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Evaluation of in vitro antioxidant and antidiabetic activity on various extracts of *Camellia sinensis*

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ABSTRACT

Alzheimer's disease and Diabetes are on the increase to find out treatments to cure or prevent them as major targets in research. Surprisingly, both share several molecular processes that underlie the respective degenerative developments like Aβ deposition, Glycogen synthase kinase-3 (GSK-3β), τ phosphorylation, oxidative stress, inflammation, ApoE4. Type 2 diabetes (T2DM) is a major risk factor which leads to AD. One recent study suggests that in people who have diabetes, the risk of developing Alzheimer's disease is more than double the risk of people without diabetes. Therefore, an attempt is being made to develop a monotherapy for both Alzheimer's disease and Diabetes. *Camellia sinensis* is chosen for the present study to evaluate the anti-oxidant and anti-diabetic activity by in vitro studies. As a part of the study, the preliminary screening was done to analyze the phytoconstituents present in it. For this, the extraction was carried out by using 95% alcohol and hydro alcoholic solvent. The extracts were obtained by both maceration and soxhlation. The extracts were analyzed for the presence of various constituents like alkaloids, glycosides, phenols, saponins, tannins, fixed oils etc. The antidiabetic activity was carried out by evaluating the glucose uptake by yeast cells, while the antioxidant activity by hydrogen peroxide scavenging and Phosphomolybdenum assay. Our results suggested that *Camellia sinensis* has antidiabetic and antioxidant activity. With this data we further extend our study by isolating each constituent to establish a mechanistic based evidence for both diabetes and Alzheimer's disease.

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INTRODUCTION

Alzheimer's disease (AD) is the most common cause of death among the elderly aged above 60 years. As the world is trying to increase the life expectancy, Alzheimer's disease has become the major hindrance. According to the world Alzheimer's report 2016, 46.8 million people in the world and 3.7 million in India suffer from this form of dementia. Two- thirds of them are female patients (Prince, M. *et al.,* 2016; IANS, 2016). The major pathological characteristics are deposition of Amyloid beta plaques, hyperphosphorylation of τ Protein and loss of cholinergic neurons (Morrison Ann, S. *et al.,* 2005). Unfortunately the available therapies are only symptomatic, that is, either they increase the levels of acetyl choline (Tacrine, Donepezil) or inhibit the activity of glutamate (Memantine) (Sergio, A.R. *et al.,* 2012). Hence there is a need for the drug which eliminates the root cause

of the disease. There are many risk factors for AD like presence of $APOE_{\varepsilon 4,}$ BACE genes genetically and life style modifications such as diabetes, obesity, smoking. Among these, Diabetes mellitus is the major risk factor (Baumgart, M. *et al.,* 2015). Insulin and its receptors also play a major role in cognition. There are many common pathogenesis for both AD and Diabetes (Durate, A.I. *et al.,* 2013). One of those is Glycogen synthase kinase 3 (GSK-3). This protein kinase disrupts the insulin signaling pathway and also abnormally phosphorylates τ protein and leads to amyloid beta plaque formation (Corinne, G.J. *et al.,* 2009; Al-Nashi *et al.,* 2013). The present study is therefore carried out to develop a monotherapy for both AD and Diabetes with least adverse effects. The plant selected for the study is *Camellia sinensis* as it is the most popular beverage worldwide (Al-Grawi *et al.,* 2018).

MATERIALS AND METHODS

Plant material

Dried leaves of *Camellia sinensis* were obtained as coarse powder from the local market as marketed product [Tetley].

Solvents

Petroleum ether (60-80) (Molychem), Ethanol (95%), Distilled water.

Reagents

Phosphate buffer (7.4 pH), H_2O_2 reagent, Phosphomolybdenum reagent, Glucose solution (1%w/v), Yeast solution (2%w/v)

Extraction

The extraction was carried out by both cold maceration and soxhlation. The dried leaves weighing about 600gm were taken and divided equally into two parts. One part is used as it is without defatting whereas the other is used after defatting using petroleum ether.

Defatting

Dried leaves weighing about 300gm were divided into 3 portions each containing 100gm and placed in an iodine flask. For 100g of the plant material, 300ml of hot petroleum ether was added and kept for 2 nights with intermittent shaking. The solution was drained and the plant material was air dried in shade for the evaporation of the left-over solvent. 1.6%w/w of the dried leaves was lost after defatting (Saraf, S. *et al.,* 2011; Sarwa, K.K. *et al.,* 2013).

Maceration

The maceration was carried out by taking defatted as well as fatted leaves by using both alcoholic and hydro alcoholic solvents. For 100gm of the plant material 400ml of 95% ethanol was added. To another portion of 100gm, 1:1 ratio of water and 95% ethanol was added. The extracts were collected with intermittent shaking for about 6, 12, 18, 24, 48, 72 and 80 hours respectively (Saraf, S. *et al.,* 2011; Sarwa, K.K. *et al.,* 2013; Al-Thahab *et al.,* 2018).

Soxhlation

The soxhlation was carried out by taking defatted as well as fatted leaves by using both alcoholic and hydro alcoholic solvents. 50g of the plant material was placed in the thimble in the Soxhlet chamber. The apparatus was assembled in a heating mantle and 300ml of the solvent was added into the chamber until 3 cycles were completed and the soxhlation was carried out till the solution turned colorless in the siphon tube. It took 12 cycles for the conversion of olive green color to colorless (Saraf, S. *et al.,* 2011; Sarwa, K.K. *et al.,* 2013).

% Yield

The extracts obtained from maceration and soxhlation were taken in china dishes and evaporated on a hot plate. The percentage of yield obtained was calculated by using the formula (Pavithra, K *et al.,* 2015) and results were shown in Figure 1.

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

Solubility

The extracts obtained were dissolved in distilled water by vigorous shaking for about 5 min followed by sonication for 15 min. The solutions were filtered to obtain a clear solution.

Phytochemical screening

Different extract solutions thus obtained from maceration and soxhlation were tested for the presence of phytoconstituents by adopting the suitable standard phytochemical tests (Kokate, C.K. *et al.,* 2008).

In vitro Antioxidant studies

The clear extracts were analyzed for their total antioxidant activity by using H_2O_2 scavenging method and Phosphomolybdenum assay.

H_2O_2 Scavenging activity

40mM H_2O_2 reagent was prepared by adding 1.32ml of H_2O_2 in 100ml of 50mM phosphate buffer (PH 7.4). Ascorbic acid was taken as standard. The concentrations of extracts and standard studied were 10, 20, 30, 40 and 50 µg/ml in 3.4ml of phosphate buffer. To this 0.6ml of H_2O_2 reagent was added and the absorbance values were read using in UV-visible spectrophotometer (Labindia 3000+) at 230nm. The blank solution used was 50mM

Table 1: Physical characteristics

No. of hours	Colour				pH				Appearance			
	Defatted		Fatted		Defatted		Fatted		Defatted		Fatted	
	Alcoholic	Hydro alcoholic	Alcoholic	Hydro alcoholic	Alcoholic	Hydro alcoholic	Alcoholic	Hydro alcoholic	Alcoholic	Hydro alcoholic	Alcoholic	Hydro alcoholic
6h	olive green	brownish green	olive green	brownish green	6	5	6	5	Glossy	Glossy	Glossy	Glossy
12h	olive green	brownish green	olive green	brownish green	6	5	6	5	Glossy	Glossy	Glossy	Glossy
18h	olive green	brownish green	olive green	brownish green	6	5	6	5	Glossy	Glossy	Glossy	Glossy
24h	dark green	greenish brown	dark green	greenish brown	6	5	6	5	Glossy	Glossy	Glossy	Glossy
48h	dark green	reddish brown	dark green	reddish brown	6	5	6	5	Glossy	Glossy	Glossy	Glossy
72h	dark green	reddish brown	dark green	reddish brown	6	5	6	5	Glossy	Glossy	Glossy	Glossy
80 h	dark green	brown	dark green	brown	6	5	6	5	Glossy	Glossy	Glossy	Glossy
soxhlation	dark green	dark green	dark green	dark green	5	5	5	5	Glossy	Glossy	Glossy	Glossy

Table 2a: Presence of phytochemical constituents

Phytochemicals	Maceration/ Defatted Maceration	6h	12h	18h	24h	48h	72h	80h
Alkaloids	MA	+	+	+	+	+	+	+
	MH	+	+	+	+	+	+	+
	DMA	+	+	+	+	+	+	+
	DMH	+	+	+	+	+	+	+
Glycosides	MA	+	+	+	+	+	+	+
	MH	+	+	+	+	+	+	+
	DMA	+	+	+	+	+	+	+
	DMH	+	+	+	+	+	+	+
Tannins	MA	+	+	+	+	+	+	+
	MH	+	+	+	+	+	+	+
	DMA	+	+	+	+	+	+	+
	DMH	+	+	+	+	+	+	+
Polyphenols	MA	+	+	+	+	+	+	+
	MH	+	+	+	+	+	+	+
	DMA	+	+	+	+	+	+	+
	DMH	+	+	+	+	+	+	+
Saponins	MA	+	+	+	+	+	+	+
	MH	+	+	+	+	+	+	+
	DMA	+	+	+	+	+	+	+
	DMH	+	+	+	+	+	+	+
Carbohydrates	MA	+	+	+	+	+	+	+
	MH	+	+	+	+	+	+	+
	DMA	+	+	+	+	+	+	+
	DMH	+	+	+	+	+	+	+
Proteins	MA	+	+	+	+	+	+	+
	MH	+	+	+	+	+	+	+
	DMA	+	+	+	+	+	+	+
	DMH	+	+	+	+	+	+	+
Fixed oils and fats	MA	+	+	+	+	+	+	+
	MH	+	+	+	+	+	+	+
	DMA	–	–	–	–	–	–	–
	DMH	–	–	–	–	–	–	–
Gums and Mucilage	MA	–	–	–	–	–	–	–
	MH	–	–	–	–	–	–	–
	DMA	–	–	–	–	–	–	–
	DMH	–	–	–	–	–	–	–

phosphate buffer and the % of peroxide free radicals scavenged was calculated after subtracting the absorbance of test from the positive control by the formula (Malik, A. *et al.,* 2011; Alam, Md. N. *et al.,* 2013) and results were shown in Figure 2 – 6.

$$\% \text{ scavenged} = \frac{\text{Absorbance of control} - \text{Absorbance of test}}{\text{Absorbance of control}} \times 100$$

Table 2b: Presence of phytochemical constituents

Phytochemicals	Soxhlation		Defatted Soxhlation	
	SA	SH	DSA	DSH
Alkaloids	+	+	+	+
Glycosides	+	+	+	+
Tannins	+	+	+	+
Polyphenols	+	+	+	+
Saponins	+	+	+	+
carbohydrates	+	+	+	+
Proteins	+	+	+	+
Fixed oils and fats	+	+	–	–
Gums and Mucilage	–	–	–	–

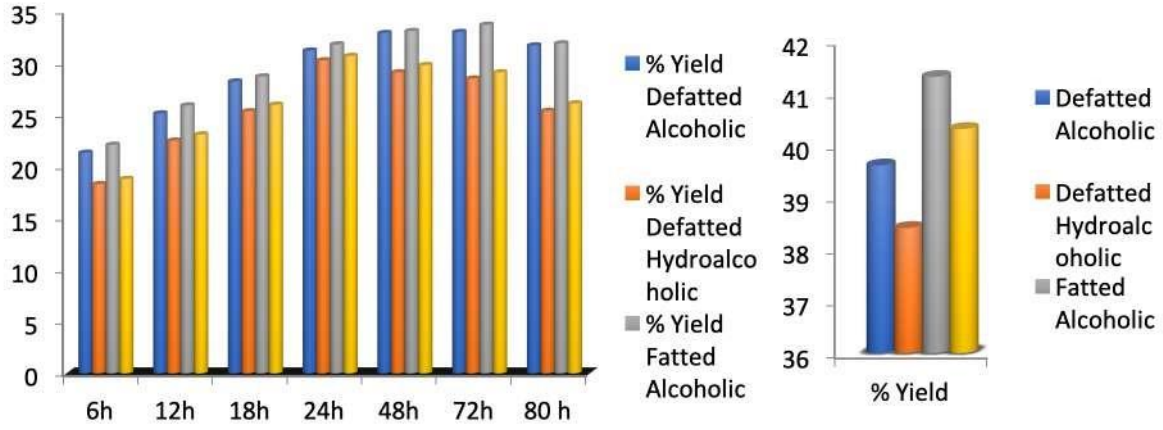

Figure 1: % Yield of various extracts obtained by maceration and Soxhlation

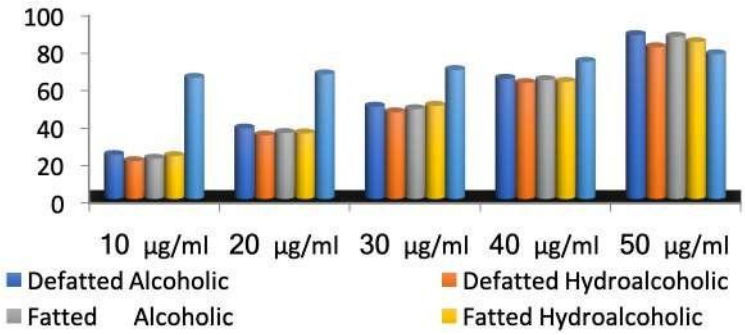

Figure 2: % scavenging of H_2O_2 of various extracts by soxhlation

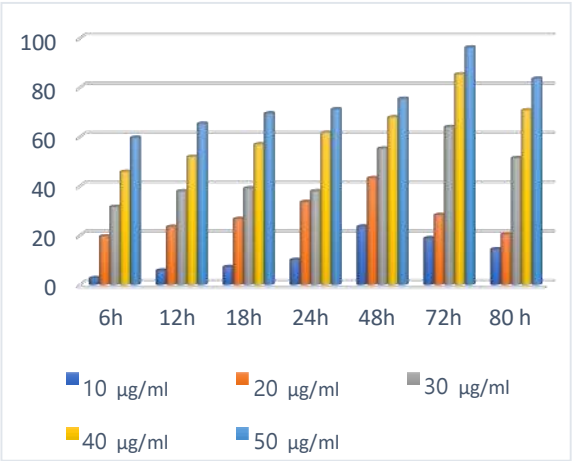

Figure 3: % scavenging of H_2O_2 of defatted alcoholic extracts by maceration

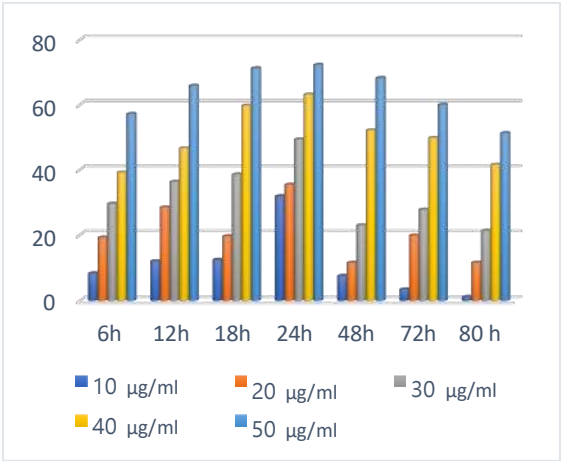

Figure 4: % scavenging of H_2O_2 of defatted hydro alcoholic extracts by maceration

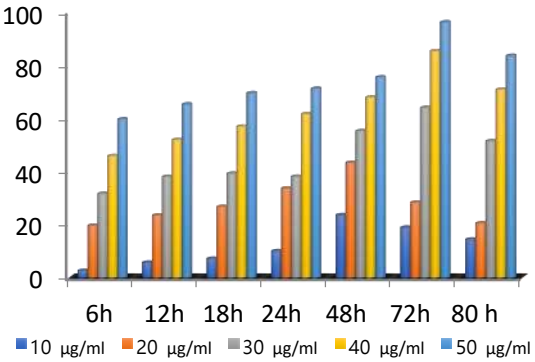

Figure 5: % scavenging of H_2O_2 of fatted alcoholic extracts by maceration

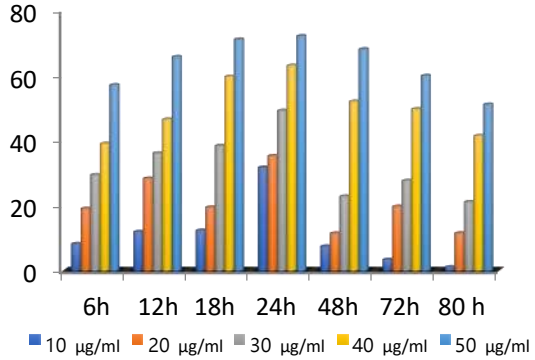

Figure 6: % scavenging of H_2O_2 of fatted hydro alcoholic extracts by maceration

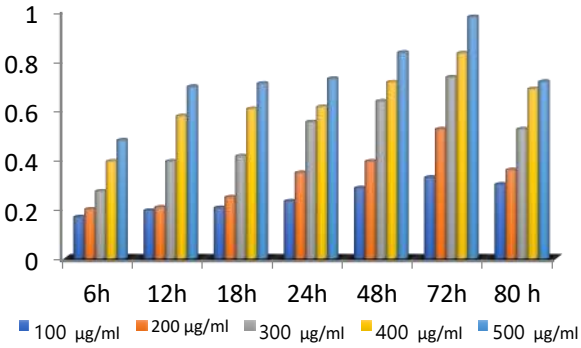

Figure 7: Effect of Defatted alcoholic extracts by maceration on total antioxidant activity by Phosphomolybdenum assay

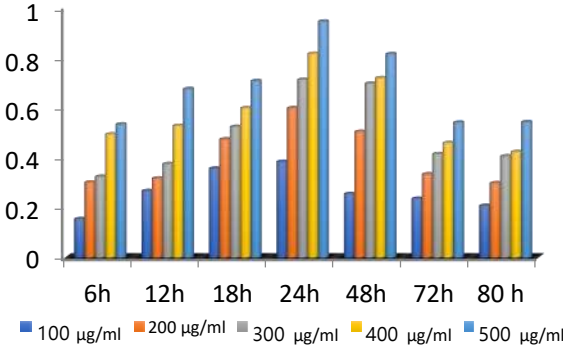

Figure 8: Effect of Defatted hydro alcoholic extracts by maceration on total antioxidant activity by Phosphomolybdenum assay

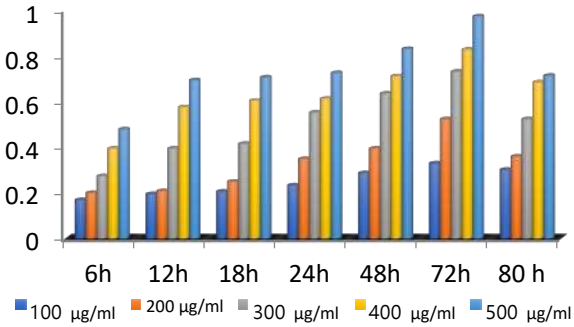

Figure 9: Effect of fatted alcoholic extracts by maceration on total antioxidant activity by Phosphomolybdenum assay

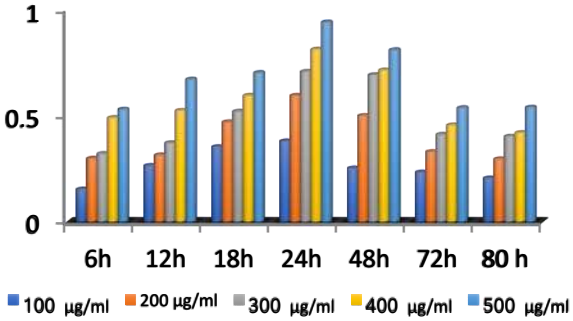

Figure 10: Effect of fatted hydro alcoholic extracts by maceration on total antioxidant activity by Phosphomolybdenum assay

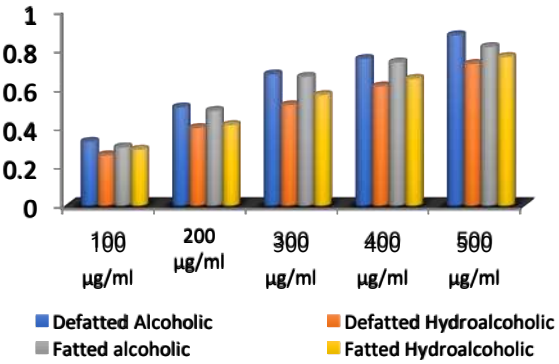

Figure 11: Effect of various extracts by soxhlation on total antioxidant activity by Phosphomolybdenum assay

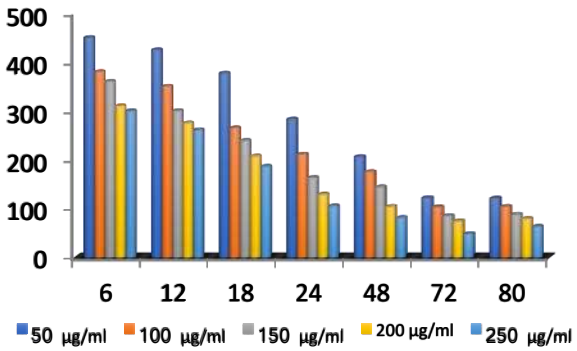

Figure 12: Glucose levels of Defatted alcoholic extracts by maceration

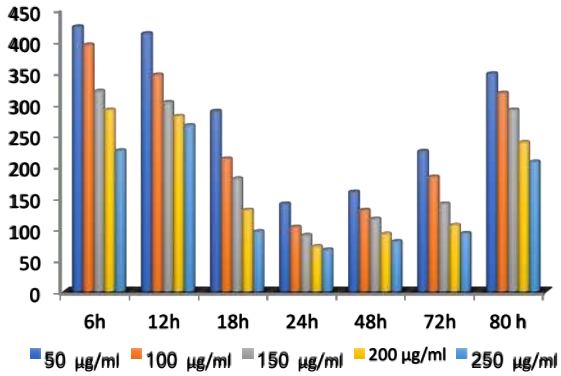

Figure 13: Glucose levels of defatted hydro alcoholic extracts by maceration

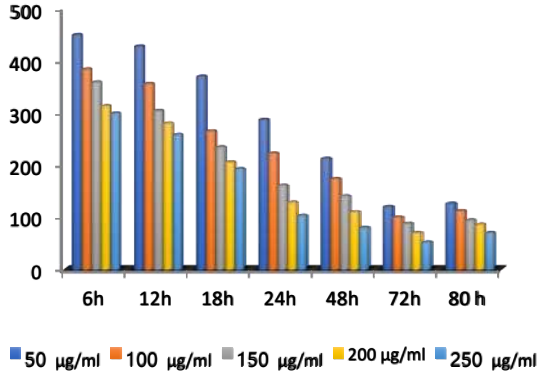

Figure 14: Glucose levels of fatted hydro alcoholic extracts by maceration

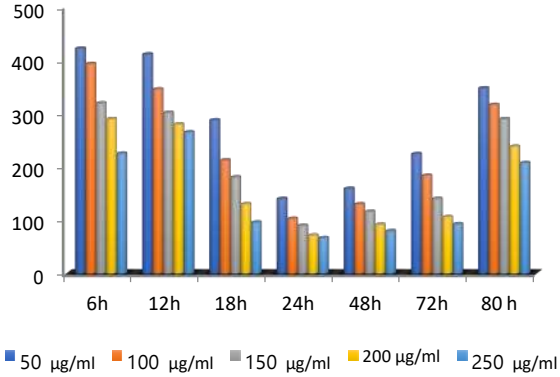

Figure 15: Glucose levels of fatted hydro alcoholic extracts by maceration

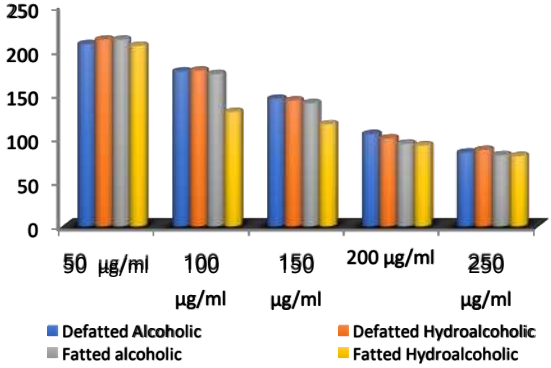

Figure 16: Glucose levels of different extracts by soxhlation

Phosphomolybdenum assay

The Phosphomolybdenum reagent was prepared by adding 20ml each of 0.6 M sulfuric acid, 28 mM sodium phosphate and 4mM ammonium molybdate diluted to 1000ml distilled water (Phatak, R.S. *et al.,* 2014). The different concentrations of extracts and standard solutions studied were 100, 200, 300, 400 and 500µg/ml respectively. To 0.5ml of the sample, 4.5ml of the reagent was added. Blank was prepared by adding 0.5ml of water to 4.5ml of reagent. The solutions were incubated in a water bath for 90 min at 95°C and cooled for 30 min for the development of color. The absorbance values were read using UV – Visible spectrophotometer (Labindia 3000+) at 695nm and is directly proportional to the antioxidant activity (Wan C *et al.,* 2011; Alam, Md. N. *et al.,* 2013) and results were shown in Figure 7-11.

In vitro antidiabetic activity using glucose uptake by yeast cells

2%w/v yeast solution was prepared from commercially available yeast by collecting the supernatant after centrifugation at 3000xg for 5 min. 1%w/v solution of glucose was prepared as stock solution. To 10ml of various concentrations of the extracts, 1ml of glucose solution and 0.1 ml of yeast solution were added and kept aside for 10min at room temperature. The supernatant is collected and observed for the amount of glucose present using the strips of digital glucometer (Accu-Chek). Decrease in the glucose levels represents increase in the uptake of glucose by the yeast cells (Sagadevan, E. *et al.,* 2013; Lateef *et al.,* 2018; Azhar Omaran, 2017) and results were shown in Fig no. 12-16.

RESULTS AND DISCUSSION

1.% Yield

The percentage yield was found to be highest with soxhlation method of extraction when compared to extraction by maceration. Extraction of fatted plant material using alcohol by soxhlation was found to have highest yield (41.3%), followed by hydro alcoholic extract (40.3%). The highest yield with maceration method of extraction was obtained by using fatted plant material using alcohol as solvent for 72hrs (33.7%). Defatted plant material showed less % yield when compared to fatted plant material.

Physical characteristics

The color of the alcoholic extracts obtained by maceration was dark green whereas the hydro alcoholic extracts showed greenish brown color. The intensity of the color increased as the time period

of extraction increased. The color of both alcoholic and hydro alcoholic extracts obtained from soxhlation was dark green. The pH of all the alcoholic extracts obtained from maceration method was found to be 5 whereas the hydro alcoholic extracts of maceration showed a pH of 6. The pH of all the extracts obtained from soxhlation was found to be 5. All the extracts of both maceration and soxhlation showed glossy appearance.

Phytochemical Screening

All the extracts showed the presence of same constituents irrespective of the solvent, method of extraction and time except for fixed oils which were absent after defatting. The constituents present were alkaloids, glycosides, polyphenols, saponins, fixed oils, carbohydrates and amino acids as shown in Table 2.

Evaluation of in vitro antioxidant activity by H_2O_2 Scavenging method

The % scavenging activity of peroxide radicals was found to be highest for alcoholic extract obtained from maceration of fatted plant material for 72hrs (96.2%) as compared with defatted plant material which was found to be 92.47% at 50µg/ml, even showed highest activity than ascorbic acid which was used as standard. The % scavenging activity of peroxide radicals by the alcoholic extract of defatted plant material obtained by soxhlation method was found to be 87.43% at 50µg/ml as compared to fatted plant material (86.52%). The hydro alcoholic extract of both fatted and defatted plant material obtained by maceration for 24hrs showed prominent % scavenging activity (fatted – 72.22%; defatted – 71.3%) and % scavenging activity decreased as the time period for maceration increased for both plant materials.

Evaluation of in vitro antioxidant activity by Phosphomolybdenum method

The antioxidant activity was found to be similar for both alcoholic extract obtained from maceration of fatted ((Absorbance - 0.989) and defatted (Absorbance -0.974) plant material for 72hrs at 500µg/ml. The antioxidant activity of the alcoholic extract of defatted plant material obtained by soxhlation method was found to be high (Absorbance – 0.875) at 500µg/ml as compared to fatted plant material ((Absorbance - 0.814). The hydro alcoholic extract of both fatted and defatted plant material obtained by maceration for 24hrs showed prominent antioxidant activity (fatted – 0.943 abs; defatted – 0.895 abs) and antioxidant activity decreased as the time period for maceration increased for both plant materials.

Evaluation of in vitro antidiabetic activity by glucose uptake by yeast cells

The hypoglycemic effect of alcoholic extract obtained from maceration of fatted plant material for 72hrs showed significant decrease in glucose levels (69mg/dl) when compared to defatted plant material (74mg/dl) at 200µg/ml. This may be due to the increase in the carbohydrate levels during defatting. The antidiabetic effect of alcoholic extract obtained from soxhlation of fatted plant material showed that glucose levels were lowered (93mg/dl) when compared to defatted plant material (104mg/dl) at 200µg/ml. The hydro alcoholic extract of both fatted and defatted plant material obtained by maceration for 24hrs showed prominent antidiabetic activity (fatted – 52mg/dl; defatted – 59mg/dl) and antidiabetic activity decreased as the time period for maceration increased for both plant materials.

CONCLUSION

The alcoholic maceration of the plant material for 72 hrs was found to be more effective in antioxidant and antidiabetic activity when compared to all others methods of extraction by maceration and soxhlation, whereas the % yield was found to be highest for the fatted plant material obtained by soxhlation using alcohol. Hence the recommended method of extraction for Camellia sinensis to carry further studies is macerating it for 72hrs without defatting. Further studies will be extended for evaluation of time effect on hypoglycemic effect and to the in vivo studies on rats for evaluating the antioxidant and antidiabetic activity.

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