SC.Parija. *et al.* / *International Journal of Phytopharmacology.* 3(1), 2012, 36-41.

e- ISSN 0975 – 9328
Print ISSN 2229 – 7472

International Journal of Phytopharmacology

Journal homepage: www.onlineijp.com

IJP

HPTLC DETECTION OF POLYPHENOLS AND FLAVONOIDS OF *CAREYA ARBOREA* LEAVES AND STUDY OF ANTIMICROBIAL EFFECT

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ABSTRACT

Careya arborea belongs to the family Lecythidaceae, is wellknown as Kumbhi in Ayurveda and has been employed in Ayurvedic treatment. A countable studies showed that the methanolic extract of stem bark of this plant exhibited antioxidant and antimicrobial effect.As in medicinal herbs the secondary metabolites are mainly polyphenols and flavonoids and their quality and quantity depends on parts of the plant. Considering that the leaves of this palnt could contain some polyphenols and flavonoids that may have different pharmacological properties, we have extracted the dry leaves using different individual and mixture of organic solvents such as water, acetone, methanol, methanol + water and ethyl acetate + methanol + water. HPTLC analysis showed that leaves extracted by ethyl acetate + methanol + water contains the highest percent of plolyphenols than by methanol. Such leaf extract of *c.arborea* exhibited a significant antimicrobial effect in disc diffusion test against bacterial culture under test.

Key-words: HPTLC analysis, careya arborea, leaves, polyphenols, antimicrobial effect.

INTRODUCTION

Emergence of new strains of pathogens and development of drug resistance are threats to growing population where ethno-medicine naturally safeguards normal health by boosting body immune system. The secondary metabolites in plants exhibit diverged biological effects as 'drugs'/ 'lead structures' for the development of synthetic molecules (Bravo, 1999; Chung *et al*., 1998; Crozier *et al*., 2000). Polyphenols derived from phenyl alanine in plants are oxidized / polymerized by their oxidases (PPOs) and peroxidases (PODs) to form covalent complexes with proteins which participate in wound repair, signaling analogous systems and act as anti-allergic, anti-artherogenic, anti-inflammatory, anti-microbial, antioxidant, anti-thrombotic and cardio-protective agents.

The inherited use of plants as a source of medicine is an important component of the health care system in India where most practitioners formulate and dispense their own recipes without any documentation and systematic research because the country is the largest producer of medicinal herbs and is appropriately called the botanical garden of the world. About 45,000 plant species concentrate in the hotspots of Eastern Himalayas, Western Ghats and Andaman and Nicobar Island. Out of which the scientific stream have officially documented 3000 plants with medicinal potency leaving more than

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6000 species used by traditional practitioners. Orissa province has the oldest and richest culture of traditional ethno-medicine (Mudgal and Pal, 1980; Saxena and Brahmam, 1985; Rout, 2004) where among several others *Careya arborea* (Kumbhi) is used for multiple ailments by rural people. This project was designed to screen and evaluate anti-microbial effects of polyphenols extracted from leaves of the plant.

METHODS

Leaf powder

The plant leaves were collected after identification from tribal region of Telkoi block of Keonjhar district in the month of April-May and classified following the description of Saxena and Brahman 1985; Rout, 2004. The fresh leaves were shade dried at 30-35°C for 3 days after washing/ rinsing in distilled water and were pulverized to powder in an electric blender to store in airtight glass jars.

Solvents

Different individual and mixture of organic solvents such as water in Gr-A, acetone in Gr-B, methanol in Gr-C, methanol (70%) + Water (30%) in Gr-D and Ethyl acetate (60%) + Methanol (30%) + Water (10%) in Gr-E were used.

Chlorophyll free extract

The extraction was done in Multiwave 3000 (Anton Par) digestion system at 80^0 C for 25 minutes with 2 g leaf powder and 20 ml of solvent taken in each vessel. Equal volumes of filtered extract and hexane were mixed, kept for 2 minutes and aspirated the superficial fluid carefully to separate chlorophyll.

Detection and estimation

The qualitative detection of phytochemical constituents was made following the methods of Edeoga *et al*., 2005, whereas quantitative estimations of total phenolics and flavonoids were made according to the methods of Singh *et al*., 2002 and Chang *et al*., 2002 respectively.

HPTLC detection of flavonoids

Chlorophyll devoid extracts of *Careya arborea* leaves were used to separate flavoinoids by HPTLC method by using silica gel 60 F_{254} plates of 20 x 10 cm as stationary phase and Butanol : Acetic acid : water (4:1: 5) as mobile phase. Development was made in 20x10 cm Twin Trough Chamber by saturating the chamber for 10 min with a distance of 70 mm followed by application of 5 µl of test and standard solutions in 6 mm bands with space 10 mm from lower edge. The peaks of samples were detected at UV-366 nm wave length after spraying anisaldehyde (9 ml 98% H_2SO_4 + 85 ml methanol + 10 ml acetic acid + 0.5 ml anisaldehyde), drying in cool air and heating at 120°C for 2 min.

Antimicrobial study

Agar cultures of 10^5 colony-forming units ($1X10^5$ cfu/ ml) of test microorganisms were prepared as described by Maceen *et al*., 1997. The chlorophyll free leaf extracts were concentrated in rotary evaporator at 40^o C and 250 mbar pressure and freeze dried. Sterile filter paper discs of 10-mm diameter were impregnated with 250 and 500 µg of dried polyphenol dissolved in 20 µl of DMSO and placed in nutrient agar containing inoculums after shade drying. DMSO (20 µl/ disc) and Ciprofloxacin (25 mg/ disc) were taken as control and reference standard, respectively. The results were recorded by measuring the growth inhibition zones surrounding the discs.

Statistical analysis

The data was statistically analyzed for analysis of variance with reference to Snedecor and Cochran, 1994.

RESULTS

Detection and estimation of phenolics and flavonoids

The qualitative detection of phenolic compounds in chlorophyll free leaf extracts of *Careya arborea* in five different solvents (Table-1) revealed that, water in Gr-A extracted all phenolic components except flavonoid, saponin, steroids and phytosterols. All the components were detected in Gr-B and D solvents whereas Gr-C and E solvents could not extract phytosterols and steroids, respectively.

The concentration of total phenolics was maximum in Gr-E solvent followed Gr-C, D, B and A whereas that of flavonoid was maximum in solvent of Gr-E followed by Gr-D, B, C and A (Table-2). The percent of flavonoids in respect to total phenolics was in descending order from solvents of Gr-E, B, D, and C to A. Although, the conc. of phenolics and flavonoids varied between solvents but mixture of solvents in Gr-E and water in Gr-A extracted highest and lowest components, respectively.

The leaf extract of *Careya arborea* depicted variable number of peaks in different solvents under HPTLC. Acetone in Gr-B displayed 22 peaks followed by mixture of solvents in Gr-E with 19 peaks, Gr-D and A with 17 peaks and Gr-C with 15 peaks (Fig 1-5). Acetone and methanol extracts claimed for highest and lowest number of flavonoids components.

Table 1. Detection of phenolic constituents of leaf extracts *Careya arborea* in different solvents

Phytoconstituents	Gr-A	Gr-B	Gr-C	Gr-D	Gr-E
Tannin	+ ve	+ ve	+ ve	+ ve	+ ve
Flavonoid	- ve	+ ve	+ ve	+ ve	+ ve
Terpenoid	+ ve	+ ve	+ ve	+ ve	+ ve
Cardiac glycosides	+ ve	+ ve	+ ve	+ ve	+ ve
Saponins	- ve	+ ve	+ ve	+ ve	+ ve
Anthraquinons	+ ve	+ ve	+ ve	+ ve	+ ve
Steroids	- ve	+ ve	+ ve	+ ve	- ve
Phytosterols	- ve	+ ve	- ve	+ ve	+ ve

Table 2. Total phenolics and flavonoids in chlorophyll free leaf extracts of *Careya arborea* in different solvents (Unit/ g of sample ± SE)

Solvents	Phenolics (mg of Gallic acid Eqv)	Flavonoids (mg of Rutine Eqv)
Gr-A	35.86 ± 1.15	16.50 ± 1.95 (46.01 %)
Gr-B	41.10 ± 2.16	26.28 ± 2.68 (63.94 %)
Gr-C	50.29 ± 1.23	24.85 ± 2.27(49.41 %)
Gr-D	48.80 ± 2.29	27.62 ± 1.26 (56.59 %)
Gr-E	56.20 ± 3.41	36.59 ± 2.88 (65.10 %)
(%) denotes the value of flavonoids out of 100 units of phenolics.		

Table 3. Zone of Inhibition of ciprofloxacin and polyphenols of *Careya arborea* leaf at different doses (mm ± SE)

Organisms	Ciprofloxacin (25 µg / disc)	Polyphenols (250 µg / disc)	Polyphenols (500 µg / disc)
Staphylococcus aureus	25.3 ^{a} ± 0.14	12.1 ^{b} ± 0.12	16.2 ^{c} ± 0.11
Escherchia coli	28.0 ^{a} ± 0.15	14.9 ^{b} ± 0.14	19.6 ^{c} ± 0.13

Means bearing different superscripts between columns within a row differ significantly (p< 0.05).

Fig 1. Gr-A (Water) extract of *Carea arborea* leaf showing 17 peaks of flavonoids in HPTLC resolution

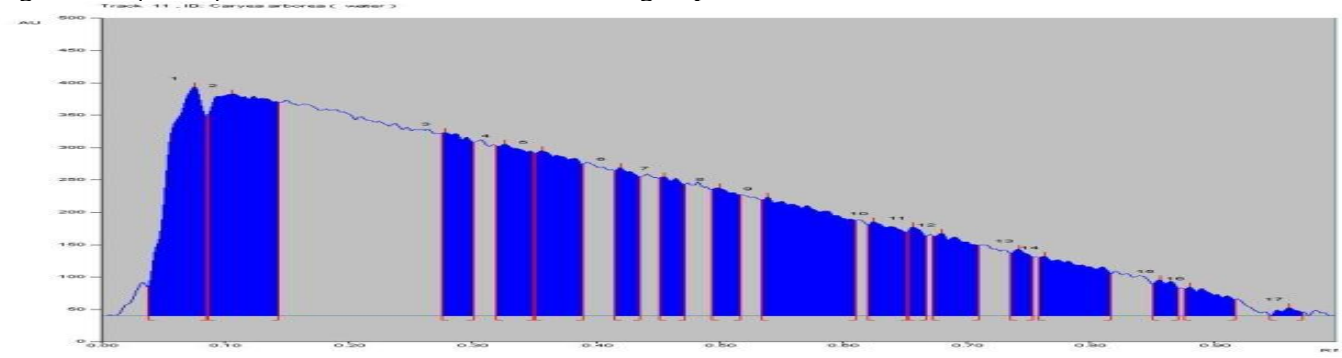


Fig 2. Gr-B (Acetone) extract of *Carea arborea* leaf showing 22 peaks of flavonoids in HPTLC resolution

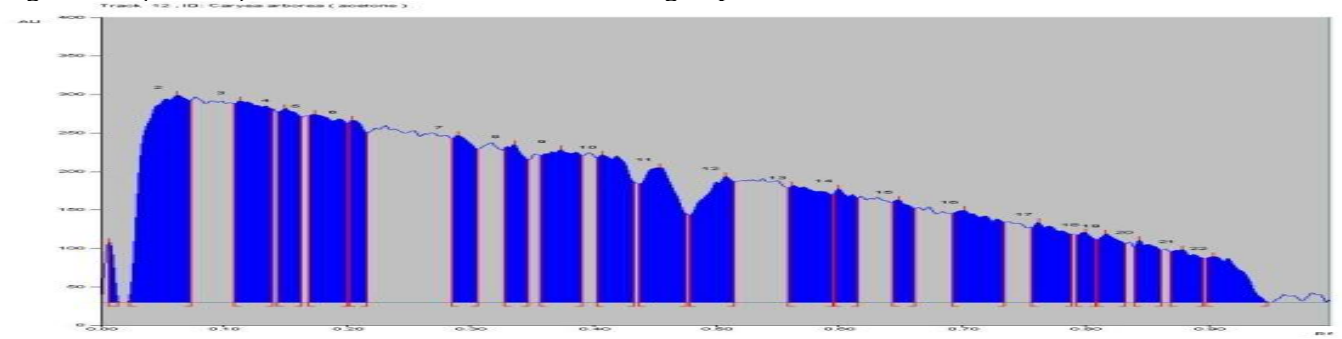


Fig 3. Gr-C (Methanol) extract of *Carea arborea* leaf showing 15 peaks of flavonoids in HPTLC resolution

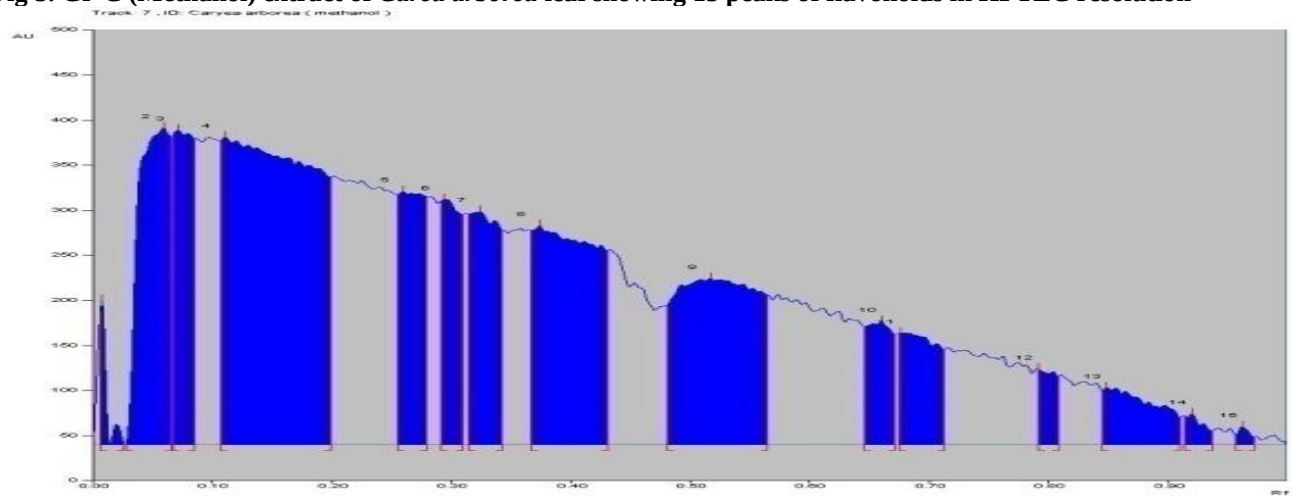


Fig 4. Gr-D (Methanol:Water 70:30) extract of *Carea arborea* leaf showing 17 peaks of flavonoids in HPTLC resolution

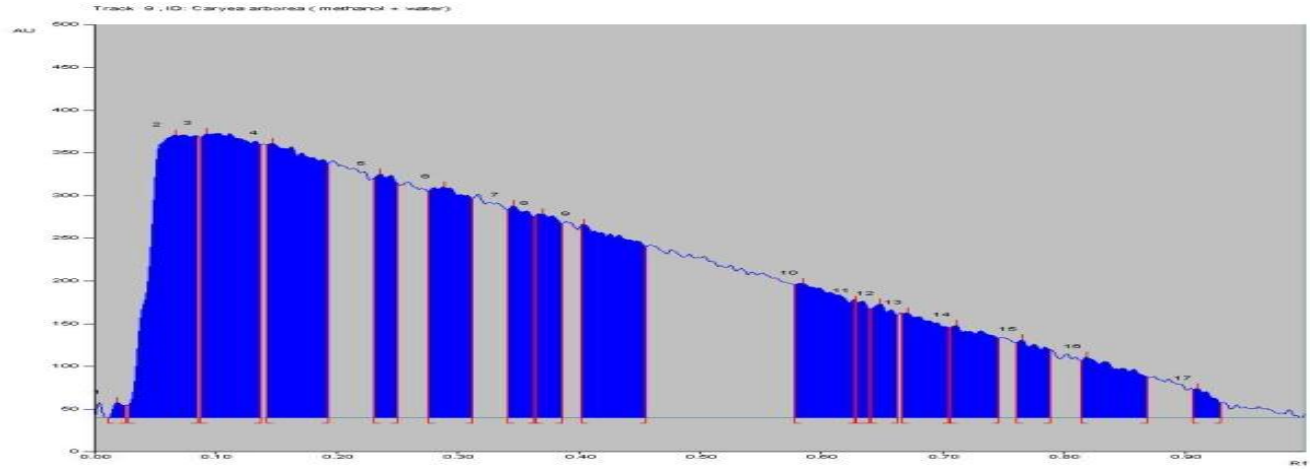


Fig 5. Gr-E (Ethyl acetate: Methanol:Water 60:30:10) extract of *Carea arborea* leaf showing 19 peaks of flavonoids in HPTLC resolution

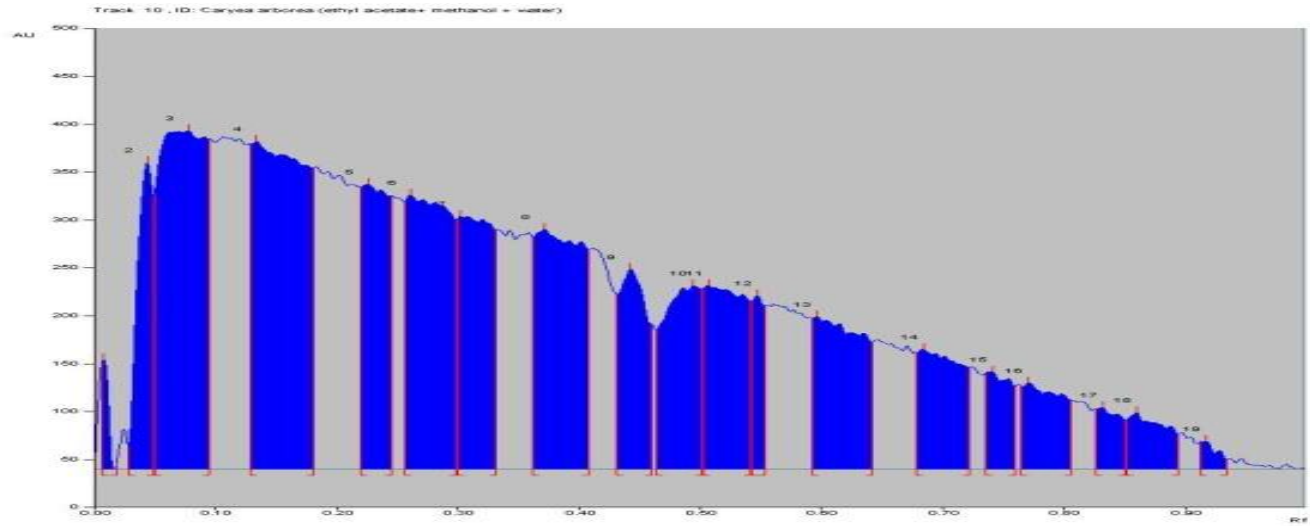


Fig 6. Anti-microbial effect of polyphenols and flavonoids of *Careya arborea* leaves on growth of *E. coli*

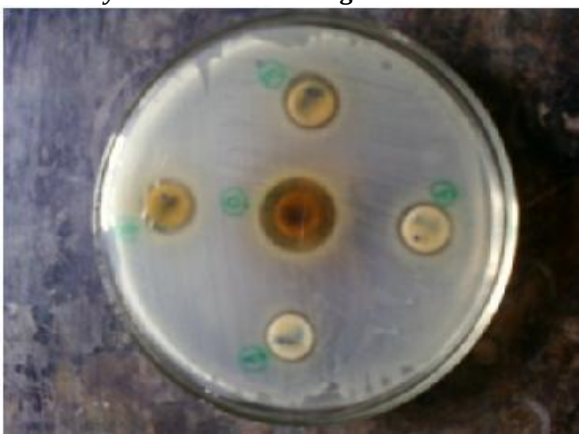
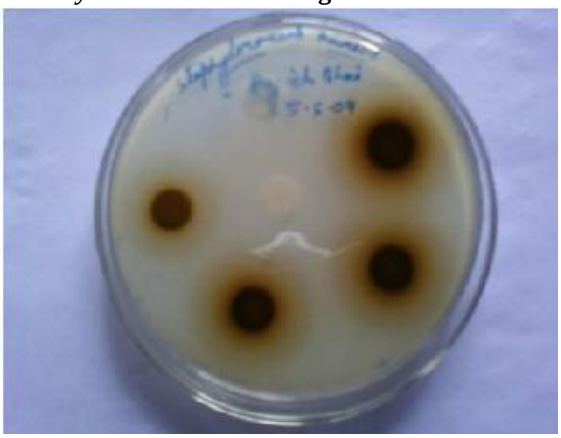


Fig 7. Anti-microbial effect of polyphenols and flavonoids of *Careya arborea* leaves on growth of *S. aurius*



DISCUSSION

Extraction of phenolics and flavonoids in microwave assisted digestion and extraction strongly depends on solvent type and concentration where solubility of components varies with polarity of solvents and physical and chemical properties of components (Turkmen *et al*., 2006; Yilmaz *et al*., 2006). In the present study, more phenolics and flavonoids are extracted and estimated in organic solvents and their mixtures in Gr-B, C, D and E than that of aqueous one in Gr-A because non-polar solvents are not affected by microwave energy for which polar solvents dominate over aqueous ones for extraction (Desotillo *et al*., 2004). Mixtures of polar and non-polar solvents effectively recover phenols and flavonoids from plants (Tsuda *et al*., 2009) for which Gr-D solvent exhibits presence of almost all components and estimates higher concentration because water in methanol protects and prevents phenolic compounds from being oxidized by phenol-oxidase enzymes (Harborne and Williams, 2000). The missed components in other solvents are recovered in mixture of solvents in Gr-E because water and ethyl acetate modifies the solvent polarity and increases yield of phenolic compounds (Geissman, 1963). Besides, high content of flavonoids and more number of peaks detected in HPTLC in Gr-E solvent may be attributed to presence of constituents like quercetin, sebiferine and litseferine. A range of 46.01-65.10% of flavonoid in total phenolics may be due to presence of anthocyanidines along with other flavonoids. The variations of flavonoids as number of peaks in different solvents may be due to their occurrence as glycosides or aglycones where polyhydroxy-flavones have lower Rf values (0.00–0.25) than oligohydroxylated and methoxylated flavones and flavonols (0.5–0.75). Most of the flavonoids like quercetin-3-glucosides (Rf-0.33), quercetin-3-arabinosides (Rf-0.35), quercetin-3-rhamnoglucosides (Rf- 0.29) and Kaempferol glucoside (Rf- 0.54) were found in all samples due to their compatible Rf values.

The leaf extract in mixture of solvents at Gr-E was used for anti-microbial study due to higher contents of total phenolics and flavonoids. The leaf polyphenols at both the doses exhibited significantly lower zone of inhibition as compared to ciprofloxacin against *S. aurius* and *E. coli.* The inhibition zones between doses differed significantly ($p<0.05$) and was ascending in dose dependant manner. Plants contain over 8,000 structural variants of polyphenols and about a dozen classes of flavonoids as secondary metabolites to exhibit multiple and diverged biological activities. The anti-microbial activity of different components of polyphenols and flavonoids has dissimilar mechanisms. Incorporation of more number of hydroxyl groups in phenol molecule by hydroxylation increases the intensity of toxicity to microorganisms (Mason and Wasserman, 1987) such as catechol with two and pyrogallol with three hydroxyl groups are responsible for toxicity through enzyme inhibition either by the oxidized compounds / by reaction with sulfhydryl groups or through nonspecific interaction with proteins (Bastidas, 1998). That is why the phenols such as magnolol, honokiol, and 3.5'-diallyl-2'- hydroxy-4-methoxybiphenyl and terpenoids of *Magnolia grandiflora* exhibit cidal effect against mycobacterium (Clark, 1981), gram-positive and acid-fast bacteria and fungi (Chang, 1998; Ho *et al*., 2001) and those of *Peltophorum africanum* exhibit antimicrobial activities against *E. coli, S. aureus, A. hydrophila, S. soneian and C. jejuni* (Obi *et al*., 2003). But, *Bridelia micrantha* extract inhibits growth of different bacterial species including *S. flexneri and S. plesiomonas* and exhibits potent anti-diarrhoeic activities (Lin *et al*., 2002). The

anti-microbial activity against *S. aurius* and *E. coli* in the present study may be due to interaction of more lipophilic flavonoids with extracellular and soluble proteins to complex with bacterial cell wall for disruption where quinone targets to surface-exposed adhesins, cell wall polypeptides and membrane bound enzymes and tannins complex with polysaccharide and alkaloids intercalate with DNA. The findings corroborate with that of terpenoid and petalostemumol from purple prairie clover against *Bacillus subtilis* and *Staphylococcus aureus* (Hufford *et al*., 2003).

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