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Phytochemical screening, total phenolics content and antioxidants activity of *Cassia Singueana*

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Abstract
The leaves and seeds of *Cassia Singueana* (*Leguminosae*), one of the most popular herbal products in tropical countries, are used in traditional medicine for treatment of several diseases and ailments. The present study was conducted to investigate the anti-oxidative activities of different solvent extracts of *Cassia Singueana* leaves and seeds. The results indicate that all the extracts have reducing power DPPH radical scavenging abilities. Ethyl acetate and methanol extract of the leaves extracts have the highest total reducing power whereas, the methanol and ethyl acetate extracts of the seeds part have more potent free radical scavenging activity than all the other extracts using DPPH free radical scavenge capacity assay. IC_{50} was calculated and compared with propyl gallate as standard. Qualitative Phytochemical screening of the two parts indicates the presence of alkaloids, coumarins, flavonoids, sterols, saponins and tannins with different concentration. Quantitative analysis of the two parts of *Cassia Singueana* for phenolic flavonoids and tannins compounds revealed that the total phenolic content ranged from 122.75 to 376.1 and 286.94 to 1990 mg/g of dry weight of leaves and seeds extracts respectively which expressed as gallic acid equivalents. The total flavonoid concentrations varied from 42 to 1020 mg/g and 188.34 to 449 mg/g of dry weight of leaves and seeds extracts respectively that expressed as Qurestin equivalents. The total tannins concentrations varied from 126.21 to 1323 and 309.88 to 1710 mg/g for leaves and seeds respectively. It could be conducted that leaves and seeds parts of *C. Singueana* Possessed anti-oxidative activities and can be used as a potential alternative medicine for oxidative stress related to non-communicable chronic diseases. Further experimental and clinical studies including laboratory animals are warranted.

Keywords: anti-oxidative, *Cassia Singueana*, free radicals, phytochemical, leaves and seeds

Introduction
Herbal medicines play a major role in primary health care, mainly in the developing countries. Therapeutic potential of herbal drugs are attributed to the present of bioactive phytochemicals. Plants are biosynthetic laboratories of a wide spectrum of chemicals of various physiological functions.

These phytochemicals are believed to have better compatibility with the human body and possess medicinal properties. Herbal drugs got a successful history as old as human civilization and today herbal medicines are coming back into prominence because of decreasing efficacy and serious side effects of the modern medicines. Oxidation is necessary for energy production in all living systems. However it can produce free radicals, which can start chain reactions that may damage cells. Antioxidants terminate these chain reactions by removing radical intermediates, and inhibit other oxidation reactions by being oxidized themselves. Generally antioxidants of plant origin are of often reducing agents such as thiols or polyphenols. (Jinu *et al*., 2012) [11].

Cassia species belong to the family *Caesalpiniaceae* which is often treated as a sub-family, *Caesalpinioideae*, of the large family *Leguminosae*. It is closely related to *Mimosaceae* and *Papilionaceae*, but can be distinguished by few stamens and five free petals. *Caesalpinioideae* consist of trees, shrubs and a few woody herbs found in the tropics. Economically, woody *Caesalpiniaceae* is important for its timber. *Cassia* and *Tamarindus* species are used for medicinal purposes. (Mau and Lin, 2002) [12].

Some species of *Caesalpiniaceae* yield dyes (Hutchinson and Dalziel, 1958 [9]; Hutchinson, 1973) [8]. *Cassia* species have been of keen interest in phytochemical and pharmacological research due to their excellent medicinal values 7.

All *Cassia* species are rich source of secondary metabolites, notably anthraquinone derivatives and has been used in Chinese and Ayurvedic preparations. *C. Singueana* has many medicinal uses throughout Africa (Kawanga and Bosch, 2007). Other applications of the plant, in Ethiopia; is that the inner bark is chewed fresh to soothe stomach spasm and smoke from its wood and bark is used for purposes of smoke baths to containers of milk and milk products. Scientific reports indicate that the plant has anthelminthic properties (Kawanga, and Bosch, 2007), antiprotozoal activity against cestodes of *Hymenolepis diminuta* (Mølgaard *et al*., 2001) [16], antiplasmodial, antinociceptive, antipyretic (Adzu *et al*., 2003) [2],

In *vivo* antioxidant and hepatoprotective properties (Ottu *et al*., 2011) [21], in *vitro* free radical scavenging activity (Gebrelibanos *et al*., 2007) [6], enzyme inhibition activities (strong- acetylcholinestrase and carboxylesterase inhibitory activities and weak glutathione-S-transferase and xanthine oxidase inhibitory activities) (Bangou *et al*., 2011) [3], antiulcer effects (Ode and Asuzu, 2011) [18] and reduce both gastric free-HCl and total acids (Ode and Onakpa, 2010) [19]. The plant is reported to contain anthraquinones, quinoids, sterols, alkaloids, terpenes, saponins, phenols, tannins (Adzu *et al*., 2003) [2], flavonoids, glycosides and carbohydrates (Adeyanju *et al*., 2011) [20]. *In vivo* antioxidant and hepatoprotective properties (Ottu *et al*., 2011) [21], in *vitro* free radical scavenging activity (Gebrelibanos *et al*., 2007) [6], enzyme inhibition activities (strong- acetylcholinestrase and carboxylesterase inhibitory activities and weak glutathione-S-transferase and xanthine oxidase inhibitory activities) (Bangou *et al*., 2011) [3], antiulcer effects (Ode and Asuzu, 2011) [18] and reduce both gastric free-HCl and total acids (Ode and Onakpa, 2010) [19]. The plant is reported to contain anthraquinones, quinoids, sterols, alkaloids, terpenes, saponins, phenols, tannins (Adzu *et al*., 2003) [2], flavonoids, glycosides and carbohydrates (Adeyanju *et al*., 2011) [20]. In *vivo* antioxidant and hepatoprotective properties (Ottu *et al*., 2011) [21], in *vitro* free radical scavenging activity (Gebrelibanos *et al*., 2007) [6], enzyme inhibition activities (strong- acetylcholinestrase and carboxylesterase inhibitory activities and weak glutathione-S-transferase and xanthine oxidase inhibitory activities) (Bangou *et al*., 2011) [3], antiulcer effects (Ode and Asuzu, 2011) [18] and reduce both gastric free-HCl and total acids (Ode and Onakpa, 2010) [19]. The plant is reported to contain anthraquinones, quinoids, sterols, alkaloids, terpenes, saponins, phenols, tannins (Adzu *et al*., 2003) [2], flavonoids, glycosides and carbohydrates (Adeyanju *et al*., 2011) [20]. In *vivo* antioxidant and hepatoprotective properties (Ottu *et al*., 2011) [21], in *vitro* free radical scavenging activity (Gebrelibanos *et al*., 2007) [6], enzyme inhibition activities (strong- acetylcholinestrase and carboxylesterase inhibitory activities and weak glutathione-S-transferase and xanthine oxidase inhibitory activities) (Bangou *et al*., 2011) [3], antiulcer effects (Ode and Asuzu, 2011) and reduce both gastric free-HCl and total acids (Ode and Onakpa, 2010) [19]. The plant is reported to contain anthraquinones, quinoids, sterols, alkaloids, terpenes, saponins, phenols, tannins (Adzu *et al*., 2003), flavonoids, glycosides and carbohydrates (Adeyanju *et al*., 2011) [20]. In *vivo* antioxidant and hepatoprotective properties (Ottu *et al*., 2011) [21], in *vitro* free radical scavenging activity (Gebrelibanos *et al*., 2007) [6], enzyme inhibition activities (strong- acetylcholinestrase and carboxylesterase inhibitory activities and weak glutathione-S-transferase and xanthine oxidase inhibitory activities) (Bangou *et al*., 2011) [3], antiulcer effects (Ode and Asuzu, 2011) and reduce both gastric free-HCl and total acids (Ode and Onakpa, 2010) [19]. The plant is reported to contain anthraquinones, quinoids, sterols, alkaloids, terpenes, saponins, phenols, tannins (Adzu *et al*., 2003) [2], flavonoids, glycosides and carbohydrates (Adeyanju *et al*., 2011) [20].

Isolated constituents of this plant include: the anthraquinones - chrysophanol, physcion and 7-methylphyscion; cassiamin A, a dimmer of chrysophanol, (Icraf, 2011) [10]; four tetrahydroanthracene derivatives from the root-torosdchrysone, germichrysone, singueanol - I and singueanol-II (Endo and Naoki, 1980) [5]; the pentacyclic triterpene lupeol, and the sterols- campesterol, β-sitosterol and stigmasterol.

The leaves contain the flavonoid leucopelargonidin, which has dyeing properties (Kawanga and Bosch, 2007). Thus, both traditional and scientific report claim indicate that the plant possesses a number of medicinal uses and can be a potential phyto-drug of multiple medicinal values. Owing to the previous research this study was conducted on *Cassia Singueana* and it is benefits in medical field to provide scientific evidence related to antioxidant properties of the plant, since it can be a potential source of antioxidant based therapies.

Materials and Methods

Chemicals and reagents

Gallic acid, tannic acid, Querstin, 1, 1-dipheny l-2-picrylhydrazy l radical (DPPH), sodium hydroxide, sodium nitrite, ferric chloride, potassium ferrous cyanide, sodium bicarbonate, aluminum chloride and Folin Ciocalteu reagent were obtained from Sigma-Aldrich, USA.

Collection of the plant material

The fresh plant of *Cassia Singueana* was collected from the Blue Nile State, Savanna, (Baw). The plant was identified and was authenticated by the herbarium unit of National Research Institute, The plant material was cleaned, separated into leaves, seeds and pods and they were shade dried. When the plant materials were thoroughly dried, they were coarsely powdered using a Manuel grinder. The powder was stored in an air tight, light resistant container for further analysis.

Preparation of the plant extracts

Forty grams of the fine powdered plant parts were separately defatted with petroleum ether (60-80). The defatted materials were sequentially extracted with chloroform, ethyl acetate and methanol by soxhlet extractor in 200 mL of the relevant solvent, After filtration through Whatman filter paper (No.1), respective solvents were evaporated under reduced pressure using a rotary evaporator (Buchi rotavapor II) at 40 ºC to obtain the solvent extracts. The solvent extract in each case was weighed, transferred to small container and stored in a refrigerator at 4 ºC until tested.

Qualitative Tests for secondary metabolites

Phytochemical screening of the prepared extracts

The prepared extracts were tested for their presence or absence of alkaloids, saponins, cardiac glycosides, flavonoids, sterols and triterpenes, sesquiterpene lactones, tannins and sugars according to methods described by Harbone (1984) and Sofowora (1993) [25].

Determination of total phenols, flavonoids and tannins contents in *C. Singueana* leaves and seeds extracts

1- Total Phenolics Content

The total phenolic content of each extract was determined by adopting the method as described by wolfe *et al*. (2003) [26]. The total phenolic contents were expressed as gallic acid

equivalents (mg/l) using the following equation based on the calibration curve: y = 0.001x + 0.136 where x = concentration of gallic acid (mg/l) corresponding to y the absorbance with R=0.995. A calibration curve was prepared using gallic acid (100-800 mg/l) as standard and used for calculation of total phenolic compounds.

2-Total flavonoids content

The total flavonoids content was determined by adopting the method described by Shanukha *et al.* (2012). Absorbance was measured at 415 nm against a reagent blank. Using Shimadzu model 1800 double beam spectrophotometer. Total flavonoids content was expressed as quercetin (mg/l) using the following equation based on the calibration curve Y = 0.000x + 0.064, where y was the absorbance and R = 0.997, calibration curve was constructed, using quercetin (50-700 mg/l) as standard and total flavonoids content of the extracts (mg/l) expressed as quercetin equivalents.

3- Total tannins content

The tannins content was determined by using $FeCl_3$ and gelatin test (Shivakumar *et al*., 2012) [24]. Absorbance was measured at 510 nm against a reagent blank using Shimadzu model 1800 double beam spectrophotometer. The total tannins content was calculated using the following equation y = 0.001x + 0.066, where x = concentration of tannic acid (mg/l) corresponding to optical density. A calibration curve was constructed, using tannic acid (100-800 mg/l) as standard with R = 0.9936 and total tannins content of the extracts (mg/l) expressed as tannic acid equivalents

Determination of antioxidant activity

DPPH radical scavening assay

The DPPH radical scavenging was determined according to the method of Shimada *et al.* (1992) [23]. The test samples were dissolved in DMSO while DPPH was prepared in ethanol. After incubation, decrease in absorbance was measured at 517nm using multiplate reader spectrophotometer. Percentage radical scavenging activity by samples was determined in comparison with a DMSO treated control group. All tests and analysis were run in triplicate.

IC_{50} calculation

The IC_{50} (the concentration of test material, which possess 50% inhibition of free radicals) of all the extracts and their fractions was determined by monitoring the effect of different concentrations ranging from 500-62.25 µg/ml. the IC_{50} of the extracts and their fractions were calculated using EZ-FIT Enzyme kinetic program (Perrella scientific, U.S.A).

Results and Discussion

Quantity of extracts

Successive extraction of leaves of *Cassia Singueana* gave the highest yield with methanol followed by ethyl acetate, petroleum ether and finally chloroforms: 20.55; 15.67; 3.063 and 2.58% respectively. Regarded to seeds, the highest yield was observed with methanol followed by ethyl acetate, chloroform and finally petroleum ether 17.4; 13.74; 3.44; 1.74% respectively.

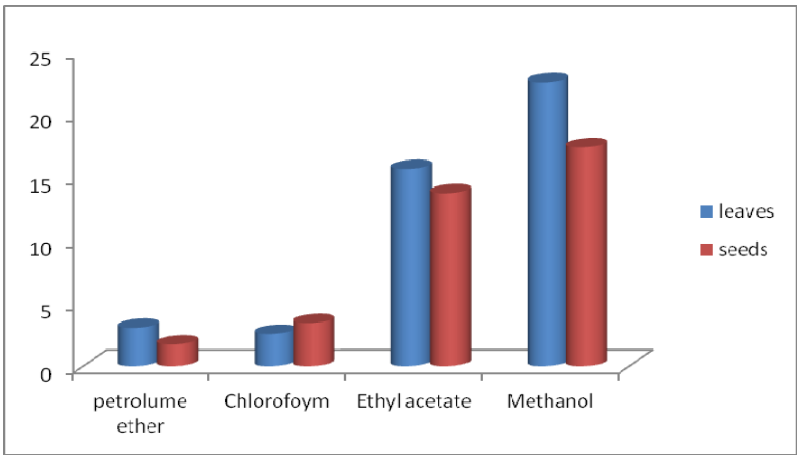

Fig 1: Extractive yield of *C. Singueana* leaves and seeds

Phytochemical screening of seeds and leaves of Cassia *Singueana* extracts revealed the presence of alkaloids, coumarins, flavonoids, sterols, saponins and tannins (Table 1)

Table 1: Preliminary phytochemical screening of leaves extracts of *Cassia Singueana*

Class of compound	Test Reagent	Extracts			
		PE	$CHCl_3$	EtOAc	MEOH
Alkaloids	Wagner	-	-ve	+ ++	++ +
	Mayer	+	++	+	+++
	Dragendorff	-	+	+++	+ ++
Flavonoids	Lead acetate	-ve	-ve	+++	+++
	Fecl3	-ve	-ve	+++	++
	KOH 1%	-ve	-ve	+++	+++
Sterols	Salkowski	+ + +	+ +	+ + +	+ + +
	Liebermann	+ +	+ +	+ +	+ +
Triterpenes	Salkowski	+++	-ve	-ve	+++
	Liebermann	+++	-ve	+++	++
Tannins	$FeCl_3$	-ve	-ve	++	+++

	Lead acetate Gelatin	-ve -ve	-ve + + +	++ + +	+++ ++
Glycosids	conc. H_2SO_4 Keller	+++ +++	-ve -ve	+++ +++	+++ +++
Lignin	Labat	-ve	+ +	-ve	++
Saponin (powder of leaves)			+ +		
Coumarin (powder of leaves)			+ +		

Note: "+" low, "++" average, "+++" high, "-" Not detected

Table 2: Preliminary phytochemical screening of Seeds extracts of *Cassia singueana*

Class of compound	Test Reagent	Extracts			
		PE	$CHCl_3$	EtOAc	MEOH
Alkaloids	Wagner	-ve	+++	+ +	-ve
	Mayer	-ve	+	+ +	-ve
	Dragendorff	-ve	++	+ + +	-ve
Flavonoids	Lead acetate	-ve	++ +	+ ++	+++
	KOH 1%	-ve	++ +	+ ++	+++
	FeCL3	-ve	+++	+++	+ +
Sterols	Salkowski	-ve	+ + +	+ + +	+ + +
	Liebermann	-ve	+ + +	+ + +	+ + +
Triterpenes	Salkowski	++	-ve	-ve	-ve
	Liebermann	++	-ve	+ + +	-ve
Tannins	$FeCl_3$	-ve	-ve	+++	+++
	Lead acetate	-ve	+++	++ +	+++
	Gelatin	-ve	++	+++	+++
Lignin	Labat	-	+ + +	+ +	+++
Saponin (powder of seeds)	+ +				
Coumarin (powder of seeds)	+ + +				

Note: "+" low, "++" average, "+++" high, "-" Not detected

Phytochemical screening of the leaves indicates the presence of alkaloids and sterols in all leaves glycosides and triterpences found in all extracts expect chloroform extract table (1). whereas analysis of seeds indicates the presence of sterols, flavonoids and tannins in all extracts except in the petroleum ether extract showed in Table (2) Several phyto-chemial studies have been previously carried out on *Cassia Singueana* Leaves (Olusola, *et al.*, 2011), Luteolin isolated from methanol extract from leaves of the plant (Ode and Asuzu, 2014) [17].

Quantitative analysis for total phenols, flavonoids and tannins content in leaves and seeds extracts of *Cassia Singueana*

The total phenolic, flavonoid and tannin contents of leaves and seeds extracts of *Cassia Singueana* were evaluated and results are presented in Table (2). The total phenolic contents in the examined plant extracts using the Folin-Ciocalteu's reagent is expressed in terms of gallic acid equivalent (the standard curve equation: y =.0008x + 0.0397, R = 0.999). The values obtained for the concentration of total phenols are expressed as mg of GA/l of extract. The total phenolic contents in the examined leaves extracts ranged from 122.75 to 752. 313 mg GA/l. The highest concentration of phenols was measured in methanolic, ethyl acetate, chloroform and petroleum ether extracts. Moreover the highest concentration of phenols in part seeds was determined in methanol, ethyl acetate, chloroform and petroleum ether extracts respectively. The total phenolic contents in plant extracts of the species *M. peregrinum* depends on the type of extract, i.e. the polarity of solvent used in extraction. High solubility of phenols in polar solvents provides high concentration of these compounds in the extracts obtained using polar solvents for the extraction (Mohsen and Ammar, 2008 [15]; Zhou. 2004) [27].

The concentration of flavonoids in various plant extracts of the *Cassia Singueana* was determined using spectrophotometric method with aluminum chloride. The content of flavonoids was expressed in terms of Querstin equivalent. The standard curve equation that used in calculation was: y =0.0007x+ 0.0537. The concentration of flavonoids in plant extracts of *Cassia Singueana* ranged from 42.857 to 2361.857 mg/L. Ethyl acetate extract contains the highest flavonoid concentration whereas the lowest flavonoid concentration was measured in petroleum ether extract. The concentration of flavonoids in plant extracts depends on the polarity of solvents used in the extract preparation (Min and Chun-Zhao, 2005) [13]. The concentration of flavonoids in seeds part ranged from 275 to 1710.938 mg/l also the highest concentration of flavonoids was found in methanol, ethyl acetate, petroleum ether and chloroform extracts respectively.

Tannin content was calculated as tannic acid equivalent (the standard curve equation:

Y = 0.0028X + 0.0824 and values ranged between 126.21 mg/l to 1323.21 mg/l for leaves part. The highest tannin content (1323.2 mg/l) was observed in methanol extract followed by ethyl acetate (242.68 mg/l), chloroform (128.964 mg/l) and petroleum ether extracts (126.21 mg/l), respectively. On the other part (seeds) the value ranged from 188.34 to 454.54 mg/l. The highest tannin content (453.54 mg/l) was observed in ethyl acetate followed by methanol (449.89 mg/l), chloroform (243.63 mg/l) and petroleum ether extracts (188.339 mg/l), respectively.

Table 3: Concentration of total polyphenols, flavonoids and total tannins of *Cassia Singueana* leaves - seeds extracts

Phenolic	Part of plant	Petroleum ether Mean	$CHCL_3$ Mean	Ethyl acetate Mean	Methanol Mean
Total polyphenol	Leaves	122.75	147.313	1683	3761
	Seeds	286.94	328.75	1700.1	1990.88
	P-value	0.170	0.224	0.498	0.132
Total tannins	Leaves	126.21	128.96	242.6786	1323.214
	Seeds	309.88	275.25	1555.313	1710.94
	P-value	0.01	0.021	0.565	0.715
Total flavonoids	Leaves	42.86	239.357	2361.86	1020.64
	Seeds	188.34	243.625	453.536	449.089
	P-value	0.533	0.369	0.802	0.126

Total polyphenol is expressed as mg Gallic acid/g of dry plant material. Total Flavonoids is expressed as mg quercetin/g of dry plant material. Total tannin is expressed as mg of tannic acid/g of dry plant material.* significantly different from the other at $P < 0.05$.

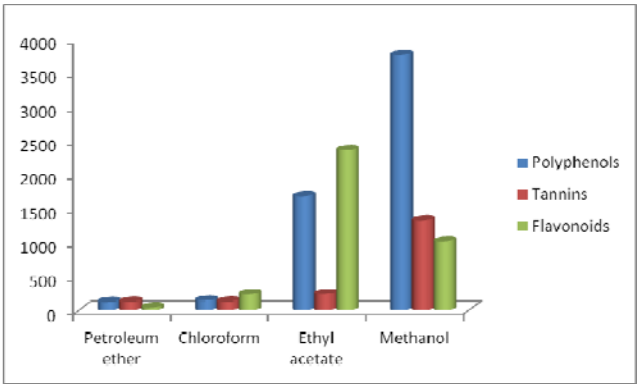

Fig 2: Total phenols, flavonoids and tannins contents in leaves extracts of *Cassia Singueana*

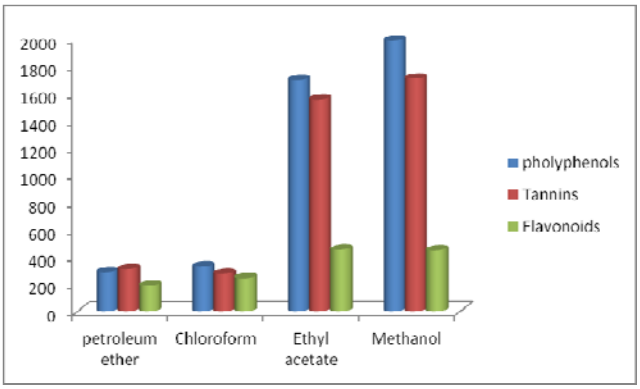

Fig 3: Total phenols, flavonoids and tannins contents in seeds extracts of *Cassia Singueana*

Antioxidant activity of *Cassia Singueana* leaves and seeds

The antioxidant activity of different plant extracts from *C. singueana* was determined using a methanol solution of DPPH reagent. DPPH is very stable free radical. Unlike *in vitro* generated free radicals such as the hydroxyl radical and superoxide anion, DPPH has the advantage of being unaffected by certain side reac . A freshly prepared DPPH solution exhibits a deep purple colour with an absorption maximum at 517 nm. This purple colour generally fades when antioxidant molecule quench DPPH free radicals (i.e. by providing atoms or by electron donation, conceivably via a free-radical attack on the DPPH molecule) and convert them into a colorless-/bleached product (i.e. 2, 2-dipheny l-1-hydrazine, or a substituted analogous hydrazine), resulting in a decrease in absorbance at 517 nm band (Amarowicz *et al*., 2003).

The antioxidant activity of four different extracts of *C. Singueana* is expressed in term of percentage of inhibition (%) and IC_{50} values (µg/ml). the standard values standard were obtained and compared to the values of the antioxidant activity. The standard substances that used in the test was propyl gallate.

The in-*vitro* antioxidant activity of the petroleum ether, chloroform, ethyl acetate and methanol extracts from leaves and seeds of *C. Singueana* was evaluated using DPPH assays (Table 3). The ethyl acetate of leaves and seeds extracts showed the highest activity 91 and 84% and the IC_{50} value was found to be 0.032 and 0.046 µg/ml, respectively. The methanol extracts of leaves and seeds showed high DPPH scavenging activity with inhibition percentage of 89 and 85 % with IC_{50} 0.118 and 0.119 µg/ml, respectively. The petroleum ether and chloroform leaves extracts showed moderate DPPH scavenging activity with inhibition percentage of 56 and 51%, respectively. While petroleum ether and chloroform seeds extracts were found to have low DPPH scavenging activity with inhibition percentage 36 and 32 leaves and seeds of the plant, IC_{50} were not calculated.

The high DPPH radical scavenging activities of the various solvent extracts which are comparable to standard antioxidants used suggest that the extracts have compounds with high proton donating ability and could serve as free radical inhibitors. However, the organic solvent extract from the leaves and seeds demonstrated a more remarkable anti-radical activity with IC_{50} values lower than propyl gallate. The EtOH extracts of the stem bark and root had a consistently higher DPPH radical scavenging ability than other extracts in these parts. (Mohammed *et al.*, 2013) [14].

Table 9: Antioxidants activity of seeds- leaves extracts of *Cassia Singueana*

Extract	%RSA ± SD (DPPH) (leaves)	IC_{50} (µg/mL) (Leaves)	%RSA±SD (DPPH) (seeds)	IC_{50} (µg/mL) (seeds)
Petroleum ether	56 ± 0.28	-	36 ± 0.22	-
Chloroform	51 ± 0.18	-	32± 0.31	-
Ethyl acetate	91 ± 0.02	0.032 ± 0.00	84 ± 0.05	0.046 ± 0.00
Methanol	89 ± 0.02	0.119 ± 0.07	85 ± 0.03	0.119 ± 0.01
Propyl gallate	84 ± 0.02	0.055 ± 0.00	84 ± 0.02	0.055 ± 0.00

Conclusion

It was noticed that the highest concentration of total phenolic compounds in the extracts were obtained using solvents of high polarity; the ethyl acetate and methanolic extract were revealed greater power of extraction for phenolic compounds from *C. Singueana*. The high contents of phenolic compounds and significant linear correlation between the values of the concentration of phenolic compounds and antioxidant activity. It could be concluded that *C. Singueana* is a natural source as an antioxidant substance. Further investigations to determine the other medicinal active compounds of the plant and experimental as well as clinical studies are warranted

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