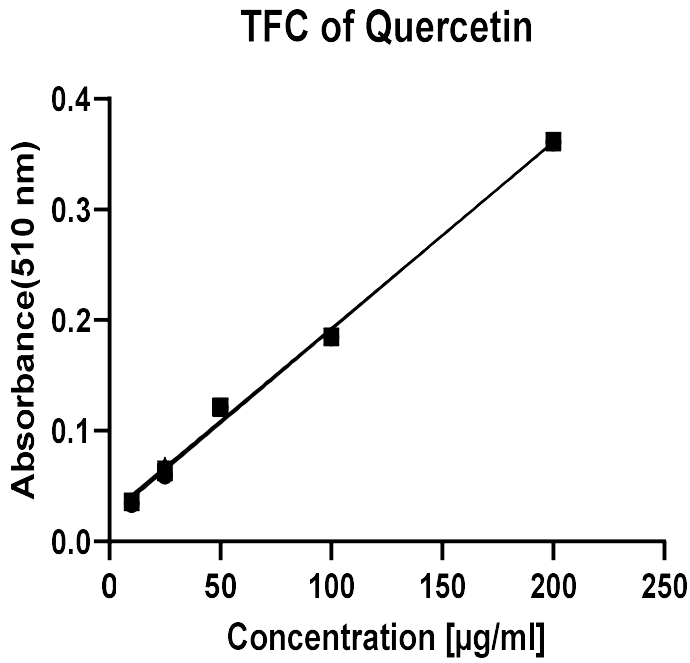

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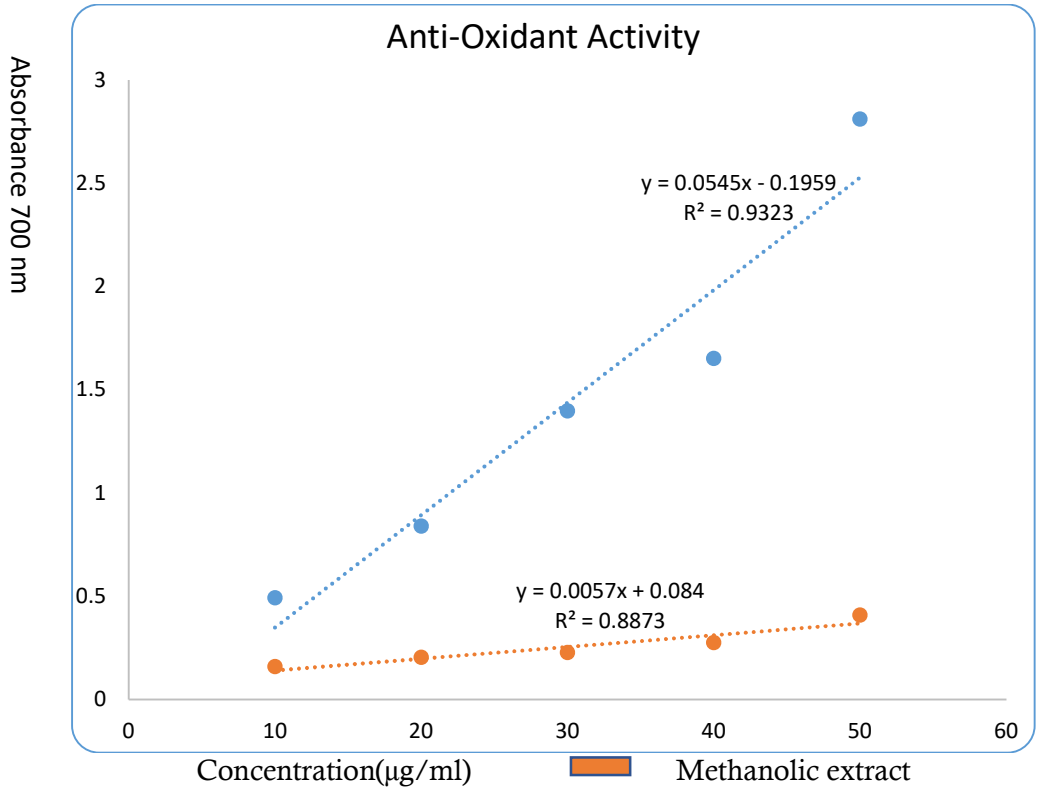
Antioxidant activity: Reducing power assay: Increased absorbance indicates the increase in the reducing power activity. The methanolic extract of *Cynodon dactylon* (L.) Pers. showed the absorbance of 0.41. In the previous study conducted by Melindakrishanti P et al. showed the absorbance of 0.72 (Krishanti et al., 2010). As the absorbance of ascorbic acid is greater in comparison to methanolic extracts of *Cynodon dactylon* (L.) Pers., ascorbic acid has greater reducing activity than methanolic extracts of *Cynodon dactylon* (L.) Pers. The graphical representation of the reducing power activity of ascorbic acid and methanolic extract are shown below:



DPPH assay: The % inhibitions were plotted against concentration and IC_{50} value of methanolic extract and ascorbic acid was found to be 24.13 µg/ml and 6.72 µg/ml respectively. Though the values are less than ascorbic acid, the study revealed that methanolic extracts of the plants showed DPPH radical scavenging activity. In the previous study conducted by Melinda krishanti P et al. showed the antioxidant values 10.48 ug/ml (Krishanti et al., 2010).

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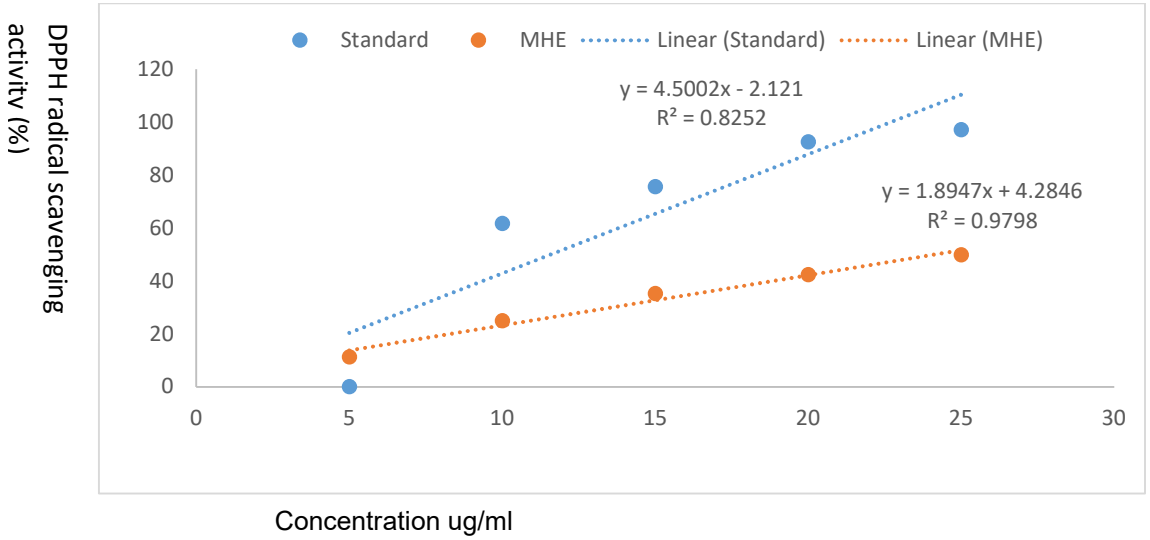

*In-vivo antiulcer activity of extracts of Cynodon dactylon (L.) Pers.:*Effect of *Cynodon dactylon*(L.) Pers. grass extract and Standard (Ranitidine) on Gastric content and Gastric pH in Indomethacin induced ulcers in rats. The gastric content was decreased in comparison to positive control in the doses (250 mg/kg and 500 mg/kg) of extracts which was also decreased in standard control. The extracts showed significant decrease in gastric content and significant increase in gastric pH. Photographs showing ulceration in Indomethacin induced ulcer in rats:

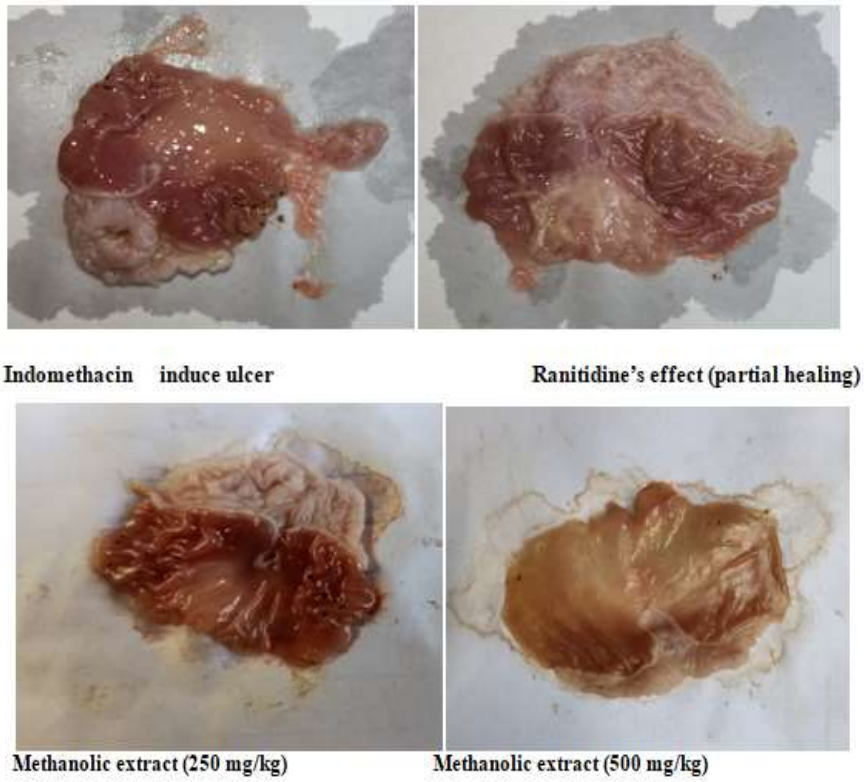

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Table 2 Effect of extracts on gastric content and gastric pH.

Treatment Group	Gastric content (ml)[Mean±SEM]	pH [Mean±SEM]
Negative Control (Normal diet)	1.500±0.1732	3.768±0.1309
Positive Control (Indomethacin 30 mg/kg b.w,p.o)	4.220±0.3693****	2.164±0.2158**
Standard Control (Ranitidine 38 mg/kg b.w,p.o)	1.760±0.2112****	5.226±0.3744****
MLD(250 mg/kg b.w,p.o)	2.860±0.09274**	4.084±0.3074**
MHD(500 mg/kg b.w,p.o)	2.220±0.2200****	4.866±0.2557****
Values are expressed as mean ± SEM (n = 5)		

Effect of Cynodon dactylon (L.) Pers. aerial extracts and Standard (Ranitidine) on free acidity and total acidity in Indomethacin induced ulcers in rats: The free acidity and total acidity were decreased in comparison to positive control in different doses of extracts which was also decreased in standard control. The dose of 500 mg/kg extracts showed greater decrease in free acidity and total acidity in comparison to 250 mg/kg of extracts of *Cynodon dactylon* (L.) Pers.

Table 3 Effect of extracts on free acidity and total acidity

Treatment Group	Free acidity (mEq/L)[mean±SEM]	Total acidity (mEq/L)[mean±SEM]
Negative Control (Normal diet)	25.60±2.600	45.20±6.689
Positive Control (Indomethacin 30 mg/kg b.w,p.o)	56.20±2.691****	101.0±8.792****
Standard Control (Ranitidine 38 mg/kg b.w,p.o)	35.20±6.598****	46.00±4.000****
MLD (250 mg/kg b.w,p.o)	44.00±2.121*	35.20±6.598*
MHD (500 mg/kg b.w,p.o)	29.80±2.871****	74.00±3.240****
Values are expressed as mean ± SEM (n = 5)		

*Effect of Cynodon dactylon (L.) Pers. aerial extracts and Standard (Ranitidine) on ulcer index and percent inhibition in Indomethacin induced ulcers in rats:*The ulcer index was decreased in comparison to positive control in different doses of extracts which was also decreased in standard control. The extracts showed significant decrease in ulcer index and significant increase in percent inhibition.

Table 4 Effect of extracts on ulcer index and % inhibition

Treatment Group	Ulcer index [mean ± SEM]	% Inhibition
Positive Control (Indomethacin 30 mg/kg b.w, p.o)	25.98±1.132	0
Standard Control (Ranitidine 38 mg/kg b.w, p.o)	12.54±0.269****	51.73
MLD(250 mg/kg b.w,p.o)	13.92±0.775****	46.42
MHD(500 mg/kg b.w,p.o)	8.46±0.291****	67.44
Values are expressed as mean ± SEM (n = 5)		

Values are expressed in terms of mean ± S.E.M. statistical analysis was carried by Graph Pad Prism through one-way ANOVA, followed by posthoc test. $P<0.05$ was considered significant. It may be due to the phenol, flavonoids and several compounds that are obtained by GC-MS analysis of the extracts. In addition, the anti-oxidant activity shown by the extracts may also be responsible for the anti-ulcer activity

CONCLUSION

The present study demonstrated that *Cynodon dactylon* (L.) Pers. grass protected gastric mucosa and has ulcer-protective activity that is evident by the significant inhibition of ulcers in indomethacin induced ulcer in rat models. *Cynodon dactylon* (L.) Pers. grass extract produces a significant, dose dependent gastroprotective effect in indomethacin induced ulcer model. Thus, the methanolic extract of grass of *Cynodon dactylon* (L.) Pers. can be used for the search of new antiulcer agent. Recommendation: Further investigation is suggested for the search of novel antiulcer agent taking samples from the different geographical regions of Nepal.

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DECLARATION OF CONFLICT OF INTEREST

We do hereby declare that the study does not encompass any conflict of interest.

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