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Research Article

COMPARATIVE ANALYSIS OF PHYTOCONSTITUTIONAL AND PHARMACOLOGICAL ACTIVITIES OF DIFFERENT SEASONAL TEA LEAVES EXTRACTS

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ABSTRACT

Methanolic extract of tea leaves (*Camellia sinensis* Linn.) of autumn and rain collections (S_A and S_R respectively) were investigated for *in vitro* antioxidants, antibacterial and wound healing activities to correlate with the impact of seasonal variation on phytoconstitutional content and commercial disparity. Both the extracts were subjected for quantitative and qualitative analysis and different concentrations (S_{A100}, S_{A200}, S_{R100} and S_{R200}) were investigated for *in vitro* antioxidant activity performing DPPH, super oxide anion, hydrogen peroxide, hydroxyl radical scavenging assay and measurement of reductive ability. The antibacterial activity of S_A and S_R was evaluated against eight human pathogens (two Gram positive and six Gram negative) using disc diffusion method and minimum inhibitory concentration of each was determined. The influence of S_A and S_R in 250 and 500 mg/Kg body wt. on rate of wound closure were investigated using the excision wound model on female wister rats (Sprague Dawley) and histological investigations of treated and untreated wound tissues were performed to observe epithelialisation, fibroblast proliferation, neovascularisation, neutrophil infiltration, and collagen deposition. The S_R extract showed higher antioxidant activities compared to S_A. Both the extracts showed significant antibacterial activities. The topical administration of S_{R500} caused significantly faster healing (99.05%), more epithelialisation, less neutrophils infiltration, higher deposition of collagen and better angiogenesis in wound area as compared to the S_{A500} and standard. These results limpidly showed that the S_R extract possess superior wound healing properties due to its higher anti oxidant and better microbial spectrum coverage probably by containing sesquiterpene derivative and higher flavonoid analogues.

Keywords: *Camellia sinensis*, seasonal variation, antioxidant, wound healing, antibacterial, sesquiterpene.

INTRODUCTION

Nature has been a source of medicinal treatments for thousands of years and plant-based systems continue to play an essential role in the primary health care of 80% of the world's underdeveloped and developing countries[1]. A source of safest and efficient medicinal agents like flavonoids, terpenoids, steroids etc has been provided in nature for traditional medicine since ancient civilisations[2]. About 1–3% of modern drugs being used for the treatment of wounds and skin disorders, almost one-third of all traditional medicines in use are for this treatment[3]. Herbs contain polyphenols which are most powerful natural antioxidants and are highly valued for their antioxidant and anti-aging effects. Antioxidant compounds like phenolic acids, terpenes, polyphenols and flavonoids inhibit the mechanism that leads to degenerative diseases[2]. Under certain conditions, oxygen can seriously affect our well being through the formation of reactive oxygen species (ROS) representing both free radical and non-free radical species which leads to the potential deleterious effects such as atherosclerosis, ischaemic heart disease, aging, inflammation, diabetes, immunosuppression, neurodegenerative diseases, cancer and other diseases[1]. Therefore, antioxidants with free radical scavenging activities may have great significance in the prevention and therapeutics of free radical mediated diseases such as wound. Studies have shown that several natural products, containing active compounds like triterpenes, alkaloids, flavonoids, and other biomolecules, promote the process of wound healing by influencing one or more phases of the healing process[4, 5].

Second only to water, tea is one of the most consumed beverages in the world[6]. The chemical compositions of *C. sinensis* is not completely known but is understood to be quite complex[7]. *C. sinensis* has been reported to have physiological and pharmacological effects strengthening capillary, slows down catabolism of catecholamine, exert anti inflammatory effects by enhancing effect of Vitamin C[8, 9]. It acts as an anti oxidant, also as hypocholesterolemic action. It inhibits angiotensin converting enzyme and the growth of implanted malignant cells[10]. One of the earliest reports of *C. sinensis* regarding its anti microbial activity is its recommendation by an army surgeon in soldiers' water bottles as a prophylactic against typhoid[11]. The antibacterial activities of tea extract are already established in several *in vitro* and *in vivo* models against intestinal pathogen[12,13,14]. The Japanese and others in a series of well conducted systemic studies showed that tea extract inhibit and kill various species of *Staphylococcus*, *Salmonella*, *Shigella* and *Vibrio*[15]. However, based on the literature survey the present study was designed to investigate the *in vitro* antioxidant activity, wound healing activity and antibacterial activity of different seasonal tea leaves extracts (*Camellia sinensis* Linn.).

MATERIALS AND METHODS

Sample collection and extraction

Fresh young leaves were collected from Jalpaiguri district (East Himalayan range known as Terai), West Bengal, India during the month of November 2010 and July 2011 for preparing autumn (S_A) and rain (S_R) extract respectively. The voucher specimens were identified and authenticated by Dr. Avoy Pada Das, Professor,

Taxonomy and Environmental Biology Laboratory, Department of Botany, North Bengal University (NBU), Darjeeling, West Bengal, India (Ref herbarium no. 09658; dated: 30.8.12, NBU). The leaves were washed with soft soap water to remove external dirt, pesticides. It was shade dried, powdered and sieved through sieve no.22 and finally macerated in a shaker subsequently with different solvents in their increasing order of polarity such as with petroleum ether, chloroform for 72 hours and with methanol for seven days at pH 7. Then the filtrate of both S_A and S_R extracts were concentrated to dryness under reduced pressure and controlled temperature below 45˚C in a rotary vacuum evaporator and the yield was preserved in a refrigerator at 4˚C for further use[16].

Preliminary phytochemical screening

The methanolic extracts of both S_A and S_R were subjected to preliminary phytochemical screening for alkaloids, flavonoids, carbohydrates, saponins, tannins, phytosterol, triterpenoids and sesquiterpenes in accordance to the procedure laid in Trease and Harbone with few modification[17,18].

HPLC-IR spectral analysis

Following materials and methodology were used for HPLC analysis

Shimadzu Model LC-10AT liquid chromatograph with Model SPD-10A UV-VIS detector, Tandem reversed-phase cartridge columns (C-18); Pecosphere, 4.6 x 33 mm, 3 micron,particles coated with octadecylsilane groups, 25-µL flat nose syringe,Methanol/water mobile phase for elution (filtered and sonicated) (70:30).

Following materials and methodology were used for IR analysis

Same eluant, obtained from chromatography separation, passed through a UV detector and then deposited as a solid phase track on the Zn-selenide collection disk of the DiscovIR. Following operating condition was adopted for IR for analysis like, Nebulizer power 14W, Gas flow 350 cc/min, Disk speed 3 mm/min, Disk temperature 0°C, Condenser Temperature 4°C, Chamber/ Condenser pressure 4 torr, Cyclone temperature 220 – 210°C[19].

Determination of total flavonoids content

The total flavonoids content was estimated using aluminium chloride method in accordance to the method described by Ordonez et al. (2006) with slight modification and was calculated as mg quercetin equivalent/g of extract powder, in reference to the standard curve of quercetin[20].

Experimental animals

24 healthy adult female Sprague dawley rats, 8 weeks old and weighing 210 to 234 g were obtained from the animal house, Department of Pharmaceutical Technology, Jadavpur University, Kolkata. All the studies were approved by the Animal Ethical Committee, Jadavpur University (Ref no. 0367/01/C/CPCSEA). Thirty five female Sprague dawley rats were housed in stainless steel cages and fed with normal commercial rat diet, given water *ad libitum* and maintained under laboratory conditions (temperature 28-30˚C, relative humidity 60-70%, and normal 12 hrs light and dark cycle). A day before the experiments the rats were brought to the laboratory and habituated to experimenter handling and the apparatus to minimize the effect of stress and novelty. All procedures and techniques used in these studies were in accordance with the National Institute of Health Guidelines for the Care and Use of Laboratory Animals (NIH, Department of Health Services publication No. 83-23, revised 1985). The protocols for the study were approved by the Departmental Ethics Committee.

Skin irritation test

This was carried out by the method of Gfeller *et al*., on rats with little modification[5]. About 500 mm^2 area on the dorsal fur of each animal was shaved. The shaved area was cleaned and the emulsion formulations of S_{A500} and S_{R500} were applied to different groups of animals. After 4 h of emulsion applications, the skin of each animal was observed for sign of inflammation.

Determination of in vitro antioxidant activity

DPPH Free radical scavenging assay

Different concentration of S_A, S_R (100 and 200 µg/ml of each test solution) and standard (Quercetin 25 and 50µg/ml) solutions were prepared in methanol. The samples were incubated at room temperature (in dark) for 30 minutes after adding the freshly prepared DPPH (30 mM). Thereafter the absorbance of each sample was measured at 518 nm. The percentage of antioxidant activity was calculated using the following formula[21]:

$$AA\ \% = 100\ \{[Abs_{sample} - Abs_{control}) \times 100] / Abs_{control}\}$$

The experiment was carried out using the method of Oyaizu *et al.,* 1986 with little modification[22]

Measurement of reductive ability

For the measurement of the reductive ability, we investigated the Fe^{+3} to Fe^{+2} transformations in the presence of S_A and S_R extracts and quercetin in different concentrations using the method of Oyaizu *et al*., 1986 with little modification[22]. Definite concentrations of S_A and S_R extracts (at 100 and 200 µg/ml for each) and quercetin (25 and 50 µg/ml), 2.5 ml phosphate buffer pH – 6.6), 2.5 ml 1% $K_3Fe(CN)_6$ were incubated at 50˚C for 20 minutes. 2.5 ml of 10% trichloro acetic acid (TCA) were added to the mixture and centrifuged for 10 minutes at 3000 r.p.m. After centrifugation, 2.5 ml of the supernatant were diluted with 2.5 ml water and shaken with 0.5 ml freshly prepared $FeCl_3$ (0.1%). The absorbance was measured at 700 nm. An increase in absorbance indicated higher reductive ability.

Super oxide anion scavenging assay

The superoxide scavenging ability was assessed according to the method of Oyaizu *et al*., 1986 with slight modifications[22]. 1 ml of nitroblue tetrazolium (NBT) solution and 1 ml of NADH solution were mixed with 0.1 ml of different tests and standard solutions. Then, 100 µm (prepared in phosphate buffer, pH 7.4) of phenazine methosulphate (PMS) solution was added to each sample. The samples were then incubated at room temperature for 5 minutes. The absorbance of the samples was measured at 560 nm. Decrease of absorbance indicated superoxide scavenging activity.

Hydroxyl radical scavenging activity

The assay was done by Elizabeth and Rao method with few modifications[23]. It is based on quantification of the degradation product of 2-deoxyribose by condensation with TBA. Hydroxyl radical was generated by the Fe^{3+}-ascorbate-EDTA-H_2O_2 system (the Fenton reaction). The reaction mixture contained, in a final volume of 1 ml, 2-deoxy-2-ribose (2.8 mm); KH_2PO_4 -KOH buffer (20 mm, pH 7.4); $FeCl_3$ (100 µ); EDTA (100 µm); H_2O_2 (1.0 mM); ascorbic acid (100 µm) and concentrations (250 and 500 µg/ml) of the test sample or reference compound. After incubation for 1 h at 37°C, 0.5 ml of the reaction mixture was added to 1 ml 2.8% TCA, then 1 ml 1% aqueous TBA was added and the mixture was incubated at 90°C for 15 min to develop the color. After cooling, the absorbance was measured at 532 nm against an appropriate blank

solution. All tests were performed for three times. Quercetin was used as a positive control. Percentage inhibition was evaluated by comparing the test and standard solutions with control.

Evaluation of wound healing activities

Excision wound model

About thirty five animals (female Sprague Dawley rats) weighing 210 to 234 g was anaesthetized with pentobarbitone (30 mg/kg body weight) intraperitoneal prior the creation of the wounds. The dorsal fur of the animals was shaved nearly to a circular diameter of about 2 cm by means of razor blades and the anticipated area of the wound to be created was outlined on the shaved skin by picric acid. Incision of the muscle layer was avoided during the procedure so that the tension of the skin would not be affected. The wound area was measured immediately by placing transparent scale. This was subsequently placed on a 1 mm^2 graph sheet. The area of the counted squares was calculated in square millimetres and the area was recorded. The entire wounds were left opened and the animals divided into seven groups of five animals each[24].

Topical wound application

The Group I animals were topically treated with 0.2 ml of povidone iodine solution twice daily as a reference standard control. Wounds of Group II rats were topically untreated as a negative control, Wounds of Group III rats were topically treated with 0.2 ml emulsion vehicle per application (10% CMC +10% Tween 20 + 50% DMSO + distilled water quantity sufficient to 100ml) alone twice daily as positive control group. The Groups IV and V animals were topically treated with 0.2 ml emulsion per application of 500 mg/ml and 250 mg/ml of S_R extract in vehicle, respectively. The Groups VI and VII animals were topically treated with 0.2 ml emulsion per application of 500 mg/ml and 250 mg/ml of S_A extract in vehicle, respectively. Wound treatment commenced from the 1st day i.e. the day next to the day of wounding surgery (referred as day one). The standard and tests preparations were topically applied to the wounds 12 hourly for 24 days. In the course of treatment, scaled photographs of the wound areas were taken alongside a millimetre scale measurement the 1st and 24th day of wound treatment.

Estimation of wound healing (Wound Closure)

The wound closure area of each animal was assessed by tracing the outline of the wound on day 0, 15 and 24 after wounding surgery and the wound closure rate was expressed as the percentage of wound area compared with that on post-operative day; that is, the change in wound size was expressed as a percentage of the original wound size contraction.

$$\text{Percentage wound contraction} = (\text{Initial wound size} - \text{Specific day wound size}) / (\text{Initial wound size}) \times 100.$$

Histological evaluation of healed wounds

Skin tissue samples of all groups were immediately fixed after animal dissection, in 10% buffered formalin, dehydrated, and processed using microtome for paraffin block sectioning. The wound tissues were cut 5-7 µm thick perpendicular to the block, stained with standard haematoxylin and eosin (H and E) dye, and examined using an Olympus BX60 light microscope. The parameters examined in each histology slide were epithelialisation, inflammatory cell infiltration, fibroblast proliferation, angiogenesis and collagen deposition.

Determination of antibacterial susceptibility

The two gram positive and six gram negative micro organisms were selected for the determination of antibacterial effect of S_A and S_R extracts. The study was performed using disc diffusion technique on nutrient agar medium as described by Vincent and Vincent with few modifications[25]. The discs were prepared with Whatman filter paper (6 mm in diameter) and after proper sterilization it was impregnated with 20 µl (equivalent to 0.2mg of extract) of S_A and S_R extracts for 2 h and subsequently dried at 50°C. About 4 % DMSO solution was used to dissolve S_A and S_R extracts in distilled water. About 0.2 ml of a 24 h broth culture (10^6cfu/ml) of the isolated bacteria species was spread on the surface of the sterile nutrient agar plates with sterile cotton wool swabs. The inoculated plates were incubated at 37°C or 30°C for 48 h depending upon the type of organism under optimum condition. Antibacterial activity of the S_A and S_R extracts against the test bacteria was indicated by growth free "zone of inhibition" near the respective disc which were compared with both negative control (without applying any drug on plate), positive control (4% DMSO alone in the disc) and standard (reference drug Gentamicin 10 µg/disc) in accordance to NCCLS method and zone measuring 18 mm or more was considered as effective against test organisms and 13 mm or more was considered as resistant.

Minimum Inhibitory Concentrations (MIC)

The S_A and S_R extracts of the plant was subjected for MIC determination on six (*Klebsiella pneumonia*-725*, Bacillus brevis*-NCTC7096*, Staphylococcus aureus*-ML411*, Shigella dysenteriae, Pseudomonas aeruginosa*-71 and *Streptococcus pneumonae*) susceptible organisms depending upon the sensitivity test results. Both the S_A and S_R extracts were dissolved in sterile water with 4% DMSO and prepared different concentration of solutions ranging from 1050-3800 µg/ml by serial dilution method and transferred about 1.0 ml to each individual sterile test tube. About 1.0 ml of sterile nutrient broth was poured in all those test tubes and mixed thoroughly. The selected bacterial strain suspension (1.0 ml) was inoculated in each test tube and incubated at 37°C for 24 h. The MIC of the extracts was regarded as the lowest concentration of the extract that did not permit turbidity or growth of the test organism[26]. The test tube containing only nutrient broth was assigned as negative control whereas the nutrient broth along with bacterial culture and 4% DMSO alone in the disc as positive control for this study.

Statistical analysis

The results were expressed as mean ± SEM. Statistical differences between the treated and control groups were determined by one way ANOVA followed by Dunnet's test using the computer software, Graph pad Prism 5. $P < 0.05$ was considered for statistically significant.

Table 1: Preliminary phytochemical screening

	Alkaloids	Flavonoids	Saponins	Tannin	Phytosterol	Triterpenoids	Sesquiterpenes
S_R	+	++	+	+	+	+	+
S_A	+	+	+	+	+	-	-

Table 2: Phytochemicals screening by IR spectra

Sample Type	S_A	S_R	Type of bond-functional groups
frequency, cm–1	-	1592.3	1600–1585 (medium) C–C stretch (in–ring) aromatics

Table 3: DPPH free radical scavenging activity of S_A and S_R

Groups	Mean ± SEM	% inhibition	P-value
Control	0.6317±.0044	-	-
Quercetin-25 µg/mL	0.1933±.0044	69.39	<.01
Quercetin-50 µg/mL	0.1233±.0088	80.47	<.01
Tea Extract S_{A100}	0.4000±.0265	36.68	<.01
Tea Extract S_{A200}	0.6033±.0260	04.49	<.01
Tea Extract S_{R100}	0.3267±.0033	48.28	<.01
Tea Extract S_{R200}	0.1900±.0058	69.92	<.01

Values are expressed as Mean ± SEM; n= 3
S_A and S_R stands for sample autumn and rain respectively

Table 4: Superoxide anion scavenging activity of S_A and S_R

Groups	Mean ± SEM	% inhibition	P-value
Control	0.8283±.0017		-
Quercetin-25 µg/mL	0.3223±.0017	61.09	<.01
Quercetin-50 µg/mL	0.1970±.0017	76.22	<.01
Tea Extract S_{A100}	0.6633±.0075	19.92	<.01
Tea Extract S_{A200}	0.7550±.0029	8.85	<.01
Tea Extract S_{R100}	0.2083±.0046	74.85	<.01
Tea Extract S_{R200}	0.7550±.0029	86.72	<.01

Values are expressed as Mean ± SEM; n= 3
S_A and S_R stands for sample autumn and rain respectively

Table 5: Reductive ability of S_A and S_R

Groups	Mean ± SEM	% inhibition	P-value
Control	0.0417±.0060	-	-
Quercetin-25 µg/mL	0.8883±.0017	95.31	<.01
Quercetin-50 µg/mL	0.8977±.0043	95.36	<.01
Tea Extract S_{A100}	0.3223±.0015	87.07	<.01
Tea Extract S_{A200}	0.3257±.0012	87.21	<.01
Tea Extract S_{R100}	0.6963±.0009	94.02	<.01
Tea Extract S_{R200}	0.7057±.0035	94.10	<.01

Values are expressed as Mean ± SEM; n= 3
S_A and S_R stands for sample autumn and rain respectively

Table 6: Hydroxyl radical scavenging activity of S_A and S_R

Groups	Mean ± SEM	% inhibition	P-value
Control	0.8350±.0126	-	-
Quercetin-25 µg/mL	0.1590±.0021	80.96	<.01
Quercetin-50 µg/mL	0.1220±.0012	85.39	<.01
Tea Extract S_{A100}	0.7883±.0060	5.59	<.01
Tea Extract S_{A200}	0.7800±.0029	6.59	<.01
Tea Extract S_{R100}	0.7590±.0046	9.10	<.01
Tea Extract S_{R200}	0.7033±.0060	15.77	<.01

Table 7: Determination of zone of inhibition by disc diffusion technique
(Zone diameter was expressed in millimetre)

	Gr.I EC	Gr.II VC	Gr.III KP	Gr.IV BB	Gr.V SA	Gr.VI SD	Gr.VII PA	Gr.VIII SP
S_A	8.10 ± 0.06	10.67 ± 0.24	18.27 ± 0.22	11.00 ± 0.12	23.40 ± 0.21	19.9 ± 0.06	18.47 ± 0.15	18.2 ± 0.06
S_R	8.33 ± 0.20	12.73 ± 0.20	19.13 ± 0.19	11.70 ± 0.12	24.50 ± 0.12	20.17 ± 0.03	19.57 ± 0.28	20.33 ± 0.09
Standard	28.67 ± 0.09	21.17 ±0.09	23.60 ± 0.67	11.87 ± 0.09	24.67 ± 0.19	25.00 ± 0.06	19.63 ± 0.33	20.87 ± 0.09
Negative Control	NG	NG	NG	NG	NG	NG	NG	NG
Positive Control	GO	GO	GO	GO	GO	GO	GO	GO

EC: *E.coli*-ATCC25938; VC: *Vibreo choleriae*-1403; KP: *Klebsiella pneumonia*-725*;* BB: *Bacillus brevis*-NCTC7096;
SA: *Staphylococcus aureus*-ML411; SD: *Shigella dysenteriae;* PA: *Pseudomonas aeruginosa*-71; SP: *Streptococcus pneumonae*;
NG: Negative Growth; GO: Growth Observed
Values are expressed as mean ± SEM of three determinations (n=3), P< 0.01.

Table 8: Determination MIC against six human pathogenic organisms

Plant extract (µg/ml)	Gr.III KP	Gr.IV BB	Gr.V SA	Gr.VI SD	Gr.VII PA	Gr.VIII SP
SA 1000	+	+	+	+	+	+
SA 1200	+	+	+	+	+	-
SA 1500	+	+	+	+	+	-
SA 2000	+	+	+	+	+	-
SA 2500	+	+	+	+	+	-
SA 3000	-	+	-	+	+	-
SA 3500	-	-	-	+	+	-
SA 4000	-	-	-	-	+	-
SR 1000	+	+	+	+	-	-
SR 1200	+	+	+	+	-	-
SR 1500	+	+	+	+	-	-
SR 2000	-	-	-	+	-	-
SR 2500	-	-	-	+	-	-
SR 3000	-	-	-	+	-	-
SR 3500	-	-	-	+	-	-
SR 4000	-	-	-	-	-	-
Standard	-	-	-	-	-	-
Negative control	-	-	-	-	-	-

KP: *Klebsiella pneumonia*-725; BB: *Bacillus brevis*-NCTC7096; SA: *Staphylococcus aureus*-ML411; SD: *Shigella dysenteriae*; PA: *Pseudomonas aeruginosa*-71*;* SP: *Streptococcus pneumonae;* '+' indicate bacterial growth, '-' indicate no bacterial growth,

Table 9: Determination of S_R and S_A on excision wound model

Groups	Wound area zero day (sq.cm)	Wound area on day 15th (sq.cm)	Wound area on day 24th (sq.cm)	% Wound healing on day 24th
Standard	3.70 ± 0.42	2.08 ± 0.23	0.54 ± 0.22	85.4
Negative control	4.57 ± 0.21	4.44 ± 0.45	3.77 ± 0.21	4.80
Positive control	3.71 ± .23	3.34 ± 0.41	3.16 ± 0.15	14.82
S_{R250}	4.61 ± 0.11	3.03 ± 0.40	0.56 ± 0.14	87.85
S_{R500}	4.20 ± 0.19	1.59 ± 0.21	0.04 ± 0.03	99.05
S_{A250}	4.27 ± 0.18	2.21 ± 0.31	1.93 ± 0.28	54.80
S_{A500}	4.14 ± 0.26	2.84 ± 0.22	1.32 ± 0.26	68.11

Values are expressed as Mean ± SEM; n= 3

S_A and S_R stands for sample autumn and rain respectively

Table 10: Year wise prices of tea leaves at Indian auction in rainy and autumn season

Year	Price in Rupees/kg tea leaves	
	Rain season	Autumn season
2011	100.92	103.79
2010	94.55	103.38
2009	98.63	105.22
2008	77.08	86.36
2007	65.81	66.19
2006	64.72	66.27

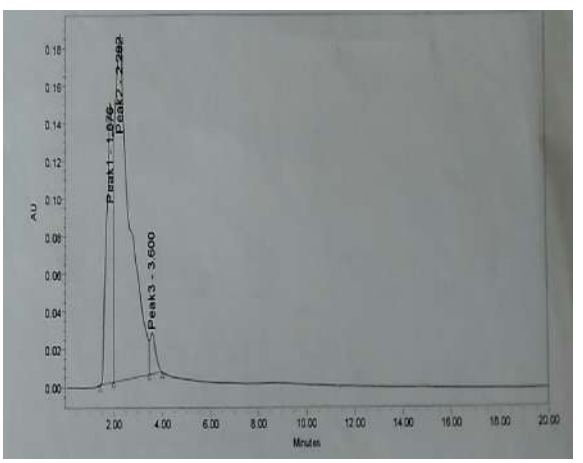


Figure 1: HPLC curves for autumn sample (S_A)

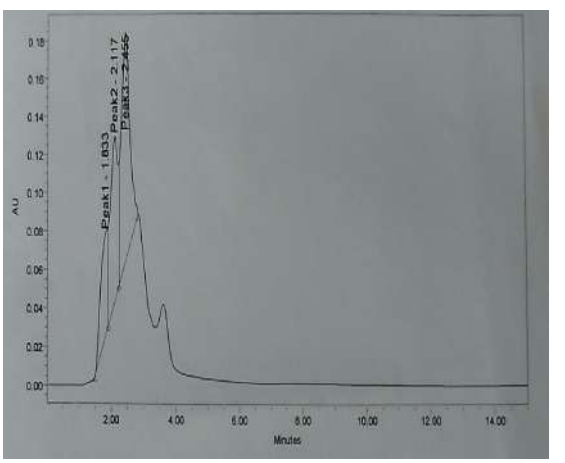


Figure 2: HPLC curves for rain sample (S_R)

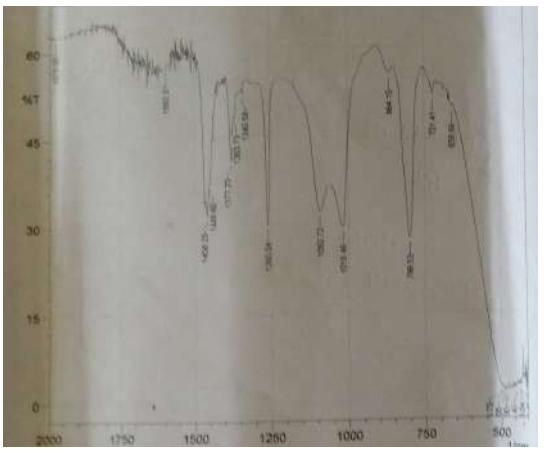


Figure 3: Rain sample IR spectrum

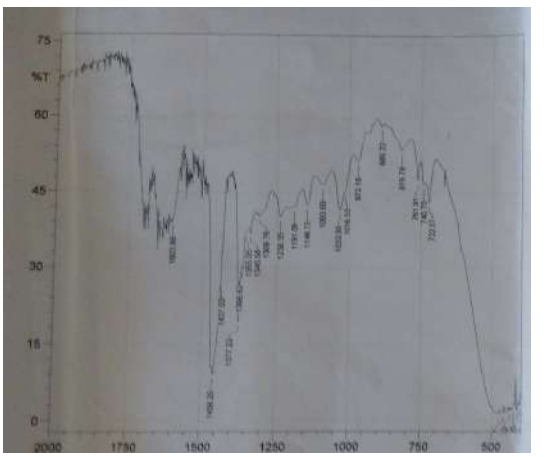


Figure 4: Autumn sample IR spectrum

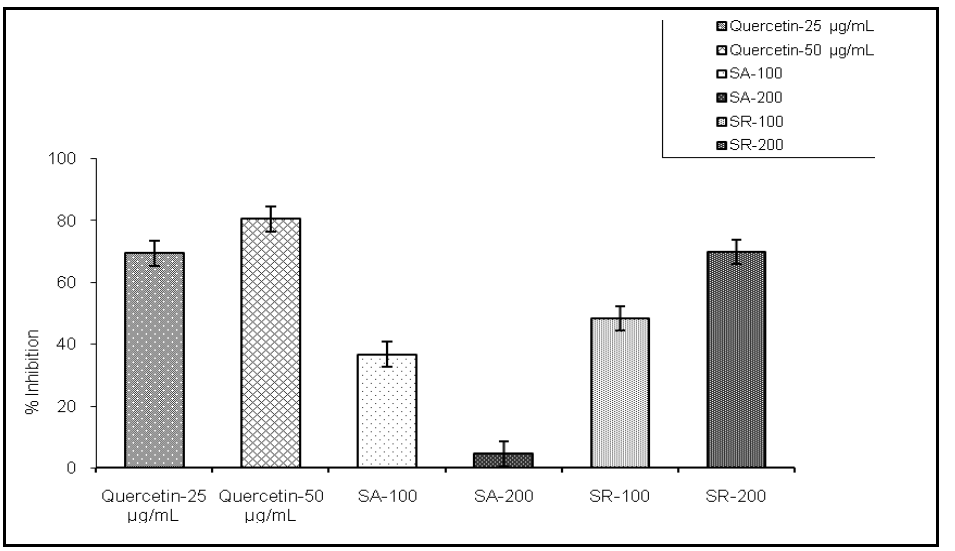


Figure 5: DPPH free radical scavenging activity of S_A and S_R

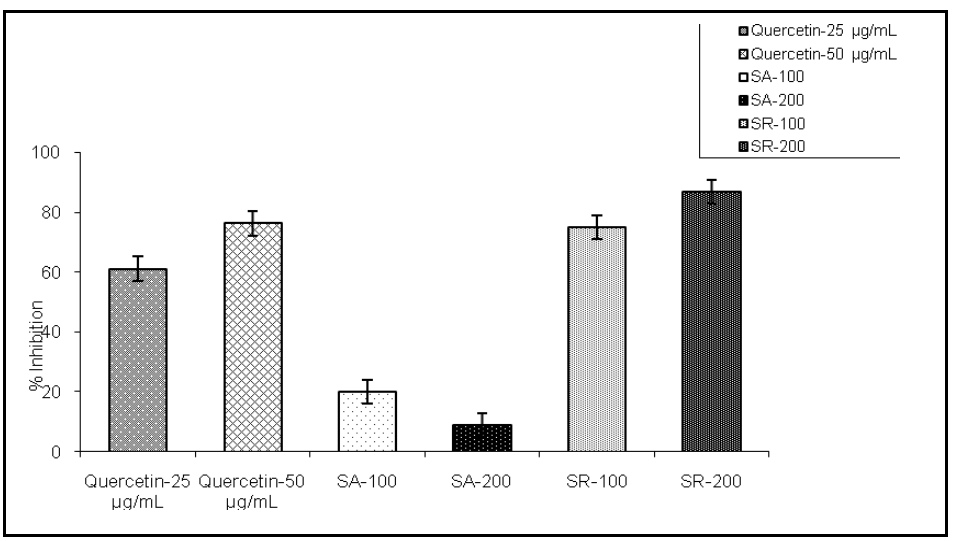


Figure 6: Superoxide anion scavenging activity of S_A and S_R

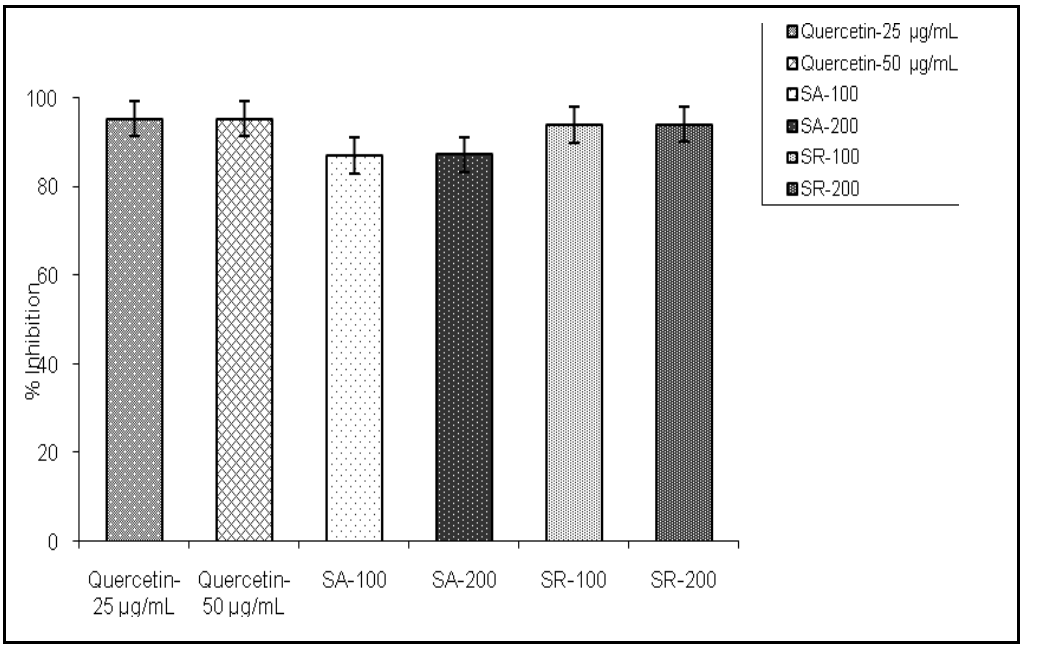


Figure 7: Reductive ability of S_A and S_R

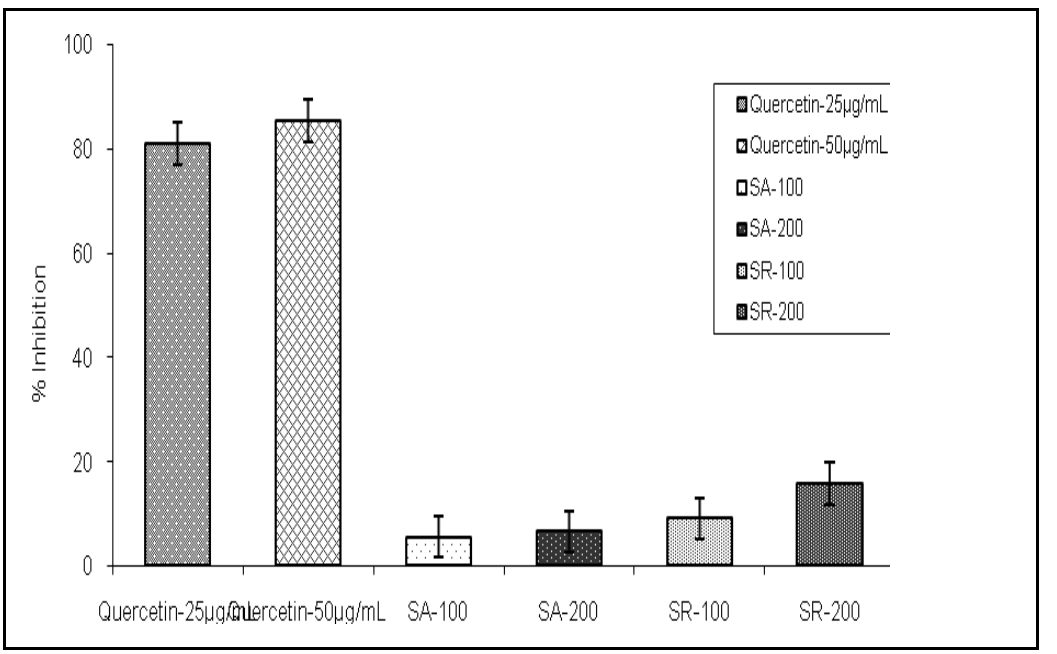
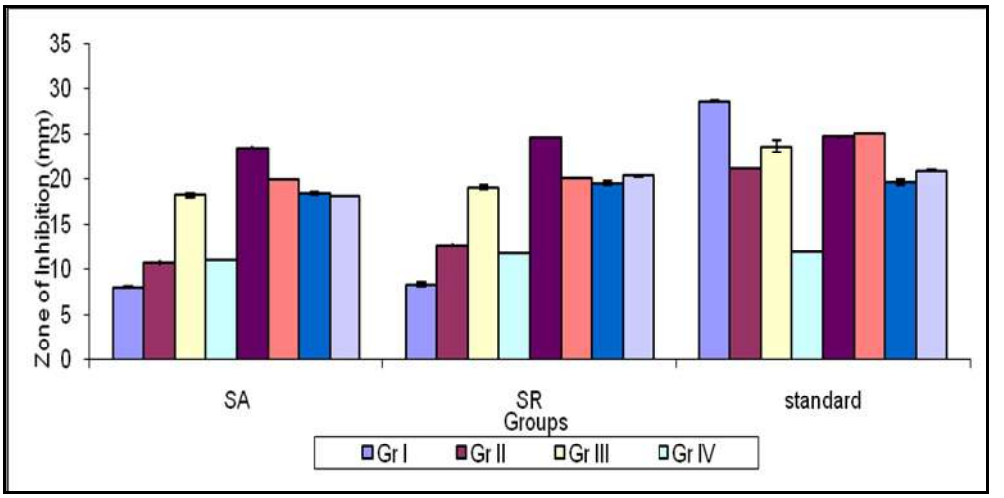


Figure 8: Hydroxyl radical scavenging activity of S_A and S_R



Values are expressed as Mean ± SEM; n= 3; Where, $P< 0.01$.

Figure 9: Determination of zone of inhibition in mm by disc diffusion technique

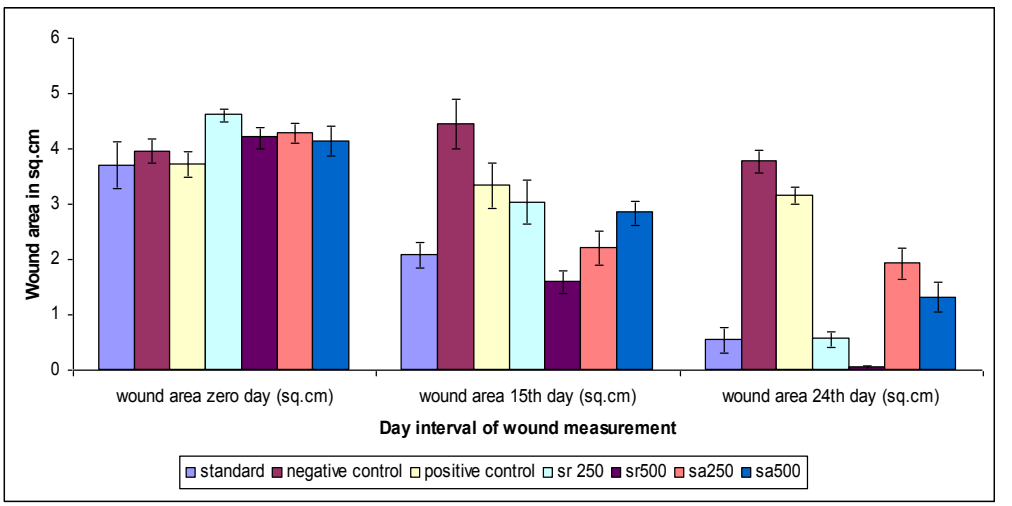


Figure 10: Determination of excision wound area for S_R and S_A on day 15

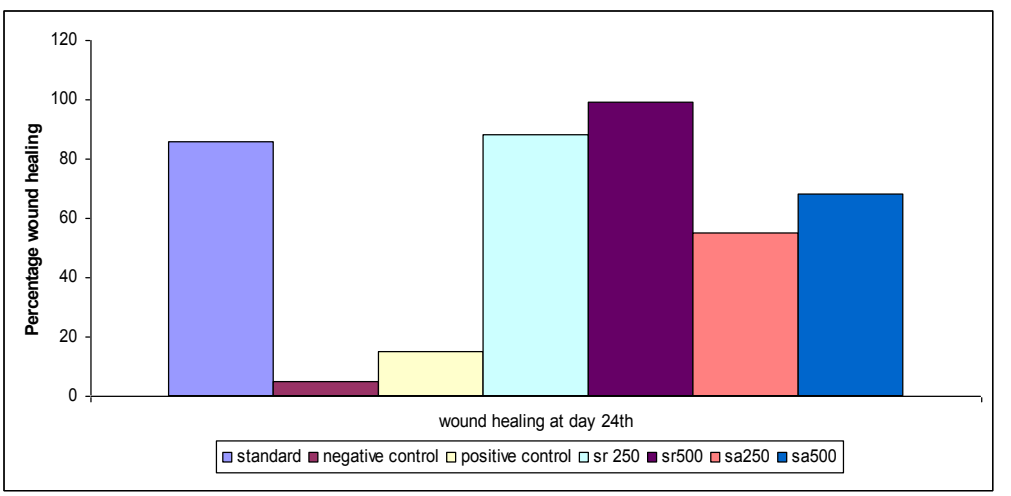


Figure 11: Determination of excision wound area for S_R and S_A on day 24

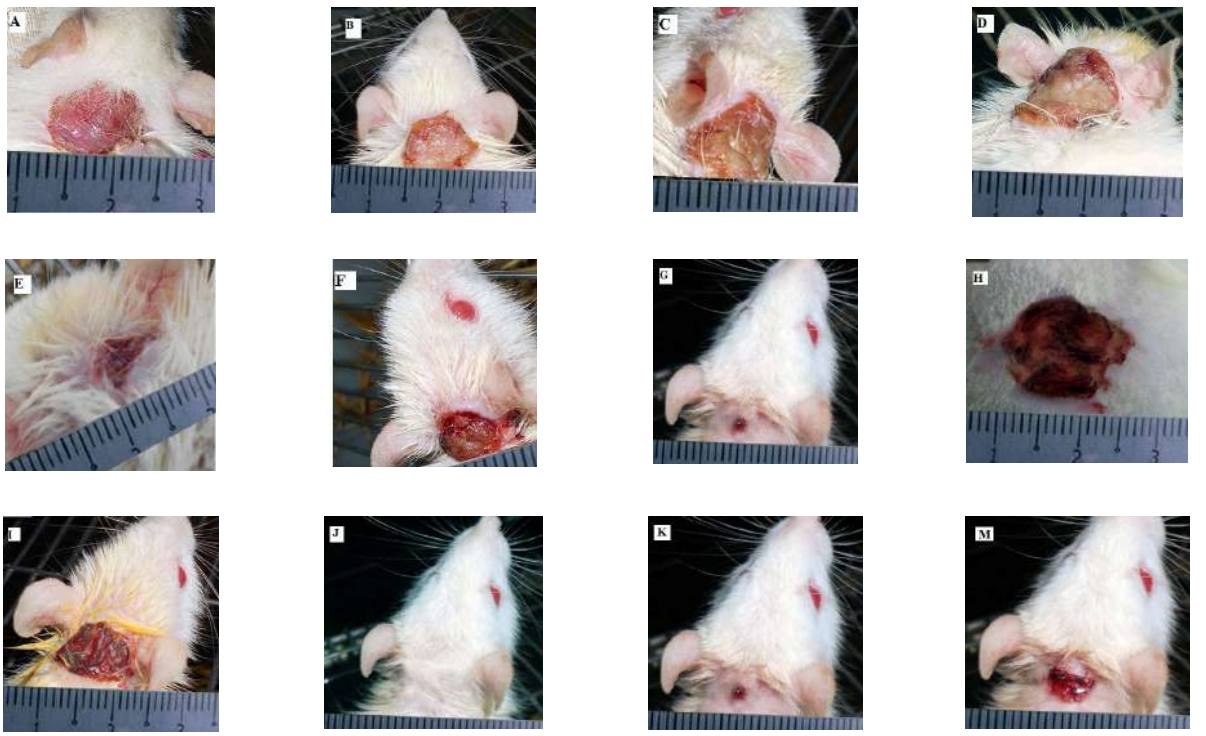


Figure 12: A to M: Photographical representation of wound showing A; Day-15 standard, B; Day-15 positive control, C; Day-15 SA_{500}, D; Day-15 SA_{250}, E; Day-15 SR_{500}, F; Day-15 SR_{250}, G; Day-24 standard, H; Day-24 positive control, I; Day-24 SA_{250}, J; Day-24 SR_{500}, K; Day-24 SR_{250}, M; Day-24 SA_{500}

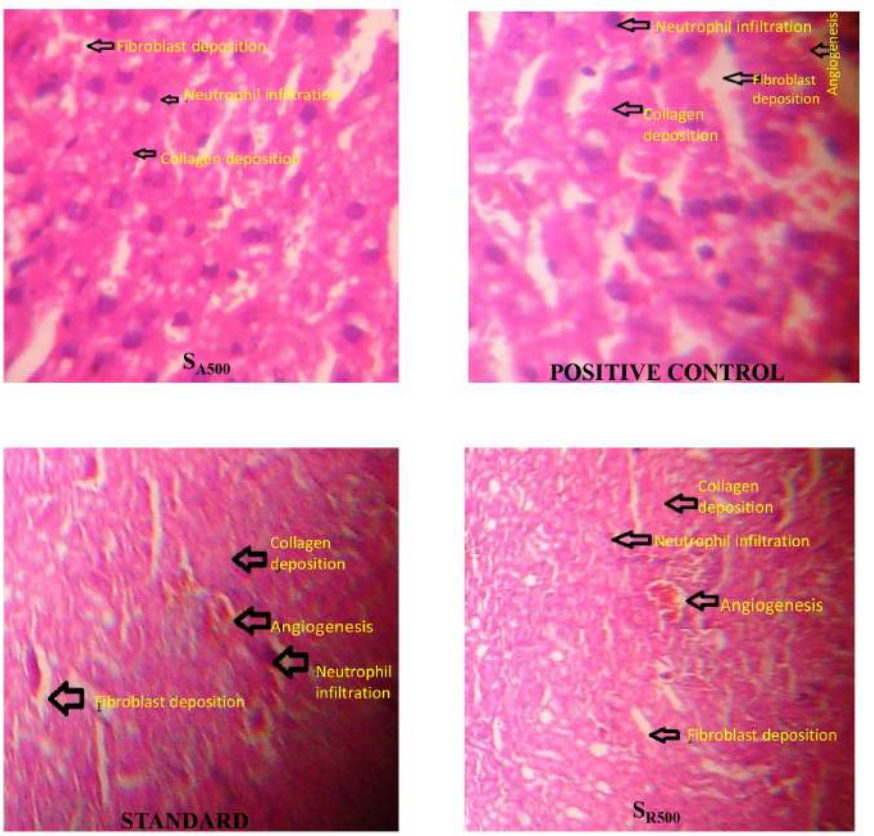


Figure 13: Histological representation of wound on day 24

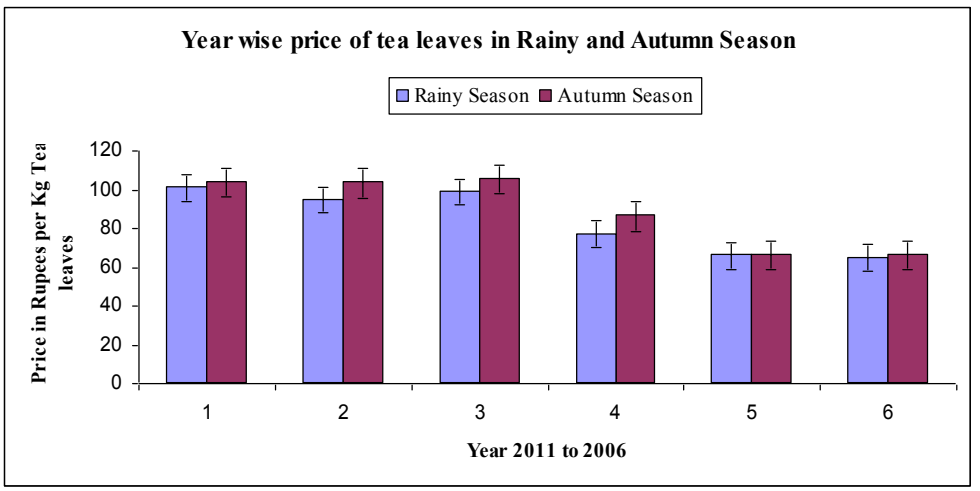


Figure 14: Year wise prices of tea leaves at Indian auction in rainy and autumn season

RESULTS

Phytochemical screening and total flavonoids content

Preliminary phytochemical screening of both, S_A and S_R extracts showed the presence of various phytochemicals, as depicted in Table 1. It revealed the presence of sesquiterpene derivatives in S_R extract but not in S_A. Analysis was further extended to include HPLC and IR study (Table -2, Figure 1-4) of the same extracts. HPLC study report showed the presence of an additional peak in S_R curve towards 4 minutes retention time and was further corroborated by IR analysis. It revealed that at frequency of 1592.31cm^{-1}, there was an absorbance in S_R spectra showing the presence of an aromatic C–C bond stretching, which was absent in S_A extract. When compared with standard library data bank, it revealed the probable presence of sesquiterpene derivatives. On quantitative assay, the total flavonoids content was found to be significantly higher in S_R extract compared to S_A in terms of quercetin equivalent/g of extract powder in reference to the standard curve. Presence of additional phytochemicals and high content of flavonoids in S_R extract can be attributed to its better pharmacological activity compared to that of S_A extract.

Acute skin irritation test

The formulated emulsion of S_{A500} and S_{R500} did not show any type of irritation and noticeable inflammation, eruption, lesion, swelling or any other change on the skin.

In vitro antioxidant activity of both extract

All the extracts showed significant level of antioxidant properties with P value< 0.01. Among all, S_{R200} extracts showed highest degree of activity in the various selected in vitro antioxidant models such as DPPH, superoxide, reductive, H_2O_2 and hydroxyl free radical etc. The antioxidant property of S_{R200} is comparable to that of the high dose (50 µg/ml) of standard (Quercetin) in all the selected models except DPPH (Table 3-6 and Figure 5-8).

Antimicrobial activity

Wound healing is greatly hampered by the microbial activity (especially *Staphylococcus aureus* and *Pseudomonas aeruginosa*) that is colonized on the wounds. So the antimicrobial activity (zone of inhibition) was tested against eight human pathogens and the results were depicted in Table 7 and Figure 9. The susceptibility test result for all eight human pathogens showed sensitivity towards the selected standard drug but only six organisms out of those i.e. group no. III, IV, V, VI, VII, and VIII showed susceptibility and exhibited higher sensitivity towards the methanolic S_R extract compared to S_A extract. MIC of both the extracts was determined against the susceptible organisms and the result was shown in the Table 8 indicating that the extracts having moderate antimicrobial activity compared to standard drug with MIC 10 µg/ml. Among the plant extracts, S_R extract with MIC ranging from 1000-4000 µg/ml showed better activity compared to S_A extract with MIC ranging from 1200-4000 µg/ml.

Wound healing activity (percentage of wound closure)

Grossly, wounds dressed with the S_{R500} extract of *Camellia sinensis* group showed considerable sign of dermal healing and significantly (P< 0.05) healed faster compared to groups received the standard drug treatment (Table 9 and Figure 10, 11). Throughout the experiment, the percentage of healing in both the autumn extract treated groups showed significantly lower healing activity when compared with both the rain extract treated groups. Macroscopic examination of wound as on day 15 and 24 of post surgery showed that wound dressed with S_{R500} extract showed less scar width compared to the standard and other extracts treated groups (Figure12; A to M) and histological evaluation (Figure13) revealed that, wound dressed with S_{R500} extract contained more granulation tissue at wound area, few inflammatory cells, and more collagen deposition and angiogenesis compared to standard and other extracts treated groups.

DISCUSSION

A major problem with open wound or injury is the high risk of infection. The swab culture of the untreated open wound revealed the presence of *Staphylococcus aureus.* It is very easy for bacteria to enter through the abraded or bruised skin and penetrate to systemic circulation. Bacteria colonize wounds within 48 hours after injury and opportunistic bacterium and their several strains, such as *Staphylococcus aureus, Pseudomonas aeruginosa, E. Coli* and *Streptococcus* sp. may cause infection and this may prolong inflammatory phase of wound healing[27]. Hence, this study was extended to include the antimicrobial activity of both the extracts against eight selected human pathogens. The test results revealed that both the extracts, i.e. S_A and S_R showed antimicrobial activity, but S_R possessed greater anti microbial spectrum coverage and lower MIC value compared to that of S_A. The process of wound

healing normally leads to the chemotaxis of neutrophils, leucocytes and monocytes, initiating the phagocytic engulfment of colonized microorganisms and removal of cell debris. This often leads to the production of oxygen-free radicals such as hydrogen peroxide, superoxide anion, and hydroxyl anion and uncontrolled production of these agents cause tissue damage if they overwhelm the natural antioxidants of the host cell such as catalase, superoxide dismutase, and glutathione peroxidase. Therefore, antioxidants prevent the activity of the free radicals and thereby prevent the damage to cells enhancing healing of infected and non infected wounds[27, 28]. The overall antioxidant status plays an important role in the development and healing of wound. Wound healing, a complex sequence of events, is initiated by the stimulus of injury to the tissues. A positive stimulus may result from the release of some factors by wounding of tissues. It has been found that plants have high cicatrizing and vulnerary properties. Impairment at the cellular level may be prevented by different antioxidants[29, 30]. Hence, if any antioxidant, having wound healing property and active against the microorganisms causing the infection, is used either systemically or topically to aid in healing process by significantly reducing the overall time for wound healing. Plant phenolics and sesquiterpenes are the major group of compounds that act as primary antioxidant[31] and are responsible for better antioxidant activity of S_R. However, these agents are known to promote the wound healing process mainly due to their astringent and antimicrobial property, which seems to be responsible for faster wound contraction, lesser necrotized cell turn over, and improved rate of epithelialisation[25, 32]. The test results of *in-vitro* antioxidant assay, and antimicrobial analysis were correlated to the wound healing activity of S_A and S_R. In this case also, the study result revealed the superiority of S_R compared to S_A and was further corroborated by quantitative and qualitative estimations of phytochemicals. Usually tea leaves are collected for almost nine months round the year. Season's first collection is made in the month of March through April which is known as 'First flush', the collection made in the month of May and June is 'Second flush', collection from July through September is referred as 'Rain collection' and from October through November, is 'Autumn collection'. Gardens remain closed for three months i.e. from December through February, during which tea bushes are pruned for new production, to maintain better yield throughout the year, and to ensure the longevity of the bush. In the present study, rain and autumn seasons have been selected to observe the impact of rain on phytoconstitutional content variation and its associated pharmacological activities in tea leaves extracts. As rainfall plays a major role in vegetative property, crop quality, so these two seasons have precisely been selected instead of all four because significant difference in rain fall is observed during these two selected seasons only. Commercially autumn tea is much more expensive and better appreciated than its rain counterpart, since its better aesthetic properties, higher astringency.(Table 10, Figure14) The underlying cause of this, might be its higher tannin content, slow pooling, and controlled higher rate of withering of tendered autumn leaves. But, from pharmacological point of view it was reverse. In rainy season, young leaves grow at faster rate and so the plucking is done. Quantitative assay for total flavonoids estimation revealed that, young leaves extract of rain collection, such as S_R contained higher quantity of flavonoids compared to that of autumn collection, such as S_A. Moreover, HPLC-IR study revealed that S_R extract could have contained additional sesquiterpene derivatives, which along with flavonoids, might have elicited a synergistic or additive effect in terms of antimicrobial, antioxidant, and wound healing activities rendering S_R a superior pharmacological agent than that of its S_A counterpart.

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